

Characterisation of phytochrome using
monoclonal antibodies

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Leicester for the degree of Doctor of
Philosophy

by

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Abbreviations

ANS	8-anilino-naphthalene-1-sulphonate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
EDTA	ethylene diamine tetraacetate
ELISA	enzyme-linked-immunosorbent assay
FACS	fluorescent activated cell sorter
FMN	flavin mononucleotide
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HIFCS	heat inactivated foetal calf serum
IgG	immunoglobulin G
IMS	industrial methylated spirit
kDa	kilodalton
LF	low fluence
mAb	monoclonal antibody
MOPS	3-(N-morpholino) propane sulphonic acid
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium
PBS	phosphate buffered saline
PEG	polyethylene glycol
Pfr	far-red absorbing form of phytochrome
PMSF	phenylmethyl sulphonyl fluoride
Pr	red-absorbing form of phytochrome
RIA	radioimmunoassay
TBS	tris buffered saline
TMB	3, 3', 5, 5' tetramethyl benzidine
Tris	tris(hydroxymethyl)aminomethane
SAR	specific absorbance ratio, the ratio of the absorbance of Pr at 665 nm to that at 280 nm

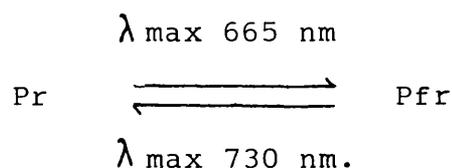
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error = $\frac{\text{standard deviation}}{\sqrt{N}}$
VLF	very low fluence
$\Delta A_r/\Delta A_{fr}$	SCR, the ratio of the maximum absorbance change in the red to the maximum change in the absorbance in the far-red, from a phytochrome absorbance difference spectrum.
$\Delta(\Delta A)$	the difference in absorbance of Pr (665 minus 730 nm) and Pfr (665 minus 730 nm)
φ_{\max}	the mole fraction of Pfr in a solution of phytochrome following red irradiation
λ	wavelength

CHAPTER 1

INTRODUCTION

1.1 Introduction

Phytochrome is a photochromic chromoprotein which plays a central role in the regulation of numerous aspects of plant development (Shropshire and Mohr 1983, Kendrick and Kronenberg 1986). The chromoprotein exists in two stable isomeric forms, Pr which absorbs maximally in the red region of the spectrum, at about 665 nm and Pfr which absorbs maximally in the far-red region of the spectrum, at about 730 nm. These two forms are readily interconvertible by light according to the scheme:-



The photoconversion of Pr to Pfr in the cell induces numerous morphogenic responses, whilst reversion to Pr cancels these inductions. Thus, it is a widely held view that Pfr is the active form and Pr the inactive form of phytochrome (Pratt 1982). However, the mechanism by which phytochrome exerts its action is unknown and the characteristics which distinguish Pr and Pfr are ill-defined.

Much of the research concerned with understanding the mechanism of phytochrome action has focussed on the study of phytochrome regulated responses. Hence, by selecting an appropriate developmental response, it may be possible to trace back through the transduction chain and pin-point the initial event. A second, complementary strategy, is to study the start of the transduction chain by characterising the phytochrome molecule, and

more importantly, identify those properties which distinguish Pr from Pfr. Although these two approaches provide valuable information on phytochrome action, neither, alone, will define the molecular mechanism of this action. The solution to this problem probably awaits the identification and characterisation of a biologically meaningful in vitro phytochrome response. One possible candidate for a biologically meaningful in vitro response to phytochrome, is the recently reported regulation of run-off transcription in isolated barley nuclei by exogenously applied native Pfr, isolated from either oat or rye seedlings (Mösinger et al 1987). In this system the addition of Pfr (from oat), accurately mimics the effect of an in vivo red-light treatment on transcription of a positively and negatively regulated gene. However, it is not clear whether the mode of action of added Pfr on transcription in vitro, presumably involving mRNA elongation rather than initiation (Schäfer et al 1986, Mösinger et al 1987), is the same as its action in vivo following red-irradiation.

This thesis is concerned with one approach towards understanding phytochrome action, namely the application of monoclonal antibody technology to characterising the phytochrome molecule. For this reason, the following Introduction deals only with properties of the phytochrome molecule and not with the physiology of its action. In addition, the chromophore is not discussed in detail as this thesis is concerned principally with the characterisation of the protein moiety.

1.2 The phytochrome molecule

1.2.1 Phytochrome purification; the problem of proteolysis.

The characterisation of the phytochrome molecule in vitro relies on the ability to purify it to homogeneity and in its native state. The major problem associated with phytochrome purification is the presence of proteases in crude plant extracts, which rapidly degrade phytochrome (Gardner et al 1971; Vierstra and Quail 1982a). The problem of phytochrome proteolysis appears to be particularly severe for extracts of oat (Avena sativa L.) seedlings; the most favoured starting material for phytochrome isolation.

Initial isolation attempts were conducted with the phytochrome as Pr, the form shown to be more stable in vivo, and in the absence of protease inhibitors. Early isolations of oat phytochrome yielded a relatively stable monomeric species of ~60 kDa molecular weight (Mumford and Jenner 1966). However, with modifications to purification protocols designed to limit in vitro proteolysis it emerged that the ~60 kDa species in fact represented a stable proteolytic fragment of a much larger multimeric species with a monomeric molecular weight of ~120 kDa (Gardner et al 1971). Phytochrome of ~120 kDa was therefore referred to as "large" phytochrome and the ~60 kDa species referred to as "small" phytochrome. "Large" phytochrome has now been isolated from several plant species and has formed the core of many investigations into the molecular characterisation of

the molecule (Pratt 1982). However, "large" phytochrome has been demonstrated to be comprised of a mixture of 118 + 114 kDa species, degradative products of a larger native molecule. The native oat phytochrome molecule has a monomeric molecular weight of 124 kDa (Vierstra and Quail 1982a). Several pieces of evidence suggest that it is unlikely that the 124 kDa species also represents a digestion product of an even larger molecule. First, in vitro translation of phytochrome mRNA yields a product with an apparent monomeric molecular weight of 124 kDa (Bolton and Quail 1982). Second, phytochrome extracted from freeze-dried tissue into boiling SDS buffer, followed by rapid immunoprecipitation co-migrates with the in vitro translation product on SDS-PAGE (Bolton and Quail 1982). Extraction of phytochrome in this way precludes the possibility of post-homogenisation proteolysis. Finally, the amino acid sequence of oat phytochrome derived from the cDNA sequence, demonstrates the molecule has a monomeric molecular weight of 124.6 kDa (Hershey et al 1985).

Several protocols now exist for the purification of native oat phytochrome (Litts et al 1983; Vierstra and Quail 1983a; Datta and Roux 1985; Grimm and Rüdiger 1987). Proteolytic cleavages are minimized by the incorporation of high concentrations of the protease inhibitor PMSF and the protein stabilisers ethylene glycol and ammonium sulphate, in extraction buffers. Also, isolation of native oat phytochrome generally exploits the observation that in vitro

proteolysis is form specific, with Pfr being more resistant to proteolytic cleavage than Pr (Vierstra and Quail 1982a; Kerscher 1983; Vierstra et al 1984). Undegraded phytochrome has also been purified from rye (Secale cereale L.) seedlings (Ernst and Oesterhelt 1984; Ernst et al 1987; Lagarias et al 1987). Peas (Pisum sativum L.) remain the only dicot from which native phytochrome has been purified to homogeneity (Lumsden et al 1985).

Proteolysis alters the spectral characteristics of native oat phytochrome. Degradation of native oat phytochrome to "large" phytochrome results in a shift in the absorbance maxima for Pfr in both the far-red and blue regions to shorter wavelengths i.e. 730 to 724 nm and 400 to 390 nm (Hunt and Pratt 1979a; Vierstra and Quail 1982b). The absorbance of native phytochrome at 730 nm is much greater than that of "large" phytochrome at 724 nm. As a consequence the $\frac{\Delta A_r}{\Delta A_{fr}}$ (spectral change ratio, [SCR]) which is close to unity for isolated native phytochrome and phytochrome in vivo, is increased to 1.2 for "large" phytochrome (Vierstra and Quail 1982b). These differences in the spectral characteristics of "large" and native phytochrome can be used as a diagnostic clue to the size and integrity of isolated phytochrome. The Pr-specific partial proteolysis of phytochrome in vitro and the accompanying alterations in the spectral properties have been shown to occur in a number of plant species, although there is some variation in the size of the native molecule and the degradation products (Vierstra et al 1984).

1.2.2. Primary and secondary structure

The complete nucleotide and derived amino acid sequences for oat and Cucurbita phytochromes have been elucidated (Hershey et al 1985; Sharrock et al 1986). Oat and Cucurbita phytochromes each consist of two identical monomers of 1128 and 1123 amino acids, respectively. Sequence analyses of chromopeptides have demonstrated that each monomer carries one chromophore which is linked to the peptide at a cysteine residue via a thioether group (Lagarias and Rapoport 1980) (cysteine 321 and cysteine 322 for oat and Cucurbita respectively). The presence of a single chromophore per monomer was confirmed by Hershey et al (1985) who found that the undecapeptide demonstrated to be common to all chromophore bearing peptides (Lagarias and Rapoport 1980) occurred only once in the oat phytochrome amino acid sequence.

Although, overall the oat and Cucurbita sequences are 65% homologous, the regions of homology are not evenly distributed over the length of the molecule. Localized regions of high homology are observed, but the extreme NH₂-terminus and COOH-terminal third of the molecule exhibit little homology. The most highly conserved region of the molecule is the 29 consecutive amino acids around cysteine 321 (oat) and cysteine 322 (Cucurbita), the chromophore attachment site, which are identical in oat and Cucurbita phytochrome (Sharrock et al 1986).

Ultraviolet circular dichroism (CD) spectroscopy has facilitated predictions to be made concerning the

secondary structure of the phytochrome molecule. Using this technique Tobin and Briggs (1973) estimated that "large" rye phytochrome contained 20% alpha-helix, 30% beta structure, with 50% random coil structure. "Large" oat phytochrome consists of 35% alpha-helix, 23% beta structure and 42% random coil (Hunt and Pratt 1980). Recently, the availability of the complete, derived amino acid sequence has facilitated Chou-Fasman analysis (Chou and Fasman 1978) and the construction of hydropathy profiles, allowing predictions to be made concerning the secondary structure of the native molecule (Hershey et al 1985). These analyses indicate that the chromophore is attached at a beta-turn in a mildly hydrophilic segment housed between two strongly hydrophobic regions. This region of the secondary structure is particularly highly conserved between oat and Cucurbita phytochrome. The larger of these hydrophobic regions between residues 80 and 315 may represent a cavity in which the chromophore resides (Hershey et al 1985). This prediction is consistent with earlier observations that the chromophore is relatively inaccessible to the external hydrophilic medium and could therefore be located in a cavity (Eilfeld and Rüdiger 1984; Song 1985).

A popular hypothesis concerning the primary site of phytochrome action proposes that phytochrome functions at the level of the membrane (Marmé 1977). However, the mean hydropathic index for phytochrome calculated across a window of 19 amino acids never exceeds 1.06, consistent with it being a water-soluble protein.

Transmembrane proteins would be expected to have a mean hydrophobic index approaching 2 (Kyte and Doolittle 1982). Thus, it seems improbable that phytochrome could interact directly with the lipid phase of cellular membranes.

1.2.3. Tertiary and quaternary structure

Sedimentation equilibrium centrifugation and cross-linking experiments, under both denaturing and non-denaturing conditions, have confirmed earlier suggestions that phytochrome exists as a dimer in solution (Jones and Quail 1986a). Analyses concerning the shape of the molecule have shown that native phytochrome does not behave as an ideal globular protein, but has an elongated shape (Lagarias and Mercurio 1985). Under non-denaturing conditions phytochrome has an apparent molecular mass of 300-350 kDa as determined by size exclusion chromatography (SEC) (Lagarias and Mercurio 1985). From these data Lagarias and Mercurio (1985) calculated the Stokes radius for the molecule to be 5.6 nm, which is in close agreement with other derived radii using analytical chromatography (5.6 nm Jones and Quail 1986a) Sarkar et al (1984) employing a quasi-elastic light scattering technique, determined the Stokes radius of native oat phytochrome to be 8.1 nm. The reason for the discrepancy between these values has yet to be resolved.

The elongated shape of the phytochrome dimer has been deduced using frictional ratio analyses (f/f_0),

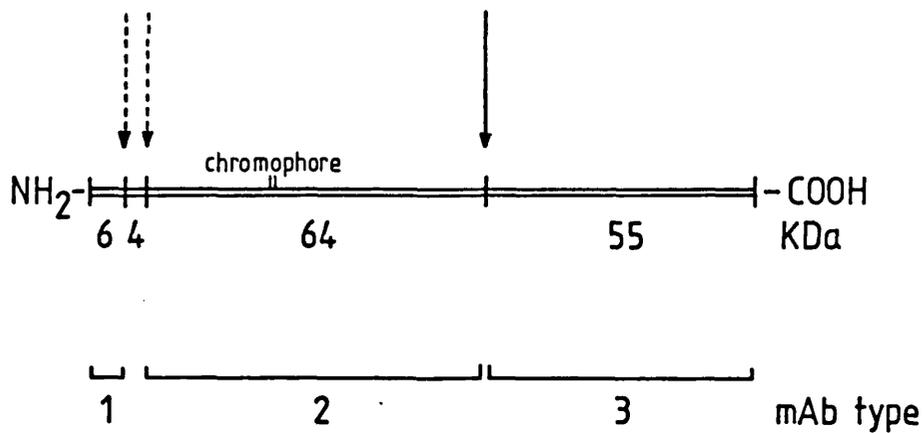
a coefficient which indicates the degree of deviation of the molecule from a sphere. Using a Stokes radius of 5.6 nm Jones and Quail (1986a) calculated this value to be 1.37, for the undegraded dimer, indicating that the molecule had an elongated shape. This elongated dimer could result from the dimerisation of two globular monomers, or, each monomer could itself have an elongated shape. The latter suggestion has been confirmed by SEC and frictional ratio analyses of dissociated phytochrome (Jones and Quail 1986a).

1.2.4. Analysis of functional domains

Native, 124 kDa oat phytochrome can be divided into three specific domains connected by protease sensitive regions. The arrangement of these domains has been mapped by exploiting the fact that digestion of phytochrome as either Pr or Pfr, by endogenous proteases generates a characteristic, but limited array of peptides (Vierstra et al 1984 ; Daniels and Quail 1984; Lagarias and Mercurio 1985). The major peptides observed following digestion of phytochrome, as Pr, by endogenous proteases are 118, 114, 68, 64 and 55 kDa species. Digestion of phytochrome as Pfr, characteristically produces peptides of 74 and 55 kDa (Daniels and Quail 1984; see section 4.3). The orientation of these peptides has been deduced independently by two laboratories (Daniels and Quail 1984; Lagarias and Mercurio 1985). Monoclonal antibodies (mAbs) (designated type 1, 2, 3) have been mapped to defined regions of the molecule

(Daniels and Quail 1984; see Fig. 1.1).

Fig 1.1 Schematic representation of antigenic domains on oat phytochrome recognised by monoclonal antibodies.



The major proteolytic cleavage sites are indicated with arrows. The dashed lines indicate cleavage sites which are kinetically favoured in the Pr form.

Type 1 antibodies recognise the 124 - and 74 kDa species only, and are raised against an antigenic determinant on the 6 kDa NH₂-terminal fragment. Type 2 antibodies recognise 124, 74, 68 and 64 kDa peptides and are raised against the chromophore-containing-half of the molecule. Type 3 antibodies

recognise 124, 118, 114 and 55 kDa peptides and are raised against the chromophoreless COOH-terminal domain.

Structure function studies concerning these principal domains have demonstrated that the 6-10 kDa NH₂-terminal domain is vital for the maintenance of the spectral integrity of the native molecule (Jones et al 1985). Loss of the NH₂-terminal peptides to produce "large" phytochrome causes a shift in the peak absorbance of Pfr from 730 nm to 724 nm and instability of Pfr so that it undergoes thermal dark reversion to Pr (Vierstra and Quail 1982b; Litts et al 1983; Jones et al 1985). The importance of the 6-10 kDa NH₂-terminus is further underlined as many studies on the physico-chemical differences between Pr and Pfr have demonstrated that loss of this domain dramatically affects the properties of the two forms, as a result of increased exposure of the chromophore (Pratt 1982).

As already described, cleavage of phytochrome as Pfr by endogenous proteases generates a 74- and 55 kDa species. The 74 kDa peptide represents the NH₂-terminal half of the molecule which contains the chromophore (Jones et al 1985). It has a compact globular conformation as shown by SEC (Jones and Quail 1986a) and is resistant to further proteolysis, except at the extreme NH₂-terminus (Lagarias and Mercurio 1985). This peptide exhibits the same spectral characteristics as 124 kDa phytochrome (Jones et al 1985). This suggests that the COOH-terminal is not involved in protein-chromophore interactions and therefore may not be directly involved in the molecular action of phytochrome

(Jones et al 1985). However, Lagarias and Mercurio (1985) report regions on the 55 kDa domain which are cleaved more rapidly in Pfr than in Pr. This indicates that there must be some interactions between the 74- and 55 kDa domains and that the COOH-terminal domain could play a role in the light mediated transduction process (Lagarias and Mercurio 1985).

Chromopeptides of 68- and 64 kDa can be generated from the 74 kDa species by sequential cleavage of 6 and 4 kDa from the NH₂-terminus, following conversion to Pr. This directly mimics the proteolytic cleavages that occur at the NH₂-terminus of 124 kDa Pr when it is digested by endogenous proteases. In this case, sequential loss of 6 and 4 kDa yields the 118- and 114 kDa species (Jones et al 1985). This is a significant observation since Grimm et al (1987) have recently reported that digestion of Pr, by endogenous proteases, can generate 118- and 114 kDa peptides which have been clipped at both the NH₂- and COOH-termini. This observation conflicts with the fact that the 74 kDa species, which does not carry any of the COOH-terminus, can undergo the same sequential cleavages, and, has the same spectral properties as 124 kDa phytochrome (Jones et al 1985). This inconsistency has yet to be resolved.

The 55 kDa COOH-terminal domain characteristically behaves as a molecule with 2-3 times its monomeric mass in SEC. As the domain becomes cross-linked with glutaraldehyde at lower concentrations than peptides derived from elsewhere on the molecule, it has been

concluded that this domain carries the site(s) for dimerisation (Jones and Quail 1986a). It is unlikely that the apparent increase in the molecular mass on SEC stems from the formation of trimers or an elongate monomer. If it were due to trimer formation then cross-linking would be observed at even lower concentrations of glutaraldehyde. Conversely, a higher concentration of glutaraldehyde would be required to cross-link an elongate monomer. Tryptic digest analyses have identified that the dimerisation site lies within 42 kDa of the COOH-terminus (Jones and Quail 1986a). High salt conditions induce partial subunit dissociation, indicating that dimerisation involves ionic bonding. Although the presence of dithiothreitol does not alter the elution position of the phytochrome on SEC, the additional involvement of disulphide bridging cannot be totally excluded (Jones and Quail 1986a).

The COOH-terminal domain acts as an elongate molecule in SEC, having a large f/f_0 of 1.44 for the 110 kDa dimer. It is possible that the dimerised COOH-domain may consist of two globular monomers, or that each monomer may be elongate. The most likely possibility is that the monomers are themselves elongate, having an open conformation, as this region of the molecule is relatively susceptible to proteolysis, unlike the globular NH_2 -terminus (Lagarias and Mercurio 1985). Thus, the phytochrome monomer consists of two major structural domains, one globular and one elongate, which are dimerised in the elongated parts

of the monomers. This structure has been described as the "Double lollipop model" (Jones, A.M., personal communication).

1.2.5 The chromophore

Phytochrome is a conjugated protein, composed of apoprotein and chromophore. The phytochrome chromophore is an open chain tetrapyrrole i.e. a bilin. The two forms of phytochrome have distinct spectral properties which in part relate to changes in the chromophore structure which occur on photoconversion.

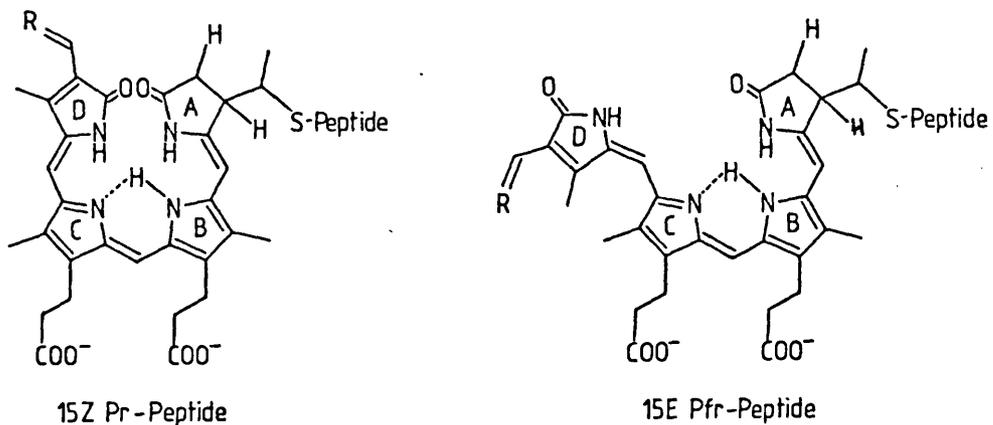
1.2.5.1 The Pr chromophore

The putative chemical structure of the chromophore was deduced using chromic acid oxidation (Rüdiger 1986). This structure was found to be very similar to that of phycocyanobilin i.e. the chromophore of phycocyanin, an accessory pigment of photosynthesis occurring in red and blue-green algae, differing only in the D ring where a vinyl group is exchanged for an ethyl group at C-3 (Fig 1.2). Chromic acid-ammonia degradation of the phytochrome chromophore (phytochromobilin) resolved the structure of the A ring and the thioether linkage between the protein and the chromophore (Rüdiger 1986). Confirmation of the chromophore structure and the thioether linkage on the A ring came from studies of a Pr chromopeptide using high resolution ¹HNMR spectroscopy (Lagarias and Rapoport 1980). Analysis of the chromophore in small chromopeptides demonstrate that the chromophore has a cyclic-conformation (Song 1984) (Fig 1.2). However, the native Pr chromophore probably

has an extended conformation (Rüdiger 1986).

Rüdiger (1986) proposes that the native phytochrome molecule stabilises the chromophore by stretching it to hold the extended conformation and protonating the chromophore so that the cation is stabilised.

Fig 1.2 Structures of the chromophores in small chromopeptides of phytochrome. 15Z = Pr chromophore, 15E = Pfr chromophore.



R = CH=CH₂ : Phytochromobilin

R = CH₂CH₃ : Phycocyanobilin

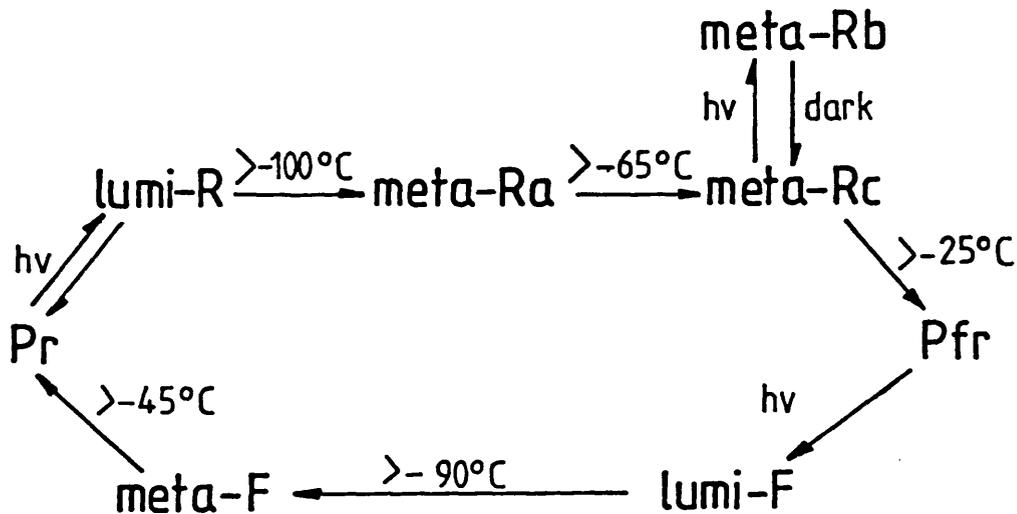
1.2.5.2 The Pfr chromophore

Photoconversion of Pr to Pfr causes a shift in the spectral characteristics of the molecule. Upon photoconversion there is a photoisomerisation, with the changes in spectral properties resulting from non-covalent Pfr-chromophore interactions (Rüdiger 1986). This Z,E isomerisation is believed to occur about the bonds C-15 and C-16 on rings C and D (Rüdiger 1986) (Fig 1.2). That is, the Pr and Pfr chromophores are considered to be geometrical isomers of one another and do not involve changes in chemical structure. There are other possibilities for internal conversions but they would not yield a stable photo-product (Rüdiger 1986).

1.2.5.3 Photoconversion intermediates

A number of short-lived intermediates have been identified between the two stable forms. Recognition of these intermediates has come principally from low-temperature spectroscopy and flash photolysis studies. From recent low-temperature spectroscopy studies with native 124 kDa oat phytochrome the following pathway of intermediates has been determined:-

Fig 1.3 Scheme of phytochrome intermediates derived from low-temperature spectroscopy studies (Eilfeld and Rüdiger 1985).



1.3 Differences between Pr and Pfr

In the context of laboratory experiments with etiolated plants, photoconversion of phytochrome from Pr to Pfr can be thought to act as a regulatory switch. However, under natural conditions this may be too simplistic a view, since plants are never exposed to brief pulses of monochromatic light. Nevertheless, even under prolonged polychromatic irradiation it is assumed that the biological action of phytochrome stems from the recognition of some molecular differences between Pr and Pfr. The differences that

do exist between the two forms of phytochrome result from changes in both the chromophore (see 1.2.5) and the protein.

1.3.1 The chromophore

This section does not deal with structural differences between the Pr and Pfr chromophore (see 1.2.5) but with reactivity and exposure of the chromophore in the two forms.

The Pfr chromophore of 124 kDa phytochrome is oxidised more rapidly by tetranitromethane (TNM) than that of Pr. On proteolytic degradation to "large" phytochrome, the Pfr chromophore is oxidised at an accelerated rate when compared to 124 kDa Pfr, but the rate of oxidation of the Pr chromophore is not altered (Hahn et al 1984). Such differences indicate that the chromophore of Pfr is intrinsically more reactive and/or more physically accessible than the Pr chromophore, and that these effects are accentuated on proteolytic degradation. Recently, Thümmler et al (1985) have used ozone to probe the native Pr and Pfr chromophores. As ozone is a small molecule it should have easy access to both the Pr and Pfr chromophore, hence allowing a comparison of the reactivity of the two chromophores. Both the Pr and Pfr chromophores bleached at the same rate, indicating that the effects observed with TNM are the result of changes in accessibility and not an effect of intrinsic reactivities of the respective chromophores. From this it could be concluded that in native phytochrome the chromophore becomes more

exposed upon photoconversion of Pr to Pfr. Also, removal of the 6-10 kDa NH₂-terminus increases chromophore exposure in both forms, but differences still exist.

Similar studies of differential sensitivity to permanganate oxidation, which also bleaches the chromophore directly, suggest that in the case of "large" phytochrome the chromophore of Pr is buried in a hydrophobic crevice in the protein, whilst that of Pfr is exposed (Hahn et al 1980). However, in contrast, studies with native Pr and Pfr indicate equal sensitivity to permanganate oxidation (Baron and Epel 1983). Thus, Baron and Epel (1983) conclude that only on degradation does the chromophore of Pfr become more exposed. The 6 kDa NH₂-terminal peptide may therefore act directly in protecting the chromophore from the external medium, or it may act indirectly as a result of alteration in a chromophore-protein interaction. The reason for the apparent discrepancy between TNM and permanganate results has yet to be resolved.

The phytochrome chromophore can also be bleached by modification of the protein moiety. Photoconversion of "large" phytochrome to Pfr results in enhanced binding of the hydrophobic probe, 8-anilino-naphthalene-1-sulphonate (ANS), with attendant chromophore bleaching, suggesting that a localised photoconversion-induced change in the surface of the protein leads to exposure of a hydrophobic site in the vicinity of the chromophore (Hahn and Song 1981, Thümmler and Rüdiger 1984). However, when the molecule is not degraded, there is

only limited bleaching of the Pfr form and a slight blue shift in the Pr absorption spectrum in the presence of ANS (Eilfeld and Rüdiger 1984). Following photoconversion of ANS-treated-Pr, the ANS-Pfr product is completely bleached and has similar spectral characteristics to P^{640} , a bleached intermediate which can be formed along the normal Pr to Pfr photoconversion pathway (Eilfeld and Rüdiger 1984). Consequently, Eilfeld and Rüdiger (1984) suggest that ANS binds to a hydrophobic site which is normally involved in stable protein-Pfr-chromophore interaction. This site is largely inaccessible following phototransformation to Pfr, but is accessible in Pr and during photoconversion such that, once bound to Pr ANS prevents normal Pfr-chromophore-protein interaction, so altering the spectral characteristics of Pfr. This model can also reconcile the findings with "large" phytochrome. Eilfeld and Rüdiger (1984) propose that in native phytochrome the Pfr chromophore interacts with the 6-10 kDa NH_2 -terminal domain. When this region of the molecule is lost on proteolytic degradation, the hydrophobic site to which ANS binds becomes generally more accessible. Hence, ANS has a much greater effect on Pfr than Pr.

1.3.2 The protein moiety

In addition to changes in the exposure of the chromophore, alterations elsewhere on the protein have been demonstrated to accompany photoconversion. As already described the two forms of phytochrome generate different peptide patterns following

proteolysis by endogenous proteases (Vierstra et al 1984 , Daniels and Quail 1984 see section 4.3). In particular, the NH₂-terminus of Pr is much more susceptible to proteolysis by either endogenous proteases or exogenous endoproteases (Lagarias and Mercurio 1985). This indicates that the region of the molecule is much more exposed when phytochrome is in the Pr form. The chromophore bearing domain, and COOH-terminal domain also contain cleavage sites which are kinetically favoured in either Pr or Pfr (Lagarias and Mercurio 1985). This indicates that form-specific conformational changes occur throughout the protein moiety.

Analyses of gross changes in conformation following photoconversion are inconclusive. Lagarias and Mercurio (1985) using SEC-HPLC analyses reported significant differences in the retention times of native oat Pr and Pfr, with Pfr eluting in advance of Pr. Such observations have been interpreted as being indicative of a change in the shape (i.e. molecular dimensions) of the molecule following photoconversion. Jones and Quail (1986a) however, only observe the increased retention time of Pr using Tris buffer and not with phosphate buffer. Jones and Quail (1986a) did however observe the differential retention with different chromatographic supports, which suggests that the differences for Pr and Pfr are not merely due to the two forms interacting differently with the chromatographic matrix. Quasi-elastic scattering measurements with native oat phytochrome have detected

no significant difference in the Stokes radius for Pr and Pfr (Sarkar et al 1984). However, the Stokes radius derived from these analyses is much higher than those calculated using analytical chromatography (Lagarias and Mercurio 1985; Jones and Quail 1986a). As quasi-elastic scattering measurements seem to overestimate the Stokes radius there may be a methodological problem associated with this technique. Alternatively, this inconsistency may relate to the integrity of the phytochrome used.

Structural differences between Pr and Pfr have also been analysed by spectroscopic techniques. Circular dichroism spectra measurements demonstrate differences between Pr and Pfr in the far-UV and UV-visible regions (Litts et al 1983; Vierstra et al 1987). Comparison of CD spectra of "large" and native oat phytochrome shows that the 6-10 kDa NH₂-terminal segment is vital in order to observe a photoreversible difference in the UV region of the spectrum interpreted as a change in protein conformation (Vierstra et al 1987). Secondary structure predictions from the CD spectra suggest that following photoconversion from Pr to Pfr there is a 3% increase in the alpha-helical content of phytochrome (Vierstra et al 1987). However, these differences were not observed in previous CD studies with native oat phytochrome (Litts et al 1983).

Despite a number of inconsistencies among the results obtained by different laboratories, using a range of techniques, it is apparent that photoconversion

of Pr to Pfr results in significant alterations in protein/chromophore conformations. There is no strong evidence for gross conformational changes, but several lines of evidence support the view that photoconversion is accompanied by several localised protein conformational changes in different regions of the molecule. One or more of these changes could be expected to be responsible for the biological activity of Pfr.

Other studies concerning physico-chemical differences between Pr and Pfr have been conducted solely on "large" phytochrome, a proteolytically altered molecule. The Pfr form of "large" phytochrome has been demonstrated to bind more tightly to hydrophobic sites of agarose immobilised blue dextran (Smith and Daniels 1981). Only the Pfr chromophore is reversibly bleached by Cu^{2+} , Co^{2+} and Zn^{2+} ions (Lisansky and Galston 1974) and dehydration (Tobin et al 1973). Finally hydrogen-tritium exchange increase on Pr- Pfr conversion suggests that the Pfr form exposes additional peptide segments (Hahn and Song 1982). Thus, all the results concerning "large" phytochrome are consistent with the concept that the chromophore is more exposed as Pfr and/or segmental conformational changes involving exposure of hydrophobic regions, occur on Pr to Pfr photo-transformation. However, these studies are of limited value, as demonstrated by the analysis of "large" and native phytochrome in the presence of TNM, permanganate and ANS, which show the dramatic effect that loss of the 6-10 kDa NH_2 -terminal fragment can

have on the different reactivities of Pr and Pfr.

1.4 Biosynthesis and degradation

In etiolated plants phytochrome is synthesised as Pr at a constant rate. Since Pr is relatively stable in vivo phytochrome accumulates in the dark until a steady-state is reached, presumably reflecting a balance between synthesis and degradation (Quail et al 1973). Irradiation of etiolated plants produces Pfr which is much less stable and is rapidly degraded. Thus, after irradiation the total amount of phytochrome is much reduced due to preferential degradation of Pfr (Quail et al 1973). If plants are transferred to continuous white light a new steady-state level is reached, representing 1-3% of the phytochrome initially present (Hunt and Pratt 1979b). If these plants are then returned to the dark, phytochrome reaccumulates due to de novo synthesis of Pr (Quail et al 1973; Shimazaki et al 1983). From this the regulation of phytochrome abundance was understood to be at the level of the protein, involving a constant synthesis of Pr, slow degradation of Pr and rapid degradation of Pfr.

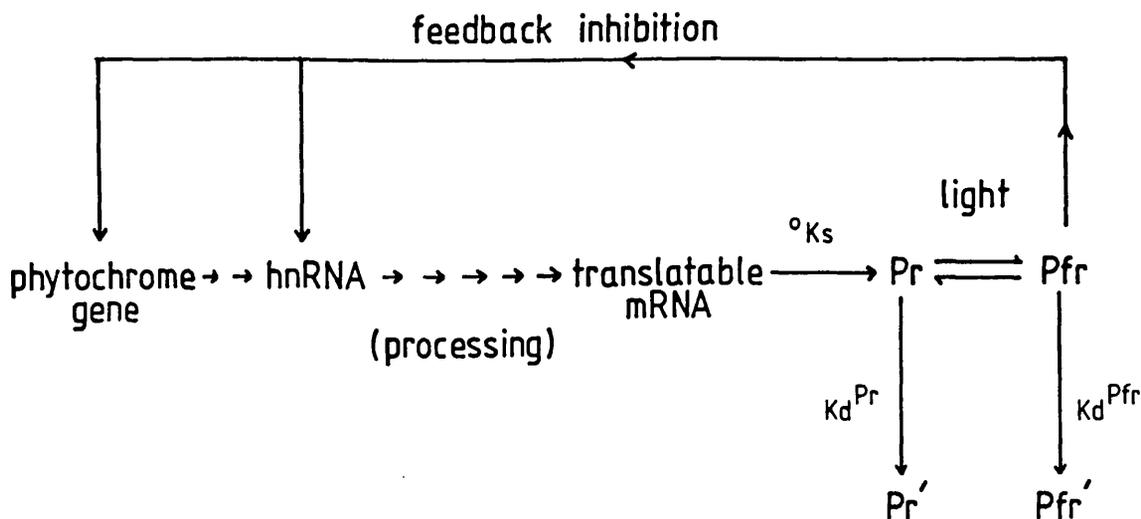
Recently, an additional level of control of phytochrome content has been described. The chromo-protein exerts rapid autoregulatory control over the activity of its own translatable mRNA. This feedback control has been observed for oat, sorghum and pea (Colbert et al 1983; Otto et al 1983, 1984).

Following red-irradiation of dark grown oat seedlings, there is a decline in the level of translatable mRNA specific for phytochrome, as judged by in vitro translation analyses (Colbert et al 1983), which is correlated with a decline in hybridisable phytochrome-mRNA (Colbert et al 1985). The red-light effect on phytochrome mRNA abundance is observed within 15-30 min and results in a greater than 10 fold decrease in abundance after 2 h. The negative feedback on expression of the phytochrome gene by red light is wholly or partly reversible by far-red irradiation (Colbert et al 1983; Otto et al 1983, 1984; Colbert et al 1985) and is released on depletion of the phytochrome concentration, as translatable mRNA levels have been reported to increase on returning light grown oats to darkness (Gottman and Schäfer 1982). Findings from studies of in vitro transcription by oat nuclei indicate that the Pfr-mediated decrease in mRNA levels results, at least partially, from regulation at the level of transcription, but post-transcriptional events may also be involved (Quail 1986).

Although auto-regulation has been observed for a number of other dark grown seedlings there are significant differences in the extent to which it occurs. In vitro translation analyses demonstrate that far-red light alone decreases levels of translatable phytochrome-mRNA in etiolated oat seedlings (Colbert et al 1983) whilst it has no effect in etiolated pea seedlings (Otto et al 1984). Similarly, Northern blot analyses have shown that in both maize

and tomato seedlings, red light leads to only limited and comparatively short-lived decreases in phytochrome-mRNA abundance, in contrast to the dramatic reductions observed with oat seedlings (Quail 1986). Similar transient depressions of the abundance of mRNA for Cucurbita phytochrome are also observed in response to red light pulses (Lissemore et al 1987). In this instance there are two hybridisable mRNA species, which show co-ordinate regulation by red light but do not display classical red/far-red reversibility (Lissemore et al 1987). Clearly, the extent to which autoregulation may control the levels of phytochrome is species dependent. Fig 1.4 shows a complete scheme for the regulation of phytochrome levels in etiolated plants.

Fig 1.4 Regulation of phytochrome levels in dark grown seedlings.



K_s = the zero-order rate constant of phytochrome synthesis.
 K_d^{Pr} and K_d^{Pfr} = the rate constants of *Pr* and *Pfr* degradation, respectively.

Regulation of phytochrome synthesis at the protein level may result from co-ordinated synthesis of apoprotein and of the tetrapyrrole chromophore. Such regulatory systems involving co-ordinated synthesis of protein and prosthetic group have already been described for haemoglobin (Kruh and Borsook 1956) and cytochrome c (Colleran and Jones 1973). Gardner and Gorton (1985) investigated the possibility of co-ordinated synthesis of chromophore and apoprotein by treating seedlings with gabaculine (5-amino-1,3-cyclohexadienyl carboxylic acid) which inhibits chlorophyll biosynthesis by irreversibly blocking the transamination of glutamate-1-semialdehyde to 5-aminolaevulinic acid, the porphyrin precursor (Flint 1984). They report that gabaculine inhibits the initial synthesis and resynthesis of spectrophotometrically detectable phytochrome in pea, maize and oat seedlings. However, Jones et al (1986) and Konomi et al (1986) using immunochemical techniques have independently shown that gabaculine does not effect the accumulation of the apoprotein in peas. The apoprotein must therefore be as stable as Pr, and more importantly although the production of Pr involves the co-ordinated synthesis of the apoprotein and prosthetic group, their syntheses are not obligatorily coupled. The alternative possibility that synthesis of the chromophore is dependent on that of the apoprotein has yet to be tested.

The precise mechanism by which photoconversion of Pr to Pfr in the cell leads to proteolytic degradation of phytochrome is not known. A number of possibilities

may be considered. First, degradation could result from the action of a Pfr-specific protease ("Pfr-ase"). The observation that, Pr derived from Pfr by irradiation of seedlings with red followed by far-red light (Pr'), is degraded at the same rate as Pfr, i.e. red-induced degradation is not readily far-red reversible (Speth et al 1986), could be taken as evidence against this view. Second, proteolytic degradation could result from photoconversion-mediated change in the compartmentation of phytochrome, such that it was brought into contact with non-specific proteases. If such a change in compartmentation was not readily far-red reversible then Pr' would be expected to be degraded, as is observed. Third, photoconversion to Pfr could lead to a "marking" reaction in which phytochrome is targetted for degradation. Again, if the marking reaction was not readily far-red reversible, Pr' would be degraded.

Recently, Shanklin et al (1987) have demonstrated that ubiquitination may be responsible for the photoconversion-induced degradation of phytochrome. Ubiquitin is a polypeptide of 8.5 kDa known to be involved in targetting proteins for cellular destruction. Ubiquitin-dependent protein degradation has been well characterised in animal systems (Hershko et al 1980). It involves an ATP-dependent coupling of ubiquitin to the target protein and subsequent recognition of the ubiquitin-protein complex by the cells proteases, which degrade the attached protein and release free ubiquitin, which can be used again (Hershko et al 1983).

Shanklin et al (1987) irradiated seedlings with red light, extracted phytochrome and probed replica blots with antibodies raised against oat phytochrome and human ubiquitin. A series of peptides with a higher molecular weight than native phytochrome were immunostained by both the anti-phytochrome and anti-ubiquitin antibodies. These peptides are thought to represent phytochrome-ubiquitin complexes. The half life of these complexes is shorter than that of phytochrome which is consistent with the hypothesis that they may be degradation intermediates.

It is perhaps significant that photoconversion of Pr to Pfr in vivo does lead to a change in the cellular location of phytochrome. This change in location, from Pr, diffusely distributed throughout the cytoplasm, to Pfr, in discrete regions of the cytoplasm is termed sequestering (McCurdy and Pratt 1986a). Circumstantial evidence that sequestering may be related to degradation comes from observations that the kinetics of red/far-red reversibility of the two phenomena are rather similar (McCurdy and Pratt 1986b). Since anti-ubiquitin antibodies immunostain the structures with which phytochrome becomes sequestered following red-irradiation in vivo (Schäfer, E., unpublished), it is possible that both sequestering and conjugation of phytochrome to ubiquitin are intermediates in degradation.

1.5 Phytochrome from light grown plants

Little is known about phytochrome from light grown plants because the very low concentrations of phytochrome make attempts at isolation unattractive and difficult. Also, since chlorophyll interferes with the spectral assay for phytochrome the problems of low abundance are further compounded by the lack of a practical detection system. Consequently, attention has focussed on characterising phytochrome from etiolated plants as this is present in much higher concentrations. However, this approach was based on the tacit assumption that phytochrome from dark and light grown tissue is the same molecule.

Early attempts to isolate phytochrome from light grown tissues used plant material treated with the herbicide SAN 9789. This carotenoid biosynthesis inhibitor prevents chlorophyll accumulation which interferes with the spectral assay for phytochrome (Jabben and Dietzer 1978). However, this widely used chemical affects the synthesis of phytochrome (Shimazaki et al 1981). An alternative method for the removal of chlorophyll from extracts containing phytochrome has been developed (Bolton and Quail 1982; Tokuhisa and Quail 1983; Tokuhisa et al 1985).

Shimazaki et al (1981) isolated and partially purified phytochrome from light grown pea seedlings. They found no significant differences in either the spectral properties or, using polyclonal antibodies to phytochrome from etiolated peas, in the immuno-chemical reactivity, between phytochrome extracted

from light and dark grown material. However, phytochrome extracted from light and dark grown oats has been demonstrated to be spectrally and immunochemically distinct. Shimazaki et al (1983) and Thomas et al (1984a) have independently demonstrated that antibodies raised against phytochrome from dark grown oats failed to accurately quantitate spectrophotometrically detectable phytochrome in extracts of light grown oats. Phytochrome from light grown plants exhibits a Pr absorbance maximum at 652 nm ~13 nm shorter wavelength than phytochrome from dark grown plants (Tokuhisa and Quail 1983). The Pfr absorbance maximum is unchanged. This compares favourably with the spectral characteristics of phytochrome extracted from herbicide-bleached light grown seedlings (Jabben and Dietzer 1978).

Phytochrome from light grown oat shoots has been further characterised (Tokuhisa et al 1985; Shimazaki and Pratt 1985; Cordonnier et al 1986b). Dark and green-tissue phytochrome have distinct proteolytic maps (Cleveland maps), suggesting that the two (or more) proteins have different primary structures (Tokuhisa et al 1985; Cordonnier et al 1986b). Immunoprecipitation analyses shows that only a maximum of 30% of phytochrome from green tissue can be precipitated by saturating amounts of either polyclonal or monoclonal antibodies raised to etiolated oat phytochrome (Tokuhisa et al 1985; Shimazaki and Pratt 1985). Tokuhisa et al (1985) therefore propose that green oat shoots contain two distinct pools of phytochrome. The most abundant pool (70%) comprises of phytochrome with a monomeric

molecular weight of 118 kDa and a minor pool (30%) of 124 kDa phytochrome. The 124 kDa pool may represent "contaminating" dark-tissue-type phytochrome, or a third type of phytochrome yet to be characterised. Cordonnier et al (1986b) have provided immunochemical evidence which indicates that green oat shoots contain two different pools of phytochrome which are distinct from that found in dark grown oats, although this has to be confirmed.

Cordonnier et al (1986b) report that phytochrome isolated from dark grown and green tissue are the same size and do not observe the molecular weight differences observed by Tokuhisa et al (1985). Like its dark grown counterpart, phytochrome from green tissue is susceptible to proteolytic degradation in vitro. However, in extracts of green tissue this proteolysis is particularly rapid and occurs equally well with phytochrome either in the Pr or Pfr form (Cordonnier et al 1986b). The initial cleavage yields a molecule with a monomeric molecular weight of 118 kDa (Cordonnier et al 1986b), which Cordonnier et al (1986b) suggest could be the species detected by Tokuhisa et al (1985). The inconsistencies between the two groups relating to the sizes of phytochrome extracted from light grown oats have yet to be resolved, although Tokuhisa et al (1985) maintain that their 118 kDa species is not a proteolytic fragment (unpublished data).

Shimazaki and Pratt (1985) have now reported that there are antigenically distinct pools of

phytochrome in dark and light grown pea shoots.

Abe et al (1985) have also demonstrated that light grown peas contain two distinct pools of phytochrome which are spectrally identical but produce different Cleveland maps following digestion by V8 protease. One of the phytochrome species in light grown peas is apparently identical to that found in etiolated peas.

The origin of the multiple species of phytochrome in light grown material is unknown. They may be derived from separate genes, or the same gene. If they are the product of the same gene the differences which are observed between the molecule may result from post-transcriptional or post-translational modification. Colbert et al (1985) using Northern blot analysis have demonstrated that mRNA isolated from light grown oat seedlings, contains a single hybridisable mRNA species of 4.2 kb, the same length as that isolated from dark grown seedlings. The abundance of this mRNA species is approximately 50 times less than that found in etiolated plants, which correlates well with the differences in the physical abundance of the protein (Colbert et al 1985). These data could mean that either phytochrome from dark and light grown oat shoots are the products of a single gene, or they are the products of two or more distinct genes, which must have some homology and give rise to transcripts of the same size.

1.6 Immunochemistry of phytochrome

Immunochemical techniques have been used to study the phytochrome molecule and have provided information which is complementary to the chemical and physical analyses.

1.6.1 Polyclonal vs monoclonal antibodies

Polyclonal antisera are, by definition, heterogeneous in nature being derived from a large number of immunochemically competent cell lines. For certain purposes, such as immunopurification and quantitation polyclonal antisera are adequate, but are of restricted use because they differ in immunochemical determinants and antigenic specificities and so cannot be used as structural probes.

Monoclonal antibodies (mAbs) represent a much more precise probe, as each mAb represents the product of a single antibody secreting cell, which can be selected for low or high affinity and can be produced in unlimited quantities. Thus, mAbs represent a unique structural probe and consequently have several applications in the study of the molecular function of phytochrome. In particular mAbs can be used to probe for any conformational differences which may exist between Pr and Pfr. In addition antibodies which are specific for only one of the isomers could facilitate the independent immunolocalisation and quantitation of each form of the photoreceptor. Also since domains and sequences related to molecular function are likely to have been conserved throughout evolution (Cordonnier et al 1983), mAbs which recognise

phytochrome regardless of source may be binding to regions essential to biological function. Such analyses coupled with epitope mapping of the molecule can identify the domains of particular interest.

1.6.2 Structure/function studies

As already described mAbs have been used to delineate three specific domains on the oat phytochrome molecule (Daniels and Quail 1984). Proteolytic cleavage maps have also been proposed for Cucurbita and pea phytochrome (Vierstra and Quail 1985; Nagatani et al 1984).

Initial structure function studies were conducted with conventional polyclonal sera raised against "large" phytochrome (Cordonnier and Pratt 1982b). Cordonnier and Pratt (1982b) provided evidence that certain domains were common to phytochrome regardless of source, although in general phytochrome from monocots and dicots formed two antigenically distinct groups. Further studies by the same group with mAbs to oat and pea phytochrome tested cross reactivity against six plant species, by ELISA (Cordonnier et al 1984). Some of the antibodies cross reacted regardless of source, whilst others only reacted with the antigen used for their production. Saji et al (1984) using the radioimmunoassay (RIA) tested six anti-rye mAbs and six anti-pea mAbs against phytochrome from eight plant species. They reported no monocot x dicot cross reactivity in either direction, although one of the anti-pea mAbs cross reacted with every dicot tested. Daniels and Quail (1984) describe very poor cross

reactivity with anti-oat mAbs using SDS-PAGE and immunoblots. Only one mAb from 46 crossed with Zucchini phytochrome. This mAb maps to an epitope on the structurally important NH₂-terminal segment. Whitelam et al (1985) describe six mAbs raised against "large" oat phytochrome which show relatively broad cross reactivity amongst the monocots and dicots. These results suggest that there may be several conserved antigenic sites on the phytochrome molecule. The relatively poor cross reactivity observed indicates that there is an apparent lack of conservation of the phytochrome molecule as a whole. Comparison of the sequences of oat and Cucurbita phytochrome have also demonstrated that regions of homology are not evenly distributed throughout the molecule (Sharrock et al 1986). However, regions of high homology do exist between oat and Cucurbita phytochrome (Sharrock et al 1986). A mAb raised against pea phytochrome which maps to the COOH-half of the molecule cross reacts with a diverse range of plant species (Cordonnier et al 1986a). This mAb must be raised against one of these regions of high homology.

1.6.3 Differences between Pr and Pfr

Polyclonal antisera have failed to find differences between Pr and Pfr (Pratt 1973; Rice and Briggs 1973; Cundiff and Pratt 1975). Monoclonal antibodies raised against "large" oat, pea and rye phytochrome have also failed to discriminate between the two forms of the photoreceptor (Cordonnier et al 1983; Nagatani et al 1983, 1984). Likewise anti-oat mAbs raised against

124 kDa phytochrome also failed to discriminate between Pr and Pfr (Daniels and Quail 1984).

Thomas et al (1984b) were the first to describe mAbs which exhibited different affinities for Pr and Pfr. They describe eight mAbs raised against "large" oat phytochrome, two of which showed a higher affinity for Pr than Pfr and one which showed a higher affinity for Pfr. Cordonnier et al (1985) have isolated three mAbs, which map to the NH₂-terminus of oat phytochrome, and exhibit a higher affinity for Pr. Spectral analyses in the presence of these mAbs show that they alter the spectral characteristics of native phytochrome. The Pfr peak is blue shifted and dark reversion proceeds (Cordonnier et al 1985). These altered spectral properties, normally characteristic of "large" phytochrome, occur in the absence of proteolytic degradation, and probably result from interference in stable protein-chromophore interactions of Pfr, resulting in conversion to the more stable Pr conformation. Monoclonal antibodies which have a higher affinity for Pfr and map to the 64 kDa domain, have also been isolated (Shimazaki et al 1986). Together these data confirm that conformational changes occur at several sites on the protein moiety upon photoconversion.

1.6.4 Immunolocalisation

Immunocytochemical investigation of phytochrome distribution was originally conducted with polyclonal antibodies to oat phytochrome (Coleman and Pratt 1974a, b). However, more recently these studies have been extended by the use of mAbs to oat and pea phytochrome

(Saunders et al 1983; McCurdy and Pratt 1986a,b) and polyclonal antibodies to 60 kDa oat phytochrome (Speth et al 1986).

Phytochrome is evenly distributed in the cytoplasm in non-irradiated tissue, but following red-irradiation the photoreceptor becomes localised in discrete regions of the cytoplasm (i.e. sequestered). This distribution is slowly reversed by far-red light (Saunders et al 1983; McCurdy and Pratt 1986a, b; Speth et al 1986). This sequestering occurs in two phases, a rapid aggregation into small loci followed by a slower association of these aggregates into larger areas (McCurdy and Pratt 1986a). The electron dense structures to which phytochrome associates are composed of unidentified amorphous granular material which are not found in unirradiated plant tissue (McCurdy and Pratt 1986a; Speth et al 1986). The reorganisation of phytochrome may represent a mechanism by which phytochrome is targeted for destruction as, following a red-light pulse, phytochrome and the electron dense structures gradually disappear (McCurdy and Pratt 1986b). Similar structures with sequestered phytochrome have also been observed in pelletable material following homogenisation of red-irradiated tissue in the presence of Mg^{2+} (McCurdy and Pratt 1986b). It may be that sequestering and pelletability result from the same primary cellular event.

1.7 Aims

The aim of this study was to produce a panel of monoclonal antibodies to undegraded oat phytochrome and to characterise them as potentially useful probes to investigate the phytochrome molecule. Of special interest was the possible identification of mAbs which are form specific, and which would therefore be able to precisely identify localised differences between Pr and Pfr. Such mAbs might be expected to be valuable tools in the search for those characteristics which are unique to Pfr and as such may be related to its activity i.e. its active site(s). An alternative approach to this problem would be the identification and characterisation of mAbs recognising highly conserved domains, in the expectation that they represent regions of biological importance e.g. the active site. Monoclonal antibodies could also be expected to be useful tools for characterising the differences between phytochrome from dark and light grown plants and hence assist in elucidating the origin of the observed differences.

CHAPTER 2

PURIFICATION OF PHYTOCHROME

2.1 Introduction

In order to characterise the phytochrome molecule it is important to purify phytochrome in an undegraded and undenatured form. The biggest limitation to successful isolation of native phytochrome is proteolytic degradation by endogenous proteases in plant extracts.

The first successful isolation of native, 124 kDa oat (*Avena sativa*. L) phytochrome employed a combination of hydrophobic- and hydroxyapatite-chromatography (Litts et al 1983). Proteolytic degradation was prevented by inclusion of high concentrations of the protein stabilisers ethylene glycol and ammonium sulphate in the extraction buffers, as well as the use of high concentrations of the serine protease inhibitor, PMSF, throughout the purification. Vierstra and Quail (1983a) also developed a purification protocol for 124 kDa oat phytochrome in which proteolysis was limited by the use of similar buffer systems to Litts et al (1983), and by maintaining the phytochrome in the Pfr form throughout the procedure. This purification procedure involved a combination of hydroxyapatite-, affinity- and gel filtration-chromatography steps.

Two other procedures for the purification of native 124 kDa oat phytochrome have been published. Datta and Roux (1985) adapted the procedure of Litts et al (1983) and produced a much faster protocol for the purification of native phytochrome. Principally, the time reduction is achieved by removal of the

hydrophobic column step and the use of fast-flow hydroxyapatite. More recently, Grimm and Rüdiger (1987) report an even more rapid protocol for the purification of 124 kDa oat phytochrome. Their procedure adopts the early part of the method of Vierstra and Quail (1983a) i.e. up to, and including hydroxyapatite chromatography. The main advance of the procedure is the exploitation of the differential solubility of phytochrome and the other proteins present in an ammonium sulphate pellet, following hydroxyapatite chromatography. Final purification is achieved by differential precipitation with polyvinylpyrrolidone (PVP).

A similar protocol for the purification of oat phytochrome has also been reported (Chai et al 1987). However, this method does not involve PVP precipitation and final purification is achieved on Bio-Gel filtration chromatography.

Immunoaffinity chromatography, developed by Hunt and Pratt (1979a), using immobilised polyclonal antibodies, has also been applied to the purification of native oat phytochrome (Vierstra and Quail 1983a). Although this procedure is highly specific and yields phytochrome which is ~95% homogeneous, the elution conditions are so stringent that they cause spectral denaturation of the molecule. In particular, exposure of the phytochrome to 3 M $MgCl_2$, on elution, leads to a 20-30% loss in absorbance of the phytochrome

at 666 nm following photoconversion. Thus, immunopurified oat phytochrome is only useful for amino acid analyses and raising antibodies.

There has been less success in efforts to isolate native phytochrome from other plant species. Native rye (Secale cereale. L) has been purified employing a combination of anion exchange, hydroxyapatite and gel filtration chromatography steps (Ernst and Oesterhelt 1984). This protocol was an adaptation of the procedure of Kerscher (1983), and took four days to complete. Recently, a faster protocol for the purification of native rye phytochrome which can be completed within 24 h has been reported (Ernst et al 1987). This procedure employs similar chromatography steps as Ernst and Oesterhelt (1984), but the procedure is significantly faster due to the incorporation of FPLC. Native rye phytochrome has also been purified employing a combination of brushite-, affinity and gel filtration chromatography steps (Lagarias et al 1987).

Native Cucurbita pepo phytochrome has been partially purified using a modification of the Affi-Gel Blue oat procedure (Vierstra and Quail 1985). Native pea (Pisum sativum. L) phytochrome has only been purified to homogeneity by immunoaffinity chromatography, using columns of immobilised monoclonal antibodies. In this instance it appears that the spectral properties of pea phytochrome are

unaffected by the stringent conditions for elution from immunoaffinity columns (Lumsden et al 1985).

In this chapter the development of two successful protocols for the purification of native 124 kDa oat phytochrome, as well as techniques for extracting partially degraded and "light-grown-tissue-type" phytochrome from dark grown oat seedlings are described. In addition, a procedure for the isolation of pea phytochrome is described.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

Oat (Avena sativa cv Dula) seeds were soaked overnight in 4 mM CaCl_2 (Litts et al 1983) at 4C in darkness and then sown in moist vermiculite. Seeds were germinated and grown in total darkness at 25C for four days prior to harvesting. Peas (Pisum sativum var Onward) were soaked overnight in cold running water and sown on moist vermiculite. Seeds were germinated and grown in total darkness at 25C for seven days prior to harvesting.

2.2.2 Buffers

Buffer A = 100 mM Tris-HCl, pH 8.3 at 4C, containing 50% (v/v) ethylene glycol (ethanediol), 140 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM EDTA, 20 mM sodium sulphite, 4 mM PMSF and Aprotinin (20,000 kallikrein units l^{-1});
Buffer B = 50 mM Tris-HCl, pH 7.8 at 4C, containing 5 mM EDTA, 14 mM 2-mercaptoethanol, 0.5 mM PMSF and Aprotinin (10,000 kallikrein units l^{-1});
Buffer C = Buffer B containing 1 mM PMSF;
Buffer D = 50 mM Tris-HCl, pH 7.8 at 4C, containing 5 mM EDTA, 70 mM $(\text{NH}_4)_2\text{SO}_4$, 14 mM 2-mercaptoethanol, and 0.2 mM PMSF; Buffer E = 5 mM KPB, pH 7.8 at 4C, containing 5 mM EDTA, 14 mM 2-mercaptoethanol and 0.2 mM PMSF; Buffer F = 100 mM KPB, pH 7.8 at 4C, containing 5 mM EDTA, 14 mM 2-mercaptoethanol and 0.2 mM PMSF; Buffer G = 10 mM KPB, pH 7.8 at 4C, containing 5 mM EDTA, 14 mM 2-mercaptoethanol and 0.2 mM PMSF; Buffer H = Buffer G containing 1 M KCl;
Buffer I = Buffer G containing 250 mM KCl;

Buffer J = Buffer H containing 10 mM FMN;
 Buffer K = Buffer F minus PMSF; Buffer L = 10 mM
 KPB, pH 7.8 at 4C, containing 5 mM EDTA, 14 mM 2-
 mercaptoethanol, 25% (v/v) glycerol; Buffer M =
 Buffer G minus PMSF; Buffer N = 100 mM Tris-HCl,
 pH 8.3 at 4C, containing 70 mM $(\text{NH}_4)_2\text{SO}_4$, 25% (v/v)
 ethylene glycol, 5 mM EDTA, 20 mM sodium sulphite,
 4 mM PMSF and Aprotinin (20,000 kallikrein units l^{-1});
 Buffer O = 25 mM KPB, pH 7.8 at 4C, containing 5 mM
 EDTA, 14 mM 2-mercaptoethanol and 1 mM PMSF;
 Buffer P = 75 mM KPB, pH 7.8 at 4C, containing
 5 mM EDTA, 14 mM 2-mercaptoethanol and 1 mM PMSF;
 Buffer Q = 50 mM Tris-HCl, pH 7.8 at 4C, 5 mM EDTA,
 150 mM KCl, and 14 mM 2-mercaptoethanol.

2.2.3 Affi-Gel Blue protocol for the purification of 124 kDa oat phytochrome

Undegraded, 124 kDa oat phytochrome was isolated
 from 1 kg freshly harvested shoots using a modified
 version of the Vierstra and Quail (1983a) procedure.
 Prior to harvest, seedlings were chilled to 4C and the
 phytochrome converted to Pfr by irradiating the
 seedlings with 2.5 min red light. Red light was
 provided by a bank of 40 W Atlas Natural Deluxe
 fluorescent tubes filtered through 1 cm of 2% (w/v)
 CuSO_4 solution and 1 layer of No. 14 (Ruby Red)
 Cinemoid, photon fluence rate $10 \mu\text{mol m}^{-2} \text{s}^{-1}$.
 The remaining procedures were performed under dim
 green safelight at 4C. Whole shoots were homogenised
 in a Wareing Blender with Buffer A at a ratio of
 0.75 ml buffer per g tissue. The resulting crude

extract was filtered through two layers of cheesecloth and one layer of Miracloth.

