The acetyl Coenzyme A binding site of

chloramphenicol acetyltransferase.

by

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Contents.

Abbreviations.

APS	Ammonium persulphate
CAT	Chloramphenicol acetyltransferase
CoA	Coenzyme A
ddH_2O	Distilled, deionised water
dNTP	Deoxynucleotide
ddNTP	Dideoxynucleotide
DTNB	5,5'-Dithiobis-(2-nitrobenzoate)
EDTA	Diaminoethanetetracetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate
IPTG	Isopropyl β-D-thiogalactoside
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
SDS	Sodium dodecylsulphate
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside.

Chapter 1. Introduction.

1.1 Alteration of protein structure by covalent modification and site-directed mutagenesis.

In order to alter enzyme specificity in a rational manner the forces involved in substrate recognition, binding and catalysis must be understood. The role of individual amino acid side chains can be investigated in several ways. Covalent modification with specific chemical reagents is a useful tool for identification of residues potentially involved in binding and catalysis, especially in the absence of a crystal structure. Determination of the high resolution structure of a protein in parallel with site-directed mutagenesis studies is however, a more definitive means of assigning roles to specific residues. With the aid of computer modelling, site-directed mutagenesis has the potential to allow alteration of protein structure to change specific aspects of function, either by modification of existing functions or by introduction of new ones.

1.1.1 Covalent modification of proteins.

In the absence of detailed structural information, the involvement of specific amino acid side chains in substrate binding and catalysis can be inferred from inactivation profiles for selective chemical reagents. Two general types of modification reagent are generally used for such studies, group-specific modification reagents and active site-directed reagents.

1.1.2 Group-specific modification reagents.

Most functionally important amino acid side chains can be specifically modified by chemical reagents under relatively mild conditions (see Lundblad and Noyes, 1984). Inactivation of enzymes by modification with reagents which are specific for a residue type is suggestive of involvement of such a residue in substrate binding or catalysis. Protection by substrates against inactivation with such reagents may allow identification of the residue whose modification results in inactivation. For example, treatment of alkaline phosphatase with 2,3-butanedione or phenylglyoxal results in rapid loss of enzyme activity and inactivation is proportional to the modification of 15 of the enzyme's 24 arginyl residues (Daemen and Riordan, 1974). In the presence of a pseudosubstrate (sodium phenylphosphonate) or an inhibitor (phosphate), the reaction results in almost no loss in activity and one less arginyl residue (per subunit) is modified, strongly suggesting that alkaline phosphatase contains an arginyl residue which is essential for enzymic activity. Subsequent site-directed mutagenesis experiments confirmed that such an arginyl residue (Arg 166) is important, although not essential for activity, and removal of this side chain (R166A) renders the enzyme insensitive to inactivation by 2,3-butanedione (Chaidaroglou et al., 1988).

A major drawback with group-specific modification reagents is that these reagents often modify many residues, consequently relating inactivation to modification of a single residue can be difficult. Furthermore, many reagents are not absolutely specific for a particular side chain and

under certain conditions can react with other side chains, especially ones that are unusually reactive (e.g. many active site residues). Thus when employing group-specific modification reagents it is essential to determine which residue type is responsible for loss of activity on modification, and to identify the residue by employing labelled (e.g. radioactively) reagent.

1.1.3 Active site-directed reagents.

Some reagents that modify proteins obey saturation kinetics, initially forming a freely reversible non-covalent complex which in turn forms a stable, but inactive covalent complex (Main, 1964; Meloche, 1967). Modification reagents can be targeted towards the active site of enzymes using the specific binding interactions of substrate analogues which are intrinsically reactive or become so in a mechanism-based fashion. The active site histidyl residue of the serine proteases can be specifically modified with methyl ketone derivatives of truncated substrates. Treatment of chymotrypsin with TPCK (tosyl-L-phenylalanyl chloromethyl ketone) results in total loss of activity (Schoellman and Shaw, 1963) with specific alkylation of N3 of His 57 (Ong et al., 1965). Similarly, trypsin is inhibited by TLCK (tosyl-L-lysyl chloromethyl ketone, Shaw et al., 1965) with specific alkylation of N3 of His 46 (Petra et al., 1965). Treatment of enzyme denatured in 6M urea with TLCK results in no incorporation of reagent, suggesting that the native structure of the enzyme is required for the reaction to take place. Furthermore, trypsin is not inhibited by TPCK nor

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chymotrypsin by TLCK. Both enzymes are however, inactivated by diisopropylfluorophosphate (DFP) which specifically modifies the active site serine residue of many serine proteases (Jansen et al., 1949).

Active site-directed reagents are generally more useful than group specific modifying reagents as they react with one or only a few residues in the active site rather than with all groups accessible to the reagent. However, the vast majority of covalent modification experiments have the same major flaw. On reaction, the size of the modified side chain is generally increased, thus loss of activity could be due to introduction of steric hindrance rather than to the loss of an essential functional group. In the absence of a high resolution structure, covalent modification is a useful tool for identification of residues which could be involved in binding and catalysis, but the results of such experiments can only be used as a guide and can often be misleading.

1.1.4 Site-specific modification of protein structure.

Recent advances in molecular biology now permit almost any amino acid of a protein to be changed to any other by directed mutagenesis of cloned genes (Hutchison et al., 1978). Modification of protein structure by site-directed mutagenesis has a number of advantages over covalent modification techniques and structural studies for study of the function of specific amino acids. Unlike covalent modification, mutagenesis can be used to both remove a functional group and reduce the size of the residue thus reducing ambiguity due to the introduction of steric effects.

For example, loss of activity on reaction of glycyl-tRNA synthetase with N-ethylmaleimide (NEM) strongly suggests a role for a cysteine residue in the catalytic mechanism of this enzyme (Ostrem and Berg, 1974). Removal of cysteine 395 by mutagenesis (C395A) results in no decrease in activity, but makes the enzyme insensitive to inactivation by NEM, suggesting that this residue is in fact not important for activity (Profy and Schimmel, 1986). Furthermore, introduction of a larger side chain at position 395 (C395Q) mimics the effect of covalent modification suggesting that loss of activity is simply due to steric effects.

Structural studies can also yield misleading results as the observed crystal structure is a fixed conformation. The structure of staphylococcal nuclease suggests that Arg 87 is important for substrate binding (Cotton et al., 1979). However, removal of this side chain by mutagenesis (R87G), results in only a modest decrease in substrate binding, whereas k_{cat} is reduced by ~10^5-fold suggesting that the residue is in fact involved in catalysis, probably via stabilisation of an anionic transition-state (Serpersu et al., 1987).

Site-directed mutagenesis can also be used to implicate residues in catalysis which appear from the crystal structure to be far from the active site. Lys 230 and Lys 233 of tyrosyl-tRNA synthetase appear from the apoenzyme crystal structure to be 0.8nm from the active site and therefore unlikely to interact with the reaction transition-state (Brick and Blow, 1987). However, the substitutions K230N and K233N cause dramatic decreases in catalytic activity suggesting that these residues are important for

transition-state stabilisation and that substrate binding results in a conformational change which brings residues 230 and 233 close to the active site (Fersht et al., 1988).

Probably the greatest advantage of site-directed mutagenesis is the ability to change the structure of a protein in a defined manner. This ability to change specific residues makes the 'ultimate goal' of redesigning enzymes to suit a specific function more realistic. However, at present mutagenesis only allows substitution with the 20 natural amino acids specified by the genetic code, although methods for introducing unnatural amino acids into proteins in vitro are being developed (for a review see Anthony-Cahill et al., 1989). To study the relationship between structure and function it is best to make conservative substitutions which simply remove function without significantly changing structure. Unfortunately, few such substitutions are possible (except Asp ↔ Asn and Glu ↔ Gln), thus substitution with smaller amino acids is preferable to reduce the likelihood of unwelcome structural changes. In order to make rational deductions from the results of mutagenesis experiments, the technique must be used in conjunction with structural studies, as interpretation of the results relies heavily on the assumption that on mutation structural changes occur only at the site of mutation and are not propagated throughout the protein. A further factor which must be considered is the effect of mutation on the overall stability of the protein.

1.1.5 Protein stability.

As the function of a protein is related to its tertiary structure, so the latter is related to the relative stability of its units of secondary structure and their interactions with each other. Thus, in order to 'engineer' proteins for specific functions, the forces involved in the folding and stabilisation of protein structure must be understood.

Kellis et al. (1988, 1989) calculated a contribution to stability of 1.0-1.6 kcal/mol/methylene group for residues in the hydrophobic core of bacterial ribonuclease (barnase) on the basis of changes in stability on mutation. Substitution of Ile 88 or Leu 14 by alanine results in a >4 kcal/mol decrease in stability equivalent to an almost 50% decrease in the total free energy of folding for the enzyme. The reduced in vitro stability of these mutant proteins is reflected in vivo by the considerably lower yields obtained (Kellis et al. 1989). From studies with T4 lysozyme, Matsumura et al. (1988a) estimated the strength of the hydrophobic effect to be ~20 cal/mol/$\text{\AA}^2$, in good agreement with the results of model studies (Chothia, 1976). In several systems the contribution to overall stability of a particular residue is found to be directly related to the hydrophobicity of the residue side chain (calculated from the free energy of transfer from aqueous solution to an organic phase (Nozaki and Tanford, 1971) or from the accessible surface areas (Chothia, 1976)), provided that the volume of the side chain does not exceed that of the 'binding site' (Matsumura et al., 1988a/b, Yutani et al., 1987 and Kellis et al., 1989). The position of temperature sensitive mutations in T4 lysozyme

are also found to correlate with regions of protein of low mobility and low solvent accessibility (Alber et al., 1987).

The stability of proteins is also affected by irreversible covalent changes to specific amino acids. Deamidation of two asparaginyl residues (14 and 78) to yield charged aspartyl residues at the subunit interface of triose phosphate isomerase was postulated to be responsible for irreversible denaturation on prolonged heating (Ahern et al., 1987). Replacement of these residues with isoleucine results in a 2-fold increase in the enzyme half-life at 100°C, whereas the substitution N78D causes a dramatic decrease in stability confirming the proposed role of deamidation. Similarly, oxidation of sulphur containing residues to sulphoxides can also result in reduced stability and activity. Substitution of Met 222, the residue responsible for the oxidative inactivation of subtilisin, with Ala or Ser yields oxidation resistant mutants which are however, less active than wild type. In comparison, the mutant M222C is more active than wild type, but is, not surprisingly, still sensitive to oxidation (Estell et al., 1985). Human α_1-antitrypsin, which is an important inhibitor of neutrophil elastase, is also inactivated by oxidation of a methionine residue (Met 358). Substitution with Val (M358V) again yields an oxidation resistant mutant which has only slightly reduced activity and thus, may be useful for treatment of a number of illnesses which result in damage to lungs via uncontrolled elastase activity (Rosenberg et al., 1984; Courtney et al., 1985).

Attempts to increase the stability of proteins by the introduction of disulphide bridges have met with limited

success. Some engineered disulphides have successfully increased the resistance of proteins to both thermal and chemical denaturation (Perry and Wetzel, 1986; Sauer et al., 1986), while others make no difference to or even reduce protein stability (Wells and Powers, 1986; Scrutton et al., 1988; Villafranca et al., 1987). The effect of such introduced disulphides on protein stability is probably very sensitive to the precise geometry of the interaction, moreover the presence of other 'unpaired' cysteine residues can also cause problems (Perry and Wetzel, 1986). The stabilising effect of disulphide bonds is also greatly reduced if formation of the bond disrupts other interactions (Sauer et al., 1986).

1.1.6 Substrate specificity.

Site-directed mutagenesis has been widely applied to investigation of the residues involved in binding and catalysis of many enzymes. With the aid of high resolution crystal structures the contributions of hydrogen bonds (Wells and Fersht, 1985), hydrophobic interactions (Estell et al., 1986; Benkovic et al., 1988) and electrostatic interactions (Gráf et al., 1988; Wells et al., 1987; Russell et al., 1987) to enzyme activity and specificity have been studied. Numerous attempts have been made to alter the specificity of enzymes for particular substrates. Changes in surface charges more than 1.3nm from the active site histidyl residue (His 64) of subtilisin results in significant changes in the pH-activity profile, with the pK_a of His 64 decreased by as much as 1 pH unit for the E156K, D99K double mutant (Russell

and Fersht, 1987; Russell et al., 1987). Such mutant enzymes have higher activities than the wild type enzyme at low (non-physiological, but commercially important) ionic strengths and also show altered substrate specificities. Furthermore, substrate specificity towards charged P1 substrates is radically altered, with up to 1900-fold increases in k_{cat}/K_m, by introduction of complementary charges into the P1 binding site of subtilisin (Wells et al., 1987). The specificity of subtilisin can also be modified by altering the size and hydrophobicity of residue 166 (Gly in the wild type sequence) which is in the P1 binding site (Estell et al., 1986). Changes in specificity are directly related to the hydrophobicity of side chain 166 for any particular non-polar P1 substrate, unless the volume of the P1 binding site is exceeded.

The substrate specificity of trypsin, another serine protease, has also been investigated by site-directed mutagenesis. Replacing the aspartyl residue (Asp 189) predicted to be responsible for the charge specificity of trypsin, with serine (D189S, the corresponding residue in chymotrypsin) results in a $\sim 10^5$-fold decrease in activity with arginyl and lysyl substrates (Gráf et al., 1988). However, rather than the expected large increase in K_m for such substrates, loss of activity is due almost entirely to a large decrease in k_{cat}. The activity of the D189S mutant is 10-50-fold greater with chymotrypsin-like substrates than with arginyl/lysyl substrates, but is considerably less active than wild type trypsin with its normal substrates.

Similarly, introducing steric hindrance (via the G216A and G226A substitutions) into the substrate binding site of trypsin results in increased discrimination between arginyl and lysyl substrates, but again the major effect is on k_{cat} rather than K_m (Craik et al., 1985).

Scrutton et al. (1990) successfully changed the coenzyme specificity of E. coli glutathione reductase from NADPH to NADH by multiple amino acid substitutions in the binding site of the 2-phosphate group. The residues to be altered were selected by consideration of the high resolution crystal structure of the homologous human erythrocyte enzyme, which uses NADPH (Karplus and Schultz, 1987) and the amino acid sequences of several NADH dependent dihydrolipoamide reductases. The cumulative effect of seven substitutions is an enzyme with an 8-fold preference for NADH compared to the wild type enzyme which has a >2000-fold preference for NADPH. The mutant enzyme however, is a less efficient reductase with NADH than is the wild type enzyme with NADPH. In contrast, a single point mutation (Q102R) in B. stearothermophilus lactate dehydrogenase (LDH) effectively changes the specificity of the enzyme from pyruvate to oxaloacetate (OAA), creating an efficient malate dehydrogenase (MDH) (Wilks et al., 1988). This 'new' MDH has an 8400-fold preference for OAA (compared with the original LDH which has a 1050-fold preference for pyruvate) and importantly retains the high catalytic activity of LDH. Thus large changes in substrate specificities can be achieved using site-directed mutagenesis, but the above examples also illustrate the point that it is often difficult to divorce the effects of substitutions on substrate binding from those on catalysis.

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1.1.7 Enzyme catalysis.

Enzymes can use the binding energy of residues far from the site of reaction to increase catalytic rates both by changing substrate K_m values and by increasing k_{cat}. The large rate enhancement derived from such residues is due to three types of binding interaction: Uniform binding, where the free energies of all the bound states (ES, ETS and EP) are optimised; differential binding, where there is discrimination between substrates (S) and products (P), such that for enzymes that keep their substrates at equilibrium, the free energies of ES and EP are roughly equal; and catalysis of the elementary steps, where the enzyme preferentially binds the transition-state (TS) rather than substrates or products (Albery and Knowles, 1976).

Site-directed mutagenesis experiments have provided examples of residues that show each of the above type of binding interaction (Ho and Fersht, 1986; Serpersu et al., 1987; Fersht et al., 1985; Leatherbarrow et al., 1985). For example, substitution of Tyr 169 of tyrosyl-tRNA synthetase with Phe (Y169F) results in a uniform 2.6-2.8 kcal/mol decrease in binding energy of all the bound states. In contrast, Cys 35 makes little or no contribution to substrate binding, but stabilises the transition-state and enzyme/product complexes by 1.2 and 1.6 kcal/mol respectively (Wells and Fersht, 1986). The most discriminating type of binding interaction involves specific stabilisation of the reaction transition-state. Substitution of Asn 155 with Leu (N155L) in subtilisin results in no change in substrate K_m, but k_{cat} is reduced 200-300-fold (Bryan et al., 1986).

Crystallographic studies show that Asn 155 can not interact with the substrate, but can donate a hydrogen bond to the oxyanion that develops as the reaction proceeds (Robertus et al., 1972). A similar role has also been proposed for Ser 148 in CAT_{III} (Lewendon et al., 1990, see section 1.2.12).

The direct participation of residues in the chemical reaction of enzymes has been extensively studied by covalent modification and crystallography, but again mutagenesis experiments are required to confirm such roles. Removal of any of the residues of the catalytic triad of the serine proteases as expected results solely in large decreases in k_{cat} confirming their central role in catalysis and the interdependence of their action (Carter and Wells, 1988; Craik et al., 1987). Similarly, removal of His 195, the putative general base of CAT_{III} results in a $>10^7$-fold decrease in activity (A.Lewendon, unpublished results). His 134 of aspartate transcarbamoylase was proposed, on the basis of crystallographic studies to play a similar role to that of His 195 of CAT. However, the mutant H134A retains ~6% of the wild type activity and subsequent NMR studies suggested that His 134 probably plays a role in substrate binding rather than catalysis (Kleanthous et al., 1988).

Site-directed mutagenesis experiments are providing valuable insights into the mechanisms of enzyme catalysis and the use of binding energy to enhance both substrate binding and reaction rates. However, much more information is required before derived structure/function relationships can be applied to useful alteration of enzyme activity and to ab initio protein design.

The work described in this thesis makes use of some of

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the techniques discussed above to investigate substrate binding to chloramphenicol acetyltransferase, the enzyme largely responsible for high level resistance to the antibiotic chloramphenicol in many prokaryotic organisms.

1.2 Bacterial resistance to the antibiotic chloramphenicol mediated by chloramphenicol acetyltransferase (CAT).

1.2.1 Isolation and structure of chloramphenicol.

Chloramphenicol was first isolated from Streptomyces venezuelae in 1947 and was identified as the antibiotic produced by this organism (Ehrlich et al., 1947). The structure of chloramphenicol was subsequently shown to be D(-)threo 2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol (fig. 1.1; Rebstock et al., 1949) and the first efficient chemical synthesis achieved by Controulis et al. (1949). Of the four possible diastereoisomers of chloramphenicol only the naturally occurring D(-)threo isomer is active as an antibiotic (Maxwell and Nickel, 1954).

Chloramphenicol is uncharged at physiological pH, reasonably soluble in aqueous solution and as a result of its amphiphilic nature can readily pass through biological membranes. Coupled with straight-forward chemical synthesis and a broad spectrum of sensitivity among pathogenic bacteria, chloramphenicol appeared to be an ideal antibiotic and received extensive clinical use. However, identification of a number of potentially life threatening side-effects has curtailed its wide-spread use, although chloramphenicol is

still used to treat certain serious infections.

1.2.2 Mode of action of chloramphenicol.

The bacteriostatic effect of chloramphenicol is due to inhibition of bacterial protein synthesis (Gale and Paine, 1951). Chloramphenicol binds to 70S ribosomes (Vazquez, 1964) at two sites. A low affinity site (K_d=200μM) on the 30S subunit has no known function, but high affinity binding (K_d=2μM) to the 50S subunit (Lessard and Petska, 1972) results in inhibition of the peptidyl transferase activity of such ribosomes (Traut and Monro, 1964). Chloramphenicol does not prevent binding of mRNA to ribosomes or inhibit binding of aminoacyl-tRNA to the ribosomal A site (Vazquez and Monro, 1967). It does however, inhibit peptide bond transfer, possibly by preventing recognition of acceptor substrates in the ribosomal P site (a detailed review of the mechanism of action of chloramphenicol may be found in Gale et al., 1981).

From the results of semi-aqueous nmr studies Jardetzky (1963) suggested that chloramphenicol might act as a structural analogue of uridine by forming an intra-molecular hydrogen bond between the C1 and C3 hydroxyl groups of the antibiotic. Subsequent solution nmr studies suggested that no such hydrogen bond was likely to occur in solution, casting doubt on this proposed mode of action (Bustard et al., 1973).

1.2.3 Resistance to chloramphenicol.

Soon after its introduction into clinical practice bacterial strains resistant to chloramphenicol began to

emerge. Such resistance was often associated with resistance to other antibiotics and was shown to be plasmid-borne (Watanabe, 1963). Miyamura (1964) demonstrated that E. coli strains made resistant to chloramphenicol by transfer of R-determinants from Shigellae could inactivate chloramphenicol and the mechanism of inactivation was later identified as enzymic acetylation (Shaw, 1967; Suzuki and Okamoto, 1967). Most high level (>100µg/ml) resistance to chloramphenicol is due to acetylation of the 3-hydroxyl group of chloramphenicol by the enzyme chloramphenicol acetyl-transferase (CAT; EC 2.3.1.28) using acetyl Coenzyme A as the acetyl donor (fig. 1.2; Shaw and Unowsky, 1968). The 3-acetyl chloramphenicol product can undergo a non-enzymic, pH-dependent rearrangement to give 1-acetyl chloramphenicol (Nakagawa et al., 1979; Thibault et al., 1980) which is a substrate for re-acetylation at the 3-hydroxyl position to give 1,3-diacetyl chloramphenicol. Neither the mono- nor di-acetylated products bind to 70S ribosomes and are therefore inactive as antibiotics (Shaw and Unowsky, 1968). The acetylation of chloramphenicol by CAT is reversible such that if provided with 3-acetyl chloramphenicol and free CoA, CAT carries out the acetylation of CoA (Kleanthous and Shaw, 1984). In the absence of chloramphenicol CAT catalyses the slow hydrolysis of acetyl CoA (Zaidenzaig and Shaw, 1978; Kleanthous and Shaw, 1984).

Although acetylation by CAT is the most common mode of resistance to chloramphenicol other mechanisms have been identified including exclusion from the cell (Backman et al., 1976), inactivation by reduction of the nitro group (Smith and Worrel, 1953) and mutation of a ribosomal protein

(Baughman and Fahnestock, 1979).

1.2.4 Chloramphenicol acetyltransferase variants.

Resistance to chloramphenicol mediated by CAT has been observed in many genera of prokaryotes, both Gram positive and Gram negative and several variants of CAT have been identified (fig. 1.3). Expression of CAT is constitutive and unregulated in most Gram negative organisms (a notable exception being type I CAT which is under catabolite control by the CRP/cyclic AMP system [LeGrice and Matzura, 1981; LeGrice et al., 1980]) but inducible in Gram positive organisms. The nucleotide sequences of eleven cat genes have been determined (fig. 1.3) which all code for proteins of between 210 and 220 residues with subunit molecular weights of ~25 kDa. An amino acid sequence numbering system for CAT variants based on the type I sequence has been proposed (Shaw and Leslie, 1989). The amino terminus of CAT_{III} thus corresponds to residue number 6 in the standard alignment (see fig. 1.3). The eleven variants are highly homologous, with 22 residues absolutely conserved in all known variants and many conservative substitutions suggesting that they may have similar tertiary structures. The formation of stable and active hybrids between the type I and type III enzymes in vivo also suggests structure similarity (Packman and Shaw, 1981b). Homology between variants is particularly great in the region of residues 188-202 which contains a conserved histidyl residue (His 195) which has been implicated in catalysis (Fitton and Shaw, 1979).

The best-characterised CAT variants are the type I and type III enzymes. Both are insensitive to inhibition by 5,5'-dithiobis-(2-nitrobenzoate) which is used in the standard enzyme assay for CAT (2.5). Crystals of the type III enzyme have been obtained (Leslie et al., 1986) and the structure solved at 0.175nm resolution (Leslie et al., 1988, Leslie, 1990 in press).

1.2.5 The kinetic mechanism of CAT.

Kinetic analysis of CAT_I suggested that CAT does not follow a double displacement (ping-pong) mechanism (Tanaka et al., 1974). Zaidenzaig and Shaw (1978) found no evidence of an acetyl-enzyme intermediate nor exchange between $[^3H]$-CoA and $[^{14}C]$-acetyl CoA which would be expected for a double displacement mechanism. Subsequent extensive kinetic analysis on CAT_{III} using alternative acyl donors and acceptors suggests that CAT follows a rapid equilibrium random order sequential (ternary complex) mechanism, with a slight preference for CoA as the leading substrate (fig. 1.4; Kleanthous and Shaw, 1984). Such a mechanism may involve a general base abstracting a proton from the 3-hydroxyl of chloramphenicol and facilitating nucleophilic attack on the carbonyl group of the acetyl CoA thioester (fig. 1.5). Such a reaction probably proceeds via a charged tetrahedral (oxyanion) intermediate which breaks down to release 3-acetyl chloramphenicol and CoA.

Although inhibition studies with thiol specific reagents implicated one or more cysteine residues in catalysis

(Zaidenzaig and Shaw, 1978), the cysteine modified by such reagents in CAT_I (Cys 31) is absent in the staphylococcal CAT variants which are resistant to inactivation by thiol specific reagents (Fitton and Shaw, 1979). All CAT variants are inhibited by iodoacetamide and pre-incubation with chloramphenicol protects against loss of activity, suggesting that the residue whose modification results in inactivation is close to the active site. Subsequent studies with iodoacetamide and the histidine-specific reagent diethyl pyrocarbonate identified His 195 as the likely catalytic residue (Fitton and Shaw, 1979; Corney, 1983).

Reaction of CAT with iodoacetamide results in modification of only N3 of His 195 suggesting that a specific tautomer of His 195 is stabilised by, for example, hydrogen bonding of N1-H to a carboxylate group as seen in chymotrypsin and phospholipase A2 (Blow et al., 1969; Verheij et al., 1980). Reaction of CAT_{III} with 3-(bromoacetyl) chloramphenicol, an active site-directed inhibitor based on 3-acetyl chloramphenicol, the substrate for the reverse reaction, abolishes both transacetylation and hydrolytic reactions and, as with iodoacetamide, only the N3 of His 195 is modified (Kleanthous et al., 1985). Thus His 195 is the likely general base of CAT.

1.2.6 The structure of CAT_{III}.

The structures of the CAT_{III} apoenzyme and the enzyme/chloramphenicol binary complex have been solved at 0.175nm resolution, whilst that of the CAT/CoA binary complex has been solved at 0.24nm resolution (Leslie et al., 1988).

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Earlier hydrodynamic and preliminary structural studies had shown that CAT_{III} is a trimer of identical subunits (Harding et al., 1987; Leslie et al., 1986). Although crystallisation of CAT_{III} requires the presence of cobalt (III) hexamine chloride in the crystallisation medium, cobalt (II) ions play an important structural role in the formation of the crystal lattice. Trimers pack face to face with three Co^{2+} ions bound between them. Each Co^{2+} ion is hydrogen bonded to the imidazole N1 of His 27 and the carboxylate of Glu 23 of one subunit and the same residues of the crystallographically two-fold related subunit to form tetrahedral coordination geometry. The trimer forms a disc shaped molecule ~6.5nm in diameter and 4.0nm thick. Trimeric soluble enzymes are relatively uncommon, but other notable examples are the catalytic trimer of aspartate transcarbamoylase, ornithine transcarbamoylase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase. Whilst the crystal structure of CAT_{III} shows no convincing homology with other known structures, Guest (1987) proposed homology between the primary structures of CAT_{III} and the dihydrolipoamide acetyltransferase (E2p) subunit of the E. coli pyruvate dehydrogenase complex. A number of regions of homology were identified which contain several conserved residues which are important for catalysis in CAT, most notably Arg 18, Ser 148, His 195 (the catalytic residue of CAT) and Asp 199 (residues 406, 550, 602 and 606 respectively in E2p). The homologies do not however, appear to extend to regions of CAT involved in binding of the substrate common to both systems, CoA.

Each CAT monomer is composed of 10 β-strands and 5 α-helices. The β-strands form two predominantly anti-parallel β-sheets, one consisting of 3 strands and a second of 6 strands. The α-helices are packed against one face and the ends of the largest β-sheet (fig. 1.6). On formation of the trimer the 6 stranded β-sheet is extended by a single strand from the adjacent subunit $\beta_H^{\#}$, (which appears from the monomer structure (fig. 1.6) to be rather isolated) to form a 7 stranded sheet (fig. 1.7). Interactions between $\beta_H^{\#}$ and β_B in this sheet potentially allows formation of 16 inter-subunit hydrogen bonds involving both main chain and side chain atoms, only four of which involve bridging water molecules. Thus it is likely that this 7-stranded β-sheet makes a significant contribution to the striking resistance of CAT to thermal and chemical denaturation (Packman and Shaw, 1981b).

Interactions between adjacent subunits are evenly distributed between polar and hydrophobic residues. There is only one direct inter-subunit ion pair, involving Glu 101 and Arg 205, both of which are conserved in 8 of the 11 variants. A buried lysyl residue (Lys 38) proposed by Packman and Shaw (1981a), on the strength of its unreactivity under native conditions with methyl acetimidate, to be involved in an inter-subunit interaction is indeed found at the subunit interface, but it forms an *intra*-subunit ionic interaction with the side chain of Asp 156 via a bridging water molecule.

1.2.7 Binding of chloramphenicol.

Chloramphenicol binds in the cleft between subunits, but

Each CAT monomer is composed of 10 β-strands and 5 α-helices. The β-strands form two predominantly anti-parallel β-sheets, one consisting of 3 strands and a second of 6 strands. The α-helices are packed against one face and the ends of the largest β-sheet (fig. 1.6). On formation of the trimer the 6 stranded β-sheet is extended by a single strand from the adjacent subunit $\beta_H^{\#}$, (which appears from the monomer structure (fig. 1.6) to be rather isolated) to form a 7 stranded sheet (fig. 1.7). Interactions between $\beta_H^{\#}$ and β_E in this sheet potentially allows formation of 16 inter-subunit hydrogen bonds involving both main chain and side chain atoms, only four of which involve bridging water molecules. Thus it is likely that this 7-stranded β-sheet makes a significant contribution to the striking resistance of CAT to thermal and chemical denaturation (Packman and Shaw, 1981b).

Interactions between adjacent subunits are evenly distributed between polar and hydrophobic residues. There is only one direct inter-subunit ion pair, involving Glu 101 and Arg 205, both of which are conserved in 8 of the 11 variants. A buried lysyl residue (Lys 38) proposed by Packman and Shaw (1981a), on the strength of its unreactivity under native conditions with methyl acetimidate, to be involved in an inter-subunit interaction is indeed found at the subunit interface, but it forms an *intra*-subunit ionic interaction with the side chain of Asp 156 via a bridging water molecule.

1.2.7 Binding of chloramphenicol.

Chloramphenicol binds in the cleft between subunits, but

the majority of binding contacts involve residues from only one subunit. One face of the chloramphenicol binding site consists of predominantly hydrophobic residues (Leu 160, Phe 24, Phe 103, Tyr 25) whereas the other face consists of largely polar residues (Thr 174, Ser 148, Asn 146, Gln 192) complementing the amphiphilic nature of the substrate (fig. 1.8). There are three hydrogen bonds involving chloramphenicol. The phenolic hydroxyl group of Tyr 25 is hydrogen bonded to the carbonyl oxygen of chloramphenicol and N3 of His 195 to the primary (C3) hydroxyl group. The secondary (C1) hydroxyl group is hydrogen bonded to the side chain of Thr 174 via a bridging water molecule. The para-nitro group of chloramphenicol and one of the two chlorine atoms of the dichloroacetamide side chain are solvent accessible as might be expected due to the wide variety of permissible substitutions at these positions (Shaw, 1983).

1.2.8 The CoA binding site.

Binding of chloramphenicol completely blocks access to the active site from the 'top' surface of the trimer (Leslie et al., 1988). Kinetic studies showed that the substrates of CAT bind independently suggesting that CoA must gain access to the active site via a different route to that of chloramphenicol (Kleanthous and Shaw, 1984). The structure of the CAT/chloramphenicol binary complex reveals a 'tunnel' 0.3-0.4nm in diameter and ~1.2nm in length leading from the catalytic centre to the 'lower' surface of the trimer (Leslie et al., 1988). Subsequent solution of the structure of the CAT/CoA binary complex confirmed that this 'tunnel' is indeed

the CoA binding site. The pantetheine arm of CoA binds in the tunnel such that the sulphur atom of CoA is only 0.33nm from N3 of His 195, with the adenosine moiety of CoA binding on the enzyme surface. As in the case of chloramphenicol, most of the interactions with CoA involve residues from only one subunit (the same one for both substrates) whilst the catalytic histidine is on the facing subunit. The main residues involved in interactions with CoA are shown in figure 1.9.

The conformation of bound CoA is similar to that observed for CoA bound to citrate synthase (Remington et al., 1982). The pantetheine arm binds in an extended conformation and the adenosine phosphate moiety adopts a folded conformation such that the adenine ring is in van der Waals' contact with the dimethyl group of the pantetheine arm. However, CoA bound to CAT adopts a much less folded conformation than that bound to citrate synthase and the characteristic internal hydrogen bonds between N7 and O52, and O20 and O51 (via a water molecule) are not present in CoA bound to CAT (The numbering system of CoA is that used by Remington et al., 1982 [fig. 1.10]). The CoA binding sites of CAT and citrate synthase also differ significantly. In CAT there is no equivalent of the proposed 'adenine recognition loop' and there are no ion pairs between the three phosphoanions of CoA and arginyl residues of the enzyme. Such ion pairs have been proposed for several other CoA binding enzymes on the strength of covalent modification experiments with arginyl specific diketone reagents (Ragione et al., 1983; Mautner et al., 1981; Ramakrishna and Benjamin, 1981), but none have yet been confirmed by structure determination.

1.2.9 The adenosine binding site.

The adenosine moiety of CoA binds in a well-defined pocket close to the protein surface. The binding surface is composed of the hydrophobic residues Tyr 178, Phe 55 and Pro 151 and also main chain atoms of residues 176-178 (see fig. 1.4). Pro 151 is conserved in all 11 known CAT sequences and residue 178 is an aromatic amino acid in 10 of the 11 known sequences. Phe 55 is not conserved. The dimethyl group of the pantetheine arm of CoA also forms a major part of the adenine binding pocket. N1 and N10 of the adenine ring form hydrogen bonds with the main chain amide of residue 178 and the main chain carbonyl oxygen of residue 176 respectively. The ribose ring and 3'-phosphate group are solvent accessible and form no significant interactions with the enzyme although the 2' hydroxyl group of the ribose ring forms a hydrogen bond to the main chain amide of residue 55 via an ordered water molecule.

1.2.10 The pantetheine binding site.

As noted above the pantetheine arm of CoA binds in an almost cylindrical tunnel leading from the active site to the 'lower' surface of the trimer. The tunnel is composed of predominantly hydrophobic residues several of which are well conserved (Phe 103, Tyr 56, Trp 152, Phe 96 see fig. 1.9). The pantetheine arm binds between β_E and β_G and thus forms interactions with several residues from these β-strands. There are several hydrogen bonds to pantetheine, but only 3 of these do not involve bridging water molecules (Gly 200

amide → S81, His 195 N3 → S81-H and Ser 148 hydroxyl → N71-H).

1.2.11 The pyrophosphate moiety of CoA.

As already mentioned there are no ion pairs between the charged pyrophosphate group of CoA and positively charged residues on the enzyme. Neither are the pyrophosphoryl anions positioned at the positive poles of any α-helices. There are however, two well conserved lysyl residues (Lys 54 and Lys 177) close to the CoA binding site which may be involved in long-range electrostatic interactions with the phosphate groups of CoA (see chapter 4). In the crystal structure of the CAT/CoA binary complex there is evidence for a metal ion (probably cobalt) bound to the pyrophosphate moiety of CoA although there is no requirement for metal ions for CAT activity.

1.2.12 The active site of CAT_{III}.

The proposed mechanism of CAT involves His 195 acting as a general base, abstracting a proton from the 3-hydroxyl of chloramphenicol and thereby promoting nucleophilic attack at the carbonyl group of acetyl CoA (see fig. 1.5). Such a mechanism almost certainly proceeds via an oxyanion tetrahedral intermediate which would be expected to be stabilised by hydrogen bonding to a suitable group on the enzyme (Lewendon et al., 1990). The structure of the active site of CAT_{III} shows that N3 of His 195 is in hydrogen bonding distance from both the 3-hydroxyl of chloramphenicol

(0.28nm) and the thiol of CoASH (0.33nm) and is thus ideally situated to deprotonate either group to promote the forward or reverse reactions respectively.

Covalent modification studies showed that only N3 of His 195 is reactive, suggesting that a specific tautomer of His 195 is stabilised (Fitton and Shaw, 1979; Corney, 1983; Kleanthous et al., 1985). Unlike chymotrypsin (Blow et al., 1969) and phospholipase A_2 (Verheij et al., 1980) in which the active site histidyl group is stabilised by hydrogen bonding to a carboxylate group, in CAT the N1-H of His 195 is hydrogen bonded to its own main chain carbonyl oxygen, a conformation not observed for a histidyl residue of any other protein in the Brookhaven structural data base (Leslie et al., 1988). Tautomeric stabilisation of His 195 may also by aided by stacking of its imidazole ring with the aromatic ring of Tyr 25.

Modelling of the transition-state (A.G.W.Leslie and P.C.E.Moody, unpublished results) suggested that the oxyanion of the tetrahedral intermediate could be stabilised by hydrogen bonding to a conserved serine (Ser 148). The substitution S148A results in a 53-fold decrease in k_{cat} with only small changes in the substrate K_m values suggesting that Ser 148 is involved primarily in transition-state binding. Complete removal of the side chain (S148G) results in a smaller decrease in k_{cat} suggesting that an ordered water molecule may be able to compensate for the absence of the seryl hydroxyl (Lewendon et al., 1990).

The general architecture of the active site is stabilised by a network of hydrogen bonds centred on the buried ion pair between Arg 18 and Asp 199 (fig. 1.11).

Disruption of this network by mutagenesis has profound effects on both catalysis and enzyme stability (Lewendon et al., 1988). The substitutions D199A and R18V both result in a ~10-fold decrease in k_{cat} and a large decrease in thermal stability. Substitution of Asp 199 with Asn allows formation of an alternative hydrogen bonding network which retains high thermal stability, but at the price of a ~1500-fold decrease in k_{cat}.

1.3 The aims of this project.

At the start of this project the crystal structure of CAT$_{III}$ was not available, and little or nothing was known about the residues involved in the binding of acetyl CoA. The initial aim of this project was therefore, to identify residues involved in CoA binding. By analogy with other CoA binding proteins, it was proposed that arginyl residues may be important for CoA binding, and two such residues (Arg 18 and Arg 74) are conserved in all known CAT sequences. Early experiments concentrated on the use of covalent modification techniques to identify possible interactions between acetyl CoA and positively charged amino acid side chains.

Once the structure of the CAT/chloramphenicol binary complex became available, a potential CoA binding site was identified and site-directed mutagenesis and substrate analogues were employed to (i) confirm the proposed position of the CoA binding site and (ii) probe the role of electrostatic interactions in the binding of CoA to CAT$_{III}$. Subsequently, the role of hydrophobic interactions in CoA binding and in enzyme catalysis was investigated using site-

directed mutagenesis and steady-state kinetic analysis.
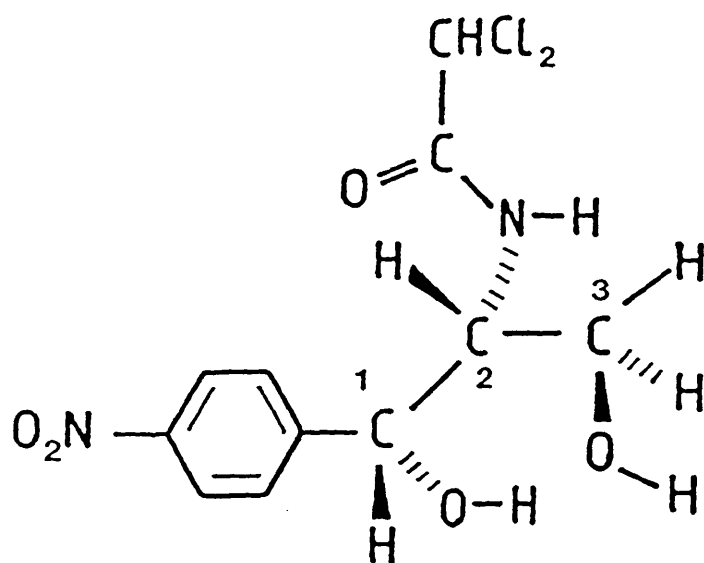
Figure 1.1. The structure of chloramphenicol.

Figure 1.2. Acetylation of chloramphenicol by chloramphenicol acetyltransferase (CAT).

CAT catalyses the acetyl CoA dependent acetylation of chloramphenicol (reaction (1), Shaw, 1967, Shaw and Unowsky, 1968). The product 3-acetyl chloramphenicol can non-enzymically rearrange (2), in a pH dependent manner, to produce 1-acetyl chloramphenicol (Nakagawa et al., 1979) which can undergo further enzymic acetylation (3).

(1) Acetyl CoA + chloramphenicol ⇄ CoA + 3-acetyl chloramphenicol

(2) 3-acetyl chloramphenicol ⇄ 1-acetyl chloramphenicol

(3) Acetyl CoA + 1-acetyl chloramphenicol ⇄ CoA + 1,3-diacetyl chloramphenicol
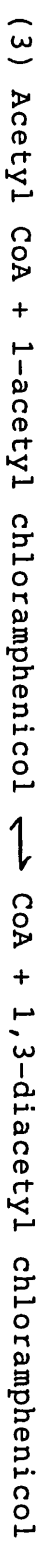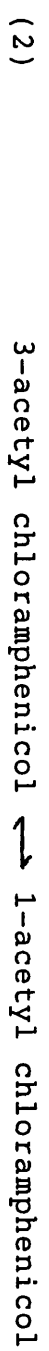
Figure 1.3. The primary structures of chloramphenicol acetyltransferase variants.

The amino acid sequences of eleven CAT variants are shown, and are aligned to maximise homology between the sequences (Shaw and Leslie, 1989). Altogether there are 22 absolutely conserved residues. All sequences are derived from nucleotide sequences: Type I, Alton and Vapnek, 1979; Proteus mirabilis, Charles et al., 1985; Type II, Haemophilus influenzae, Murray,I.A., Martinez-Suarez,J.V., Close,T.J. and Shaw,W.V. unpublished results; Bacteroides uniformis, Martinez-Suarez,J.V., Murray,I.A., Shaw,W.V. and Perez-Diaz,J.C. unpublished results; Type III, Murray et al., 1988; Clostridium difficile, Wren et al., 1989; Bacillus pumilis, Hopwood et al., 1983; Staphylococcus aureus pC221, Brenner and Shaw, 1985, pC194, Horinouchi and Weisblum, 1982 and Streptomyces acrimycini, Murray et al., 1989. Conservation of residues is indicated by the shading and * indicates the catalytic residue (His 195).

Protein sequence alignment (three blocks)

Block 1 (residues 1–80)

```
                    1    5    10   15   20   25   30   35   40   45   50   55   60   65   70   75   80
I
  Proteus       MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFIHILARLMNAHFEFRMAMK D
II
  Haemophilus   MDTKRVGYTVVDLSQWGRKEHPEAFQSFAQCTFSQTVQLDITSSLKTVKQNGYKFYPTFIYIISLLVNKHAEFRMAMK D
III
  Bacteroides   MNFTRIDLNTWNREHFALYRQQIKCGFSLTTKLDITALRTALAEGTGYKFYPLMIYLISRAVNQFPEFRMAMK D
  Clostridium   MNFTRIDLNTWNREHFALYRQQIKCGFSLTTKLDITALRTALAETDYKFYPVMIYLISRAVNQFPEFRMAIK D
  Bacillus      MNYTKFDVKNMVRREHFEFYRHRLPCGFSLTSKIDITTLKKSLDDSAYKFYPVMIYLIAQAVNQFDELRMAIK D
  pC221         M FKIIDRNMWKRNPYFDHYFDKIPCTYSITVNIDISRVIRFKTEQNTKIYPLLLVVALSKAVNCYDEFRTDINEN
  pC194         MVFEKIDKNSWNRKEYFDMYFASVPCTYSMSLKVDITQ    IKEKGMKLYPAMLYIAMIVRHSEFRTAINQD
  Bacillus      M FKQID ENYLKEHFHHYMTLTRCSYSLVINLDITKLHAILKEKKLKVPVQIYLLARAVQKIPEFRMDQVND
  Clostridium   MTFNIIKLENWDRKEYFEHY FNQQTTYSITKEIDITLFKDMIKKKGYEIYPSLIYAIMEVVNRNKVFRTGINSE
  pC221         MNFNKIDLDNWKRELFNHY LNQQTTFSITTEIDISVLYRNIKQEGYKFYPAFIFLVTRVINSNTAFRTGYNSD
  S'myces       MDAPIPTPAFILDTWPRQHFDHYRRRVPCTYAMTVEVDVTAFAAALRRSPRKSYIAQWNALATVVNRHEEFRMCINSS
```

Block 2 (residues 85–160)

```
                 85   90   95   100  105  110  115  120  125  130  135  140  145  150  155  160
I
  Proteus       GELVIWDSVHPCYTVFHEQTETFSSLWSEYHDFRQFLHIYSQDVACYGENLAYFPRGFI ENMFFVSANPWVSFTSFDL
II
  Haemophilus   GELVIWDSVNPGYNIFHEQTETFSSLWSYYHKDINRFLKTYSEDIAQYGDDLAYFPKEFI EMFFVSANPWVSFTSFNL
III
  Bacteroides   NELIWDQSDPVITVFHKETETFSALSCRYFPDLSEFMAGYNAVTAEYQHDTRLFPQGNLPENHLNISSLPWVSFDGFNL
  Clostridium   AELIWDQTDPVFIVFHKETETFSALFCRYCPDISEFMAGYNAVMAEYQHNTALFPQGALPENHLNISALPWVNFDSFNL
  Bacillus      DELIWDSVDPQFIVFHQETETFSALSCPYSSDIDQFMVNYLSVMERYKSDTKLFPQGVTPENHINISSLPWTTFTGFNI
  Bacteroides   GEVGAWETLHPCYTVFHKESETFSNIWTEWDKDLNTFLSNYNRDIEIYGNNTAIDAKPQAPKNVFPISSLPWTTFTGFNI
  Clostridium   VELGIYDEMIPSYTLFHNDLETFSSLWTECKSDFKSFLADYESDTQRYENNHRMEGKPNAPENIFNVSMIPWVTFDGFNI
  Bacillus      NKLGYWDKINPLYIVFHKQTEKFTNIWTESDNNFTSFYNNYKNDLLEYKDKEEMFPKKPIPENTIPISMIPWIDFSSFNL
  pC221         GELGYWDKLEPLYIIFDGVSKIFSGIWTPVKNDFKEFYDLYLSDVEKYNGSGKLFPKTPIPENAFSISIIPWTSFTGFNL
  S'myces       GDPAWPVVHPAFTVFNPERETFACWAPYDPFGTFHDTAAPLLAEHSRATDFFPQGNPPNAFDVSSLPWVSFTGFTL
```

Block 3 (residues 165–240)

```
                 165  170  175  180  185  190  195  200  205  210  215  220  225  230  235  240
I
  Proteus       NVANMDNFFADVFTMGRYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNEIQQYCDEWQGGA
II
  Haemophilus   NMANINNFFAPVFTIGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRLLNEIQQYCDEGCK
III
  Bacteroides   NITGNDDYFAPVFTMAKFQQEGDRVLLPVSVQVHHAVCDGFHAARFINTLQMCDNILK
  Clostridium   NVANFTDYFAPIITMAKYQQEGDRLLLPLSVQVHHAVCDGFHAARFINTLQMCDNILK
  Bacillus      NIKPDTGYLLPIFTGKYFKQEDKFYIPISIQVHHAVCDGFEVARFINRIQELCNSKLK
  pC221         NLQKGYCYLIPIFTMGKIIKKDNKIIIPIAIQVHHAVCDGFFICRFVNELQELIIVTQVCL
  pC194         NVSTDEAYLLPIFTIGKFVEEGKIIIPVAIQLHHAVCDGYHASLFMNEFQDIIHKVDDWI
  Bacillus      NIGNNSNFLLPIITIGKFYSENNKIYIPVAIQVHHAVCDGYHAGLFMNSIQELSDRPNDWLL
  Clostridium   NINNNSNYLLPITTAGKFINKGNSIYLPLSLQVHHAVCDGYHARLTNELQTLLADPAWL
  S'myces       DIRDGWDHLAPIFTLGRYTERDTRLLLPLSVQIHAAADGFHTARLTNELQTLLADPAWL
```

*

Figure 1.4. The kinetic mechanism of chloramphenicol acetyltransferase (CAT).

CAT follows a ternary complex mechanism, with a random order of addition of substrates. The free enzyme (E), both binary complexes (ECm and EAcCoA) and the ternary complex (EAcCoACm) are in rapid equilibrium. (Kleanthous and Shaw, 1984). K_m and K_d are the substrate dissociation constants for the ternary and binary complexes respectively, and k is the catalytic rate constant.

E

K_dAcCoA K_dCm

EAcCoA ECm

K_mCm K_mAcCoA

ECmAcCoA

k

E + AcCm + CoA
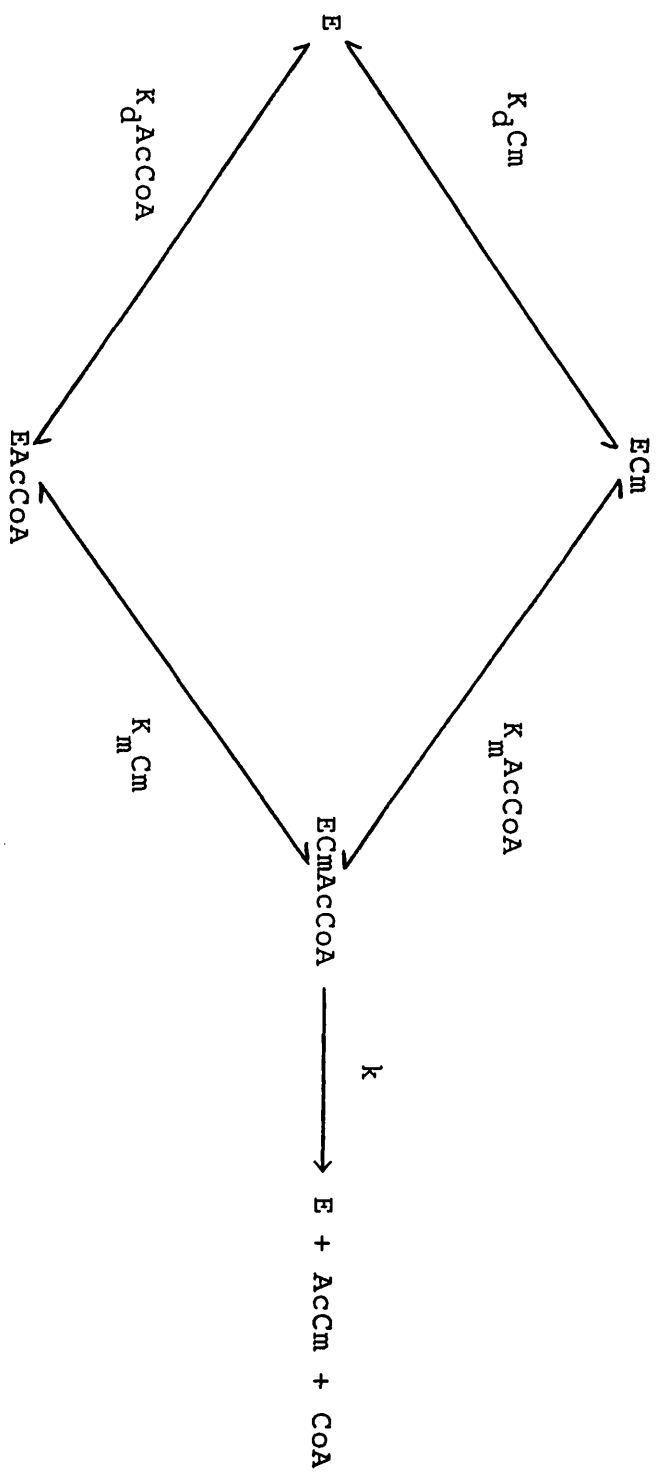
Figure 1.5. The chemical mechanism proposed for the acetyl CoA dependent acetylation of chloramphenicol by CAT.

Histidine 195 acts as a general base, abstracting a proton from the 3'hydroxyl group of chloramphenicol and thereby promoting nucleophilic attack at the thioester of acetyl CoA. The reaction proceeds via a charged (oxyanion) tetrahedral intermediate, which is stabilised by formation of a hydrogen bond to Ser 148 (Lewendon et al., 1990), and which finally breaks down to yield the products, 3-acetyl chloramphenicol and CoA.
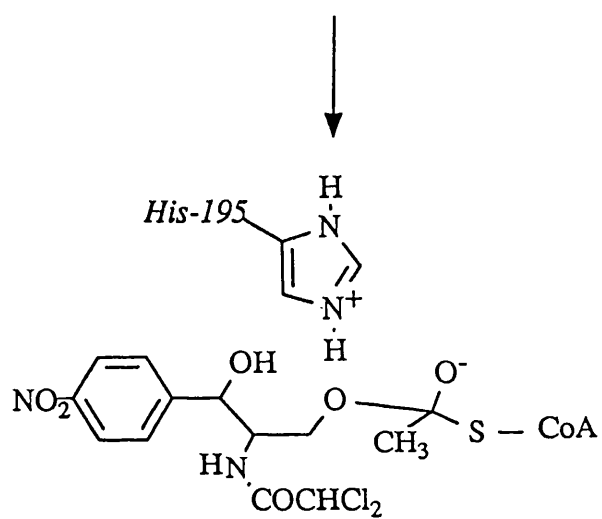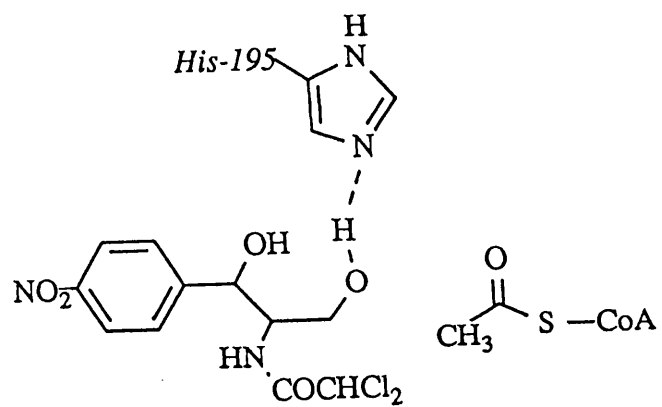
Tetrahedral Intermediate
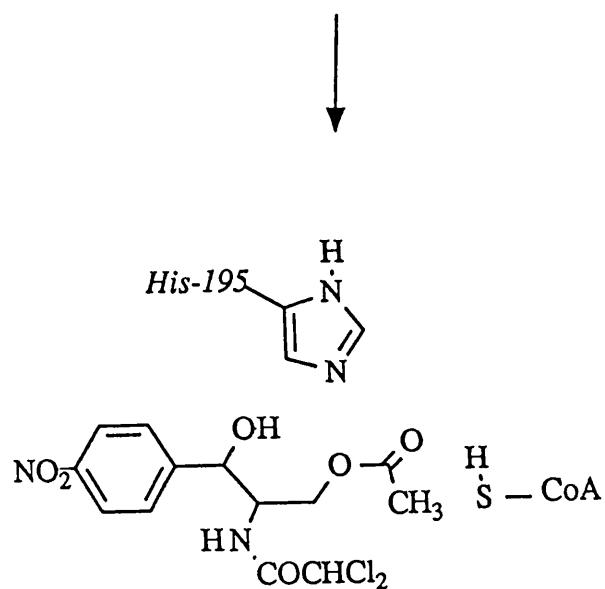
Figure 1.6. The structure of a single CAT subunit.

Each CAT monomer is composed of 5 α-helices ($\alpha_1-\alpha_5$) and 10 β-strands (A-J). The β-strands form two sheets, a small three stranded sheet and a major six stranded sheet. β_H appears from the monomer structure to be somewhat isolated, but on formation of the trimer is involved in inter-subunit interactions (fig. 1.7). The α-helices are packed against one side and the ends of the large β-sheet. The catalytic histidyl residue (His 195) is found on the loop between β_J and α_5.