Nucleic acids, pectins and acidic proteins were precipitated by the addition of 10 ml l^{-1} of a 10% (v/v) poly(ethylenimine) solution pH adjusted to 7.8 at 4C (Jendrisack and Burgess 1975). Following addition of the poly(ethylenimine) the extract was stirred for 15 min and then centrifuged at 8,000 x g for 15 min. The supernatant was decanted and the phytochrome precipitated by the addition of 250 g l^{-1} of dry $(NH_4)_2SO_4$, with constant stirring. Once the $(NH_4)_2SO_4$ was fully dissolved stirring was continued for a further 20 min and then the extract centrifuged at 8,000 x g for 20 min. The resultant pellets were carefully drained and then resuspended in 200 ml Buffer B. The resulting solution was clarified by centrifugation at 10,000 x g for 15 min and then applied to a 2.4 x 10 cm column of hydroxyapatite (Bio-Rad DNA-Grade Gel HTP) previously equilibrated in Buffer D. After sample application, the column (flow rate $3ml\ min^{-1}$) was washed with two column volumes of Buffer B, followed by five column volumes of Buffer E. The phytochrome was eluted with an exponential gradient from 100 ml Buffer E to 200 ml Buffer F. The peak phytochrome-containing fractions were pooled and precipitated by addition of 0.61 ml of 3.3 M $(NH_4)_2SO_4$ (Tris-buffered) per ml of eluate, with constant stirring. Following centrifugation for 15 min at 8,000 x g, the resulting pellet was resuspended in 20 ml Buffer G. After complete

resuspension the phytochrome was converted to Pr by irradiation via a Schott RG9 filter. The sample was then applied to a 1.2 x 26 cm column of Affi-Gel Blue (Bio-Rad, 100-200 mesh) previously equilibrated in Buffer G. Following sample application, the column (flow rate = 0.7 ml min^{-1}) was sequentially washed with four column volumes of Buffer G, four column volumes Buffer H, and finally two column volumes Buffer I. The bound phytochrome was then eluted with Buffer J. Phytochrome in the eluate was precipitated by the addition of 0.9 ml of 3.3 M Tris-buffered $(\text{NH}_4)_2\text{SO}_4$ per ml of eluate. Following centrifugation at $10,000 \times g$ for 20 min, the pelleted phytochrome was resuspended in about 5 ml Buffer K and applied to a 2.5 x 45 cm column of Bio-Gel A-1.5 M (Bio-Rad, 20-400 mesh) previously equilibrated in Buffer K. The column was run overnight (flow rate = 0.4 ml min^{-1}) in Buffer K. Phytochrome containing fractions were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation with 3.3 M Tris-buffered $(\text{NH}_4)_2\text{SO}_4$ ^{10.61 ml $(\text{NH}_4)_2\text{SO}_4$ per ml eluate}. The pelleted phytochrome was resuspended in Buffer L, frozen in liquid nitrogen and stored at -80°C as Pr.

2.2.4 Fast purification of 124 kDa oat phytochrome

124 kDa oat phytochrome was also purified using a similar protocol to that described by Grimm and Rüdiger (1987). The initial stages of this procedure were as described above, up to and including hydroxyapatite chromatography. The hydroxyapatite eluate was precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 42% saturation by the addition of 1.5 ml of 3.3 M Tris-

buffered $(\text{NH}_4)_2\text{SO}_4$ per ml of eluate with constant stirring and then centrifugation at $10,000 \times g$ for 20 min. The pellet was washed by mixing with Buffer M at a ratio of 0.65 ml buffer per $1000 \times 10^{-3} \Delta(\Delta A)$ units, for 10 min at 4C and then centrifugation at $36,000 \times g$ for 20 min. The pellet was further washed with Buffer K at a ratio of 0.85 ml per $1000 \times 10^{-3} \Delta(\Delta A)$ units. The resultant pellet was resuspended into Buffer M and precipitated with PVP at 37 mg per $1000 \times 10^{-3} \Delta(\Delta A)$ units. Following centrifugation at $10,000 \times g$ for 20 min the pellet was carefully rinsed with distilled water and then resuspended into Buffer L. The resuspended phytochrome was converted to Pr, frozen in liquid nitrogen and stored at -80C .

2.2.5 Partial purification of "large" oat phytochrome

"Large" oat phytochrome was partially purified by following the above procedure to hydroxyapatite chromatography, but using buffers without protease inhibitors and maintaining phytochrome as Pr.

2.2.6 Isolation of "light-grown-tissue-type" phytochrome

"Light-grown-tissue-type" phytochrome was isolated according to Whitelam and Atkins (1987). Four day old etiolated oat seedlings were treated with three red-light pulses (5 min) at four hour intervals on the day before harvest. Extraction, poly(ethylenimine) and $(\text{NH}_4)_2\text{SO}_4$ precipitation were as described for the Affi-Gel Blue purification of etiolated oat phytochrome.

2.2.7 Partial purification of pea phytochrome

All procedures were conducted under dim green safelight at 4C. Seven day old etiolated peas were

chilled to 4C and the upper halves of the shoots homogenised in a Wareing Blender, with Buffer N, at a ratio of 1 ml buffer per g fresh tissue. The homogenate was filtered through two layers of cheesecloth and one layer of Miracloth. Nucleic acids, pectins and acidic proteins were precipitated by the addition of 10 ml l^{-1} of a 10% (w/v) solution of poly(ethylenimine), pH 7.8. The filtrate was stirred for 15 min and then centrifuged at 8,000 x g for 15 min. The supernatant was decanted and the phytochrome precipitated by the addition of 250 g l^{-1} dry $(NH_4)_2SO_4$, with constant stirring. The extract was centrifuged for 20 min at 8,000 x g. The resulting pellets were drained and resuspended in 200 ml Buffer C. The resuspended sample was clarified by centrifugation at 10,000 x g for 15 min and applied to a 2.4 x 8 cm hydroxyapatite column (Bio-Rad DNA-Grade Gel HTP), previously equilibrated in Buffer D. Following sample application, the column was sequentially washed with two column volumes Buffer C, followed by five column volumes of Buffer O. Phytochrome was eluted from the column with Buffer P. The phytochrome containing fractions were pooled and precipitated by the addition of 15 g PEG 6000 per 100 ml of extract. Phytochrome was pelleted by centrifugation at 10,000 x g for 10 min. After draining and rinsing with distilled water the pellets were resuspended in Buffer C. The resuspended phytochrome was then applied to a 2 x 7 cm DEAE Agarose column, previously equilibrated in Buffer C.

After sample application, the column was washed with two column volumes of Buffer C. Phytochrome was eluted with an exponential gradient from 100 ml of Buffer C to 300 ml of Buffer Q. Phytochrome containing fractions were pooled and precipitated with 3.3 M Tris-buffered $(\text{NH}_4)_2\text{SO}_4$. The sample was centrifuged at 8,000 x g for 15 min. The resultant pellet was resuspended in Buffer M and applied to a 2.5 x 45 cm column of Bio-Gel A-1.5 M (Bio-Rad, 20-400 mesh) equilibrated in Buffer M. The column was run overnight (flow rate = 0.4 ml min^{-1}) in Buffer M. Phytochrome containing fractions were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The final pellet was resuspended in Buffer L, frozen in liquid nitrogen and stored at -80°C as Pr.

2.2.8 Spectrophotometry

Phytochrome absorption spectra were recorded with an Aminco DW-2a (uv/vis) spectrophotometer, with the cuvette chamber cooled to 3°C . Rates of Pfr dark reversion at 3°C in the presence of sodium dithionite were determined as described by Pike and Briggs (1972a). Sodium dithionite was added to a final concentration of 5 mM immediately following preparation of 0.5 M stock.

A Δ (ΔA) assay was used to determine the amount of phytochrome present at each stage of the purification procedure. The phytochrome containing samples were measured with the Aminco DW 2a spectrophotometer in the dual wavelength mode, with measuring beams set at 665 nm and 730 nm. Actinic light sources were

empirically determined to be saturating. Red and far-red irradiations of the sample were conducted via an Oriel broadband interference filter (peak 650 nm) and a Schott RG9 filter, respectively.

All the amounts of phytochrome stated in this thesis were calculated using the extinction coefficient reported by Kelly and Lagarias (1985) of 1.21×10^5 mole⁻¹ cm⁻¹, which determines $1 \times 10^{-3} \Delta(\Delta A)$ unit to be the equivalent of 0.942 µg oat phytochrome. However, recently Lagarias *et al* (1987) have re-evaluated the extinction coefficient of phytochrome and determined it to be 1.32×10^5 mole⁻¹ cm⁻¹. Thus, $1 \times 10^{-3} \Delta(\Delta A)$ unit is now calculated to represent 0.863 µg phytochrome, which is 91.6% of the original value. Therefore, the amounts of phytochrome quoted in this thesis are slight over estimations of the actual value.

2.2.9 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was conducted according to Laemmli (1970) using 7.5% acrylamide resolving gels (acrylamide:bisacrylamide ratio of 30:0.8) and 3% acrylamide stacking gels. Gels were stained in 0.5% kenacid blue dissolved in 30% (v/v) IMS and 12% acetic acid for 1 h at 37C. Gels were destained in 25% (v/v) IMS and 10% (v/v) acetic acid.

2.3 Results and Discussion

2.3.1 Purification of 124 kDa oat phytochrome using the Affi-Gel Blue procedure

Proteolysis of phytochrome during purification was minimized by extraction of the photoreceptor as Pfr, into ice-cold buffer, containing high concentrations of the serine protease inhibitors PMSF and Aprotinin, as well as the protein stabilisers ethylene glycol and $(\text{NH}_4)_2\text{SO}_4$. The protease inhibitor PMSF inhibits protease activity by covalently reacting with the serine residue at the active site of the protease. This protease inhibitor has to be added regularly throughout the purification procedure as it is readily hydrolysed in aqueous buffers. The extent of hydrolysis is pH-dependent, being extremely rapid at pH 9, but less so at pH 7 (James 1978). Aprotinin, also known as kallikrein inhibitor, is a protein serine protease inhibitor and is much more stable than PMSF. The presence of high concentrations of ethylene glycol and $(\text{NH}_4)_2\text{SO}_4$ are necessary to afford full protection against proteolysis as extraction in the absence of these chemicals leads to rapid degradation of 124 kDa phytochrome to the 118 + 114 kDa species (Vierstra and Quail 1983a; Litts et al 1983). Sodium sulphite is used early in the procedure to produce a reducing environment necessary to prevent oxidative side reactions with phenolics and phytochrome. It is used in preference to 2-mercaptoethanol as high concentrations of this reductant stimulate protease activity and so leads to significant degradation of phytochrome

(Pike and Briggs 1972a; Litts et al 1983). This suggests that the proteases responsible for phytochrome degradation are thiol requiring i.e. sulphhydryl proteases. The presence of sulphhydryl proteases in oat extracts has been known for several years. Pike and Briggs (1972a) partially purified a neutral endoprotease from etiolated oat shoots whose activity was inhibited by high ionic strength buffers, HgCl_2 , and PMSF and was dependent on sulphhydryl groups for activity. Although PMSF is principally used as a serine protease inhibitor it can also inhibit sulphhydryl proteases (Gray 1982). However, the inhibitory effect of PMSF may be overcome by the addition of non-ionic reducing compounds such as 2-mercaptoethanol (Pike and Briggs 1972a). It seems that these significant observations have been largely ignored, which may account for the lapse of over 10 years before native phytochrome was purified.

The precipitation of nucleic acids, pectins and acidic proteins by poly(ethylenimine) did not significantly increase the A_{665}/A_{280} (specific absorbance ratio, SAR) (Table 2.1, Fig 2.1.2). However, this step clarified the crude extract without precipitating phytochrome and improved the resolubilisation of phytochrome following the $(\text{NH}_4)_2\text{SO}_4$ precipitation (Vierstra and Quail 1983a). The $(\text{NH}_4)_2\text{SO}_4$ fractionation step resulted in a four fold increase in the SAR value for the extract (Table 2.1; Fig 2.1.3). Typically, hydroxyapatite chromatography (Fig 2.2) yields 60-65% of the

Table 2.1 Summary of purification of 124 kDa phytochrome from 1 kg of etiolated oat seedlings using the Affi-Gel Blue procedure

Step	Volume (ml)	Activity (μml^{-1})	total (units)	SAR	% recovery	
					per step	overall
1. Crude extract	1500	17	25,000	-		
2. 10% PEI (10 ml^{-1})	1390	14	19,460	0.007	100	100
3. $(\text{NH}_4)_2\text{SO}_4$ I	198	72	14,256	0.027	73.3	73.3
4. Hydroxyapatite	117	86	10,062	0.14	70.6	52
5. $(\text{NH}_4)_2\text{SO}_4$ II	20	500	10,000	-	94.2	94.2
6. Affi-Gel Blue	48	153	7,344	-	73.4	37.7
7. $(\text{NH}_4)_2\text{SO}_4$ III	3.4	1987	6,756	-	92	35
8. BioGel A 1.5M	46.5	100	4,650	0.91	69	24
9. $(\text{NH}_4)_2\text{SO}_4$ IV	6.4	400	2,560	-	55	13

1 phytochrome unit = $1 \times 10^{-3} \Delta(\Delta A)_{665:730 \text{ nm}}$.

Figure 2.1 Absorption spectra of phytochrome
containing fractions at various stages of the
Affi-Gel Blue purification protocol

1 = crude extract

2 = supernatant following PEI precipitation
(SAR = 0.007)

3 = resuspended $(\text{NH}_4)_2\text{SO}_4$ precipitate
(SAR = 0.027)

4 = hydroxyapatite pool (SAR = 0.14)

SAR = specific absorbance ratio $A_{665\text{nm}}/A_{280\text{nm}}$

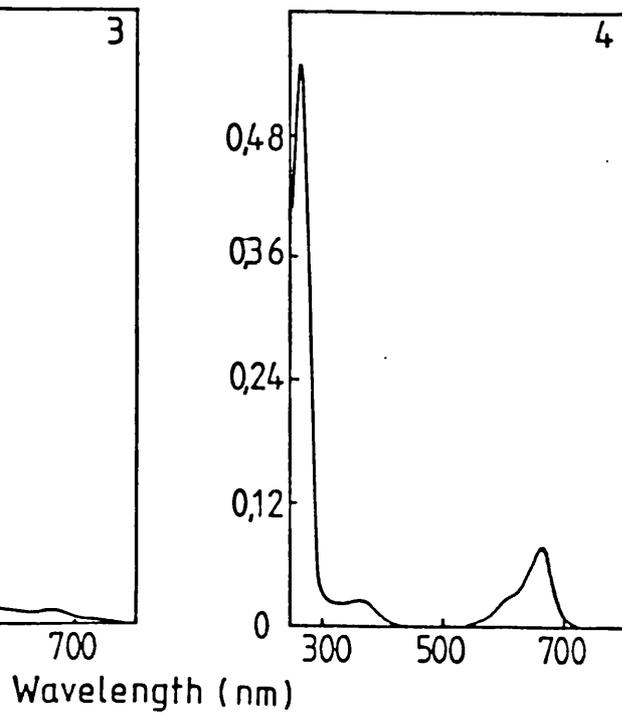
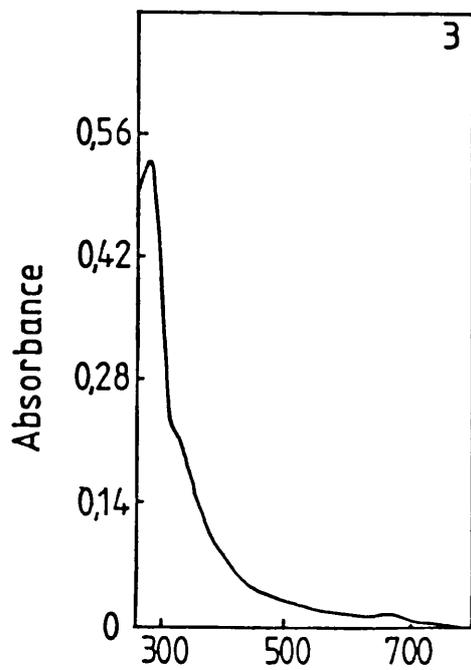
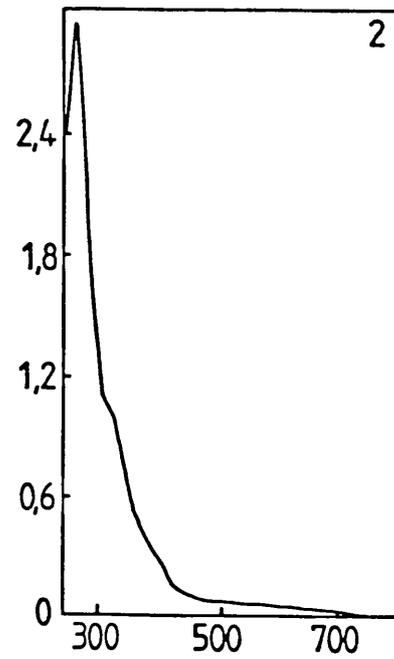
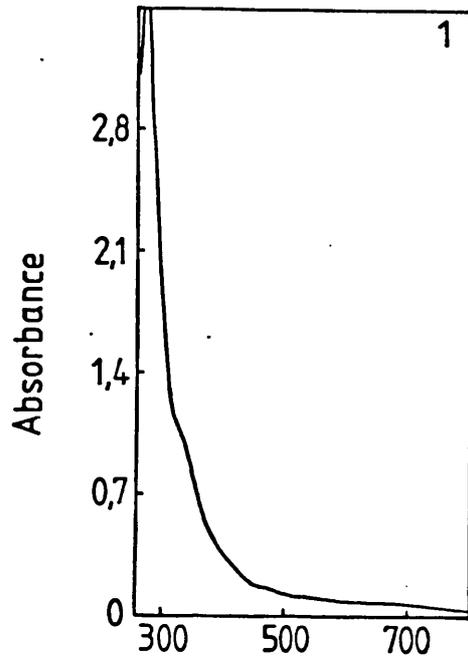
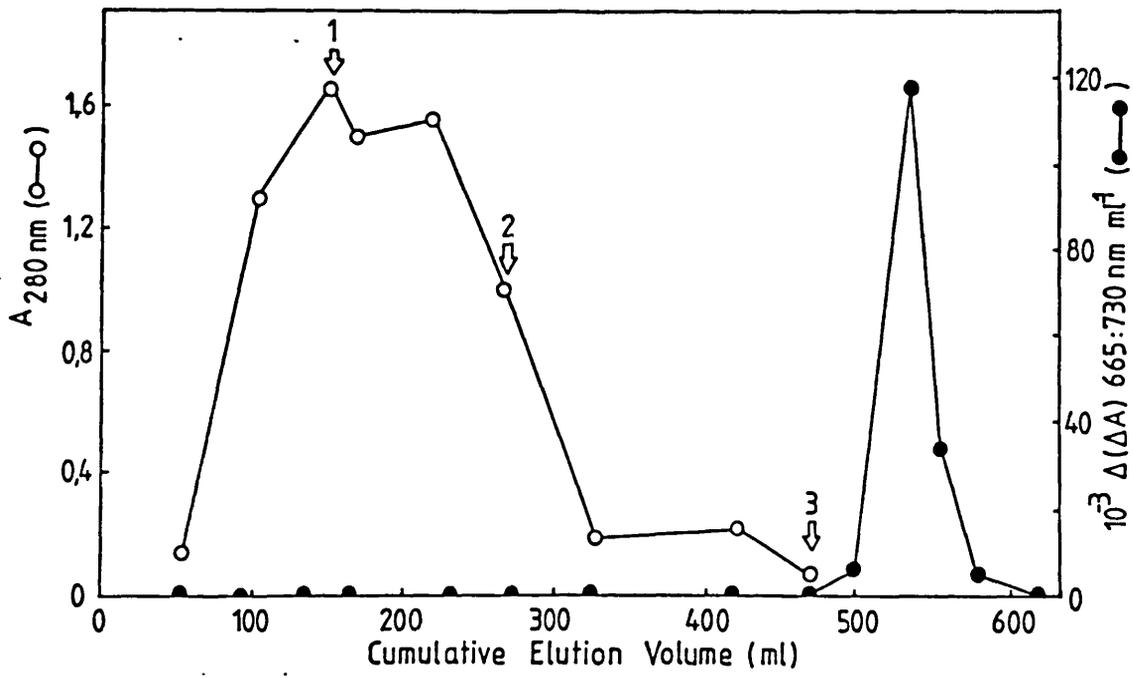


Figure 2.2 Hydroxyapatite chromatography of extract following $(\text{NH}_4)_2\text{SO}_4$ fractionation

The sample was applied in Buffer D to a 2.4 x 10 cm hydroxyapatite column. The column was washed sequentially with (1) two column volumes of Buffer B and (2) five column volumes Buffer E. Phytochrome was eluted by (3) an exponential gradient of 100 ml Buffer E to 200 ml Buffer F.



phytochrome applied with an approximately six-fold increase in SAR (Table 2.1; Fig 2.1.4). In contrast to Vierstra and Quail (1983a) hydroxyapatite chromatography was conducted in the absence of ethylene glycol as its presence significantly slowed the column. Preliminary experiments demonstrated that at this stage ethylene glycol was not essential for the prevention of proteolytic cleavage of the phytochrome. Elution of phytochrome using an exponential gradient rather than isocratic elution as described by Vierstra and Quail (1983a) provided a good compromise between speed, yield and purity.

Following $(\text{NH}_4)_2\text{SO}_4$ concentration the phytochrome sample was converted to Pr, before application to the Affi-Gel Blue column. This is because this form of the photoreceptor has been demonstrated to elute more efficiently from the Affi-Gel Blue affinity column (Smith and Daniels 1981). Moreover, at this stage problems associated with preferential proteolysis of phytochrome as Pr are very much reduced.

Affi-Gel Blue consists of the dye, Cibacron blue 3GA, covalently linked to agarose. The Cibacron dye has a high affinity for specific binding domains on proteins. Thus, this matrix binds proteins which have a binding domain which is compatible with the dye. Proteins which are bound specifically can only be eluted by their natural ligand or cofactor. Hence proteins bound non-specifically can be removed by increasing the ionic conditions of the washing conditions. The cofactor FMN is an analogue of the

Cibacron blue dye and is used to specifically elute phytochrome, once contaminants have been removed. The observation that FMN could specifically elute phytochrome suggests that phytochrome may carry a flavin binding site. However, no flavin remains bound to the eluted phytochrome (Smith 1983). Sarkar and Song (1982) reported interactions of flavins and phytochrome in solution, however, there has been no direct demonstration of complex formation. The nature of the FMN elution of phytochrome from Affi-Gel Blue remains unknown.

The protocol for Affi-Gel Blue chromatography routinely gives considerable purification with several contaminants being removed prior to FMN elution (Fig 2.3). The elution conditions from this affinity column are very specific with only one major and two minor bands being detectable by SDS-PAGE (Fig 2.5). Typically, 60-65% of the phytochrome applied is recovered (Table 2.1). The use of Bio-Gel A-1.5 M chromatography (Fig 2.4) removes the remaining contaminants and allows the separation of FMN and the phytochrome, phytochrome eluting well in advance of the FMN.

This protocol routinely purifies 124 kDa oat phytochrome with SAR ranging from 0.86-0.91 and with an overall recovery of 12% of the phytochrome present in the starting material (Table 2.1; Fig 2.6).

Figure 2.3 Affi-Gel Blue affinity chromatography of Pr following hydroxyapatite chromatography

The sample in Buffer G was applied to a 1.2 x 26 cm Affi-Gel Blue column which had been equilibrated in the same buffer.

The column was washed sequentially with Buffer G, Buffer H and Buffer I.

Phytochrome was eluted with Buffer J (\equiv Buffer I containing FMN).

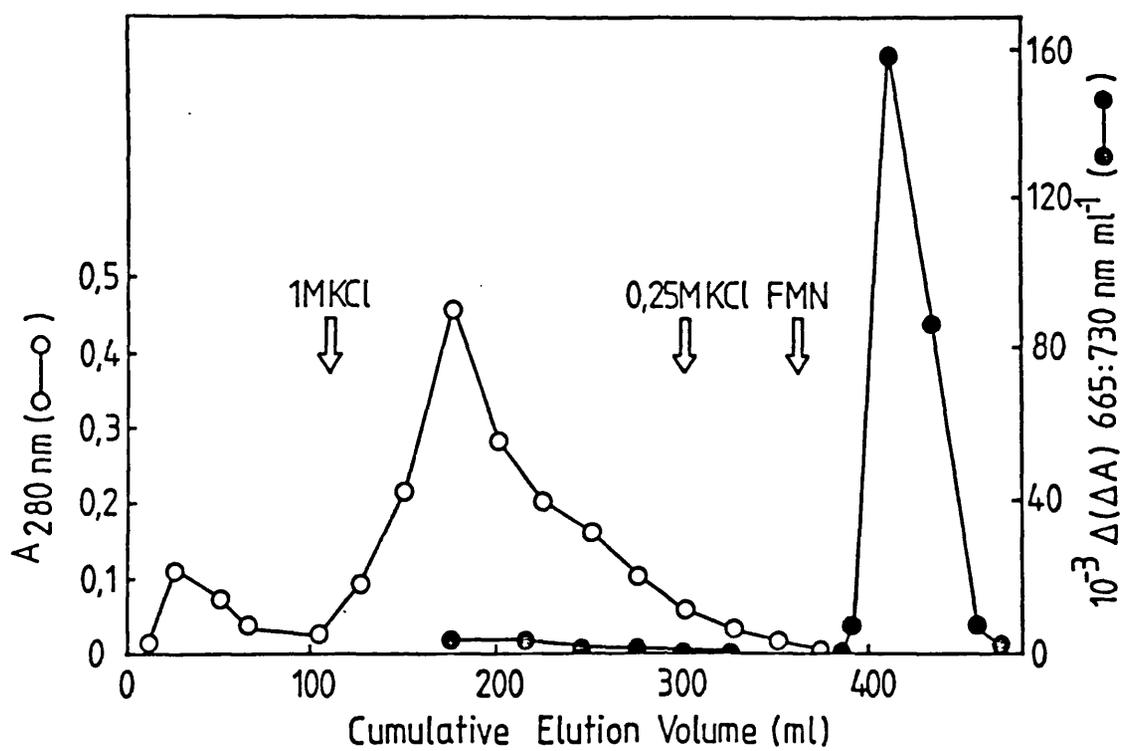


Figure 2.4 Bio-Gel A-1.5M column
chromatography

The phytochrome sample was resuspended in Buffer F and applied to a 2.5 x 45 cm column of Bio-Gel A-1.5M equilibrated in the same buffer. The column was run overnight with the flow rate maintained at 0.4 ml min^{-1} , and 3 ml fractions were collected.

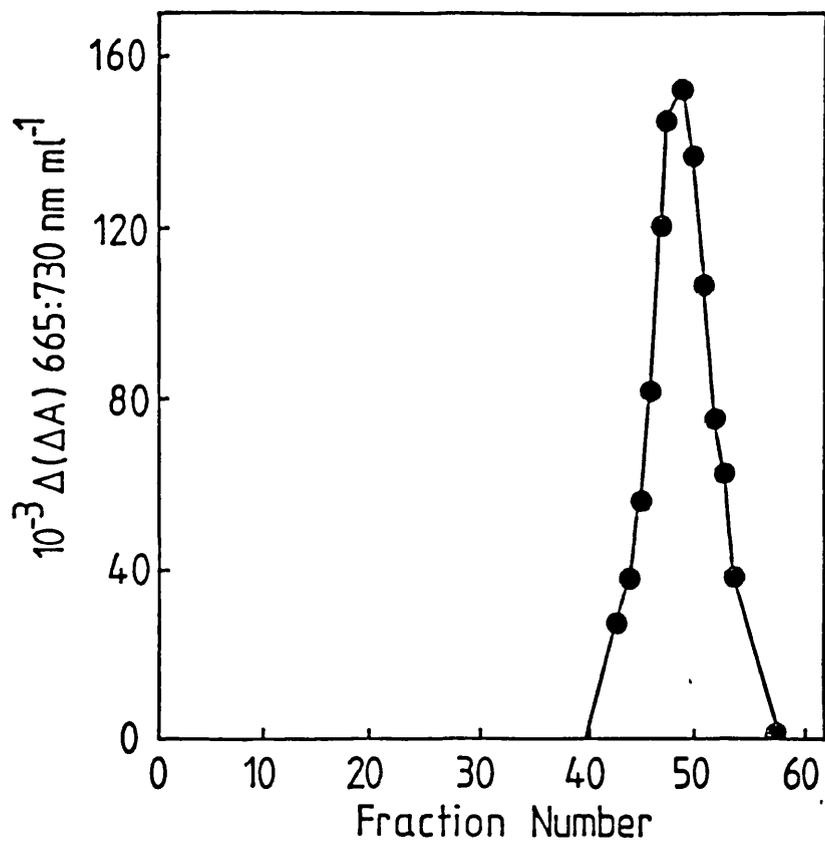


Figure 2.5 SDS-PAGE of aliquots of phytochrome-
containing fractions at various stages of the
Affi-Gel Blue purification procedure

The gel was stained with 0.5% kenacid blue in
30% (v/v) IMS and 12% (v/v) acetic acid.

The gel was destained in 25% (v/v) IMS and
10% (v/v) acetic acid.

Lane 1 = crude extract containing 400 ng
phytochrome per lane.

2 = Supernatant following PEI
precipitation containing 400 ng
phytochrome per lane.

3 = Resuspended $(\text{NH}_4)_2\text{SO}_4$ pellet
containing 400 ng phytochrome
per lane.

4 = Hydroxyapatite pool containing
400 ng phytochrome per lane.

5 = Affi-Gel Blue pool containing
400 ng phytochrome per lane.

6 = Bio-Gel A-1.5M pool containing
400 ng phytochrome per lane.

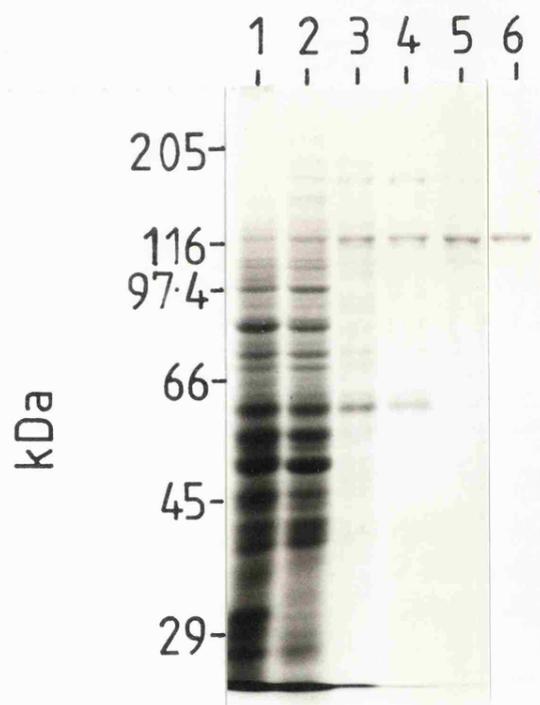


Figure 2.6 SDS-PAGE of purified phytochrome.

a) Affi-Gel Blue procedure final product.

b) Fast procedure final product.

The gel was stained with 0.5% kenacid blue dissolved in 30% (v/v) IMS and 12% (v/v) acetic acid. The gel was destained with 25% (v/v) IMS and 10% (v/v) acetic acid.

a) Lane 1 = 500 ng phytochrome

2 = 1 μ g phytochrome

3 = 2 μ g phytochrome

4 = 5 μ g phytochrome

5 = 10 μ g phytochrome

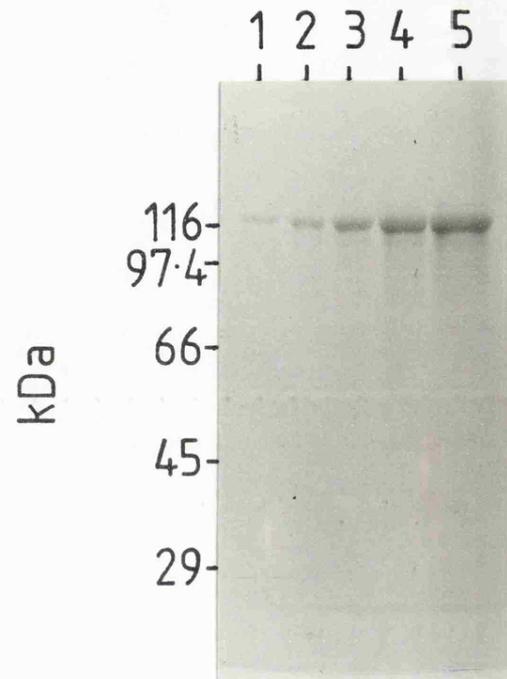
b) Lane 1 = 300 ng phytochrome

2 = 1 μ g phytochrome

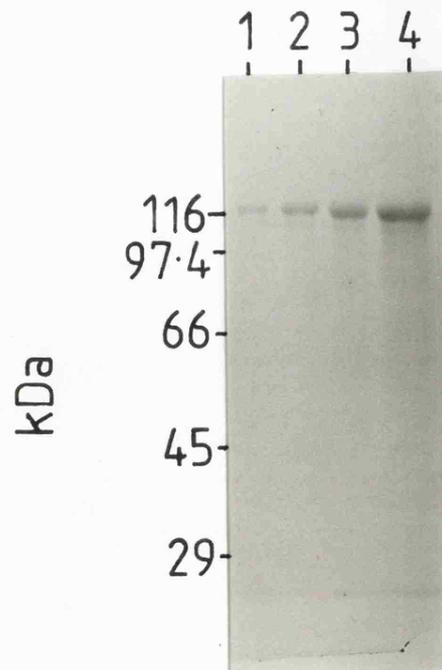
3 = 3 μ g phytochrome

4 = 6 μ g phytochrome

2.6 a



2.6 b



2.3.2 Purification of phytochrome by fast protocol

Following hydroxyapatite chromatography, phytochrome containing fractions were precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 42% saturation which maintains the $(\text{NH}_4)_2\text{SO}_4$ concentration at 2% for the subsequent washing steps. At this stage in the purification procedure, phytochrome is scarcely soluble at 2% $(\text{NH}_4)_2\text{SO}_4$ due to the relative purity of the sample (Grimm and Rüdiger 1987). The washing steps exploit the differential solubility of the phytochrome and the remaining contaminants. The 10 mM KPB wash releases many contaminating proteins and a small amount of phytochrome (Fig 2.7). The subsequent 100 mM KPB wash releases further contaminating proteins from the pellet, but the majority of the phytochrome remains insoluble (Fig. 2.7). Less phytochrome is lost at this stage as phytochrome is inherently less soluble in 100 mM KPB. The washing steps remove any contaminating 118 + 114 kDa or 60 kDa species of phytochrome, as well as the proteases which are normally associated with phytochrome degradation (Grimm and Rüdiger 1987). Typically, 60-70% of the phytochrome is recovered at this stage and typical SAR values are in the range 0.4-0.67 (Table 2.2). The final purification of the sample is achieved by differential precipitation with PVP. The concentrations of PVP required to precipitate the phytochrome were found to be much higher than those reported by Grimm and Rüdiger (1987). Routinely, 37 mg PVP per $1000 \times 10^{-3} \Delta (\Delta A)$ units is required. This precipitation step yields 124 kDa phytochrome with

Figure 2.7 SDS-PAGE of phytochrome
containing fractions at various stages
of the fast procedure

The gel was stained with 0.5% kenacid blue in 30% (v/v) IMS and 12% (v/v) acetic acid. The gel was destained in 25% (v/v) IMS and 10% acetic acid.

Lane 1 = Hydroxyapatite pool.

2 = Supernatant following 10 mM KPB wash of $(\text{NH}_4)_2\text{SO}_4$ pellet.

3 = Supernatant following 100 mM KPB wash of $(\text{NH}_4)_2\text{SO}_4$ pellet.

4 = Sample of resuspended pellet following 100 mM KPB wash.

5 = Supernatant following PVP precipitation.

6 = Resuspended PVP pellet containing 200 ng of phytochrome per lane.

7 = Resuspended PVP pellet containing 400 ng of phytochrome per lane.

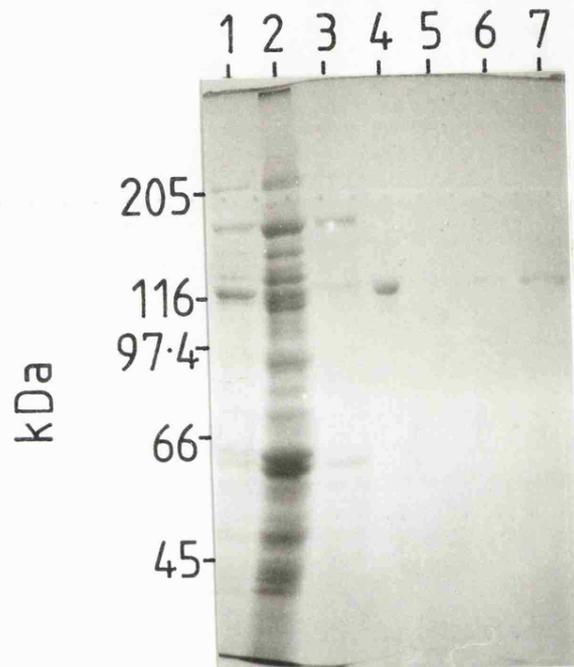


Table 2.2 Summary of purification of 124 kDa phytochrome from 1 kg of etiolated oat seedlings using the fast procedure

Step	Volume (ml)	Activity (μml^{-1})	total (units)	SAR	% recovery per step	overall
1. Crude extract	1700	15	24,750	-	-	-
2. PEI (10 ml^{-1})	1380	14	19,320	-	100	100
3. $(\text{NH}_4)_2\text{SO}_4$ I	200	71	14,200	-	73	73
4. Hydroxyapatite	110	86	9,500	-	67	49
5. $(\text{NH}_4)_2\text{SO}_4$ II after washing with phosphate	13	420	5,460	0.67	57	28
6. PVP precipitate	3.4	1240	4,216	0.93	77	22

1 phytochrome unit = $1 \times 10^{-3} \Delta(\Delta A)_{665:730 \text{ nm}}$.

SAR values in the range 0.89-0.93 (Table 2.2; Fig 2.6). The overall recovery is high with this technique, typically 25% of the initial phytochrome is recoverable. (Table 2.2).

2.3.3 Spectral analyses and photochemical properties of purified phytochrome

Phytochrome purified by the Affi-Gel Blue procedure typically displays absorbance maxima for Pr at 665, 379 and 280 nm, whilst Pfr exhibits maxima at 730, 673, 400 and 280 nm (Fig 2.8a). The Pr minus Pfr difference spectrum exhibits maxima and minima at 665 and 730 nm and has a SCR of 1.06-1.12 (Table 2.3; Fig 2.8b). Similarly, phytochrome purified by the fast protocol has absorbance maxima for Pr at 280, 378 and 665 nm whilst Pfr exhibits absorbance maxima at 280, 400, 673 and 730 nm. These preparations have SCR's ranging from 1.01-1.09 (Table 2.3, Fig 2.9a, b). These spectral characteristics are similar to those observed in vivo and in crude extracts immediately after extraction (Vierstra and Quail 1982b) and compare favourably with those reported for other preparations of 124 kDa oat phytochrome (Table 2.3).

Recently, Lagarias et al (1987) have demonstrated that there are significant differences in the in vitro photochemical parameters of 124 kDa oat phytochrome depending on whether the phytochrome is isolated as Pr or Pfr (Table 2.4). The principal differences are that phytochrome that has been isolated as Pfr exhibits a Pr absorbance maximum that is blue shifted by about 2 nm in comparison with phytochrome that has been

Table 2.3 Spectral characteristics of "large" and 124 kDa
oat phytochrome

	SAR	<u>Pfr 730</u> Pr 665	<u>Pfr 730</u> Pfr 673	<u>Pfr 724</u> Pfr 673	<u>Pfr 730</u> Pfr 667	Δ Ar Δ Afr	Ref
118 + 114 kDa phytochrome	-	0.47		1.07	1.07	1.23	a
	0.96	0.50		1.21	1.26	1.18	b
	0.15	0.52		1.10	1.10	1.17	c
124 kDa phytochrome	0.88	0.57	1.43		1.40	1.09	d
	0.94	0.58	1.45		1.50	1.07	e
	0.89	0.56	1.27		1.35	1.12	f
	0.99	0.60	1.43		1.46	1.01	g
	0.83	0.57	1.40		1.32	1.12	h ¹
	0.91	0.59	1.43		1.45	1.06	h ²
	0.85	0.58	1.45		1.48	1.10	h ³
	0.86	0.58	1.46		1.50	1.12	h ⁴
	0.83	0.56	1.41		1.45	1.10	h ⁵
	0.86	0.58	1.48		1.41	1.03	i ¹
	0.93	0.57	1.45		1.43	1.09	i ²
	0.90	0.59	1.38		1.40	1.01	i ³
	0.87	0.59	1.36		1.38	1.07	i ⁴

a = Song et al (1980)

f = Datta and Roux (1985)

b = Smith and Daniels (1981)

g = Grimm and Rüdiger (1987)

c = This work

h = This work (Affi-Gel Blue protocol)

d = Litts et al (1983)

i = This work (fast protocol)

e = Vierstra and Quail (1983b)

Figure 2.8 Spectral properties of 124 kDa oat phytochrome purified by the Affi-Gel Blue procedure. a) Absorption spectrum. b) Difference spectrum (Pr-Pfr)

Absorption spectra were measured at 30°C after saturating red (Pfr) and far-red (Pr) irradiation.

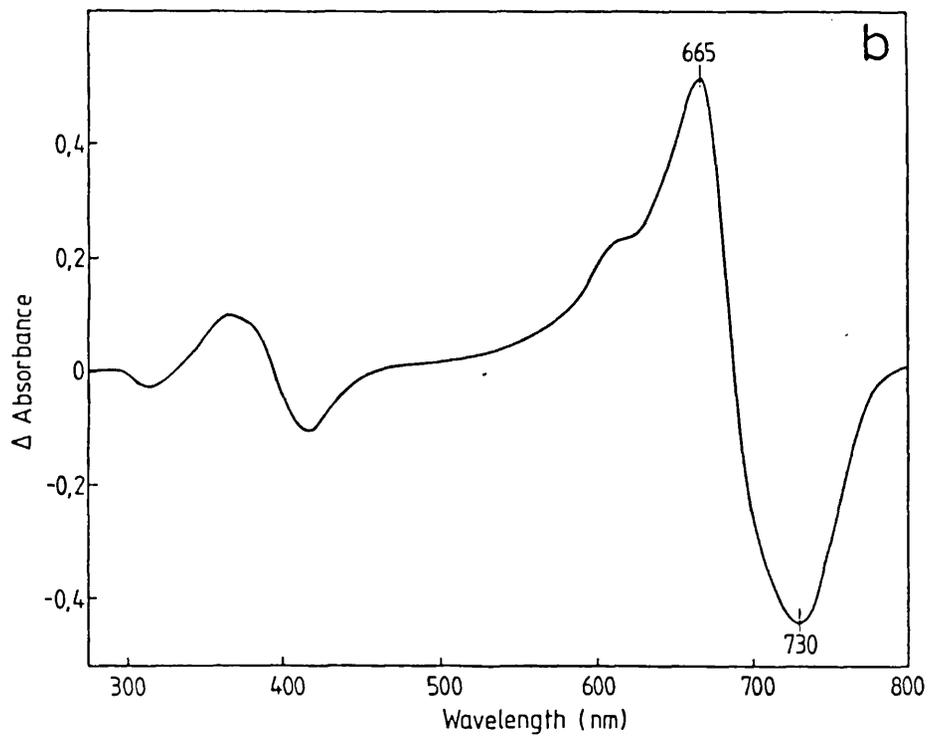
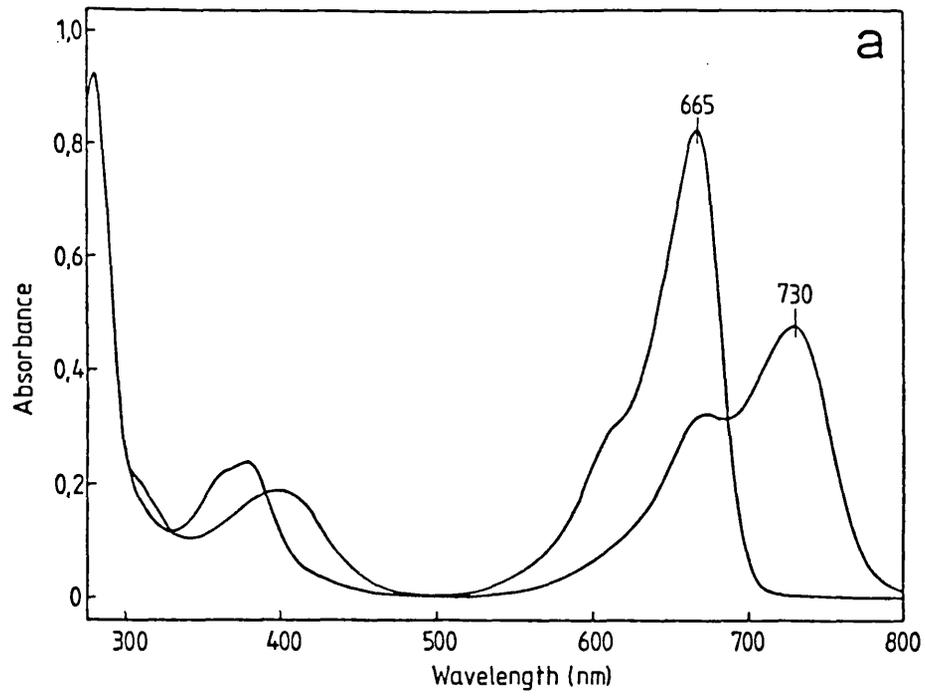
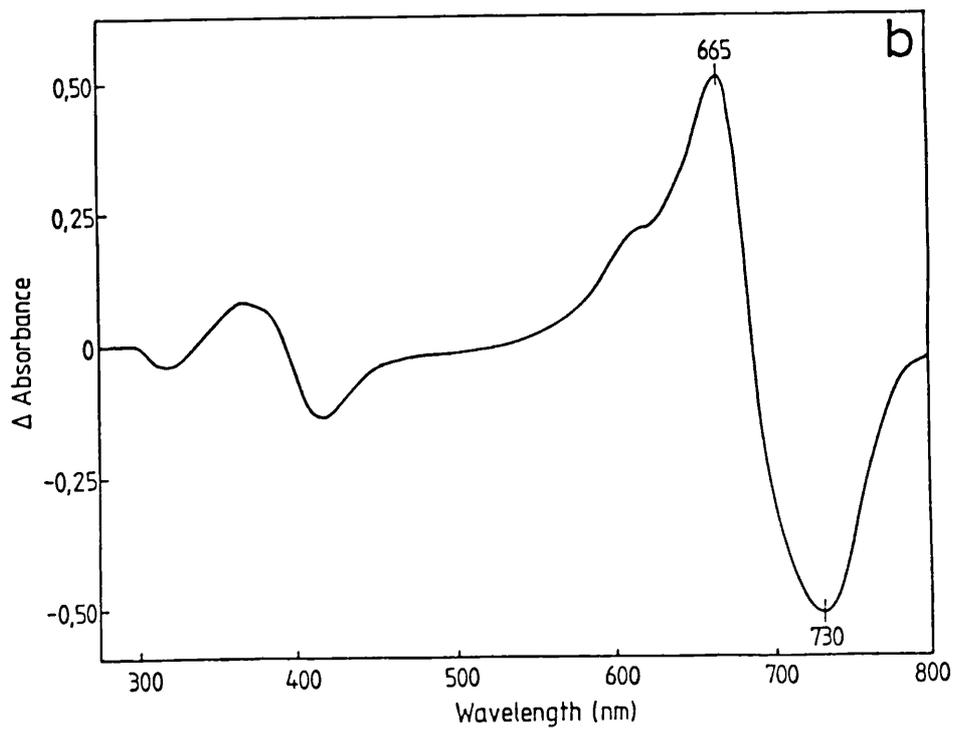
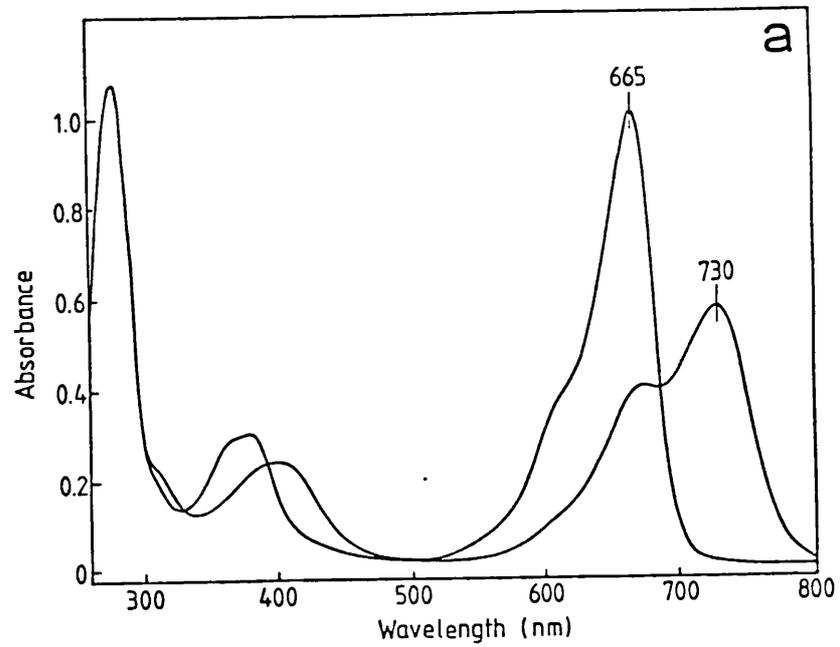


Figure 2.9 Spectral properties of 124 kDa
oat phytochrome purified by the fast protocol.
a) Absorption spectrum. b) Difference
spectrum (Pr-Pfr).

The absorption spectra were measured at 3C
after saturating red (Pfr) and far-red (Pr)
irradiation.



extracted as Pr. In addition, there is a larger ratio of absorbance loss at the irradiation wavelength to the gain of absorbance at the far-red absorbance maximum of Pfr (i.e. $\frac{A_{\lambda} \text{Pfr max}}{A_{\text{Pfr 673 nm}}}$) upon Pr to Pfr phototransformation for phytochrome isolated as Pfr (Table 2.4). The spectral properties of the phytochrome isolated here are consistent with these observations, being typical of Pfr-purified phytochrome (Table 2.4). Lagarias et al (1987) suggest that the blue shift in the Pr spectrum of phytochrome purified as Pfr may be due to sulphhydryl modification, as Pfr is apparently more susceptible to this type of post-homogenisation modification. However, it is not possible to dismiss the possibility that phytochrome isolated as Pr is also modified during purification, although the good correlation between these in vitro and in vivo analyses suggest that the phytochrome extracted as Pr may be more representative of the phytochrome molecule found in vivo. Interestingly, no differences in photochemical properties are observed when rye phytochrome is isolated as either Pr or Pfr (Lagarias et al 1987).

Comparison of the spectral properties of 124 kDa oat phytochrome purified by both the Affi-Gel Blue and the fast protocol with that of "large" phytochrome demonstrate the significant alterations in spectral characteristics which occur on proteolysis. "Large" phytochrome exhibits absorbance maxima for Pr at 665 nm but the Pfr peak is blue shifted to 725 nm (Fig 2.10). The absorbance of Pfr at 730 nm for the

Table 2.4 Summary of spectral characteristics of 124 kDa oat phytochrome extracted as either Pr or Pfr

	Phytochrome isolated as Pr			Phytochrome isolated as Pfr		
	a (nm)	b (nm)	c (nm)	d (nm)	e (nm)	f (nm)
Pr λ max (red)	668	669	668	666	668	665
Pr λ max (blue)	378	378	381	379	379	379
Pfr λ max (red)	730	731	730	730	730	730
Pfr λ max (blue)	402	402	402	400	400	400
$A_{\lambda}^{Pfr\max}/A_{673}^{Pfr}$	1.43	1.27	1.33	1.45	1.43	1.42
$\Delta A_{\lambda}^{Pfr\max}/\Delta A_{\lambda}^{Pr\max}$	0.57	0.56	0.56	0.58	0.60	0.58
Far-red minus red (Pr-Pfr) difference spectra.						
$\lambda(\Delta A^{r\max})$	-	-	668	666	-	665
$\lambda(\Delta A^{fr\max})$	-	-	732	730	-	730
$\Delta A^r/\Delta A^{fr}$	1.07	1.12	1.14	1.07	1.01	1.07

a Litts et al (1983)

b Datta and Roux (1985)

c Lagarias et al (1987)

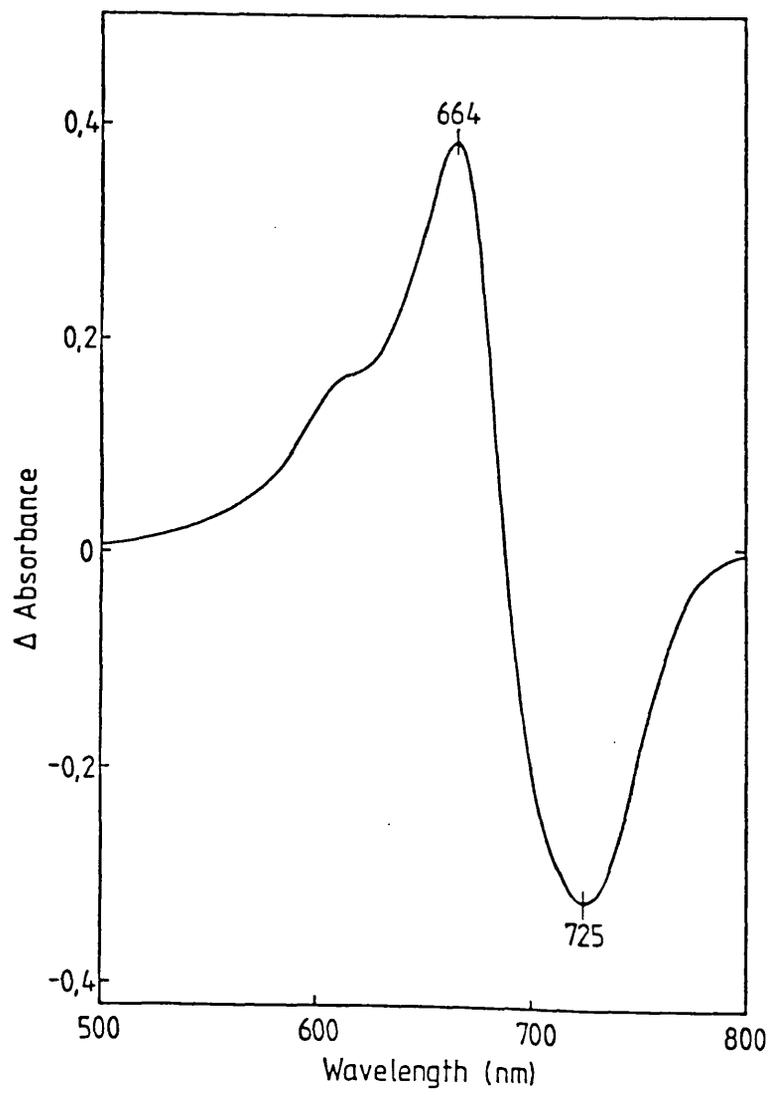
d Vierstra and Quail (1983b)

e Grimm and Rüdiger (1987)

f This work

Figure 2.10 Difference spectrum of "large"
oat phytochrome

Absorption spectra for Pr and Pfr were
measured at 3C after saturating red (Pfr)
and far-red (Pr) light.



124 kDa molecule is substantially larger than the far-red maximum at 725 nm for the partially degraded molecule. This results in an increase in the SCR to 1.17-1.2 for "large" phytochrome (Fig 2.10; Table 2.3). The $A_{730}^{Pfr}/A_{673}^{Pfr}$ for 124 kDa phytochrome is 1.27-1.46 compared to only 1.07-1.10 for 118/114 kDa preparations (Table 2.3). The ratio of $A_{730}^{Pfr}/A_{665}^{Pr}$ ranges from 0.56-0.60 for the 124 kDa but is only 0.47-0.52 for the degraded, 118-114 kDa species (Table 2.3). Finally, $A_{730}^{Pfr}/A_{667}^{Pfr}$ for 124 kDa phytochrome is 1.35-1.50 compared to 1.07-1.10 for 118/114 kDa species (Table 2.3).

Phytochrome purified by both the Affi-Gel Blue and the fast protocols exhibit negligible dark reversion, i.e. non photochemical reversion of Pfr to Pr, in the presence of sodium dithionite, over a period of 90 min (Fig 2.11). However, the preparation of "large" phytochrome showed more than 60% reversion in the presence of sodium dithionite over a similar time course (Fig 2.11). These observations are consistent with those reported for native and "large" phytochromes, where dark reversion is considered to be an artefact of proteolytic degradation of the 124 kDa molecule (Vierstra and Quail 1983a).

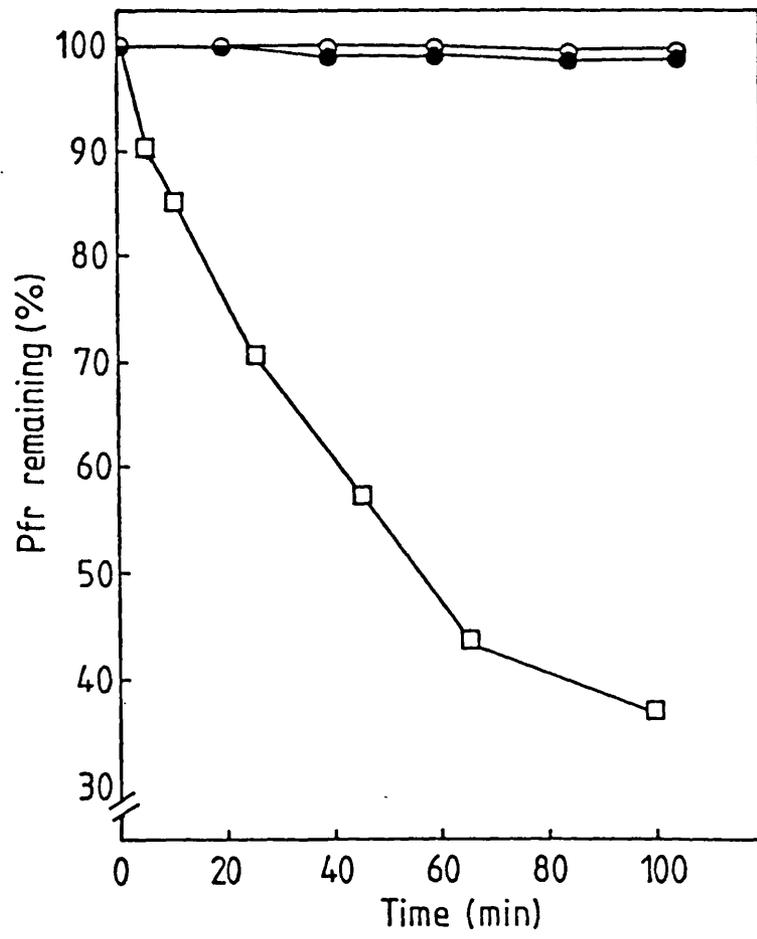
Phytochrome purified by both the Affi-Gel Blue and the fast protocols yield only 124 kDa oat phytochrome. The fast protocol, first described by Grimm and Rüdiger (1987), and used here, has several advantages over the Affi-Gel Blue protocol. Only one chromatography step is included which facilitates

Figure 2.11 Dark reversion analyses of phytochrome, isolated by different procedures, in the presence of 5 mM sodium dithionite at 4C

(○) 124 kDa oat phytochrome purified by the Affi-Gel Blue procedure (SAR = 0.89)

(●) 124 kDa oat phytochrome purified by the fast protocol (SAR = 0.91)

(□) Partially purified "large" oat phytochrome (SAR = 0.15).



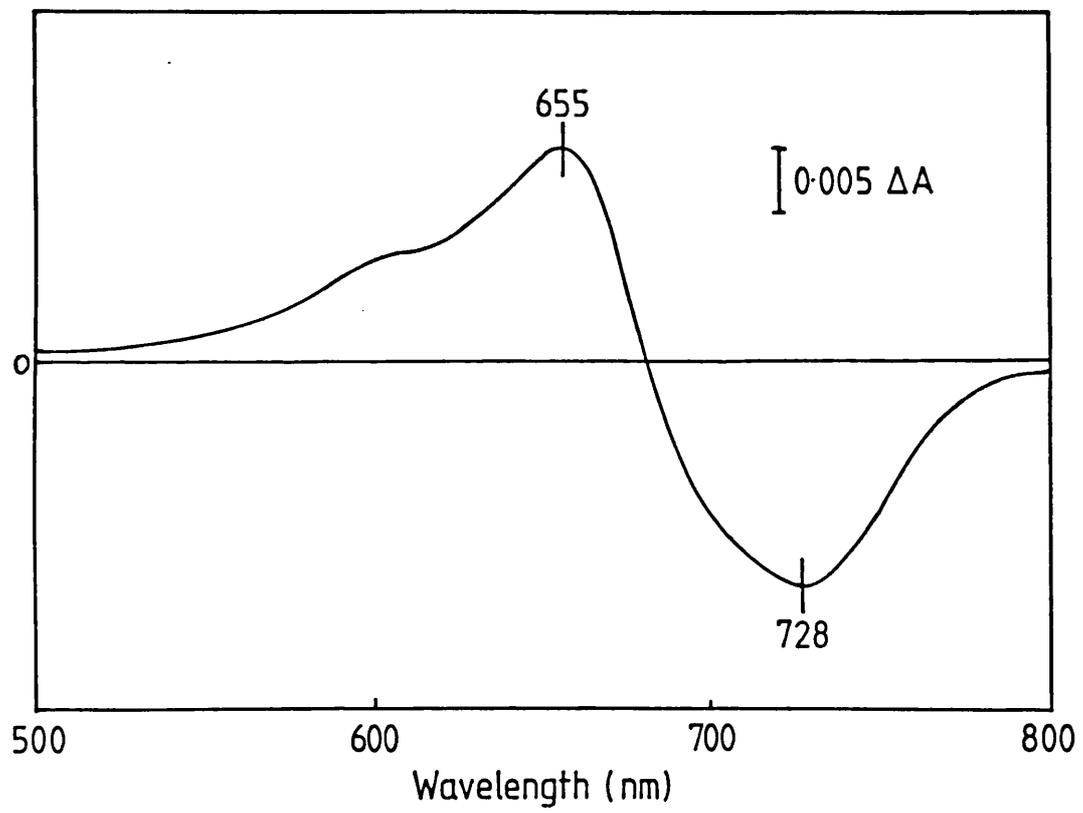
the purification of phytochrome in a single working day. Affi-Gel Blue chromatography is avoided, which although very specific, involves the elution of phytochrome by FMN. Since FMN is a photosensitizer, exposure of phytochrome plus FMN to light can cause photooxidation of the phytochrome molecule (Sarkar and Song 1982). Consequently, Affi-Gel Blue chromatography and Bio-Gel 1.5 M chromatography have to be conducted in complete darkness. Although the phytochrome purified by the Affi-Gel Blue and the fast protocols are very similar in terms of purity and spectral integrity, the fast protocol has a much higher overall recovery of phytochrome, i.e. it is a more efficient way of purifying phytochrome than the Affi-Gel Blue procedure.

2.3.4 Isolation of "light-grown-tissue-type" phytochrome

Isolation of "light-grown-tissue-type" phytochrome followed the same extraction, poly(ethylenimine) and $(\text{NH}_4)_2\text{SO}_4$ precipitation as described in the Affi-Gel Blue protocol for etiolated oat phytochrome purification. The pre-treatment of the etiolated seedlings with red-light pulses removes essentially all the etiolated type phytochrome by initiating Pfr destruction and preventing resynthesis of etiolated-tissue type phytochrome (Whitelam and Atkins 1987). The absorption spectrum for this phytochrome exhibits maxima at 655 and 730 nm with a SCR of 1 (Fig 2.12). These spectral characteristics are similar to those described for phytochrome extracted from green tissue (Tokuhisa et al 1985; Shimazaki and Pratt 1985).

Figure 2.12 Difference spectrum of hydroxy-apatite purified "light-grown-tissue-type" oat phytochrome

Absorption spectra for Pr and Pfr were measured at 3C after saturating red (Pfr) and far-red (Pr) irradiations.