α1

α5

α2

α3

α4

A

B

C

D

E

F

G

H

I

J

N

C
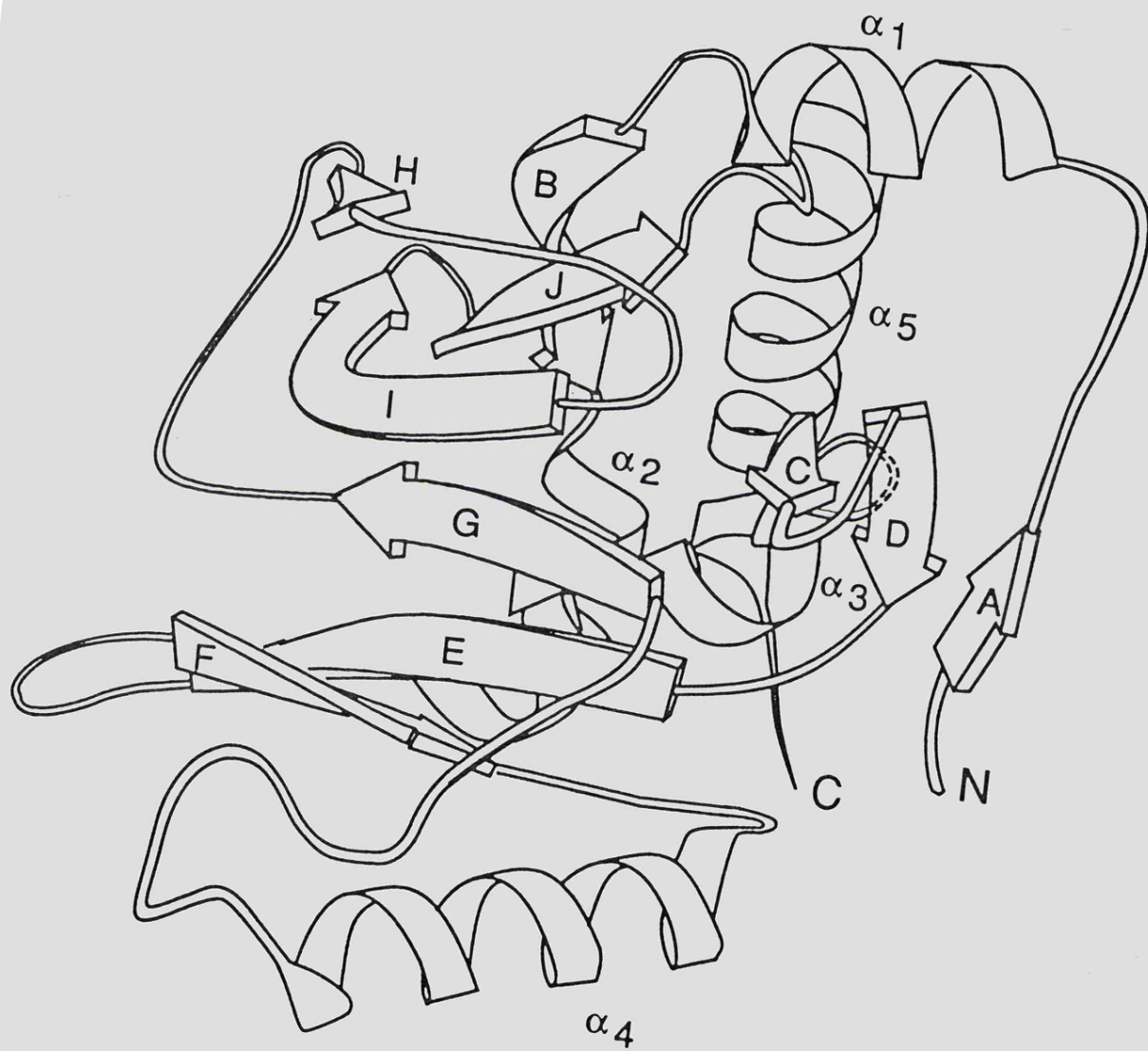
Figure 1.7. The oligomeric structure of CAT$_{III}$.

Chloramphenicol acetyltransferase is a trimeric enzyme composed of identical 25 kDa subunits (Leslie et al., 1986, Harding et al., 1987). On formation of the trimer the large six stranded β-sheet of the monomer is extended by a single strand (β_H, *) from the facing subunit (shaded). The active site is at the subunit interface, with the catalytic residue (His 195) on one subunit and both substrate binding sites on the facing subunit (Leslie et al., 1988).

Figure 1.8. The chloramphenicol binding site of CAT$_{III}$.

A. The active site of CAT$_{III}$ with chloramphenicol bound. the
3'hydroxyl group of chloramphenicol forms a hydrogen bond
(dashed line) with N3 of the general base catalyst, His 195.
The 'tunnel' leading from the active site to the protein
surface is the acetyl CoA binding site. In the absence of
chloramphenicol, the acetyl CoA and chloramphenicol binding
sites form a continuous, solvent filled tunnel through the
subunit. Residues names in orange belong to an adjacent
subunit, and the blue 'mesh' represents the amino acid
surfaces.

B. Stereoview of the chloramphenicol binding site.
Chloramphenicol is shown in bold, ordered water molecules by
the double circles and predicted hydrogen bonds by the dashed
lines. Residue names preceded by # belong to an adjacent
subunit.

(Both figures were kindly provided by A.G.W.Leslie).

A
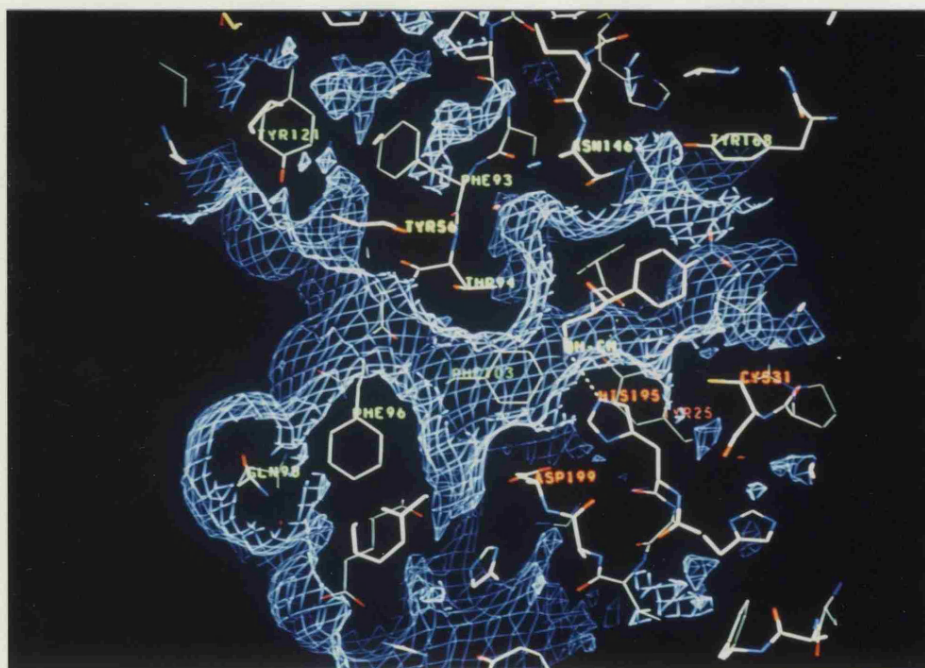
B
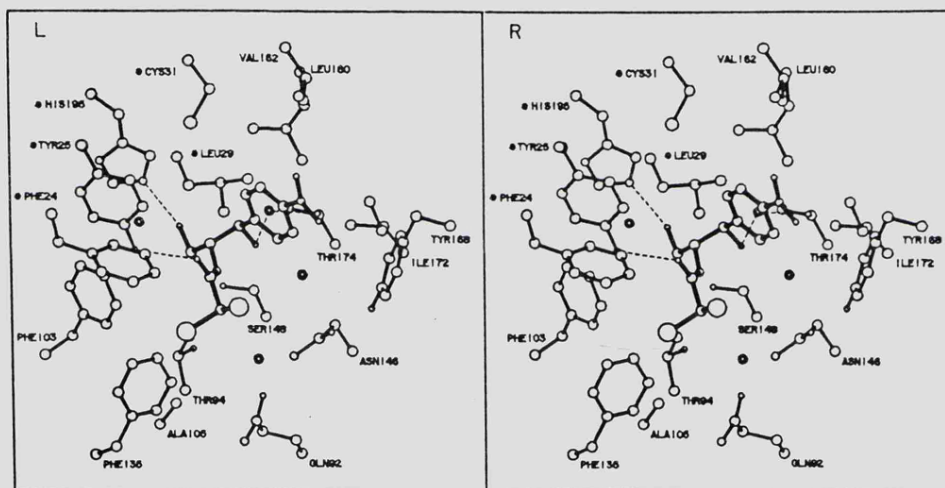
Figure 1.9. The Coenzyme A binding site of CAT$_{III}$.

A. A schematic diagram showing the residues involved in interactions with acetyl CoA (taken from Leslie et al., 1988).

   Predicted hydrogen bonds are shown as dotted lines and ordered water molecules indicated by W. Residue names preceeded by # belong to an adjacent subunit.

B. Stereoview of the coenzyme A binding site of CAT$_{III}$.

A

TYR 178

PHE 55

LYS 54

LYS 177

176

PRO 151

TRP 152

H₃C

H₃C

96

TYR 56

SER 148

PHE 103

#ASP 199

#200

#HIS 195
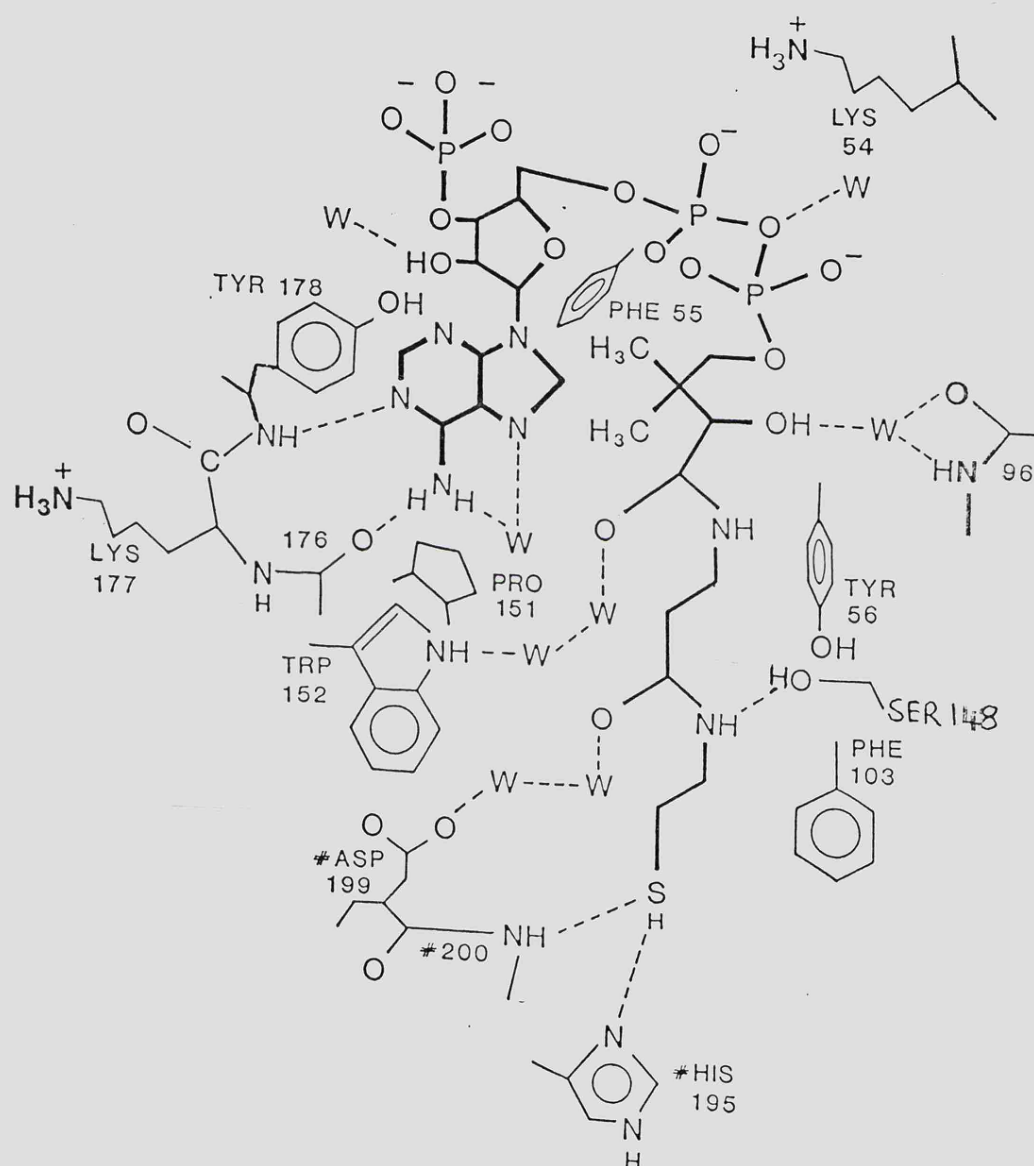
B
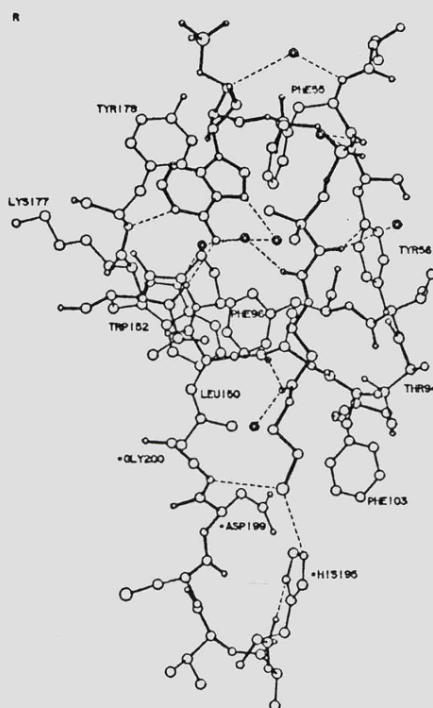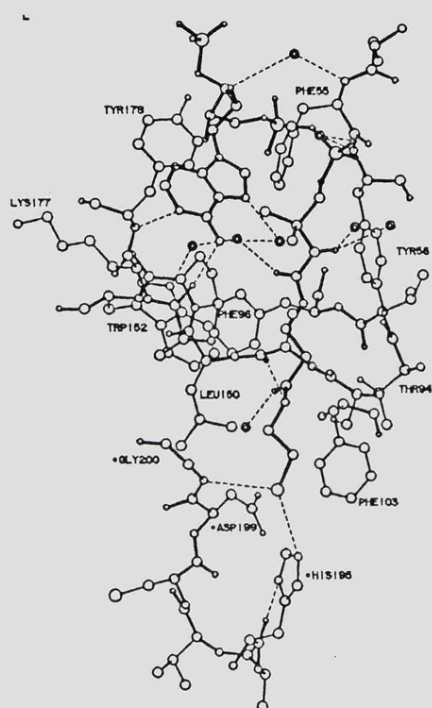
Figure 1.10. The structure of Coenzyme A.

The numbering system is that used by Remington et al.(1982).
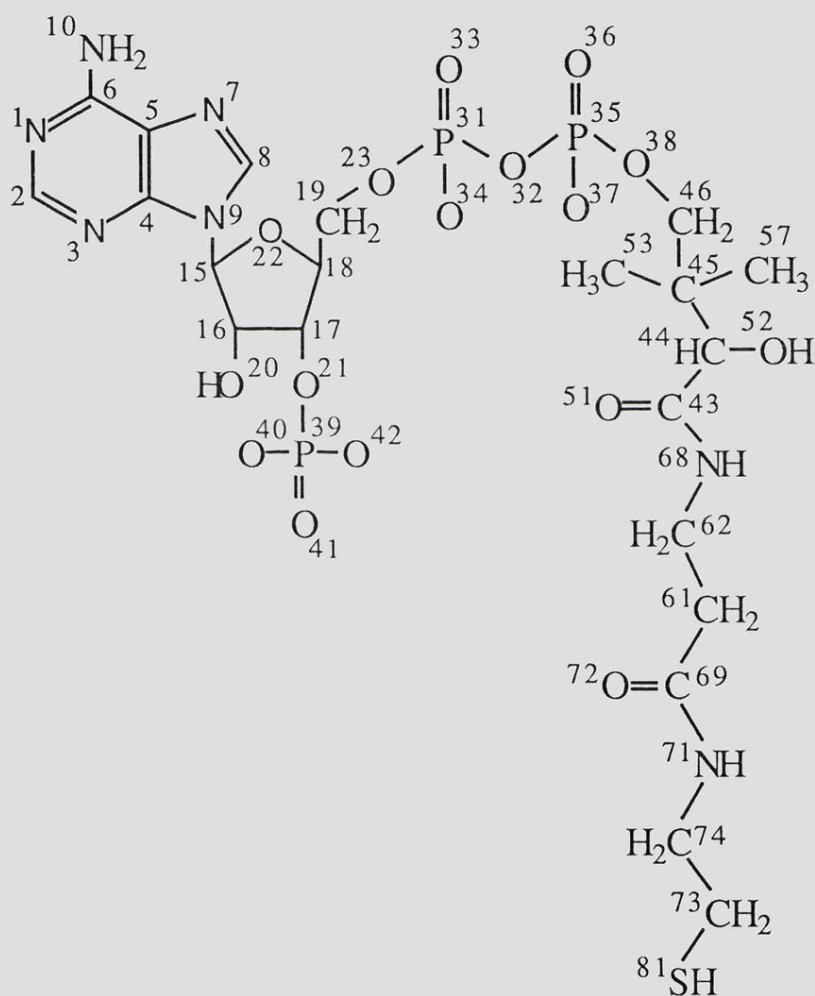
Figure 1.11. The structure of the active site of CAT_{III}.

The high resolution structure of CAT_{III} (Leslie et al., 1988) shows that Asp 199 plays an important role in retaining the architecture of the active site (Lewendon et al., 1988), rather than stabilising the required conformer of His 195 as previously thought (Kleanthous et al., 1985). Asp 199 and Arg 18 are involved in a complicated web of hydrogen bonds (dotted lines) in and around the active site, which includes interactions with the catalytic histidine residue (His 195). Removal of either Arg 18 or Asp 199, by mutagenesis, results in a decrease in k_{cat} and greatly reduced thermostability (Lewendon et al., 1988).
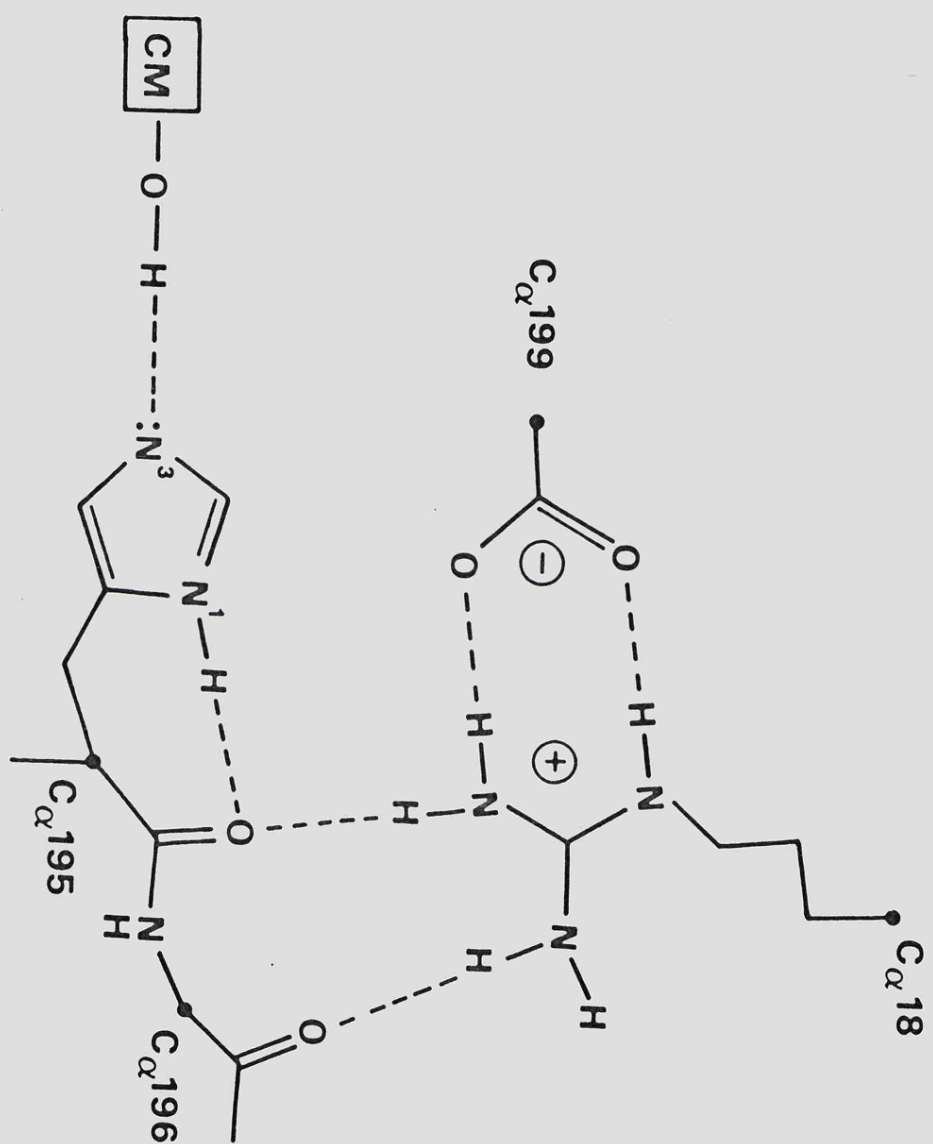
Chapter 2. Materials and methods.

Materials.

Aldrich Chemical Co. Ltd.

Phenylglyoxal, 2,3-butanedione, triethylamine, butyric anhydride, succinic anhydride.

Bachem Inc.

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal).

BDH Biochemicals.

Amberlite MB-1, bromophenol blue, dimethylformamide, xylene cyanol FF.

Bethseda Research Laboratories/Gibco.

Restriction endonucleases, E. coli. DNA polymerase I Klenow fragment, T4 polynucleotide kinase.

Difco.

Bactoagar, bactotryptone, yeast extract.

Fisons Plc.

Phenol, ethanol, methanol, chloroform, isoamylalcohol.

May and Baker.

Acetic anhydride.

Miles laboratories.

Agarose.

New England Biolabs.

T4 DNA ligase.

Pharmacia.

Coenzyme A, Sephadex G10, G25, Sepharose 4B, deoxy and dideoxy nucleotide triphosphates.

Serva.

Acrylamide, N,N'-methylenebisacrylamide, urea.

Sigma Chemical Co. Ltd.

5'AMP, sodium salt (type II, from yeast), 5'ADP, potassium salt (from equine muscle), 5'ATP, sodium salt (grade I), 3'AMP, sodium salt (from yeast), ammonium persulphate, ampicillin sulphate, bovine serum albumin (fraction V), cyanoethylphosphate dihydrate, barium salt, chloramphenicol, diaminoethanetetracetic acid, disodium salt (EDTA), dimethylaminopyridine (DMAP), 5,5'-dithiobis (2-nitrobenzoic acid), dithiothreitol, Dowex 50W, D-pantethine, ethidium bromide, ethyl acetimidate, ficoll, guanidine hydrochloride, isopropyl β-D-thiogalactopyranoside (IPTG), L-cysteine, lysozyme, 2-mercaptoethanol, N-2-hydroxyethylpiperazine-N' -2-ethanesulphonic acid, N,N'-dicyclohexylcarbodiimide, N,N,N',N'-tetra-methylethylenediamine (TEMED), polyethyleneglycol 8000, tetracycline, thiamine.HCL, triethanolamine hydrochloride, Trizma base.

The Radiochemical Centre, Amersham.

Ethyl [1-^{14}C] acetimidate, γ[^{32}P] ATP, α[^{35}S] dATP.

2.1 Solutions and buffers.

2.1.1 Solutions for enzymology.

TME   50mM Tris.HCl (pH 7.5)

0.1mM EDTA

0.1mM 2-mercaptoethanol. Stored at 4°C.

TSE   50mM Tris.HCl (pH 7.5)

0.1mM EDTA

100mM NaCl. Stored at 4°C.

TEA   0.2M Triethanolamine.HCl (pH 8.5)

0.1mM 2-mercaptoethanol. Stored at 4°C.

HEPES   50mM N-2-Hydroxyethylpiperazine-N'

-2-ethanesulphonic acid. Stored at 4°C.

TEAB   1M Triethylamine, bubble CO_2 through until pH

reaches 7.5. Store at room temperature.

Chloramphenicol   5mM aqueous solution stored at 4°C, made

fresh every 3-4 weeks.

2.1.2 Solutions for DNA manipulation.

ddH$_2$O distilled, deionised water, autoclaved.

Phage buffer.

KH_2PO_4 3g

Na_2HPO_4 7g

NaCl   5g make to 1 litre with ddH$_2$O, autoclaved. When cooled
to room temperature add 1ml of each of the following:

1M $MgCl_2$, 0.1M $CaCl_2$ and 1% (w/v) gelatin, all autoclaved and cooled.

Phenol solution.

Freshly distilled phenol containing 0.1 % (w/v) 8-hydroxy-quinoline, equilibrated with 0.1M Tris.HCl (pH 7.5) and mixed with an equal volume of chloroform/isoamylalcohol (24:1) equilibrated with 0.1M Tris.HCl (pH 7.5).

10x TM   100mM Tris.HCl (pH 8.5)

                  50mM $MgCl_2$ autoclaved, stored at -20°C.

10x TBE   900mM Tris.HCl (pH 7.5)

                  90mM boric acid

                  0.25mM EDTA

10x React 3 (supplied with restriction endonucleases)

                  50mM Tris.HCl (pH 8.0)

                  10mM $MgCl_2$

                  100mM NaCl stored at -20°C.

10x Denhardt's solution

                  1g Ficoll

                  1g Polyvinylpyrrolidine

                  1g BSA to 500ml with ddH_2O.

6x SSC   0.9M NaCl

                  0.09M Trisodium citrate (pH 7.5).

5x Ligase buffer

> 250mM Tris.HCl (pH 7.6)
>
> 50mM $MgCl_2$
>
> 5mM ATP
>
> 5mM DTT
>
> 25% (w/v) polyethyleneglycol-8000 stored at -20°C.

10x Kinase buffer

> 1M Tris.HCl (pH 8.3)
>
> 0.1M $MgCl_2$ autoclaved, stored at -20°C.

TE   10mM Tris.HCl (pH 8.0)

> 1mM EDTA autoclaved.

Lysis solution

> 25mM Tris.HCl (pH 8.0)
>
> 50mM sucrose
>
> 10mM EDTA autoclaved, stored at -20°C. Add lysozyme (0.1% w/v) immediately before use.

Acetate solution

> 60ml 5M potassium acetate
>
> 11.5ml glacial acetic acid
>
> 28.5ml ddH_2O stored at 4°C.

$CaCl_2$ 1M solution, autoclaved. Dilute to 50mM with ddH_2O before use.

10x Agarose gel loading buffer

> 0.25% (w/v) bromophenol blue
>
> 0.25% (w/v) xylene cyanol FF
>
> 25% (w/v) Ficoll.

2.1.3 Solutions for site-directed mutagenesis.

10mM ATP — filter sterile, stored at -20°C in 5μl aliquots.

2mM dNTPs — dATP,dCTP,dGTP,dTTP in ddH$_2$O. Filter sterile, stored at -20°C.

10x A

> 0.2M Tris.HCl (pH 7.5)
>
> 0.1M MgCl$_2$
>
> 0.5M NaCl autoclaved. Mix 99:1 with 1M DTT before use.

10x B

> 0.2M Tris.HCl (pH 7.5)
>
> 0.1M MgCl$_2$ autoclaved. Mix 9:1 with 1M DTT before use.

Oligo dye mix

> 8ml deionised formamide
>
> 0.1ml 0.5M EDTA
>
> 2mg bromophenol blue
>
> 5mg xylene cyanol FF to 10ml with ddH$_2$O.

2.1.4 Growth media and supplements (Maniatis et al., 1982).

2YT   16g bactotryptone

                10g yeast extract

                5g NaCl to 1 litre with ddH$_2$O, autoclaved.

L-broth (Lennox, 1955)

                10g bactotryptone

                5g yeast extract

                5g NaCl to 1 litre ddH$_2$O, autoclaved.

M9 salts

    Stock solution were made by mixing the following four sterile solutions:

    (1)6g Na$_2$HPO$_4$

        3g KH$_2$PO$_4$

        0.5g NaCl

        1g NH$_4$Cl to 1 litre with ddH$_2$O, set to pH 7.4,

        autoclaved. When cool add the following:

    (2)2ml 1M MgCl$_2$

    (3)10ml 20% (w/v) glucose

    (4)0.1ml 1M CaCl$_2$.

LB agar   1.5% (w/v) agar in L-broth, autoclaved.

L-Top agar   0.8% (w/v) agar in L-broth, autoclaved.

2YT agar   1.5% (w/v) agar in 2YT, autoclaved.

Minimal agar   1.5% (w/v) agar in M9 salts (part 1 only)
               autoclaved. Add $CaCl_2$, $MgCl_2$, glucose and
               thiamine to set plates.

Thiamine   10% (w/v) filter sterile.

Ampicillin   100mg/ml filter sterile, store at -20°C.

Tetracycline   12.5mg/ml in ethanol, store at -20°C.

IPTG   24mg/ml filter sterile, store at -20°C.

X-gal   24mg/ml in dimethylformamide, store at -20°C.

2.1.5 Oligonucleotides.

Mutagenic oligonucleotides (Site of mutation underlined).

PDK177A 5' C TTG CTG ATA T<u>GC</u> TGC CAT TGT TA 3'

PDK177Q   C TTG CTG ATA TT<u>G</u> TGC CAT TGT TA

PDK177E   C TTG CTG ATA TT<u>C</u> TGC CAT TGT TA

PDK54Q   AC CGG ATA AAA TT<u>G</u> ATA CGC TG

PDY56A   CAT TAC CGG <u>AGC</u> AAA CTT ATA

PDY56V   T CAT TAC CGG <u>AAC</u> AAA CTT A

PDY56I   T CAT TAC CGG <u>AAT</u> AAA CTT A

PDY56F   T CAT TAC CGG <u>AAA</u> AAA CTT A

PDY178A   TTC TTG CTG <u>AGC</u> TTT TGC CAT

PDY178V C TTC TTG CTG <u>AAC</u> TTT TGC C

PDY178I   TTC TTG CTG <u>AAT</u> TTT TGC CAT

PDY178F C TTC TTG CTG <u>GAA</u> TTT TGC C

Oligonucleotide primers for DNA sequencing.

20mer 5' CG ATC CCA AAC TCC ATG ATG 3'

IAM39   AAC AGT CGG TTC TAT G

IAM40   TGA GTA TGG GCA ACT C

IAM41   ATA AAA CTT ATA CAC T

2.2 Bacterial strains.

JM101 Δ(lac-pro),thi,supE/F'traD36,proAB$^+$,lacI^q,lacZΔM15

             Store on minimal agar plates with added thiamine.

             (Messing, 1979).

RZ1032   HFrKL16,Po/45,Zbd-279::Tn10,lysA,thi-1,relA1,spoT1,

             supE44,dut-1,ung-1.

             Grow under constant tetracycline selection in the

             dark at 30°C.

             (Kunkel et al., 1987).

TGII   K12,Δ(lac-pro),supE,thi,recA$^-$,Srl::Tn10Tc^R,hsdD5/

             F'traD36, proAB$^+$,lacI^q,lacZΔM15

             (Gibson, 1984).

BMH 71-18 MutL   K12,Δ(lac-pro),supE,thi,mutL::Tn10/F'proAB$^+$,

             lacI^q,lacZΔM15.

             (Kramer et al., 1984).

2.2.1 Maintenance of bacterial strains.

All strains were stored at -70°C in 15% (v/v) glycerol. A

single colony, checked for the correct phenotype was grown overnight at 37°C in 1.5ml 2YT. To 850μl of this culture was added 150μl glycerol and the culture stored at -70°C. Strains which were used regularly were also maintained on agar plates and were subcultured every 2 months.

2.2.2 Plasmids and vectors.

pUC18	Ap^r	Yannisch-Perron et al., 1985
pUC18:III:ME1	Ap^r Cm^r	Murray et al., 1988
M13 mp18		Yannisch-Perron et al., 1985

Ap = ampicillin, Cm = chloramphenicol

2.3 Purification of type III Chloramphenicol acetyl transferase.

Type III CAT was purified from E. coli extracts by affinity chromatography by the method of Zaidenzaig and Shaw (1976) as modified by Packman and Shaw (1981a).

Sterile 2YT medium (500ml) containing 200μg/ml ampicillin was inoculated with 25μl of an overnight culture of E. coli JM101 bearing plasmid pUC18:III:ME1 (Murray et al., 1988) and incubated for 12-16 hours at 37°C with vigorous shaking. Cells were harvested by centrifugation for 10 minutes at 10,000rpm in a Beckman J21B centrifuge at 4°C. Pelleted cells were resuspended in 60ml TME (pH 7.5) and sonicated (on ice; 8 microns peak to peak) in 1 minute bursts. Release of activity was monitored by assaying an appropriately diluted aliquot by the standard assay (2.5). Sonication was continued until further sonication yielded no further release of enzyme activity. Cell debris was then pelleted by centrifugation (1

hour, 15,000rpm, 4°C) and the supernatant collected and loaded directly onto a chloramphenicol substituted Sepharose 4B column (~50-100ml resin volume) equilibrated with TME (pH 7.5). After washing with 200ml TME, unbound protein was washed from the column with TME containing 0.3M sodium chloride until the absorbance at 280nm of the column eluent was less than 0.02. CAT was eluted with the same buffer containing 5mM chloramphenicol, and 10ml fractions collected. Fractions containing peak CAT activity were pooled and exhaustively dialysed against TME. Purity was checked by SDS-PAGE (2.8, fig. 2.1) and purified protein stored at -20°C in 1 or 5ml aliquots. Typically 120-150mg CAT could be purified from a 500ml culture of E. coli JM101/pUC18:III:ME1.

Chloramphenicol Sepharose resin was routinely washed with TME containing 8M urea after use and stored in TME containing 0.02% (w/v) sodium azide at 4°C.

2.4 Protein determination.

Determination of protein concentrations of crude extracts and purified protein were carried out by the method of Lowry et al. (1951). The value obtained from the Lowry assay using bovine serum albumin as a standard, is related to the true CAT_{III} protein concentration as follows:

Lowry value = 1.11x true value for CAT_{III}

Concentrations of purified CAT dialysed against TME to remove chloramphenicol could be determined directly from the absorbance at 280nm using dialysis buffer as a blank (ε = 1.314 ml/mg/cm).

2.5 Standard CAT assay (Kleanthous and Shaw, 1984).

Enzyme activity was measured by monitoring the generation of product (CoA), from acetyl CoA in a linked assay system containing 5,5'-dithiobis (2-nitrobenzoate) [DTNB]. Reduction of the DTNB disulphide by released CoA produces the yellow 5-thio-2-nitrobenzoate dianion which can be monitored spectrophotometrically at 412nm (ε = 13.6 $mM^{-1}.cm^{-1}$).

Reaction was initiated by addition of 10μl of appropriately diluted sample to the following in a 1ml cuvette:

> 930μl DTNB (1mM) in TSE (pH 7.5)
>
> 40μl Acetyl CoA (10mM)
>
> 20μl chloramphenicol (5mM)

Final substrate concentrations were 100μM chloramphenicol ($\sim$8xK_m) and 400μM acetyl CoA ($\sim$5xK_m). Activity was calculated from the initial linear rate of change of absorbance at 412nm measured on a PYE Unicam SP1800 double beam spectrophotometer. One unit of enzyme activity is the amount converting 1μmol of substrate to product per minute at 25°C.

The Hydrolysis of acetyl CoA

Enzyme catalysed hydrolysis of acetyl CoA was measured as described above except that chloramphenicol was omitted from the assay. Enzyme concentrations approximately 1000-fold greater than those required to measure the transacetylation reaction (above) were needed to measure the hydrolytic reaction catalysed by CAT.

2.6 Steady-state kinetic analysis.

Kinetic analyses were carried out as described for the standard assay (2.5) except that the concentrations of substrates were varied, ideally in the range $0.3-5.0 \times K_m$. For each of four fixed concentrations of acetyl CoA, the concentration of chloramphenicol was varied (4 concentrations) to form a 4x4 matrix. Linear initial rates were measured in triplicate and the kinetic parameters calculated from slope and intercept replots of double reciprocal plots (fig. 2.2, Lineweaver and Burk, 1934). Enzyme concentrations were typically in the range 0.2-2nM.

For determination of the kinetic parameters for the hydrolysis of acetyl CoA, chloramphenicol was omitted from the assay, and up to 10 different acetyl CoA concentrations assayed. Kinetic parameters were calculated from double reciprocal plots (fig. 2.3).

Inhibitors.

For inhibitors expected to be competitive with respect to acetyl CoA, the concentration of chloramphenicol in each assay was fixed at $25 \mu M$. For each concentration of inhibitor (usually 6 in the range $0-10 \times K_i$) the concentration of acetyl CoA was varied and initial rates measured. Inhibitor constant (K_i) values were determined from slope replots of double reciprocal plots (fig. 2.4).

Errors in kinetic parameters.

Typical errors in kinetic parameters determined as described above were $\pm 5\%$ for rate constants (k_{cat}) and $\pm 10\%$ for binding constants.

2.7 Synthesis of acyl CoAs (Simon and Shemin, 1953).

To 100mg CoA (0.14mmol) in 8ml 125mM potassium bicarbonate was added a 1.25-fold excess of an appropriate acid anhydride, the solution vortexed immediately and placed on ice for 10 minutes. The presence of residual CoA was monitored from the absorbance at 412nm of a $10\mu l$ sample added to $990\mu l$ 1mM DTNB in TSE (pH 7.5). If the absorbance was greater than 0.02, further additions of anhydride were made (plus $400\mu l$ $KHCO_3$ per $10\mu l$ anhydride). If the absorbance was less than 0.02 the reaction was deemed to be complete and the concentration of acetyl CoA measured by adding $10\mu l$ CAT to the following:

$930\mu l$ DTNB (1mM) in TSE

$50\mu l$ chloramphenicol (5mM)

$10\mu l$ acetyl CoA prep.

The concentration of acetyl CoA was calculated from the steady absorbance at 412nm. After dilution to 10mM, acetyl CoA was stored at -20°C in 0.4ml aliquots.

2.8 Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) was carried out using the discontinuous buffer system of Laemli (1970).

2.8.1 Solutions for polyacrylamide gel electrophoresis.

Acrylamide/bisacrylamide (30:0.8)

To 30g acrylamide and 0.8g N,N methylenebisacrylamide was

added water to 100ml. When the acrylamide had dissolved the solution was filtered and stored at 4°C in the dark.

Separating gels.

		6	8	10	12	15
				% acrylamide		
acrylamide:bisacrylamide	(ml)	6	8	10	12	15
1.5M Tris.HCl (pH 8.8)	(ml)	7.5	7.5	7.5	7.5	7.5
water	(ml)	16.05	14.05	12.05	10.05	7.05
10% (w/v) SDS	(ml)	0.3	0.3	0.3	0.3	0.3
10% (w/v) APS	(ml)	0.15	0.15	0.15	0.15	0.15
TEMED	(ml)	0.01	0.01	0.01	0.01	0.01

Stacking gel (3% acrylamide).

acrylamide:bisacrylamide	1.50ml
0.5M Tris.HCl (pH 6.8)	3.75ml
water	9.45ml
10% (w/v) SDS	0.15ml
10% (w/v) APS	0.15ml
TEMED	0.10ml

2x sample buffer (SDS-PAGE)

10% (w/v) SDS	3.0ml
0.5M Tris.HCl (pH 6.8)	2.0ml
saturated bromophenol blue	0.4ml
glycerol	2.0ml
water	2.6ml

2x sample buffer (native PAGE)

0.5M Tris.HCl (pH 6.8)	2.0ml
saturated bromophenol blue	0.4ml
glycerol	5.0ml
water	2.6ml

10x running buffer

Tris.HCl   30g

glycine   144g make to 1 litre with

water, store at room temp.

Gel stain

0.1% (w/v) PAGE blue 83

10% (v/v) acetic acid

50% (v/v) methanol

Gel destain

10% (v/v) acetic acid

10% (v/v) methanol

2.8.2 SDS-PAGE.

1. Gel pouring.

Ethanol washed gel plates (16cm x 16cm) were clamped together separated by three spacers (0.1cm thick) and molten agarose (0.1% w/v) used to form a seal. The acrylamide/Tris /water part of the separating gel was transferred to a side arm flask and degassed. After addition of SDS, ammonium persulphate and TEMED the solution was carefully poured between the plates to within 2-3cm of the top of the plates. The gel was overlaid with isopropanol and allowed to set. Once

the gel was set (~1/2 hour) the stacking gel was prepared as above, the isopropanol washed from the top of the separating gel with water and the stacking gel carefully poured. A comb was then inserted and the gel allowed to set.

2. Sample preparation.

Protein samples of 5-10μg for purified protein or 50-100μg for crude extracts in low salt buffer were routinely analysed using SDS-PAGE. An equal volume of 2x sample buffer and 5μl 2-mercaptoethanol was added to each sample and loaded onto the gel after boiling for 2 minutes. For CAT samples 12% acrylamide gels were generally used.

3. Running SDS polyacrylamide gels.

The comb and bottom gel spacer were removed and the gel assembly clamped to a vertical gel tank with 1x running buffer containing 0.1% SDS in upper and lower resevoirs. The wells were flushed with buffer and freshly boiled samples loaded. Gels were run for ~3 hours at 50mA (constant current) until the bromophenol blue marker dye reached the bottom of the gel. The gel assembly was then removed from the tank, the plates separated and the gel transferred to a tank containing gel stain. After 1-2 hours at 37°C the gel was transferred to a tank of destain and incubated at 37°C overnight.

2.8.3 Native PAGE.

Non-denaturing gels were prepared as described for SDS gels except that SDS was replaced with water in all mixes. Samples were added to an equal volume of 2x native sample buffer and loaded directly onto the gel (without boiling). Native gels were run at 5-10mA overnight at 4°C in 0.4x

running buffer and staining/destaining carried out as described for SDS-PAGE. Typically 6-8% native acrylamide gels were used when investigating charge differences in CAT mutants.

2.9 Covalent modification of CAT with phenylglyoxal.

Modification reactions were carried out in 45mM HEPES buffer (pH 8.0) and stock solutions of phenylglyoxal were made fresh each day in the same buffer. Reactions were initiated by addition of 10μl enzyme to the following:

> 890μl 11mM phenylglyoxal in 50mM HEPES (pH 8.0)
> 100μl water

to give final concentrations of 10mM phenylglyoxal and 45mM HEPES. Reactions were carried out at 25°C and inactivations followed by removal of 10μl samples at timed intervals, quenched by dilution into TSE containing 1mg/ml BSA and activity measured by the standard assay (2.5). Results were plotted as log (% activity remaining) against time.

For protection experiments, the 100μl water was replaced with either 100μl of 10mM acetyl CoA or 5mM chloramphenicol (final concentrations of 1mM acetyl CoA or 0.1mM chloramphenicol). Enzyme concentrations varied between 0.4μM and 4.0μM when monitoring loss of transacetylation activity or 45μM for the hydrolytic activity.

2.10 Covalent modification of CAT with 2,3-butanedione.

Reactions were performed in 50mM sodium borate buffer (pH 7.8) and stock solutions of various concentrations of

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2,3-butanedione were prepared immediately before use. Enzyme samples were dialysed into borate buffer prior to study.

Modification reactions were initiated by addition of 0.5ml 2,3-butanedione solution (pH 7.8) to an equal volume of enzyme solution, and allowed to proceed at 25°C. Inactivation was monitored as described for modification with phenylglyoxal (2.9). For protection experiments the enzyme was preincubated for 10 minutes with substrates (either 2mM acetyl CoA or 0.2mM chloramphenicol). Final enzyme concentrations were in the range 1-2μM.

2.11 Amidination of CAT (Packman and Shaw, 1981a).

Amidination reactions were carried out in TEA buffer (pH 8.5). Stock solutions of 1M ethyl acetimidate were made immediately before use by adding reagent to an appropriate volume of TEA buffer, containing 0.75M sodium hydroxide to neutralise the hydrochloride. The pH of the solution was tested and if the pH was greater than pH 8.5 it was adjusted with dilute HCl until in the range 8.0-8.5. If the pH was <8.0 the solution was discarded. The reagent was used within 5 minutes of preparation by dilution into the protein solution.

Native CAT was amidinated with 100mM ethyl acetimidate for 1 hour after which time a fresh addition of reagent was made. Aliquots (10μl) were removed at timed intervals, diluted into TSE to quench the reaction, and activity measured using the standard assay (2.5). A 2-fold excess (over reagent) of 0.5M ammonium acetate (pH 5.5) was added to stop the reaction and the reagents removed by dialysis against water or buffer.

2.11.1 Amidination of CAT with ethyl [1-^{14}C] acetimidate.

Protein treated as described above was dialysed against water prior to lyophilisation and resuspension in TEA buffer containing 8M guanidine hydrochloride. Modified and denatured protein was amidinated with 0.1M ethyl [1-^{14}C] acetimidate (prepared as above) for 1 hour, the reaction stopped by addition of an equal volume of 0.5M ammonium acetate (pH 5.5) and reagents removed by dialysis.

2.12 Chymotryptic digestion.

(i) Reduction and carboxymethylation (Lumsden and Coggins, 1978).

Protein (3mg) was exhaustively dialysed against water, lyophilised and resuspended in 1ml 0.2M Tris.HCl (pH 8.5) containing 6M guanidine hydrochloride. After transfer to a flask containing 0.57g guanidine hydrochloride, the volume was made to 2ml with water and DTT added to 2mM. The flask was flushed with nitrogen, sealed and stirred at room temperature for 2 hours in the dark. Iodoacetate (15mM final concentration) was added and the reaction allowed to proceed for a further 2 hours. Reaction was quenched by addition of excess DTT and the carboxymethylated protein dialysed exhaustively against water, lyophilised and resuspended in 1ml 0.5% (w/v) ammonium bicarbonate.

(ii) Enzymic digestion.

Chymotrypsin (1% w/w) was added to a 1ml suspension of protein from (i) above and digestion allowed to proceed for 2 hours at 37°C with stirring. A further addition (1% w/w) of

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chymotrypsin was made and digestion continued for a further 2 hours. Digested protein was lyophilised, resuspended in water and lyophilised again to remove the ammonium bicarbonate.

(iii) Separation of peptides.

If the peptides were to be separated by SDS-PAGE, the digest was resuspended in 1ml water and a sample treated as described (2.8). Where peptides were to be separated by fast protein liquid chromatography (FPLC), digests were resuspended in 0.1% (v/v) trifluoroacetic acid and filtered (0.5μ diameter Millipore filter). Peptides were separated using a Pharmacia FPLC unit on a C18 reverse phase column and eluted with an acetonitrile gradient. Peptide elution was monitored by measuring absorbance of eluent at 214nm, and 1ml fractions collected.

2.13 Purification of CoA analogues by ion exchange chromatography.

Analogue solutions were diluted to 50ml with TEAB buffer (pH 7.5) to give a final concentration of 20mM. Analogues were then loaded onto a 100ml DEAE Sephadex A25 column pre-equilibrated with 20mM TEAB and unbound material eluted by washing the column with 100-200ml 20mM TEAB. Products were eluted using a 2 litre (total volume) TEAB gradient at 4°C, with a flow rate of 50-60ml/hour. Fractions of 10ml were collected and elution of analogues monitored by measuring A_{260} for CoA derivatives or A_{412} on addition of a 10μl aliquot to 1ml of a 1mM DTNB solution for pantetheine derivatives. Peak fractions were pooled and repeatedly evaporated to dryness in the presence of methanol to remove the TEAB. Analogues were

stored as triethylammonium salts at -20°C.

2.14 Synthesis of acetyl pantetheine (Dupré et al., 1984).

To 40ml 1M cysteine (pH 8.5) was added 1g (1.8mmol) D-pantethine and the solution stirred under a nitrogen stream for 1 hour at 4°C. The reaction solution was loaded directly onto a Dowex 50W (H^+) ion exchange column pre-equilibrated with water, and D-pantetheine eluted with water. Peak thiol containing fractions were pooled and the thiol concentration of the pool calculated. Acetic anhydride (1.25 equivalents) was added with an appropriate volume of 1M potassium bicarbonate (1ml per 25μl anhydride) and the solution stirred on ice for 10 minutes. If no free thiols remained the solution was lyophilised, resuspended in 1-2ml water and passed through a Sephadex G-10 desalting column. Fractions were assayed for acetyl pantetheine using the standard CAT assay (2.5). Peak fractions were pooled and stored at -20°C in small aliquots.

Typical yield = 2.9mmol (81%).

2.15 Synthesis of 11-O-succinyl pantetheine.

To 1g (1.8mmol) D-pantethine dissolved in 10ml dry pyridine was added 433mg (1.2 equivalents) recrystallised succinic anhydride and 50mg (12.5% w/w) diethylaminopyridine (DMAP) and reaction allowed to proceed overnight at room temperature with constant stirring. A sample was evaporated to dryness, washed with methanol and analysed by thin layer chromatography on a silica plate using chloroform:methanol (80:20) as the mobile phase. If the reaction was near

completion the whole reaction was evaporated to dryness and resuspended in 35ml water. DTT (834mg, 1.5 equivalents) was added and the solution stirred for 30 minutes. The solution was made to 20mM in TEAB and products separated as described (2.13) using a 20-200mM TEAB gradient. Peak fractions were pooled, TEAB removed and the product confirmed by ^{1}H-nmr. Acetylation was performed as described for pantetheine (2.14) and acetyl 11-O-succinyl pantetheine stored at -20°C.

Yield = 1.6mmol (44%).

2.16 Synthesis of 4-phosphopantetheine (Shimizu, 1970).

To 3g (9.3mmol) cyanoethylphosphate, barium salt was added Dowex 50W (H^+) resin and the mixture stirred until the cyanoethylphosphate dissolved. The supernatant was collected and 4ml dry pyridine added. After evaporating to dryness and resuspending in dry pyridine several times to produce the pyridine salt, the product was finally resuspended in 30ml dry pyridine. To this was added 1.9g (9.2mmol) dicyclocarbodiimide (DCCD) and 2g (3.6mmol) D-pantethine and the reaction allowed to proceed for 24 hours at room temperature. The reaction mixture was filtered and 25ml water added to the filtrate. After 1 hour the mixture was filtered again and the filtrate evaporated to dryness. After resuspending in 20ml water the solution was neutralised with barium hydroxide and evaporated to dryness. Ethanol (40ml) was added, the solution filtered and the filtrate evaporated to dryness. Sodium hydroxide (30ml, 2M) was added and after incubation on ice for 30 minutes, Dowex 50W (H^+) resin added until the pH was less than 2. After filtering again, the filtrate was brought to pH 7.5

by addition of potassium hydroxide (3M). The product, 4-phosphopantethine, potassium salt was reduced by addition of 3.6mmol DTT, the solution made to 20mM in TEAB and the products separated as described (2.13) using a 20-200mM TEAB gradient. Peak fractions were pooled and the presence of a single phosphate containing species confirmed by ^{31}P-nmr. Product was stored at -20°C as the acetylated form.

Yield = 0.8mmol (11%).

2.17 Synthesis of acetonyl CoA (Rubenstein and Dryer,1980).

To a stirred solution of CoA (200mg, 0.24mmol) in 10ml methanol was added 2 equivalents of triethylamine. Freshly distilled chloroacetone was added stepwise in 0.04mmol aliquots until no free thiol groups remained (as judged from A_{412} on addition of a 10μl sample to 1ml 1mM DTNB in TSE). The reaction mixture was then evaporated to dryness, resuspended in 50ml 20mM TEAB and the products separated as described (2.13) using a 20-700mM TEAB gradient. Peak A_{260} containing fractions were pooled and TEAB removed (2.13) to leave a colourless oil, which was shown to be the desired product by ^{1}H-nmr.

Yield = 0.2mmol (83%), <0.5% free CoA.

2.18 Synthesis of 2-hydroxypropyl CoA.

To 120μmol acetonyl CoA (from 2.17) in 20ml water was added 240μmol sodium borohydride in small aliquots. After stirring for 2 hours at room temperature the solution was diluted to 50ml with TEAB (20mM TEAB final concentration) and

the products separated as described (2.13,2.17).

Yield = 75μmol (63%).

2.19 Phenol extraction of nucleic acid solutions.

Protein was removed from solutions of nucleic acids by extraction with phenol/chloroform/isoamylalcohol (25:24:1) saturated in Tris.HCl (10mM, pH 7.5). Phenol solution (0.5ml) was added to 0.5ml nucleic acid solution in a 1.5ml Eppendorf tube, the solutions mixed by vortexing for 30 seconds and the phases separated by centrifugation at 12,000rpm for 5 minutes. The upper aqueous layer (450μl) was collected, care being taken not to disturb the layer of denatured protein at the phase interface, and an equal volume of chloroform/ isoamylalcohol (24:1) added. After vortexing for 30 seconds the phases were separated by centrifugation and the aqueous phase collected.

2.20 Ethanol precipitation of nucleic acids.

Nucleic acids were precipitated from aqueous solution by addition of sterile sodium chloride to a final concentration of 0.3M and two volumes of ice cold ethanol. After incubation at -70°C for 30 minutes precipitated nucleic acid was pelleted by centrifugation at 12,000rpm and the pellet carefully washed with ice cold 70% ethanol, followed by further centrifugation. The supernatant was removed with a drawn-out pipette and the pellet dried under vacuum before being resuspended in an appropriate volume of sterile ddH$_2$O or TE buffer (pH 7.5) and stored at -20°C.

2.21 Transformation of E. coli JM101, TGII and MutL.

Sterile 2YT medium (20ml) was inoculated with 50μl of an overnight culture of an appropriate E. coli strain and incubated with vigorous shaking at 37°C until the optical density at 600nm reached 0.3-0.4. Cells were pelleted by centrifugation ay 3000rpm for 5 minutes, the supernatant discarded and the cells resuspended in 10ml sterile ice cold 50mM calcium chloride. After 30 minutes on ice the cells were again pelleted and resuspended in 2ml sterile ice cold calcium chloride and 0.2ml aliquots transferred to 1.5ml Eppendorf tubes. Transforming DNA (10-100ng ligation products or 1-5ng of a known plasmid) was added and after incubation on ice for 30 minutes the cells were heat stepped to 45°C for 2 minutes.

Cells transformed with plasmid DNA were gently pelleted, resuspended in 50μl 2YT and applied to dried agar plates containing appropriate nutrient and antibiotic supplements, and incubated overnight at 37°C. When M13 phage DNA (single stranded or RF form) was used, transformed cells were added to 3ml molten (45°C) 0.8% L-Top agar and carefully poured over appropriate dried agar plates. When the L-Top had set the plates were inverted and incubated at 37°C overnight. For transformations of BMH 71-18 MutL cells with M13 phage DNA 200μl of an overnight culture of E. coli TGII were added to the L-Top agar to ensure that the plaques formed in a TGII background. (mutL cells are repair deficient and are therefore susceptible to high rates of mutation).

2.22 Isolation of plasmid dna.

Small scale ('miniprep') isolation of plasmid DNA for analysis with restriction endonucleases was carried out by the method of Birnboim and Doly (1979).

Overnight cultures (1.5ml) of E. coli bearing the desired plasmid were transferred to sterile Eppendorf tubes and centrifuged for 30 seconds at 12,000rpm. Cell pellets were resuspended in 0.1ml ice cold lysis solution (2.1) and chilled on ice for 5 minutes. 0.2ml 0.2M NaOH, 1% (w/v) SDS was added and the solutions carefully mixed by inversion of the tubes. After chilling for a further 5 minutes 0.15ml acetate solution added (2.1), the solutions vortexed and replaced on ice for 5 minutes. Precipitated high molecular weight DNA, protein and cell debris was pelleted by centrifugation and 0.45ml supernatant collected. After phenol extraction, the DNA was ethanol precipitated, resuspended in 50μl sterile TE containing 25μg/ml pre-boiled ribonuclease A and stored at -20°C.

2.23 Isolation of bacteriophage M13 single stranded DNA (Winter and Coulson, 1982).

Single plaques from M13 transformation plates (2.21) were picked into 1ml phage buffer (2.1) and stored at 4°C. To 1.5ml 2YT medium was added 1μl of an overnight culture of E. coli JM101 or TGII and 0.1ml phage stock from above and incubated overnight at 37°C with vigorous shaking. Cells were pelleted by centrifugation at 12,000rpm for 10 minutes and 1ml of the supernatant collected and transferred to a 1.5ml Eppendorf

tube. Phage were precipitated by addition of 0.33ml 10% (w/v) PEG 8000, 2.5M NaCl and collected by centrifugation after incubation for 30 minutes at room temperature. The supernatant was carefully removed, ensuring that no PEG remained and the phage pellet resuspended in 0.5ml sterile ddH$_2$O. After phenol extraction (2.19) to remove the phage protein coat, single stranded phage DNA was ethanol precipitated (2.20), resuspended in 21μl ddH$_2$O and stored at -20°C. Single stranded DNA prepared in this way was used directly in sequencing reactions (2.26).