2.3.5 Purification of pea phytochrome

Extraction of the phytochrome was conducted into a buffer system designed to minimize proteolysis i.e. buffer containing high concentrations of ethylene glycol, $(\text{NH}_4)_2\text{SO}_4$ and protease inhibitors. Although Shimazaki et al (1981) demonstrated that poly(ethylenimine) precipitates 66% of phytochrome from crude extracts of etiolated peas, preliminary experiments showed that less than 10% of the phytochrome was precipitated from a crude pea extract by the addition of poly(ethylenimine) to a final concentration (v/v) 0.05%. Consequently poly(ethylenimine) was used to clarify the sample prior to $(\text{NH}_4)_2\text{SO}_4$ fractionation. Following $(\text{NH}_4)_2\text{SO}_4$ precipitation it was not possible to determine the SAR of the sample due to the high and variable background absorption at 280 nm.

Hydroxyapatite chromatography yielded 60% of the phytochrome applied and removed several contaminants (Fig 2.14). The conditions used for washing and elution of the phytochrome from the column were derived empirically (data not shown). The hydroxyapatite pool had a SAR of 0.07 (Table 2.5). Spectral analyses of the hydroxyapatite pool indicated that the phytochrome may have been partially degraded. Native pea phytochrome has absorbance maxima at 665 and 730 nm and a SCR of 1.09 (Fig 2.13; c.f. Vierstra et al 1984). Here the phytochrome exhibited absorbance maxima at 665 and 725 nm and had a SCR of 1.4 (Table 2.6), these spectral characteristics may be due to the presence of a triterpenoid saponin, which

Figure 2.13 In vivo difference spectrum
of pea phytochrome

Pea epicotyl tissue was carefully packed into 2,3 ml plastic cuvettes, one of which served as a reference. Absorption spectra of the sample cuvette were recorded at 3C after saturating red (Pfr) or far-red (Pr) irradiations.

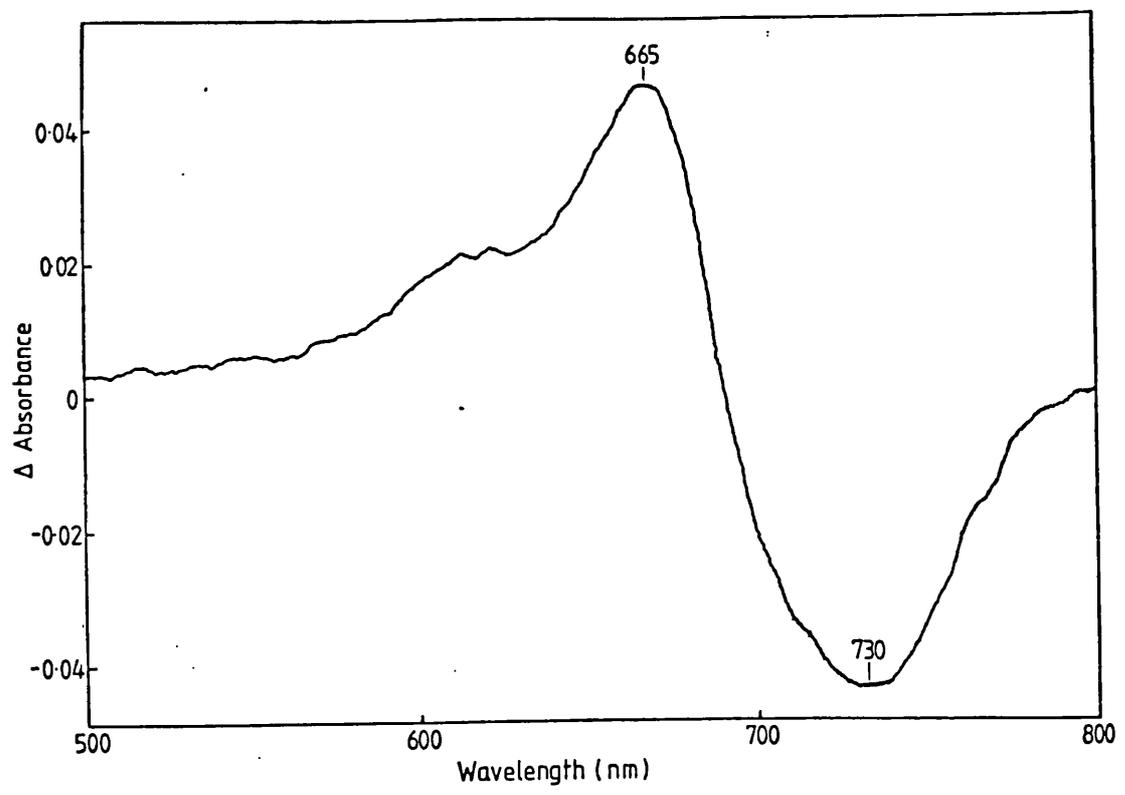


Figure 2.14 SDS-PAGE of pea phytochrome
containing fractions during the purification
procedure

The gel was stained with 0.5% kenacid blue dissolved in 30% (v/v) IMS and 12% (v/v) acetic acid. The gel was destained in 25% (v/v) IMS and 10% (v/v) acetic acid.

- Lane 1 = Crude extract containing 200 ng phytochrome per lane
- 2 = Supernatant following PEI precipitation containing 200 ng phytochrome per lane
- 3 = Resuspended $(\text{NH}_4)_2\text{SO}_4$ pellet containing 600 ng of phytochrome per lane
- 4 = Hydroxyapatite pool containing 700 ng of phytochrome per lane
- 5 = Resuspended PEG precipitate containing 800 ng of phytochrome per lane
- 6 = DEAE chromatography pool containing 400 ng of phytochrome per lane
- 7 = Final sample containing 400 ng of phytochrome per lane
- 8 = Final sample containing 5 μg of phytochrome per lane

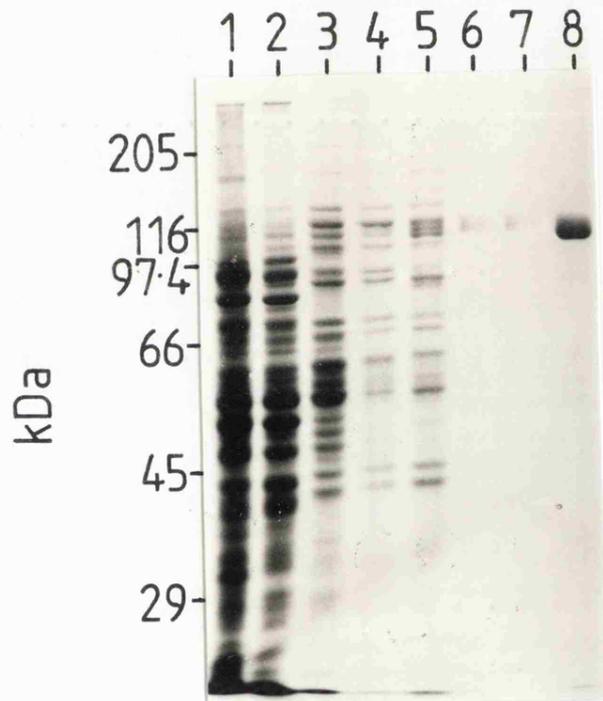


Table 2.5 Summary of purification of "large" phytochrome from 700 g of etiolated pea seedlings

Step	Volume (ml)	Activity (μml^{-1})	total (units)	SAR	% recovery per step	% recovery overall
1. Crude extract	1350	8	10,800	-	-	-
2. PEI (10 ml^{-1})	1250	6.5	8,125	-	100	100
3. $(\text{NH}_4)_2\text{SO}_4$ I	200	33	6,600	-	81	81
4. Hydroxapatite	108	39	4,212	0.01	64	52
5. $(\text{NH}_4)_2\text{SO}_4$ II	32	122	3,904	-	93	48
6. PEG precipitation	6.6	525	3,465	-	89	43
7. DEAE Agarose	31	59	1,836	0.52	53	23
8. $(\text{NH}_4)_2\text{SO}_4$ III	2	820	1,640	0.75	89	19
9. BioGel A 1.5M	32	30.5	979	-	60	12
10. $(\text{NH}_4)_2\text{SO}_4$ IV	1	910	910	0.85	93	11

1 phytochrome unit = $1 \times 10^{-3} \Delta(\Delta A)_{665:730 \text{ nm}}$.

causes spectral denaturation of the phytochrome so that it resembles that of partially degraded phytochrome (Yokota et al 1982; Vierstra et al 1984). The saponin was thought to be specific for Pfr, the so called "Pfr killer", but subsequently it has been demonstrated to have a greater effect on Pr (Konomi et al 1982).

During the development of this protocol the phytochrome sample was applied to an Affi-Gel Blue column. However, this column proved to be unsuitable. Following application of the sample to the column, the column was washed according to the procedure described for oat phytochrome. However, washing with 100 mM KPB containing 250 mM KCl caused the phytochrome to streak off the column. Phytochrome was therefore eluted by the application of FMN. Following removal of the FMN the SAR was determined to be 0.13, comparable to the SAR of oat phytochrome following hydroxyapatite chromatography. The FMN therefore was not specifically eluting phytochrome but co-chromatographed many other proteins. It may be that in order for Affi-Gel Blue to function properly the phytochrome applied has to be significantly purer than SAR 0.07. Clearly, pea phytochrome does not "act" like oat phytochrome with this affinity matrix where significant purification is achieved (Vierstra and Quail 1983a).

Differential precipitation of phytochrome by PEG has been demonstrated to significantly enrich oat extracts for phytochrome (Litts et al 1983). Differential PEG precipitation also enriches pea

extracts for phytochrome. However, it is not possible to accurately quantitate the SAR value for the extract at this stage due to scattering in the sample, which is particularly problematical at shorter wavelengths and hence distorts the spectrum.

Following PEG precipitation the spectral characteristics of the preparation were comparable to those of the preparation following hydroxyapatite chromatography. The sample had a Pr absorbance maximum at 665 nm and Pfr absorbance maximum at 725 nm and a SCR of 1.5 (Table 2.6). Resolution of this sample on SDS-PAGE indicates that the putative phytochrome band at ~120 kDa also has two slightly smaller peptides associated with it in the form of a triplet. However, as this gel is only stained for protein it is not possible to identify these peptides as proteolytic products of phytochrome (Fig 2.14). The spectral characteristics observed may therefore be a consequence of partial proteolysis or may result from saponin-dependent spectral denaturation, or a combination of these two phenomena.

Phytochrome was further purified by anion exchange chromatography on DEAE-agarose. This step yields 50% of the phytochrome applied with a SAR of 0.42, a substantial increase in purity (Table 2.5). At this stage the phytochrome sample has absorbance maxima at 667 and 725 nm with a SCR of 1.76 (Table 2.6). On concentration of this sample before application to Bio-Gel A-1.5 M the SAR increases to 0.75 and there are distinct changes in

Table 2.6 Summary of spectral properties of pea phytochrome at various stages of the purification protocol

Stage	$\lambda_{Pr \text{ max}}$ (nm)	$\lambda_{Pfr \text{ max}}$ (nm)	$\frac{\Delta Ar}{\Delta Afr}$
Hydroxyapatite	665	725	1.4
PEG	665	725	1.5
DEAE Agarose	667	725	1.76
$(NH_4)_2SO_4$	667	725	1.38
BioGel A-1.5M	665	723	1.33

the spectral characteristics which reduce the SCR to 1.38 (Table 2.6). The large increase in the SAR value presumably results from purification due to the $(\text{NH}_4)_2\text{SO}_4$ fractionation step. However, following Bio-Gel A-1.5 M chromatography the SAR of the sample was less than the SAR for the sample that had been applied to the column, but increased again to 0.85 following $(\text{NH}_4)_2\text{SO}_4$ concentration. The reason for this apparent concentration dependence on SAR and spectral properties is not known. Nevertheless the final product of this purification protocol is almost pure, as judged by SDS-PAGE (Fig 2.14).

The spectral characteristics of this partially degraded sample demonstrate absorbance maxima for Pr at 663 nm and for Pfr at 723 nm. The sample has a SCR of 1.33 (Fig 2.15). Problems were encountered with the spectral analyses of the preparations, due to the sample dark reverting very rapidly (Fig 2.16), a further indication that the preparation was partially degraded. Consequently, the recorded relative peak heights of the Pfr spectrum are probably distorted since dark reversion of Pfr during sample scanning will have the effect of reducing $A_{725 \text{ nm}}$ and increasing $A_{665 \text{ nm}}$ and $A_{673 \text{ nm}}$.

This purification protocol therefore yields partially degraded pea phytochrome despite the inclusion of protease inhibitors and protein stabilisers. The apparently rapid partial proteolysis of the phytochrome may be prevented by the inclusion of

Figure 2.15 Spectral properties of highly purified pea phytochrome. (SAR = 0.85)
a) Absorption spectrum. b) Difference spectrum (Pr-Pfr).

Absorption spectra for Pr and Pfr were measured at 3C after saturating red (Pfr) and far-red (Pr) irradiations.

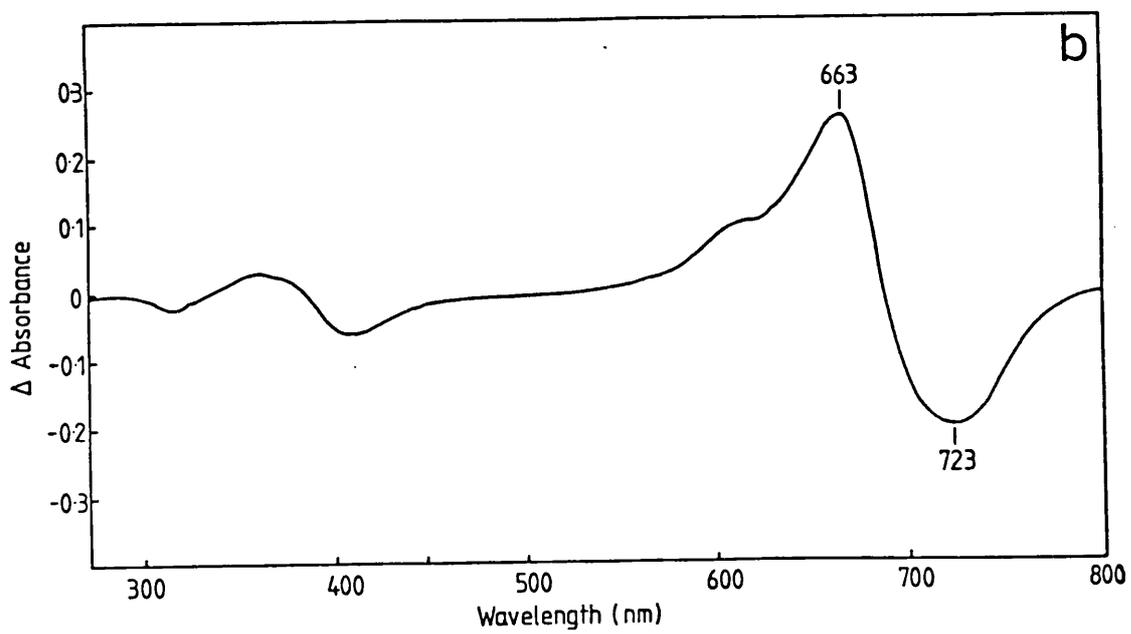
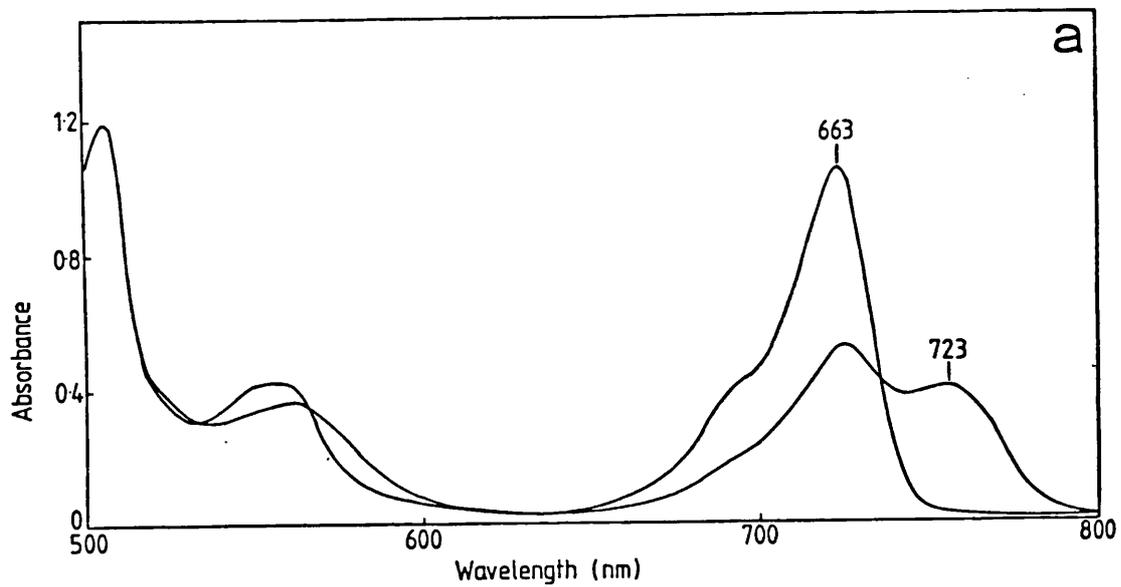
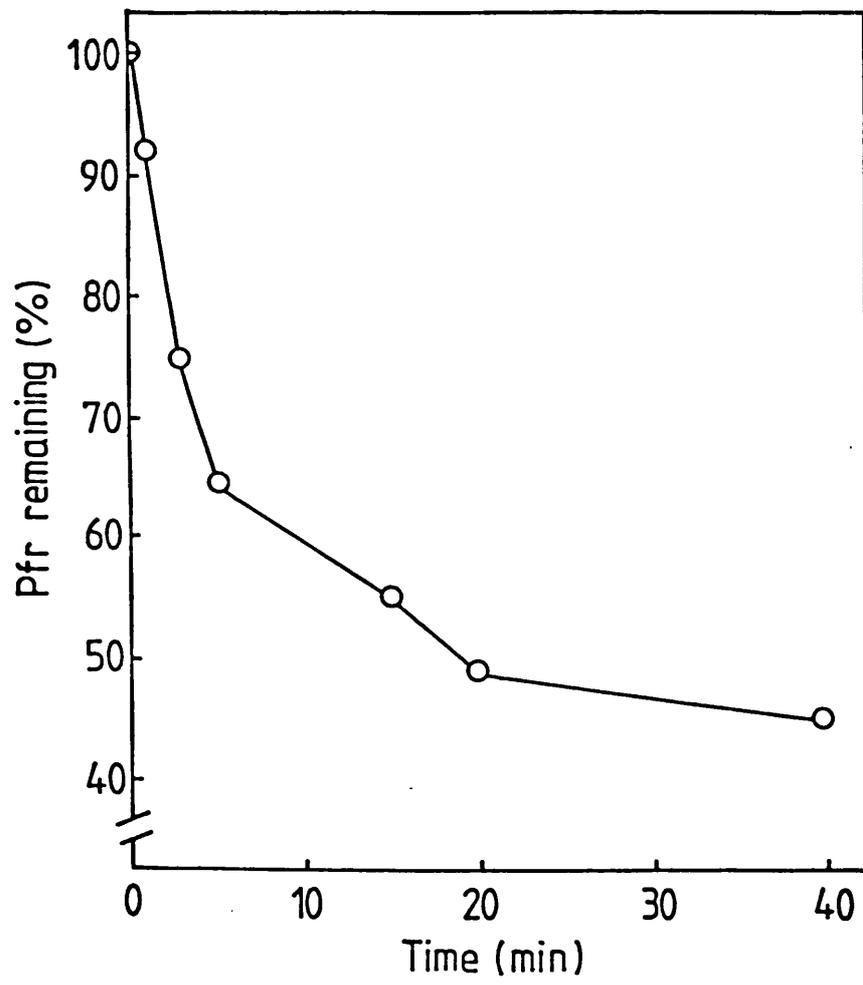


Figure 2.16 Dark reversion analysis of
purified (SAR = 0.85) pea phytochrome
at 3C



sulphydryl protease inhibitors such as p-Chloro-mercuriobenzoic acid (PCMB) and the competitive inhibitor Leupeptin in the buffer systems. Also, phytochrome extraction could be conducted as Pfr, the form of phytochrome known to be less susceptible to in vitro proteolysis (Vierstra et al 1984) and the form which is less effected by the triterpenoid saponin present in crude pea extracts. The problem of precipitation of Pfr by poly(ethylenimine) could be overcome by the inclusion of an alternative CaCl_2 precipitation step which also clarifies the extract. These suggested modifications are untested.

2.3.6 Summary

The Affi-Gel Blue and the fast protocol for the purification of oat phytochrome, routinely yield highly purified (SAR = 0.85-0.93), native, 124 kDa phytochrome. It was necessary to purify intact oat phytochrome, primarily so that it could be used as the immunogen for the generation of mAbs and, more importantly, so that it could be used in the subsequent ELISA screening assays. It was also necessary that the purified phytochrome was spectrally native as it was to be used to characterise whether mAbs exhibited differential binding to Pr or Pfr, and also whether the binding of mAbs altered the spectral properties of the phytochrome molecule.

The pea purification protocol also described here yields highly purified (SAR = 0.85) but partially degraded phytochrome. However, the

purification of pea phytochrome was not intended to be a major contribution to this thesis. It was purified merely as a tool to be used in early screening assays of the hybridomas. Of course, since the pea phytochrome was partially degraded there were some limitations on its use as a screening tool.

CHAPTER 3

PRODUCTION AND INITIAL CHARACTERISATION OF
MONOCLONAL ANTIBODIES TO 124 kDa OAT
PHYTOCHROME

3.1 Introduction

3.1.1 Theory of monoclonal antibodies

A monoclonal antibody (mAb) is the product of a single clone of B lymphocyte cells. However, B cells are terminally differentiated and therefore cannot be cultured indefinitely in vitro. In contrast, myelomas which are tumours consisting of a malignant form of B cell are immortal and can be successfully cultured in vitro. Köhler and Milstein (1975) exploited the potential of individual B cells to secrete mAbs and the immortal properties of myelomas, by constructing hybrid myelomas (hybridomas) which secreted antibodies to antigens of choice. In principle, a compatible myeloma cell line is fused to individual spleen lymphocytes, which have been stimulated to secrete useful antibodies and hence produce immortal hybridomas, which secrete defined mAbs. These hybridomas are thus a source of very specific antibodies, each antibody having precise binding properties and, unlike conventional polyclonal antibodies, they can be grown in continuous culture, or as ascitic tumours in animals, providing an unlimited supply of antibodies.

Following cell fusion, hybridomas continue to produce all of the immunoglobulin polypeptide chains synthesised by each of the parent cell lines. As the expression of the immunoglobulin genes are co-dominant and the pairing of the respective immunoglobulin chains is random, then a mixed species of immunoglobulin, carrying immunoglobulin chains from both parent cell lines may be secreted. This problem has been overcome

by the selection of myeloma lines which have lost the ability to produce their own immunoglobulin chains, or at least the heavy chains (for review see Westerwoudt 1985). In addition, drug marked myeloma cell lines are used, which provide a selective marker for those myeloma cells which do not fuse with B cells. The myeloma cell lines usually lack the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT) or thymidine kinase (TK), which are required in one of the salvage pathways of nucleotide biosynthesis.

At the time of fusion, cells are cultured in a selective medium containing hypoxanthine, aminopterin and thymidine (HAT medium) (Littlefield 1964). As the myeloma cell lines used lack HGPRT, these cells die because aminopterin a toxic analogue of folic acid, blocks de novo biosynthesis of purines and pyrimidines and they are not able to utilize the exogenous supply of hypoxanthine or thymidine via the salvage pathway. However, if mutant myeloma cells fuse with spleen cells, which are wildtype, the resulting hybridomas can utilize hypoxanthine and thymidine, and so grow in HAT medium. The unhybridised spleen cells are terminally differentiated and normally die within a few days.

3.1.2 Immunisation

Many different immunisation protocols have been described which relate to the suitability of a particular antigen to generate an immune response. In the case of phytochrome which is a water-soluble

protein it is necessary to use an adjuvant to invoke a good immune response. Adjuvants non-specifically promote immune responses by delaying the release of antigen after injection into the animal. The most commonly used adjuvant is Freund's complete adjuvant which consists of an emulsifying agent in mineral oil with killed mycobacteria. This is mixed with the antigen to produce an emulsion which is then used to immunise the animal. Alternative methods of immunisation include a single intrasplenic immunisation (Gearing et al 1985). Although only one injection is required, which is economical with the antigen, it tends to generate mAbs which belong to the IgM class. Until relatively recently anti-IgM enzyme labelled conjugates have not been commercially available, which has limited the use of IgM mAbs. In addition IgM antibodies which are pentamers and are therefore relatively large, can have difficulty in penetrating histochemical sections and therefore are not necessarily suitable for immunohistochemical analysis (Campbell 1986). In vitro immunisation also predominantly produces hybridomas which secrete IgM as only the primary immune response is stimulated (Reading 1982). This method is particularly suitable for the generation of mAbs to toxic products, which would kill the host animal if immunised in the standard way. Intraperitoneal implantation of paper disks which carry small peptides, which are normally not very antigenic, has also been used successfully to generate an immune response (Viamontes et al 1986). This method is an alternative to the production of carrier-hapten

conjugates, the traditional method of increasing the antigenicity of small peptides. A further single-step immunisation protocol has also been described. This involves the stimulation of the immune system due to the controlled release of antigen from a biodegradable antigen device, which degrades into L-tyrosine and its derivatives, which themselves have adjuvant properties (Kohn et al 1986). However, it has not been necessary to adopt any of the one-step immunisation protocols in the production of mAbs to phytochrome. This is because the antigen is not particularly scarce, or toxic. It is not necessary to derivatize the protein onto nitrocellulose or construct carrier-hapten conjugates as the phytochrome molecule is relatively large and is generally considered to be a good antigen (Pratt 1982). Likewise, it is not necessary to construct a complex delivery device c.f. Kohn et al (1986), as the adjuvant activity of Freund's complete adjuvant is sufficient to generate a strong immune response. Consequently, in the case of phytochrome it has been possible to adopt a simple approach and induce antibody activity by a series of injections of phytochrome emulsified in Freund's complete adjuvant (Cordonnier et al 1983; Nagatani et al 1983, 1984; Daniels and Quail 1984; Whitelam et al 1985; Thomas et al 1984b).

3.1.3 Fusion

Many fusion protocols have been reported which all adopt the same basic approach (for reviews see Galfré and Milstein 1981; Westerwoudt 1985; Goding 1983; Campbell 1986). All these protocols incorporate the

use of polyethylene glycol (PEG) as the fusing agent first described by Galfré et al (1977). The concentration of PEG used varies from 25% (Hoffman et al 1980) to 50% (Galfré and Milstein 1981). Few hybrids are formed when the PEG concentration is below 30% but the cells can tolerate this concentration for longer periods. In general fusion protocols use a PEG concentration of 40-50% as this represents the most favourable balance between fusion efficiency and toxicity. Hybridisation frequency is also highly dependent on the pH of the PEG solution, with the greatest number of clones being formed when the pH is at 8.0-8.2 (Sharon et al 1980). Little is known about how PEG brings about the fusion of the cells. Membrane fusion occurs in two distinct phases; cell agglutination where the cell membrane of adjacent cells are brought together, and the formation of cytoplasmic bridges between cells. This is followed by osmotic swelling and heterokaryon formation. Polyethylene glycol decreases the surface potential of membranes and causes charge neutralisation of the cell membrane. In addition, PEG has a slight negative charge in solution and is hydrophilic so that water binds to it, consequently dehydration appears to play a role in PEG mediated fusion (Knutton and Pasternak 1979).

An alternative method for fusing cells involves electro-fusion and has been used in the production of human hybridomas (Bischoff et al 1982). In this case the fusion of cells is observed under the microscope

and the resulting hybridoma cells are removed using a micromanipulator. It is therefore not necessary to use drug marked myeloma cells. However, the general applicability of this method has yet to be tested.

3.1.4 Cloning

Once the hybridomas have become established it is necessary to clone the cell lines to ensure that they are monoclonal. Cells may be cloned by growth in soft agar (Coffino et al 1972). This method is relatively popular and has been used to clone mAbs to oat phytochrome (Thomas et al 1984b). However, this method carries the disadvantage that cells have to be recultured in liquid medium before biological activity may be assessed (Goding 1983) but, as specific clones can be removed from the agar, it probably yields clonal lines at the first round of cloning. An alternative cloning protocol using a semi-solid support involves the simultaneous growth and cloning of cell lines in methylcellulose which is impregnated with HAT medium (Davis et al 1982). As single colonies can be picked from the methylcellulose the clonality of the cell lines is assured. This protocol was initially adopted by Cordonnier et al (1983) but subsequent fusions and cloning by these workers were conducted in liquid medium (Cordonnier et al 1986a). The reason for this change in approach is not known. Further cloning protocols involve cloning the cell lines in liquid medium. Cell lines may be cloned using a fluorescence-activated cell sorter (FACS) (Parks et al 1979). Using this rather specialised

procedure healthy cells are detected by their unique light scattering characteristics, and the cell containing droplet is electrostatically deflected into the appropriate culture well. A further cloning technique is that of cloning by limiting dilution. Cells are plated out at very low densities so that the probability of achieving clonal lines is high. In general this is the preferred method of cloning cell lines and has been used in the majority of cases to clone cell lines which secrete antibodies to phytochrome (Nagatani et al 1983, 1984; Cordonnier et al 1985; Silberman et al 1985).

3.1.5 Why more monoclonal antibodies to phytochrome?

Although several panels of mAbs to oat, pea and rye phytochrome have been produced (Cordonnier et al 1983, 1985; Nagatani et al 1983, 1984; Thomas et al 1984b; Daniels and Quail 1984; Silberman et al 1985; Whitelam et al 1985), there is still considerable scope for the production of new panels of mAbs to phytochrome. In general, the production of previous panels of mAbs has resulted in the generation of novel mAbs, which have provided additional information concerning the properties of the phytochrome molecule. This suggests that there is still great potential for the use of mAbs as specific probes for the further characterisation of the phytochrome molecule. For instance, the 6 kDa NH₂-terminus of the molecule has been fairly well characterised but the contribution of the adjacent 4 kDa domain in the maintenance of the spectral integrity of the molecule is unknown.

In addition there are still large regions on the rest of the molecule that have to be characterised. Monoclonal antibodies which discriminate between Pr and Pfr have been isolated, but as yet form specific mAbs have not been produced. Only one mAb which cross reacts with phytochrome from a diverse array of plant species has been characterised. Although this is the best candidate for a mAb recognising a domain of biological significance, since it is so widely conserved, the general use of this mAb in characterisation of the molecule and more specifically in the purification of phytochrome from a range of plant species is rather limited as this mAb does not react well with native phytochrome (Cordonnier et al 1986a).

In this chapter the production and initial characterisation of a panel of mAbs to 124 kDa oat phytochrome are described. The production of polyclonal antibodies to 124 kDa oat phytochrome are also detailed.

3.2 Materials and Methods

3.2.1 Production of polyclonal antibodies

Polyclonal antibodies were raised in a 1.5 kg New Zealand White rabbit by intramuscular injection at several sites of 1 mg 124 kDa oat phytochrome (SAR = 0.91) emulsified with 1 ml of Freund's complete adjuvant. Two booster injections of 0.5 mg 124 kDa oat phytochrome in Freund's complete adjuvant were given at two week intervals. Test bleeds were taken at the time of boosting to monitor the anti-phytochrome antibody titre. Two weeks after the final boost the rabbit was bled out. Blood was collected in polycarbonate tubes, incubated at 37C for 1 h and then incubated at 4C overnight. Serum was recovered by centrifugation at 1,000 x g for 10 min. Rabbit polyclonal antibodies were partially purified by differential $(\text{NH}_4)_2\text{SO}_4$ precipitation according to Goding (1983). Saturated $(\text{NH}_4)_2\text{SO}_4$ was added to 50%, and the mixture stirred for 15 min and then centrifuged at 10,000 x g for 15 min. The resultant pellet was washed three times with 40% saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was resuspended into phosphate buffered saline (PBS) pH 7.4 and thoroughly dialysed against several changes of PBS.

3.2.2 Production of immobilised phytochrome column and purification of polyclonal antisera

Polyclonal antibodies were purified by immunoadsorption to immobilised phytochrome. Highly purified (SAR = 0.85) 124 kDa oat phytochrome was coupled to CNBr-activated agarose (Sigma).

The phytochrome containing solution was made 0.5 M NaCl and the pH was adjusted to pH 8.3 with 1N NaOH. The CNBr-agarose was washed extensively (200 ml g⁻¹) with 1 mM HCl and immediately added to the phytochrome solution at a ratio of 120 mg dry weight agarose per mg of protein. The agarose and phytochrome were mixed overnight at 4C. The agarose beads were collected by centrifugation at 1500 x g for 5 min. Remaining reactive sites on the agarose were blocked by the addition of 4 ml 0.1 M monoethanolamine (pH 9) for every ml of swollen agarose. The agarose was mixed overnight at 4C, transferred to the column support and washed with 10 column volumes of 25 mM MOPS-Tris, pH 7.8 containing 5 mM EDTA, to remove the monoethanolamine and equilibrate the column. Twenty ml of antisera was mixed for 1 h with the immobilised phytochrome. The agarose was allowed to settle and the column was washed with 10 mM MOPS-Tris, pH 7.8, containing 1 M NaCl and 5 mM EDTA. Washing continued until the $A_{280}^{1\text{cm}}$ of the eluate was below 0.03. The column was then equilibrated with 25 mM MOPS-Tris, pH 7.8, containing 5 mM EDTA. The outlet of the column was attached to the inlet of a Sephadex G15 column, also equilibrated in 25 mM MOPS-Tris, pH 7.8, containing 5 mM EDTA. One column volume of 1 N HCOOH was layered on top of the agarose column and percolated into the agarose, immediately followed by 25 mM MOPS-Tris, pH 7.8, containing 5 mM EDTA. Immunoglobulin containing fractions from the G15 column were detected by A_{280} nm and pooled.

The immunoglobulin concentration was determined by assuming $E_{280\text{ nm}}^{1\%, 1\text{cm}} = 13.6$ (Pratt 1984).

3.2.3 Monoclonal antibody production

3.2.3.1 Immunisation

Six week old BALB/C mice were injected intraperitoneally with 200 μg of 124 kDa oat phytochrome (SAR = 0.91), emulsified in Freund's complete adjuvant. Three weeks later they were injected intraperitoneally with a further 100 μg of phytochrome in Freund's complete adjuvant. Finally, two weeks later the selected mouse was injected intravenously with 50 μg of phytochrome in PBS. Test bleeds were taken when the mice were boosted to determine the anti-phytochrome antibody titre.

3.2.3.2 Cell culture

Established hybridoma cell lines were grown in RPMI-1640 (Gibco), containing 10% (v/v) heat inactivated foetal calf serum (HIFCS) (Gibco) in a humidified chamber at 37C and in air supplemented to 10% CO_2 . The P3-NSI-Ag4 (NSI) myeloma cell line, generously provided by Dr. T. Harrison, Biochemistry Dept., Leicester University, Leicester, U.K., was cultured in RPMI-1640, containing 10% (v/v) HIFCS and 8-azaguanine ($15\ \mu\text{g}\ \text{ml}^{-1}$). All media contained 100 units ml^{-1} penicillin and $100\ \mu\text{g}\ \text{ml}^{-1}$ streptomycin. Feeder cells (macrophages) were obtained from 180 g Wistar rats by peritoneal lavage with 20 ml RPMI-1640 containing 10% (v/v) heat inactivated newborn calf serum.

3.2.3.3 Fusion

The fusion was performed three days after the final boost as described by Billett et al (1984).

Cells from one spleen ($\sim 10^8$) were washed with RPMI-1640 and then mixed with 10^7 NSI cells that had been harvested during mid-log growth. The cells were pelleted by centrifugation at 200 x g for 5 min. Fusion was achieved by the addition of poly(ethylene glycol) (PEG) 1500 (BDH) adjusted to approx. pH 7.8 with NaOH, in RPMI-1640 containing 5% (v/v) Dimethyl sulphoxide (DMSO) at 37C. The PEG was added slowly, incubated for 1 min, and then diluted out slowly by the addition of warm RPMI. Cells were collected by centrifugation for 5 min at 200 x g and resuspended directly into RPMI-1640 containing 15% (v/v) HIFCS, 0.4 μ M aminopterin, 0.1 mM hypoxanthine and 16 μ M thymidine (HAT medium) (Littlefield 1964). Cells were distributed onto four 96-well culture plates (NUNC), which had previously been coated with feeder cells. Cells were fed with HAT medium for 7 days, at which stage selection was terminated. Hybridomas were then cultured in RPMI-1640 containing 10% (v/v) HIFCS, 0.1 mM hypoxanthine and 16 μ M thymidine (HT medium).

3.2.3.4 Cloning

Cell lines were cloned twice by limiting dilution. Hybridomas were plated out onto feeder cells at a concentration of 10, 3 and 0.5 cell per well, onto 96 well culture plates. Wells containing only one centre of growth were selected at each round of cloning.

Cell lines were screened at each stage for the secretion of anti-phytochrome antibodies in solid-phase ELISA and on mini-western blots.

3.2.3.5 Storage

Aliquots of hybridoma cells ($\sim 10^7$ per vial) were resuspended into 1 ml of an ice-cold mixture of 90% (v/v) HIFCS and 10% DMSO. Vials were frozen to -80°C and then transferred to liquid nitrogen.

3.2.4 Screening

3.2.4.1 ELISA 3.1

Hybridomas were routinely screened for antibody production using a solid-phase enzyme linked immunosorbent assay (ELISA). For the assay 96 well poly vinyl plates (Dynatech) were precoated with 50 μl per well of 2 $\mu\text{g ml}^{-1}$ pure (SAR = 0.93) 124 kDa oat phytochrome in 50 mM bicarbonate buffer, pH 9.2, overnight at 4°C . The plates were then washed extensively with PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20). Remaining protein binding sites were blocked by filling the wells with PBS-Tween containing 1% (w/v) bovine serum albumin (BSA) and incubating for 1 h at room temperature. After washing, 50 μl of hybridoma medium was added to each well and the plates incubated at room temperature for 2 h. Following a further wash, 50 μl of peroxidase-conjugated sheep antibodies against mouse IgG (Sigma), diluted in PBS-Tween containing 0.1% (w/v) BSA was added to each well and incubated at room temperature for 1 h. After washing, bound

peroxidase activity was visualized with $1 \mu\text{g ml}^{-1}$ 3, 3', 5, 5', tetramethyl benzidine in 50 mM citrate acetate buffer, pH 6.0. Reactions were stopped by the addition of $50 \mu\text{l}$ $2.5 \text{ M H}_2\text{SO}_4$.

3.2.4.2 ELISA 3.2

A second ELISA assay configuration was used to characterise mAbs which were raised to conformationally determined epitopes. ELISA assay plates (96-well) (Dynatech) were coated with $5 \mu\text{g ml}^{-1}$ goat anti-mouse IgG (Sigma) in 50 mM bicarbonate buffer, pH 9.2, and incubated overnight at 4C. The plates were then washed extensively with PBS-Tween. Remaining protein-binding sites were blocked by filling the wells with PBS-Tween containing 1% (w/v) BSA and incubated for 1 h at room temperature. After washing, $50 \mu\text{l}$ of the respective mAbs were added at $5 \mu\text{g ml}^{-1}$ in PBS-Tween and incubated for 2 h at room temperature. Following a further wash an appropriate dilution of 124 kDa oat phytochrome was added to the wells, and incubated for 2 h at room temperature. Following a further wash, immunoaffinity-purified-rabbit polyclonal antibodies to 124 kDa oat phytochrome, at a concentration of $5 \mu\text{g ml}^{-1}$ in PBS-Tween, was added to the wells and incubated for 1 h at room temperature. After washing, a commercial peroxidase conjugate of goat anti-rabbit antibodies (Sigma) was added at a dilution of 1/500 in PBS-Tween, and incubated for 30 min at room temperature. Bound peroxidase activity was detected as described above.

3.2.4.3 ELISA 3.3

The relative affinities of the mAbs was determined using a modification of ELISA 3.1. Following coating of the ELISA plate with $2 \mu\text{g ml}^{-1}$ 124 kDa oat phytochrome and blocking as described for ELISA 3.1, the appropriate dilution of the respective mAbs were added to the assay wells and incubated for 2 h at room temperature. Following washing, 50 μl of peroxidase conjugated sheep antibodies against mouse IgG (Sigma) diluted in PBS-Tween containing 0.1% (w/v) BSA ^{were added} and incubated at room temperature for 45 min. After washing bound peroxidase activity was detected as described above.

3.2.4.4 "Mini-western blotting"

A partially pure (SAR = 0.15) mixture of 124, 118 and 114 kDa phytochrome was mixed with an equal volume of 125 mM Tris, pH 6.8, 40% (v/v) glycerol, 1.4 M 2-mercaptoethanol, 0.02% (w/v) bromophenol blue, 4% (w/v) SDS (double strength SDS sample buffer) and incubated at 96C for 2 min. Discontinuous SDS-PAGE was carried out according to Laemmli (1970) using 7% polyacrylamide resolving gels and 3% stacking gels. Following electrophoresis, proteins (100 ng phytochrome protein per lane) were transferred electrophoretically onto nitrocellulose at 320 mA for 1 h in a Bio-Rad Transblot Cell in 25 mM Tris, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol. Remaining protein-binding sites on the nitrocellulose were blocked by incubating the nitrocellulose for 3 h at room temperature in 100 mM Tris-HCl, pH 9, containing 150 mM NaCl, 0.05% (v/v) Tween 20 and 3% (w/v) Marvel

(skimmed milk powder, Cadbury). The nitrocellulose was then cut into 2 mm x 2.4 cm strips which covered the area of the gel containing proteins of 150-100 kDa. Each strip was incubated overnight at 4C with the supernatant from a different cell line. The strips were then washed in several changes of 50 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 0.1% (v/v) Tween 20 (TBS-Tween). The strips were then incubated for 1 h at room temperature with a commercial biotin-conjugate of goat anti-mouse antibodies (Sigma) at a dilution of 1/1000 in TBS-Tween containing 1% (w/v) Marvel. Following a further wash, strips were incubated for 20 min at room temperature with a 1/500 dilution of avidin-peroxidase (Sigma) in TBS-Tween containing 1% BSA. Following a final wash bound peroxidase was visualized with 0.6 mg ml^{-1} 4-chloronaphthol in PBS containing 0.02% (v/v) hydrogen peroxide.

3.2.5 Subclass determination

The IgG subclass of the mAbs was determined using a modification of ELISA 3.1. ELISA plates were coated with a 1/1000 dilution of goat anti-IgG₁, -IgG_{2a}, -IgG_{2b}, -IgG₃ (Sigma subclass kit). Following blocking, 50 μ l culture supernatant from each cell line was added to each of the anti-mouse subclass wells and incubated for 2 h at room temperature. Following washing, a 1/1000 dilution of sheep anti-mouse peroxidase conjugate (Sigma) in PBS-Tween containing 0.1% (w/v) BSA was added and incubated for 1 h at room temperature. Bound peroxidase was visualized as described above.

3.2.6 Binding of Protein A to mAbs

In brief ELISA plates were coated with each mAb at a concentration of $10 \mu\text{g ml}^{-1}$, and incubated at 4°C overnight. Following a wash with PBS-Tween and blocking, a peroxidase-conjugate of Protein A (Sigma) at a concentration of $10 \mu\text{g ml}^{-1}$ in PBS-Tween pH 7.5, 8.0, 8.5 or 9.0 was added. Following incubation of the Protein A for 1 h at room temperature, bound peroxidase was detected with TMB.

3.2.7 Ascites production

Ascitic tumours in BALB/C mice were induced by priming mice by intraperitoneal injection of 0.5 ml pristane (2, 6, 10, 14-tetrapentadecane; Sigma). Seven days later 10^6 - 10^7 hybridoma cells were injected intraperitoneally. Ascitic tumours developed in 10-20 days. Ascitic fluid was harvested and centrifuged at $200 \times g$ for 15 min to remove cell debris. The ascites was partially purified by differential $(\text{NH}_4)_2\text{SO}_4$ precipitation (see above).

3.2.8 Purification of Immunoglobulins from ascites

3.2.8.1 Purification of immunoglobulins on immobilised anti-mouse antibodies

Mouse immunoglobulins were purified on a column of immobilised anti-mouse antibodies (Sigma) using a modification of the procedure of Cordonnier *et al* (1983). Ammonium sulphate purified ascites was mixed with immobilised anti-mouse antibodies for 1 h at 4°C. The agarose was allowed to settle and washed with several column volumes of PBS until the $A_{280 \text{ nm}}$ fell below 0.03. One column volume of 0.1 M glycine-HCl, pH 2.5,

was layered onto the column and followed immediately with PBS. Fractions (1 ml) were collected into test tubes which already contained 0.2 ml 0.5 M Tris-HCl, pH 8.3, to neutralise the effect of the acidic glycine. Fractions with significant $A_{280 \text{ nm}}$ were pooled and precipitated with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at $30,000 \times g$ for 15 min. The resultant pellet was resuspended into PBS and thoroughly dialysed. Mouse IgG concentration was determined by solid phase ELISA using IgG₁ kappa MOPC 21 (Sigma) as standard.

3.2.8.2 Purification of immunoglobulins by Protein A chromatography

Ammonium sulphate purified ascitic fluid was applied to immobilised Protein A (Sigma) and mixed for 1 h at 4°C. The column of Protein A was then washed with 10 column volumes of 100 mM sodium phosphate, pH 8.5, until the $A_{280 \text{ nm}}$ fell below 0.03. Mouse antibodies were eluted with 100 mM sodium phosphate, pH 5. Fractions with significant $A_{280 \text{ nm}}$ were rapidly pooled and precipitated with $(\text{NH}_4)_2\text{SO}_4$ as described above.

3.2.8.3 Purification of immunoglobulins by cation exchange chromatography

Cation exchange was conducted on a zeta-prep cation exchange disc (LKB). Mouse antibodies were purified using a modification of the protocol outlined by Boonekamp and Pomp (1986). The ascitic fluid was diluted 20 fold in 50 mM sodium acetate pH 5.5 and applied to the disc at a flow rate of

5 ml min⁻¹. After application the disc was washed with 20 times the sample volume of 50 mM sodium acetate, pH 5.5 by which time the A_{280 nm} had dropped to 0. Mouse antibodies were eluted with 50 mM sodium acetate, pH 5.5 containing 1 M NaCl. Mouse antibodies were concentrated with (NH₄)₂SO₄ as described above.

3.3 Results

3.3.1 Production and purification of polyclonal antibodies

Antibody titre of the immunized rabbit was periodically determined by solid phase ELISA 3.1 (Fig 3.1). After 6 weeks when the antibody titre was greater than 1/100,000, the rabbit was sacrificed to recover the polyclonal antisera. Polyclonal antibodies were purified by immunoadsorption to a column of immobilised 124 kDa oat phytochrome. Antibodies were specifically eluted with 1 M HCOOH (Fig 3.2) which breaks the bonds between immunoglobulins and phytochrome. This protocol yielded 25 mg of purified polyclonal antibodies from 20 ml of polyclonal serum.

3.3.2 Production of monoclonal antibodies

Three BALB/C mice were immunized with 124 kDa oat phytochrome. Following the first booster injection the titre of anti-phytochrome antibody activity was determined by solid-phase ELISA (Fig 3.3). As mouse 3 exhibited the highest antibody titre this mouse was chosen to be used in the fusion.

3.3.2.1 Fusion products

Ten days after fusion 90% of the microtitre wells contained hybridomas. The supernatants from these wells were screened against 124 kDa pure (SAR = 0.91) oat phytochrome in a solid phase ELISA 3.1. All the wells tested contained anti-phytochrome activity. The supernatants from the wells were also screened against partially degraded pea phytochrome (SAR = 0.85).

Figure 3.1 Anti-oat phytochrome polyclonal antibody titre determined by ELISA a) two weeks after the primary immunisation b) two weeks after the secondary immunisation c) two weeks after the tertiary immunisation

Rabbit anti-phytochrome polyclonal antibodies were added to immobilised 124 kDa phytochrome and the bound polyclonal antibody detected with a commercial peroxidase-conjugate of anti-rabbit antibodies.

Each reading is the average of three replicate assays from a single experiment.

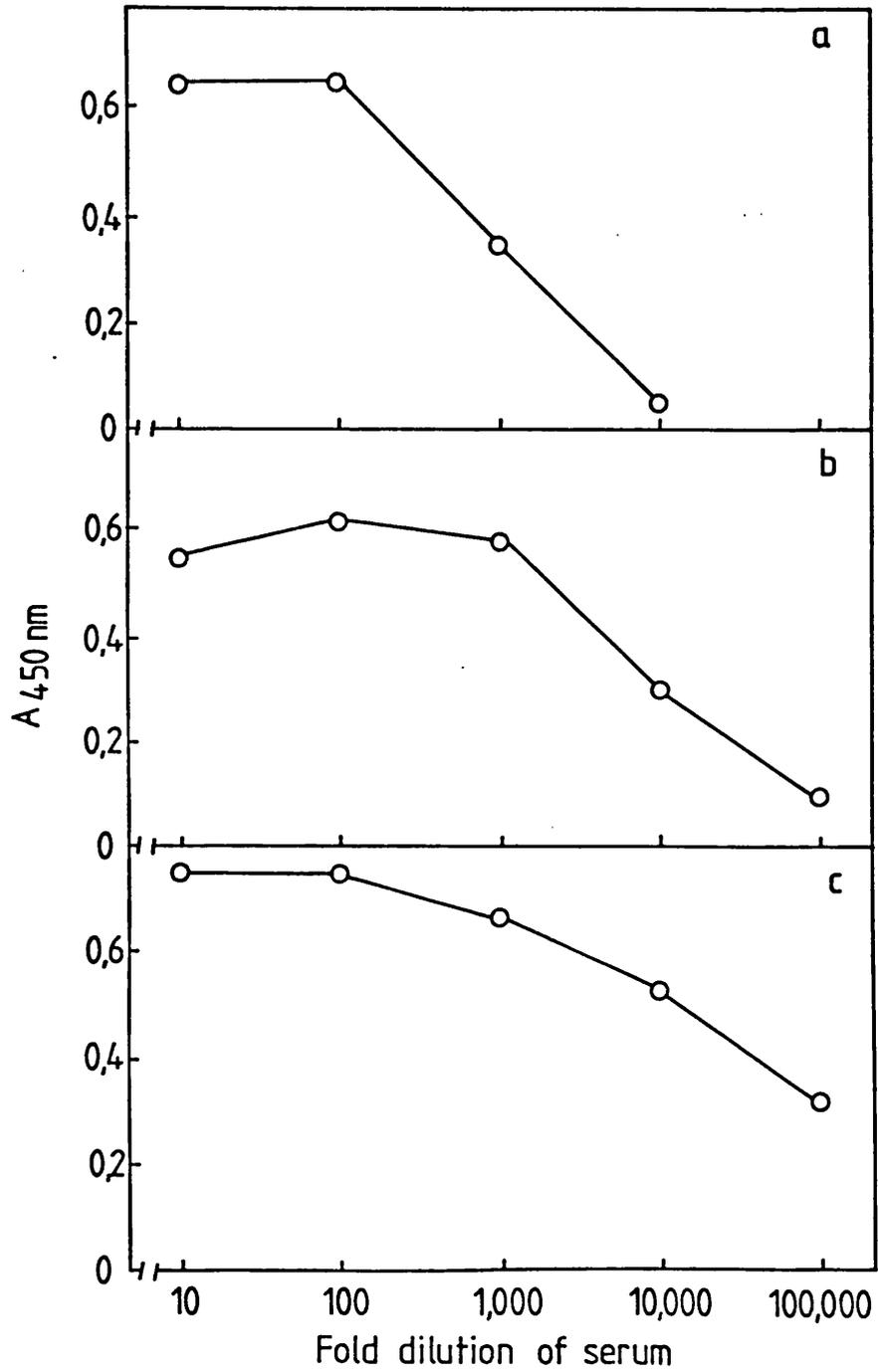


Figure 3.2 Immunoaffinity-purification of polyclonal anti-oat phytochrome antibodies

Polyclonal antiserum, after $(\text{NH}_4)_2\text{SO}_4$ fractionation, was applied to a column of immobilised 124 kDa oat phytochrome. The column was washed with 10 mM MOPS-Tris, pH 7.8 containing 1M NaCl and 5 mM EDTA until the $A_{280 \text{ nm}}$ value fell below 0.03. Anti-phytochrome polyclonal antibodies were eluted with 1 N HCOOH.

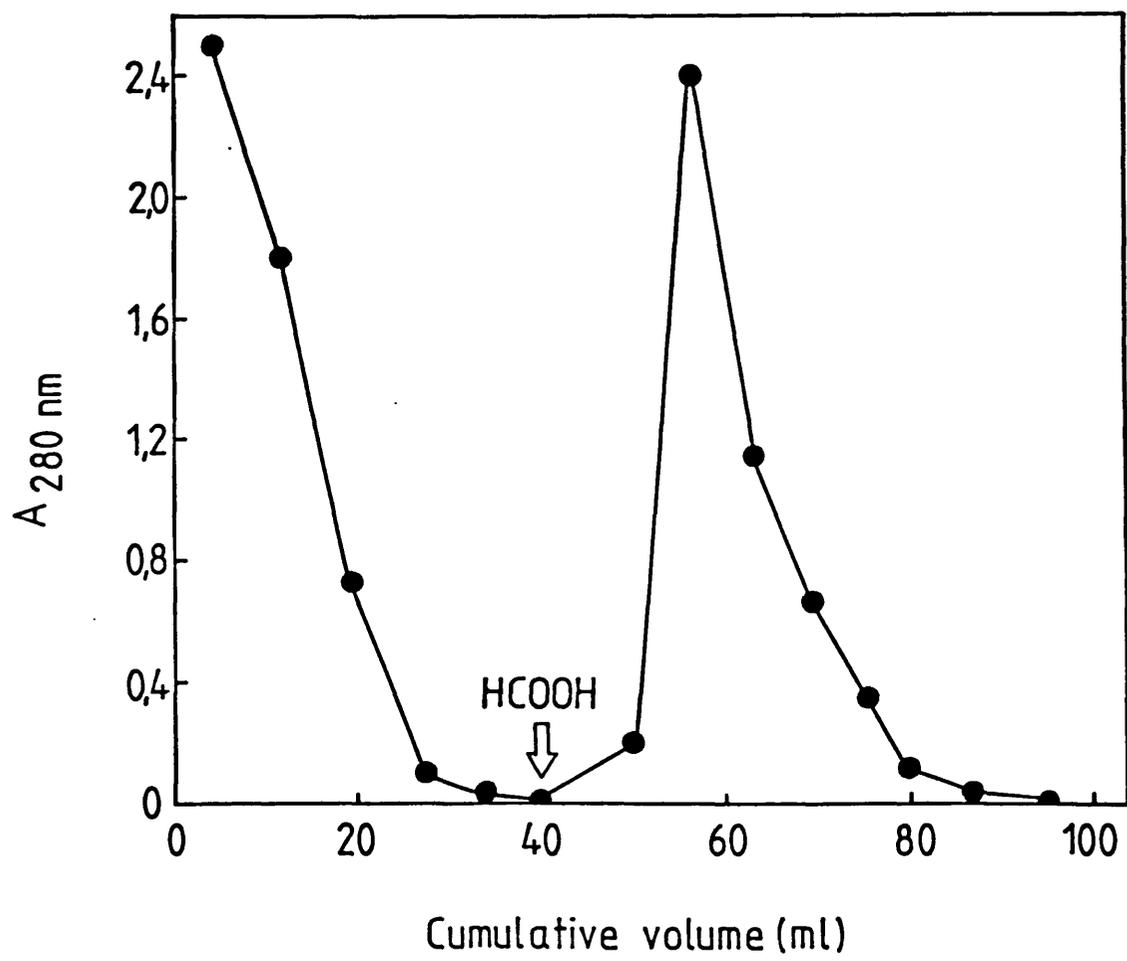


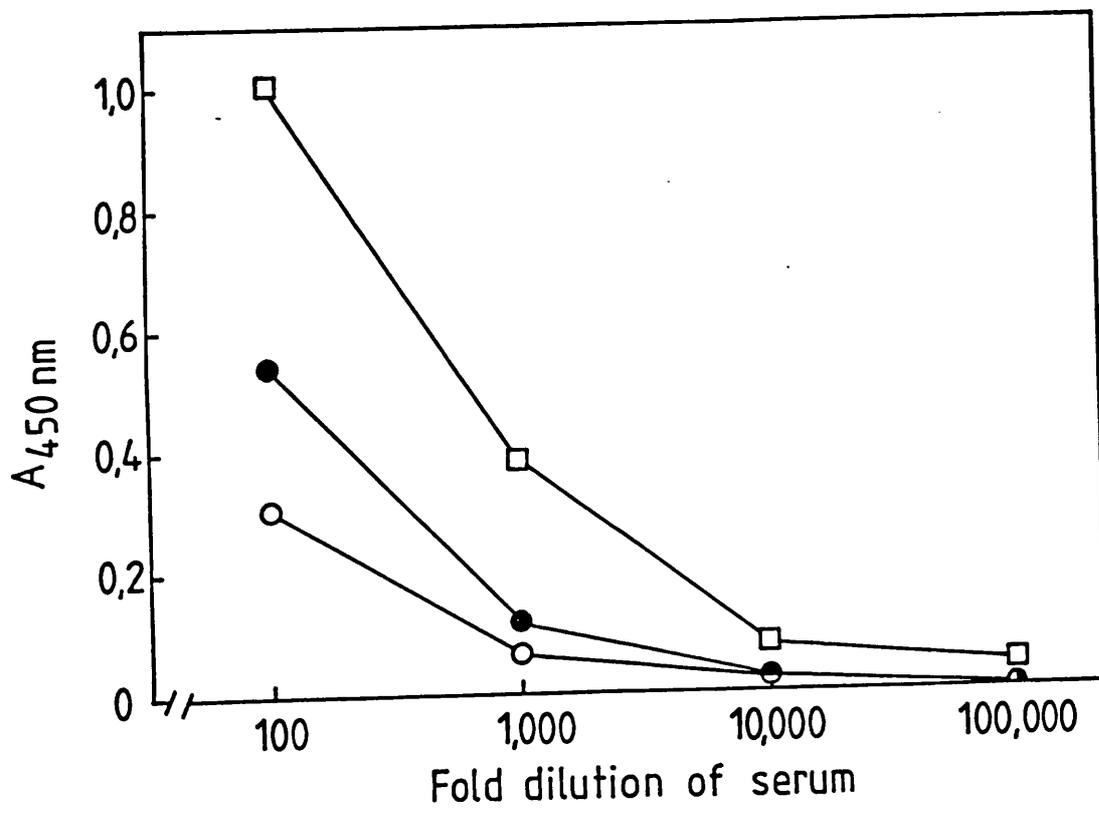
Figure 3.3 ELISA for anti-phytochrome
antibody titre of the three mice immunised
with 124 kDa oat phytochrome

Dilutions of mouse sera were added to immobilised 124 kDa oat phytochrome and bound antibody detected by the addition of peroxidase-conjugated sheep anti-mouse antibodies.

(○) = mouse 1, (●) = mouse 2,

(□) = mouse 3.

Each reading is the average of three replicate assays from a single experiment.



Only 10% of the wells positively reacted with pea phytochrome. Amongst the cell lines that positively recognised oat and pea phytochrome there was a range in the reactivity observed. Unused hybridoma medium and supernatant from the NS1 cells gave very low backgrounds i.e. ELISA $A_{450 \text{ nm}} = 0.02$. These control levels were considered to be indicative of negative cell lines. Positive reactivity was arbitrarily classified by ELISA $A_{450 \text{ nm}}$ values above 0.1. The majority of the ELISA readings were of 0.8 or higher.

On the basis of the first round of screening against oat and pea phytochrome 100 cell lines were chosen to be grown on. A repeat of the initial screen against oat and pea phytochrome showed no loss of antibody secreting activity from the selected lines. Significantly, 3B₁₀ i.e. cell line(s) on plate 3 at position B,10, gave the same ELISA reading for oat and pea phytochrome (Table 3.1).

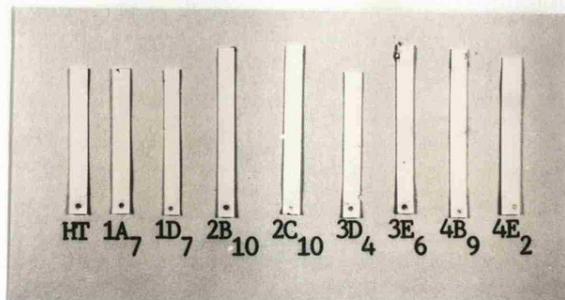
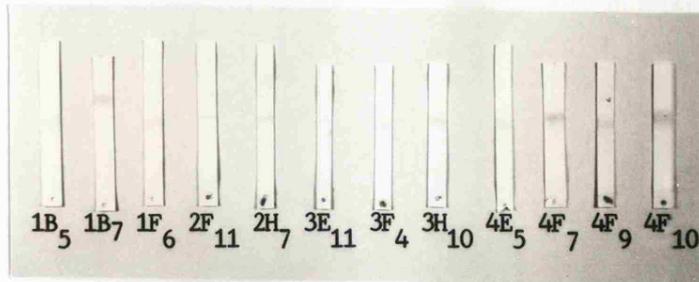
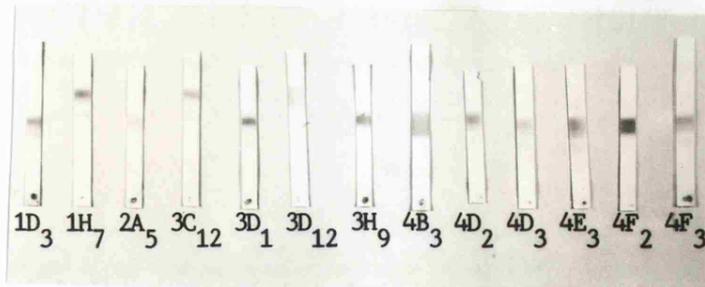
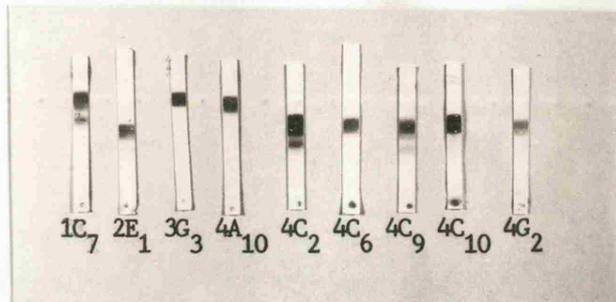
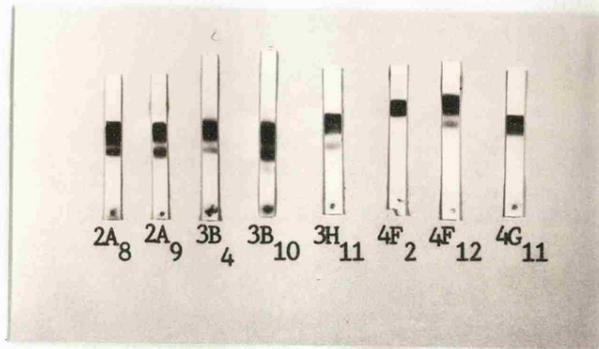
The second round of screening tested for the ability of the secreted antibodies from the respective cell lines to recognise partially purified (SAR = 0.15) 124, 118, and 114 kDa oat phytochrome which had been denatured in SDS, separated on SDS-PAGE and electroblotted onto nitrocellulose. The supernatants tested fell into five categories ranging from very strong recognition, through to an inability to immunostain SDS-denatured phytochrome (Fig 3.4). Only a representative number of the cell lines which failed to recognise SDS-denatured phytochrome are shown. Amongst the cell lines which did immunostain

Table 3.1 Summary of early screening data for hybridomas that secrete antibodies against oat phytochrome

Cell line	ELISA value		Peptides recognised on mini western blot (kDa)
	oat	pea	
1A ₆	1.21	-	-
1A ₇	1.09	-	-
1C ₇	0.68	-	124, 118, 114
1D ₂	1.18	-	-
1D ₃	1.10	0.36	124, 118?
1D ₄	1.04	0.34	-
1D ₉	1.12	-	-
1E ₂	1.10	-	-
1E ₁₁	1.05	-	-
2A ₈	1.14	-	124, 118, 114
2A ₉	1.07	0.80	124, 118, 114
2C ₁₀	0.74	-	-
2G ₈	0.76	-	-
2H ₇	0.72	-	124
3B ₄	1.11	-	124, 118?
3B ₁₀	1.13	1.13	124, 118, 114
3G ₃	1.09	-	124, 118?
3G ₉	0.96	0.42	-
3H ₈	1.07	-	-
3H ₁₁	1.18	-	124, 118?
4A ₁₀	0.92	-	124, 118?
4B ₃	1.01	-	124, 118?
4C ₂	1.06	-	124, 118, 114
4C ₆	0.95	-	124, 118?
4C ₉	1.10	-	124, 118?
4C ₁₀	0.98	-	124, 118?
4C ₁₁	0.88	-	-
4D ₃	0.90	-	124, 118?
4D ₁₁	1.02	-	-
4E ₃	1.02	-	124, 118?
4E ₄	0.92	-	-
4F ₂	1.17	-	124, 118?
4F ₇	0.88	-	124
4F ₉	0.94	-	124
4G ₁₁	0.94	-	124, 118?