2.24 Isolation of M13 replicative form (RF) DNA.

Infection of sensitive E. coli by bacteriophage M13 is followed by replication of the ssDNA genome to form a double stranded replicative-form (RF). The RF is amplified by bidirectional replication to 50-100 copies per cell, before replication is switched to a rolling-circle mechanism to produce ssDNA, which is packaged prior to phage release. The RF DNA is suitable for cloning of DNA fragments ready for production of ssDNA, which can be used in sequencing or mutagenesis reactions.

A single plaque from a fresh M13 transformation plate was picked into phage buffer and stored at 4°C. To 1.5ml 2YT was added 1μl of an overnight culture of JM101 and 100μl phage stock from above, and the culture incubated overnight at 37°C with vigorous shaking. Cells were pelleted by centrifugation at 12,000rpm for 30 seconds and the supernatant removed. The presence of phage was tested by addition of 330μl PEG/NaCl to 1ml supernatant. A white pellet on centrifugation of the above

at 12,000rpm for 5 minutes was indicative of phage. If phage were present RF DNA was isolated from the pelleted cells using the same protocol described for isolation of plasmids (2.22).

2.25 Large scale preparation of M13 single stranded DNA with misincorporation of deoxyuridine residues (Sagher and Strauss, 1983).

E. coli RZ1032 is an F^+, dUTPase$^-$, uracil-N-glycosylase$^-$ strain which is deficient in thymidine biosynthesis and which misincorporates deoxuridine in place of some thymidine residues on DNA synthesis. DNA containing deoxyuridine is selectively degraded by 'wild type' E. coli (e.g. JM101), thus the use of single stranded DNA containing deoxyuridine as the template in mutagenesis experiments (2.31) results in strong selection for the newly synthesised (potentially mutated) strand.

Sterile 2YT (1.5ml) was inoculated with 1μl of a JM101 overnight culture and 0.1ml of an appropriate phage stock and incubated overnight at 37°C with vigorous shaking. A culture of E. coli RZ1032 was also set up an incubated overnight at 30°C in the dark with 12.5μg/ml tetracycline present in the medium. The JM101 culture was transferred to a sterile 1.5ml Eppendorf tube and centrifuged for 10 minutes at 12,000rpm. 1ml of the supernatant collected and phage precipitated by addition of 0.33ml 10% (w/v) PEG 8000, 2.5M NaCl. After 30 minutes phage were harvested by centrifugation and the supernatant removed and discarded. The phage pellet was resuspended in 1ml phage buffer. 500ml 2YT medium containing tetracycline (12.5μg/ml) and deoxyuridine (0.25μg/ml) in a 2

litre (foil wrapped) baffled flask was inoculated with 0.5ml of an RZ1032 overnight culture and 1ml phage stock from above, and incubated overnight at 30°C with vigorous shaking. Cells were pelleted by centrifugation at 10,000rpm for 30 minutes at 4°C and 400ml of the supernatant collected. 140ml 10% (w/v) PEG 8000, 2.5M NaCl was added and after 60 minutes precipitated phage were harvested by centrifugation. The supernatant was carefully removed and the phage pellet resuspended in 5ml sterile ddH_2O and transferred to Eppendorf tubes. After phenol extraction (x3, 2.19) and ethanol precipitation (2.20), single stranded DNA was resuspended in 1-2ml sterile ddH_2O. An aliquot was scanned at 220-300nm and the concentration of DNA calculated (assuming 1 A_{260nm} unit ~37μg single stranded DNA). After dilution to 50pmol/ml, single stranded DNA was stored at -20°C in 10μl aliquots.

2.26 DNA sequencing.

Sequencing of M13 single stranded DNA was carried out by the method of Sanger et al. (1977) using α[^{35}S] dATP (Biggin et al., 1983).

2.26.1 Reagents for DNA sequencing.

dNTP solutions	dATP,dCTP,dGTP,dTTP 50mM and 0.5mM stocks in sterile TE stored at -20°C.
ddNTP solutions	ddATP,ddCTP,ddGTP,ddTTP 4mM stocks in sterile TE stored at -20°C.
Chase solution	0.25mM dATP/dCTP/dGTP/dTTP in sterile TE stored at -20°C as 100μl aliquots.

Dideoxy nucleotide mixes (volumes in μl).

Reagent	A	C	G	T
0.5mM dCTP	250	12.5	250	250
0.5mM dGTP	250	250	12.5	250
0.5mM dTTP	250	250	250	12.5
4mM ddATP	1.25	–	–	–
4mM ddCTP	–	10	–	–
4mM ddGTP	–	–	20	–
4mM ddTTP	–	–	–	62.5
sterile TE	239	494	478	462

all stored at -20°C in 25μl aliquots.

2.26.2 Annealing of template and primer.

To 5μl single stranded template DNA from 2.23 was added 1μl 10x TM buffer (2.1), 1pmol of an appropriate primer oligonucleotide and ddH$_2$O to 10μl. the solution was boiled for 2 minutes and allowed to slowly return to room temperature.

2.26.2 Sequencing reactions.

2μl annealed template and primer was added to each of 4 capless Eppendorf tubes and one of the following added to each tube:

Tube	1	2	3	4
Template/primer (i)	2	2	2	2
A mix	2	–	–	–
C mix	–	2	–	–
G mix	–	–	2	–
T mix	–	–	–	2
Klenow mix	2	2	2	2

(volumes in μl).

Klenow mix = 1μl $\alpha[^{35}S]$ dATP and 5 units of DNA polymerase I Klenow fragment made to 12μl with ddH$_2$O.

The reactions were started by brief centrifugation and allowed to proceed at room temperature for 20 minutes. Chase mix (2μl) was then added to each tube and the reaction allowed to proceed for a further 20 minutes after which time the reactions were stored at -20°C or 2μl formamide dye added and the samples boiled for 2 minutes prior to separation of products by polyacrylamide gel electrophoresis (2.27).

2.27 Separation of products of sequencing reactions by polyacrylamide gel electrophoresis.

Products of sequencing reactions were separated on 6% (w/v) acrylamide/43% (w/v) urea buffer gradient gels (Biggin et al., 1983).

2.27.1 Solutions for sequencing gels.
1) 40% acrylamide stock solution.

38g acrylamide, 2g N,N′ methylenebisacrylamide made up to

100ml with ddH$_2$O and deionised by stirring with Amberlite MB1 resin for 10 minutes. After filtering to remove the Amberlite the solution was stored in the dark at 4°C.

2) 0.5x gel mix.

215g ultrapure urea, 25ml 10x TBE, 75ml 40% acrylamide stock made up to 500ml with ddH$_2$O and stored as 1).

3) 2.5x gel mix.

215g ultrapure urea, 125ml 10x TBE, 75ml 40% acrylamide stock, 25g sucrose, 25mg bromophenol blue made up to 500ml with ddH$_2$O and stored as 1).

4) Formamide dye mix.

100ml deionised formamide, 0.1g xylene cyanol FF, 0.1g bromophenol blue, 2ml 0.5M EDTA.

2.27.2 Pouring and running sequencing gels.

Siliconised sequencing gel plates (33cmx38cm) were taped together separated by 0.4mm spacers. To 60ml 0.5x gel mix 15ml 2.5x gel mix in side-arm flasks was added 0.4ml and 0.1ml 10% ammonium persulphate respectively and the solutions degassed briefly under vacuum. TEMED (21μl and 7μl respectively) was added and 8ml of the 0.5x mix drawn up into a 25ml pipette followed by 12ml of the 2.5x mix. A partial gradient was formed by drawing a few bubbles through the pipette and the solution slowly poured between the plates. The rest of the 0.5x mix was then poured between the plates, 'sharkstooth' combs inserted, the plates clamped and the gel allowed to set. Once the gel had set the gel assembly was clamped to a vertical gel apparatus, 0.5x TBE added to the upper resevoir and 1.0x TBE added to the lower resevoir. The combs were

removed, the top of the gel flushed with 0.5x TBE and the combs replaced inverted to form wells. 1μl formamide dye was added to alternate wells (to check for seepage between wells) and the gel pre-run for 30 minutes at 1600V, 45Watts.

After flushing the wells with 0.5x TBE, boiled samples (containing 2μl formamide dye, from 2.26) were loaded onto the gel in the order A,C,G,T for each set of reactions. The gel was run at 1600V and 45Watts until the bromophenol blue marker dye reached the bottom of the gel. After removing the gel assembly from the tank the plates were carefully separated. The plate to which the gel adhered to was immersed in 10% methanol/10% acetic acid fixing solution for 40 minutes and then the gel was blotted onto a sheet of Whatman 3MM paper. The gel was dried at 80°C under vacuum on a Bio-Rad slab gel drier prior to autoradiography overnight using Fuji RX X-ray film.

2.28 Purification of oligonucleotides by polyacrylamide gel electrophoresis.

Siliconised plates (20cmx38cm) were taped together separated by 0.4mm spacers. 0.3ml 10% (w/v) ammonium persulphate and 20μl TEMED was added to 40ml 20% acrylamide stock (100g ultrapure urea, 100ml 40% acrylamide stock from 2.27, 20ml 10x TBE made up to 200ml with ddH$_2$O and stored as 2.27 1).), and the solution poured between the plates using a 25ml pipette. A 'sharkstooth' comb was inserted, the plates clamped and the gel allowed to set. Once set, the gel assembly was clamped to a vertical gel apparatus containing 1.0x TBE in both tanks. The comb was removed, the top of the gel flushed

with 1.0x TBE and the comb replaced inverted to form wells.

Formamide oligo dye (2μl) was added to oligonucleotides (2-4μg in 4μl water) which were then boiled for 1 minute and loaded onto the gel. Gels were run at 1400V/30Watts for 2-3 hours. The gel was removed from the tanks, the plates separated and the gel coated on both sides with clingfilm. Oligonucleotides were viewed by UV shadowing against a fluorescent silica tlc plate and their positions marked. Gel slices containing oligonucleotides were immersed in 100μl ddH$_2$O overnight and desalted using a G-25 spun column (2.29).

2.29 Desalting of DNA solutions using spun columns.

A sterile 1ml syringe was plugged with sterile siliconised glass wool, filled with sterile pre-swollen Sephadex G-25 and placed in a disposable test-tube before centrifugation for 30 seconds at 3000rpm to compact the Sephadex. More Sephadex was added and packed by centrifugation until a column of 1ml was produced. The column was washed several times by passing 100μl ddH$_2$O through it by centrifugation. When equilibrated the column was transferred to a fresh tube, the sample (100μl) applied and DNA eluted by centrifugation for 30 seconds at 3000rpm. Desalted DNA was diluted to 1ml with ddH$_2$O and the concentration determined from the absorbance at 260nm.

1 A$_{260nm}$ = 31μg single stranded DNA

1 A$_{260nm}$ = 50μg double stranded DNA.

2.30 Phosphorylation of oligonucleotides.

Salt free oligonucleotide (50pmol) was dried down from aqueous solution and 10μl 1x kinase buffer containing 1mM ATP and 1mM DTT added. After mixing, 4 units of T4 polynucleotide kinase was added and the reaction allowed to proceed at 37°C for 30 minutes. The kinase was then inactivated by heating at 65°C for 15 minutes and the phosphorylated oligonucleotide stored at -20°C.

2.30.1 5' End labelling of oligonucleotides with ^{32}P (Zoller and Smith, 1984).

Purified oligonucleotide (20pmol) dried down from salt free aqueous solution was resuspended in 20μl 1x kinase buffer containing 1mM DTT and 50μCi γ[^{32}P] ATP @ 3000Ci/mmol and 4 units of T4 polynucleotide kinase. After 60 minutes at 37°C the kinase was inactivated by heating to 65°C for 15 minutes. Labelled oligonucleotide was either stored at -20°C (in a lead pot) or diluted with 4ml 6xSSC, 10x Denhardt's solution (2.1) and used in hybridisation experiments.

2.31 Site-directed mutagenesis.

Site-directed mutagenesis was performed with 21-24mer oligonucleotides and mutants selected by the deoxyuridine selection method of Kunkel (1985), or by probing with ^{32}P radiolabelled oligonucleotides.

2.31.1 Kunkel mutagenesis (Kunkel, 1985).

a) Annealing of template and primer oligonucleotide.

To 0.5pmol single stranded template DNA containing deoxyuridine residues (2.25) was added 10μl buffer A (2.1) containing 10pmol kinase treated oligonucleotide (2.30), the solution boiled for 3 minutes and allowed to cool to room temperature slowly.

b) Extension and ligation.

To each annealed template and primer from a) was added 10μl of the following:

> 1μl 10x buffer B (2.1)
>
> 1μl 10mM ATP
>
> 4μl 2mM dNTPs
>
> 2 units DNA polymerase I Klenow fragment
>
> 3 units T4 DNA ligase
>
> ddH$_2$O to 10μl.

After gentle mixing, the reaction was incubated at 15°C overnight. Extension/ligation reactions were diluted to 1:5 with ddH$_2$O and 5μl used to transform competent E. coli JM101 as described (2.1). Four of the resulting plaques were picked into phage buffer, single stranded DNA prepared (2.23) and mutants identified by DNA sequencing using an appropriate primer (2.1, fig. 2.5). After sequencing the entire cat gene of identified mutants to ensure no other mutations were present, M13 replicative form DNA was prepared (2.24) and the cat gene cloned into pUC18 as a BamHI/HindIII fragment (2.33).

2.31.2 <u>Alternative mutagenesis strategy.</u>

Single stranded template DNA was annealed to kinase treated mutagenic oligonucleotide as described 1a) except that TM buffer was used in place of buffer A. To each annealed template and primer was added the following:

> 1μl 10x TM
>
> 2.5μl 2mM dNTPs
>
> 0.5μl 10mM ATP
>
> 1μl 100mM DTT
>
> 4μl ddH$_2$O
>
> 5 units T4 DNA ligase
>
> 2.5 units DNA polymerase I Klenow fragment

After incubation overnight at 15°C the reaction was stopped by addition of 100μl 10mM EDTA, 10mM Tris.HCl (pH 8.1) and 50μl used to transform competent <u>E. coli</u> BMH 71-18 MutL cells as described (2.21). Mutants were identified using ^{32}P radiolabelled oligonucleotides as described (2.32), confirmed by DNA sequencing and cloned into pUC18.

2.32 <u>Screening for potential mutants.</u>

Desired mutants were identified by DNA:DNA hybridisation with specific radiolabelled oligonucleotides. Transfer of M13 DNA onto nitrocellulose filters was achieved using an adaptation of the colony hybridisation method of Grunstein and Hogness (1975).

Sterile toothpicks were used to pick plaques from M13 transformation plates onto duplicate 2YT agar plates which were then incubated at 37°C overnight. Up to 200 plaques could

be picked onto each plate. One of the duplicate plates was stored at 4°C. A nitrocellulose filter was carefully laid onto the other plate and left for one minute at room temperature. The filter was orientated with pin pricks, gently lifted from the plate and placed, colonies uppermost, onto blotting paper soaked in 0.5M NaOH, 1.5M NaCl for 5 minutes to denature the phage coats. The filter was then transferred to blotting paper soaked in neutralisation solution (0.5M Tris.HCl (pH 7.5) containing 1.5M NaCl) for 2x 5 minutes. After washing for 5 minutes in 6x SSC the filter was dried and then baked at 80°C for 1 hour. The colonies were then carefully washed from the filters prior to prehybridisation at 65°C in 6x SSC, 10x Denhardt's solution, 0.2% (w/v) SDS for 30 minutes in a heat-sealable envelope. After washing in 100ml 6x SSC, hybridisation with ^{32}P radiolabelled oligonucleotide was carried out at 65°C for 30 minutes in 6x SSC, 10x Denhardt's solution. The filter was then allowed to slowly cool to room temperature and was washed with several changes of 6x SSC until the number of counts on the filter remained constant. Washed filters were sealed in a fresh envelope and exposed against Fuji X-film for 1 hour at -70°C. If the filter was uniformly labelled it was washed in 6x SSC at a temperature half way between the calculated Tm for the wild type and mutant templates (Tm = 2x A/T bases + 4x C/G bases °C, Suggs et al., 1981) until no further decrease in bound counts was observed. After further autoradiography for several hours, positive colonies were identified, and streaked out onto minimal agar plates and dried for 5-10 minutes at 37°C. The plates were then overlaid with 3ml L-Top agar containing 200μl of an overnight culture of E. coli TGII and incubated

overnight at 37°C. Single plaques were picked into phage buffer, single stranded DNA prepared and the presence of the desired mutant confirmed by DNA sequencing.

2.33 Subcloning of mutant cat genes.

Mutated genes were cloned into pUC18 as BamHI/HindIII fragments.

2.33.1 Digestion with restriction endonucleases.

M13 replicative form DNA bearing the mutated cat gene was ethanol precipitated and resuspended in 50μl TE containing 25μg/ml pre-boiled RNase. To 8μl of this was added 1μl 10x React 3 buffer (2.1), 5 units of BamHI and 5 units of HindIII and digestion allowed to proceed at 37°C for 4 hours. Vector DNA (pUC18, 5μg) was digested as above except that 0.1 units of calf intestinal phosphatase (CIP) was also added to remove free 5′ phosphate groups from the linearised plasmid. Digests were analysed by agarose gel electrophoresis (5μl of the RF digestion and 1μl of the pUC18 digestion). If digestion was complete, the reaction was stopped by dilution to 500μl with ddH$_2$O and enzymes removed by phenol extraction (2.19). After ethanol precipitation (2.20), digested DNA was resuspended in 20μl (for RF) or 100μl (for pUC18) ddH$_2$O.

2.33.2 Ligation of fragments.

The products of BamHI/HindIII digested M13 RF DNA were ligated into similarly digested pUC18 by incubation at 15°C in

the presence of T4 DNA ligase. Ligations were set up as follows:

Addition	control(1)	2	3
pUC18xBamHI, xHindIII, xCIP	10	10	10
M13 RfxBamHI, xHindIII	0	2	5
5x ligase buffer (2.1)	4	4	4
ddH$_2$O	5.5	5.5	5.5
T4 DNA ligase (10units/μl)	0.5	0.5	0.5

(volumes in μl)

After incubation at 15°C overnight, 10μl of each ligation reaction was used directly to transform competent E. coli JM101 and cells plated on LB agar plates containing 200μg/ml ampicillin, 24μg/ml X-gal and 24μg/ml IPTG and incubated overnight at 37°C. White colonies from plates 2 and 3 (plate 1 should contain a few blue colonies only) were picked into 1.5ml 2YT medium and plasmid DNA prepared as described (2.22). Plasmid DNA was digested with BamHI/HindIII and analysed by agarose gel electrophoresis along side similarly digested pUC18:III:ME1 (to provide a marker). Cell free extracts of likely candidates were prepared, assayed for CAT activity and analysed by SDS-PAGE. Glycerol freezes prepared of cultures producing CAT.

2.34 Agarose gel electrophoresis.

DNA samples were routinely analysed by electrophoresis on 1% (w/v) agarose gels. Molten agarose (1% w/v in 1x TBE) was poured onto a gel plate (10cmx10cm) and a comb inserted. Once the gel was set the comb was removed and the gel transferred

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to a horizontal electrophoresis tank containing 1x TBE. To each DNA sample (~5μl) was added 1μl 10x dye mix (2.1) and the samples loaded into separate wells. Gels were run at 40mA until the bromophenol blue marker dye reached the end of the gel. The gel was then transferred to a tank of water containing 1.0μg/ml ethidium bromide for 10 minutes and DNA viewed under UV illumination.

Figure 2.1. Purification of CAT$_{III}$ by affinity chromatography.

A. Purification table for the preparation of CAT$_{III}$ from a 500ml culture of E. coli. JM101 bearing plasmid pUC18:III:MEI.

B. Monitoring the progress of CAT purification by electrophoresis through a 12% denaturing (SDS) polyacrylamide gel.

Lane	sample
1 and 5	molecular weight markers
2	purified CAT$_{III}$ (10μg)
3	proteins eluted by 0.3M NaCl (50μg)
4	E. coli. crude extract (100μg)

molecular weight markers: Bovine serum albumin, 66kDa

Trypsinogen, 24kDa

β-lactoglobulin, 18kDa.

A

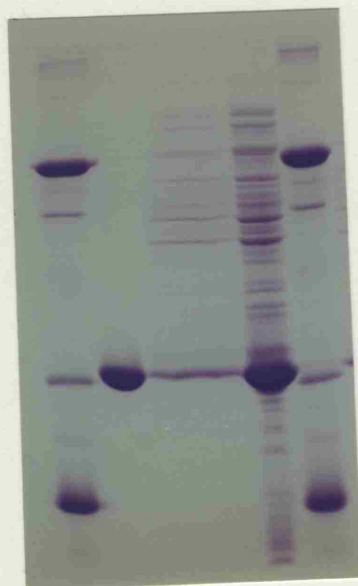| Fraction	Volume (ml)	[Protein] (mg/ml)	Activity (U/ml)	Sp.Act. (U/mg)	Purification factor
Crude extract	65	22	2769	126	1
Cm-Sepharose	25	5.6	5150	911	7

Figure 2.2. Lineweaver-Burk plot for the acetyl CoA dependent acetylation of chloramphenicol by CAT.

Assays were performed as described (2.5) at 25°C over a range of chloramphenicol concentrations (5, 10, 20 and 100μM) for each of four fixed acetyl CoA concentrations a) 25μM, b) 50μM, c) 100μM and d) 400μM. Reciprocal initial rates were plotted against reciprocal chloramphenicol concentration for each concentration of acetyl CoA (A) and steady-state kinetic parameters calculated from intercept and slope replots (B). Data was also plotted as reciprocal rates against reciprocal acetyl CoA concentration for each chloramphenicol concentration (not shown).

$$K_m \text{ Acetyl CoA} = 93\mu M$$
$$K_m \text{ Chloramphenicol} = 12\mu M$$
$$k_{cat} = 600s^{-1}$$
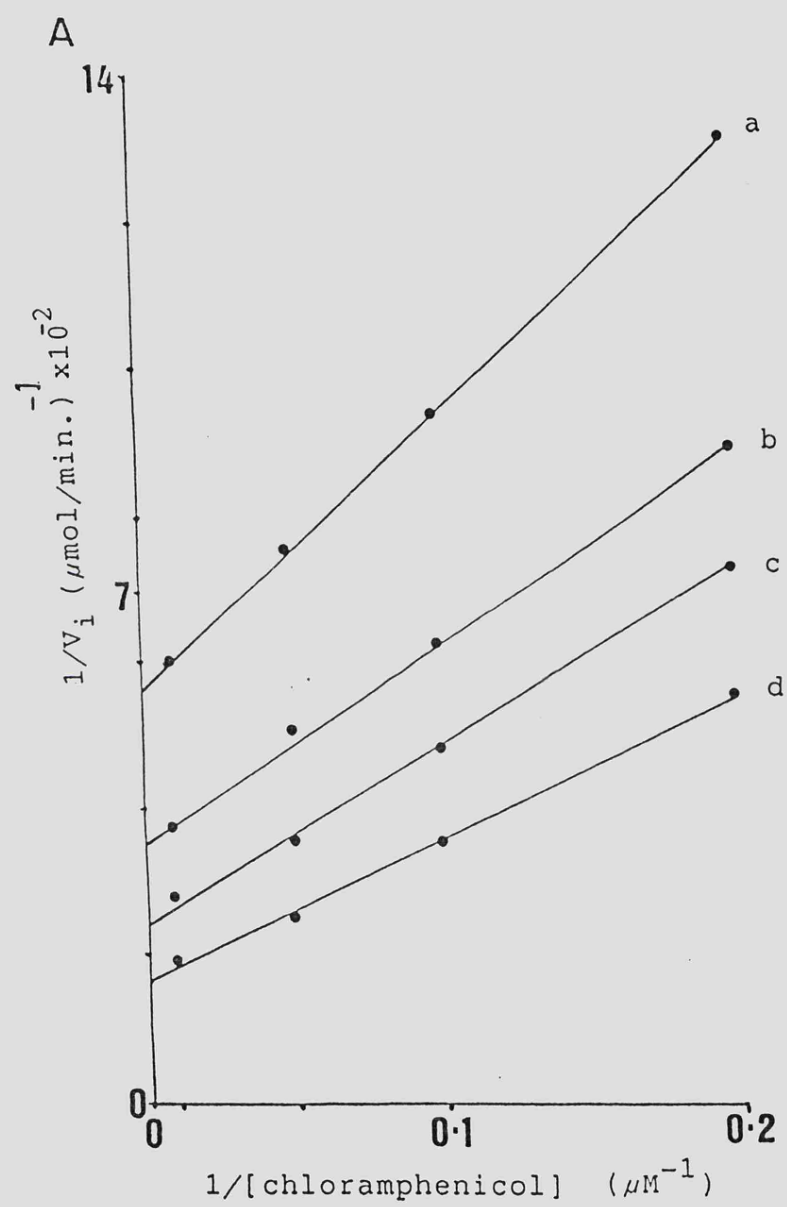
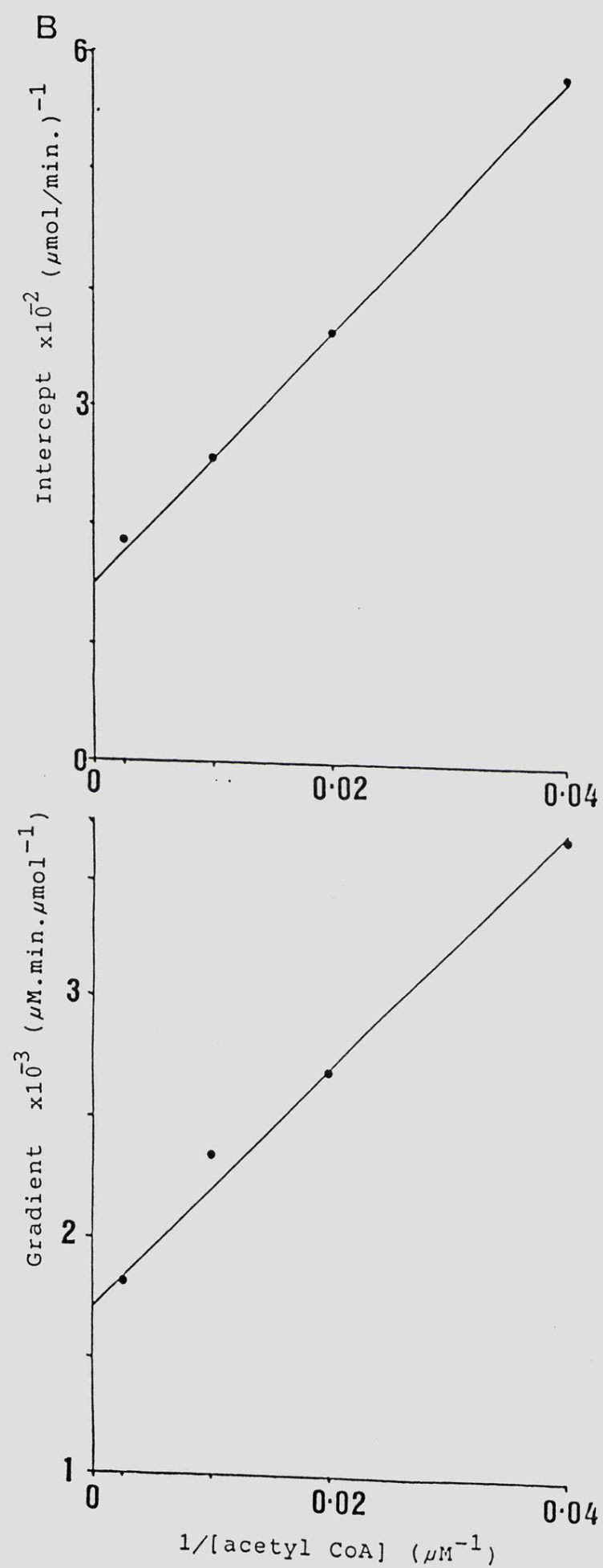**Figure** <u>2.3.</u> <u>Lineweaver-Burk</u> <u>plots</u> <u>for</u> <u>the</u> <u>hydrolysis</u> <u>of</u> <u>acetyl</u> <u>CoA</u> <u>by</u> <u>CAT.</u>

Initial rates were measured as described (2.5) with the acetyl CoA concentration varied in the range $10-400\mu M$ and in the absence of chloramphenicol. The final enzyme monomer concentration was $1\mu M$.