Figure 3.4 Screen of cell culture
supernatants for the recognition of SDS-
denatured oat phytochrome

50 ng 124 kDa and 50 ng 118 + 114 kDa
oat phytochrome (per lane) were resolved
on 7.0% SDS-gels and electroblotted onto
nitrocellulose. The nitrocellulose was cut
into strips corresponding to proteins of
100-150 kDa. Individual strips were
incubated with the respective culture
supernatants from the cell lines. Bound
mouse antibody was detected by the addition
of a commercial peroxidase-conjugate of
anti-mouse antibodies.



electro-blotted phytochrome there is a range of staining patterns e.g. 3B₁₀ immunostains 3 bands whilst 4A₁₀ at most stains 2 bands (Table 3.1).

On the basis of these early findings 35 lines were selected for further characterisation (Table 3.1). Cell lines were selected on their ability to:

- (1) recognise SDS-denatured oat phytochrome;
- (2) cross-react with pea phytochrome in ELISA;
- (3) the intensity of reactivity with oat phytochrome in ELISA;
- (4) the healthiness of the cell line.

These cell lines were expanded by growth in flasks and routinely screened to monitor antibody secretion in solid phase ELISA 3.1. Eighteen of these cell lines were cloned immediately by limiting dilution. The remaining cell lines were frozen down and stored under liquid nitrogen. The clonal nature of the cell line was ensured by selection of wells with only one centre of growth. These wells were screened for positive secretion of anti-phytochrome antibodies in ELISA and on mini-western blots. Following the first round of cloning 14% of the cell lines no longer secreted anti-phytochrome antibodies. The remaining cell lines were all positive in ELISA, but 46% (2A₈, 4E₃, 4C₆, 4C₂, 4C₁₀, 4F₂ and 4F₃) had lost the ability to immunostain SDS-denatured blotted phytochrome (Fig 3.5, Table 3.2). Following the second round of cloning 74% of the cell lines were still actively secreting anti-phytochrome antibodies. None of these cell lines had lost the ability to immunostain

Figure 3.5 Screen of cell culture
supernatants for the recognition of SDS-
denatured phytochrome following the first
round of cloning

50 ng 124 kDa and 50 ng 118 + 114 kDa
oat phytochrome (per lane) were resolved
on 7.0% SDS- gels and electroblotted onto
nitrocellulose. The nitrocellulose was cut
into strips corresponding to proteins of
100-150 kDa. Individual strips were
incubated with the respective culture
supernatants from the cell lines. Bound
mouse antibody was detected by the addition
of a commercial peroxidase-conjugate of
anti-mouse antibodies.

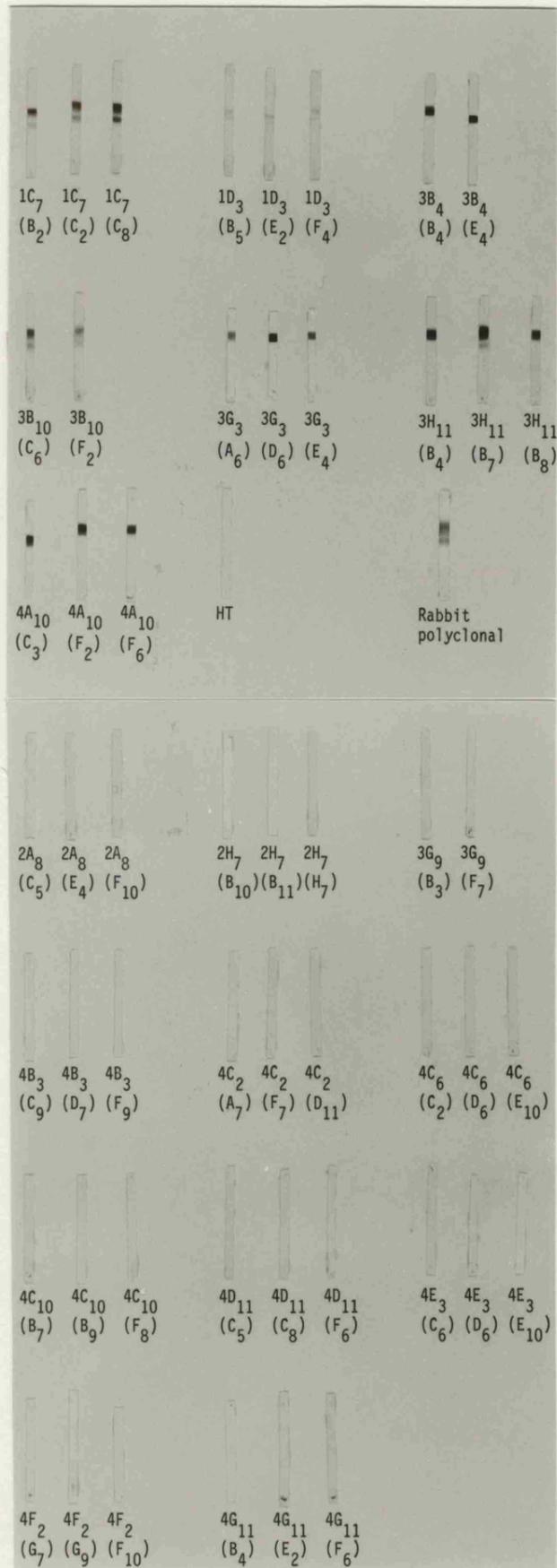


Table 3.2 Summary of recognition of phytochrome
following first round of cloning

Cell line	ELISA value	Recognition of phytochrome on western blot
1C7-B2	1.77	+
1C7-C2	1.68	+
1C7-C8	1.82	+
1D3-E2	1.45	+
1D3-B5	1.50	+
1D3-F4	1.35	+
2A8-F10	0.97	-*
2A8-C5	0.94	-*
2A8-E4	0.79	-*
2H7-H7	0.03	-*
2H7-B10	0.03	-*
2H7-B11	0.05	-*
3B4-E4	1.74	+
3B4-B4	1.71	+
3B10-F2	1.77	+
3B10-C6	1.64	+
3G3-A6	1.68	+
3G3-D6	1.77	+
3G3-E4	1.70	+
3G9-B3	0.28	-
3G9-F7	0.17	-
3H11-B4	1.77	+
3H11-B7	1.70	+
3H11-B8	1.70	+
4A10-F2	1.75	+
4A10-F6	1.96	+
4A10-C3	1.90	+
4B3-D7	0.34	-*
4B3-C9	0.39	-*
4B3-F9	0.14	-*
4C2-A1	0.13	-*
4C2-F7	0.11	-*
4C2-D11	0.16	-*
4C6-E2	0.23	-*
4C6-C2	0.20	-*
4C6-F2	0.17	-*
4C10-B7	0.13	-*
4C10-F8	0.16	-*
4C10-B9	0.10	-*
4D11-C5	0.09	-
4D11-F6	0.08	-
4D11-C8	0.04	-
4E3-D6	0.77	-*
4E3-C6	0.52	-*
4E3-E10	0.59	-*
4F2-F10	0.06	-*
4F2-G7	0.04	-*
4F2-G9	0.04	-*
4G11-F6	0.60	-*
4G11-E2	0.54	-*
4G11-B4	0.20	-*

* = indicates change in recognition of phytochrome
on western blots following the first round
of cloning.

SDS-denatured blotted phytochrome (Table 3.3).

Following cloning the now clonal cell lines were re-named (Table 3.4).

The 11 cell lines that had been cloned were expanded by growth in flasks. Each cell line was then injected into pristane-primed BALB/C mice in order to raise ascitic fluid. LAS 43 failed to induce ascitic tumours. LAS 12 yielded ascitic fluid with no anti-phytochrome activity.

3.3.3 Purification of ascites

Ascites from each cell line was partially purified by differential $(\text{NH}_4)_2\text{SO}_4$ precipitation. The $(\text{NH}_4)_2\text{SO}_4$ purification gave substantial purification of the immunoglobulins as determined by SDS-PAGE (Fig 3.9). LAS 41 was chosen as a model candidate in order to determine the most efficient purification protocol. However, there are limitations to this approach as each mAb has its own unique properties and ideally should be tested individually.

3.3.3.1 Anti-mouse IgG chromatography

Mouse immunoglobulins were purified from ascites by immunoabsorption to immobilised anti-mouse IgG (Fig 3.6). This method gave good purification as determined by SDS-PAGE (Fig 3.9) but the binding capacity of the column was so low that a maximum of 0.25 mg could be purified on each run.

3.3.3.2 Protein A chromatography

Mouse immunoglobulins were also purified from ascites by immunoabsorption to Protein A (Fig 3.7).

Table 3.3 Summary of recognition of phytochrome
following the second round of cloning

Cell line	ELISA value	Recognition of phytochrome on western blots
1C7.B2.G4	1.20	+
1C7.B2.F1	1.07	+
ID3.B5.B6	0.85	+
ID3.B5.F2	1.06	+
2A8.F10.D6	0.29	-
2A8.F10.E4	0.19	-
3B4.B4.E7	1.40	+
3B4.B4.F5	1.41	+
3B10.F2.B4	1.40	+
3B10.F2.F2	1.50	+
3G3.D6.B6	1.07	+
3G3.D6.E2	1.06	+
3G9.B3.A2	0.39	-
3G9.B3.C2	0.18	-
3H11.B8.B3	1.50	+
3H11.B8.E3	1.57	+
4A10.F2.A3	1.29	+
4A10.F2.D3	1.40	+
4B3.G9.E4	0.40	-
4B3.G9.F6	0.45	-
4C6.E2.D1	0.03	-
4C6.E2.E6	0.03	-
4C10.F8.D3	0.02	-
4C10.F8.F2	0.04	-
4E3.D6.B3	0.05	-
4E3.D6.C2	0.03	-
4G11.F6.B2	0.29	-
4G11.F6.B5	0.17	-

Table 3.4 Renaming selected cell lines

Original cell line identification	Final Designation
1C ₇ .C ₂ .G ₄	LAS 11
1D ₃ .B ₅ .F ₂	LAS 12
2A ₈ .F ₁₀ .D ₆	LAS 21
3B ₄ .B ₄ .E ₇	LAS 31
3B ₁₀ .F ₂ .F ₂	LAS 32
3G ₃ .D ₆ .B ₆	LAS 33
3G ₉ .B ₃ .A ₂	LAS 34
3H ₁₁ .B ₈ .E ₃	LAS 35
4A ₁₀ .F ₂ .D ₃	LAS 41
4B ₃ .G ₉ .F ₆	LAS 42
4G ₁₁ .F ₆ .B ₂	LAS 43

Figure 3.6 Purification of LAS 41 from ascites by anti-mouse IgG chromatography

Ammonium sulphate purified ascites were applied to a 2 ml column of anti-mouse IgG (Sigma). The column was washed with several column volumes of PBS to elute non-specifically bound proteins. Mouse IgG were eluted with 0.1 M glycine-HCl, pH 2.5.

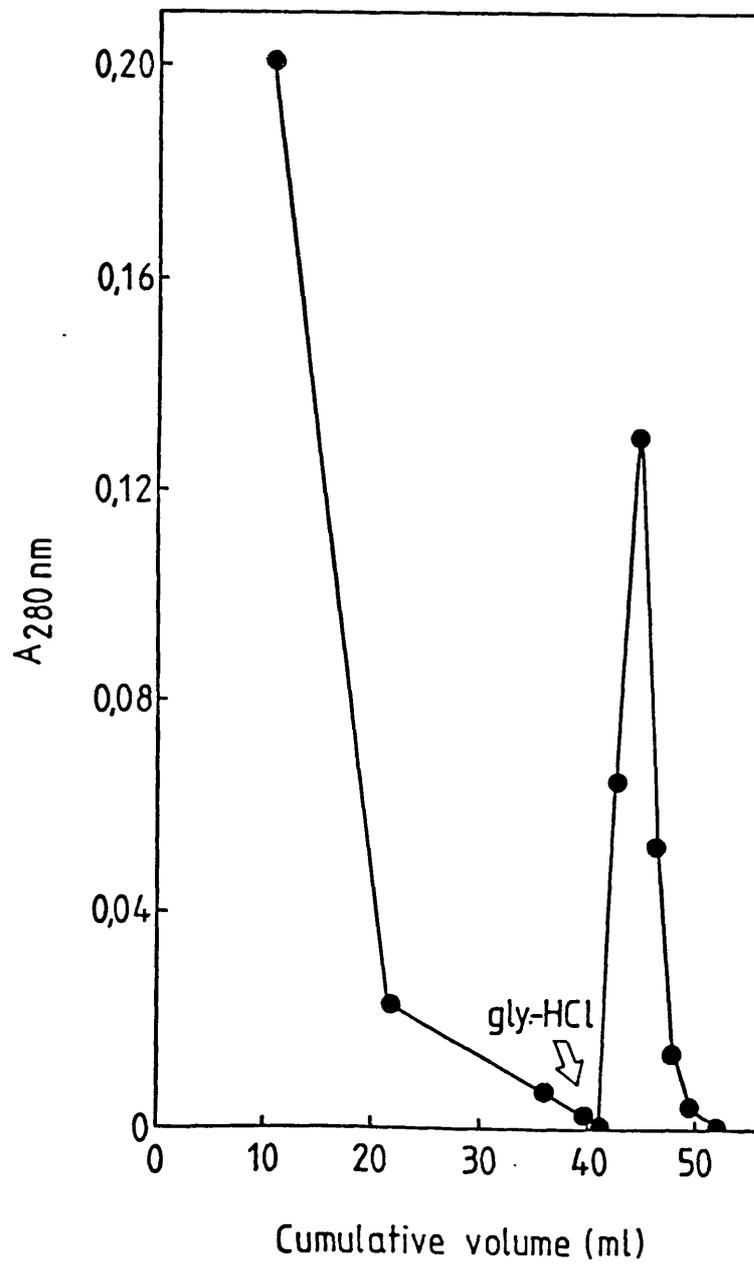
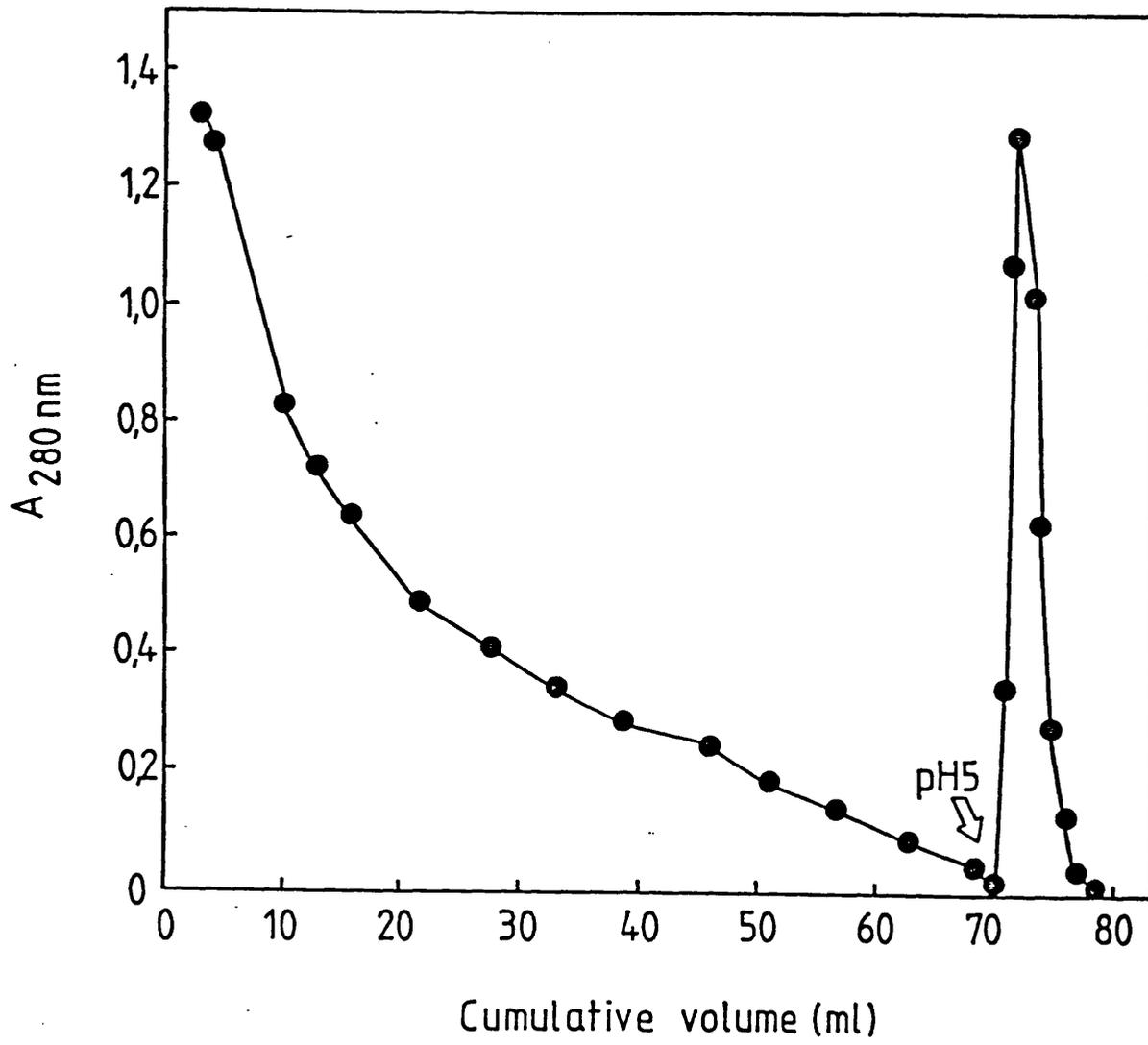


Figure 3.7 Purification of LAS 41 from ascites by Protein A chromatography

Ammonium sulphate purified ascites were applied to a 2 ml column of immobilised Protein A (Sigma). The column was washed with several column volumes of 100 mM sodium phosphate, pH 8.5 to elute non-specifically bound proteins. Mouse IgG were eluted with 100 mM sodium phosphate, pH 5.



This one step purification method also yielded pure IgG as determined by SDS-PAGE (Fig 3.9). The binding capacity of the 2 ml Protein A column was determined to be 2 mg mouse IgG.

3.3.3.3 Cation exchange chromatography

Mouse IgG were also purified to homogeneity as determined by SDS-PAGE (Fig 3.9) by cation exchange chromatography (Fig 3.8). This method typically yielded 10 mg of pure antibody. The capacity of this procedure was not tested.

3.3.4 Characterisation of mAbs

3.3.4.1 Subclass determination

Each cell line was screened against goat anti-mouse IgG subclass antibodies in a solid phase ELISA. All the cell lines were found to secrete antibodies which belong to the subclass IgG₁ (Fig 3.10).

3.3.4.2 Relative affinities of mAbs

The relative affinity of each mAb was determined using the solid phase ELISA 3.3 (Fig 3.11). LAS 42 has the lowest affinity and LAS 41 has the highest affinity for oat phytochrome.

3.3.4.3 Characterisation of mAbs raised to the native conformation of the molecule

The three mAbs (LAS 21, 34 and 42) which do not recognise SDS-denatured oat phytochrome were screened using two different ELISA configurations. In the first assay a modification of ELISA 3.1 was used. In this case a range of phytochrome concentrations were coated onto the plate. When less than 50 ng of phytochrome is coated onto the plate LAS 21, 34

Figure 3.8 Purification of LAS 41 from ascites by cation exchange chromatography

Cation exchange was conducted on a zeta-prep cation exchange disc (LKB). Ascitic fluid was applied to the disc in 50 mM sodium acetate pH 5.5, and washed with the same buffer until the $A_{280 \text{ nm}}$ dropped to 0. Mouse antibodies were eluted with 50 mM sodium acetate pH 5.5 containing 1 M NaCl.

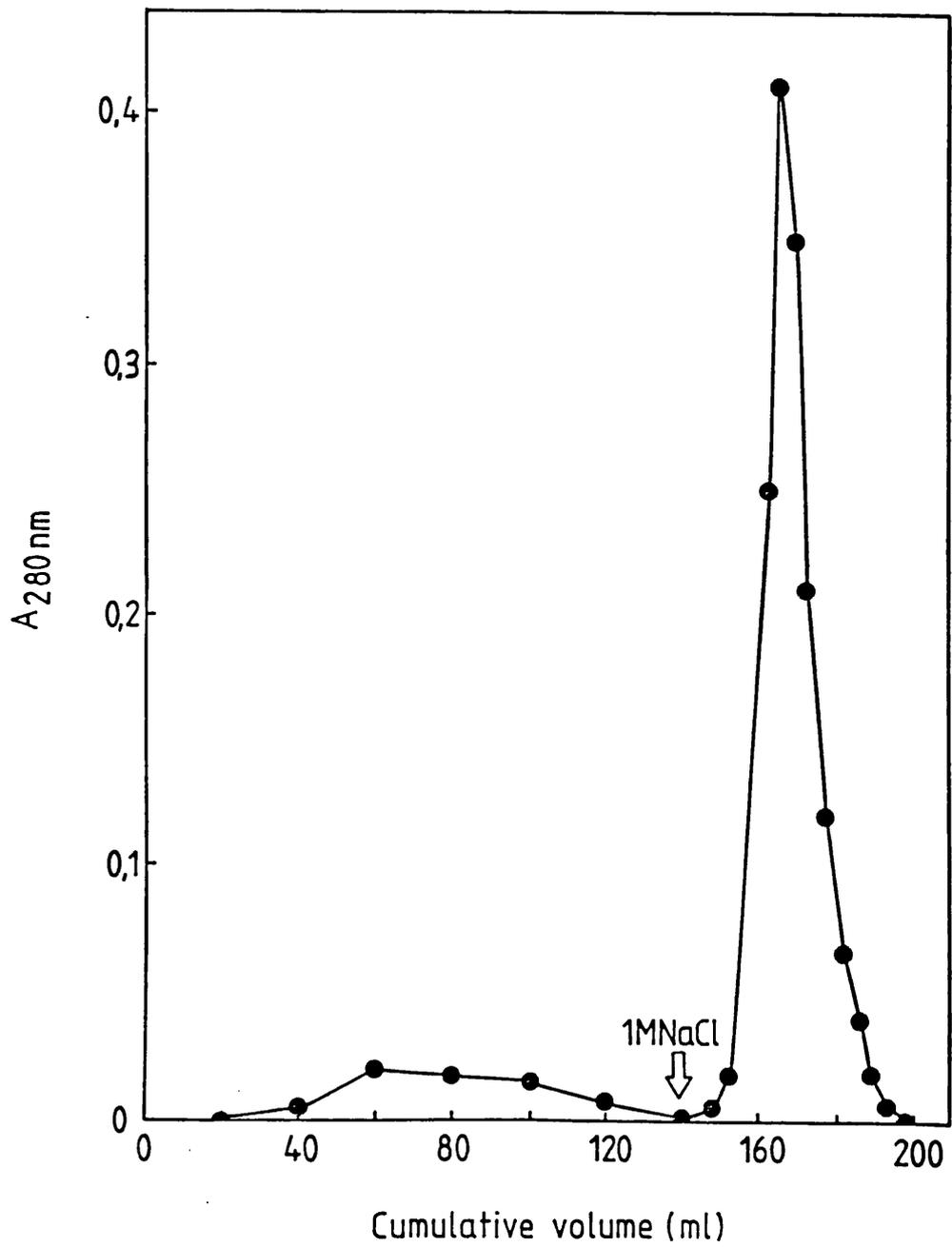


Figure 3.9 SDS-PAGE of samples of LAS 41
from ascites at various stages of purification

Each sample was resolved on a 10% SDS-gel.
The gel was stained with 0.5% Coomassie Brilliant Blue
dissolved in 30% (v/v) IMS and 12% (v/v)
acetic acid. The gel was destained in
25% (v/v) IMS and 10% (v/v) acetic acid.

Lane 1 = crude ascites

2 = following $(\text{NH}_4)_2\text{SO}_4$ purification

3 = following anti-mouse IgG
purification (10 μg)

4 = following Protein A purification
(10 μg)

5 = following cation-exchange
purification (10 μg)

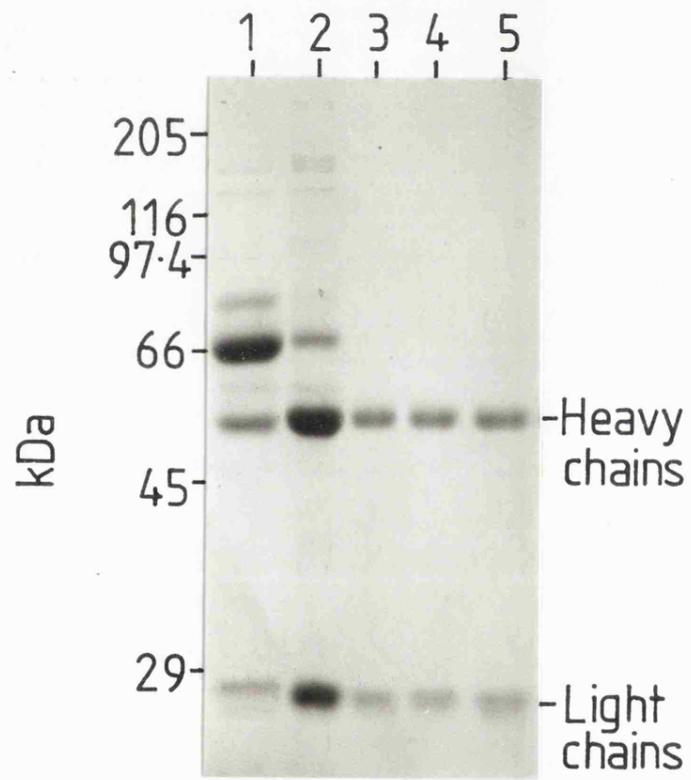


Figure 3.10 IgG subclass determination of
the mAbs by ELISA

ELISA wells were coated with anti-IgG₁,
-IgG_{2a}, -IgG_{2b} or -IgG₃. Individual culture
supernatants were added to each of the
anti-IgG- isotypes and bound mouse antibody
was detected by the addition of a
commercial peroxidase-conjugate of anti-
mouse antibodies.

a = anti-mouse IgG₁

b = anti-mouse IgG_{2a}

c = anti-mouse IgG_{2b}

d = anti-mouse IgG₃

Each reading is the average of three
replicate assays from a single experiment.

1 = LAS 11, 2 = LAS 21, 3 = LAS 31

4 = LAS 32, 5 = LAS 33, 6 = LAS 34

7 = LAS 35, 8 = LAS 41, 9 = LAS 42

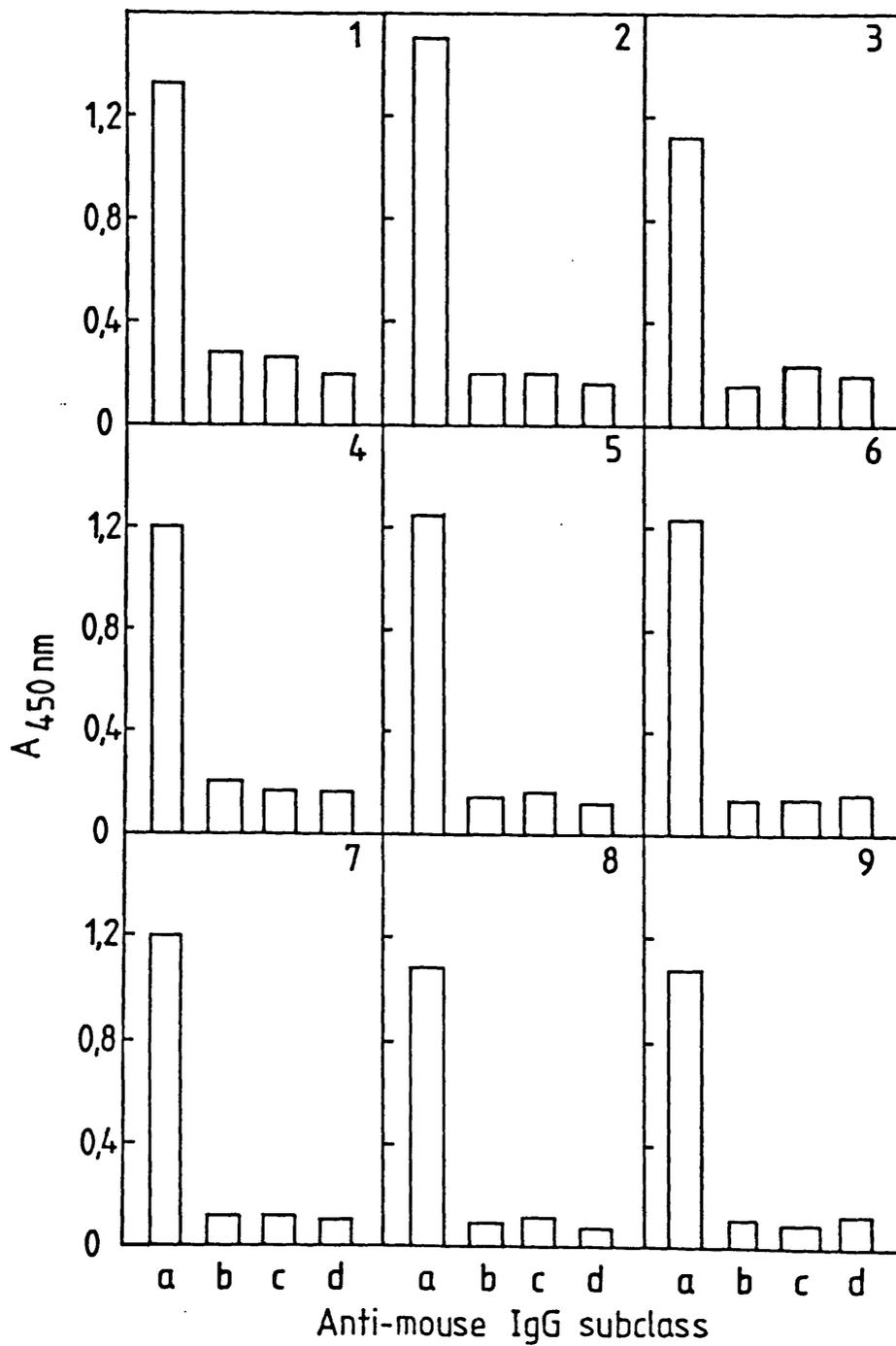
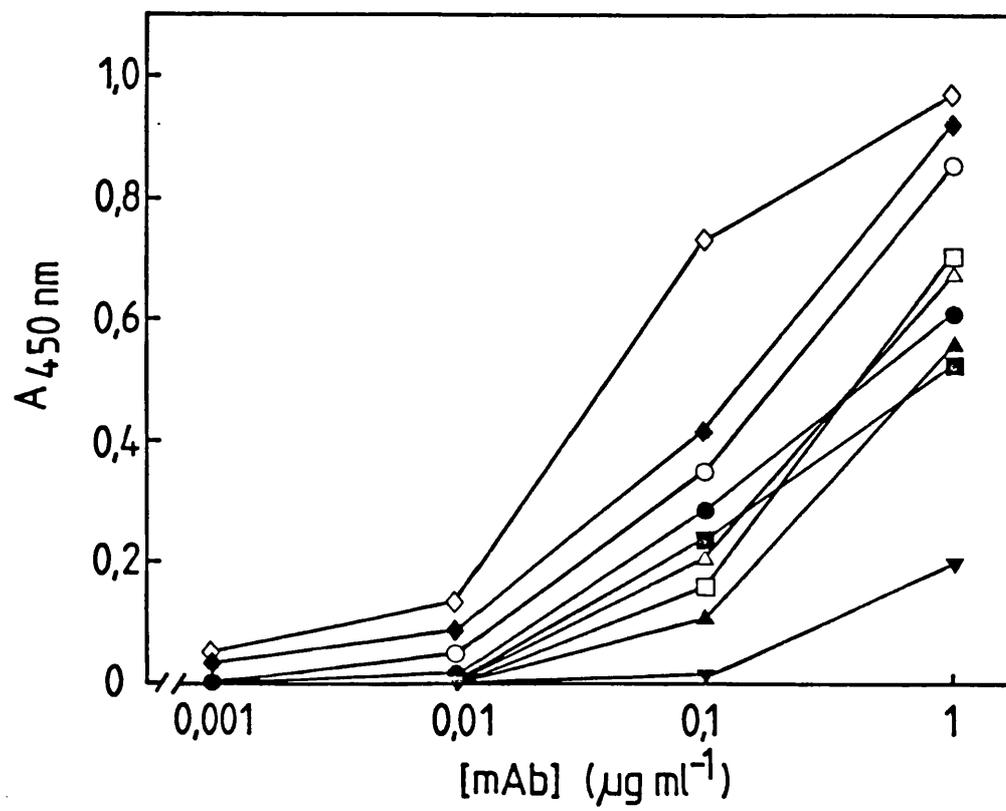


Figure 3.11 Relative affinities of the monoclonal antibodies for native 124 kDa oat phytochrome as determined by ELISA

Dilutions of the mAbs were added to immobilised 124 kDa oat phytochrome. Bound mAb was detected by the addition of peroxidase-conjugated sheep anti-mouse antibodies.

(○) LAS 11, (●) LAS 21, (□) LAS 31,
(■) LAS 32, (△) LAS 33, (▲) LAS 34,
(◇) LAS 35, (◆) LAS 41, (▼) LAS 42.

Each reading is the average of three replicate assays from a single experiment.



and 42 do not give a positive ELISA signal (Fig 3.12). However, when 50 ng or more phytochrome is coated onto the plate significant ELISA values are observed. When an alternative configuration of assay, ELISA 3.2, is used in which the mAb is immobilised and incubated with phytochrome in free solution, LAS 42 can detect 1 ng and LAS 21 and 34 can detect less than 500 pg of phytochrome (Fig 3.13). LAS 35, a mAb which immunostains SDS-denatured and electroblotted oat phytochrome, gave a high ELISA signal (0.75) with 5 ng of oat phytochrome in ELISA 3.1 (Fig 3.12). In ELISA 3.2 LAS 35 detected phytochrome down to a very similar concentration i.e. less than 500 pg, as LAS 21 and 34 (Fig 3.13).

3.3.4.3 Binding of Protein A to mAbs

Binding of Protein A to mAbs was tested in ELISA over a pH range of 7.5-9.0, as binding by Protein A has been determined to be pH dependent (Ey et al 1978). LAS 11, 21, 32, 35 and 42 were not appreciably bound by Protein A at any of the pH values tested (Fig 3.14). LAS 31, 33, 34 and 41 were bound by Protein A but this binding was not apparently pH dependent over the range tested (Fig 3.14).

Figure 3.12 Recognition of "unwound" 124 kDa
oat phytochrome, by LAS 21, 34, 35 and 42
in ELISA

LAS 21, 34, 35 and 42 were added to dilutions of immobilised 124 kDa oat phytochrome. Bound mAb was detected by the addition of peroxidase-conjugated sheep anti-mouse antibodies.

(●) LAS 21, (▲) LAS 34, (◇) LAS 35,
(▼) LAS 42.

Each value is the average of three replicate assays from a single experiment.

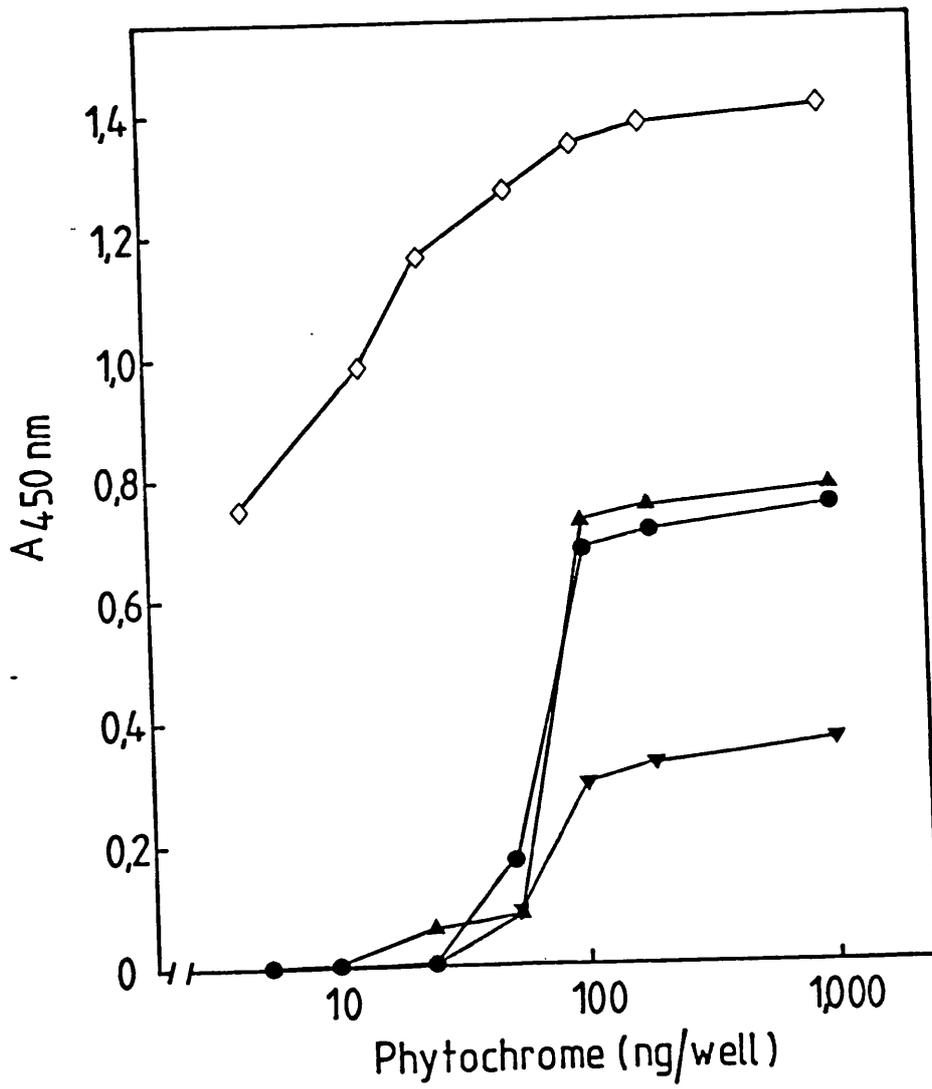


Figure 3.13 Recognition of "native" 124 kDa
oat phytochrome by LAS 21, 34, 35 and 42
in ELISA

Dilutions of 124 kDa oat phytochrome were added to immobilised LAS 21, 34, 35 and 42. Bound phytochrome was detected by the addition of rabbit anti-phytochrome polyclonal antibodies and the subsequent addition of a peroxidase-conjugate of anti-rabbit antibodies.

(●) LAS 21, (▲) LAS 34, (◇) LAS 35,
(▼) LAS 42.

Each value is the average of three replicate assays from a single experiment.

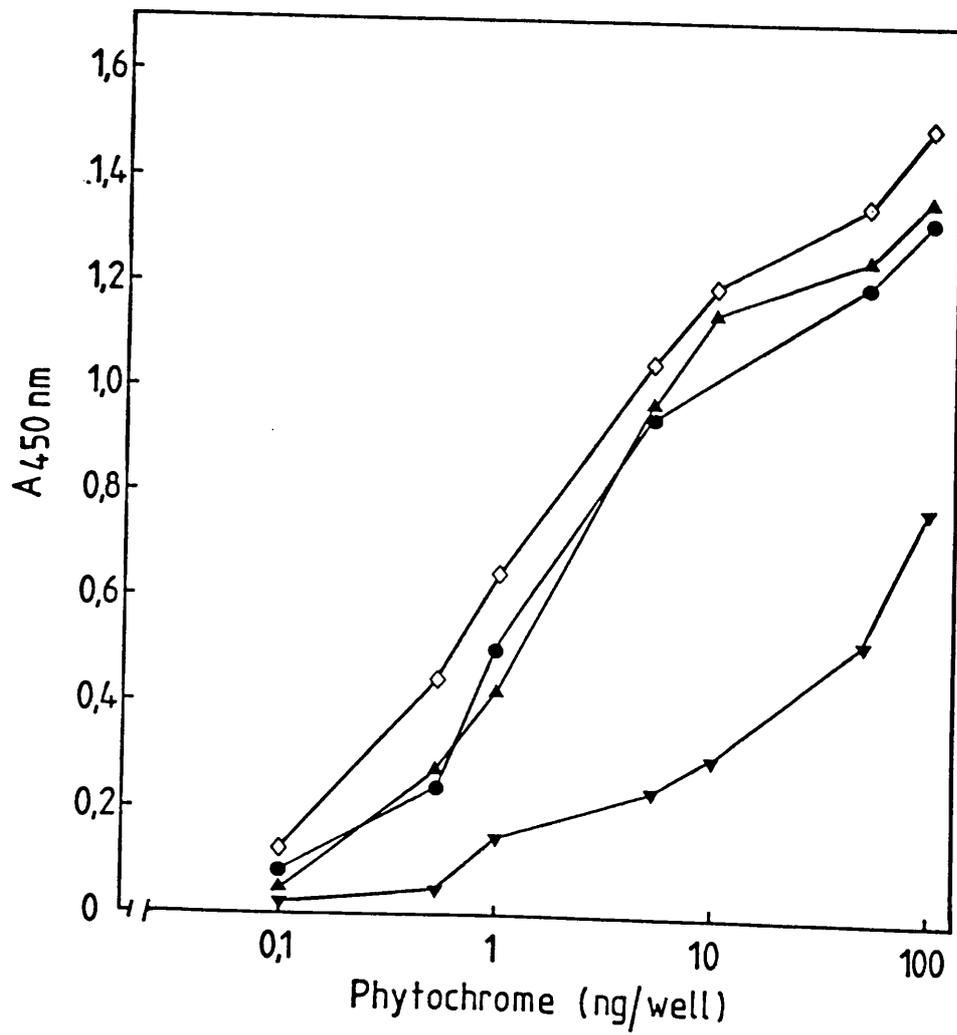


Figure 3.14 ELISA for the determination of binding of Protein A to mAbs

A peroxidase conjugate of Protein A was added to each immobilised mAb at pH 7.5, 8.0, 8.5 or 9.0. Bound peroxidase was detected with TMB.

a = pH 7.5

b = pH 8.0

c = pH 8.5

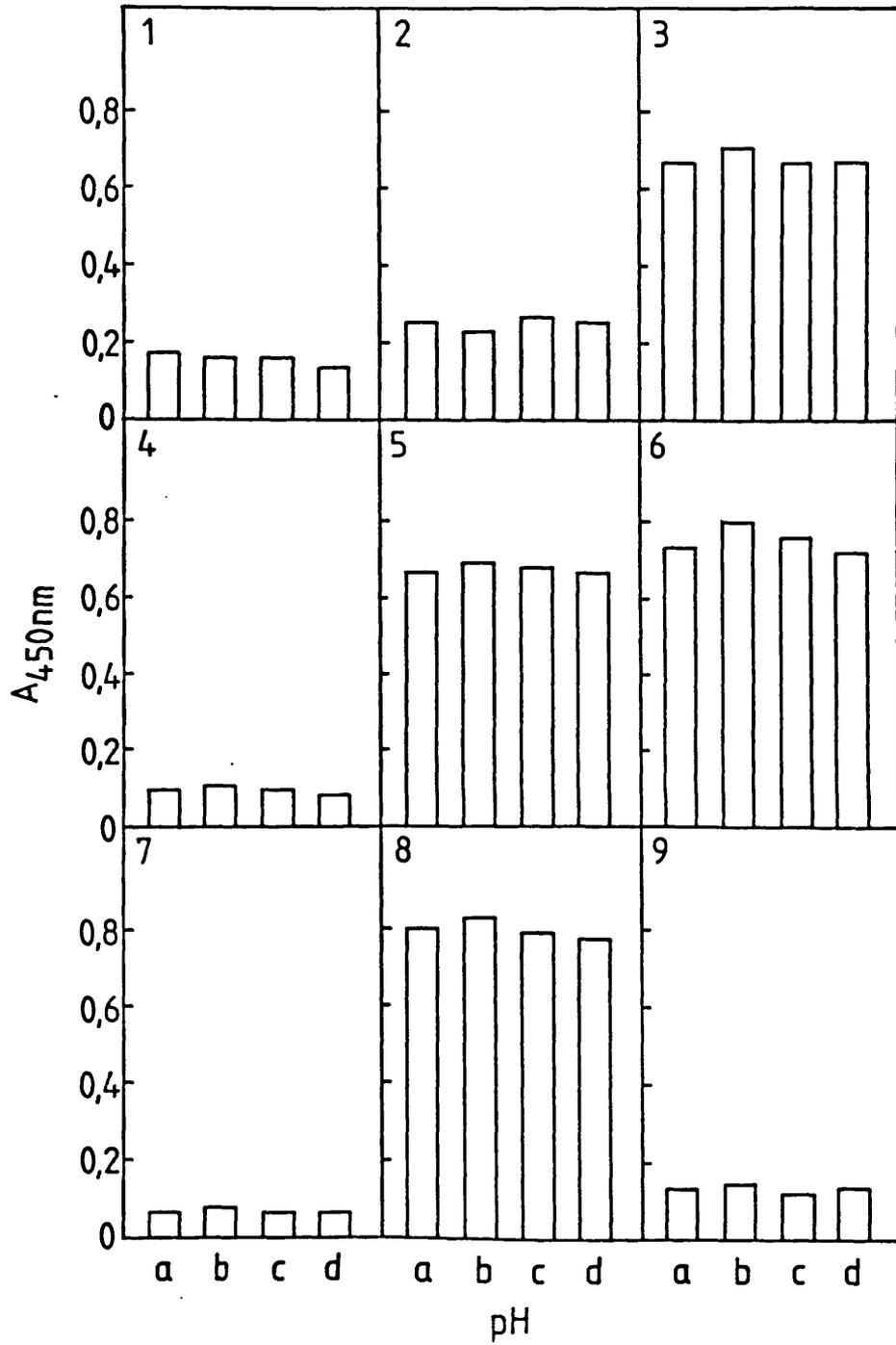
d = pH 9.0

Each reading is the average of three replicate assays from a single experiment.

1 = LAS 11, 2 = LAS 21, 3 = LAS 31

4 = LAS 32, 5 = LAS 33, 6 = LAS 34

7 = LAS 35, 8 = LAS 41, 9 = LAS 42



3.4 Discussion

3.4.1 Fusion product

A very high number of hybridomas were formed following cell fusion, which all secreted antibodies to oat phytochrome. Of these cell lines 10% also secreted antibodies which cross-reacted with partially degraded pea phytochrome. Since these cell lines are secreting antibodies which cross-react with phytochrome from a distantly related species, they are candidates for probes for conserved domains.

Cell lines which had given relatively high (0.65+) ELISA values when screened against oat phytochrome were selected for the second round of screening. This selection procedure would therefore be expected to select against "slow secretors" which may produce antibodies with high affinity, but do not give strong positive values due to the low titre, and against cell lines secreting antibodies with low affinity. However, when so many cell lines are positive it is necessary to compromise over selection strategy due to the practical limitations of expanding all the cell lines for further characterisation. At this stage cell lines were screened regularly, as the cell lines are relatively unstable and may stop secreting antibody due to chromosome loss. Several cell lines lost the ability to secrete antibodies to phytochrome, and others failed to thrive when taken from the 96 well plates and put onto 24 well plates.

Several factors are thought to be involved in the loss of chromosomes. These include genetic control of mitosis, asynchronous DNA replication, premature chromosome condensation, disturbances in the control of protein metabolism and faulty interaction between chromosome and spindle fibres, which results in unequal distribution of chromosomes to the two daughter cells (Westerwoudt 1985). Loss of detectable anti-phytochrome activity and a failure to thrive can also result from over growth of the cell line(s) by "non-secretors". As 50% of the protein synthesis in hybridomas is directed towards the secretion of antibodies these cell lines grow relatively slowly allowing them to be "overtaken" by hybridomas which are not secreting antibodies. In order to minimize the possibility of "choking" by non-secreting cell lines it is necessary to clone the cell lines at the earliest opportunity.

At the second round of screening 3B₁₀ (LAS 32) emerged as a particularly interesting cell line. This cell line secreted antibodies which gave the same ELISA reading for oat and pea phytochromes (Table 3.1). This antibody must be recognising a conserved region of the molecule which may be biologically significant. This antibody can also immunostain SDS-denatured blotted oat phytochrome. Since the proteins in crude extracts of plant material can be separated by SDS-PAGE and be electroblotted onto nitrocellulose this mAb is a good

candidate for looking for a conserved domain on the primary sequence of other phytochromes. 3G₉ (LAS 34) also cross-reacted with pea phytochrome but did not immunostain SDS-denatured electro-blotted phytochrome. As this mAb recognises the native conformation it may be possible to immobilise this mAb and hence immunopurify phytochrome from several plant species.

Screening against partially purified oat phytochrome on immunoblots potentially identified mAbs recognising regions of primary sequence. However, since SDS is absent during electro-blotting and in the subsequent steps, it is possible that at least partial renaturation of the molecule may have occurred, and that some of the mAbs may be specific for epitopes determined by the conformation of the molecule. However, all the mAbs which recognise SDS-denatured phytochrome can also detect phytochrome in ELISA when the phytochrome concentration coated on the plate is limiting (see later). This suggests that in the case of these mAbs the assumption that screening on immunoblots detects mAbs which are raised to primary sequence epitopes is probably upheld. In addition using 124, 118 and 114 kDa phytochrome to screen on immunoblots it is possible to determine whether the mAbs which recognise this SDS-denatured phytochrome are raised to epitopes on the 6 and 4 kDa NH₂-terminal peptides. This assay system however cannot distinguish mAbs which are raised to epitopes on the 64 kDa chromophore-bearing domain and the 55 kDa COOH-terminal domain.

3B₄ (LAS 31), 3G₃ (LAS 33), 3H₁₁ (LAS 35) and

4A₁₀ (LAS 41) all apparently map close to the NH₂-terminus (Fig 3.4).

At this stage the cell lines fall into four categories:- cell lines which (1) recognise only native oat phytochrome; (2) recognise only native oat and pea phytochrome; (3) recognise native and SDS-denatured oat phytochrome; (4) recognise native oat and pea phytochrome and SDS-denatured oat phytochrome. Within these four categories the extent of reactivity varies as determined by ELISA and the intensity and pattern of staining on immunoblots (Table 3.1).

In order to characterise these antibodies it is essential to ensure that the cell line from which they are secreted is monoclonal. During the first round of cloning 3 cell lines lost the ability to secrete antibodies to phytochrome (Table 3.2). This may stem from instability in the cell line and hence chromosome loss. However, repeated endeavours with fresh stocks of the original cell lines failed to yield clones. It may be that these cell lines were inherently unstable, were particularly sensitive to being plated out at low concentrations (even in the presence of feeders), or became over grown by non-secreting cells. However, it is not possible to distinguish between these possibilities. Other lines which were successfully cloned still showed positive reactivity to oat phytochrome but no longer recognised SDS-denatured phytochrome on immunoblots. This suggests that the original cell

line was not monoclonal but consisted of a mixture of cell lines which secreted antibodies to both region(s) of primary sequence and the native conformation of the molecule. From this mixture a monoclonal cell line has been cloned which secretes antibody raised to the native conformation of the molecule and the other cell line(s) have been lost.

The 11 cell lines that were successfully cloned were injected into pristane-primed BALB/C mice in order to produce ascites. The peritoneal cavity is preconditioned with pristane, a branch-chain alkane as this severely depresses the normal immunological function of the animal (Freund and Blair 1982). Nine of the lines generated ascitic tumours with a high recovery of mAb. Ascites fluid was collected from mice injected with LAS 12, but there was no anti-phytochrome antibody activity. LAS 43 did not establish ascitic tumours. In the cases of LAS 12 and LAS 43 these problems probably result from instability of the hybridomas. These problems are normally associated with cell lines which have been maintained in culture for several months (Hoogenraad and Wraight 1986). However, these cell lines were relatively young and had been cloned at the earliest opportunity. Since these cell lines could not be successfully grown in vivo they have not been fully characterised. It was not possible to generate large amounts of mAb in culture as there are no in-house facilities for culturing cell lines on a large scale in vitro.

3.4.2 Screening rationale

It is essential that the screening assay used will select antibodies of most use. It is therefore necessary to choose an assay system as close to the assay in which the antibody will be eventually used. A very important consideration when screening mAbs is that it is only possible to select mAbs which the screening assay detects. In this case the aim was to select both mAbs that recognise conformationally determined and primary sequence epitopes, which occur throughout the phytochrome molecule. This involves double screening with ELISA (which detects antibodies raised to both the native conformation and to epitopes on the primary sequence) and mini-western blots (which should distinguish mAbs raised to the native conformation and the primary sequence).

In the solid phase ELISA (ELISA 3.1), 100 ng of oat phytochrome was coated onto each well. Screening of a previous panel of mAbs (not described here) demonstrated that this concentration of phytochrome detects antibodies raised to both conformationally determined epitopes and primary sequence epitopes. It is unlikely that this assay will select cell lines which secrete antibodies that only recognise primary sequences, as the concentration of phytochrome used is not limiting. Only when the concentration of phytochrome coated on the plate falls below 50 ng do mAbs raised to the native conformation of the molecule fail to immunodetect the phytochrome (Fig 3.12). The

inability of the mAbs raised to the native conformation to bind the phytochrome is probably due to distortion of the molecule following unwinding on the plate. Loss of native conformation by adsorption onto vinyl plates has been described for other proteins (Campbell 1986). This assay is also not biased towards selection of antibodies which recognise conformationally determined epitopes as some unwinding of the phytochrome molecule is assumed to occur on the plate. This assumption is borne out by the selection of 6 mAbs which immunostain SDS-denatured electro-blotted phytochrome and give high ELISA values. Using this assay, antibodies recognising the native conformation of the molecule give significantly lower, but still positive values than antibodies which are recognising regions of primary sequence (Fig 3.12).

A double antibody sandwich ELISA in which phytochrome is immobilised by an antibody (polyclonal or monoclonal) and therefore likely to retain its native conformation, may bias the assay so that it tends to select lines which secrete antibodies that are raised to the native conformation of the molecule (Smith and Wilson 1986). This is the configuration of assay used by Thomas et al (1984b). Certainly using this assay configuration it is not possible to distinguish antibodies recognising the native conformation or regions of primary sequence (Fig 3.13). Nagatani et al (1983) screen for anti-phytochrome antibodies using a RIA in which

phytochrome is coupled to sheep red blood cells. This assay design is probably prone to the same drawbacks as those outlined for the double antibody sandwich ELISA.

Early screening with pea phytochrome selects mAbs of particular interest as they are also recognising a related molecule, and therefore may be raised to a conserved region. Double screening on mini-western blots facilitates early detection of cell lines which recognise SDS-denatured phytochrome and which are potentially secreting antibodies against a region of primary sequence on the molecule. Conversely, those antibodies which do not immunostain the denatured phytochrome are candidates for probes for the native conformation of the molecule. Thus, by multiple screening in this way it is possible to detect mAbs with potentially interesting properties at an early stage.

3.4.3 Purification of mAbs

Under certain circumstances such as enzyme labelling of antibodies or coupling antibodies to a column, it is necessary to purify mAbs. As each mAb has different properties it is usually necessary to test the antibodies individually in order to determine the optimum method for purification. Several protocols for the purification of mAbs have been described, including the use of purification on Protein A (Stephenson et al 1984); DEAE Affi-Gel Blue chromatography (Bruck et al 1982); anion

exchange and gel filtration chromatography (Burchiel et al 1984); cation exchange and gel filtration chromatography (Carlsson et al 1985) and hydroxyapatite chromatography (Stanker et al 1985). The majority of these protocols have been compared by Manil et al (1986). They report that DEAE chromatography in combination with Protein A gave the best compromise between yield and purity.

In this study the aim was to select a one-step purification procedure using LAS 41 as the reference mAb. Three purification protocols were compared:- affinity chromatography on an anti-mouse IgG column; affinity chromatography on a column of Protein A and cation exchange chromatography. Ascitic fluid contains a variety of host-mouse proteins including albumin and transferrin. Monoclonal antibodies usually represent approximately 10% of the total protein content of ascites (Bruck et al 1982). Ammonium sulphate fractionation preferentially removes high molecular weight and acidic proteins, including the majority of transferrin and albumin and generally renders the immunoglobulins 40-50% pure (Manil et al 1986; Fig 3.9).

The three methods of purification tested generated pure mAb as determined by SDS-PAGE (Fig 3.9). However, purification by immunoadsorption to anti-mouse IgG only yielded 0.25 mg per run due to the low binding capacity of the column. Consequently, in order to scale-up the purification it would be necessary to invest in a much larger column. Alternatively it would

be possible to produce in-house anti-mouse IgG antibodies and thus generate a column of immobilised anti-mouse IgG. A further disadvantage of this system is that in order to elute the antibodies it is necessary to expose them to acidic conditions which may effect the subsequent activity of the purified mAb. One advantage of this method is that it is generally applicable and therefore can be used for all the mAbs.

Affinity chromatography with Protein A was also successful. However, in general IgG₁ antibodies are poorly bound by Protein A, although this binding is pH dependent (Ey et al 1978). As this protocol had worked relatively well binding of Protein A to the remaining mAbs over a pH range of 7.5 to 9.0 was determined. Only LAS 31, 33 and 34 were bound by Protein A, which suggests that they also could be purified using this method. However, Protein A purification was not the most appropriate protocol tested as this column also had a relatively low binding capacity of 2 mg mAb. Using cation exchange it was possible to purify 10-15 mg of pure IgG on each run. Since mAbs may have unique properties, there are possible limitations of only testing one mAb, LAS 41, however the cation exchange purification protocol is probably generally applicable. This is because cation exchange chromatography exploits the relatively high pI of immunoglobulins compared to other ascitic proteins. Monoclonal antibody is bound to the column at pH 5.5, as below this pH

additional proteins, which would elute with the IgG, are retained on the column. Above pH 5.9 mAbs themselves are not retained on the column (Boonekamp and Pomp 1986). Due to the specific pH dependence of the binding of the mAb it is not necessary to use a gradient elution and so mAbs can be eluted isocratically with high salt buffer.

Cation exchange therefore offers several advantages over the other two purification procedures. This method is not IgG subclass specific, provides a high yield of pure antibody, is fast, inexpensive and is conducted in the absence of denaturing conditions. Cation exchange was therefore selected as the most appropriate method for purification of immunoglobulins from ascitic fluid. This method has also been used successfully to purify LAS 32.

3.4.4 Summary of characterisation

Initial characterisation of the nine mAbs to be further investigated demonstrate that they all belong to the same antibody subclass, IgG₁. Six of the mAbs immunostain SDS-denatured and immunoblotted phytochrome and also positively react with native oat phytochrome in ELISA. These mAbs are therefore raised against a putative region of primary sequence which may be located at or near the surface of the protein molecule. The remaining three mAbs only react with native phytochrome and therefore are probably raised to an epitope determined by the native conformation of the molecule. LAS 34 and LAS 32 cross-react with pea phytochrome in ELISA and

are therefore potential probes for conserved domains amongst phytochromes. Comparative affinity analyses amongst the mAbs demonstrate that LAS 41 has the highest affinity for oat phytochrome. LAS 42 has a particularly low affinity for phytochrome. The lower affinity mAbs which cross-react with pea phytochrome may be suitable for coupling to columns which can then be used to immunopurify phytochrome from other plant species. In some instances it is not possible to immunopurify proteins on columns of immobilised mAb due to the very high affinity of binding which cannot be broken without total denaturation of the protein (Billett, personal communication). Therefore, as a rule of thumb, it is better to select relatively low affinity antibodies for immunopurification. High affinity antibodies which can be bound by Protein A may be used successfully in immunoprecipitation experiments.

CHAPTER 4

EPI TOPE MAPPING AND COMPETITION ANALYSES

4.1 Introduction

In order to use mAbs to conduct structure/function analyses it is imperative that the mAbs can be mapped to precise regions of the phytochrome molecule. In addition, it is necessary to determine the relative spatial relationship of the epitopes, within the respective domains, to which the mAbs are binding.

Mapping of mAbs to regions of a molecule is dependent on the generation of unambiguous, proteolytically derived fragments of the native molecule, which can be orientated with respect to one another. Furthermore, it is necessary that the epitope to which the mAb binds occurs only once on the peptide. In the case of phytochrome, proteolytic digestion by endogenous proteases in crude extracts produces a characteristic array of peptides, whose arrangement has been elucidated (Daniels and Quail 1984). For mAbs which recognise SDS-denatured phytochrome it is possible to determine the domain to which they are raised by resolving the peptide fragments on SDS-PAGE, electroblotting the peptides onto nitrocellulose and probing with the respective mAbs. Using this technique mAbs have been assigned to the three major domains (Daniels and Quail 1984; Cordonnier et al 1985; Shimazaki et al 1986). Silberman et al (1985) have mapped mAbs which recognise SDS-denatured phytochrome, by partial digestion of phytochrome and then separation of the peptide fragments by ion exchange chromatography. The various fractions are resolved on SDS-PAGE and

probed with the respective mAbs. Recently, Thompson et al (1987) have described a method which incorporates the use of cDNA clones to phytochrome, and the use of the λ gt 11 expression vector, to assist in the mapping of mAbs which recognise the primary sequence of phytochrome. The product of the cDNA clones, which have been partially sequenced so that they can be orientated within the entire published Avena sequence (Hershey et al 1985), are probed with the respective mAbs. This method has been used to confirm that mAbs previously mapped by conventional means i.e. according to Daniels and Quail (1984), recognise the expected region of the molecule.

A further category of mAbs, which are conformation-specific and therefore do not recognise SDS-denatured phytochrome cannot be mapped using conventional methods. In this instance it is necessary to generate native (i.e. not SDS-denatured) diagnostic peptides which can be reacted with the mAb in question, and the peptide to which the mAb has bound identified. However, as yet, there are no published protocols for the mapping of mAbs which only recognise the native conformation of the phytochrome molecule.