$$K_m \text{ Acetyl CoA} = 66\mu M$$
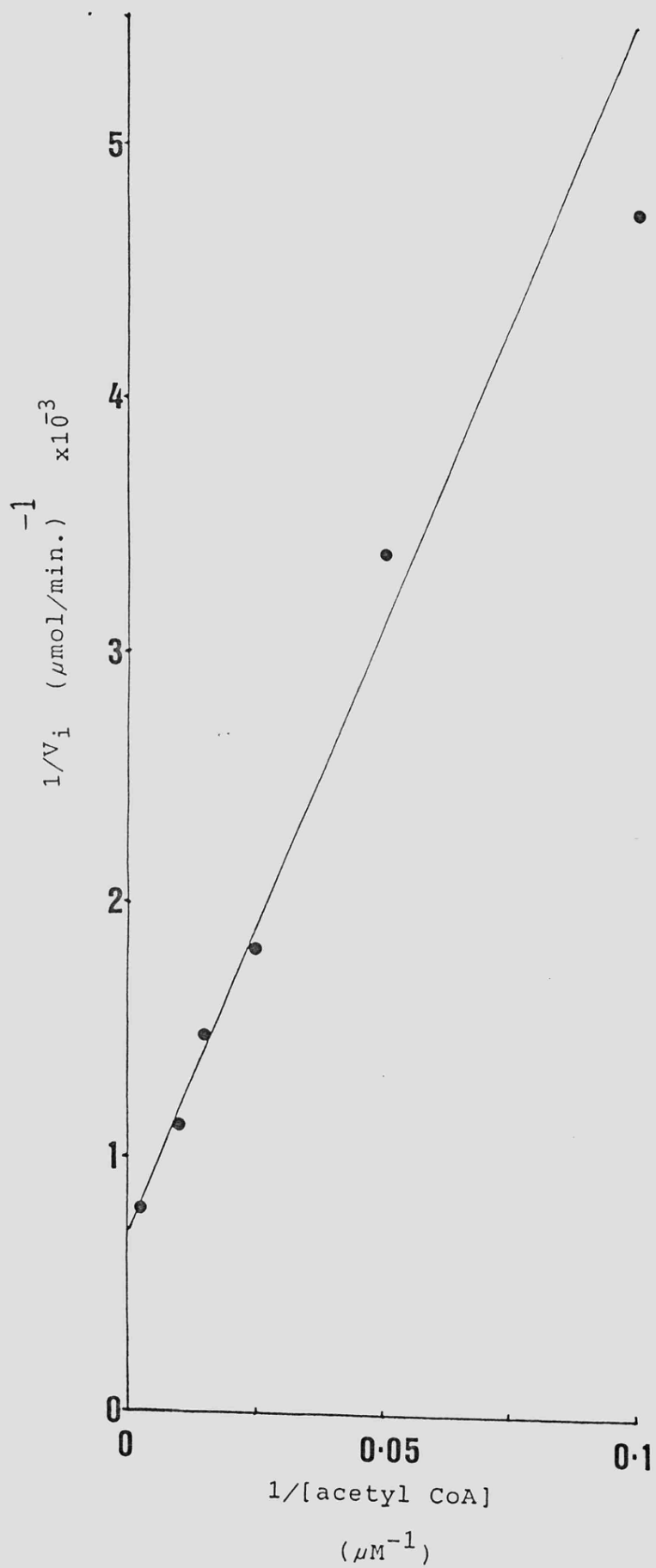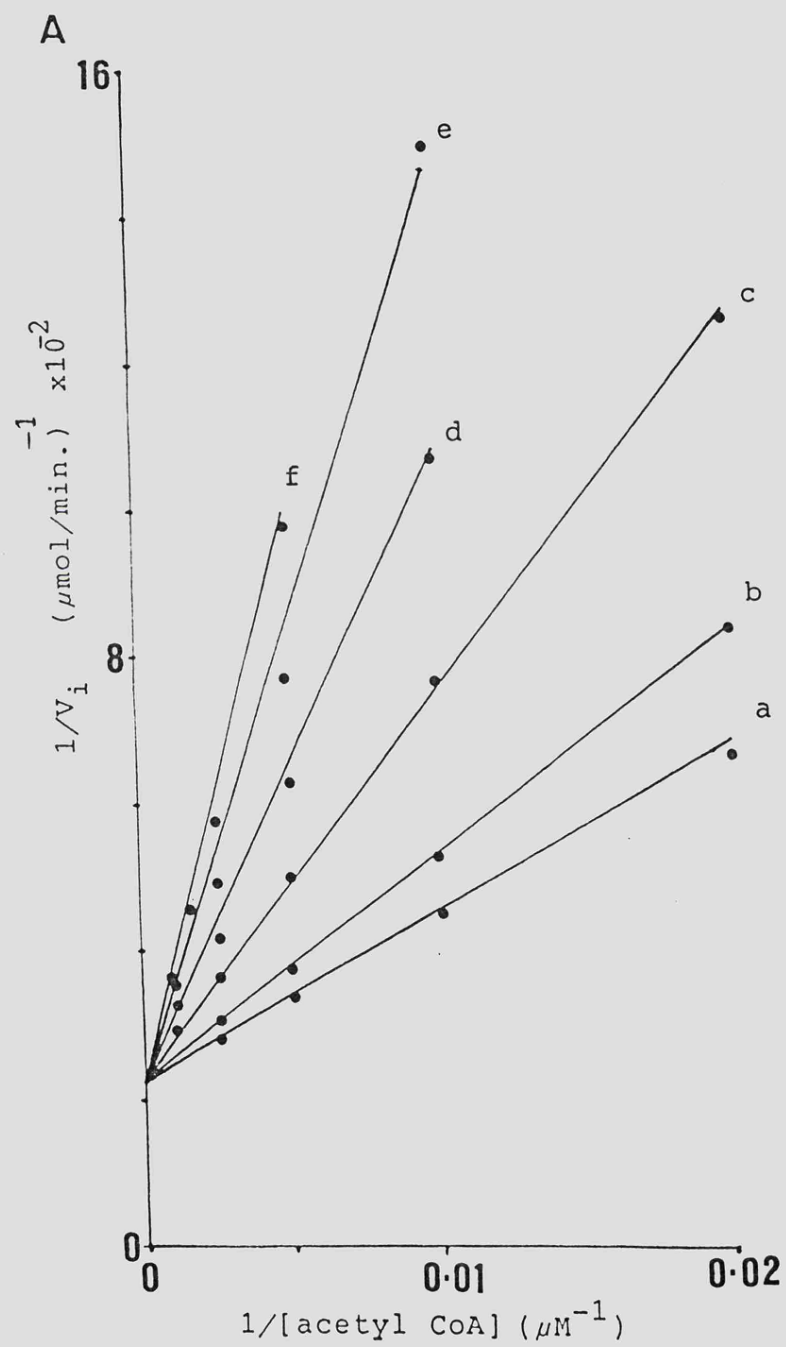$$k_{cat} = 0.13s^{-1}$$

Figure 2.4. Lineweaver–Burk plots for the acetyl CoA dependent acetylation of chloramphenicol in the presence of the competitive (with respect to acetyl CoA) inhibitor 3'AMP.

For each of six inhibitor concentrations a) no inhibitor, b) 2.5mM c) 5mM, d) 10mM, e) 15mM and f) 20mM the concentration of acetyl CoA in the assay was varied in the range 25–1000μM and initial rates measured as described (2.5). The concentration of chloramphenicol was kept constant at 25μM. Results were plotted as shown (A) and the inhibitor constant (K_i) calculated from a slope replot (B).

$$K_i = 4.6mM$$

A

B

Gradient $\times 10^{-4}$ $(\mu M.min.\mu mol^{-1})$ — (y-axis, values 2, 8, 16)

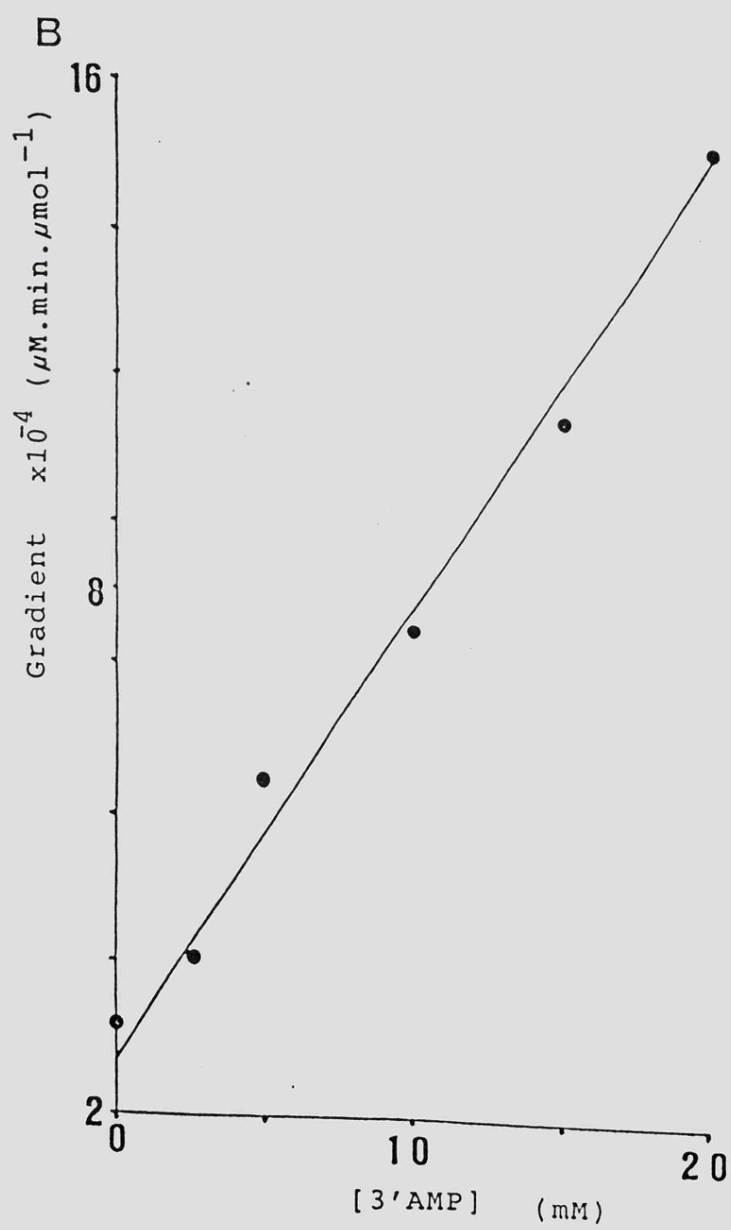[3'AMP] (mM) — (x-axis, values 0, 10, 20)

Figure 2.5. Identification of mutant cat genes by DNA sequencing.

      Genes altered by site-directed mutagenesis were identified by DNA sequencing, using the chain termination method of Sanger et al. (1977). Several examples of sequences around the sites of mutation are illustrated.

   Wild type sequences:

                    residue   176 177 178 179
                    sequence   5' GCA AAA TAT CAG 3'
                    amino acid   Lys Tyr

                    residue   53 54 55 56 57
                    sequence   5' TAT AAG TTT TAT TCC 3'
                    amino acid   Lys   Tyr

                    residue   31 32 33 34
                    sequence   5' TGT GGT TTT TAG 3'
                    amino acid   Phe

      Naming of mutants - e.g. Y56A. 56 is the residue number (in the amino acid sequence), Y is the residue type (single letter code) of the wild type enzyme and A is the new residue type.
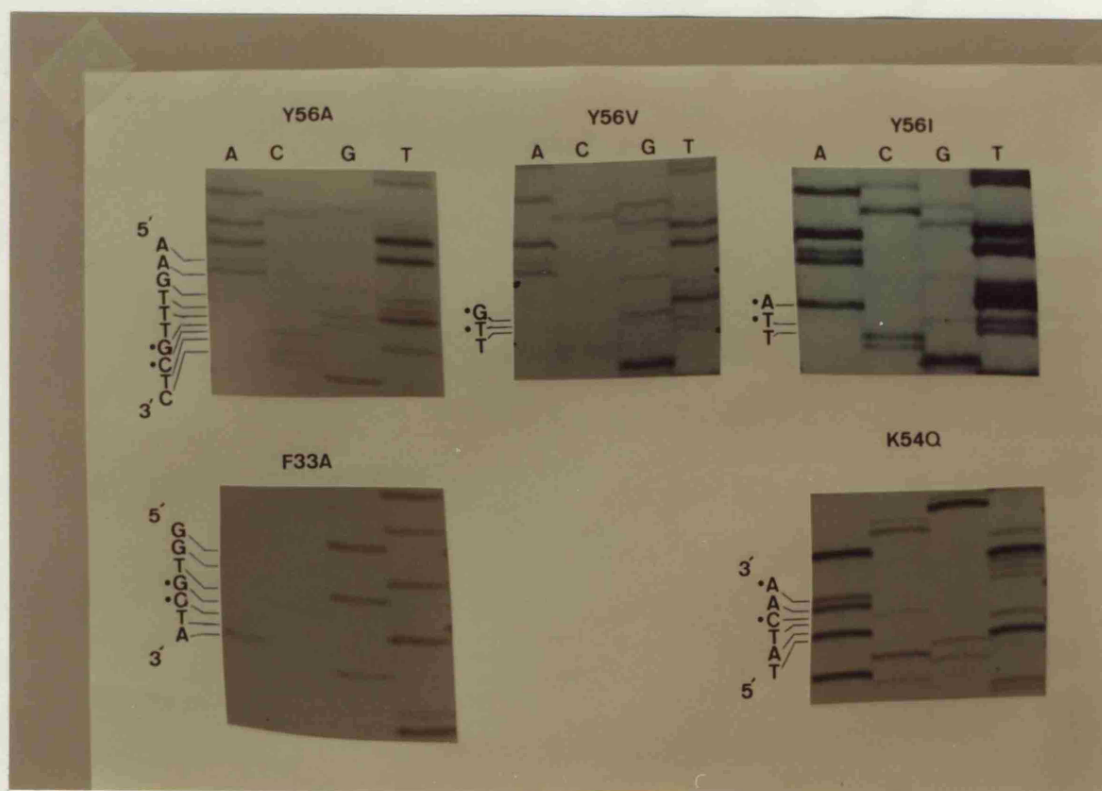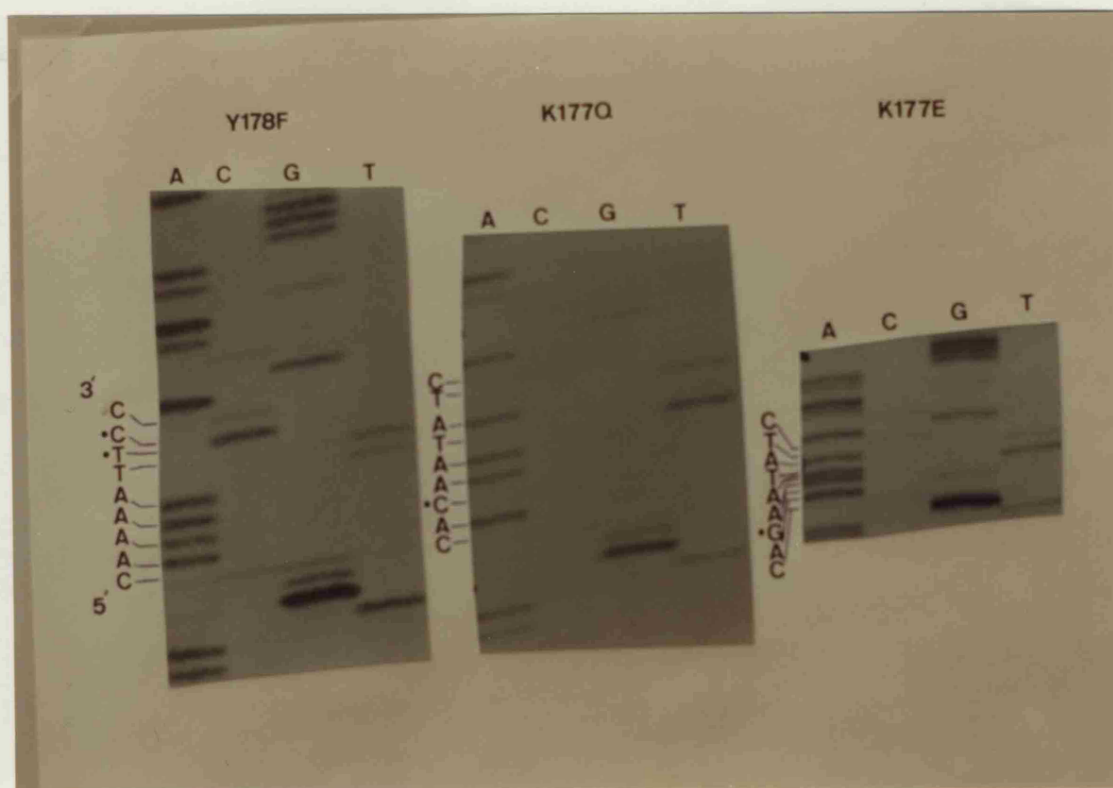
Chapter 3. Covalent modification of CAT_{III} with
arginyl specific reagents.

3.1 Introduction.

Essential arginyl residues have been found in the substrate binding sites of many enzymes which use anionic substrates or coenzymes. Riordan et al. (1977) proposed that arginyl residues may act as 'positively charged recognition sites for negatively charged substrates', particularly those that contain phosphate and diphosphate moieties. Arginine is ideally suited to forming ion pairs with phosphate groups as the guanidino group can form multiple hydrogen bonds.

Since coenzyme A has a 3' phosphate and 5' diphospho linkage it may be imagined that CoA binding might involve ion pairs between arginyl residues and such phosphoanions. Covalent modification studies have implicated arginyl residues in the binding of CoA to spermine/spermidine N1-acetyltransferase (Ragione et al., 1983), ATP citrate lyase (Ramakrishna and Benjamin, 1981) and choline acetyl transferase (Mautner et al., 1981). The crystal structure of citrate synthase has been solved at 0.17nm resolution for enzyme with bound CoA and with a competitive inhibitor, citrylthioetherCoA (Remington et al., 1982). Both structures show that each of the three phosphoanions of CoA form ion pairs with arginyl residues on the enzyme, two directly and one via a bridging water molecule.

Choline acetyl transferase (ChA) catalyses the O-acetylation of choline by acetyl CoA. Covalent modification of ChA from squid ganglia with the arginine specific reagent camphorquinone-10-sulphonic acid, results in rapid loss of enzyme activity (Mautner et al., 1981). The prior addition of CoA greatly reduces the rate of inactivation by this reagent,

whereas 3'-dephosphoCoA affords much less protection against inactivation. In contrast, choline, the other substrate, affords no protection. The K_m for acetyl 3'-dephosphoCoA (820μM) is 17-fold greater than that for acetyl CoA (47μM). These results suggest that the 3' phosphate group is important for binding of CoA to ChA and that it may interact with an arginyl residue on the enzyme.

For CAT_{III} the K_m for acetyl 3'-dephosphoCoA (850μM, A.Lewendon, unpublished results) is nearly 10-fold greater than that for acetyl CoA (93μM) implying that the 3' phosphate group of CoA may also be important for CoA binding to CAT. By analogy, the data are compatible with the hypothesis that arginyl residues could play a direct role in the binding of CoA to CAT.

Arginyl residues can be modified specifically with diketone reagents, of which the most commonly used are 2,3-butanedione and phenylglyoxal. These reagents also modify primary amines and thiol groups at a significant rate, but in many cases amino acid analysis has established that treatment of proteins with such diketone reagents results only in arginine modification (Takahashi, 1977b). The reactions of diketone reagents with the guanidinium group of arginine are poorly understood. For example, although the stoichiometry of the reaction of phenylglyoxal with arginine has been shown to be 2:1 for model systems (Takahashi, 1968) and for several enzymes (alkaline phosphatase, Daemen and Riordan, 1974; RUBISCO, Schloss et al., 1978), a stoichiometry of 1:1 has been demonstrated for other enzymes (glycine methyl transferase, Konishi and Fujioka, 1987; hexokinase, Philips et al., 1979; carbonic anhydrase, Pullan and Noltman,

1985). The reaction of 2,3-butanedione with arginyl residues is often enhanced by borate buffers, which are thought to stabilise a reaction intermediate. Proposed mechanisms for the reaction of phenylglyoxal and 2,3-butanedione with arginyl residues are shown in figure 3.1.

3.2 Covalent modification of CAT_{III} with 2,3-butanedione.

It has previously been shown that CAT_{III} is inactivated by 2,3-butanedione and that both chloramphenicol and acetyl CoA afford protection against inactivation. Inactivation by 2,3-butanedione at 37°C in phosphate buffer (pH 7.8) is biphasic, with pseudo first order rate constants of 0.37 min^{-1} and 0.04 min^{-1}. Both CoA and chloramphenicol afford protection against the fast phase of inactivation, but only CoA does so against the slow phase. The biphasic nature of the inactivation reaction was interpreted as being due to modification of two classes of arginyl residues (Corney, 1983).

In the present study borate buffer (pH 7.8) was used and modification carried out as described (2.10) at 25°C. Treatment of CAT with 2,3-butanedione results in a rapid reduction in enzyme activity, such that after 30 minutes only 1-2% of the original activity remains (fig. 3.2). As found by Corney (1983), the inactivation is biphasic with pseudo first order rate constants of 0.12 min^{-1} and 0.04 min^{-1} for the fast and slow phases respectively. Both acetyl CoA and chloramphenicol afford good protection against both phases of inactivation, but protection against inactivation by acetyl CoA is greater than that afforded by chloramphenicol.

Since in other systems phenylglyoxal gives similar results to 2,3-butanedione and is, unlike 2,3-butanedione, available commercially in radioactive form, phenylglyoxal was used in further modification experiments to allow the identification of modified arginyl residues by the techniques of protein chemistry.

3.3 Covalent modification of CAT$_{III}$ with phenylglyoxal.

Treatment of CAT with phenylglyoxal (10mM) as described (2.9) results in a rapid, biphasic loss of enzyme activity (fig. 3.3). The initial fast phase (~10 minutes) of inactivation (k_{app} = 6x10^{-2} min^{-1}) corresponds to a ~70% decrease in activity. Subsequently a much slower rate is observed (k_{app} = 5.2x10^{-3} min^{-1}). The presence of acetyl CoA during modification results in only a 2-fold decrease in the rate of the fast phase of inactivation, whereas the slow phase is unaffected. However, in the presence of chloramphenicol (0.1mM; ~8xK$_m$) the fast phase of inactivation is almost completely abolished, yielding pseudo first order kinetics with k_{app} = 5.4x10^{-3} min^{-1}, essentially that observed for the second (slow) phase of inactivation in the absence of chloramphenicol.

The biphasic kinetics of inactivation of CAT with 2,3-butanedione and phenylglyoxal suggests that these reagents modify two classes of residue. Arginyl residues in substrate binding site have often been found to be more reactive the than other arginyl residues of enzymes (Riordan et al., 1977). Thus the fast phase of inactivation could be due to modification of a substrate binding site residue, with

the slow phase being due to modification of other arginyl residues.

The pattern of protection against inactivation by substrates differs for 2,3-butanedione and phenylglyoxal, with chloramphenicol affording greatly enhanced protection against inactivation by phenylglyoxal, suggesting that the two reagents may be modifying different residues. Phenylglyoxal, by virtue of its phenyl ring, bears some resemblance to chloramphenicol and thus may bind preferentially at the chloramphenicol binding site prior to reaction. Protection by both substrates is also in keeping with the notion that the protected residue could be in the catalytic centre or that substrate binding may cause a conformational change which protects a residue from modification.

Inactivation of CAT by a range of concentrations of phenylglyoxal gives biphasic time courses in each case (fig. 3.4). However, the extent of inactivation observed at the end of the fast phase is dependent on the initial inhibitor concentration. Treatment of CAT with 10mM phenylglyoxal results in a rapid 70% decrease in activity after which inactivation proceeds at a much slower rate, but with 1mM phenylglyoxal the rapid phase of inactivation results in only a 30% decrease in activity. A plot of 1/[initial rate of inactivation] (calculated from the fast phase of inactivation) against 1/ [phenylglyoxal] is linear (fig. 3.5), suggesting that the inactivation process obeys saturation kinetics with the requirement that the inhibitor binds to form a non-covalent complex before reacting with a residue side chain. This result implies that the reaction

proceeds by the following scheme:

Equation (1)

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E*I \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E'I$$
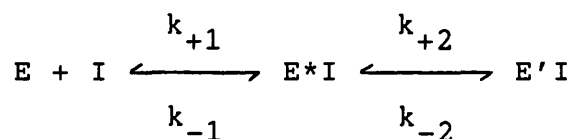
Where E = enzyme, I = inhibitor, E*I = non-covalent complex and E'I = covalently modified enzyme.

If the modification of the residue which results in the rapid phase of activity loss goes to completion ($k_{+2} \gg k_{-2}$, where the reaction is effectively irreversible), then the fast phase of inactivation with phenylglyoxal should yield the same 'final' residual activity irrespective of the concentration of inhibitor used, provided that the inhibitor itself is stable over the time course of incubation. The time taken to reach the residual activity should be dependent upon the inhibitor concentration. Although phenylglyoxal is stable in aqueous solution, and the covalent complex formed on reaction with arginyl residues of many enzymes is reasonably stable, after removal of excess reagent the covalent complex slowly decomposes at mildly alkaline pH (Takahashi, 1968, 1977a). Therefore, for modification of CAT with phenylglyoxal, k_{+2} might be expected to be very much larger than k_{-2} if modification of an arginyl residue is responsible for the rapid phase of activity loss. This is observed for inactivation of yeast hexokinase with phenylglyoxal, where partial reactivation on dilution is slow. Inactivation

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follows pseudo first-order kinetics, over a range of phenylglyoxal concentrations, until ~80% of the initial enzyme activity is lost, and prolonged incubation with reagent results in total loss of activity (Philips et al., 1979). However, this is not true for all enzymes. Modification of pyruvate oxidase with phenylglyoxal gives biphasic inactivation profiles, and yields a stable residual activity, which varies depending on the initial inhibitor concentration (Koland et al., 1982). On 16-fold dilution into buffer there is a rapid partial reactivation. Peters et al. (1981) proposed that the reversibility of the reaction of phenylglyoxal with arginyl residues is directly related to the stoichiometry of the reaction, 2:1 complexes being stable (irreversible) and 1:1 complexes being readily reversible. Furthermore, the ability of a residue to form a 2:1 complex is related to its accessibility i.e. relatively inaccessible residues will tend to form only 1:1 complexes due to steric constraints.

Modification of glutamate dehydrogenase with pyridoxal phosphate (Chen and Engel, 1975) gives similar inactivation profiles to those for modification of CAT with phenylglyoxal. Modification with a range of inhibitor concentrations yields different final residual activities, and the results were interpreted using equation (1) wherein $k_{-1} >> k_{+2} > k_{-2}$ and with E and E*I in rapid equilibrium. That is, the covalent modification step is significantly (but slowly) reversible and results in an equilibrium between E*I and E'I. Modification therefore, never reaches completion and the final equilibrium position depends on the initial concentration of inhibitor added. On dilution into an assay,

E*I rapidly dissociates to yield free enzyme (E) which is active, whereas E'I dissociates more slowly. Therefore, over the time course of an assay, all enzyme in the E'I form remains inactive.

On dilution into TSE pH 7.5 containing 1mg/ml BSA, the rapid phase of inactivation of CAT_{III} with phenylglyoxal is rapidly reversed, with almost 100% activity recovered after incubation for 40 minutes at 25°C (fig. 3.6). Thus k_{-2} is significant in the reaction of phenylglyoxal with CAT. The rapid reversibility of the covalent modification step suggests that perhaps inactivation of CAT by phenylglyoxal is due to modification of a relatively buried arginyl residue. However, good protection against inactivation by chloramphenicol, an uncharged, relatively hydrophobic compound, is unlikely to be due to direct protection of an arginyl residue against modification. It is therefore, possible that inactivation of CAT by phenylglyoxal is due to modification of an arginyl residue which becomes inaccessible on binding of substrates, but which is not directly involved in binding. Alternatively, inactivation could be due to modification of a residue other than an arginine. Both lysyl and cysteinyl residues can also react with phenylglyoxal, and both form much less stable covalent complexes with phenylglyoxal than do arginyl residues. No other amino acid side chains react significantly with phenylglyoxal (Takahashi, 1977a).

3.4 The effect of modification with phenylglyoxal on the hydrolytic reaction.

Modification of CAT with the active site-directed inhibitor 3-(bromoacetyl) chloramphenicol results in the loss of both transacetylation and hydrolytic reactions, a result in accord with the view that both activities require the same catalytic residues (Kleanthous et al., 1985). Similarly, modification by phenylglyoxal of a residue involved in either catalysis or acetyl CoA binding should result in parallel decreases in transacetylation and hydrolytic activities.

Reaction of CAT with phenylglyoxal results in a 40-50% increase in the hydrolytic activity (fig. 3.7). There is a rapid increase in the hydrolytic activity for the first 10 minutes of reaction after which time the activity increases only slowly. The time course of activation of the hydrolytic activity is similar to that for the loss of transacetylation activity (fig. 3.3), suggesting that modification of the same residue is responsible for both effects. The increase in hydrolytic activity strongly suggests that phenylglyoxal modifies neither a residue involved in catalysis nor one contributing directly to the binding of acetyl CoA. The most likely cause of loss of activity following treatment of CAT with phenylglyoxal is modification of a residue in or close to the chloramphenicol binding site.

3.5 Covalent modification of R18V CAT with phenylglyoxal.

Arginine 18, a residue conserved in all known CAT sequences, is involved in a complex web of hydrogen bonds in

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and around the active site (Leslie et al., 1988, see fig. 1.11). The substitution R18V results in a 9-fold decrease in k_{cat} with only small changes in the substrate K_m values. Unlike the wild type enzyme, the mutant protein is very sensitive to thermal inactivation probably due to the presence of a unpaired buried charge (Lewendon et al., 1988). Inactivation of R18V CAT with phenylglyoxal is much more rapid than for the wild-type enzyme with less than 2% activity remaining after 5 minutes (fig. 3.8). The enhanced inactivation of R18V CAT with phenylglyoxal suggests that inactivation of the wild-type enzyme by arginine specific reagents is unlikely to be due to modification of Arg 18. Furthermore, as the mutation is likely to cause disruption of the active site, enhanced sensitivity to phenylglyoxal suggests that modification of an active site residue may be responsible for inactivation. R18V CAT is more susceptible than wild type CAT to inactivation by thiol specific reagents such as 4,4'-dithiodipyridine (Lewendon et al., 1988). Inactivation of CAT by thiol specific reagents is thought to be due to modification of Cys 31 (fig. 3.9), a residue close to the chloramphenicol binding site and catalytic centre (Zaidenzaig and Shaw, 1978; Fitton and Shaw, 1979).

3.6 Covalent modification of C31A CAT with phenylglyoxal.

Phenylglyoxal and 2,3-butanedione, although highly specific for arginyl residues, can react reversibly with thiol groups (Takahashi, 1968, 1977a; Philips et al., 1979) usually at a much lower rate. The mutation C31A renders

CAT_{III} insensitive to thiol specific reagents (A.Lewendon, unpublished results). To investigate the possibility that inactivation of CAT by diketone reagents is due, not to modification of arginyl residues, but rather to modification of a reactive thiol group, the C31A mutant was treated with phenylglyoxal.

Inactivation of C31A CAT is ~25-fold less rapid than for the wild type enzyme (fig. 3.8). Moreover, the presence of chloramphenicol during modification affords only slight protection against inactivation. Thus it seems likely that rapid inactivation of CAT_{III} by phenylglyoxal is due to modification of Cys 31.

3.7 Discussion.

Arginyl residues have been implicated in the binding of CoA to several enzymes. Diketone reagents can be used to 'specifically' modify arginyl residues. To investigate the role of arginyl residues in CoA binding to CAT, the enzyme was treated with 2,3-butanedione and phenylglyoxal. With either reagent the result is a biphasic inactivation rate profile suggesting that two classes of arginyl residue are modified. Surprisingly, both substrates afford protection against inactivation, and chloramphenicol is much more effective than CoA at protecting against inactivation by phenylglyoxal. It thus appeared unlikely that inactivation was due to modification of a residue involved solely in the binding of CoA. The hydrolytic activity, which occurs in the absence of chloramphenicol, is enhanced, rather than
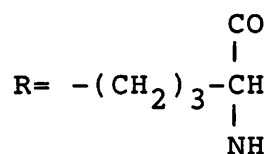
inhibited on modification with diketone reagents, thus eliminating residues involved in catalysis as well as CoA binding as the residue responsible for loss of transacetylation activity on modification. Removal of Cys 31 by site-directed mutagenesis results in greatly reduced sensitivity to phenylglyoxal, compatible with the hypothesis that inactivation of CAT by phenylglyoxal (and probably 2,3-butanedione) is due to modification of Cys 31 rather than an arginyl residue. The position of Cys 31 in the active site (fig. 3.9) suggests that both substrates can protect against inactivation. The slow phase of inactivation of CAT by phenylglyoxal is probably due to modification of surface arginyl residues.

The results of covalent modification studies suggest that, unlike several other CoA dependent enzymes, arginyl residues are not involved in binding of CoA to CAT_{III}. Also, the results of such modification studies must be interpreted cautiously due to the relatively loose specificity of diketone reagents.
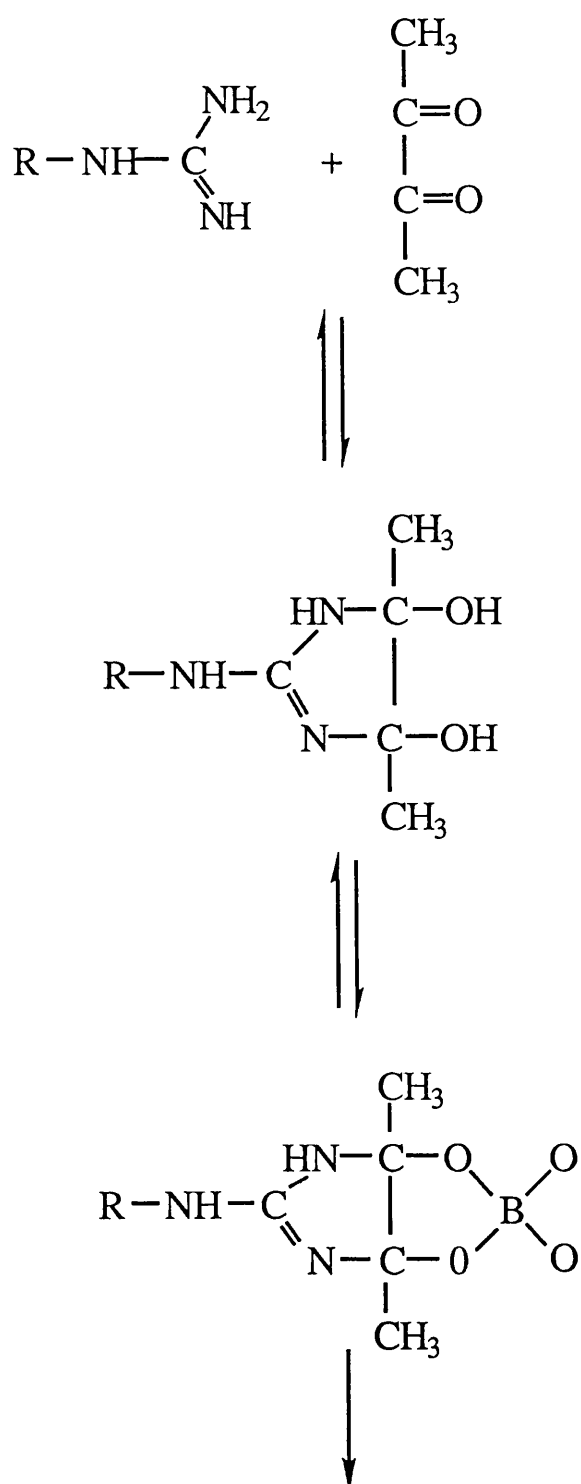
Figure 3.1. The reaction of diketone reagents with arginyl residues.

3.1A. A proposed reaction of 2,3-butanedione with arginyl residues (see Lundblad and Noyes, 1984; Takahashi, 1977). The presence of borate buffers is thought to stabilise the initial product as shown. In the early stages the reaction is reversible, but on longer incubation (>~1/2 hour) a stable unidentified product is formed.

3.1B. A proposed reaction of phenylglyoxal with arginyl residues (Takahashi, 1968). The stoichiometry of reaction with free arginine is 2:1 (Takahashi, 1968), but is variable in protein systems (2:1 Daemen and Riordan, 1974, 1:1 Borders and Riordan, 1975).
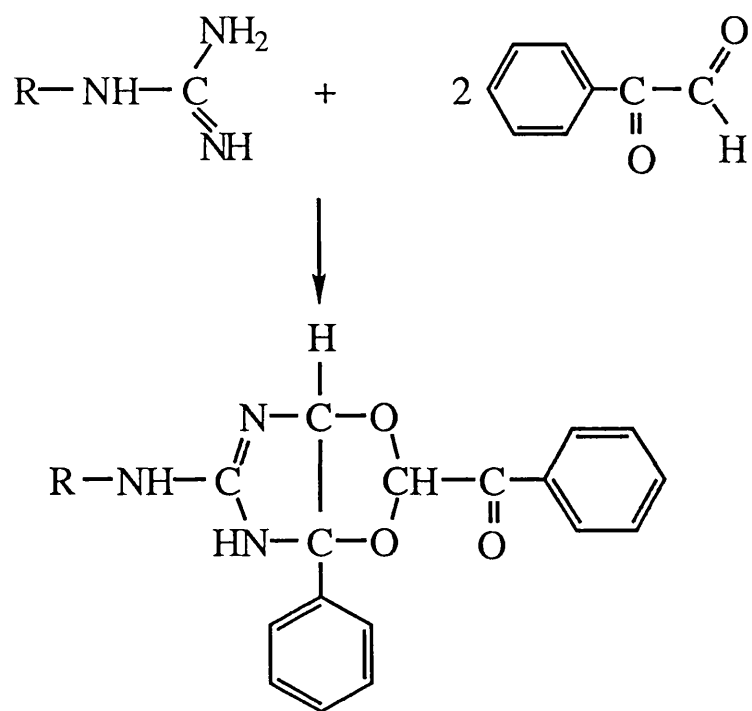
$$R= \ -(CH_2)_3-\overset{\displaystyle CO}{\underset{\displaystyle NH}{CH}}$$

A

FURTHER IRREVERSIBLE REACTION

B

$$R-NH-\overset{\displaystyle NH_2}{\underset{\displaystyle NH}{C}} \quad + \quad 2 \; C_6H_5-\overset{O}{C}-\overset{O}{C}-H$$

$\downarrow$

Structure 1 (bicyclic):

R—NH—C, with N=C and HN—C, fused oxazolidine rings bearing H, phenyl (C₆H₅), and benzoyl (C(=O)C₆H₅) substituents.

OR

Structure 2 (open chain):

R—NH—C imidazolidine with N=C, N—C—OH (phenyl), N—C—OH (H), HO—CH, and O=C—phenyl substituents.
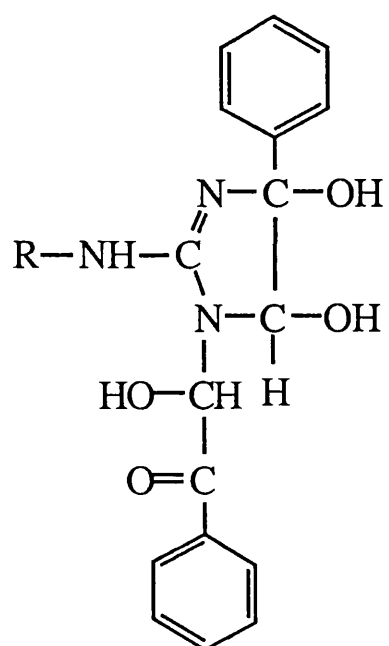
Figure 3.2. The time course of inactivation of CAT$_{III}$ (1.5μM) with 2,3-butanedione (150mM) in 50mM sodium borate (pH 7.8) at 25°C.

Inactivations were carried out as described (2.10) in the presence of acetyl CoA (1mM,●), chloramphenicol (0.1mM,○) or in the absence of substrates (▲). In the absence of reagent no significant loss of activity was observed over the incubation period.
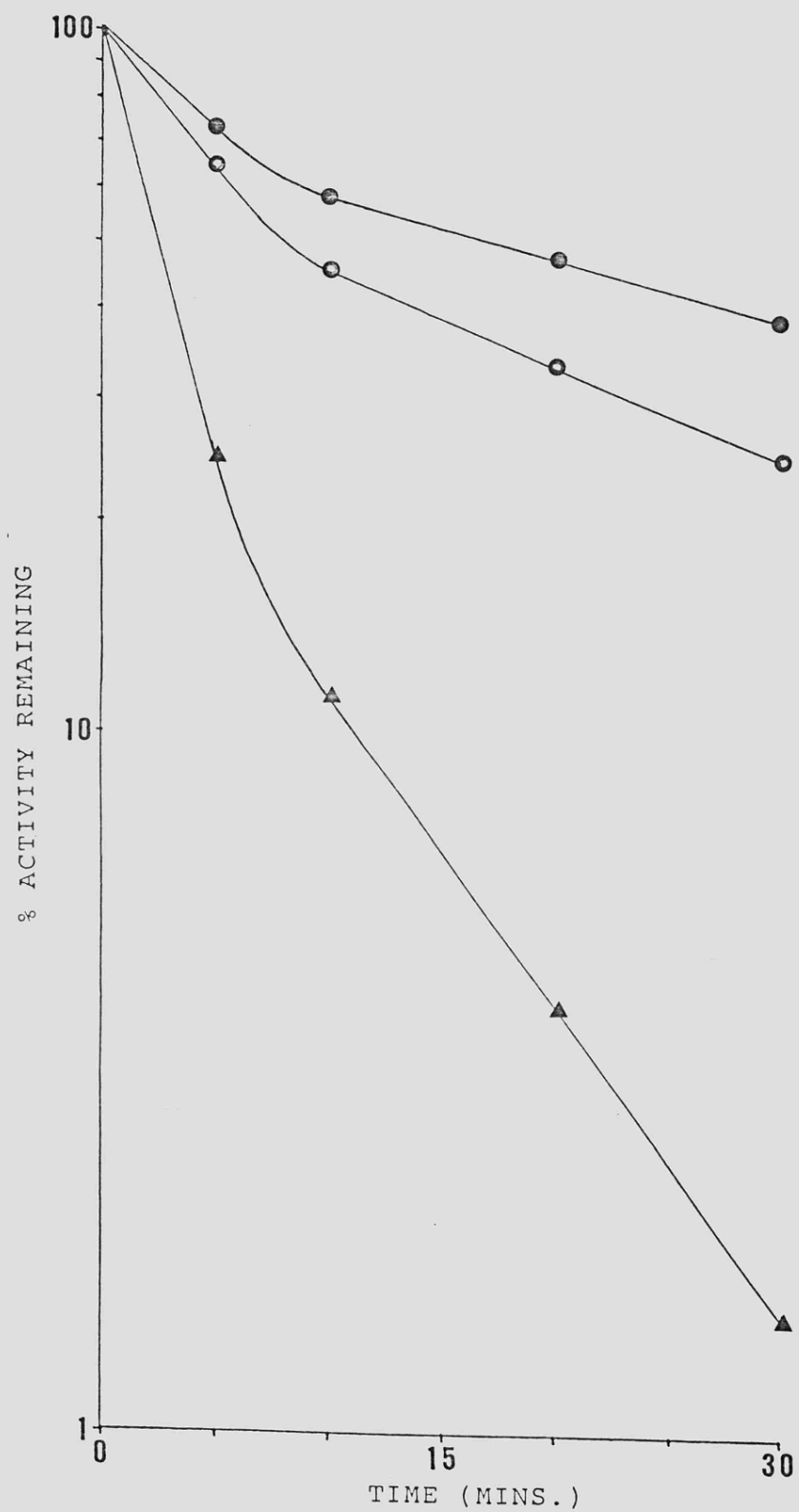
Figure 3.3. The time course of inactivation of CAT_{III} (1.5μM) with phenylglyoxal (10mM) in 45mM HEPES buffer (pH 8.0).

Inactivations were performed as described (2.9) in the presence of acetyl CoA (1mM,■), chloramphenicol (0.1mM,●), in the absence of substrates (▲) or in the absence of reagent (△).
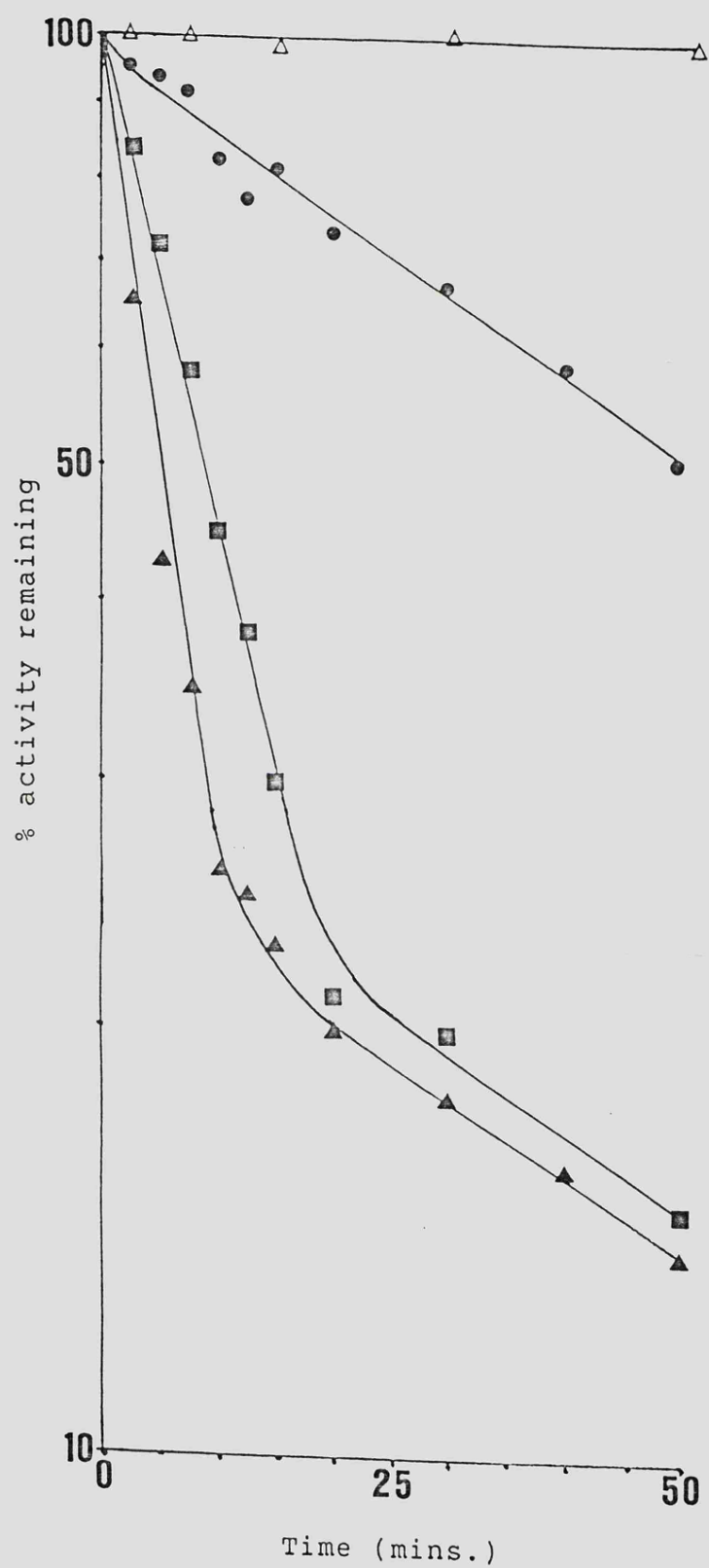
Figure 3.4. The time courses of inactivation of CAT_{III} (1.5μM) with various concentrations of phenylglyoxal.

Inactivations were performed as described (2.9) using 1mM (●), 3mM (O), 5mM (▲) and 10mM (Δ) phenylglyoxal.
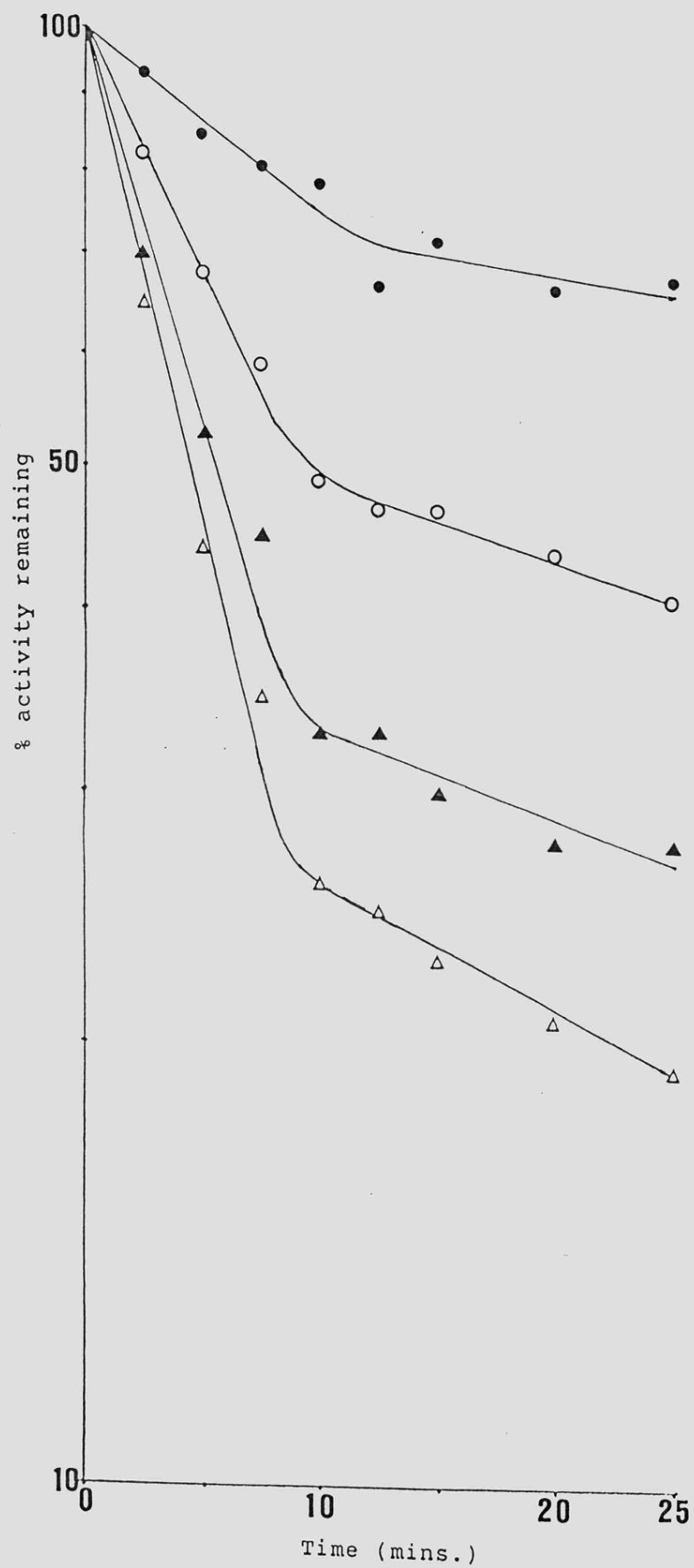
Figure 3.5. Double reciprocal plot of apparent initial rate of inactivation of CAT $(1/k_{app})$ against concentration of phenylglyoxal $(1/I)$.

Initial rates were calculated from the fast phase of inactivation from semi-log plots of log (% activity remaining) against time for several concentrations of phenylglyoxal (see fig. 3.4). A linear plot is indicative of saturation kinetics, with the inhibitor binding before reacting with an amino acid side chain. K_{inact}, the concentration yielding half maximal inhibition, can be calculated from the abscissa intercept, and k_{app}, the maximum inactivation rate at saturating inhibitor concentration can be calculated from the ordinate intercept. The reaction probably proceeds by the following scheme:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \overset{k_{+2}}{\longrightarrow} E'I$$
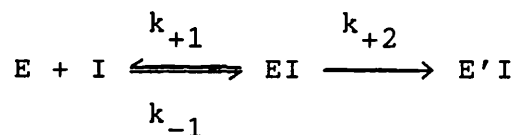
$$K_{inact} = 13\text{mM} \quad (=[k_{-1} + k_{+2}]/k_{+1})$$

$$k_{+2} = 0.18\text{min}^{-1}$$
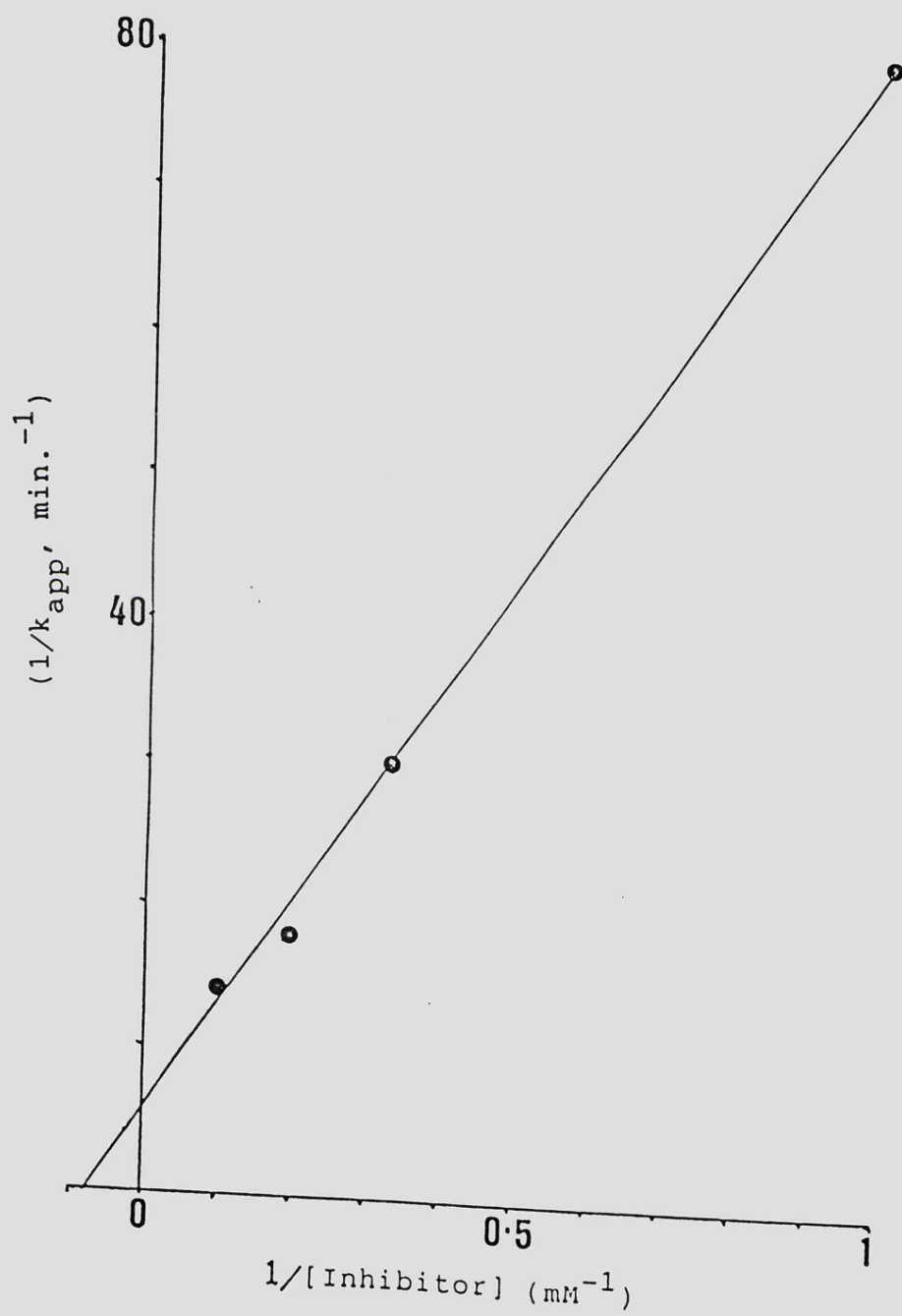
Figure 3.6. Reactivation of CAT modified with phenylglyoxal on dilution.

CAT (1.5μM) was modified with phenylglyoxal (10mM) as described (2.9). At timed intervals 10μl aliquots were removed, diluted into 990μl TSE containing 1mg/ml BSA and assayed for CAT activity (2.5). After 12.5 minutes a sample was treated as above, then incubated at 25°C and assayed at timed intervals.