Competition analyses, which determine whether or not two different mAbs can bind simultaneously to the antigen molecule, provide complementary information to that provided by epitope mapping. Such analyses can indicate whether mAbs which are raised to the same

domain are recognising the same or overlapping epitopes. Competition analyses may also be used to examine whether regions of primary sequence which are spatially distant are brought together in the native conformation.

In this chapter mAbs which recognise SDS-denatured phytochrome are mapped to the major functional domains (see section 1.2.4). In addition a novel protocol for the mapping of mAbs which only recognise the native conformation of the phytochrome molecule is described. Competition analyses between all of the mAbs, which determines the relative distribution of the mAb binding sites, is also detailed.

4.2 Materials and Methods

4.2.1 Epitope mapping of mAbs which immunostain SDS-denatured phytochrome.

4.2.1.1 Generation of peptides

Phytochrome peptides were generated in crude extracts by endogenous proteases as described by Jones et al (1985). Immediately prior to harvest 4 day old oats were irradiated with 2.5 min red light. Under dim green safelight whole shoots were homogenised in 100 mM 3-(N-morpholino) propanesulphonic acid (MOPS), pH 7.8, containing 5 mM EDTA, and 56 mM 2-mercaptoethanol, at a ratio of 1 ml buffer per g tissue. The homogenate was filtered through two layers of cheesecloth. Calcium chloride was added to the supernatant from a 3 M stock, pH 7.4, to make a final concentration of 15 mM and the total mixture stirred on ice for 5 min. Following centrifugation at 10,000 x g for 20 min the supernatant was decanted and divided into two equal aliquots. One aliquot was irradiated with 5 min saturating red light, via an Oriel broad band interference filter (peak 650 nm), and the other with 5 min saturating far-red light via a Schott RG9 filter. After irradiation the crude extracts were incubated under dim green safelight at 25C. At the appropriate times samples were removed and prepared for gel electrophoresis by boiling in double strength sample buffer for 1 min.

4.2.1.2 SDS-PAGE and immunoblotting

Samples of crude extract containing 100-200 ng phytochrome were resolved on 8% SDS-polyacrylamide gels (Laemmli 1970). Following electrophoresis, polypeptides were electro-blotted onto nitrocellulose filters in a custom-made semi-dry blotting apparatus using 25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol buffer. Remaining protein-binding sites on the nitrocellulose were blocked by incubating the filters for 30 min at room temperature in 100 mM Tris, pH 9, containing 150 mM NaCl, 0.05% (v/v) Tween 20 and 3% (w/v) Marvel. The nitrocellulose filters were then incubated at room temperature for 2 h with mAb at a concentration of $10 \mu\text{g ml}^{-1}$ in TBS containing 0.1% (w/v) Marvel. The filters were then washed with three changes of TBS-Tween and then incubated for 1 h at room temperature, with a 1/500 dilution of commercial goat anti-mouse antibodies (Sigma) in TBS containing 0.1% (w/v) Marvel. Following further washing in TBS-Tween the filters were then incubated for 1 h at room temperature with a 1/500 dilution of biotinylated sheep anti-goat antibodies (Sigma) in TBS containing 0.1% (w/v) Marvel. After washing, filters were incubated for 20 min at room temperature with a 1/500 dilution of avidin peroxidase (Sigma) in TBS. The filters were then thoroughly washed with TBS-Tween and rinsed with distilled water. Following equilibration of the filters in PBS the bound peroxidase was visualised with 0.6 mg ml^{-1} 4-chloronaphthol (Sigma) in PBS

containing 0.02% (v/v) hydrogen peroxide.

4.2.1.3 Mapping of LAS 32

4.2.1.3.1 Generation of peptides

The calcium chloride clarified crude extract was prepared as above. Pure (SAR = 0.91) 124 kDa oat phytochrome was added to the crude mixture at a concentration of $35 \times 10^{-3} \Delta (\Delta A) \text{ units ml}^{-1}$. The crude mixture was then divided into two aliquots. Following irradiation with either saturating red or far-red light the aliquots were incubated under dim green safelight at 25C. Samples were prepared for electrophoresis as described above.

4.2.1.3.2 SDS-PAGE and immunoblotting

Samples were run on SDS-PAGE and electroblotted onto nitrocellulose as described above. Filters were developed with the following modifications. Following incubation with mAb, filters were washed with three changes of TBS-Tween containing 0.05% SDS (TBS-Tween-SDS) and incubated with a 1/500 alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) in TBS containing 3% (w/v) Marvel for 1 h at room temperature. Following a further three washes with TBS-Tween-SDS the nitrocellulose filters were incubated with a 1/500 alkaline phosphatase-conjugated sheep anti-goat antibody (Sigma) in TBS containing 3% (w/v) Marvel for 1 h at room temperature. Following a final wash in three changes of TBS-Tween-SDS, blots were equilibrated in 0.75 M Tris, pH 9.5. Bound alkaline phosphatase was visualised with 0.5 mg ml^{-1}

5-Bromo-4-chloro-indolyl phosphate (BCIP)(Sigma)
in 0.75 M Tris, pH 9.5.

4.2.2 Conjugation of mAbs with biotin succinimide ester

Ammonium sulphate purified LAS 11, 31 and 32 were made 1 mg ml^{-1} in 0.1 M NaHCO_3 , pH 8.2. The biotin succinimide ester was made 1 mg ml^{-1} in DMSO and $120 \mu\text{l}$ of this were added per ml of antibody. The mixture was incubated at room temperature for 1.5 h and then extensively dialysed against PBS overnight at 4C. Conjugated antibody was screened for anti-phytochrome activity using a solid phase ELISA. In brief, $2 \mu\text{g ml}^{-1}$ of 124 kDa oat phytochrome was coated onto the plate in 50 mM bicarbonate buffer overnight at 4C. Following blocking a dilution series of the respective mAbs in PBS-Tween were added to the wells and incubated for 1 h at room temperature. Following washing bound biotin was detected by the addition of a 1/1000 dilution of avidin peroxidase (Sigma) and incubated for 15 min. Following a final wash with PBS-Tween bound peroxidase was visualised with $1 \mu\text{g ml}^{-1}$ TMB in citrate-acetate buffer, pH 6.

4.2.3 Epitope mapping of conformation-specific mAbs

4.2.3.1 Generation of peptides

Phytochrome peptides were generated in crude extracts by endogenous proteases as described above except that the extracts were incubated overnight at room temperature. Proteolysis was terminated by the addition of PMSF to 4 mM.

4.2.3.2 Mapping ELISA

Flat bottomed, 96-well vinyl plates (Dynatech) were coated with $10 \mu\text{g ml}^{-1}$ of either LAS21, 34 or 42 in 50 mM bicarbonate buffer, pH 9.2, overnight at 4C. Following the overnight incubation the assay plates were washed extensively with PBS-Tween. Remaining protein-binding sites were blocked by filling the wells with PBS-Tween containing 3% (w/v) Marvel and incubated for 30 min at room temperature. Assay wells were washed with PBS-Tween and 50 μl of proteolytic fragments ($\sim 50 \text{ ng}$ phytochrome) digested as either Pr or Pfr were added and incubated for 1 h at room temperature. Following washing with PBS-Tween 1/1000 dilution of a biotin-conjugate of LAS 31 and 32 or a 1/200 dilution of a biotin-conjugate of LAS 11 in PBS-Tween were added to the appropriate wells and incubated for 1 h at room temperature. Following a further wash a 1/1000 dilution of commercial avidin peroxidase (Sigma) in PBS-Tween was added and incubated for 15 min at room temperature. Following a final wash bound peroxidase activity was visualised with TMB.

4.3.2 Competitive ELISA assay

Competition analysis was conducted using a modification of the protocol described by Cordonnier et al (1985). Flat-bottomed, 96-well vinyl plates (Dynatech) were coated with $10 \mu\text{g ml}^{-1}$ mAb 1 in 50 mM bicarbonate buffer pH 9.2, overnight at 4C. During this time appropriate mixtures of phytochrome and mAb 2 were prepared in separate tubes and incubated

overnight at 4°C. Following the overnight incubation the assay plates were washed extensively with PBS-Tween. Remaining protein-binding sites were blocked by filling the wells with PBS-Tween containing 3% (w/v) Marvel and incubated for 30 min at room temperature. The preincubated mixtures of mAb 2 and phytochrome were added to the assay wells which had been washed with PBS-Tween, and incubated for 1 h at room temperature. Following washing with PBS-Tween $5 \mu\text{g ml}^{-1}$ immuno-affinity purified-rabbit polyclonal antibodies in PBS-Tween containing 0.1% (w/v) Marvel were added and incubated for 1 h at room temperature. Following a further wash a commercial peroxidase-conjugate of goat anti-rabbit antibodies (Sigma) was added at a dilution of 1/500 in PBS-Tween containing 0.1% (w/v) Marvel and incubated for a further 30 min at room temperature. Bound peroxidase activity was visualised as described above. Inhibition of binding of mAb 1 to phytochrome by the presence of mAb 2 was determined as follows. When mAb 2 was omitted from the assay the ELISA value obtained was considered representative of 0% inhibition. Likewise, when mAb 1 was omitted from the assay the ELISA value obtained was considered representative of 100% inhibition.

4.3 Results

4.3.1 Epitope mapping of mAbs which immunostain SDS-denatured phytochrome

Incubation of crude extracts containing Pr or Pfr produce a range of characteristic proteolytic cleavage fragments. Immunostaining of these extracts, following SDS-PAGE and blotting onto nitrocellulose, with polyclonal antibodies reveal major bands at 124, 118, 114, 74, 68, 64 and 55 kDa (Fig 4.1). LAS 31 and 33 immunostain only the 124 and 74 kDa peptides (Fig 4.2 b, d). LAS 35 and 41 immunostain 124, 118, 74 and 68 kDa peptides but not the 114, 64 or 55 kDa peptides (Fig 4.2 e, f). LAS 11 immunostains 124, 114, 118, 74, 68 and 64 kDa peptides (Fig 4.2 a) whereas LAS 32 immunostains 124, 114, 118 and 55 kDa peptides (Fig 4.2 c).

When pure phytochrome was added to the crude oat extract the same characteristic array of peptides are produced, but additional less abundant cleavage fragments may also be observed. Immunostaining of these extracts, following SDS-PAGE and blotting onto nitrocellulose, with polyclonal antibodies reveal further bands at 97, 91, 88, 81, 40 and 36 kDa (Fig 4.3). LAS 31 immunostains 124, 97, 88 and 74 kDa peptides (Fig 4.4 a). LAS 32 immunostains 124, 97, 88, 81, 40 and 36 kDa species (Fig 4.4 b).

4.3.2 Epitope mapping of mAbs which only immunostain the native phytochrome molecule

In order to conduct the mapping ELISA it was necessary to conjugate with biotin, a representative

Figure 4.1 Time course of Pr or Pfr
digestion by endogenous proteases in a
crude extract of oat shoots

A crude extract of etiolated oat shoots (irradiated with either red or far-red light) was incubated at 25C for up to 6 h. Aliquots were removed at the indicated times, heated in SDS-sample buffer and resolved on an 8% SDS-gel (200 ng per lane t = 0) and electroblotted onto nitrocellulose. The blot was probed with a mixture of mAbs raised against 124 kDa oat phytochrome.

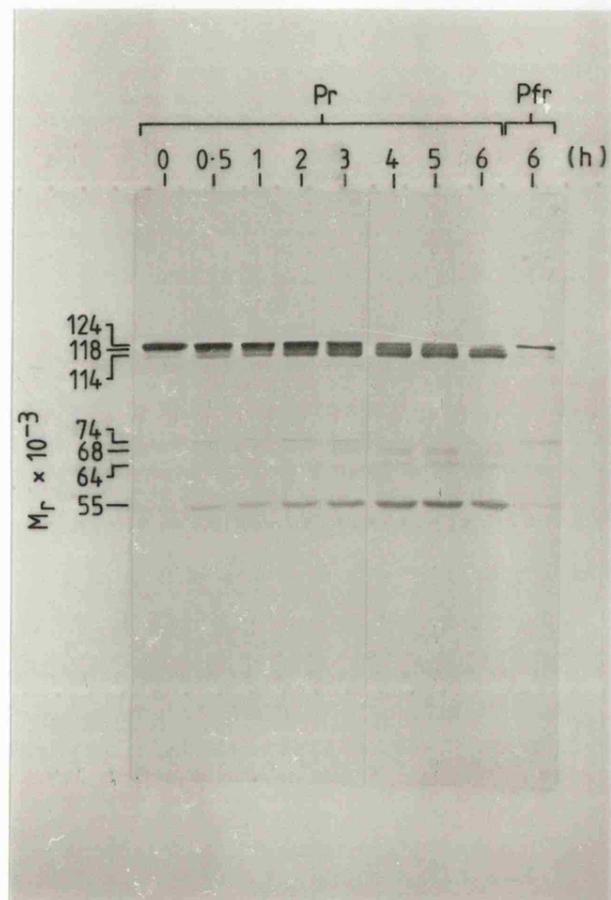
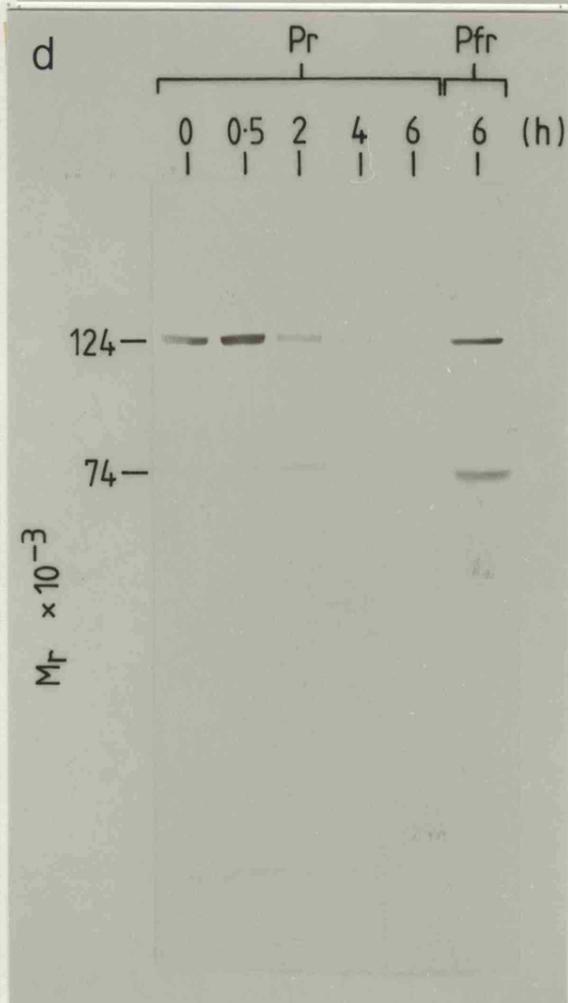
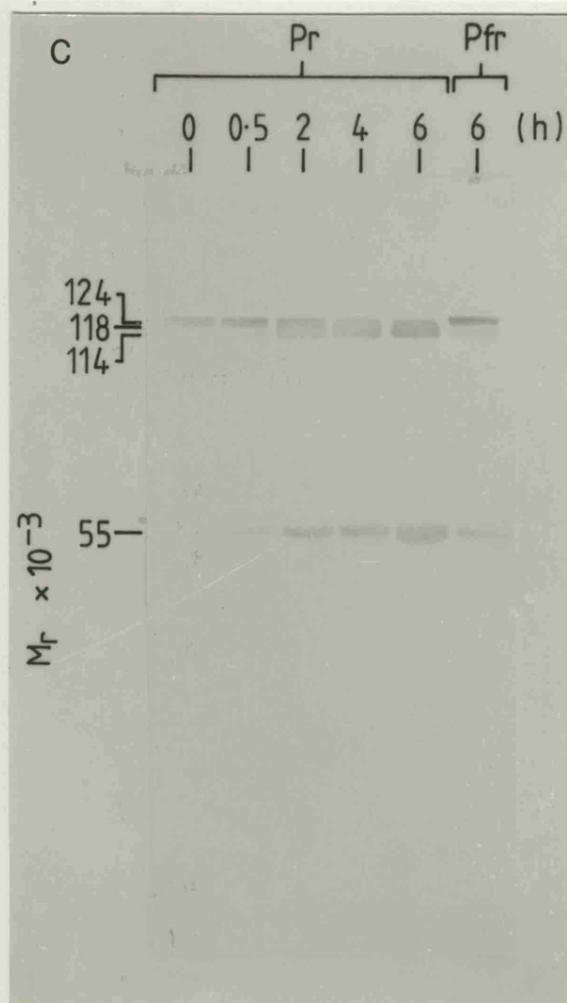
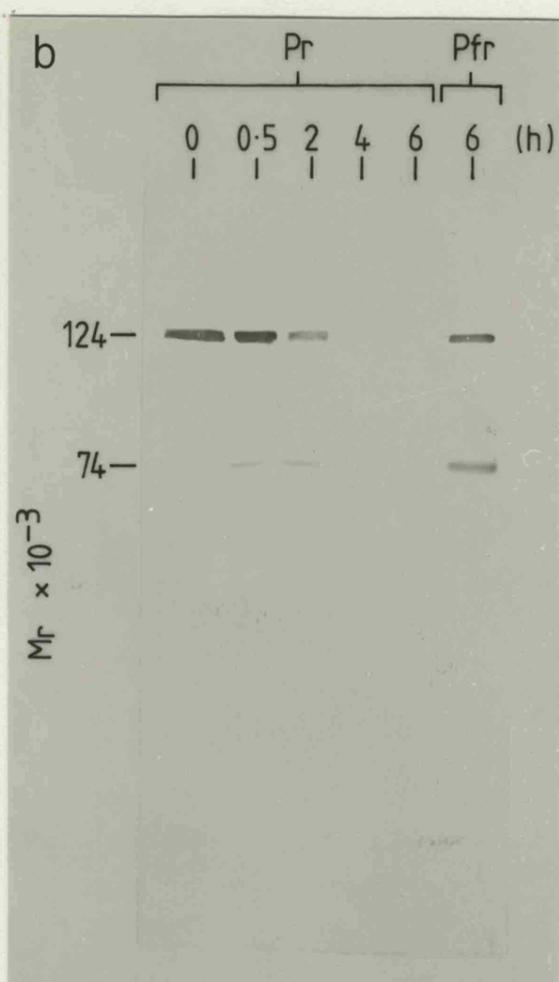
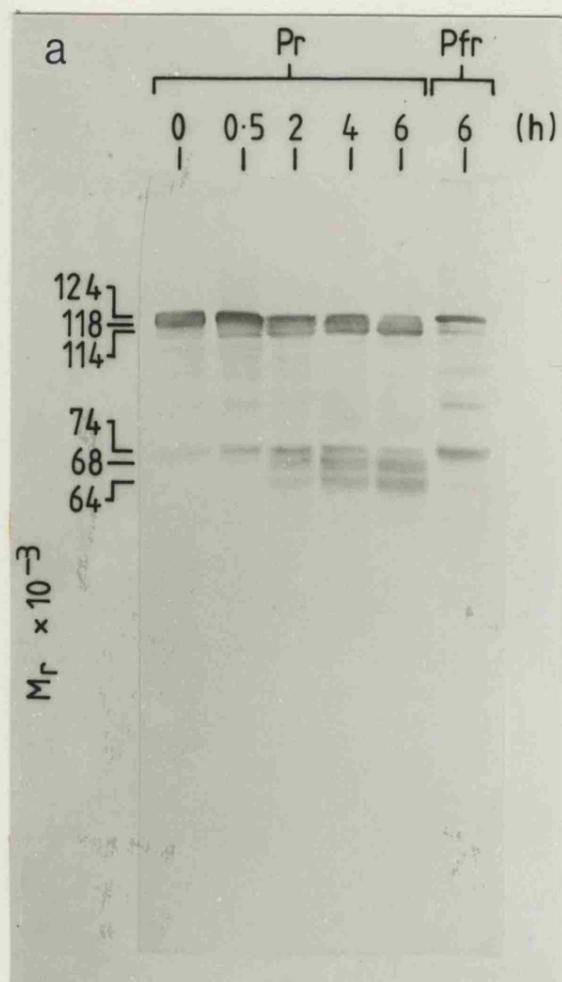


Figure 4.2 Mapping of mAbs to proteolytic fragments derived from digestion of Pr or Pfr by endogenous proteases

Samples were prepared, electrophoresed and electroblotted exactly as described in fig. 4.1. Individual blots were probed with the mAbs a) LAS 11, b) LAS 31, c) LAS 32, d) LAS 33, e) LAS 35, f) LAS 41, g) non-immune serum, h) protein stain.



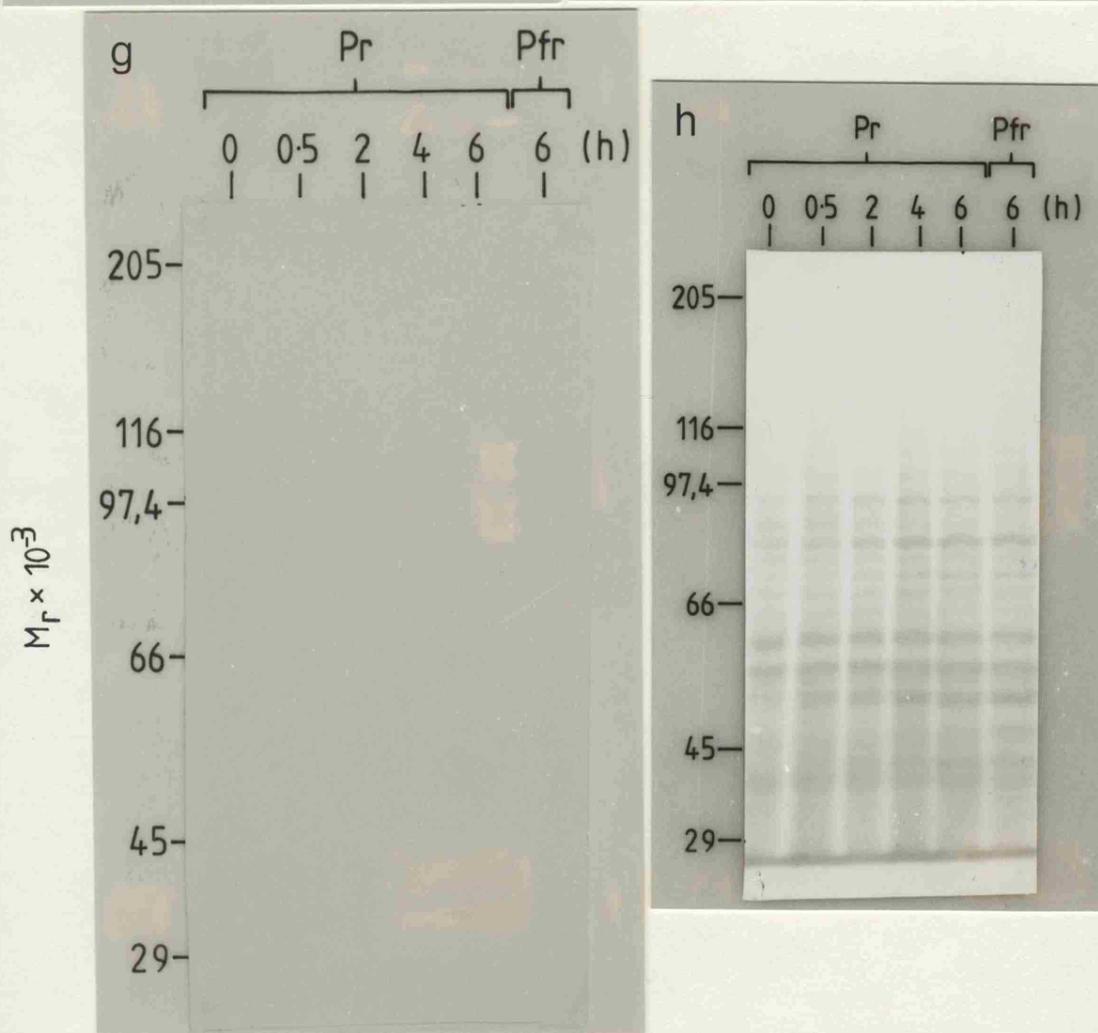
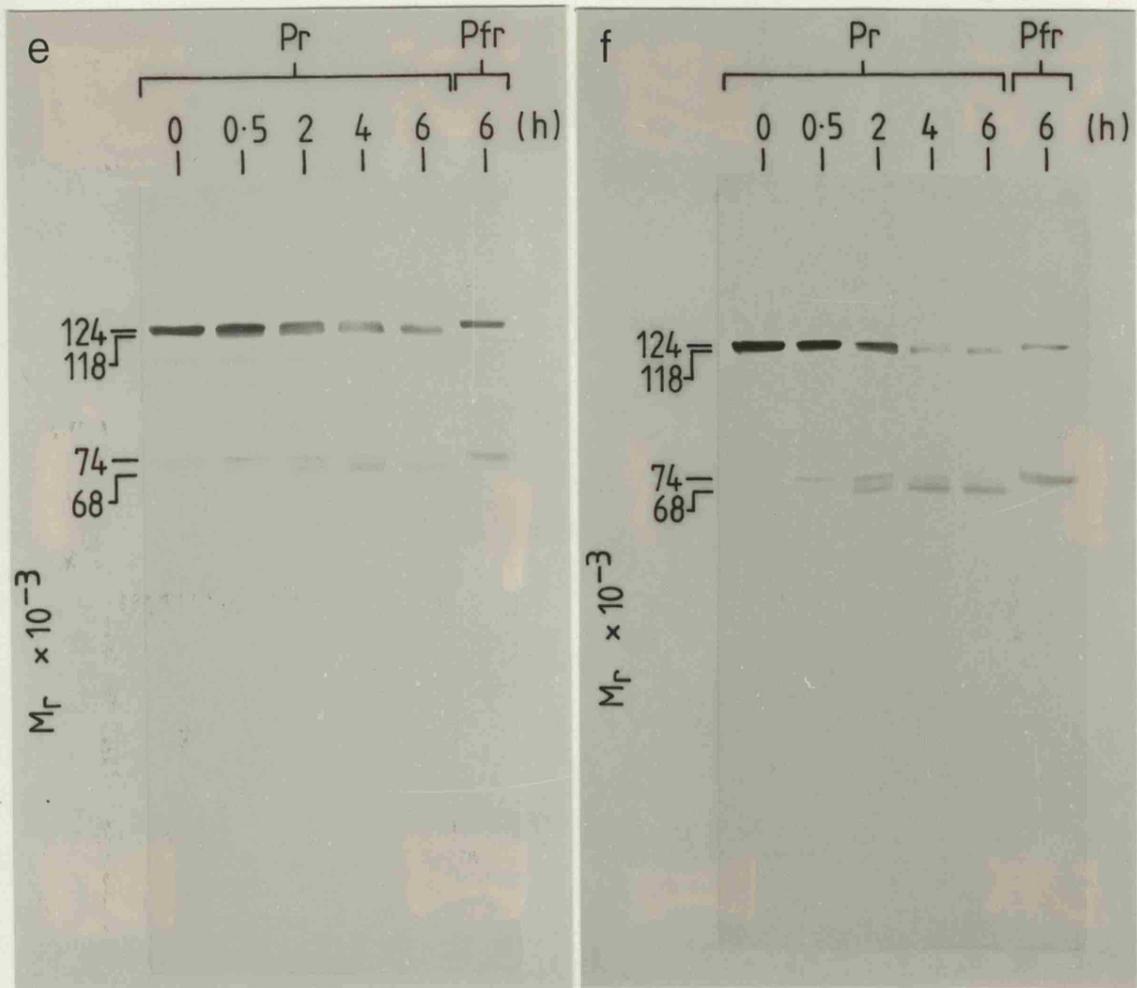


Figure 4.3 Digestion of pure 124 kDa Pr or Pfr by endogenous proteases from a crude extract of etiolated oat shoots

35 µg of pure 124 kDa Pr or Pfr was incubated with a crude extract of etiolated oat shoots (irradiated with red or far-red light) at 25C for up to 8 h. Aliquots were removed at the indicated times, heated in SDS-sample buffer and resolved on an 8% SDS-gel. (500 ng per lane t = 0) and electroblotted onto nitrocellulose. The blot was probed with a mixture of mAbs raised against 124 kDa oat phytochrome.

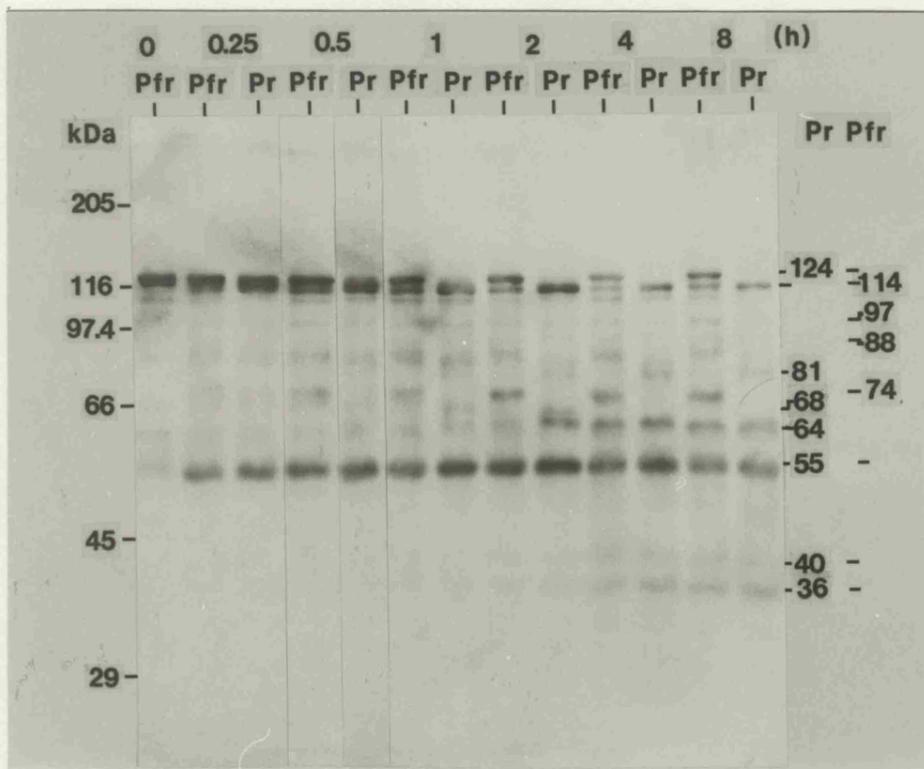


Figure 4.4 Mapping of mAbs to proteolytic
fragments derived from digestion of Pr or
Pfr by endogenous proteases

Samples were prepared, electrophoresed and electroblotted exactly as described in fig. 4.3. Individual blots were probed with a) LAS 31, b) LAS 32.

Type 1, 2 and 3 mAb. Following conjugation the respective mAbs were tested for their ability to still recognise phytochrome and whether they had been successfully labelled with biotin (Fig 4.5). As a consequence of this screen LAS 32 and 31 were used at a dilution of 1/1000 and LAS 11 which exhibits much lower reactivity at 1/200.

In the mapping ELISA the phytochrome fragments bound by the polyclonal antibodies were recognised by LAS 11 and LAS 32. LAS 31 did not give a positive signal (Fig 4.6 d). Immunoblotting of the crude extract used here demonstrated that the major peptides present were the 74, 64 and 55 kDa species (Fig 4.7). The fragments bound in the ELISA by LAS 21, 34 and 42 are only recognised by LAS 11 (Fig 4.6 a, b, c). LAS 21, 34 and 42 also positively bound partially pure 124, 118 and 114 kDa phytochrome.

4.3.3 Competition analyses

An ELISA designed to test whether two mAbs can bind simultaneously to Pr i.e. whether they compete (c.f. Cordonnier et al 1985), indicate that LAS 11, 21, 32, 34 and 42 only compete with themselves (Fig 4.8 a, b, d, f, i; Table 4.1). LAS 31 and 33 compete with ~~one another~~, but do not compete with any of the other mAbs (Fig 4.8 c, e; Table 4.1). LAS 35 and 41 also compete in either direction with one another almost as well as they compete with themselves but do not compete with any of the other mAbs (Fig 4.8 g, h; Table 4.1).

Figure 4.5 Screen for biotinylation of
LAS 11, 31 and 32 in ELISA

LAS 11, 31 and 32 were added to immobilised 124 kDa oat phytochrome. The extent of biotinylation was assessed following the addition of avidin peroxidase.

(○) LAS 32, (●) LAS 31, (□) LAS 11.

Each reading is the average of three replicate assays from a single experiment.

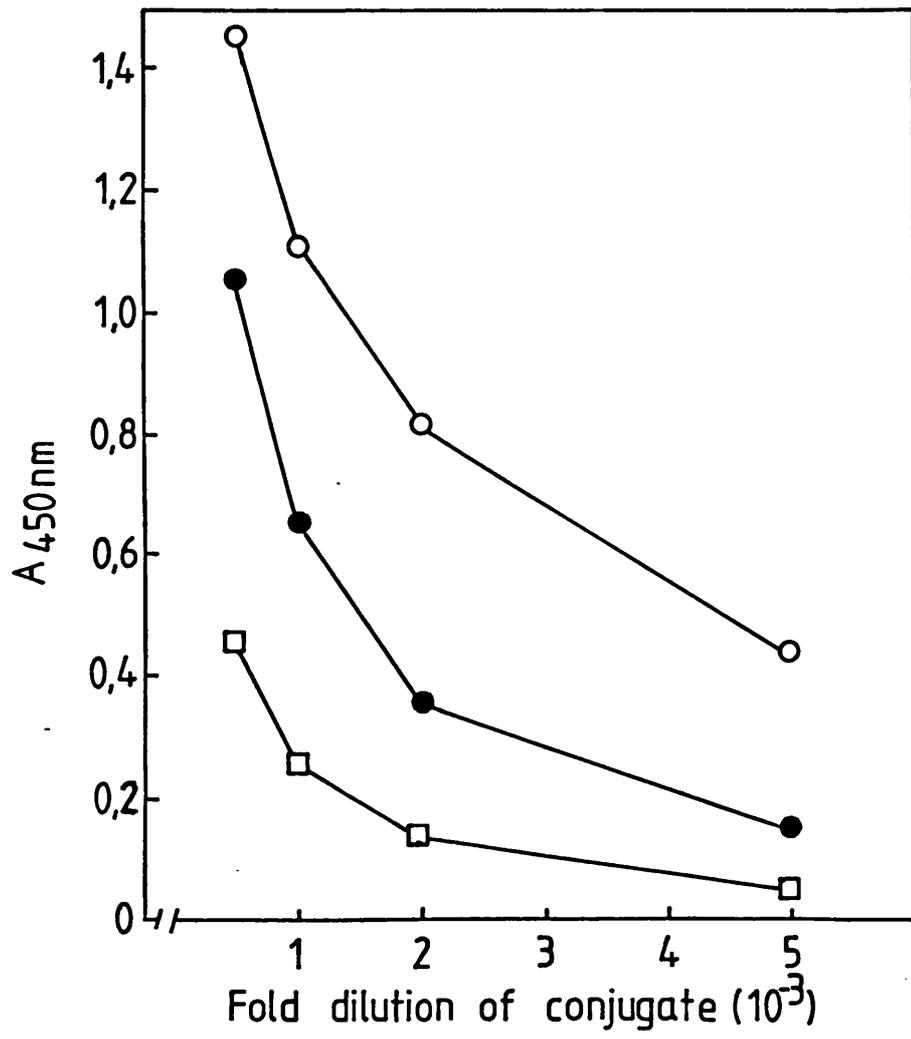


Figure 4.6 Recognition of proteolytically
derived fragments of Pr and Pfr in ELISA
by conformation-specific mAbs

Proteolytic digests of Pr (mainly 64 and 55 kDa polypeptides) or Pfr (mainly 74 and 55 kDa polypeptides) were added to immobilised LAS 21, 34, 42 or polyclonal anti-phytochrome antibodies (pAb). Additionally, samples of 124 kDa or 118 + 114 kDa oat phytochrome were added to immobilised mAb. Bound phytochrome was detected with a biotinylated Type 1, Type 2 or Type 3 mAb after subsequent incubation with avidin peroxidase.

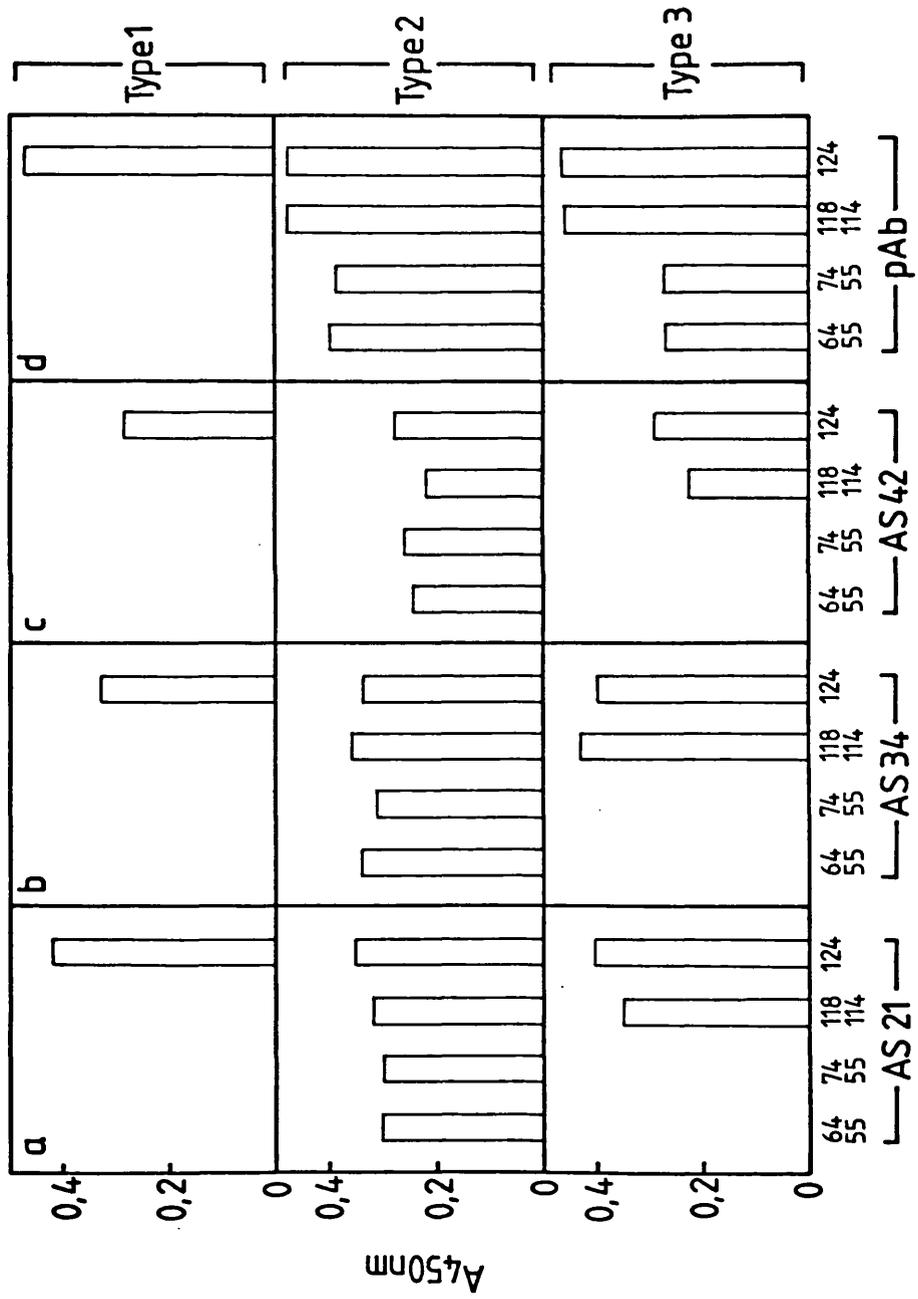


Figure 4.7 Immunoblot of proteolytic digests of Pr or Pfr used to map conformation-specific mAbs

Following incubation of crude oat extracts containing Pr or Pfr for 18 h at 25C, samples were taken and resolved on an 7.5% SDS- gel . Following electroblotting onto nitrocellulose, the phytochrome peptides were visualised with a mixture of mAbs.

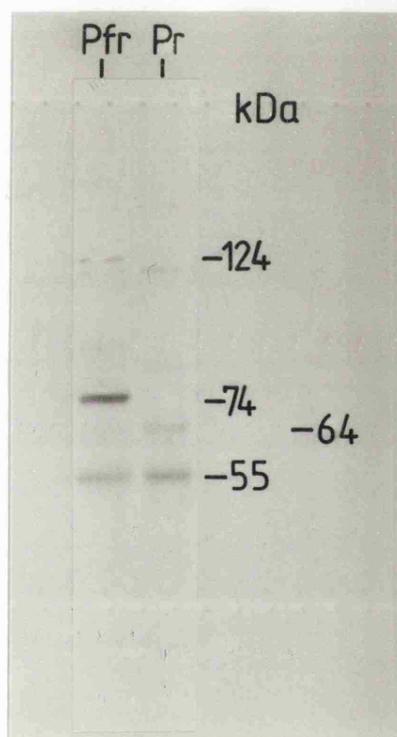


Figure 4.8 Competition among mAbs for binding to 124 kDa oat phytochrome as assayed by ELISA

12 $\mu\text{g ml}^{-1}$ of mAb 1 was bound to immobilised anti-mouse IgG₁ on the ELISA plate. Mixtures of Pr and dilutions of mAb 2 were added to the bound mAb 1. Bound phytochrome was detected with rabbit polyclonal anti-phytochrome antibodies. When mAb 2 was omitted from the assay, inhibition was set at 0%, when mAb 1 was omitted, inhibition was set at 100%. mAb 1:

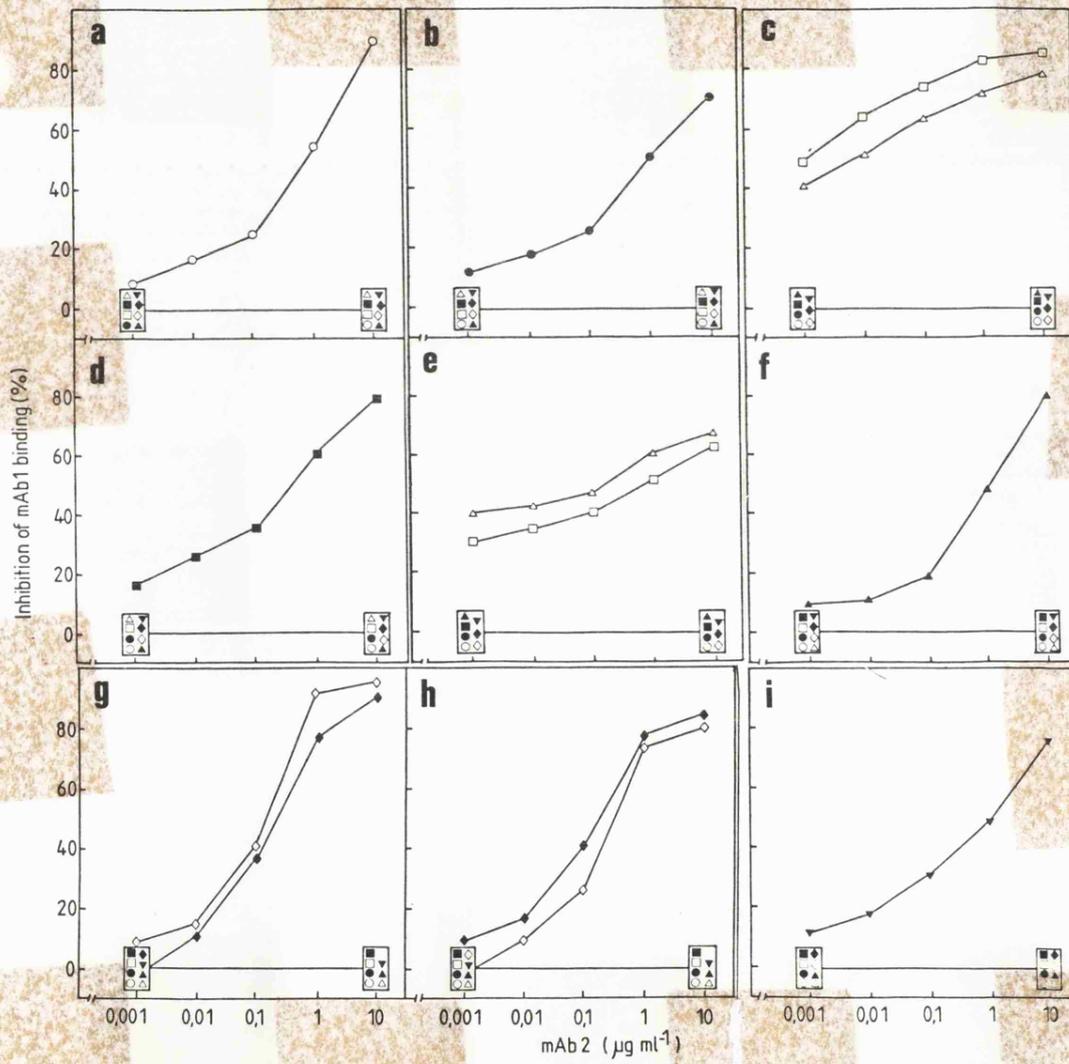
(a) LAS 11, (b) LAS 21, (c) LAS 31,
(d) LAS 32, (e) LAS 33, (f) LAS 34,
(g) LAS 35, (h) LAS 41, (i) LAS 42.

mAb 2:

(○) LAS 11, (●) LAS 21, (□) LAS 31,
(■) LAS 32, (△) LAS 33, (▲) LAS 34,
(◇) LAS 35, (◆) LAS 41, (▼) LAS 42.

Each reading is the average of three replicate assays from a single experiment.

Actual values for 0% inhibition range from 0-2% inhibition across the concentration of mAb tested.



4.4 Discussion

4.4.1 Epitope mapping mAbs which recognise SDS-denatured phytochrome

The domains to which mAbs that recognise SDS-denatured phytochrome bind, were determined by immunoblotting of peptides generated by endogenous proteases (c.f. Daniels and Quail 1984). LAS 31 and 33 immunostain the characteristic array of peptides which are diagnostic of mAbs raised to the 6 kDa NH₂-terminus i.e. they only immunostain the 124 and 74 kDa peptides. In accordance with the designations of Daniels and Quail (1984) LAS 31 and 33 are therefore classified as Type 1 mAbs. LAS 35 and 41 recognise an epitope located on the 4 kDa domain adjacent to the 6 kDa NH₂-terminal fragment. The assignment of LAS 35 and 41 to this domain derives from their ability to immunostain 124, 118, 74 and 68 kDa peptides. The 118 kDa peptide is assumed to result from a cleavage at the NH₂-terminus of the phytochrome monomer and the 68 kDa peptide is assumed to be derived from a similar NH₂-terminal cleavage of the 74 kDa peptide, which itself has an intact NH₂-terminus (Jones et al 1985). LAS 41 and 35 do not immunostain the 114 and 64 kDa peptides which are assumed to result from a further sequential cleavage at the NH₂-terminus of the 118 and 68 kDa peptides, respectively (Jones et al 1985). Grimm et al (1987) have recently reported that digestion of phytochrome by endogenous proteases can generate 118 and 114 kDa peptides, which have been clipped at both the NH₂-

and COOH-terminus. This may mean that LAS 31, 33, 35 and 41 could map to either the NH₂- or COOH-terminus. However, with respect to epitope mapping these mAbs, the diagnostic feature is the recognition in the case of LAS 31 and 33 of the 74 kDa peptide, and in the case of LAS 35 and 41 the recognition of the 74 kDa and 68 kDa peptides. Since the 74 kDa peptide has spectral properties which are identical to those of 124 kDa phytochrome, Jones et al (1985) conclude that it must carry an intact NH₂-terminus. Therefore, LAS 31, 33, 35 and 41 must bind to an epitope located close to the NH₂-terminus. In the original classification of mAbs described by Daniels and Quail (1984) those binding to the 6 kDa NH₂-terminal domain were classified as Type 1. Since no mAbs were found to bind to the adjacent 4 kDa domain such mAbs cannot have been characterised, but will be designated Type 1' here, in order to distinguish the binding sites of LAS 31 and 33 from 35 and 41. LAS 35 and 41 are therefore Type 1' mAbs.

LAS 11 immunostains all the phytochrome peptides greater than 60 kDa, which is characteristic of a mAb binding to the 64 kDa chromophore-bearing domain. LAS 11 therefore maps to an antigenic determinant on the 64 kDa chromophore-containing region of the molecule. The ability to map the epitope to which LAS 11 binds to a region within this rather large domain, is hampered by the fact that this domain represents the proteolytic resistant core of the

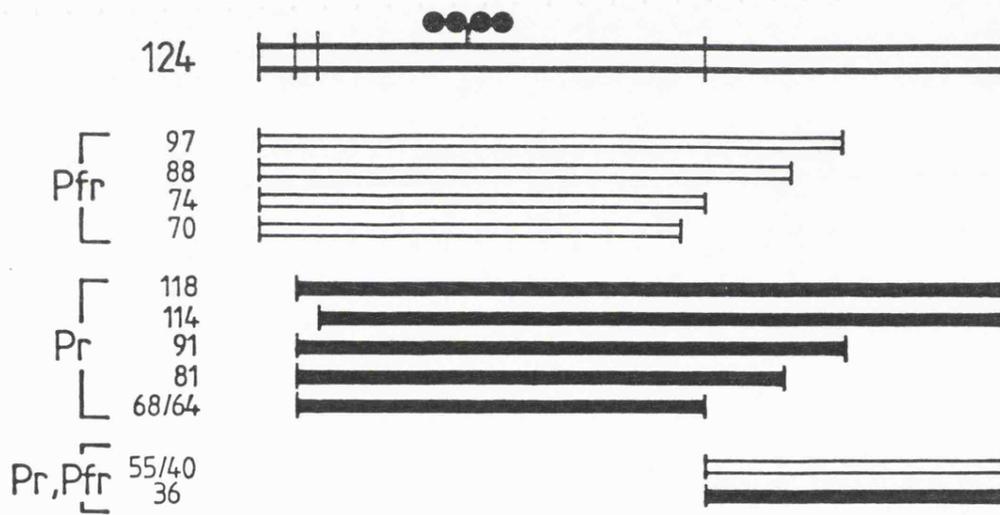
molecule. This domain carries no Pr/Pfr preferred cleavage sites for endogenous proteases. However, Lagarias and Mercurio (1985) have demonstrated that there is a Pr-preferred thermolysin cleavage site within 14 kDa of the NH₂-terminus and a Pr-preferred subtilisin BPN' cleavage site within 41 kDa of the NH₂-terminus. The subtilisin cleavage site effectively cuts the 64 kDa chromophore-bearing domain in two where one half contains the chromophore, which facilitates the orientation of the two similarly sized peptides. The chromophore-bearing peptide could be detected by zinc induced chromophore fluorescence (Berkelman and Lagarias 1986). Thus, LAS 11 could be mapped to a ~30 kDa domain. If the epitope to which LAS 11 binds lies close to the chromophore, it may be possible to more precisely map this mAb by generating small chromopeptides. Extensive digestion of phytochrome by subtilisin generates a 16 kDa chromopeptide (Jones and Quail 1986b). This peptide lies on the NH₂-terminal side of the chromophore approximately between amino acid residues 190-330 (Jones and Quail 1986b). Alternatively, if LAS 11 is not raised to an epitope close to the chromophore it would not be possible to precisely map LAS 11 to a region smaller than ~30 kDa, due to the problems of orientating peptides derived from further proteolytic cleavage of this domain. Due to the potential difficulties in more precisely mapping LAS 11 and the fact that LAS 11 does not bind to an epitope of apparent significance, further

attempts to locate the epitope to which this mAb binds were not undertaken.

LAS 32 immunostains 124, 118, 114 and 55 kDa species. It does not immunostain the 74, 68 and 64 kDa peptides. This mAb therefore is raised to an epitope on the COOH-terminal-half of the molecule and is classified as a Type 3 mAb. However, using these key proteolytic fragments it is not possible to identify whereabouts LAS 32 binds within this 55 kDa domain. Since LAS 32 is a particularly interesting mAb (see section 6.3, 6.4) a second digest was conducted so that the less abundant proteolytic peptides could be visualised, with the attendant possibility that the epitope to which LAS 32 binds could be more precisely mapped. LAS 32 recognises the 97 kDa and 88 kDa peptides which carry an intact NH₂-terminus, as indicated by the immunostaining of these peptides by LAS 31, a Type 1 mAb. The 88 kDa peptide is derived from the 97 kDa peptide through a cleavage(s) at the COOH-terminus. Therefore, the recognition of the 97 kDa and 88 kDa peptides establishes that the epitope to which LAS 32 is raised maps to a region between 74 and 88 kDa from the NH₂-terminus (Fig 4.9; (Daniels and Quail 1984)). The immunostaining of the 40 kDa and 36 kDa peptides by LAS 32 suggests that these peptides may not have an intact COOH-terminus.

Of the mAbs which recognise SDS-denatured phytochrome 67% are Type 1 or Type 1', 16.5% are Type 2 and 16.5% Type 3. If the generation of mAbs

Figure 4.9 Schematic representation of peptides generated following digestion of Pr or Pfr by endogenous proteases



to the different regions of the molecule is random, then there are a disproportionate number of mAbs raised to the NH₂-terminus of the molecule. This finding, which suggests that the NH₂-terminal segment of the molecule is immunodominant has also been observed by Daniels and Quail (1984). However, Hopp-Woods (1983) analysis, which predicts the presence of protein antigenic determinants from amino acid sequences, does not predict the presence of an antigenic determinant on the NH₂-terminal fragment. This suggests that the antigenicity of this region is determined by the tertiary conformation.

4.4.2 Epitope mapping of conformation-specific mAbs

Several parameters determine the success of mapping mAbs raised to the native conformation of the molecule. Chiefly it is necessary that the peptide fragments generated on proteolysis retain their native conformation. It is likely that this assumption is correct for the 74 kDa peptide as it retains the spectral characteristics of native 124 kDa oat phytochrome (Jones et al 1985). In addition it is necessary that the epitope to which the mAbs under analysis bind does not span a proteolytic cleavage site.

Initial attempts to map the conformation-specific mAbs were unsuccessful. Early protocols involved the immobilisation of the mAb under analysis and the subsequent immunodetection of the peptides which had been bound by the mAb on immunoblots. Initially, mAbs were immobilised onto nitrocellulose discs.

This system provides a good reactive surface area for the binding of the mAb and therefore the subsequent binding of the proteolytic fragments. Following extensive washing after incubation with the peptide fragments the bound contents on the disc were boiled in SDS- sample buffer, subjected to SDS-PAGE and electroblotted onto nitrocellulose. Immunodevelopment of the blot with polyclonal antibodies to phytochrome failed to immunostain any phytochrome peptides. This suggests that insufficient phytochrome is being bound by the mAb for subsequent detection on immunoblots, or that each mAb is raised to an epitope which spans a cleavage site. As the latter possibility seemed unlikely this procedure was modified to increase the surface area of bound mAb, by binding the mAb to polyvinyl ELISA plates. However, again the immunodevelopment of the blots failed to immunostain any phytochrome peptides. Clearly it was not possible to bind the phytochrome in sufficient amounts so that following SDS-PAGE and immunoblotting the peptides can be immunostained, although preliminary experiments had indicated that it was possible to remove bound proteins from nitrocellulose and ELISA plates by boiling in SDS-sample buffer. An alternative strategy therefore was adopted which incorporates the use of a sandwich ELISA which is a much more sensitive assay than immunoblotting.

The success of the method described here is dependent on the use of mAbs which can be mapped due to their ability to recognise SDS-denatured

phytochrome. These mAbs are used as specific probes for the phytochrome bound by the conformation-specific mAbs. As it is necessary that both the conformation-specific mAb and the second diagnostic mAb can bind to the phytochrome at the same time, a further requirement of this assay is that the mAbs under analysis do not compete (see 4.4.3). However, had the conformation-specific mAbs competed with the mAbs which can be mapped conventionally, then this could have given an indication of the region to which the test mAb was binding.

In principle the mapping ELISA involves the immobilisation onto polyvinyl ELISA plates of the mAbs to be mapped. To this, proteolytically derived peptides generated in crude extracts as Pr or Pfr (containing major peptides of 74, 64 and 55 kDa) are added. Following incubation and removal of any unbound phytochrome peptides, bound phytochrome peptides are probed with a Type 1, 2 or 3 mAb which has been conjugated with biotin. Only if the mAb under analysis has bound phytochrome peptides which also carry the epitope to which the second mAb can bind is there a positive signal. In addition to the peptide fragments, 124 kDa and a mixture of 118 + 114 kDa phytochrome were also tested.

Using this assay design each of the three mAbs under analysis was found to bind peptide fragments that only the Type 2 mAb could subsequently detect. All the mAbs under analysis also bound the 124 and 118 + 114 kDa peptides which could then be bound by

the Type 2 or Type 3 mAb and in the case of 124 kDa phytochrome by the Type 1 mAb. From this pattern of reactivity it is possible to deduce that each of the three mAbs under analysis binds to the 64 kDa domain.

This is the first demonstration of epitope mapping mAbs which only recognise the native conformation of the phytochrome molecule. The ability to map conformation-specific mAbs increases the potential usefulness of these mAbs in structure function analyses which have been confined to the use of mAbs that recognise putative regions of primary sequence on the molecule. In addition as it is possible to map conformation-specific mAbs, further amendments have to be made to the classification described by Daniels and Quail (1984). The domain to which Type 4 mAbs bind will be designated by a domain specific second number i.e. all the Type 4 mAbs described here are Type 4.2 mAbs.

4.4.3 Competition analyses

Competition analyses were conducted with Pr as several mAbs exhibit differential affinities for the two forms of phytochrome, and with the exception of LAS 42 (which does not exhibit a large affinity difference) all the mAbs have a higher affinity for Pr.

Competition analyses demonstrate that LAS 11, 21, 32, 34 and 42 do not compete with any of the other mAbs but only compete with themselves. These mAbs are therefore raised against unique epitopes that are spatially separated either in terms of primary

sequence or tertiary conformation. Analysis based on the size of an antibody arm has estimated that epitopes recognised by two simultaneously binding mAbs are at least 3.5 nm apart (Tzartos et al 1981). LAS 11, 21, 34 and 42 all map to the 64 kDa chromophore-bearing domain and therefore sites to which these mAbs bind must be distributed throughout this domain. The lack of competition in either direction, between these mAbs and the Type 1, 1' and 3 mAbs, which are raised to spatially distant regions of primary sequence, suggests that in the native conformation the epitopes to which the respective Type 1, 1' and 3 mAbs bind do not come to lie close to one another, or to those epitopes recognised by the Type 2 mAbs.

The binding of LAS 33 and 31 (Type 1 mAbs) is mutually exclusive with each mAb competing with each other nearly as well as they compete with themselves. Therefore, these mAbs must be recognising the same or an overlapping epitope. A further possibility that the binding of the first mAb may induce a conformational change which prevents the subsequent binding of the second mAb is less likely, as there have been no reports in which binding of one mAb has been shown to induce conformational changes which result in masking a spatially distant epitope recognised by a second mAb (Wilson and Smith 1984). However, further characterisation of LAS 31 and 33 concerning the effect of binding of these mAbs on the spectral properties of

124 kDa oat phytochrome (see section 5.3, 5.4) clearly indicate that they are not raised to the same, but recognise an overlapping epitope. LAS 31 and 33 do not compete, in either direction with any of the other mAbs tested.

The binding of LAS 35 and 41 (Type 1' mAbs) is also mutually exclusive, but these mAbs do not compete with any of the other mAbs tested. Competition between LAS 35 and 41 therefore suggests that these mAbs are mapping to the same or an overlapping epitope. However, further characterisation of these mAbs concerning differential binding to Pr and Pfr and the effect of binding of the respective mAbs on the spectral properties of phytochrome, demonstrate that they are recognising distinct but overlapping epitopes.

Although the Type 1 and 1' domains lie adjacent to one another on the primary sequence there is no competition between the mAbs which bind to the respective domains. This observation suggests that although they lie next to one another on the primary sequence there is no direct interaction between these domains in the native conformation.

4.4.4 Summary

LAS 31 and LAS 33 are Type 1 mAbs which recognise a distinct but overlapping epitope on the 6 kDa NH₂-terminal domain. LAS 35 and 41 are Type 1' mAbs which recognise unique but overlapping epitopes on the 4 kDa sub-NH₂-terminal domain. LAS 11 is raised against an epitope on the 64 kDa chromophore-

containing peptide and therefore is designated a Type 2 mAb. LAS 21, 34 and 42 are Type 4 mAbs which also recognise epitopes determined by the native conformation on the 64 kDa domain and are therefore classified as Type 4.2 mAbs. LAS 32 recognises an epitope which lies between 74 and 88 kDa from the NH₂-terminus. LAS 11, 21, 32, 34 and 42 do not compete with one another or with any of the other mAbs, and therefore are raised to unique, spatially distinct epitopes.

CHAPTER 5

DIFFERENTIAL BINDING OF MONOCLONAL
ANTIBODIES TO Pr AND Pfr

5.1 Introduction

One approach used in efforts to identify molecular differences between Pr and Pfr has been the application of immunochemical techniques, initially with conventional polyclonal antisera and more recently with mAbs. Although an initial study with polyclonal antisera apparently identified differences between Pr and Pfr (Hopkins and Butler 1970), subsequent analyses have failed to confirm this observation (Cundiff and Pratt 1975; Rice and Briggs 1973). These latter findings are not entirely unexpected as polyclonal antisera are known to be composed of a heterogeneous population of antibodies raised against different antigenic determinants over the whole molecule. Such antisera are therefore unlikely to identify the local, rather than gross, changes which are believed to occur on photoconversion of phytochrome (Song 1985; see section 1.3.2). The specificity and defined nature of mAbs offer a potential means of overcoming these limitations. Several groups have adopted this approach using mAbs raised against oat, pea and rye phytochromes (Cordonnier et al 1984; 1985; Shimazaki et al 1986; Thomas et al 1984b; Thomas and Penn 1986; Nagatani et al 1983; 1984).

Early attempts to isolate mAbs which exhibit differential affinities for Pr and Pfr were unsuccessful (Cordonnier et al 1984; Nagatani et al 1983; 1984). This lack of success probably stems from methodological problems, rather than the

production of mAbs which intrinsically could not discriminate between Pr and Pfr (Cordonnier et al 1985). Initial assays for determining differential affinities for the two forms involved immobilising the phytochrome, following irradiation with saturating red or far-red light, on polystyrene microtitre plates (Cordonnier et al 1984) or on the surface of sheep red blood cells (Nagatani et al 1983; 1984). However, it is known that proteins become denatured when bound to polystyrene plates (Smith and Wilson 1986). Hence, immobilising phytochrome in such a way may mask the conformational changes which occur on photoconversion. Accordingly, Thomas et al (1984b) adopted an alternative approach. Instead of immobilising phytochrome directly on the assay plate, it was immobilised by polyclonal anti-phytochrome antibodies, before probing with mAbs. Thus, differential affinities for Pr and Pfr are determined by assaying directly the amount of mAb bound to phytochrome. Using this assay, Thomas et al (1984b) described two mAbs which showed a higher affinity for Pr than Pfr and one which had a higher affinity for Pfr. Although Cordonnier et al (1985) pointed out potential methodological inadequacies with this assay design Thomas and Penn (1986) subsequently proved that this assay does detect antibodies which recognise intrinsic differences between Pr and Pfr. However, Thomas et al (1984b) have made no attempt to quantify the differences in affinity of mAbs for Pr and Pfr

that they observe. This is probably due to the fact that the titration curves they produce are not parallel, making it difficult to draw such conclusions.

Cordonnier et al (1985) have developed an alternative ELISA assay which identifies mAbs that recognise intrinsic differences between Pr and Pfr. In this assay phytochrome is added as Pr or Pfr to an immobilised test mAb, and so the native conformation of the molecule is maintained. The differential binding of mAbs to Pr and Pfr is measured by assaying the amount of mAb-bound phytochrome, with phytochrome specific polyclonal antibodies. Using this configuration of assay Cordonnier et al (1985) have isolated three mAbs which show a higher affinity for Pr and Shimazaki et al (1986) have reported two mAbs which have a higher affinity for Pfr.

In addition to conducting quantitative ELISAs to determine differential affinities for Pr or Pfr, spectral analyses conducted in the presence of mAbs which discriminate between Pr and Pfr, may delineate regions of the molecule which are crucial for the maintenance of the spectral integrity of the molecule. Cordonnier et al (1985) have demonstrated that the binding of a mAb, which exhibits a higher affinity for Pr, to the 6 kDa NH₂-terminus disrupts normal protein-chromophore interactions. Binding of this mAb alters the spectral characteristics of the phytochrome so that they resemble those of partially

degraded "large" phytochrome, although these changes occur in the absence of proteolysis. Binding of mAbs which exhibit a higher affinity for Pfr and map to the 64 kDa chromophore-bearing domain do not induce any alterations in the spectral characteristics of 124 kDa phytochrome (Shimazaki et al 1986).

In this chapter the nine mAbs under investigation were tested for differential affinities towards Pr or Pfr. In addition, the effect of the presence of these mAbs on the spectral characteristics of 124 kDa oat phytochrome were investigated. In particular the isolation of LAS 35 and 41 Type 1' mAbs allowed an assessment, for the first time, of the contribution of the 4 kDa sub-NH₂-terminal domain in the maintenance of the spectral integrity of the molecule. On the basis of findings from assays which determine differential binding of mAbs to Pr or Pfr and from the effect of mAbs on the spectral characteristics of phytochrome, a mAb which is specific for Pr has been identified. This Pr-specificity has been exploited to produce a Pfr solution which is free of contaminating Pr. This mAb has also been applied to the development of an ELISA assay for phytochrome photoequilibrium. In addition this Pr-specificity has enabled a comparison of different assay protocols which reveals a major inconsistency in the evaluation of mAbs with differential affinities for the two forms of phytochrome.

5.2 Materials and Methods

5.2.1 Determination of differential affinities of mAbs for Pr and Pfr

5.2.1.1 ELISA 5.1

Flat bottomed 96-well vinyl plates (Dynatech) were coated with $5 \mu\text{g ml}^{-1}$ immunoaffinity-purified-rabbit polyclonal antibodies to 124 kDa oat phytochrome in 50 mM bicarbonate buffer, pH 9.2, overnight at 4C. The plates were then washed with PBS-Tween and remaining protein-binding sites blocked by filling the wells with PBS-Tween containing 3% (w/v) Marvel. During this time appropriate dilutions of the mAbs and phytochrome, (124kDa, SAR=0.91) as Pr (following saturating far-red irradiation) or Pfr (following saturating red-irradiation) were prepared in separate tubes and incubated in darkness at room temperature for 1 h. All remaining manipulations were conducted under dim green safelight, with the incubations in darkness. These preincubated mixtures were added to the assay wells, which had been washed with PBS-Tween, and then incubated for 1 h at room temperature. Following a further wash a commercial, peroxidase-conjugate of sheep anti-mouse antibodies (Sigma) was added at a dilution of 1/500 in PBS-Tween and the plate incubated for a further 30 min at room temperature. Bound peroxidase activity was visualised with $1 \mu\text{g ml}^{-1}$ TMB (Sigma) in 50 mM citrate acetate buffer, pH 6.

5.2.1.2 ELISA 5.2

Wells were coated overnight at 4C with goat anti-mouse IgG₁ at a concentration of 5 $\mu\text{g ml}^{-1}$ in 50 mM bicarbonate buffer, pH 9.2. Remaining protein-binding sites were blocked with PBS-Tween containing 3% (w/v) Marvel, for 30 min at room temperature. Following washing of the plates with PBS-Tween, mAb was added at a concentration of 5 $\mu\text{g ml}^{-1}$ in PBS-Tween and the wells incubated for 1 h at room temperature. All remaining manipulations were conducted under dim green safelight, with the incubations in darkness. Following a further wash phytochrome was added at the indicated concentrations as Pr (following saturating far-red irradiation) or Pfr (following saturating red irradiation), and the wells incubated for 1.5 h at 4C. Following washing, immunoaffinity-purified-rabbit polyclonal antibodies to 124 kDa oat phytochrome were added at a concentration of 5 $\mu\text{g ml}^{-1}$ in PBS-Tween containing 0.05% (w/v) Marvel and wells incubated for 1.5 h at 4C. Following a further wash with PBS-Tween a commercial, peroxidase-conjugate of sheep anti-rabbit antibodies (Sigma) was added at a 1/500 dilution in PBS-Tween and the wells incubated for a further 45 min at 4C. Bound peroxidase activity was visualised as described for ELISA 5.1.

5.2.1.3 ELISA 5.3

This third ELISA is a modification of ELISA 5.2. In brief, instead of detecting bound phytochrome with polyclonal antisera, bound phytochrome was

detected directly by the addition of a peroxidase-conjugate of LAS 32. Detection of bound peroxidase was as described above.

5.2.2 Photoreversible binding assay

Photoreversible binding of LAS 41 to Pr was tested using a modification of ELISA 5.1. In addition to incubations of LAS 41 with Pr or Pfr, samples of LAS 41 previously incubated with Pr were subsequently irradiated with combinations of red and far-red light. Following a further 1 h incubation of LAS 41 plus phytochrome in the dark at room temperature, the solutions of phytochrome and mAb were added to immobilised polyclonal antibody raised against 124 kDa oat phytochrome. The binding of LAS 41 to phytochrome was then assayed as described in ELISA 5.1.

5.2.3 Phytochrome photoequilibrium assay

The phytochrome photoequilibrium assay is a modification of ELISA 5.1. In brief, phytochrome solutions of different photoequilibria were generated in the spectrophotometer using subsaturating doses of actinic red light and incubated with LAS 41 for 1 h in the dark at room temperature. These mixtures were subsequently added to the immobilised anti-phytochrome polyclonal antibodies. Detection of bound LAS 41 was conducted as described in ELISA 5.1.

5.2.4 Immunoprecipitation

Antibodies were first complexed with fixed Staphylococcus aureus cells (Sigma), which had been

previously washed in 100 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and Aprotinin (20,000 kallikrein units l^{-1}), by tumbling end over end for 30 min at 4C. The complexes were pelleted, washed and resuspended in the above buffer. Phytochrome was added to the complexes as Pr or Pfr and the mixtures incubated for a further 30 min at 4C. The bound phytochrome was pelleted by centrifugation for 3 min at 12,000 x g. Phytochrome remaining in the supernatant was measured using an Aminco DW-2a uv/vis spectrophotometer.

5.2.5 Peroxidase conjugation of LAS 32

LAS 32 was conjugated with peroxidase using the periodate method according to Voller et al (1979).

5.2.6 Effect of mAbs on the spectral characteristics of 124 kDa oat phytochrome

Each sample consisted of 40 μ g of 124 kDa oat phytochrome (SAR = 0.91), and 120 μ g mAb in 1 ml 100 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and Aprotinin (20,000 kallikrein units l^{-1}). Phytochrome, as Pr or Pfr was incubated with mAb for 30 min at 4C prior to the start of the analyses. Phytochrome absorbance spectra were measured with an Aminco DW-2a uv/vis spectrophotometer with the cuvette chamber cooled to 3C. All actinic irradiations were empirically determined to be saturating. Dithionite-dependent dark reversion analysis was conducted according to Pike and Briggs (1972b). Sodium dithionite was added to 5 mM from a freshly prepared 0.5 M stock and the course of dark reversion

assayed.

5.2.7 Spectrophotometric Assays

Absorption spectra of pure Pfr (Pfr^P) were measured using a Perkin Elmer Lambda 5 (uv/vis) spectrophotometer. Phytochrome photoequilibria were measured with the Aminco DW-2a in the dual wavelength mode, with measuring beams set at 665 nm and 730 nm.

5.3 Results

5.3.1 Differential affinities of mAbs for Pr and Pfr

The 9 mAbs raised against 124 kDa etiolated oat phytochrome were screened for differential affinities towards Pr and Pfr. Using an assay configuration in which mAb and phytochrome are mixed together in free solution prior to incubation with immobilised polyclonal anti-phytochrome antibodies (ELISA 5.1), LAS 31, 33, 34, 35 and 41 exhibit a higher affinity for Pr (Fig 5.1). LAS 42 exhibits a higher affinity for Pfr and LAS 11, 21 and 32 react equally well with both forms of the molecule (Fig 5.1). The greatest affinity difference is exhibited by LAS 41 which has an apparent 7-8 fold higher affinity for Pr i.e. the Pfr titration curve is shifted to 7-8 fold higher phytochrome concentrations (Fig 5.1).

Using a second ELISA assay design (ELISA 5.2) in which the differential binding of mAbs to Pr and Pfr is measured by assaying the amount of mAb-bound phytochrome with phytochrome specific polyclonal antibodies, qualitatively, the same pattern of differential binding to Pr and Pfr is observed (Fig 5.2). However, in each case, with the exception of LAS 41, each mAb which exhibited a differential affinity for either Pr or Pfr in ELISA 5.1 exhibited a greater differential affinity with ELISA 5.2. In this assay LAS 33 exhibits the greatest affinity difference having an apparent 10 fold higher affinity for Pr (Fig. 5.2).

Figure 5.1 Differential affinities of mAbs
for Pr (○—○) and Pfr (●—●) as assayed by
ELISA 5.1

ELISA wells were coated with rabbit polyclonal anti-phytochrome antibodies. Dilutions of mixtures of mAb and phytochrome, as Pr (following saturating far-red irradiation) or Pfr (following saturating red irradiation) were added to immobilised rabbit polyclonal anti-phytochrome antibodies. The extent of mAb binding was assayed with peroxidase-conjugate of sheep anti-mouse antibodies.

- a = LAS 11
- b = LAS 21
- c = LAS 32
- d = LAS 31
- e = LAS 33
- f = LAS 35
- g = LAS 34
- h = LAS 41
- i = LAS 42

Each reading is the average of three replicate assays from a single experiment.

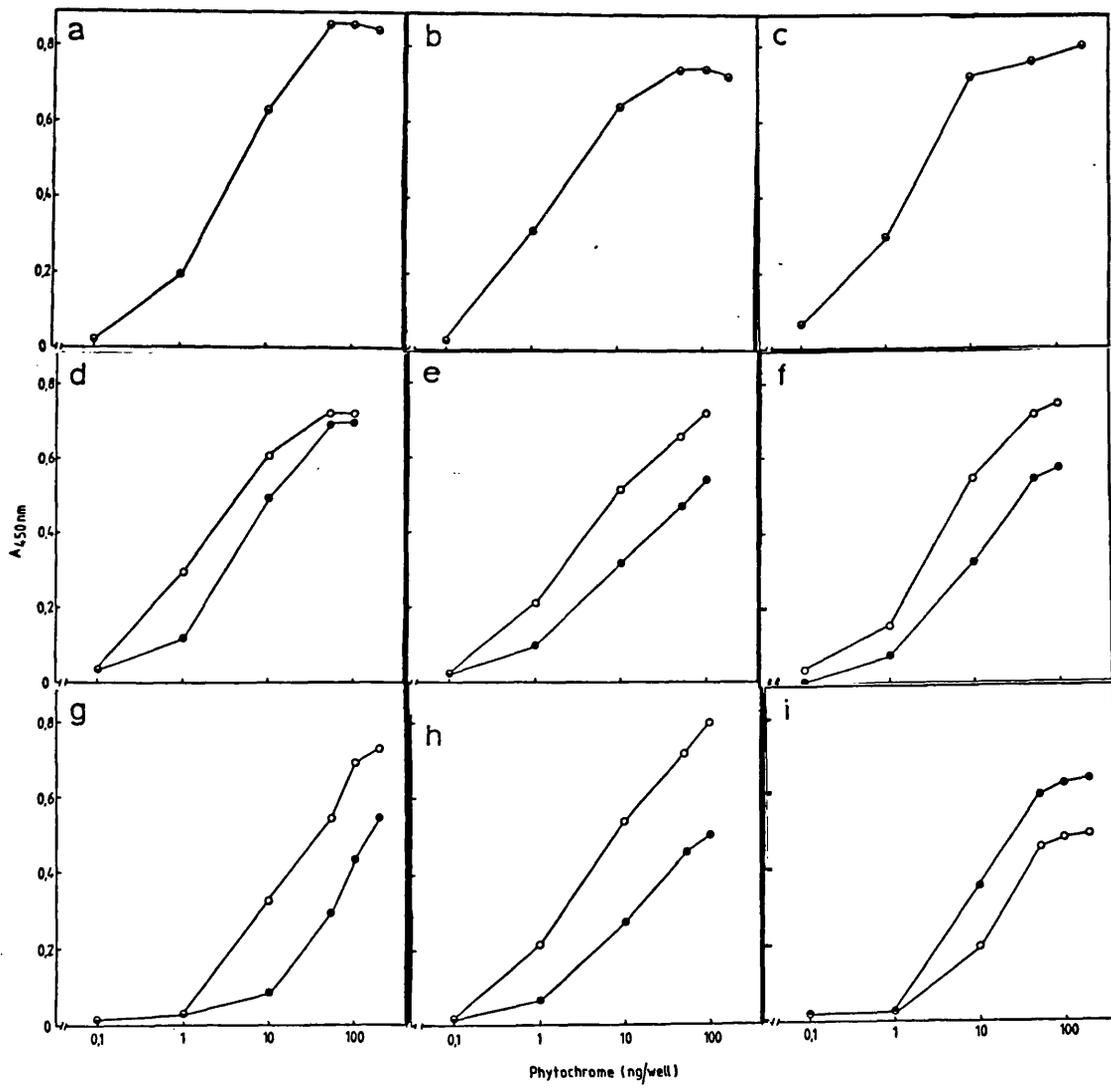
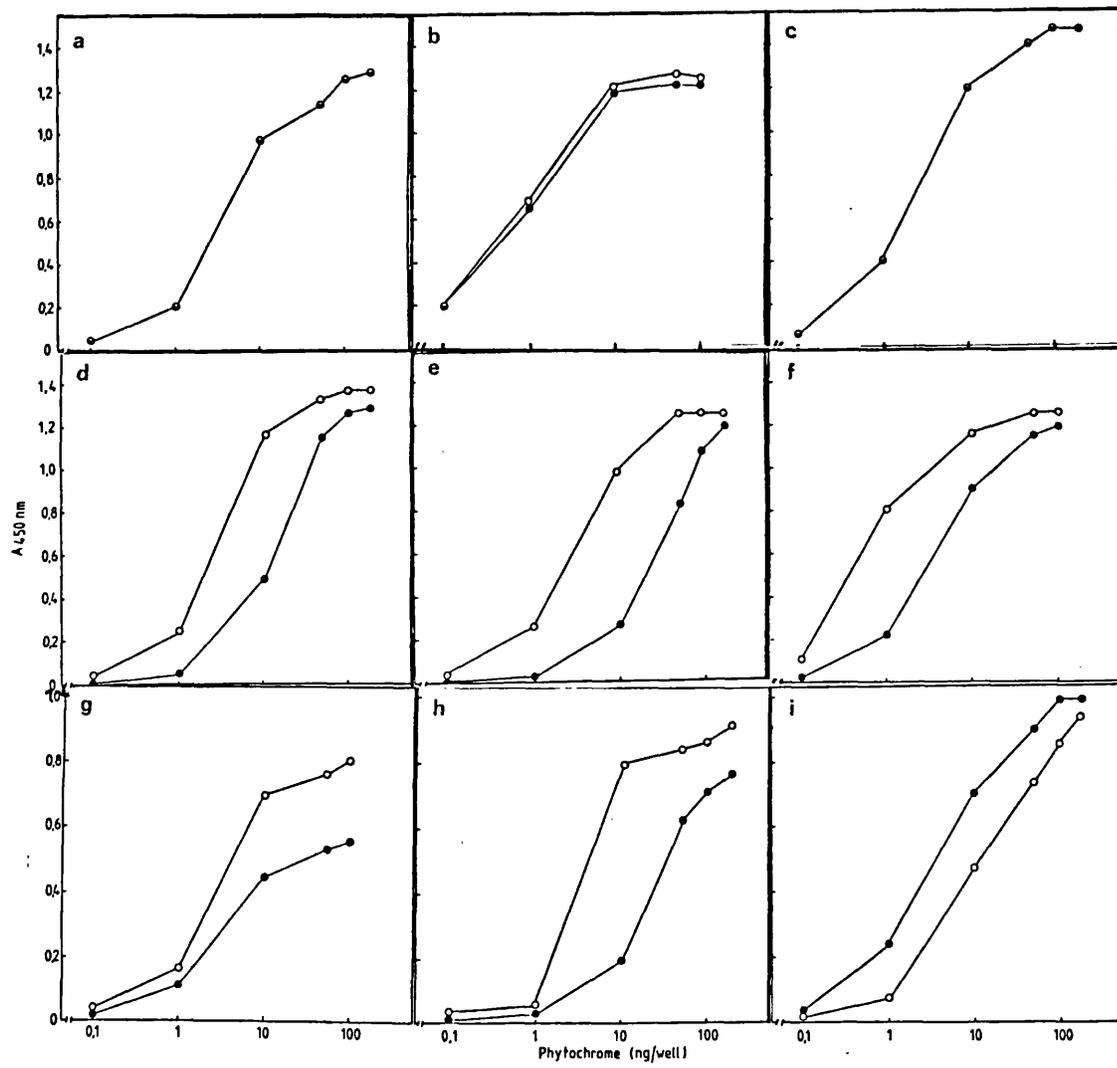


Figure 5.2 Differential affinities of mAbs
for Pr (○—○) and Pfr (●—●) as assayed by
ELISA 5.2

Phytochrome was added to the immobilised mAb at the indicated concentrations as Pr (following saturating far-red irradiation) or Pfr (following saturating red irradiation). Bound phytochrome was detected by the addition of immunoaffinity-purified rabbit polyclonal antibodies and a subsequent incubation with a commercial peroxidase-conjugate of sheep anti-rabbit antibodies.

- a = LAS 11
- b = LAS 21
- c = LAS 32
- d = LAS 31
- e = LAS 33
- f = LAS 35
- g = LAS 34
- h = LAS 41
- i = LAS 42

Each reading is the average of three replicate assays from a single experiment.



Differential binding to Pr and Pfr was also tested in a third ELISA (ELISA 5.3). In this case bound phytochrome was detected with a peroxidase-conjugate of LAS 32. LAS 31, 41 and 11 exhibit the same differential affinities for Pr and Pfr as observed using the ELISA 5.2 assay (Fig 5.3).

5.3.2 Immunoprecipitation analyses

In the case of LAS 41 differential affinities for Pr and Pfr were also assayed by immunoprecipitation titrations where the amount of phytochrome bound by an immobilised mAb is assayed. Using this assay increasing amounts of LAS 41 lead to a precipitation of ~100% phytochrome as Pr but a maximum of only ~24% phytochrome when presented as Pfr (Fig 5.4a). The saturation of the Pfr curve at a lower phytochrome concentration compared with the concentration of the Pr curve is indicative of a quantitative difference in antigen availability rather than a genuine affinity difference. Similar experiments with immuno-affinity purified rabbit polyclonal antibodies to oat phytochrome demonstrate that increasing amounts of this antibody lead to ~100% precipitation of Pr and Pfr (Fig 5.4b).

5.3.3 Effect of mAbs on the spectral properties of 124 kDa oat phytochrome

Incubation of LAS 11, 21 or 32 with Pr or Pfr for 30 min has no effect on any of the photochemical parameters of the absorption spectrum of the phytochrome (Fig 5.5, 5.6, 5.7). Since there was no effect on the absorption spectrum, and these mAbs

Figure 5.3 Differential affinities of mAbs
for Pr (○—○) and Pfr (●—●) as assayed by
ELISA 5.3

Phytochrome was added to immobilised mAb, at the indicated concentrations, as Pr (following saturating far-red irradiation) or Pfr (following saturating red irradiation).

Bound phytochrome was detected by the addition of peroxidase-conjugated LAS 32.

a = LAS 11

b = LAS 31

c = LAS 41

Each reading is the average of three replicate assays from a single experiment.

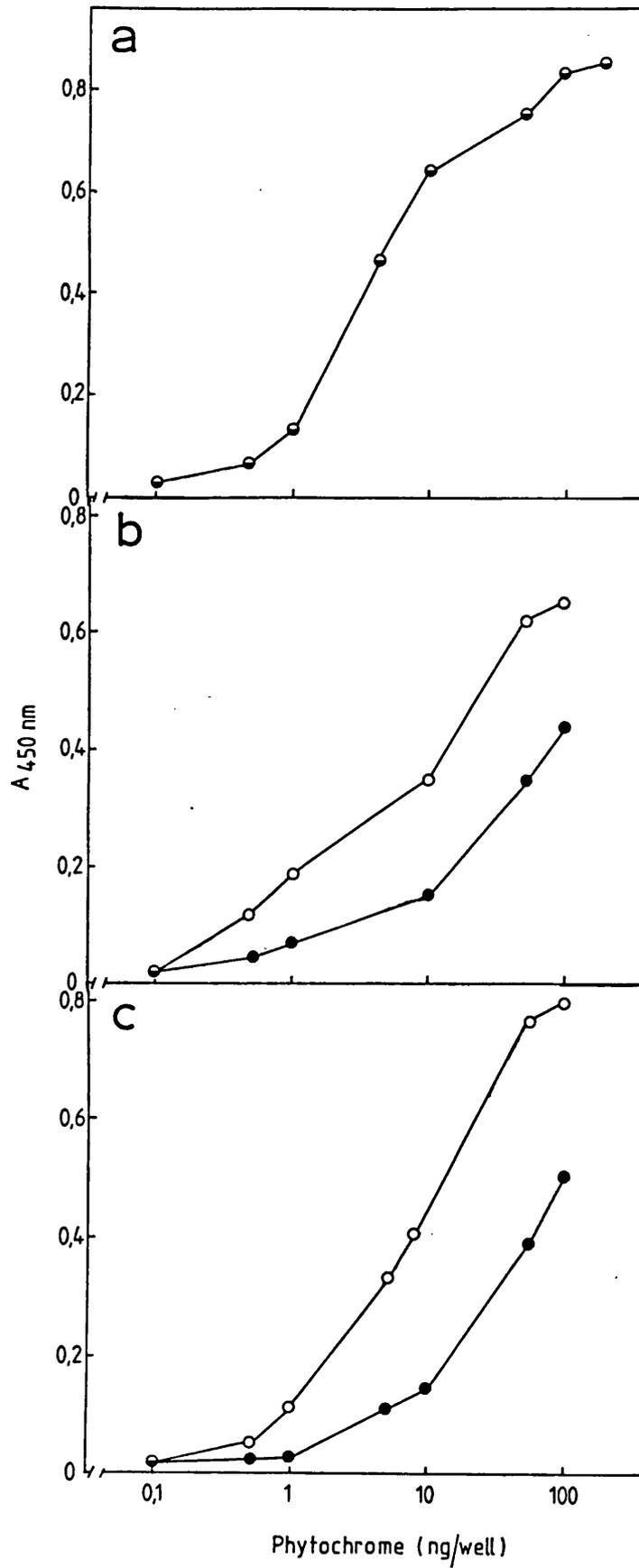


Figure 5.4 Immunoprecipitation of phytochrome
as Pr or Pfr

Increasing concentrations of LAS 41 or rabbit polyclonal antibodies were complexed with S. aureus cells and subsequently mixed with 10 μ g of 124 kDa oat phytochrome (SAR = 0.91) as Pr (○—○) or Pfr (●—●). Bound phytochrome was pelleted by centrifugation at 12,000 x g. Photoreversible phytochrome remaining in the supernatant was assayed with the Aminco DW-2a spectrophotometer.

a = Immunoprecipitation by LAS 41

b = Immunoprecipitation by rabbit
anti-phytochrome polyclonal
antibodies

Each value is the average of three replicate assays from a single experiment.

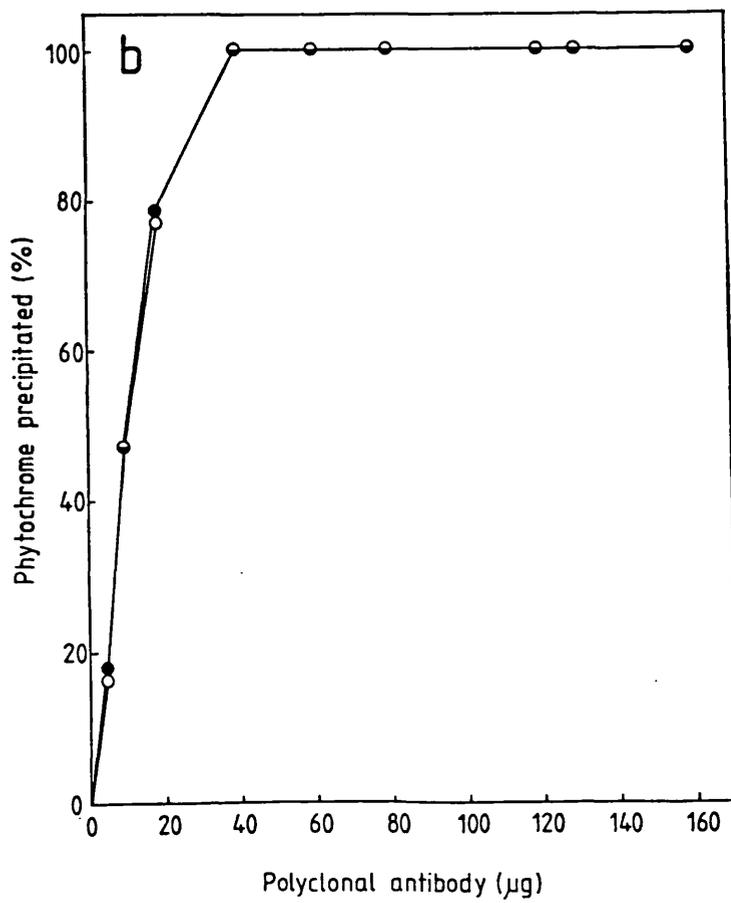
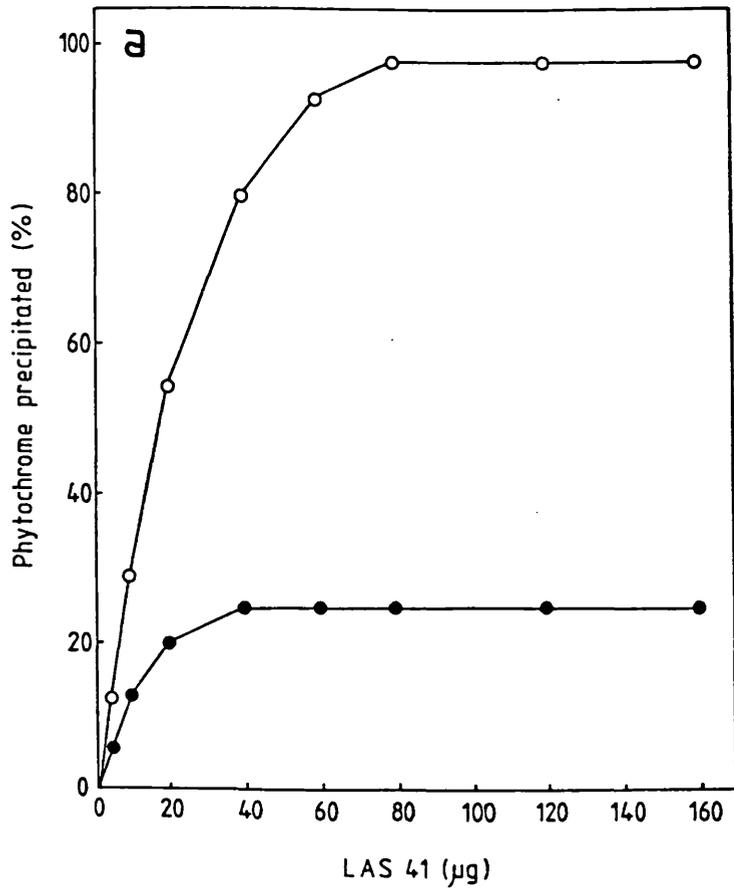


Figure 5.5 Effect of LAS 11 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome, (40 µg) as either Pr or Pfr, was incubated with 120 µg LAS 11 for 30 min at 4C in darkness, prior to start of spectral analyses.

a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 11 with Pr.

b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 11 with Pfr.

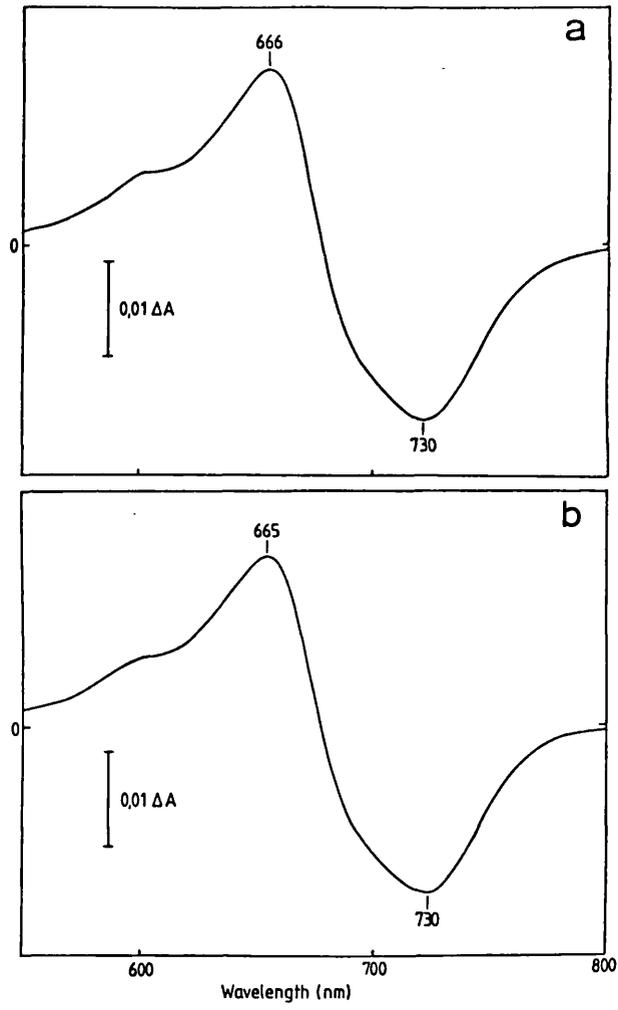


Figure 5.6 Effect of LAS 21 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome (40 µg) as either Pr or Pfr was incubated with 120 µg LAS 21 for 30 min at 4C in darkness, prior to the start of spectral analyses.

a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 21 with Pr.

b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 21 with Pfr.

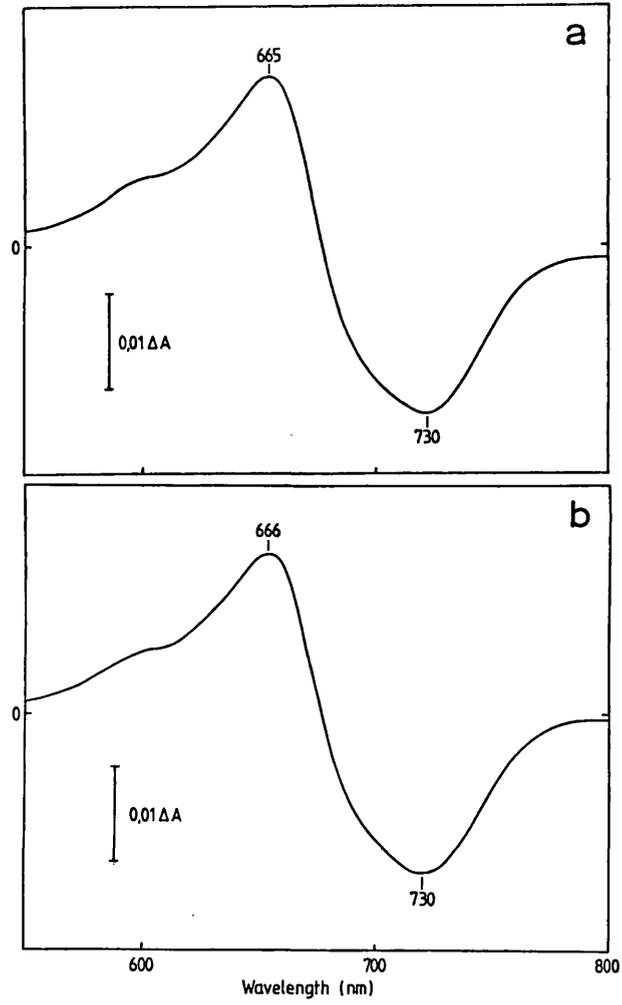
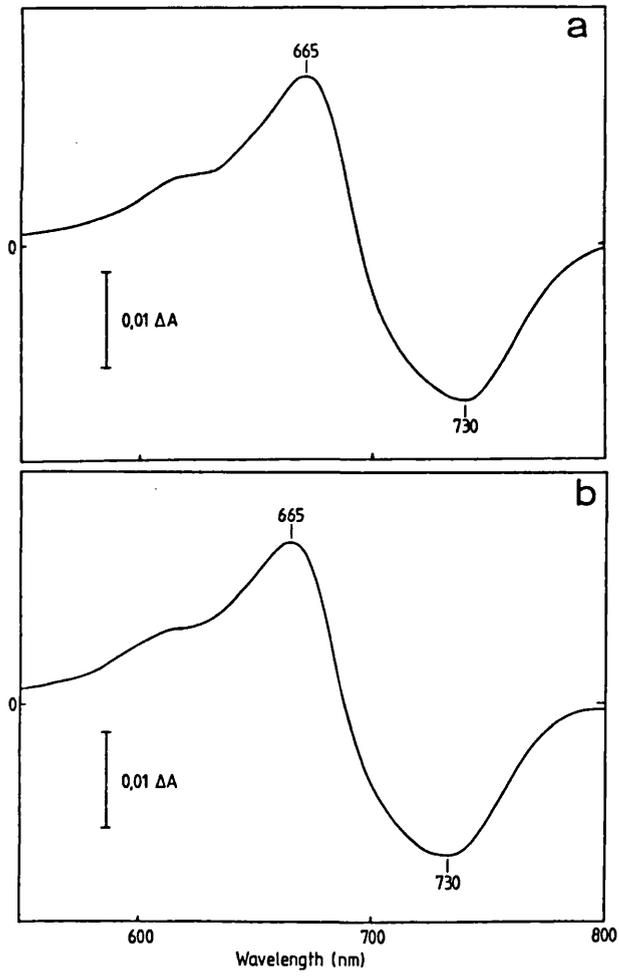


Figure 5.7 Effect of LAS 32 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome (40 µg) as either Pr or Pfr was incubated with 120 µg LAS 32 for 30 min at 4C in darkness, prior to the start of spectral analyses.

a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 32 with Pr.

b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 32 with Pfr.



do not exhibit differential affinities for either Pr or Pfr, dark reversion analyses were not conducted in the presence of these mAbs. LAS 42, a mAb which exhibits a slightly higher affinity for Pfr also did not affect the spectral properties of phytochrome, nor did it induce non-photochemical reversion of Pfr to Pr in the presence or absence of dithionite (Fig 5.8).

Incubation of LAS 31 and 33 with Pr results in a shift in the absorbance maximum from 665 nm to 667 nm (Fig 5.9e,5.10e). Similar incubations with Pfr do not affect the absorption spectrum. However, photoconversion of Pr, incubated in the presence of LAS 31 or 33, to Pfr produces an altered Pfr absorption spectrum (Fig 5.9e, 5.10e). In both cases the absorbance maximum is decreased from 730 nm to ~726 nm and this is accompanied by a reduction in the extinction at that wavelength, which results in an increase in the SCR from 1.06, which is characteristic of native phytochrome (Fig 5.14a), to 1.10-1.15 (Fig 5.9a, 5.10a). This altered spectrum is stable. The modified Pfr, produced following prior incubation of LAS 31 or 33 with Pr and photoconversion, undergoes dithionite-stimulated dark reversion. The presence of LAS 33 induces 35% dark reversion (Fig 5.10c) whereas LAS 31 induces 47% dark reversion over a period of 60 min at 4C (Fig 5.9c). Dithionite-dependent dark reversion is also observed following incubation of LAS 31 with Pfr although there is no effect on the Pfr spectrum. In this case 20% dark reversion is observed over a similar period

Figure 5.8 Effect of LAS 42 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome, (40 µg) as either Pr or Pfr, was incubated with 120 µg LAS 42 for 30 min at 4C in darkness, prior to the start of spectral analyses.

a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 42 with Pr.

b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 42 with Pfr.

c) Dark reversion, at 4C, in the presence of 5 mM sodium dithionite, following incubation of LAS 42 with Pr, and photoconversion to Pfr.

d) As in c) except following incubation of LAS 42 with Pfr.

e) As in c) but in the absence of 5 mM sodium dithionite

f) As in d) but in the absence of 5 mM sodium dithionite.

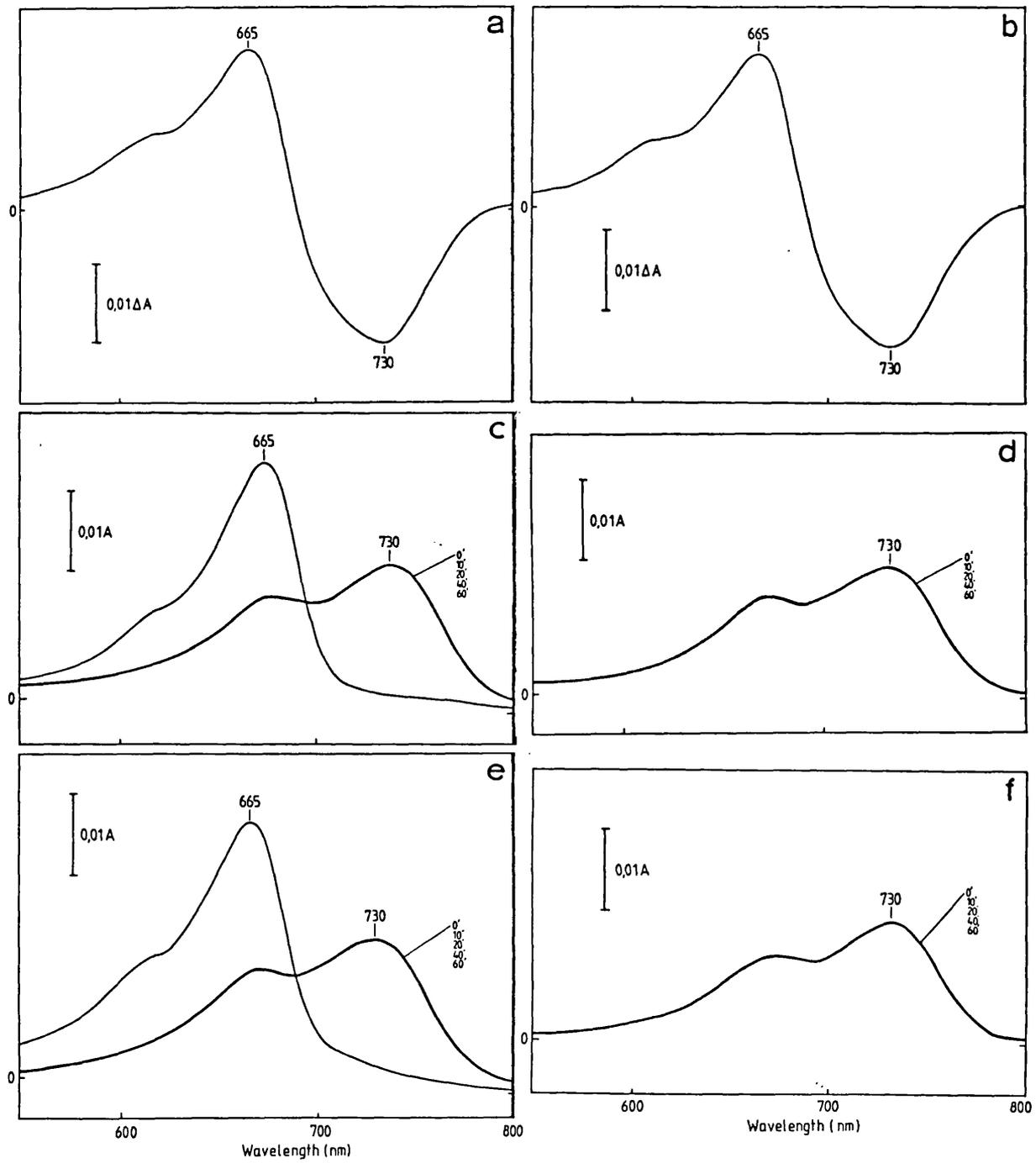


Figure 5.9 Effect of LAS 31 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome, (40 µg) as either Pr or Pfr, was incubated with 120 µg LAS 31 for 30 min at 4C in darkness, prior to the start of spectral analyses.

- a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 31 with Pr (—) and following a subsequent 10 min incubation with Pfr (---).
- b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 31 with Pfr.
- c) Dark reversion, at 4C, in the presence of 5 mM sodium dithionite, following incubation of LAS 31 with Pr, and photoconversion to Pfr.
- d) As in c) except following incubation of LAS 31 with Pfr.
- e) As in c) but in the absence of 5 mM sodium dithionite.
- f) As in d) but in the absence of 5 mM sodium dithionite.

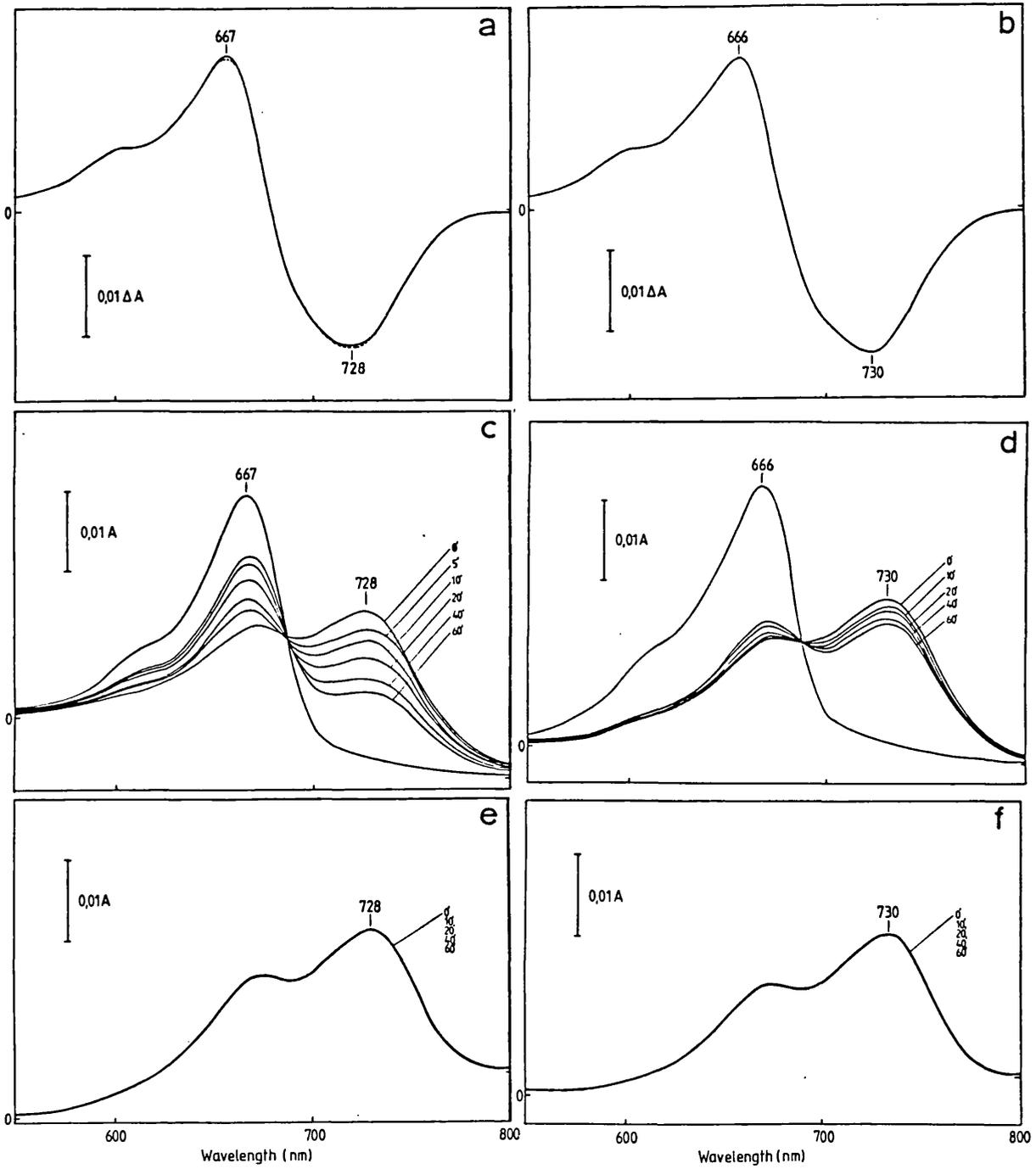
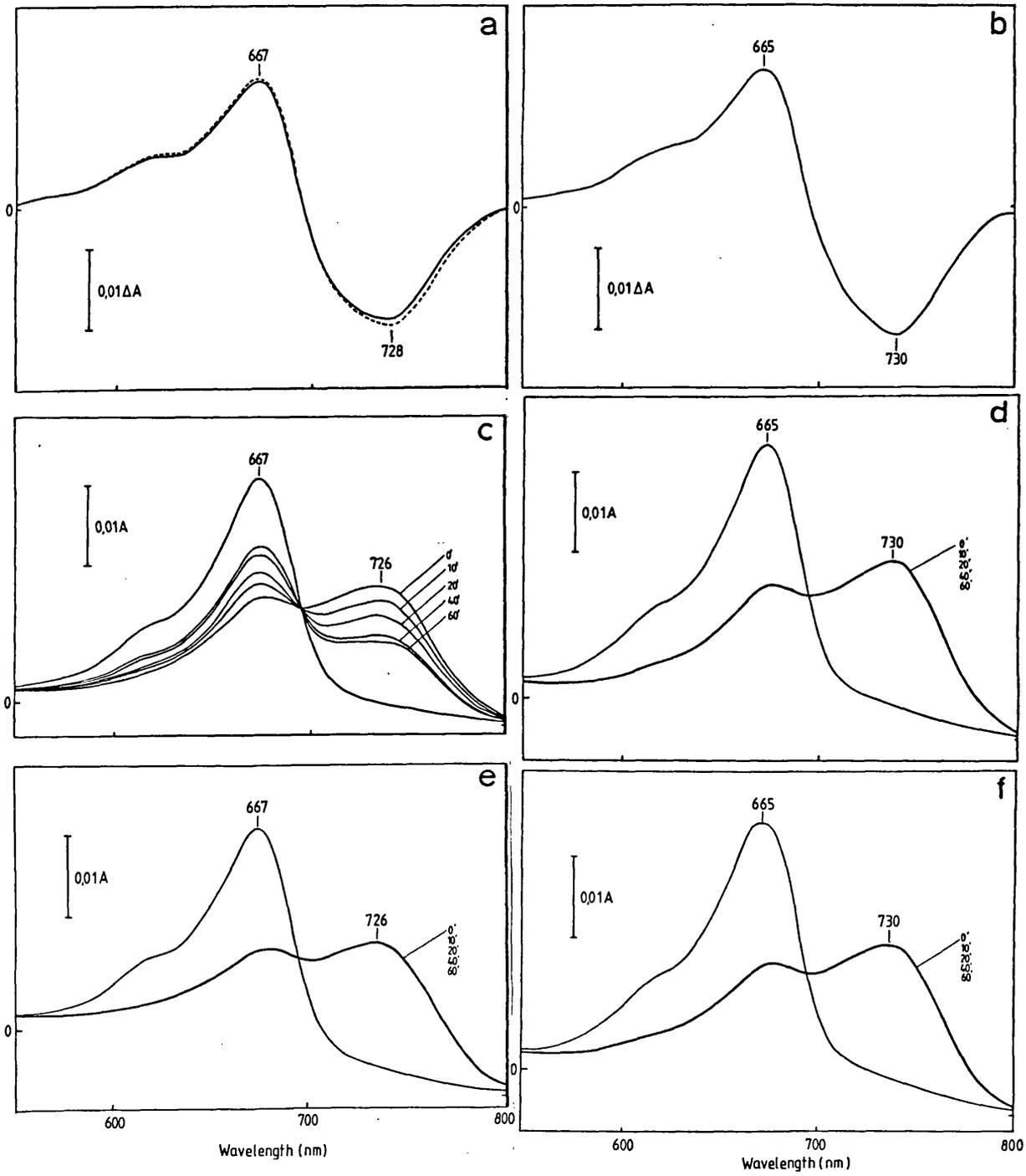


Figure 5.10 Effect of LAS 33 on the spectral characteristics of 124 kDa oat phytochrome

Phytochrome, (40 μg) as either Pr or Pfr, was incubated with 120 μg LAS 33 for 30 min at 4C in darkness, prior to the start of spectral analyses.

- a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 33 with Pr (—) and following a subsequent 10 min incubation with Pfr (---).
- b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 33 with Pfr.
- c) Dark reversion, at 4C, in the presence of 5 mM sodium dithionite, following incubation of LAS 33 with Pr, and photoconversion to Pfr.
- d) As in c) except following incubation of LAS 33 with Pfr.
- e) As in c) but in the absence of 5 mM sodium dithionite.
- f) As in d) but in the absence of 5 mM sodium dithionite.



at 4C (Fig 5.9d). In contrast LAS 33 did not accelerate dithionite-dependent dark reversion following a preincubation with Pfr. This sample exhibited negligible dark reversion which is comparable to that of 124 kDa native oat phytochrome (Fig 5.10d, 5.14b). No dark reversion was observed in the absence of dithionite whether phytochrome as Pr or Pfr had been previously incubated with LAS 31 or 33 (Fig 5.9e, f, Fig 5.10e, f).

Incubation of LAS 34 with Pr for 30 min, or subsequent photoconversion to Pfr has no effect on the properties of the absorption spectrum (Fig 5.11a). Prior incubation of LAS 34 with Pfr for 30 min does not affect the absorbance maximum of Pfr (Fig 5.11f) but causes an increase in its extinction, which consequently reduces the SCR to unity (Fig 5.11b). This altered spectral characteristic is unstable because following a further 10 min incubation with Pr and rescanning the Pfr spectrum the extinction at 730 nm is reduced, so that the SCR has returned to the standard value for native 124 kDa oat phytochrome of 1.06 (Fig 5.11b). LAS 34 did not induce dark reversion in the presence or absence of dithionite, whether incubated with Pr or Pfr, prior to the start of the dark reversion analyses (Fig 5.11c, d, e, f).

Incubation of LAS 35 with Pr results in a shift in the absorbance maximum from 665 nm to 667 nm and a decrease in the extinction at this wavelength (Fig 5.12a, e). Similar incubations of LAS 35 with Pfr produce an unaltered spectrum (Fig 5.12b, f).

Figure 5.11 Effect of LAS 34 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome, (40 µg) as either Pr or Pfr, was incubated with 120 µg LAS 34 for 30 min at 4C in darkness, prior to the start of spectral analyses.

a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 34 with Pr.

b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 34 with Pfr(—) and a further 10 min with Pr (---).

c) Dark reversion, at 4C, in the presence of 5 mM sodium dithionite, following incubation of LAS 34 with Pr, and photoconversion to Pfr.

d) As in c) except following incubation of LAS 34 with Pfr.

e) As in c) but in the absence of 5 mM sodium dithionite.

f) As in d) but in the absence of 5 mM sodium dithionite.

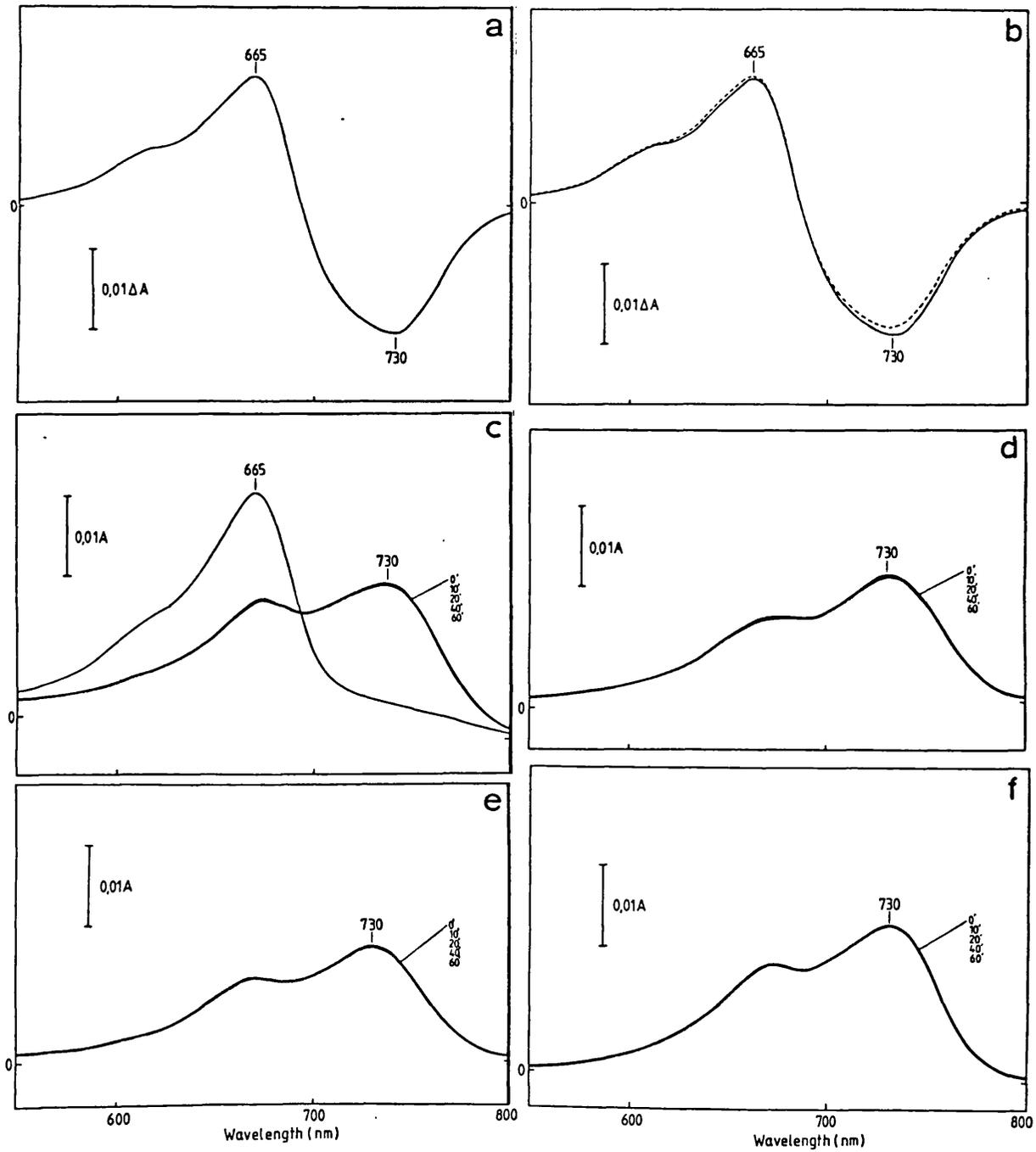


Figure 5.12 Effect of LAS 35 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome, (40 µg) as either Pr or Pfr, was incubated with 120 µg LAS 35 for 30 min at 4C in darkness, prior to the start of spectral analyses.

a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 35 with Pr (—) and following a subsequent 10 min incubation with Pfr (---).

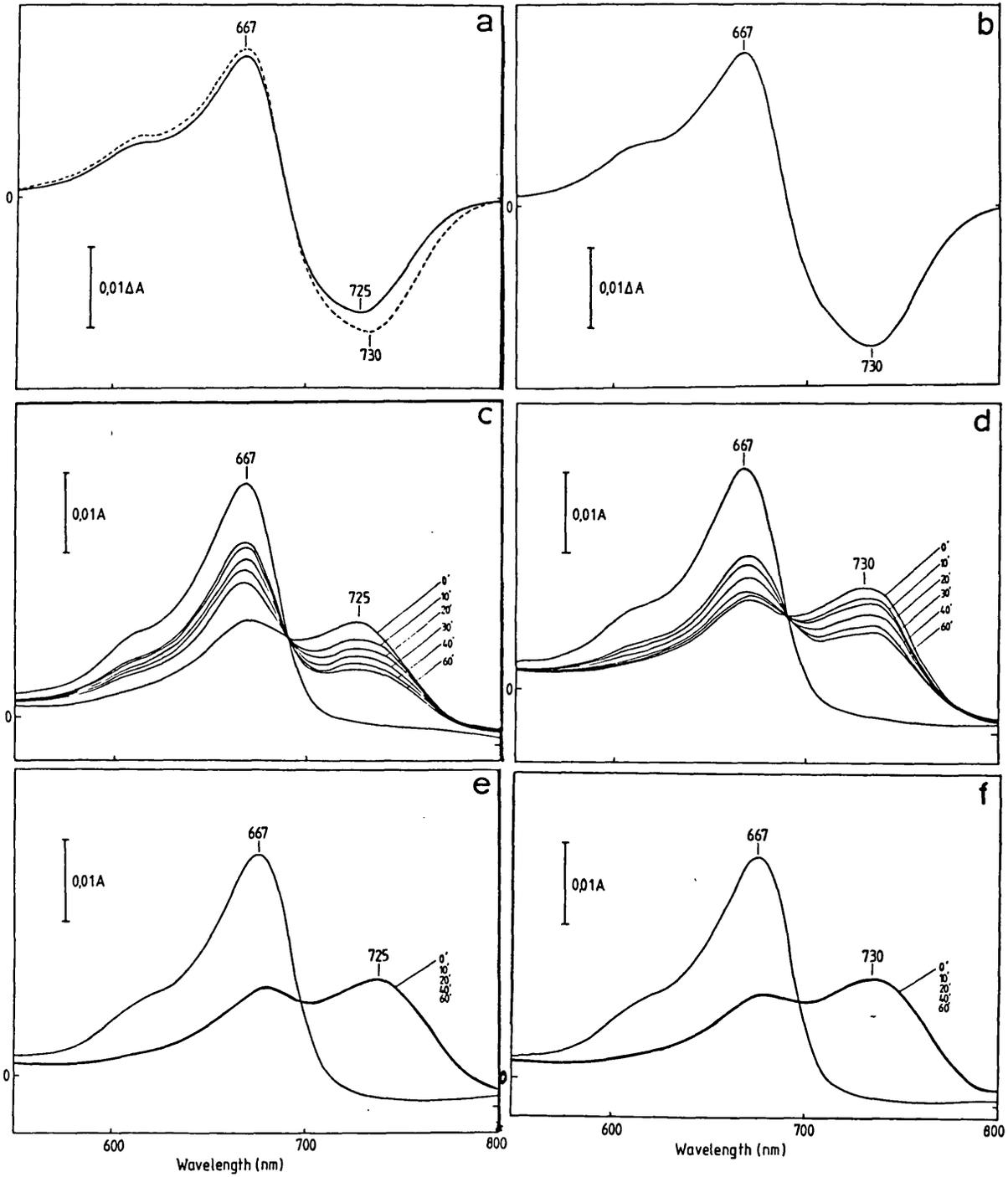
b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 35 with Pfr.

c) Dark reversion, at 4C, in the presence of 5 mM sodium dithionite, following incubation of LAS 35 with Pr, and photoconversion to Pfr.

d) As in c) except following incubation of LAS 35 with Pfr.

e) As in c) but in the absence of 5 mM sodium dithionite.

f) As in d) but in the absence of 5 mM sodium dithionite.



However, photoconversion of Pr, incubated in the presence of LAS 35, produces an altered Pfr spectrum (Fig 5.12e). The absorbance maximum is blue-shifted from 730 nm to 725 nm and this is accompanied by a reduction in extinction (Fig 5.12a, e). A consequence of these changes in the Pr and Pfr spectra is that the SCR increases to 1.25 (Fig 5.12a). The modified Pfr spectrum is not stable. Following a further 10 min incubation maintaining phytochrome as Pfr and then rescanning, the Pfr absorption spectrum exhibits an absorbance maximum at 730 nm and the extinction at this wavelength is increased (Fig 5.12a). Photoconversion of this Pfr back to Pr results in a Pr absorbance spectrum with an absorbance maximum at 668 nm and an increased extinction at this wavelength (Fig 5.12a). Consequently, the SCR is reduced from 1.25 to 1.11, but does not return to the native SCR of 1.06 (compare Figs 5.12a; 5.14a). Prior incubation of LAS 35 with Pr and photoconversion to Pfr before addition of dithionite induces 47% dithionite-dependent dark reversion over a period of 60 min at 4C (Fig 5.12c). Prior incubation of LAS 35 with Pfr induces 30% dithionite-dependent dark reversion over a similar period (Fig 5.12d). No dark reversion is observed in the absence of dithionite (Fig 5.12e, f).

Incubation of LAS 41 with Pr for 30 min induces a similar change in the Pr absorption spectrum as that observed with LAS 35 (compare Fig 5.12e, 5.13e). The absorbance maximum is shifted from 665 nm to

Figure 5.13 Effect of LAS 41 on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Phytochrome, (40 µg) as either Pr or Pfr, was incubated with 120 µg LAS 41 for 30 min at 4C in darkness, prior to the start of spectral analyses.

a) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 41 with Pr (—) and following a subsequent 10 min incubation with Pfr (---).

b) Difference spectrum (Pr-Pfr) of phytochrome following incubation of LAS 41 with Pfr.

c) Dark reversion, at 4C, in the presence of 5 mM sodium dithionite, following incubation of LAS 41 with Pr, and photoconversion to Pfr.

d) As in c) except following incubation of LAS 41 with Pfr.

e) As in c) but in the absence of 5 mM sodium dithionite.

f) As in d) but in the absence of 5 mM sodium dithionite.

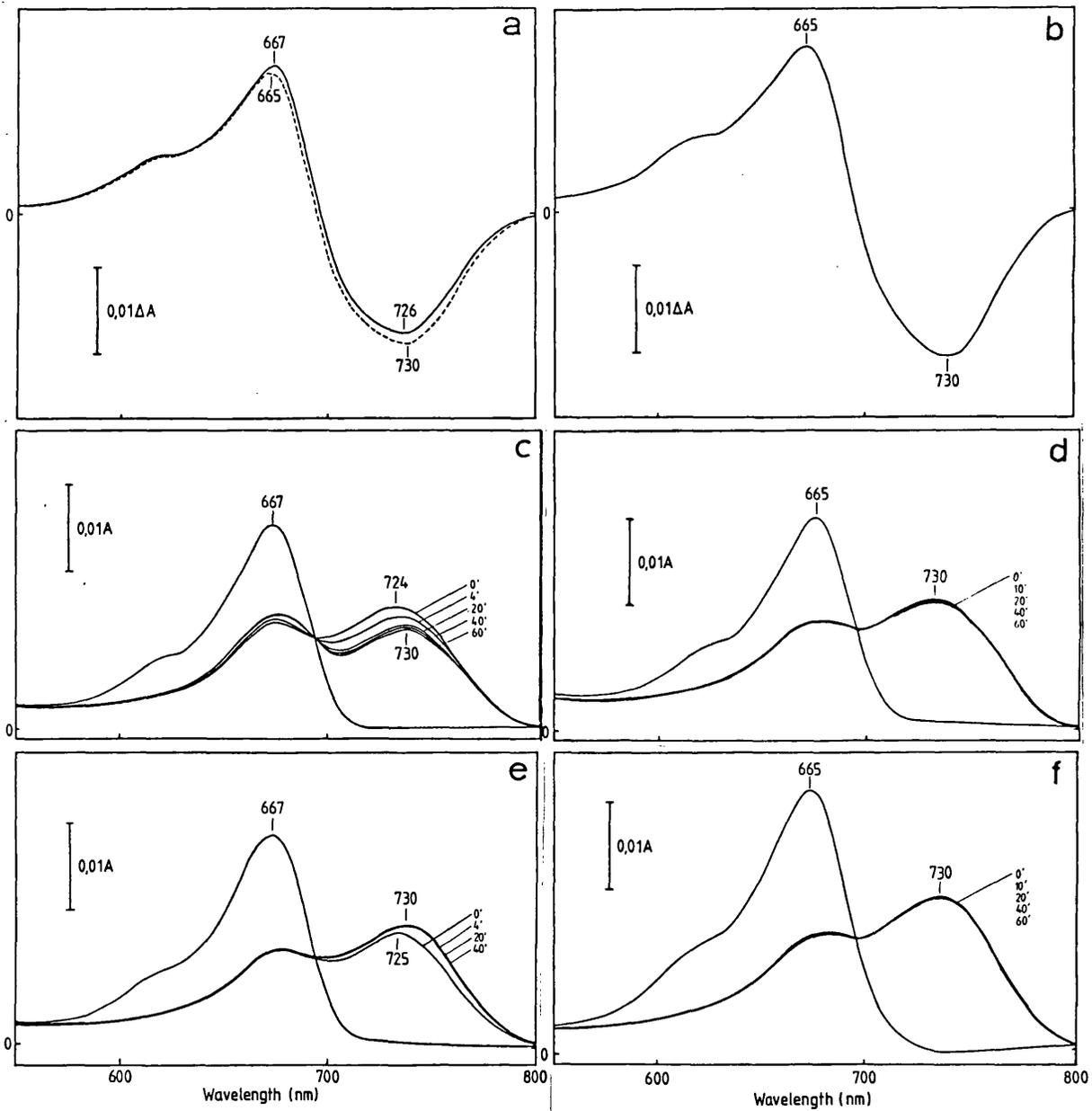
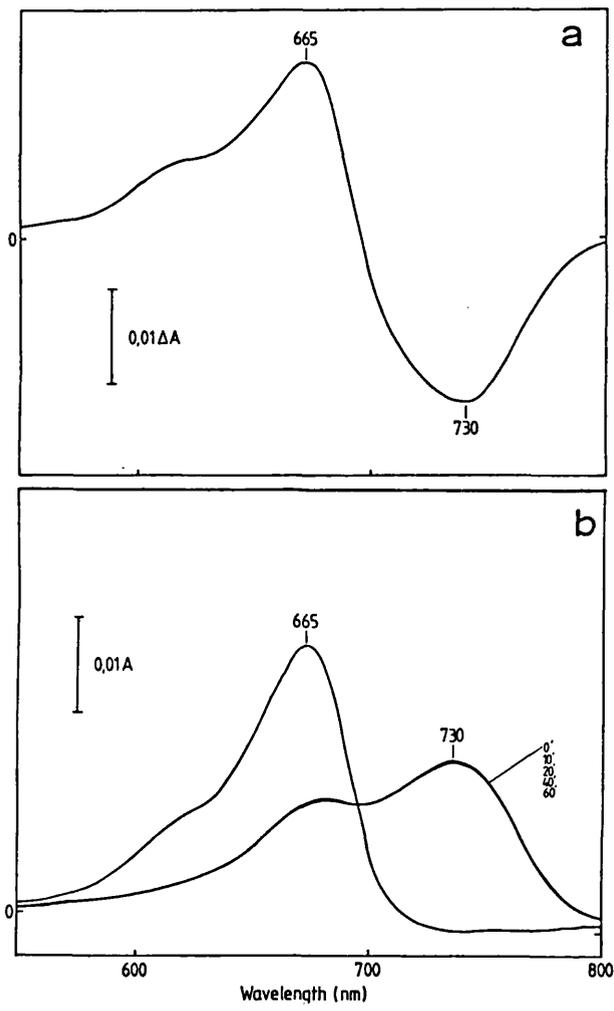


Figure 5.14 Spectral characteristics of
124 kDa oat phytochrome (SAR = 0.91)

- a) Difference spectrum (Pr-Pfr) of 124 kDa oat phytochrome measured at 4C following saturating red and far-red irradiations.
- b) Dark reversion, at 4C, of 124 kDa oat phytochrome in the presence of 5 mM sodium dithionite.



667 nm with a concomitant increase in extinction at this wavelength (compare Fig 5.12a; 5.13a). Incubation of LAS 41 with Pfr produces an unaltered Pfr spectrum (Fig 5.13f) and if this is photoconverted to Pr a native Pr spectrum is also observed, again similar to the effects of LAS 35 (compare Fig 5.12b; 5.13b). Photoconversion of Pr incubated in the presence of LAS 41, to Pfr produces an altered Pfr absorption spectrum (Fig 5.13e). The absorbance maximum is decreased from 730 nm to 726 nm and this is accompanied by a reduction in extinction at this wavelength (Fig 5.13a). A consequence of these changes in the Pr and Pfr spectra is that the SCR increases from 1.06, to 1.21 (compare Fig 5.13a; Fig 5.14a). The altered Pfr spectrum resulting from a prior incubation of LAS 41 with Pr and subsequent photoconversion is not stable. After a further 10 min incubation maintaining phytochrome as Pfr and then rescanning, a native Pfr spectrum is obtained (Fig 5.13a). Likewise, reversion of this Pfr back to Pr produces a native Pr spectrum. As a result the SCR decreases from 1.21 to 1.06 (Fig 5.13a).

The modified Pfr, produced following prior incubation of LAS 41 with Pr and photoconversion, undergoes transient dithionite-stimulated dark reversion (Fig 5.13c). This dark reversion, which is not observed when LAS 41 is incubated with Pfr (Fig 5.13d), proceeds for about 4 min during which time the Pfr absorbance maximum shifts from 724 nm to 730 nm. Once this shift is completed no further

dark reversion occurs, although there is some loss of absorbance at 730 nm, probably the result of bleaching (Fig 5.13d). In the absence of dithionite LAS 41 does not induce dark reversion of Pfr, produced by photoconversion of Pr previously incubated with LAS 41. Rather, the modified Pfr spectrum detectable immediately following photoconversion decays to a native Pfr spectrum within 4 min and then remains quite stable in the dark (Fig 5.13e). LAS 41 did not induce dark reversion in the absence of dithionite following prior incubation with Pfr (Fig 5.13f).

All the effects of the mAbs on the spectral properties are summarised in Table 5.1.

5.3.4 Photoreversible binding of LAS 41 to Pr

The photoreversible binding of LAS 41 to phytochrome was assayed using a modification of ELISA 5.1. LAS 41 bound to Pr becomes unbound following red-irradiation of the phytochrome, but is rebound following a further far-red irradiation (Fig 5.15). Thus, binding of LAS 41 to phytochrome shows classical red/far-red reversibility.

5.3.5 Purification and spectral properties of Pfr^P

The apparent specificity of LAS 41 for Pr has facilitated the purification of Pfr^P. In many instances the term Pfr is used rather loosely to refer to the population of phytochrome molecules generated by saturating red-irradiation, which contains mainly Pfr and some Pr molecules. The term Pfr^P, therefore refers specifically to a

Table 5.1 Summary of effect of mAbs on the spectral characteristics of 124 kDa oat phytochrome (SAR = 0.91)

Antibody	Type	Incubated with	$\lambda_{r_{max}}$ (nm)	$\lambda_{fr_{max}}$ (nm)	$\frac{\Delta Ar}{\Delta Afr}$	Accelerated ^a dark reversion
None	-	-	665	730	1.06	NO
LAS 11	2	Pr	666	730	1.06	NT
		Pfr	665	730	1.04	NT
LAS 21	4.2	Pr	665	730	1.05	NT
		Pfr	666	730	1.05	NT
LAS 31	1*	Pr	667	728	1.15	YES
		Pfr	666	730	1.06	YES
LAS 32	3	Pr	665	730	1.06	NO
		Pfr	665	730	1.06	NO
LAS 33	1*	Pr	667	726	1.15	YES
		Pfr	665	730	1.07	NO
LAS 34	4.2*	Pr	668	730	1.04	NO
		Pfr	665	730	1.02	NO
LAS 35	1'*	Pr	667	725	1.26	YES
		Pfr	667	730	1.07	YES
LAS 41	1'*	Pr	667	726	1.21	TRANSIENT
		Pfr	665	730	1.06	NO
LAS 42	4.2*	Pr	665	730	1.04	NO
		Pfr	665	730	1.05	NO

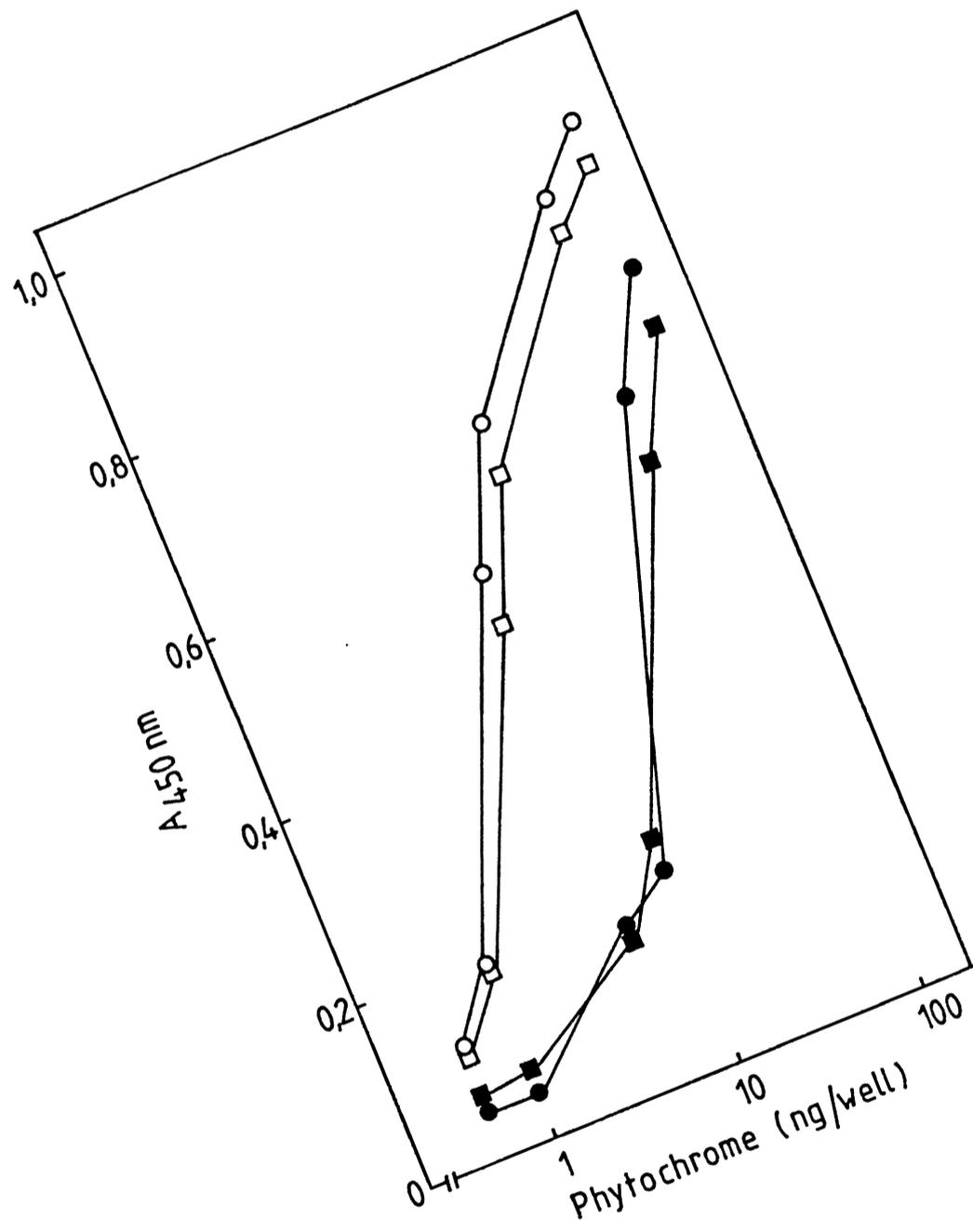
a = in the presence of dithionite

* = different affinities for Pr and Pfr

NT = not tested

Figure 5.15 Photoreversible binding of
LAS 41 to phytochrome as assayed by ELISA

Phytochrome, as either Pr (○—○) or Pfr (●—●), was mixed with LAS 41 in free solution. In addition, mixtures of LAS 41 and Pr were incubated in darkness and then irradiated with red (■—■), or red- followed by far-red light (□—□). These mixtures were added to immobilised rabbit anti-phytochrome polyclonal antibodies. Bound LAS 41 was assayed by the addition of a peroxidase-conjugate of anti-mouse antibodies.



solution of phytochrome containing only Pfr molecules.

Excess LAS 41 complexed with S. aureus cells (see Fig 5.4a) was used to immunoprecipitate Pr from a solution previously irradiated with red light. The absorption spectrum of the supernatant shows an increased extinction at 730 nm and a decreased absorbance in the 670 nm region, when compared to the absorption spectrum of the same solution following a further red-irradiation (Fig 5.16). As a consequence the Pr-Pfr^P difference spectrum exhibits a significantly higher $\Delta(\Delta A)$ than the normal Pr-Pfr difference spectrum (Fig 5.17). Since the lower $\Delta(\Delta A)$ value for the normal Pr-Pfr difference spectrum results from the presence of contaminating Pr in the red irradiated phytochrome, these measurements can be used to determine directly the mole fraction of Pfr at photoequilibrium in red light (φ max). That is φ max is given by:-

$$\frac{\Delta(\Delta A) \text{ 665:730 nm for Pr-Pfr}}{\Delta(\Delta A) \text{ 665:730 nm for Pr-Pfr}^P}$$

Eight independent assays determined this value to be 0.874 ± 0.0015 (S.E).

5.3.6 Phytochrome photoequilibrium assay

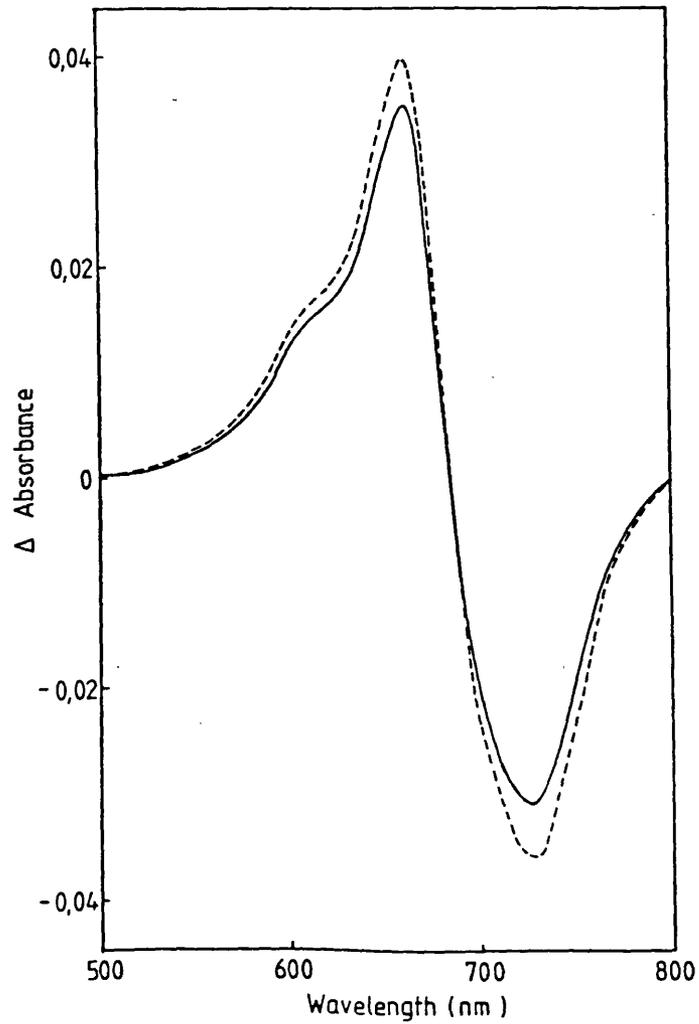
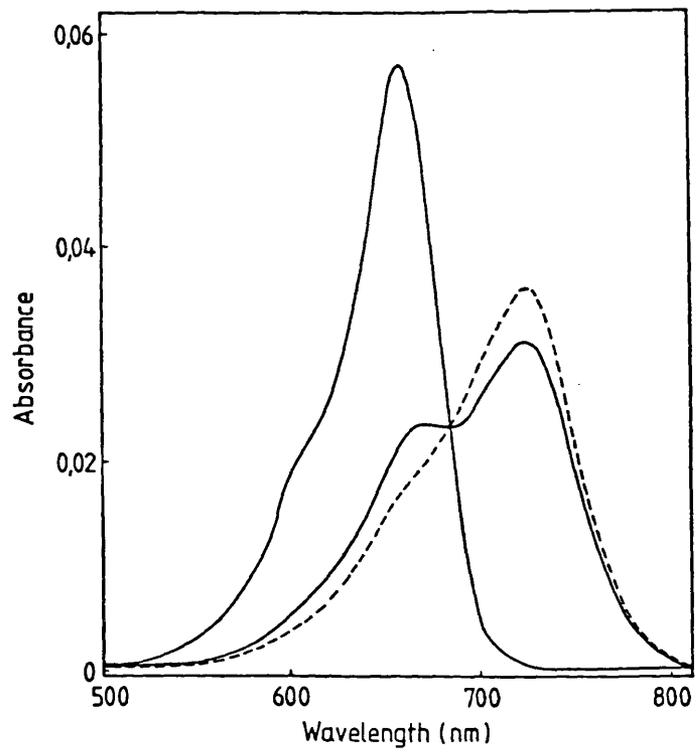
Solutions of phytochrome of different photo-equilibria were generated in the spectrophotometer. LAS 41 was incubated with each of the phytochrome containing solutions over a range of phytochrome concentrations. As the photoequilibrium of the phytochrome was increased, the titration curves

Figure 5.16 Absorption spectra of Pr, Pfr and Pfr^P

Pfr^P (---) was purified by removing contaminating Pr from Pfr by immunoprecipitation with LAS 41. Following immunoprecipitation the absorption spectrum of the supernatant was recorded immediately (---, Pfr^P), and following saturating red (Pfr) and far-red light (Pr).

Figure 5.17 Comparison of the difference spectra of Pr-Pfr(—) and Pr-Pfr^P(---)

Pfr^P was purified by the removal of Pr from Pfr by immunoprecipitation by LAS 41. The absorption spectrum of the supernatant (Pfr^P) was recorded. The sample was then irradiated with saturating far-red light (Pr) and the Pr-Pfr^P difference spectrum derived. The same sample was then irradiated with saturating red light (Pfr) and the Pr-Pfr difference spectrum derived.



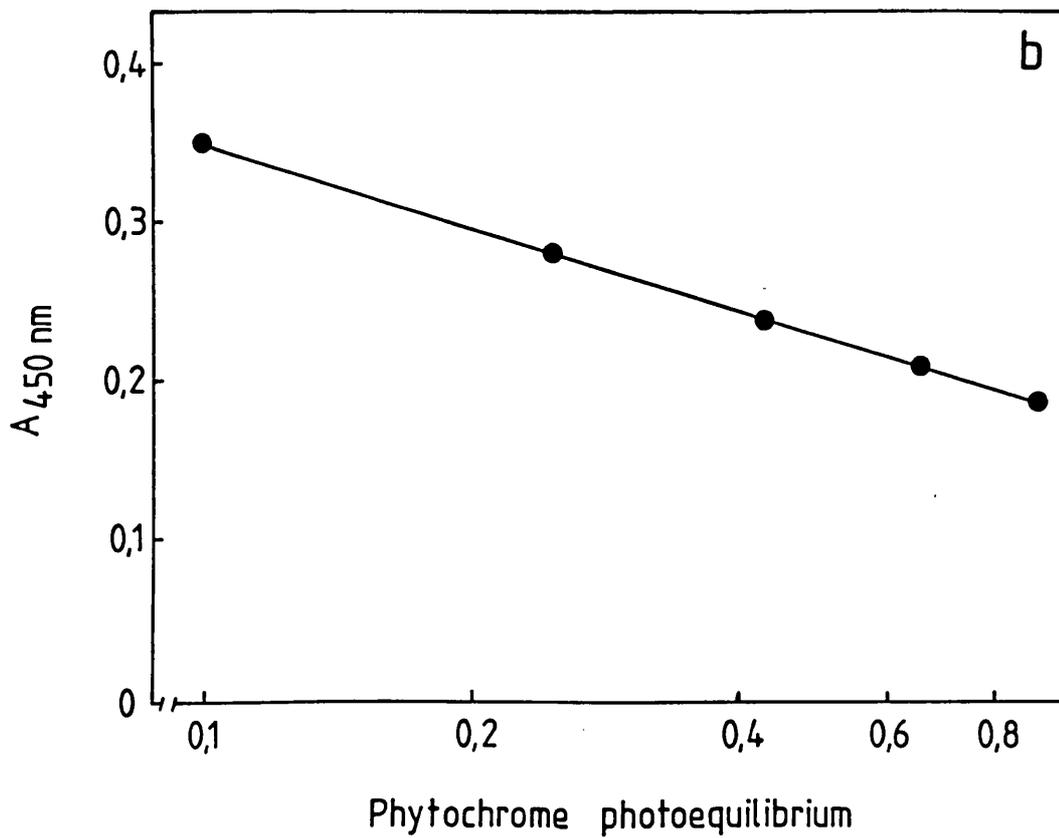
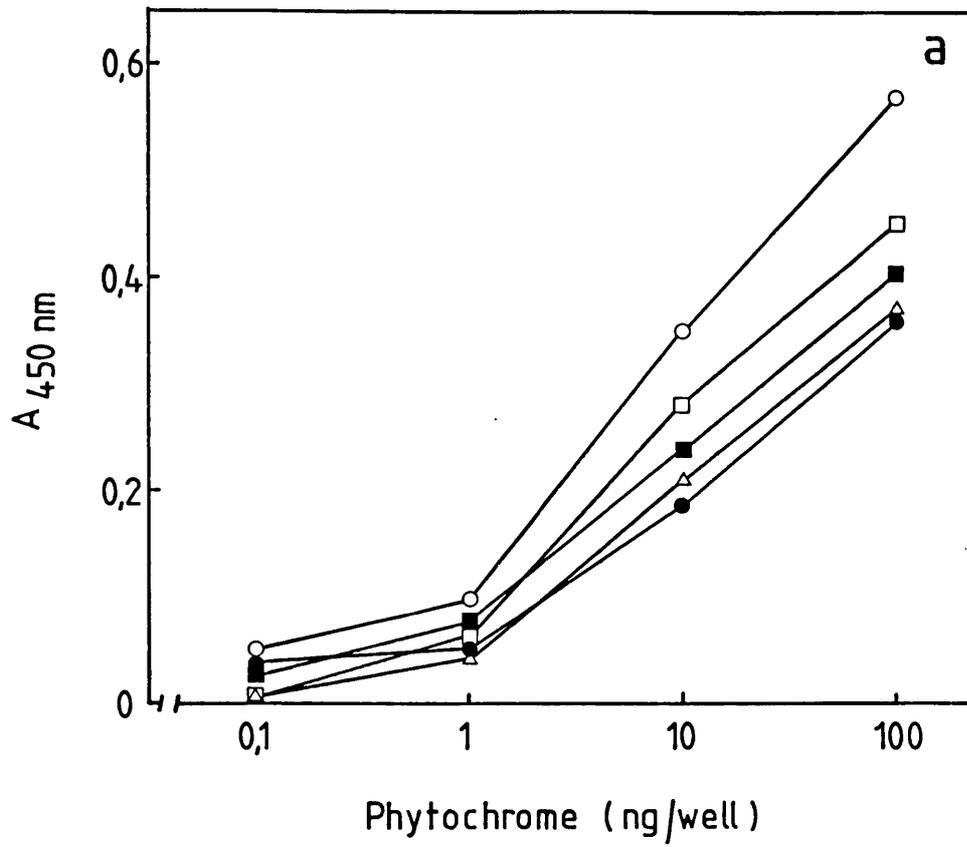
obtained with LAS 41 were shifted to slightly higher phytochrome concentrations (Fig 5.18a). Selection of the ELISA values obtained for 10 ng of phytochrome and plotting these values against phytochrome photoequilibrium demonstrates that there is a log linear relationship between ELISA value (i.e. binding of LAS 41) and phytochrome photoequilibrium (Fig 5.18b).

Figure 5.18 Phytochrome photoequilibrium
assay

Phytochrome solutions of different photo-equilibria were generated in the spectrophotometer, using sub-saturating doses of actinic red light, and incubated with LAS 41 in darkness. These mixtures were subsequently added to immobilised anti-phytochrome polyclonal antibodies. Detection of bound LAS 41 was achieved by the addition of a peroxidase-conjugate of anti-mouse antibodies.

- a) ELISA titrations of phytochrome solutions of differing photoequilibrium
- b) The ELISA value for 10 ng of phytochrome versus the photoequilibrium.

- (○) $\mathcal{P} = 0$
- (□) $\mathcal{P} = 0.25$
- (■) $\mathcal{P} = 0.45$
- (△) $\mathcal{P} = 0.67$
- (●) $\mathcal{P} = 0.87$



5.4 Discussion

5.4.1 Differential affinities for Pr and Pfr

Of the 6 mAbs to exhibit differential affinities for Pr and Pfr, 5 have a higher affinity for Pr. The observed higher affinity for Pr suggests that the epitope to which these mAbs bind is less accessible, or has a less favourable conformation in Pfr. Amongst the mAbs which exhibit a higher affinity for Pr, LAS 31 and 33 are Type 1 mAbs, LAS 35 and 41 are Type 1' mAbs and LAS 34 is a Type 4.2 mAb. Monoclonal antibodies which bind to the 4 kDa sub-NH₂-terminal domain and which exhibit differential binding to Pr and Pfr have not been reported elsewhere. This observation indicates that light induced conformational changes therefore also occur on the 4 kDa sub-NH₂-terminal domain.

None of the mAbs which map to the 6 kDa NH₂-terminus or the adjacent 4 kDa sub-NH₂-terminal domain exhibit higher affinities for Pfr. This observation is consistent with the findings of Cordonnier et al (1985) who report three mAbs which map to the 6 kDa NH₂-terminal domain and exhibit higher affinities for Pr. These observations indicate that the epitopes to which the Type 1 and 1' mAbs are binding are more exposed in Pr than in Pfr. This could mean that the region to which the mAb is binding is the same as, or overlaps with the Pr-preferred cleavage site between these regions. As binding of a Type 1 mAb to 124 kDa oat phytochrome can prevent proteolytic removal of the 6 kDa

NH₂-terminus (Cordonnier et al 1985), at least in this instance, the proposal may be correct. The prevention of proteolytic cleavage due to the binding of LAS 31, 33, 35 or 41 is however untested, so it is not possible to draw similar conclusions here.

The higher affinity for Pr exhibited by LAS 34, a Type 4.2 mAb, and the higher affinity for Pfr shown by LAS 42, also a Type 4.2 mAb, indicate that photoreversible conformational changes can also occur on the 64 kDa chromophore-bearing domain. Monoclonal antibodies which map to this domain and have a higher affinity for Pfr have already been reported (Shimazaki et al 1986). However, it is unlikely that LAS 42 is recognising the same epitope as the mAbs reported by Shimazaki et al (1986) because this mAb can only react with the native conformation of the molecule, whilst those reported by Shimazaki et al (1986) (Oat-9 and Oat-16) probably recognise a region of primary sequence on the molecule. Thomas et al (1984b) have also reported mAbs raised to "large" oat phytochrome which exhibit differential affinities for Pr and Pfr. However, whether these mAbs bind to sites on the 64 kDa chromophore-bearing domain or on the 55 kDa COOH-terminal domain is not known.

The results reported here are therefore consistent with the view that several sites on the phytochrome molecule undergo photoreversible conformational changes (see 1.3.2). They confirm

the observations that light-induced conformational changes occur on the 6 kDa NH₂-terminus and the 64 kDa chromophore-bearing domain, but also indicate that such conformational changes also occur on the 4 kDa sub-NH₂-terminal domain. Although light-induced conformational changes also occur on the 55 kDa COOH-terminal domain (Lagarias and Mercurio 1985), LAS 32 a Type 3 mAb is not raised to such an epitope.

5.4.2 Identification of mAb specific for Pr

Amongst all the previous reports which have described mAbs with different affinities for Pr and Pfr (Thomas et al 1984b; Thomas and Penn 1986; Cordonnier et al 1985; Shimazaki et al 1986), none of the mAbs were found to be specific for one of the isoforms.

The conclusion that LAS 41 is specific for Pr is based on a number of observations relating to the properties and characteristics of this mAb. First, in quantitative ELISA assays LAS 41 exhibits an apparent 7-8 fold higher affinity for Pr than for Pfr. Due to the overlapping absorption of Pr and Pfr in the red region of the spectrum, Pfr solutions inevitably contain approximately 13% Pr (Kelly and Lagarias 1985), so the ELISA titration curves for a Pr-specific antibody would be expected to be shifted to 7.69 fold higher phytochrome concentrations. The apparent affinity difference exhibited by LAS 41 could simply reflect this quantitative difference in Pr concentration. In order to observe a 7-8 fold

apparent affinity difference for a Pr-specific mAb it is essential that each monomer of the phytochrome dimer is titrated separately. The configuration of ELISA 5.1 achieves this because, the assay effectively measures the amount of mAb bound to a population of phytochrome molecules, themselves bound by a polyclonal antibody. Furthermore, following immunoprecipitation of red-irradiated phytochrome solutions with LAS 41, the remaining soluble phytochrome is found to be exclusively Pfr^P, uncontaminated by Pr (see later). Finally, the observation that the binding of this antibody to Pr is fully photoreversible by red light (Fig 15.5) suggests that LAS 41 is indeed Pr-specific.

An alternative method of detecting differential affinities of mAbs for Pr and Pfr would be to assay the amount of phytochrome bound by an immobilised mAb as in the case of ELISA 5.2, rather than assaying the amount of mAb bound to immobilised phytochrome. However, under these circumstances the apparent affinity difference for a Pr-specific mAb will be determined by the dimeric nature of phytochrome. If each of the monomers of a phytochrome dimer behaves as an "autonomous photochromic unit" (Brockmann et al 1987) i.e. if phytochrome can exist as Pr:Pfr heterodimers in vitro, then a Pr-specific mAb would be expected to show an apparent ~4 fold difference in affinity for Pr over Pfr. This is because after saturating red-irradiation, a solution of phytochrome would be composed of the

following mixture of dimers: Pfr:Pfr = 76.4%,
Pr:Pfr = 22% and Pr:Pr = 1.6% (Napier and Smith 1987).
So, an immobilised Pr-specific mAb would be expected
to bind both the Pr:Pr homodimers and the Pr:Pfr
heterodimers, that is 23.6% of the dimers present.
Using polyclonal antibodies to assay the bound
phytochrome, the maximum apparent affinity difference
for a Pr-specific mAb will be 4.2 fold. Alternatively,
if phytochrome could only exist as homodimers in vitro
then a Pr-specific mAb should show a 7-8 fold higher
affinity for Pr over Pfr, in assays which detect
bound phytochrome. The immunoprecipitation assays
performed here are analogous to this type of assay,
but the phytochrome is assayed spectrophotometrically.
In this assay, the LAS 41 immunoprecipitation titration
curve for Pfr saturates at 24.5% of the total
phytochrome, which is quantitatively consistent with
the hypothesis that phytochrome does exist as stable
heterodimers in vitro.

The finding that LAS 41 exhibits a ~4 fold
higher affinity for Pr when bound phytochrome is
assayed, is further evidence that this mAb is Pr-
specific. However, the conclusion that a 4 fold
affinity difference is indicative of a Pr-specific
mAb is dependent on the demonstration that phytochrome
can exist as stable heterodimers in vitro. LAS 41
has been used to demonstrate that heterodimers do
exist in vitro. Therefore, the argument that a 4
fold affinity difference in immunoprecipitation assays
is diagnostic of a Pr-specific mAb becomes rather
circular.

5.4.3 Effect of mAbs on the spectral properties of 124 kDa oat phytochrome

In each instance, when describing the effect of mAbs on the spectral properties of phytochrome Pr-Pfr difference spectra have been shown in order to highlight the modifications which have occurred in the absorption spectra. Although, a difference spectrum is the product of both the Pr and Pfr absorption spectra and therefore represents the properties of both forms of phytochrome, the changes observed in the red are due to effects on the Pr spectrum and those in the far-red are due to effects on the Pfr spectrum. This is clearly demonstrated when the absolute absorption spectra of Pr and Pfr from the dark reversion analyses are observed.

Spectral analyses in the presence of LAS 31 and 33, Type 1 mAbs, underline the significance of the 6 kDa NH₂-terminal domain in the maintenance of the spectral integrity of the molecule. Binding of these mAbs to Pr, followed by photoconversion to Pfr produces an altered Pfr absorption spectrum. The absorbance maximum is shifted to ~726 nm with an accompanying increase in the SCR to ~1.15 (Fig 5.9a 5.10a). These changed spectral properties are similar to those observed upon proteolytic cleavage of the 124 kDa molecule to "large" phytochrome (Vierstra and Quail 1982b; Vierstra and Quail 1983b and Litts et al 1983), but here occur in the absence of proteolysis. In addition, the binding of LAS 31 and 33 to Pfr, induces non-photochemical reversion of

Pfr to Pr in the presence of dithionite (Fig 5.9c; 5.10c). These observations are similar to the findings of Cordonnier et al (1985) who also conducted spectral analyses in the presence of Type 1 mAbs which exhibited a higher affinity for Pr. However, binding of their mAb, Oat-25, to Pr and subsequent photoconversion to Pfr, also induced non-photochemical dark reversion in the absence of dithionite, which is not observed here. Therefore, Oat-25 may be binding to a unique epitope on the 6 kDa domain, or possibly an epitope which overlaps with the site to which LAS 31 and 33 bind. These findings are also similar to those observed by Lumsden et al (1985) using mAbs to pea phytochrome. One of the mAbs which binds close to the NH₂-terminus can induce changes in the spectral properties so that they resemble those of proteolytically degraded pea phytochrome, and induce non-photochemical reversion in the absence of dithionite.

Clearly, the binding of LAS 31 and 33 is interfering with stable protein-chromophore interactions between the 6 kDa NH₂-terminal domain and the chromophore. This may be a consequence of the mAb physically blocking the chromophore-protein interaction by occupying a site which normally interacts with the chromophore. The observation that the Pfr absorption spectrum is only modified following prior incubation with Pr and subsequent photoconversion, suggests that the site to which

the mAb binds is not freely available after photoconversion to Pfr, presumably because it is already occupied by the chromophore. The effects of binding LAS 31 and 33 to 124 kDa phytochrome are similar to those reported for the binding of ANS (Eilfeld and Rüdiger 1984). The major similarity being that binding of ANS to Pr and subsequent photoconversion of this Pr to Pfr induces changes in the Pfr absorption spectrum. However, direct binding of ANS to Pfr does not affect the absorption spectrum. This is because the ANS binding site is not accessible on Pfr, but is only accessible on Pr and during phototransformation. It seems unlikely that LAS 31 is binding to the same hydrophobic site to which ANS binds, because LAS 31 does bind to Pfr, as evidenced by the fact its binding to Pfr induces non-photochemical dark reversion to Pr.

Since LAS 31 and 33, which are both Type 1 mAbs, induce different alterations in the spectral characteristics, it is unlikely that they are raised to precisely the same epitope, yet the binding of these mAbs to phytochrome is mutually exclusive. Presumably, LAS 31 and 33 must be recognising distinct but overlapping epitopes.

The effect of LAS 31 and 33 on the spectral characteristics of 124 kDa phytochrome confirm the importance of the 6 kDa NH₂-terminal domain in the retention of the spectral integrity of the molecule. However, the contribution of the adjacent 4 kDa domain in the maintenance of the spectral integrity

of the phytochrome molecule is less defined. Spectral analyses of 124 kDa phytochrome in the presence of Type 1' mAbs demonstrate that the spectral characteristics of the molecule are modified. Binding of LAS 35 and 41 to Pr induces a slight red-shift in the absorbance maximum. Following photoconversion of this Pr to Pfr, a modified Pfr spectrum is obtained. These alterations to the Pfr spectrum are similar to those induced by binding of mAbs to the 6 kDa NH₂-terminal domain. However, in the case of LAS 41 these effects are short lived, such that, after only a few minutes, a native Pfr spectrum is obtained. The effects of LAS 35 on Pfr produced following a previous incubation of mAb with Pr and subsequent photoconversion are partially photoreversible. Following a further incubation maintaining the phytochrome as Pfr and then rescanning, the Pfr absorbance maximum returns to 730 nm but the extinction at this wavelength is still reduced. Together these observations suggest that the 4 kDa domain is directly involved in stable protein-chromophore interactions. An alternative explanation is that the binding of either LAS 35 or 41 leads to modification of the Pfr spectrum due to interference with the interactions between the chromophore and the adjacent 6 kDa domain.

The transient nature of the effect of LAS 41 on the spectral properties of Pfr, produced by incubation of Pr with LAS 41 and subsequent photoconversion, is

presumably a reflection of the fact that LAS 41 is specific for Pr. It seems likely that upon photoconversion of Pr to Pfr there is a conformational change such that the epitope to which LAS 41 binds is no longer available, LAS 41 thus becomes unbound. The photoreversible loss of binding, which is clearly observable by ELISA (Fig 5.15) is, presumably, not very rapid, since the Pfr spectrum is modified for at least the time required to record the absorption spectrum (~ 2 min). Apparently the photoconversion of Pr in the presence of LAS 41 yields an unstable photoproduct with unique spectral properties, which decays to free Pfr and free antibody over a period of time.

Binding of LAS 35 to Pr or Pfr induces prolonged non-photochemical dithionite-dependent dark reversion. When LAS 35 is bound to Pr and this is photoconverted to Pfr, the Pfr absorbance maximum is transiently at 725 nm. However, when LAS 35 is incubated directly with Pfr the absorbance maximum is at 730 nm. Yet in both instances dark reversion proceeds in the presence of dithionite. These differences must reflect the accessibility of the binding site on Pfr (c.f. the findings with Type 1 mAbs). Binding of LAS 35 to Pr and subsequent photoconversion with red light yields an unstable photoproduct (λ max 725 nm) which decays to Pfr with an absorbance maximum at 730 nm. Presumably, this is the same LAS 35-phytochrome product observed when LAS 35 is bound directly to Pfr.

Although the far-red absorbance maximum of this LAS 35-Pfr complex is apparently native (albeit with reduced extinction) the ability of LAS 35 to induce dithionite-dependent dark reversion suggests that the mAb is still interfering with stable protein-chromophore interactions.

LAS 35 must be binding to Pfr and so is clearly not Pr-specific. Therefore, it cannot be binding to the same epitope as LAS 41. As the binding of these two mAbs is mutually exclusive they must be binding to unique but overlapping epitopes.

LAS 34 and LAS 42 which are both Type 4.2 mAbs and exhibit differential affinities for Pr and Pfr, have little or no effect on the photochemical parameters of the native Pr and Pfr spectra of 124 kDa oat phytochrome. Similar findings have been observed with Type 2 mAbs which exhibit a higher affinity for Pfr (Shimazaki *et al* 1986). The reason for this lack of effect is presumably due to the fact that, although the epitopes to which these mAbs bind undergo light induced conformational changes they do not interact directly with the chromophore.

The three mAbs, LAS 21, 11 and 32 which react equally well with both forms of the photoreceptor also do not affect any of the spectral properties of 124 kDa phytochrome. As these mAbs are not binding to regions of the molecule that undergo light induced conformational changes, and map to the 64 kDa chromophore-bearing and 55 kDa COOH-

terminal domains, it is unlikely that they would interfere directly with the action of the chromophore.

5.4.4 Spectral properties of Pfr^P

The specificity of LAS 41 for Pr has facilitated the purification of Pfr^P by removal of the contaminating Pr which is unavoidably produced on photoconversion of phytochrome by red light. The absorption spectrum of this Pfr^P has a small shoulder in the red region of the spectrum. This is qualitatively similar to that predicted by Kelly and Lagarias (1985) and Lagarias et al (1987) who derived the spectrum from photochemical measurements using an estimated λ max of 0.876. This differs from the derived spectrum reported by Vierstra and Quail (1983b), who calculated λ max to be 0.862, in which there is no shoulder in the red. Furthermore, the availability of Pfr^P has enabled direct determination of λ max and the value of 0.874 obtained here is in close agreement with that of Kelly and Lagarias (1985) and Lagarias et al (1987). Kelly and Lagarias (1985) have proposed that the discrepancy between the two estimates of λ max may relate to the purification protocols used, in which phytochrome is purified as either Pr (Kelly and Lagarias 1985) or as Pfr (Vierstra and Quail 1983a). Recently, it has been demonstrated that the spectral properties of oat phytochrome purified as either Pr or Pfr are distinct (Lagarias et al 1987). Nevertheless, the retention of the shoulder in the red region of the Pfr^P spectrum

is not a function of the purification protocol, as the phytochrome used in the analyses reported here was purified as Pfr, using a modification of the Vierstra and Quail (1983a) method.

5.4.5 Applications of a Pr-specific mAb

The identification of a Pr-specific antibody potentially allows the independent characterisation of the two forms of phytochrome. Of special interest is the observation that LAS 41 enables the purification of Pfr^P, which could be useful in physico-chemical characterisations of the two forms of the molecule. Previous reports of mAbs with differing affinities for Pr and Pfr have discussed the possibility of using such mAbs for the independent immunoassay of the two forms and hence for the determination of phytochrome photoequilibrium (Thomas et al 1984b; Cordonnier et al 1985). However, such assays have not yet been performed, presumably because form-specific antibodies have not yet been available. Using the ELISA (5.1) in which phytochrome titrates as monomers LAS 41 can be used to determine phytochrome photoequilibrium. However, the general use of this immunoassay for the determination of phytochrome photoequilibrium is limited. As this assay is concentration dependent it would be necessary to accurately quantify total phytochrome which would require a second immunoassay. Furthermore, although there is a log-linear relationship between ELISA value and phytochrome photoequilibrium the slope of the line

produced is rather shallow making it difficult to assign a particular photoequilibrium to unknowns. Finally, LAS 41 does not recognise "light-grown-type" phytochrome or cross react widely with phytochrome from other species (see section 6.3, 6.4) which would restrict the use of this assay to extracts from etiolated tissue from the grasses.

The possibility of using LAS 41 to independently immunolocalise Pr and Pfr in fixed oat sections has also been tested. However, preliminary experiments were unsuccessful (E. Schäfer personal communication).

5.4.6 Evaluation of methods for determining differential affinities of monoclonal antibodies for the two forms of phytochrome

The production of LAS 41, a mAb specific for Pr, has facilitated the evaluation of assay designs for the determination of differential affinities for Pr and Pfr. Using LAS 41 as a reference, differential binding to Pr and Pfr was assayed either by measuring bound phytochrome as described by Cordonnier et al (1985) and Shimazaki et al (1986), or bound mAb as described by Thomas et al (1984b).

Monoclonal antibodies were screened for differential affinities towards Pr and Pfr using a modification of the protocol described by Cordonnier et al (1985), ELISA 5.2. Of the six mAbs to show differences, LAS 33 exhibits the greatest difference, exhibiting a ten fold higher affinity for Pr than Pfr (Fig 5.2). Of the other mAbs that show affinity differences,

LAS 31, 34, 35 and 41 also have a higher affinity for Pr than Pfr, whilst LAS 42 has a higher affinity for Pfr (Fig 5.2). In each instance, with the exception of LAS 42, the Pr-preferring mAbs exhibit greater than four fold affinity differences. Likewise, Cordonnier et al (1985) also report affinity differences greater than 4.2 fold which, using this assay design is greater than the theoretical maximum, as phytochrome can exist as stable heterodimers in vitro (see section 5.4.2). Thus, following red-irradiation of a solution of phytochrome 23.6% of the dimers present will contain Pr molecules (Napier and Smith 1987). Consequently, if the phytochrome bound by a Pr-specific mAb is assayed, the Pfr curve should be shifted to a four fold higher concentration. Yet using such an assay design the observed affinity difference for a mAb specific for Pr is anomalously high (Fig 5.2).

Cordonnier et al (1985) predict that in such an assay system, a Pr-specific mAb would exhibit a 7-8 fold higher affinity for Pr over Pfr and so must have assumed that phytochrome only exists as homodimers in vitro. Alternatively, they could have assumed that phytochrome is monomeric in their assay. However, ELISA buffers do not cause dissociation of phytochrome (data not reported) and Partis et al (1984) have shown that phytochrome is still dimeric in ELISA. Certainly there are technical problems associated with the quantitative

element of this assay design. The polyclonal antibodies which detect bound phytochrome may somehow be overestimating the differential binding, or the amplification of the affinity differences may derive from the reduced binding of phytochrome heterodimers by the immobilised mAb. In order to assess whether the anomaly resulted from detection of the bound phytochrome by polyclonal antibodies LAS 32, a mAb which does not exhibit differential affinities for Pr or Pfr (Fig 5.2) was labelled with peroxidase and used to detect bound phytochrome, in place of the polyclonal antibodies. The three mAbs tested, LAS 31, 41 and 11 reproducibly exhibited the same affinity difference between Pr and Pfr as in the first assay (Fig 5.3). This may indicate that the overestimation does not stem from the detection of bound phytochrome, but may relate to the binding of the phytochrome to the mAb. However, when mAb and phytochrome are mixed together in free solution, such as in the immunoprecipitation analyses and in ELISA 5.1, there is no apparent reduction in binding to heterodimers. The overestimated affinity difference may therefore result from some other unexplained artefact of the ELISA configuration/assay.

Using an alternative ELISA configuration (ELISA 5.1) it is possible to overcome the technical problems outlined above. In this instance phytochrome and mAb are mixed together in free solution and then added to immobilised anti-phytochrome polyclonal

antibodies. As the bound mAb is assayed directly, the phytochrome molecules will titrate as monomers. As a Pr-specific mAb will bind only to Pr molecules, using this configuration of assay a Pr-specific mAb would exhibit a 7.69 fold higher affinity for Pr, as is observed for LAS 41 (Fig 5.1). In addition, all the other mAbs which exhibit differential affinities in the initial assay (ELISA 5.2) (Fig 5.2), exhibit lower affinity differences here (Fig 5.1). Thus, this assay is detecting intrinsic differences between Pr and Pfr and, significantly, is also genuinely quantitative.

In conclusion, the assay configuration adopted by Cordonnier et al (1985) does detect mAbs which are recognising intrinsic differences between Pr and Pfr but due to some anomalous character of the assay it is not quantitative. Shimazaki et al (1986) have also commented on the disparity between ELISA configurations when assaying mAbs for differential binding to Pr and Pfr. These workers also find that the ELISA of Cordonnier et al (1985) produces larger apparent affinity differences than that of Thomas et al (1984b). Hence, caution should be observed when using an assay configuration in which bound phytochrome is measured. The ELISA design (ELISA 5.1) which titrates phytochrome on the basis of monomers, however, can be used as a truly quantitative assay for mAbs which recognise intrinsic differences between Pr and Pfr.

CHAPTER 6

CROSS REACTIVITY ANALYSES

6.1 Introduction

Several workers have used immunochemical techniques to probe for conserved antigenic domains amongst phytochromes from widely different plant species. This strategy stems from the assumption that regions of biological importance on the molecule will have been conserved throughout evolution (Cordonnier et al 1983), and therefore mAbs which demonstrate wide cross reactivity may be binding to epitopes essential to the biological function of the molecule.

Initially, cross reactivity studies were conducted with polyclonal antisera raised against partially degraded phytochrome. Using double diffusion and microcomplement fixation assays, Pratt (1973) demonstrated that anti-oat antibodies could recognise rye and barley, but not pea phytochrome. Similarly, Rice and Briggs (1973) demonstrated that anti-oat antibodies recognised rye and maize phytochrome, although partial cross reactivity was also observed with pea phytochrome, in double diffusion assays. Cordonnier and Pratt (1982b) also used double diffusion assays to test for cross reactivity. Using anti-oat antibodies they provided evidence for the existence of epitopes common to phytochrome regardless of source, although in general monocot and dicot phytochromes formed two antigenically distinct groups. However, the use of polyclonal antibodies in cross reactivity studies is fairly limited. Although polyclonal antibodies can cross react with phytochromes

isolated from several plant species it is difficult to identify and separate these antibodies. It is also difficult to identify the region(s) to which the antibodies are binding, as they presumably are raised to various sites on the phytochrome molecule. These limitations can be overcome by the use of mAbs which have a defined specificity, being raised to a single epitope. Consequently, further studies have involved the use of mAbs in comparative studies to identify epitopes which are common to phytochrome irrespective of its source.

In general most of the mAbs tested have shown limited cross reactivity. Nagatani et al (1983) using mAbs to "large" rye phytochrome tested cross reactivity against oat and pea phytochrome in a radioimmunoassay (RIA). Only 2 of the mAbs cross reacted with oat phytochrome and none crossed with pea phytochrome. In contrast, Cordonnier et al (1984) using mAbs raised to "large" oat and pea phytochrome, tested cross reactivity against 6 plant species in ELISA. Some of the mAbs cross reacted with pea, zucchini, lettuce, oat, rye and barley phytochrome, whilst others merely reacted with the antigen used for their production. Less cross reactivity was observed by Saji et al (1984) using a RIA. They tested 6 anti-rye and 6 anti-pea mAbs against phytochrome from 8 plant species. Although 2 of the anti-rye mAbs cross reacted with oat phytochrome and 3 anti-pea mAbs recognised phytochrome from the other dicots, there was no

monocot x dicot cross reactivity in either direction. Daniels and Quail (1984) also describe very poor cross reactivity with mAbs raised to 124 kDa oat phytochrome, using SDS-PAGE and immunoblotting. From a panel of 46 anti-oat mAbs only 1 cross reacted with zucchini phytochrome. Whitelam et al (1985) describe 6 mAbs raised against "large" oat phytochrome which exhibit wide cross reactivity amongst the monocots, two of which also crossed with all the dicots tested on immunoblots. Using a similar technique Cordonnier et al (1985) describe 3 mAbs raised against native oat phytochrome whose cross reactivity is limited to the monocots.

Only 1 mAb, Pea-25 has been isolated that cross reacts with phytochrome from a diverse range of plant species (Cordonnier et al 1986a). Not only does this mAb cross react with phytochrome from many etiolated sources, it will also immunostain a putative phytochrome polypeptide in crude extracts from light-grown material.

In this chapter the 6 mAbs which recognise SDS-denatured phytochrome are screened for the ability to cross react with phytochrome from 12 different plant species, which include both monocots and dicots. Since immunochemical analyses with mAbs to maize phytochrome suggest that phytochrome extracted from shoots and roots of maize may be distinct (Schwarz and Schneider 1987), the mAbs were tested for their ability to recognise phytochrome extracted from oat

and maize roots. In addition the mAbs were tested for the ability to recognise "light-grown-tissue-type" oat phytochrome. This is because phytochrome extracted from green plants may be composed of more than one phytochrome species (Tokuhsa et al 1985, Cordonnier et al 1986b). Five of the mAbs exhibit a range of cross reactivity which is confined to phytochrome from etiolated grasses, whilst LAS 32 crosses with phytochrome from every plant species tested.

6.2 Materials and Methods

6.2.1 Plant materials and growth conditions

Seeds were sown in moist vermiculite, germinated and grown in total darkness at 25C for five days prior to harvesting. Seeds of Amaranthus, mustard, sugar beet and cauliflower were sown in petri dishes on moist filter paper and grown in total darkness at 25C for four days prior to harvesting. The plants tested and the tissue harvested were as follows:

Oat (Avena sativa cv Dula), whole shoots and root tips; Wheat (Triticum aestivum L.), whole shoots; Barley (Hordeum vulgare L.), whole shoots; Maize (Zea mays L.), whole shoots and root tips; Bromus (Bromus sterilis L.), whole shoots; Onion (Alium cepa L.), whole shoots; Rice (Oryza sativa L.), whole shoots; Pea (Pisum sativum cv Onward), half shoots; Amaranthus (Amaranthus caudatus), whole shoots; Marrow (Cucurbita pepo L.), hypocotyl hooks; Sugar beet (Beta vulgaris L.), whole shoots; Tomato (Lycopersicon esculentum L.), whole shoots; Sunflower (Helianthus annus L.), hypocotyl hooks; Lettuce (Lactuca sativa L.), whole shoots; Cauliflower (Brassica oleraceae L.), whole shoots; Broad bean (Vicia faba L.), hypocotyl hooks; Mustard (Sinapis alba L.), whole shoots; Mungbean (Phaseolus aureus L.), hypocotyl hooks. Mesotaenium caldariorum (CCAP, Cambridge) was grown in continuous light in batches of BG11 medium (Stanier et al 1977).

Under dim green safelight plant tissues were harvested into liquid nitrogen. Following powdering

in a pestle and mortar the samples were lyophilised. Mesotaenium was harvested by centrifugation, resuspended into a small volume of distilled water and frozen at -20C. Once frozen the sample was also lyophilised. "Light-grown-tissue-type" oat phytochrome was partially purified as described in section 2.2.6.

6.2.2 SDS-PAGE and immunoblotting

Lyophilised samples were prepared for SDS-PAGE by extraction into single strength SDS sample buffer at a ratio of 75 mg powder per ml sample buffer. The "light-grown-tissue-type" phytochrome sample was mixed with double strength SDS-sample buffer. Samples were heated to 96C for 3 min and clarified by centrifugation at 12,000 x g for 5 min. Each sample (50 µl of supernatant) was resolved on 7.5% SDS-polyacrylamide gels (Laemmli 1970). Following electrophoresis polypeptides were electroblotted onto nitrocellulose filters as described in section 4.2.1.2. For LAS 11, 31, 33, 41 and 35 the nitrocellulose filters were immunodeveloped as described in section 4.2.1.2. For LAS 32 the nitrocellulose filters were immunodeveloped as described in section 4.2.1.3 except the detection of bound alkaline phosphatase was with 0.5 mg ml^{-1} BCIP and 0.3 mg ml^{-1} nitro blue tetrazolium (NBT) (Sigma) in 100 mM Tris-HCl, (pH 9.5), containing 100 mM NaCl and 50 mM MgCl_2 .

6.3 Results

The 6 mAbs which recognise SDS-denatured oat phytochrome were screened against crude extracts from 12 different plant species on immunoblots. For 5 of the mAbs the extent of cross reactivity was limited to the grasses. LAS 11 only cross reacts with phytochrome from barley and Bromus (Fig 6.1a). LAS 31 and 33 only recognise phytochrome extracted from maize and Bromus (Fig 6.1b, c). LAS 35 and 41 exhibit a wider pattern of cross reactivity. LAS 35 cross reacts with all the monocots tested except onion and rice (Fig 6.1d). LAS 41 cross reacts with all the monocots tested except onion (Fig 6.1e). The greatest cross reactivity is observed with LAS 32. LAS 32 cross reacts with all the monocots and dicots tested, plus an additional 5 dicot species which were not used to screen the other mAbs (Fig 6.1f)

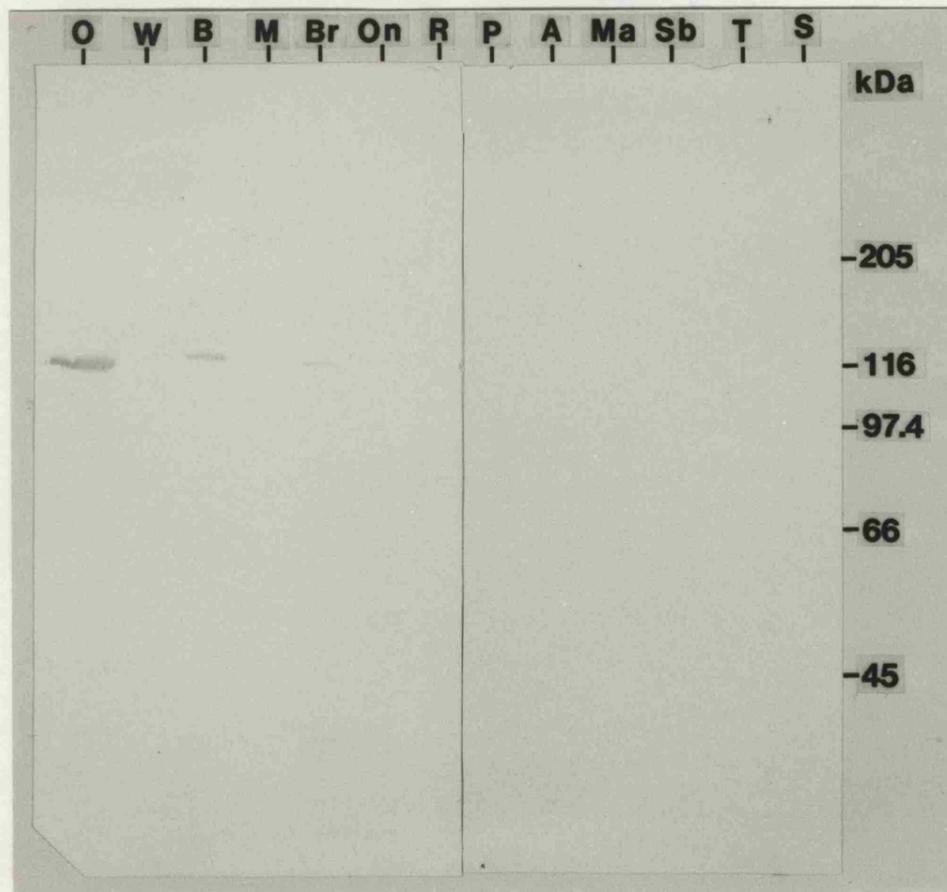
Further cross reactivity analyses tested for the ability to detect "light-grown-tissue-type" oat phytochrome and phytochrome from oat and maize roots. All the mAbs tested immunostain a single band ~124 kDa for the "light-grown-tissue-type" phytochrome (Fig 6.2a, b, c, d, e, f). However, LAS 32 immunostains an additional slightly smaller peptide (Fig 6.2f). LAS 11, 32, 33 and 41 recognise oat root phytochrome. LAS 32, 33, 35 and 41 all immunostain maize root phytochrome. LAS 31 does not recognise phytochrome from either oat or maize roots. All these results are summarised in Table 6.1.

Figure 6.1 Immunodetection of phytochrome
in SDS-denatured crude extracts from 17
plant species

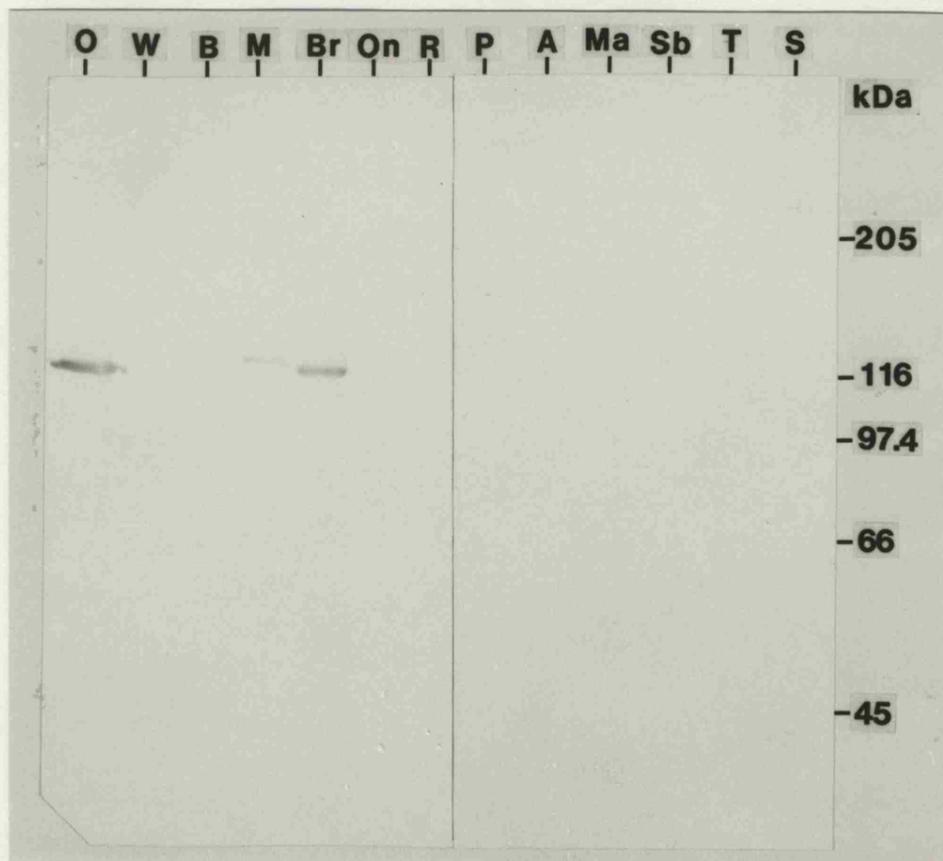
Lyophilized samples of etiolated plant material were prepared for SDS-PAGE by extraction into single strength SDS sample buffer at a ratio of 75 mg powder per ml sample buffer. Each sample (50 μ l) was resolved on 7.5% SDS-gels and electroblotted onto nitrocellulose. Replica nitrocellulose filters were immunodeveloped with a) LAS 11, b) LAS 31, c) LAS 33, d) LAS 35, e) LAS 41, f) LAS 32, g) non-immune serum, h) protein stain of extracts.

O = Oat
W = Wheat
B = Barley
M = Maize
Br = Bromus
On = Onion
R = Rice
P = Pea
A = Amaranthus
Ma = Marrow
Sb = Sugar beet
T = Tomato
S = Sunflower
L = Lettuce
C = Cauliflower
Bb = Broad bean
Mu = Mustard
Mb = Mung bean

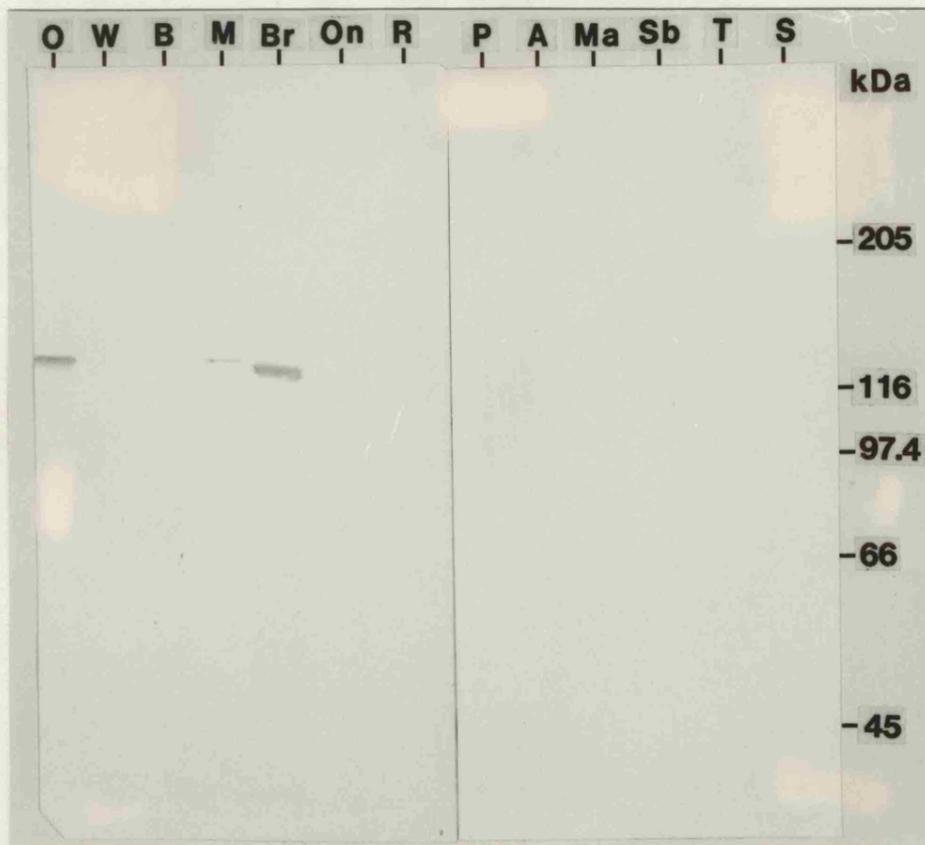
6.1a



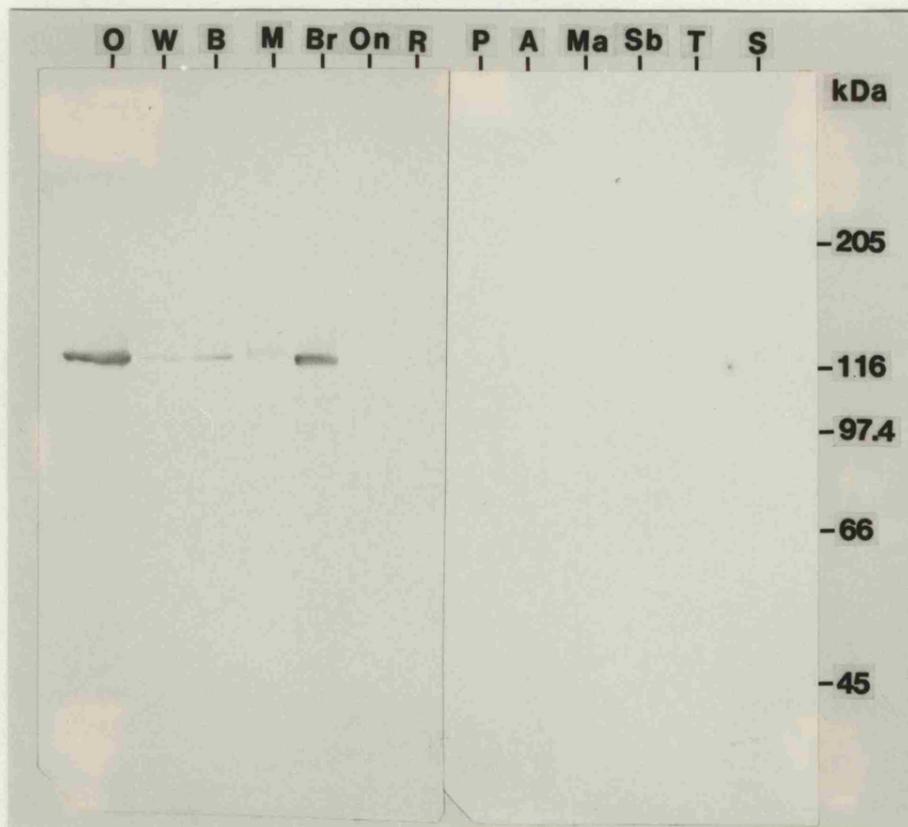
6.1b



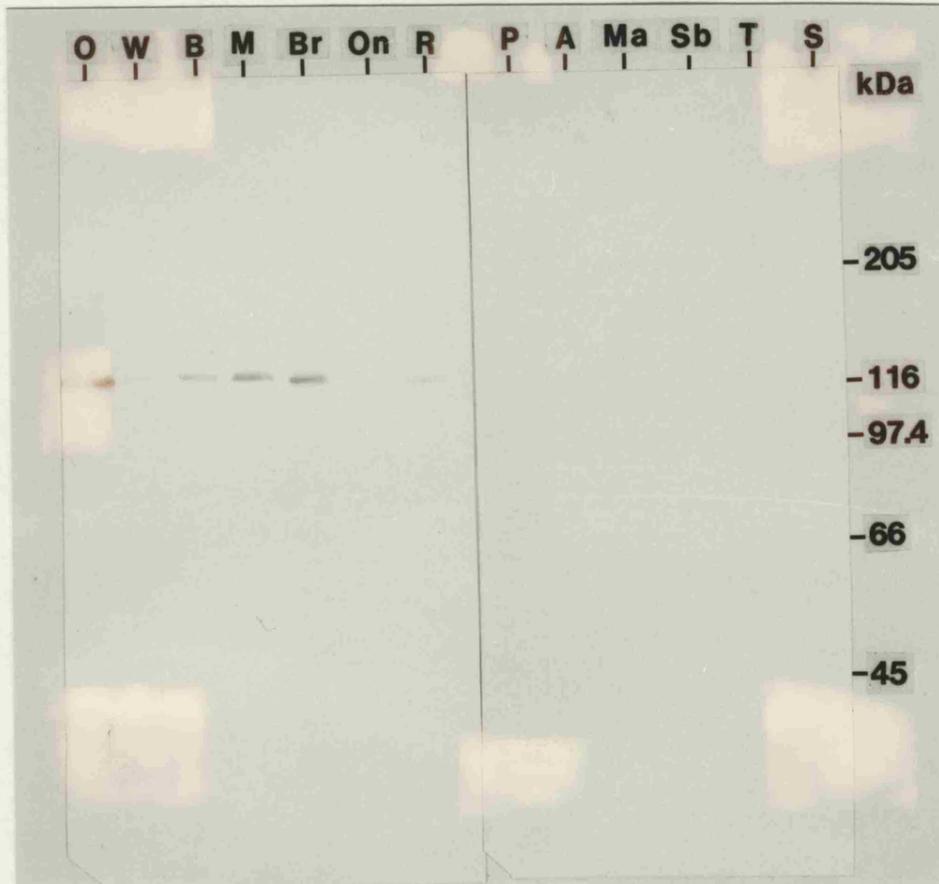
6.1c



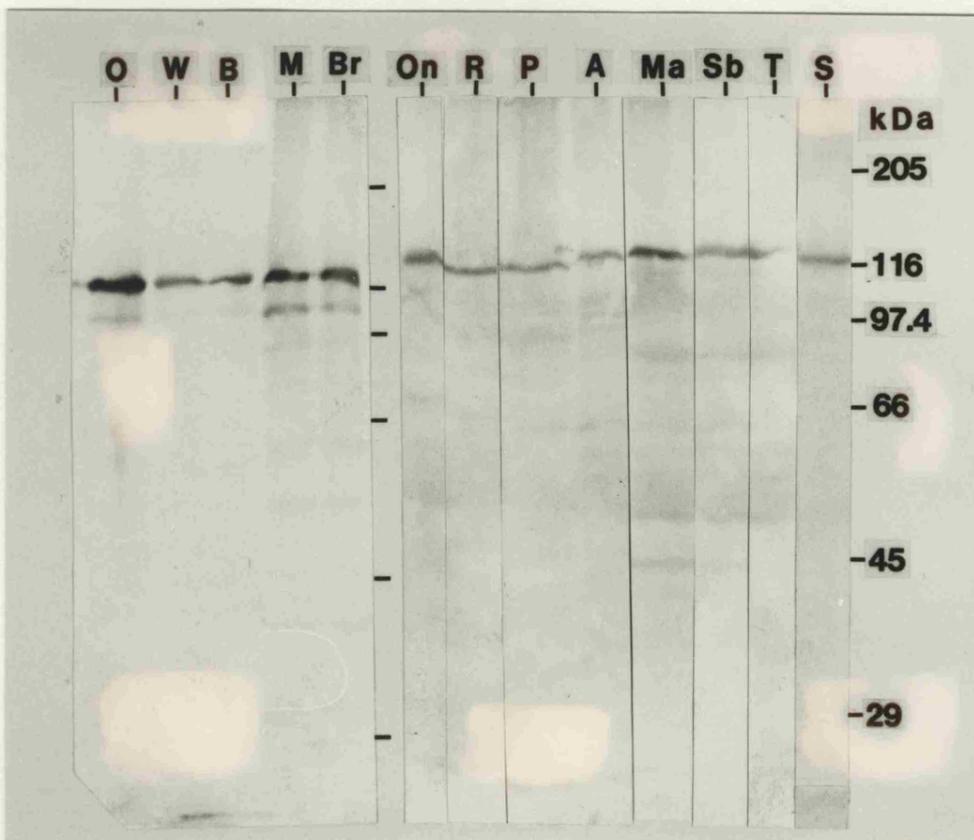
6.1d



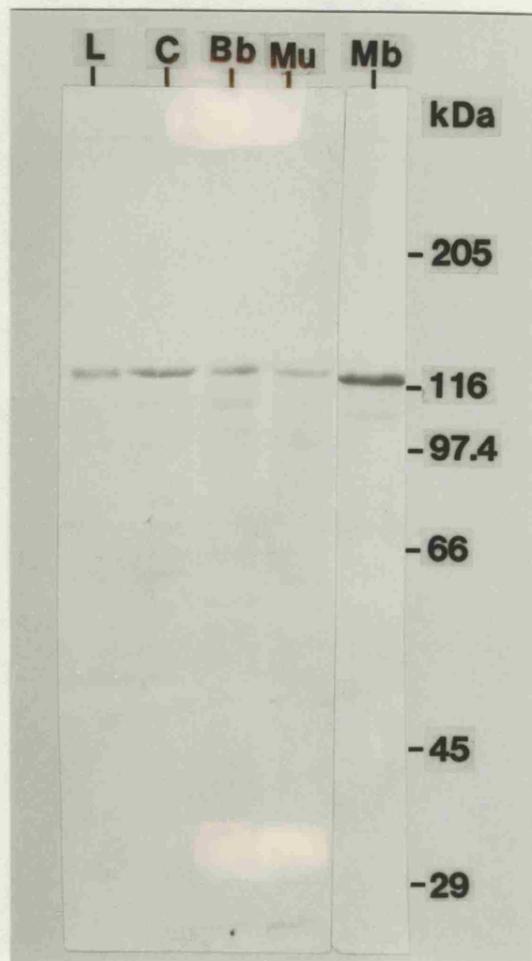
6.1e



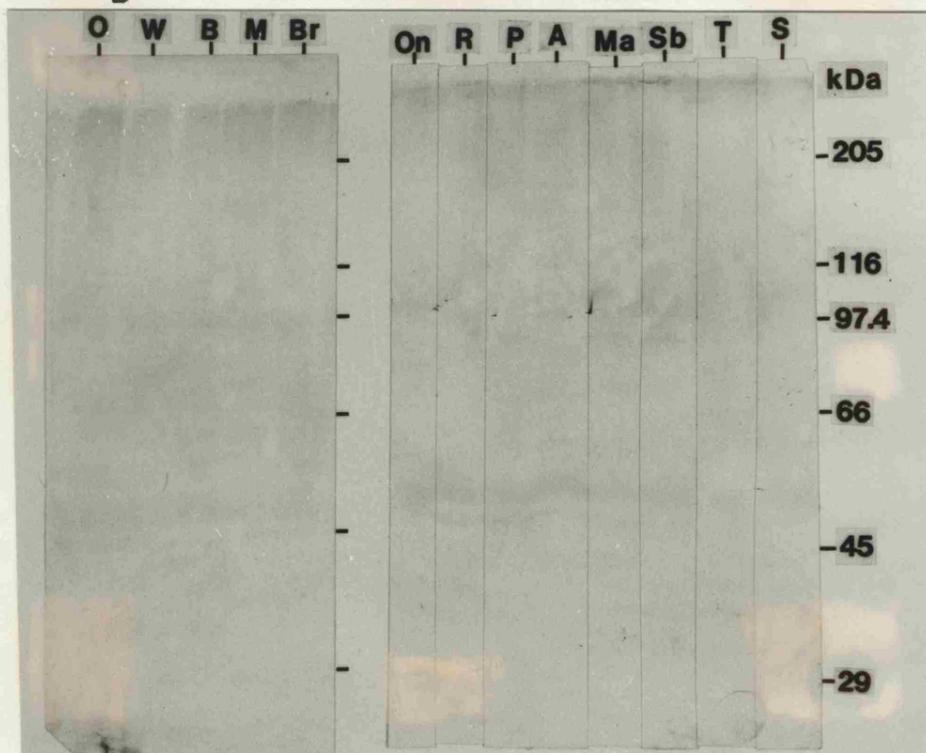
6.1f



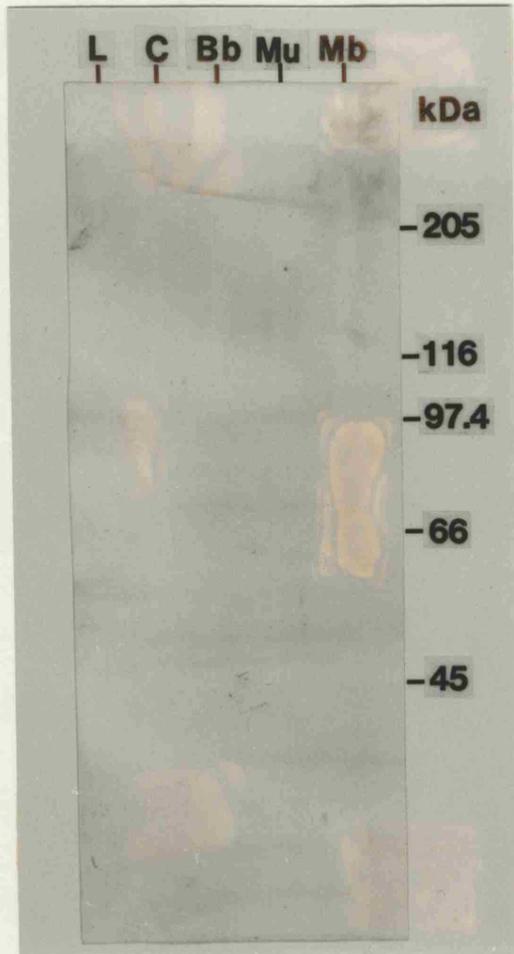
6.1f (continued)



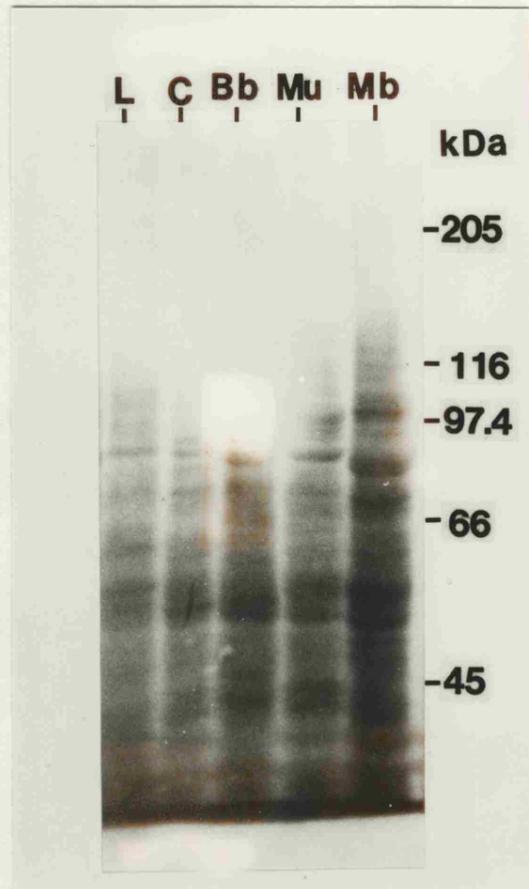
6.1g



6.1g (continued)



6.1h



6.1h (continued)

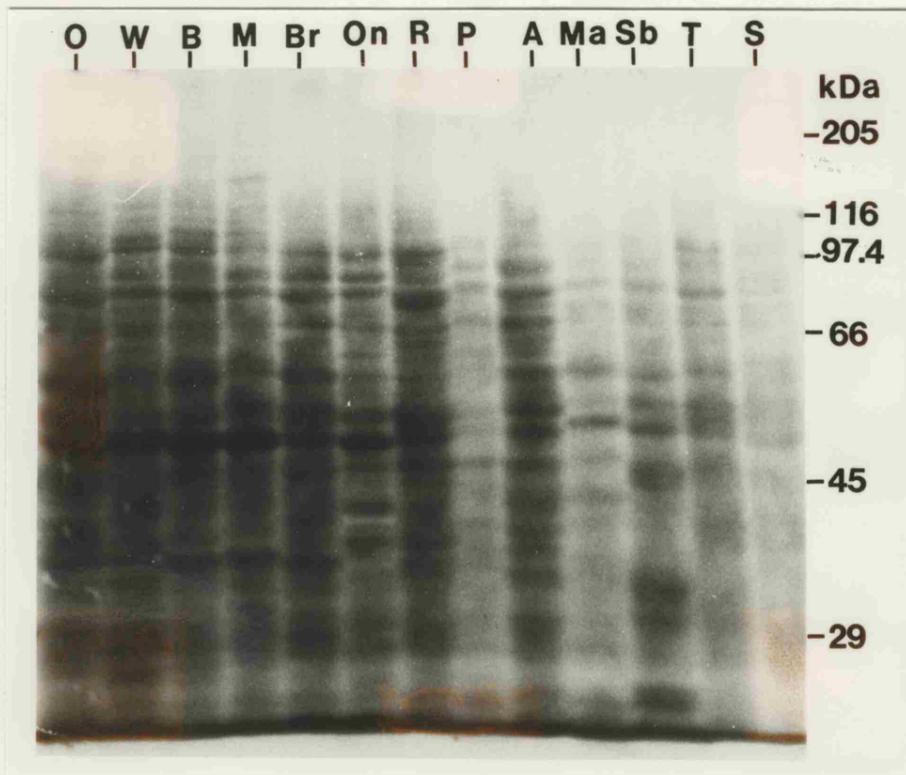


Figure 6.2 Immunodetection of "light-grown-tissue-type" phytochrome from oats, phytochrome in crude extracts of oat and maize roots and in extracts of Mesotaenium

Lyophilized samples of maize and oat roots and Mesotaenium were prepared for SDS-PAGE by extraction into single strength SDS-sample buffer at a ratio of 75 mg powder per ml sample buffer. Each sample (50 μ l supernatant) as well as samples of etiolated oat phytochrome (300 ng per lane) and "light-grown-tissue-type" oat phytochrome (300 ng per lane) were resolved on 7.5% SDS-gels and electroblotted onto nitrocellulose. Replica nitrocellulose filters were immunodeveloped with a) LAS 11, b) LAS 31, c) LAS 33, d) LAS 35, e) LAS 41, f) LAS 32, g) non-immune serum, h) protein stain.

O = etiolated oat phytochrome

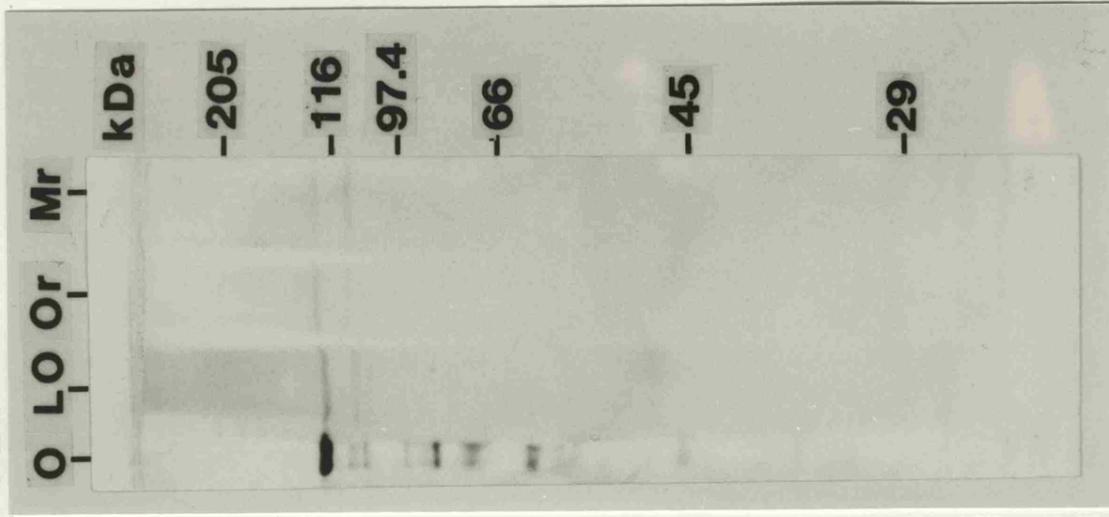
LO = "light-grown-tissue-type" oat
phytochrome

Or = oat root

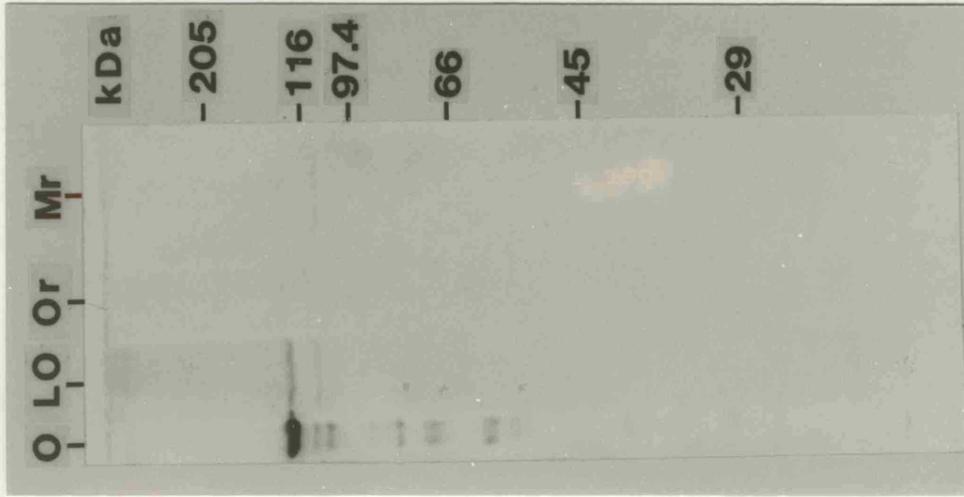
Mr = maize root

Me = Mesotaenium

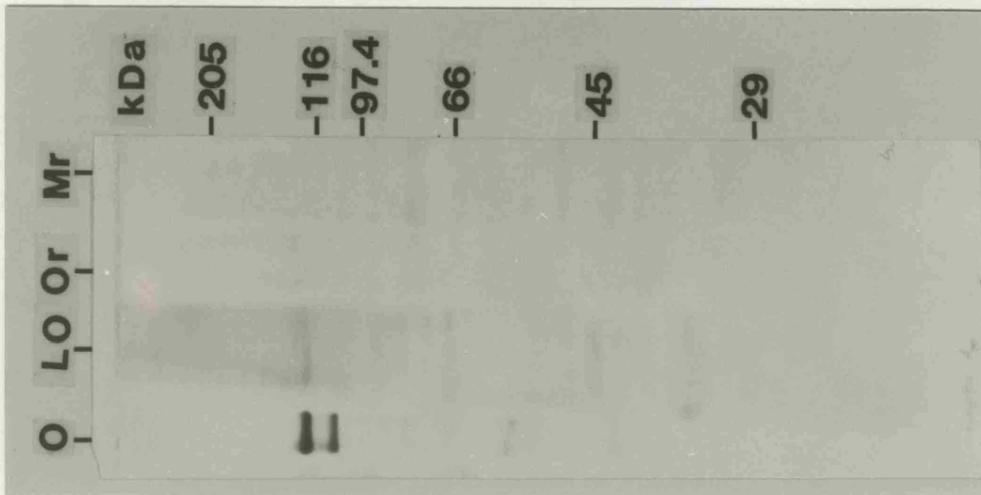
6.2c

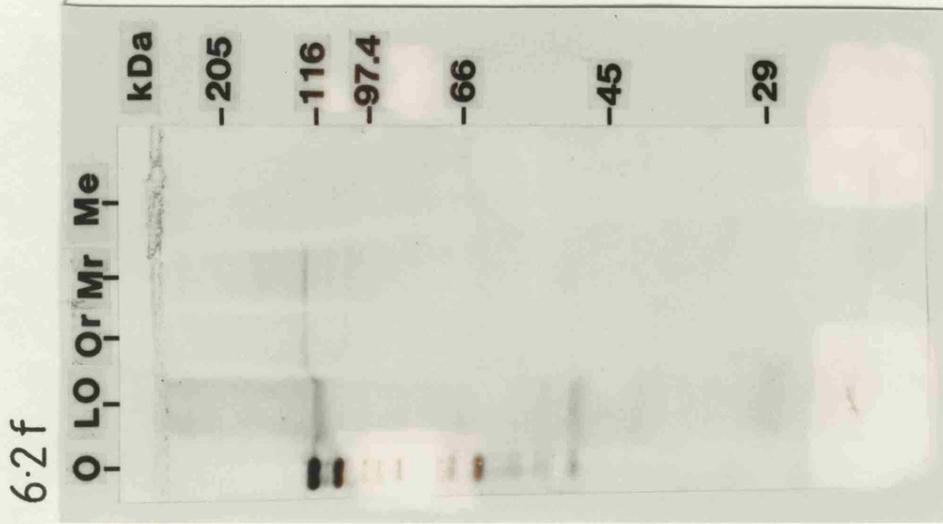
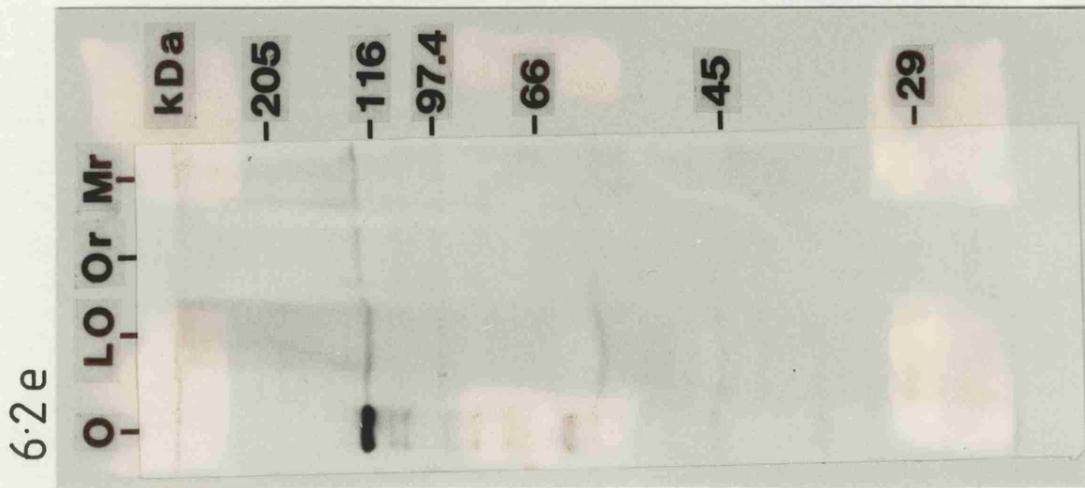
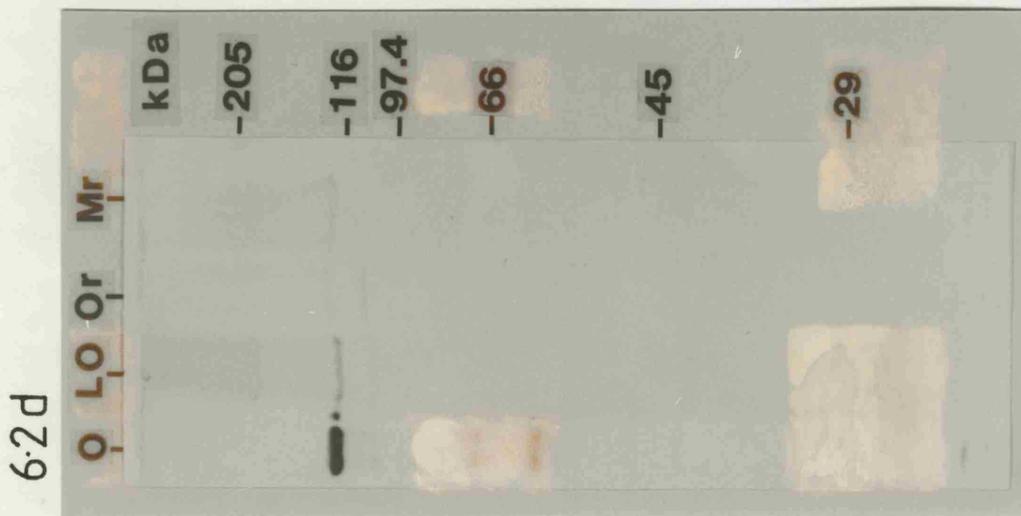


6.2b



6.2a





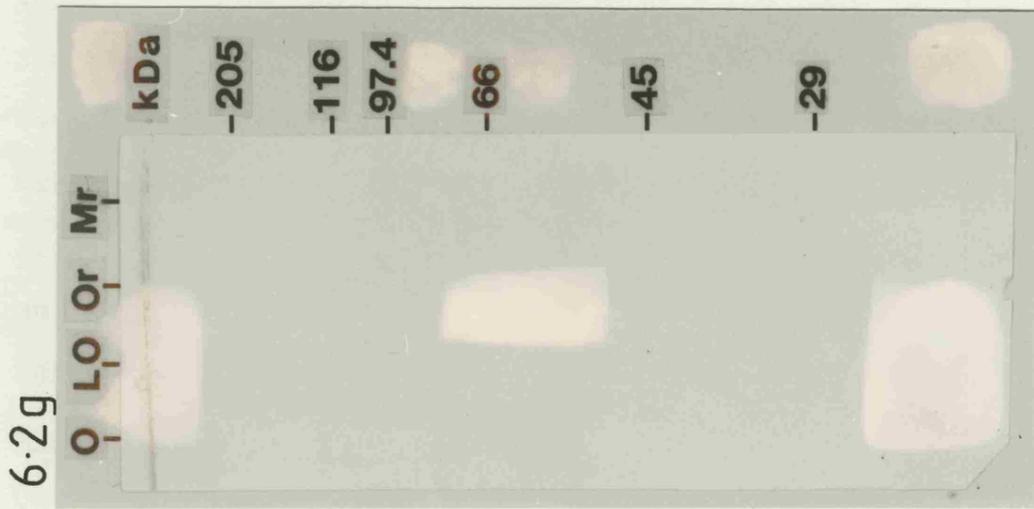
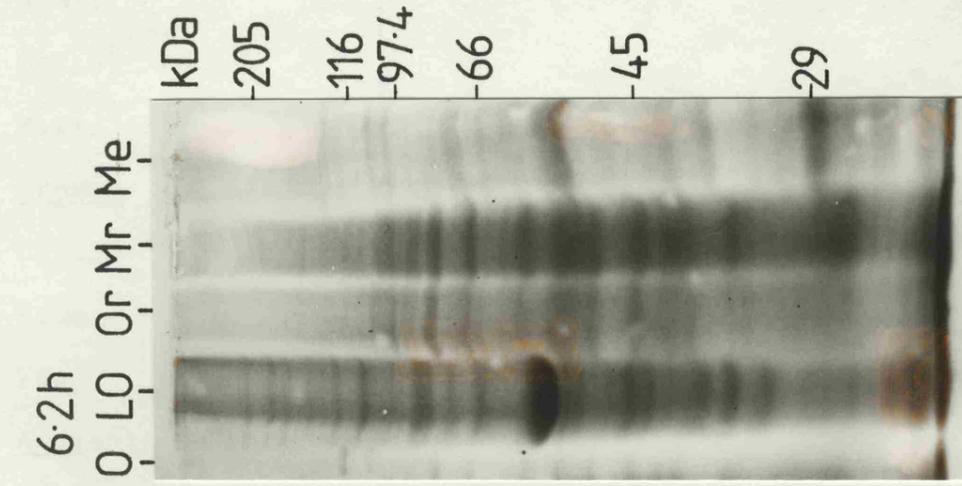


Table 6.1 Summary of cross reactivities of mAbs with phytochrome in SDS-denatured crude extracts of 17 plant species

Species tested	mAb					
	11	31	32	33	35	41
Wheat	-	-	+	-	+	+
Barley	+	-	+	-	+	+
Maize	-	+	+	+	+	+
<u>Bromus</u>	+	+	+	+	+	+
Onion	-	-	+	-	-	-
Rice	-	-	+	-	-	+
Pea	-	-	+	-	-	-
<u>Amaranthus</u>	-	-	+	-	-	-
Marrow	-	-	+	-	-	-
Sugar beet	-	-	+	-	-	-
Tomato	-	-	+	-	-	-
Sunflower	-	-	+	-	-	-
Lettuce	-	-	+	-	-	-
Cauliflower	N.T	N.T	+	N.T	N.T	N.T
Broad bean	N.T	N.T	+	N.T	N.T	N.T
Mustard	N.T	N.T	+	N.T	N.T	N.T
Mung bean	N.T	N.T	+	N.T	N.T	N.T
<u>Mesotaenium</u>	N.T	N.T	-	N.T	N.T	N.T
Oat ("light-grown-tissue-type")	+/-	+/-	++	+/-	+/-	+/-
Oat root	+	-	+	+	+	+
Maize root	-	-	+	+	+	+

N.T = not tested

6.4 Discussion

The 6 mAbs which gave good reactivity towards SDS-denatured oat phytochrome were tested to assess the extent of cross reactivity with electroblotted phytochrome from 12 other plant species. The cross reactivity studies undertaken here were conducted on crude extracts. However, any immunostaining of peptides observed are likely to result from specific staining, as peptides of a similar size to phytochrome represent a tiny proportion of the proteins present, and where cross reactivity is observed, the corresponding peptide is not immunostained by non-immune mouse immunoglobulins. Furthermore, in the case of mAbs which exhibit limited cross reactivity, where the mAbs do not immunostain a peptide of ~ 120 kDa, then the negative lanes can be considered as a set of "internal controls" for specific staining.

In each case, cross reactivity is defined as the recognition of a peptide of ~ 120 kDa, which is not stained by the non-immune serum. The recognition of a peptide which is of a similar size to phytochrome does not however, confirm that the peptide that is immunostained is phytochrome. It may merely be a peptide which carries shared epitopes. Therefore, the peptide stained can only be considered to be putatively phytochrome. In addition lack of immunostaining has been considered to be indicative of lack of cross reactivity. However, lack of immunostaining may result from very weak cross

reactivity which, if the phytochrome was present at higher concentrations, could be seen.

Amongst the mAbs tested the only mAb to cross react with phytochrome from species of dicots was LAS 32. The remaining 5 mAbs crossed with phytochrome from monocot species, but in some cases the cross reactivity was very limited. Specifically, LAS 11 which is a Type 2 mAb, only cross-reacted with phytochrome from barley and Bromus. On the basis of this observation, the epitope to which LAS 11 binds is not particularly conserved. However, there are sites on the 64 kDa chromophore-bearing domain which are highly conserved. LAS 34 a 4.2 mAb cross reacts with pea phytochrome in ELISA and therefore the site to which this mAb binds is conserved across the monocot/dicot division. In addition, using polyclonal antisera raised specifically to the undecapeptide which surrounds the chromophore attachment site, Mercurio et al (1986) have demonstrated that this region of the molecule is also highly conserved amongst monocots and dicots.

LAS 31 and 33 which are both Type 1 mAbs show a limited pattern of cross reactivity, recognising only maize and Bromus phytochrome. The lack of cross reactivity indicates that although the 6 kDa NH₂-terminus is vital for the retention of the spectral integrity of oat phytochrome, the region to which LAS 31 and 33 are binding is not very well conserved. This is consistent with the observations

of Cordonnier et al (1985) who isolated 3 mAbs which map to the 6 kDa NH₂-terminal domain and also exhibit limited cross reactivity, which is restricted to the grasses. There is only one report of a mAb which maps to the 6 kDa NH₂-terminus which cross reacts with dicot phytochrome (Daniels and Quail 1984). The reason for the lack of conservation may be that this region of the molecule is surface located and therefore under more "evolutionary pressure" than regions of the molecule that are internalised (D. Bowles, personal communication).

Unlike the 6 kDa NH₂-terminus, the adjacent 4 kDa domain is apparently more conserved. LAS 35 a Type 1' mAb, recognises wheat, barley, maize and Bromus phytochromes. LAS 41, also a Type 1' mAb, recognises the same range of species as LAS 35, but also recognises rice phytochrome. This is the first evidence that a highly conserved epitope, at least amongst the grasses, is carried on the 4 kDa sub-NH₂-terminal domain.

The greatest cross reactivity is exhibited by LAS 32. This mAb which maps between 74 and 88 kDa from the NH₂-terminus on the 55 kDa COOH-terminal domain, cross reacts with every plant species tested, with the exception of the alga Mesotaenium. The lack of cross reactivity with Mesotaenium may result from insufficient phytochrome being present in the sample, or a genuine lack of recognition. However, attempts to enrich the sample for phytochrome proved unsuccessful and therefore it is not possible to

define the reason for the lack of recognition.

Clearly, LAS 32 is raised to an extremely highly conserved region of the molecule.

LAS 32 exhibits similar cross reactivities to Pea-25 (Cordonnier et al 1986a). Pea-25 cross reacts with a putative phytochrome peptide from several green plant tissues. However, LAS 32 has only been tested against "light-grown-tissue-type" oat phytochrome, and so the extent of cross reactivity amongst other green tissue phytochromes is untested. Both these mAbs map to the same region on the 55 kDa COOH-terminal domain (M-M Cordonnier, personal communication). However, apart from their ability to cross react widely with phytochrome from different plant species these mAbs exhibit distinct characteristics. Besides being raised against different phytochromes, LAS 32 demonstrates a relatively low reactivity towards SDS-denatured oat phytochrome in comparison with the other mAbs but reacts relatively well with native oat phytochrome. Conversely, Pea-25 works very well on immunoblots but does not react particularly well with the native molecule.

The only phytochrome recognised by all the mAbs in addition to oat was Bromus. This suggests that Bromus has several epitopes spread throughout the molecule that are shared with oat phytochrome, although the plant species are not particularly closely related.

Further cross reactivity analyses assessed the ability of the mAbs to recognise phytochrome extracted from shoots and roots of oats and maize. Schwarz and Schneider (1987) have demonstrated that some anti-maize phytochrome mAbs only recognise phytochrome extracted from maize shoots but not from maize roots. They therefore propose that phytochrome from maize shoots and roots may be distinct. The fact that LAS 31 can detect shoot but not root phytochrome in maize and oats provides further evidence that shoots and roots may contain different pools of phytochrome. Since other mAbs can cross react with root and shoot phytochrome, root phytochrome may represent a slightly modified molecule which carries shared epitopes with shoot phytochrome, but is not totally homologous. Shoot and root phytochrome are the same size, as determined by SDS-PAGE (Schwarz and Schneider 1987) which is also consistent with them representing only slightly modified molecules. It may be possible to determine the extent of homology between root and shoot phytochrome by conducting Cleveland mapping on the phytochrome extracted from shoots and roots. As this method can identify as little as a 2% overall difference in structure, even if root phytochrome represents only a slightly modified molecule it may be possible to detect these differences. The differences observed between shoot and root phytochrome may be a consequence of differential expression of phytochrome genes.

It has been demonstrated that there are 4 distinct phytochrome genes which belong to a gene family and the regions which have been sequenced are 98% homologous (Hershey et al 1985). Perhaps these genes are differentially expressed in shoots and roots.

The final assessment of cross reactivity tested for the ability to recognise "light-grown-tissue-type" phytochrome. All the mAbs recognised a peptide which was ~124 kDa in the "light-grown-type" extract. However, LAS 32 immunostains a doublet with the top band of the doublet corresponding to the single band of ~124 kDa that the other mAbs are recognising. Although the same amount of etiolated oat phytochrome and "light-grown-tissue-type" phytochrome, in terms of $\Delta(\Delta A)$ measurements, were run side by side on immunoblots the intensity of staining of the "light-grown-tissue-type" phytochrome is much reduced when compared to that of etiolated oat phytochrome. In the case of mAbs which only immunostain the ~124 kDa species, reduced staining could be due to limited cross reactivity, or may merely reflect a quantitative difference in the presence of etiolated phytochrome. However, it is not possible to distinguish between these alternatives. It may be significant that when Tokuhsa et al (1985) tested Type 1, 2 and 3 mAbs which were raised to native etiolated oat phytochrome only the Type 3 mAb cross reacted with phytochrome from green tissue.

Significantly, Tokuhisa et al (1985) report that green tissue phytochrome comprises two molecular species of differing molecular masses. The most abundant species (~70% of the total) has a Mr of 118 kDa and the lower abundance species (~30% of the total) has a Mr of 124 kDa. Tokuhisa et al (1985) suggest that the lower abundance species could represent contaminating etiolated-type phytochrome, or alternatively another separate species of phytochrome. Cordonnier et al (1986b) who do not observe the size differences between the respective phytochrome species, have provided evidence that both the 70% and 30% species are distinct from etiolated-tissue phytochrome, although this has to be confirmed.

The pattern of staining observed for the mAbs tested here suggests that the ~124 kDa species may represent "carry-over" etiolated-tissue phytochrome. Nevertheless, although all the mAbs tested immunostain the ~124 kDa species it is possible that this peptide represents a third minor species of phytochrome.

The "light-grown-tissue-type" phytochrome used here was extracted under conditions to minimize proteolytic cleavage of the native molecule. In addition the sample exhibits spectral characteristics which are consistent with those reported for native green-tissue phytochrome (Tokuhisa et al 1985; Cordonnier et al 1986b). The finding that LAS 32, which exhibits wide cross reactivity, recognises a doublet of peptides of ~124 and ~118 kDa, is

comparable with the findings of Tokuhisa et al (1985) but in conflict with the observations of Cordonnier et al (1986b). As green-tissue phytochrome is particularly susceptible to proteolytic cleavage, Cordonnier et al (1986b) have suggested that the 118 kDa species is a proteolytic cleavage product of the native 124 kDa molecule. However, Cordonnier and Pratt have exchanged samples of green-tissue phytochrome which when subjected to SDS-PAGE ran at either 121 kDa (Geneva) or 125 kDa (Georgia) (Cordonnier et al 1986b). In addition Pratt and Quail have exchanged green-tissue phytochrome samples. The green-tissue phytochrome extracted by Pratt and run on SDS-PAGE by Quail ran at 118 kDa and the green-tissue phytochrome extracted by Quail and electrophoresed by Pratt ran at 124 kDa (Quail personal communication). Therefore the reported discrepancies in the size of green-tissue phytochrome may merely reflect a change in electrophoretic mobility due to slightly different buffer conditions, although in principle the running conditions were identical.

Thus, all the immunochemical evidence from cross reactivity studies are consistent with the observations of Tokuhisa et al (1985). The "light-grown-tissue-type" phytochrome is identical to that extracted from green tissue. As it is not possible to categorically state whether the ~124 kDa species represents a third phytochrome species or etiolated phytochrome, a conservative conclusion would be that only LAS 32 immunostains "light-grown-tissue-type"

phytochrome, with the ~118 kDa species corresponding to that reported by Tokuhisa et al (1985).

Clearly LAS 32 can cross react with phytochrome from a diverse array of plant species. It therefore must be recognising a very conserved region of the molecule. Comparison of the oat and Cucurbita cDNA sequence suggests that at the level of primary and secondary structure the region to which LAS 32 is raised is highly conserved (Sharrock et al 1986). However, it is unlikely that the epitope to which LAS 32 binds represents the active site of the molecule. The epitope does not undergo a light induced conformational change and does not interact directly with the chromophore. However, LAS 32 maps between 74 and 88 kDa from the NH₂-terminus, and as the dimerisation site lies within 42 kDa of the COOH-terminus it may be that LAS 32 maps at or close to the dimerisation site, and therefore is recognising a region of biological significance. Nevertheless it seems likely that there must be additional sites on the molecule which are also highly conserved which may represent the active site(s) of the molecule.

LAS 32 provides a useful probe for the immunopurification of phytochrome from a wide range of species. As it is possible to electro-elute proteins which are bound to mAb coupled to a column without causing denaturation of the molecule (Dean et al 1977), this mAb could be used to

immunopurify phytochrome which could then be further characterised. Furthermore this purified phytochrome could be used to raise new panels of mAbs in order to generate probes for the phytochrome in question which were raised to the whole of the molecule and not just to the COOH-terminus. Significantly this mAb may prove useful in the rapid purification of "light-grown-tissue-type" phytochrome. Consequently using etiolated specific and "light-grown-tissue-type" specific mAbs it would be possible to distinguish whether green-tissue phytochrome does consist of two distinct molecules. This approach has already been used to distinguish green phytochrome in peas (Abe et al 1985). Using mAbs which were specific for etiolated pea phytochrome they have demonstrated that green phytochrome from peas contains two phytochrome pools which are distinct from one another with respect to primary structure but do share common epitopes. The least abundant species corresponds to etiolated pea phytochrome.

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

This thesis has been concerned with the characterisation of phytochrome using mAbs as specific probes to help gain an understanding of the mechanism of phytochrome action. In particular, mAbs have been used to detect differences which exist between Pr and Pfr. In addition, mAbs have been used to search for conserved domains amongst phytochrome from many different plant species, as these may represent regions of biological significance.

7.2 Differences between Pr and Pfr

Type 1, 1' and 2 mAbs have been used to demonstrate that there are several sites on the phytochrome molecule that undergo light induced conformational changes. This observation which is consistent with previous findings (Cordonnier et al 1985; Shimazaki et al 1986), suggest that throughout the molecule there are significant differences between Pr and Pfr. However, despite the isolation of a large number of mAbs which exhibit differential affinities for Pr and Pfr, analytical chromatography, H'NMR and CD spectroscopy analyses suggest that there are few, if any, gross changes in structure between Pr and Pfr (Jones and Quail 1986a; Lagarias and Mercurio 1985; Vierstra et al 1987). This could mean that form-discriminating-mAbs, raised in different laboratories are in fact recognising only a limited number of epitopes on the respective domains. Therefore, there could be as few as 5 or 6

localised changes in conformation between Pr and Pfr, which would account for the lack of detection of gross changes in conformation.

If the mAbs which exhibit differential affinities for Pr and Pfr are raised to just a few regions on the molecule, then these regions are apparently immunodominant. As the domains to which discriminating antibodies are raised also contain Pr/Pfr preferred cleavage sites, it may be that these cleavage sites coincide with the epitopes to which the mAbs are binding. The immunodominancy may relate to the particular shape and exposure of these regions on the molecule. Certainly, in the case of Type 1 and 1' mAbs there is evidence that the epitopes to which the mAb binds are close to proteolytic cleavage sites. First, Type 1 and 1' mAbs tend to exhibit a higher affinity for Pr than Pfr (Cordonnier et al 1985; see section 5.3, 5.4), suggesting that the epitopes to which they bind are more exposed or have a more favourable conformation in Pr. Second, the regions of the molecule to which Type 1 and 1' mAbs bind each contain a Pr-preferred proteolytic cleavage site which presumably reflects the fact that the proteolytically vulnerable sites are more exposed on Pr. Finally, a Type 1 mAb to oat phytochrome has been demonstrated to prevent proteolytic cleavage of 124 kDa to 118 kDa phytochrome (Cordonnier et al 1985).

Spectral analyses of phytochrome in the presence of Type 1 and Type 2 mAbs have confirmed previous

findings. The binding of Type 1 mAbs causes alterations in the spectral characteristics of 124 kDa oat phytochrome so that they resemble those of "large" phytochrome (c.f. Cordonnier et al 1985). This confirms that the 6 kDa NH₂-terminal domain is crucial for the maintenance of the spectral integrity of the molecule. Binding of Type 2 mAbs to sites which undergo light-induced conformational changes, however, does not affect the spectral characteristics of the phytochrome. The epitopes to which the discriminating Type 2 mAbs are binding therefore do not appear to be involved in stable protein/chromophore interactions (c.f. Shimazaki et al 1986).

The isolation of Type 1' mAbs has, for the first time, allowed an assessment of the contribution of the 4 kDa sub-NH₂-terminal domain in the maintenance of the spectral integrity of the molecule. The binding of the Type 1' mAbs to 124 kDa oat phytochrome induces very similar alterations in the spectral characteristics as those observed with Type 1 mAbs. Since Type 1 and 1' mAbs do not compete i.e. they can both bind simultaneously to phytochrome, this may suggest that the 6 and 4 kDa NH₂-terminal domains are functioning as separate units. Thus, the 4 kDa sub-NH₂-terminal domain also interacts directly with the chromophore. However, as the binding of the Type 1 and 1' mAbs has the same affect on the absorption spectra of phytochrome it may be that the 6 kDa and 4 kDa domains constitute a single functional domain i.e. the region of protein which interacts with

the chromophore lies on both domains and encompasses the proteolytic cleavage site. Alternatively, the region of protein which interacts with the chromophore could be restricted to the 6 kDa or to the 4 kDa domain and binding to either domain interrupts normal protein/chromophore interaction.

7.3 Isolation of a Pr-specific mAb

Of particular interest and significance has been the isolation of a mAb (LAS 41) which is specific for Pr. This is the first report of a mAb which is form-specific. A form-specific mAb potentially allows the independent characterisation of the two forms of the molecule. For instance, until now it has been possible to produce Pfr solutions which only contain, at most, 88% Pfr molecules, the remainder being the contaminating Pr, which is inevitably produced on photoconversion with red light. By the selective immunoprecipitation of Pr, LAS 41 has allowed direct analysis of the spectral properties of Pfr^P i.e. a solution of ~100% Pfr molecules. LAS 41 has also allowed the first direct determination of the mole fraction of Pfr present after saturating red light (φ max). This value has been determined to be 0.874. Other workers have calculated the value for φ max and from this value have derived the spectrum for Pfr uncontaminated by Pr. The value for φ max determined here is similar to those reported by Kelly and Lagarias (1985), Lagarias et al (1987) and Chai et al (1987) who determined φ max to be 0.876,

0.878 and 0.88 respectively. The absorption spectrum for Pfr^P determined here has a small shoulder in the red region of the spectrum, similar to the derived spectra of Kelly and Lagarias (1985) and Lagarias et al (1987). This differs from the derived spectrum of Vierstra and Quail (1983b), who calculated λ_{max} to be 0.862, in which there is no shoulder in the red region of the spectrum. Kelly and Lagarias (1985) have proposed that differences observed between their results and those of Vierstra and Quail (1983b) may relate to whether the phytochrome is purified as Pr or Pfr. Lagarias et al (1987) have demonstrated that there are significant differences in the spectral properties of phytochrome which are dependent on the form in which it is purified. The differences observed are understood to relate to sulphhydryl modification of Pfr. However, it is unlikely that this explanation can account for the discrepancy in findings, as the phytochrome used here is purified as Pfr using a modification of the Vierstra and Quail (1983a) method.

LAS 41 has also facilitated the first direct demonstration that phytochrome can exist as stable heterodimers in vitro. Therefore, each monomer is photochemically distinct and apparently shows no interaction i.e. the monomers act as autonomous units. However, how or whether the monomers interact biochemically is unknown. Until now it has only been possible to speculate on the existence of heterodimers and on their potential significance for phytochrome action. Van Der Woude (1986) has

provided a theoretical model which predicts that in vivo Pr:Pr homodimers are inactive and that Pfr:Pfr homodimers and Pr:Pfr heterodimers are both active. He proposes that in VLF responses Pr:Pfr is the form of phytochrome which modulates the response but in LF responses Pfr:Pfr homodimers are the modulating agents. This is because VLF irradiations can only establish Pr:Pfr heterodimers, whilst LF irradiations are required to phototransform both monomers in the dimer. Brockmann et al (1987) have also proposed that heterodimers exist in vivo, however, as yet, there is no direct evidence that this is the case.

The photoreversible binding ELISA assay has demonstrated that the epitope to which LAS 41 binds is unique to Pr, such that upon photoconversion the epitope is modified and the antibody can no longer bind. By extension, it seems possible that there might be at least one epitope unique to Pfr which could also show reversible binding. Such an epitope would be a good candidate for the attachment site of a Pfr-specific binding partner. It may be that a Pfr-specific binding site would be located close to or at the epitope to which LAS 41 binds i.e. the "alter epitope".

7.4 Cross reactivity analyses

Amongst the grasses there seems to be a general conservation of the 6 and 4 kDa NH₂-terminal

domains, but they are not apparently conserved amongst other monocots and dicots. There is only one report of a Type 1 mAb which could cross react with phytochrome from a dicot, namely Zucchini (Daniels and Quail 1984). Thus, although this region has been shown to be vital to the maintenance of the spectral integrity of the molecule, the NH₂-terminal domains are not highly conserved. This lack of conservation may relate to the fact that this region of the molecule is fairly exposed and therefore is more likely to have become modified during evolution (D. Bowles, personal communication). However, the NH₂-terminal Pr-preferred cleavage sites are conserved amongst monocot and dicot phytochromes (Vierstra et al 1984). Comparison of the sequences of oat and Cucurbita phytochromes have shown that overall the 10 kDa NH₂-terminal domain is conserved to a lower degree than the central portion of the molecule, but there are local segments of high sequence homology (Sharrock et al 1986). The regions of high homology may represent the Pr-preferred proteolytic sites. If this is the case, then it is unlikely that the Type 1 and 1' mAbs produced here are binding to epitopes which correspond exactly to these proteolytic cleavage sites.

The 64 kDa chromophore-bearing domain has epitopes which are conserved amongst the grasses and dicots. Cordonnier et al (1984) demonstrated that 6 out of 16 Type 2 mAbs raised against oat phytochrome could cross react with dicot phytochrome in ELISA. LAS 34,

also a Type 2 mAb can also cross react with pea phytochrome. LAS 11, however, only demonstrates limited cross reactivity which is restricted to the grasses. The existence of highly conserved regions on the Type 2 domain is confirmed at both the level of nucleotide and amino acid sequences as Cucurbita and oat phytochrome share regions of high homology on this domain, particularly around the chromophore attachment site. Furthermore, polyclonal antibodies raised to a synthetic undecapeptide, comprising the chromophore attachment site, have been shown to cross react with phytochrome from monocots and dicots (Mercurio et al 1986).

The only mAbs to show broad cross reactivity amongst monocot and dicot phytochromes have been mapped to a site within the COOH-terminal domain. Also, only these Type 3 mAbs have convincingly recognised green-tissue phytochrome (c.f Cordonnier et al 1986a; Tokuhisa et al 1985). Jones et al (1985) have suggested that the COOH-terminal domain, which apparently does not interact with the other functional domains, has no biological function, except that it houses the site for dimerisation. However, since this domain does carry Pr/Pfr-preferred cleavage sites, there must be some sort of interdomain interaction and, contrary to the proposal of Jones et al (1985) this region could be involved in the light mediated transduction process (Lagarias and Mercurio 1985). However LAS 32 and Pea-25, the two mAbs which exhibit broad cross

reactivity do not exhibit differential affinities for Pr and Pfr. LAS 32 and Pea-25 map to a site which lies between 74 and 88 kDa from the NH₂-terminus and therefore may be raised against the dimerisation site as this has been located to be within 42 kDa of the COOH-terminus (Jones and Quail 1986a). If this is the case, LAS 32 could be used to pin point the exact region of the molecule involved in dimerisation.

7.5 Multiple species of phytochrome

The cross reactivity analyses conducted here are consistent with the findings that phytochrome extracted from green tissue comprises of more than one molecular species (Tokuhisa et al 1985; Cordonnier et al 1986b). Roots and shoots of oats and maize may also contain different pools of phytochrome (Schwarz and Schneider 1987). Hilton and Thomas (1985) using mAbs raised to "large" phytochrome from etiolated oats reported that seed and seedling phytochromes are also different. These differences probably correspond to the differences between etiolated- and green-tissue phytochrome. This is because phytochrome within seeds will have been accumulated during seed development and maturation i.e. during growth in the light.

Etiolated- and green-tissue phytochrome have been demonstrated to be spectrally and immuno-chemically distinct (Tokuhisa et al 1985; Cordonnier et al 1986b). It is unknown whether these

different species of phytochrome are derived from the same or distinct genes. Colbert et al (1985) using Northern blot analysis have demonstrated that green-tissue-phytochrome-mRNA has the same size transcript as etiolated-tissue-phytochrome-mRNA i.e. 4.2 kb. The green-tissue-phytochrome-mRNA is 50 times less abundant, which correlates well with the physical abundance of the protein. If the different molecular species of phytochrome represent the product of the same gene, a post-transcriptional modification must occur to account for their distinct characteristics. The differences observed may result from differential splicing of the primary transcript or from a post-translational modification such as glycosylation. Alternatively, etiolated- and green-tissue phytochrome may be the products of distinct genes which share enough homology to facilitate hybridisation and that give rise to transcripts of the same size. The only way to distinguish between these two possibilities would be to compare the sequence of phytochrome encoding cDNA clones which are derived from etiolated and green tissue. However, this comparison may be complicated by the fact that green tissue could contain a small pool of etiolated type phytochrome.

The different pools of phytochrome found in roots and shoots may represent slightly modified molecules which are organ specific. The differences between root and shoot phytochrome are apparently only minor, the difference being observed by the

inability of LAS 31, a Type 1 mAb, to cross react with root phytochrome. Four phytochrome genes have been isolated from etiolated oats and sequence analyses of two complete and one partial coding region demonstrate that they share 98% homology. It may be that root and shoot phytochrome are different members of the same gene family. Such differential expression of members of a gene family has been observed in other systems. Specifically, Silverthorne et al (1986) have demonstrated that different members of the ribulose-1,5-bisphosphate carboxylase small subunit (SSU) gene family are differentially expressed in Lemna, with some mRNA species being only expressed in the roots. Tingey et al (1987) have also demonstrated that glutamine synthetase is differentially expressed in leaves, roots and nodules of peas. Although the two phytochrome genes which have been completely sequenced are 98% homologous in the coding region, they diverge at the 3' untranslated region. Selection of appropriate sequences from the 3' divergent region could serve as specific gene probes, and therefore could assess whether the members of the gene family are expressed differentially. There is evidence that two of the phytochrome gene transcripts correspond to 80% of the phytochrome mRNA present, which suggests that there is differential expression of these genes (Hershey et al 1985). Alternatively, root and shoot phytochromes may be the product of a single gene whose product has been post-transcriptionally or post-translationally modified.

7.6 The future

The application of mAb technology has led to an increased understanding of several aspects of phytochrome structure and function. In particular mAbs have facilitated the delineation and identification of functional domains on the molecule. They have also detected regions on the protein moiety which distinguish Pr from Pfr and the identification of an epitope which is unique to Pr. Furthermore, mAbs have provided complementary information concerning conserved regions on the phytochrome molecule. Perhaps, most importantly mAbs have been used to show that green tissue and etiolated tissue contain different molecular species of phytochrome.

Monoclonal antibodies provide the "spring board" to study green-tissue-phytochrome. Monoclonal antibodies are the ideal probes as they are raised to defined regions but they can be monospecific even if the antigen used for immunisation is not pure. This is particularly important as it will be difficult to purify green-tissue-phytochrome to homogeneity in its native state due to its very low abundance and apparent susceptibility to proteolysis.

There is still scope for the production of mAbs which recognise highly conserved domains, not only because they provide a means of purifying and analysing other phytochrome species, but they may also be recognising the active site(s) of the molecule. Although the only mAbs which recognise

a highly conserved domain map to the 55 kDa COOH-terminus, it would be predicted that the 64 kDa chromophore-bearing domain would also carry conserved domains, particularly in the region of the chromophore attachment site. It may be possible to generate a ubiquitous probe for phytochrome by raising mAbs to the chromophore. However, the generation of such a probe is reliant on the fact that the chromophore of the phytochrome molecule is not entirely internalised and is also immunogenic. In addition to their use as ubiquitous probes, such mAbs could perhaps be used to study whether there is heterogeneity amongst chromophores in phytochromes isolated from different plant species.

Monoclonal antibodies may also be used to try and interrupt the action of phytochrome in in vitro assays. This may lead to the identification of the active site of the molecule. Mössinger et al (1987) have shown that 124 kDa phytochrome can regulate run-off transcription of particular messages in isolated nuclei. It is not known whether this reflects the action in vivo. Since this in vitro effect is specific for 124 kDa Pfr then perhaps only Type 1 mAbs would block the action of phytochrome. If, however, phytochrome action results from inter-domain interactions it may be that binding of mAbs to other regions of the molecule could also prevent action.

Bearing in mind all the advantages of mAb technology and the information which has already been

gleaned from its applications, it is unlikely that mAb technology, or any other analytical technique alone, will determine how phytochrome functions. However, the application of mAbs in conjunction with other biochemical analyses, such as an in vitro action of phytochrome might be expected to identify the active site(s) on the phytochrome molecule.

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Characterisation of phytochrome using monoclonal antibodies

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Native oat phytochrome has been purified to homogeneity and used to produce a panel of monoclonal antibodies (mAbs). Selection of mAbs followed early screening against native phytochrome by ELISA, and SDS-denatured phytochrome by "mini" western blotting. Six mAbs which recognised SDS-denatured phytochrome were mapped using proteolytically derived fragments of phytochrome and subsequent immunoblotting. LAS 31 and 33 map to the 6 kDa NH₂-terminus and LAS 35 and 41 map to the adjacent 4 kDa sub-NH₂-terminal domain. LAS 11 maps to the 64 kDa-chromophore-bearing domain and LAS 32 maps to between 74 and 88 kDa from the NH₂-terminus on the COOH-terminal-half of the molecule. A novel protocol for the mapping of conformation-specific mAbs has been developed and used to assign LAS 21, 34 and 42 to the 64 kDa-chromophore-bearing domain. Determination of differential affinities towards Pr and Pfr demonstrated that LAS 42 exhibited a higher affinity for Pfr, LAS 31, 33, 34 and 35 exhibited a higher affinity for Pr. LAS 41 discriminates absolutely against Pfr. LAS 41 has therefore facilitated:- (i) the purification of Pfr^P, i.e. Pfr which is free of contaminating Pr, (ii) the development of an ELISA for phytochrome photoequilibrium, (iii) the first direct experimental evidence that phytochrome can exist as a stable heterodimer in vitro and (iv) an appraisal of ELISA protocols for determining differential affinities of mAbs towards Pr and Pfr. Spectral analyses of phytochrome in the presence of mAbs have underlined the importance of the 6 kDa NH₂-terminus in the maintenance of the spectral integrity of the molecule but have also indicated that the 4 kDa sub-NH₂-terminal domain also interacts with the chromophore. Cross reactivity studies amongst phytochrome from monocots and dicots demonstrate that the epitopes recognised by LAS 11, 31, 33, 35 and 41 are not highly conserved. However, LAS 32 recognises phytochrome from every plant species tested, and is therefore recognising a highly conserved region on the molecule.