Inactivation curve ●

Reactivation curve ▲
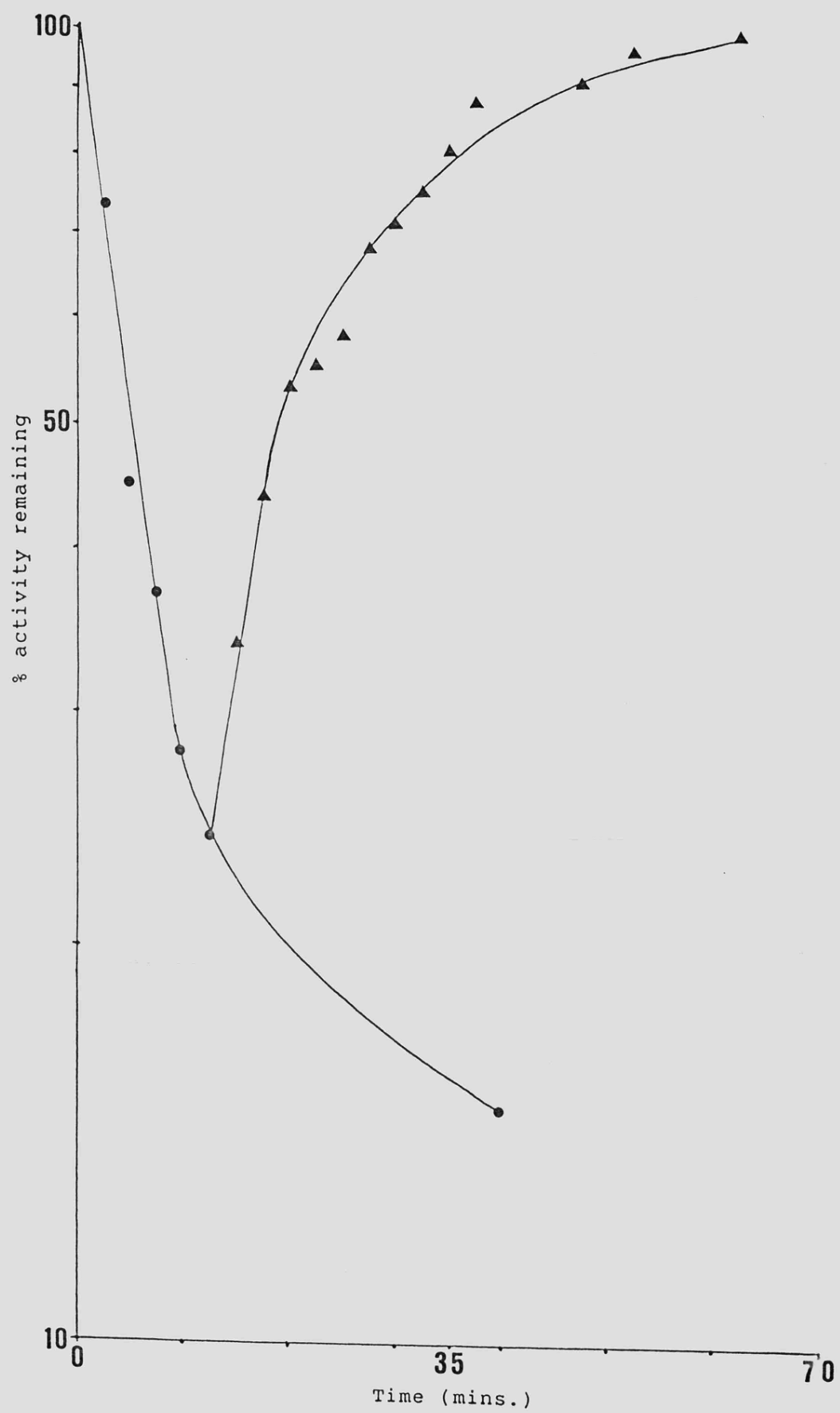
Figure 3.7. The effect of modification of CAT_{III} (45μM) with phenylglyoxal (10mM) on the enzyme catalysed hydrolysis of acetyl CoA.

The modification reaction was carried out as described (2.9) in the absence of substrates, and modified enzyme assayed for hydrolytic activity (2.5).
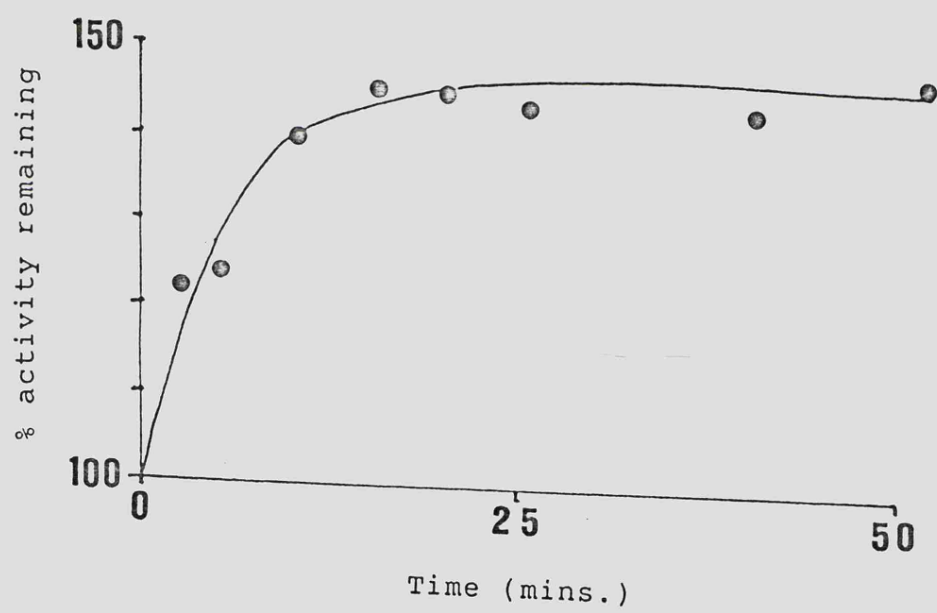
Figure 3.8. The time course of inactivation of wild type CAT$_{III}$, R18V CAT and C31A CAT with phenylglyoxal (10mM).

Inactivations were carried out in the absence of substrates as described (2.9). The enzymes were kindly provided by A.Lewendon (C31A CAT) and I.A.Murray (R18V CAT).

Wild type CAT   (1.5μM) ▲

R18V CAT   (0.4μM) ■

C31A CAT   (4.0μM) ●
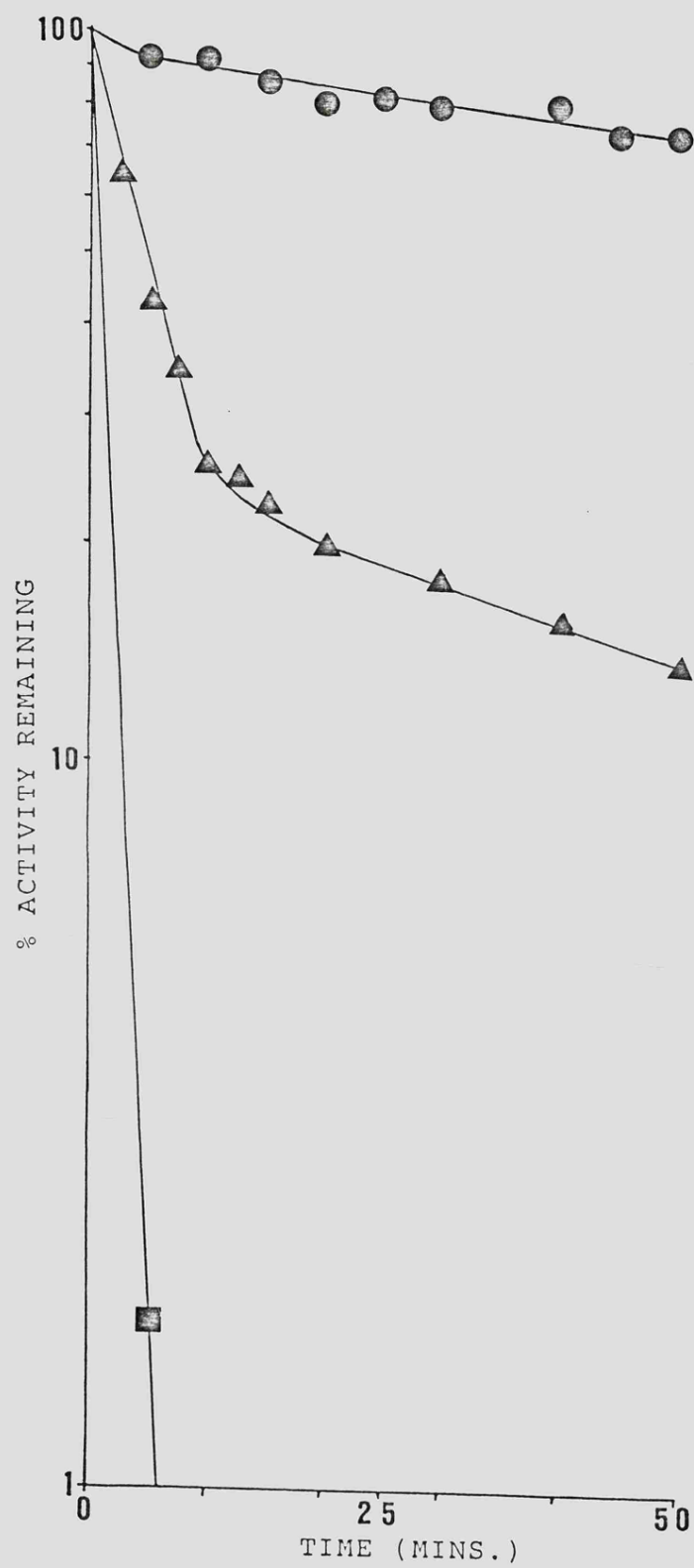
Figure 3.9. Stereoview of the active site of CAT_{III} showing the position of Cys 31 in relation to that of chloramphenicol (CLM) and CoA.

Residue names preceded by # belong to an adjacent subunit and predicted hydrogen bonds are indicated by dashed lines. Only the terminal four atoms of CoA are shown.

Chapter 4. The role of electrostatic interactions
in the binding of acetyl CoA to CAT$_{III}$.

4.1 Covalent modification of lysyl residues.

The ε-amino group of lysyl residues can be modified specifically by imido esters (Hunter and Ludwig, 1962, fig. 4.1). Although the amidination reaction, which also modifies the amino-terminus of proteins, results in an increase in side chain size, it retains the positive charge of the protonated residue at ph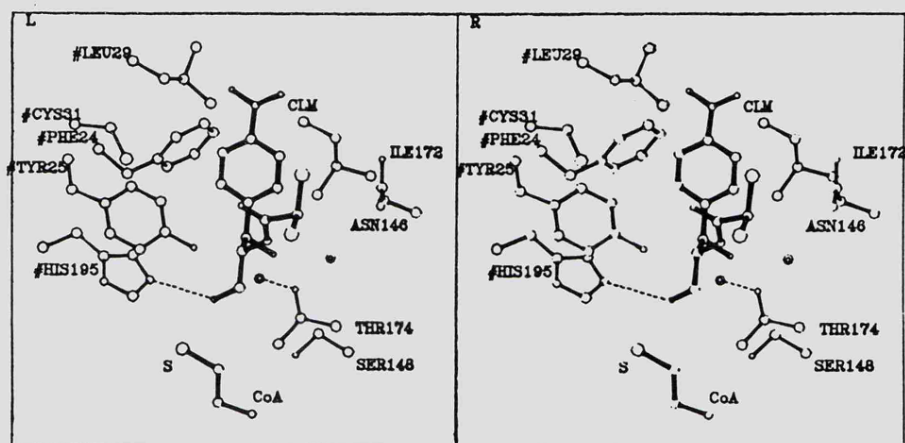ysiological pH values. As the charge of the residue is retained on modification, imido esters are less likely to cause major structural changes than are other reagents which modify lysyl residues (for example, acid anhydrides). Imido esters have been used to probe structural roles of lysyl residues, such as lysyl residues involved in ion pairs in the interior of protein subunits or inter-subunit interactions (Lambert et al., 1977). Modification of proteins in the native state, followed by denaturation and subsequent modification with $[1-^{14}C]$ acetimidate allows such residues to be identified (Lambert and Perham, 1977). Packman and Shaw (1981a) used methyl acetimidate to identify such a 'buried' lysyl residue (Lys 38) in CAT_{III}. Reaction of native CAT_{III} with methyl acetimidate results in modification of 10 of the 11 lysyl residues, with a corresponding 70% decrease in enzyme activity. The presence of either substrate during modification gave no protection against loss of activity.

4.2 Covalent modification of CAT_{III} with ethyl acetimidate.

Ethyl acetimidate which, unlike methyl acetimidate is available commercially in both radioactive and unlabelled form, was used to covalently modify CAT_{III} as described

(2.11). Treatment of CAT with ethyl acetimidate results in a rapid decrease in enzyme activity which stabilises at 55-60% of the original activity (fig. 4.2). Further addition of fresh reagent causes no further decrease in activity, and preincubation of enzyme with acetyl CoA or chloramphenicol gives no protection against loss of activity. These results agree well with those of Packman and Shaw (1981a) except that methyl acetimidate appears to cause greater loss of activity than does ethyl acetimidate. As both reagents yield the same amidinated species on reaction (fig. 4.1) and the low concentrations of by-products (methanol and ethanol) have no discernable effect on either the modification reaction or on enzyme activity, a difference in the extent of inactivation seems most unlikely. The only other difference between the two experiments was the concentration of acetyl CoA used in the standard assay (400μM in this study compared with 100μM in the study of Packman and Shaw), suggesting that modification may result in a change in the K_m for acetyl CoA. To test this hypothesis the steady-state kinetic parameters of the modified enzyme were determined.

Modification of CAT with ethyl acetimidate results in a 5-fold increase in the K_m for acetyl CoA, with no change in k_{cat} or the K_m for chloramphenicol (Table 4.1). Although the 5-fold increase in K_m for acetyl CoA is compatible with the hypothesis that lysyl residues are involved in binding of CoA, the lack of protection against loss of activity by acetyl CoA suggests that an ion pair involving a lysyl residue and a phosphoanion of CoA is unlikely. The presence of such an ion pair can be investigated using a similar approach to that used by Packman and Shaw (1981a) to identify

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a subunit interface lysyl residue in CAT.

CAT modified with ethyl acetimidate in the presence or absence of substrates was denatured in guanidine hydrochloride prior to modification with ethyl [1-^{14}C] acetimidate. Labelled enzyme was digested with chymotrypsin and the resulting peptides separated by FPLC. The radioactive peptide elution profiles are shown in figure 4.3. For each lysyl residue modified with [1-^{14}C] acetimidate 1.7x10^5 cpm should be incorporated, and each sample should have at least one radioactively labelled lysyl residue (Lys 38), as identified by Packman and Shaw (1981a).

Irrespective of whether CAT is modified in the presence or absence of substrates only a single lysyl residue remains unmodified after treatment of native enzyme with ethyl acetimidate (Table 4.2). The lack of protection of a lysyl residue from modification by acetyl CoA suggests, as did lack of protection of activity, that there are no substrate/lysine ion pairs in the binary complex of acetyl CoA and CAT$_{III}$.

4.3 Long-range electrostatic interactions.

The role of hydrogen bonds and ion pairs in substrate binding and catalysis has been studied extensively (e.g. Fersht et al., 1985; Linse et al., 1988). As the distance (r) between the interacting atoms is increased the strength of the interaction decreases rapidly (as a function of 1/Dr^6, where D is the dielectric constant). Nonetheless in subtilisin, long-range electrostatic interactions have been judged to be important in substrate binding and catalysis (Russell et al., 1987; Russell and Fersht, 1987; Sternberg et

al., 1987). Replacement of charged residues, by site-directed
mutagenesis, at single positions up to 1.3nm from the active
site of subtilisin results in changes in the pK_a of the
catalytic histidine residue of up to 0.6 pH units.
Substitutions of such residues can also alter the specificity
of the enzyme for charged substrates. Substitution of both
Asp 99 and Glu 156 with serine results in a 100-fold increase
in specificity (k_{cat}/K_m) for negatively charged substrates as
compared with positively charged substrates, even though
these residues are 1.3nm and 1.4nm respectively from the
active site of subtilisin. Long-range electrostatic
interactions are most pronounced at low ionic strengths,
being gradually reduced as the ionic strength is increased
due to the presence of excess counter ions.

The structure of the CAT/CoA binary complex shows that
two well conserved lysyl residues are close to the CoA
binding site (Leslie et al., 1988). Lys 54 is conserved in
all but one of the known CAT sequences (pC221 CAT, Shaw et
al., 1985), and Lys 177 is an arginine in Streptomyces CAT
(Murray et al., 1989). Since the ε-amino groups of Lys 54 and
Lys 177 are more than 0.6nm and 1.4nm from the 5'diphosphate
moiety and ~0.7nm and 1.2nm from the 3'phosphate moiety of
CoA respectively, neither residue is close enough to form an
ion pair interaction with any of the phosphoanionic groups of
CoA. Both lysyl residues are, however, in regions of the CAT
structure which make intimate contacts with CoA. Residues 176
and 178 are both hydrogen bonded to the adenine ring of CoA
via main-chain atoms, and the side chain of residue 56
interacts with the pantetheine arm of CoA (figs. 1.9, 6.1 and
6.3).

To investigate the possibility that Lys 54 and Lys 177 might be involved in long-range electrostatic interactions with CoA, a number of substitutions which alter the charge of the side chains of residues 54 and 177 were made. Both Lys 54 and Lys 177 were replaced with glutamine, a moderately conservative mutation which removes a positive charge whilst retaining the hydrophilic nature of the side chain. Lys 177 was also replaced with alanine, to remove all interactions between CoA and the residue side chain and with glutamate, to reverse the polarity of the side chain and thereby potentially introduce electrostatic repulsion. Mutagenesis was carried out as described (2.31) and purified proteins analysed by SDS PAGE and native PAGE to confirm the presence of charge differences (fig. 4.4).

4.4 Lysine 177.

The most significant discernable effect of removing the positive charge from the side chain of residue 177 is to increase the K_m for acetyl CoA. The K_m for chloramphenicol and k_{cat} for each of the substitutions is essentially unchanged (Table 4.3), suggesting that none of the mutations cause major global structural changes.

The substitution K177Q results in only a 2-fold increase in the K_m for acetyl CoA, with a corresponding calculated contribution to the apparent free energy of binding of CoA (ΔG_{app}) for the positive charge on the side chain of residue 177, of, only 0.4 kcal/mol. This change in ΔG_{app} is considerably smaller than would be expected for the removal of an ion pair interaction (>3 kcal/mol, Fersht et al.,

1985), in agreement with covalent modification and structural studies. Substitution with glutamate (K177E) results in a larger increase in K_m for acetyl CoA consistent with the hypothesis that Lys 177 is involved in an electrostatic interaction with CoA. The small changes in K_m on substitution of Lys 177 with Gln and Glu imply that Lys 177 plays a relatively small role in the binding of CoA and it is therefore surprising that Lys 177 should be so well conserved.

The K177A substitution was made prior to solution of the structure of the CAT/CoA complex and was designed to remove all interactions between the side chain of residue 177 and CoA. The structure of the CAT/CoA complex suggests that the K177Q and K177A substitutions should have very similar effects on the K_m for acetyl CoA as the side chain of residue 177 appears not to be involved in direct interactions with CoA. The mutation to alanine however, results in a much larger (7-fold) increase in K_m for acetyl CoA than might be expected, even greater than that observed by reversing the charge (K177E) of the side chain of residue 177. Residues 176 and 178 are both hydrogen bonded to CoA and the tyrosyl side chain of residue 178 is stacked with the adenine ring of CoA. In the event that the K177A substitution causes movement of the main chain atoms of residue 177, then interactions between CoA and residues 176 and 178 could be adversely affected. Substitution of Lys 177 with a residue more hydrophobic than alanine (for example isoleucine) might be expected to result in a still larger increase in the K_m for acetyl CoA than that observed for K177A, on the grounds that a hydrophilic residue might be required at position 177 to

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retain the correct conformation of the residue main chain atoms.

The structure of the K177A CAT/chloramphenicol binary complex at 0.22nm resolution (M.Gibbs and A.G.W.Leslie, unpublished results) shows that the substitution causes essentially no changes in conformation of the main chain atoms of residues 176, 177 or 178 in the chloramphenicol binary complex. Thus the high K_m for acetyl CoA for K177A CAT can not be explained by a structural change at least not in the chloramphenicol binary complex. Solution of the K177A/CoA complex structure may resolve this problem.

4.5 Lysine 54.

Substitution of Lys 54 with glutamine (K54Q) results in an almost 10-fold increase in K_m for acetyl CoA compared with only a 2-fold increase for the analogous substitution K177Q (Table 4.3). The calculated contribution to CoA binding of the positive charge on the side chain of residue 54, 1.3 kcal/mol, is again too small to have resulted from the removal of an ion pair. The larger changes in kinetic parameters caused by the K54Q substitution are consistent with the observation that Lys 54 is closer to the charged moieties of CoA than is Lys 177.

The results of site-directed mutagenesis experiments imply that both Lys 54 and Lys 177 are involved in electrostatic interactions with CoA. Lys 54 appears to have a greater role in binding than Lys 177, but neither residue appears to be involved in an ion pair with CoA.

4.6 Effect of ionic strength on CoA binding.

Long-range electrostatic interactions are greatly reduced as the ionic strength is increased due to the neutralising effects of excess counter ions in solution. The effect of changing ionic strength on the specificity constant (k_{cat}/K_m) for a given substrate can be used to probe for the presence of electrostatic interactions between the enzyme and the transition-state. With an enzyme for which electrostatic interactions are involved in substrate binding, k_{cat}/K_m would be expected to decrease as ionic strength was increased due to the gradual loss of a favourable interaction. A mutant enzyme where such an interaction is removed would be expected to be much less sensitive to changing ionic strength than the wild type enzyme.

The effect of changing ionic strength on the kinetic parameters of the hydrolytic activity has been determined. The hydrolytic reaction was investigated to allow whole plots to be completed in one day and to remove any effects of ionic strength on binding of chloramphenicol. Kinetic parameters for the wild type and mutant enzymes were plotted as k_{cat}/K_m against ionic strength (fig. 4.5). Increasing ionic strength from 135mM to 945mM results in a 17-fold decrease in the specificity constant for wild-type CAT, largely due to a 15-fold increase in the K_m for acetyl CoA (Table 4.4). The observed k_{cat} decreases only slowly as ionic strength is increased. The mutant enzymes are less sensitive to changes in ionic strength, K177Q yielding a 5-fold decrease in k_{cat}/K_m and K54Q only a modest (less than 2-fold) decrease. The smaller changes in k_{cat}/K_m for the Lys $\rightarrow$ Gln

substitutions confirmed that both residues 54 and 177 are involved in long-range electrostatic interactions with CoA. As expected the K177E mutant is less sensitive to changes in ionic strength than is K177Q. The difference between these two mutants is smaller than expected probably due to the greater effect of ionic strength on interactions with Lys 54, partially masking the changes for Lys 177.

4.7 Specificity of long-range electrostatic interactions.

In order to determine which of the phosphoanions of acetyl CoA interact with Lys 54 and Lys 177, a number of analogues of CoA, which have phosphate groups at only one position were employed. 3' Adenosine monophosphate (3'AMP) and 5'adenosine monophosphate (5'AMP) are competitive inhibitors of CAT which are analogous to CoA with either the 5'diphosphate or 3'phosphate groups removed. Although the ribose/phosphate moiety of such analogues is likely to be more flexible than the corresponding region of CoA, they are likely to bind to CAT in a similar manner to CoA as their solution conformational preferences have been inferred, from nmr studies, to be very similar to those of CoA (Lee and Sarma, 1974, 1975; Evans and Sarma, 1974).

4.7.1 3'AMP.

The K_i values for 3'AMP of the wild-type, K177Q and K177E enzymes are approximately equal (Table 4.5) suggesting that Lys 177 does not interact significantly with the 3'phosphate of CoA. The K177A K_i is higher than that for

K177Q again suggesting that the Ala substitution causes disruption of interactions between CAT and the adenine moiety of CoA, other than those involving the lysyl side chain (see 4.4). The K54Q substitution results in a small increase in the K_i for 3'AMP suggesting that Lys 54 may interact with the 3'phosphate group of CoA. However, the increase in K_i is considerably smaller than the observed increase in K_m for acetyl CoA implying that interaction with the 3'phosphate group of CoA is not the main contribution to CoA binding of Lys 54.

4.7.2 5'AMP/ATP.

The K_i values for 5'AMP and ATP follows the same pattern as the K_m for acetyl CoA for substitutions of Lys 177, increasing as the residue side chain becomes more negatively charged. Again K177A results in a higher than expected K_i. (Table 4.5). Thus it seems probable that Lys 177 is involved in a long-range electrostatic interaction with the 5'diphosphate moiety of CoA.

The K54Q substitution results in a smaller increase in the K_i for 5'AMP than the increase in the K_m for acetyl CoA, but a much larger increase in the K_i for ATP, suggesting that Lys 54 may be able to form novel interactions with ATP. The triphosphate 'arm' of ATP may fold in such a way so as to maximise interactions with Lys 54. If so, ATP probably binds more tightly to CAT than would be expected were the conformation of the phosphodiester moiety restricted by the pantetheine arm of CoA.

4.8 Covalent modification of mutant Chloramphenicol acetyl transferases with ethyl acetimidate.

To confirm that the increase in K_m for acetyl CoA on modification of CAT with ethyl acetimidate was due to modification of Lys 54 and Lys 177, the four mutant enzymes were treated with ethyl acetimidate and the kinetic parameters of the modified proteins determined. Loss of activity on treatment of the mutant enzymes with acetimidate follows a similar profile to that seen for the wild type enzyme, although the final residual % activities differ (fig. 4.6). Modification with ethyl acetimidate results in a ~5-fold increase in the K_m for acetyl CoA for both the wild-type and Lys 177 mutants suggesting that the loss of activity on modification probably is not due to modification of Lys 177 (Table 4.6). Modification of K54Q results in a smaller (<2-fold) increase in the K_m for acetyl CoA. Thus, loss of activity on modification of CAT_{III} with ethyl acetimidate is largely due to modification of Lys 54.

As modification with imido esters does not alter the charge of the modified side chain, a change in the K_m for acetyl CoA on modification of Lys 54 might not be expected. The increase in K_m possibly reflects the increase in distance between the charge on the side chain of residue 54 on modification and the phosphoanions of CoA.
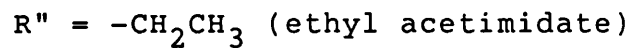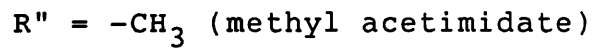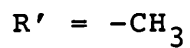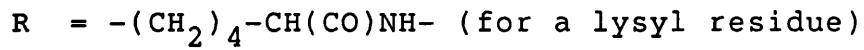
4.9 Discussion.

Chemical modification studies suggested that arginyl residues are not involved in the binding of CoA to CAT

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(Chapter 3). It follows that if the phosphoanions of CoA are ion paired with positively charged side chains on CAT, lysyl or histidyl residues must be involved. The structure of the CAT/CoA binary complex has subsequently shown that no such ion pair interactions are likely. Nonetheless, covalent modification of lysyl residues with ethyl acetimidate results in an increase in the K_m for acetyl CoA suggesting that lysyl residues are involved in binding of CoA. Although two well conserved lysyl residues (Lys 54 and Lys 177) are within 1.4nm of the CoA binding site, neither are close enough to form ion pairs with the phosphoanions of CoA. Substitution of Lys 177 with Gln results in only a 2-fold increase in the K_m for acetyl CoA and a slightly reduced sensitivity to increasing ionic strength, implying that Lys 177 makes only a small contribution to CoA binding. Lys 54 makes a much larger contribution to CoA binding, probably via a long-range electrostatic interaction with the 5'diphosphate moiety of CoA. The change in calculated ΔG_{app} on substitution of Lys 54 with Gln (K54Q) is much smaller than would be expected for the removal of an ion pair, in agreement with structural studies. Thus, in CAT_{III} electrostatic interactions between enzyme and substrate play a much smaller role than might be expected from the structure of acetyl CoA.

Figure 4.1. The reaction of imido esters with primary amines.

Imido esters can be used to rapidly and specifically modify the amino groups (both α and ε) of proteins (Hunter and Ludwig, 1962). Lysyl residues modified in this way retain a positive charge at physiological pH values, but are resistant to cleavage by trypsin.

R $= -(CH_2)_4-CH(CO)NH-$ (for a lysyl residue)

R' $= -CH_3$

R" $= -CH_3$ (methyl acetimidate)

R" $= -CH_2CH_3$ (ethyl acetimidate)

$$R{-}NH_2 \quad + \quad \overset{\displaystyle H_2\overset{+}{N}}{\underset{\displaystyle R''O}{\underset{\|}{C}}}{-}R'$$

$$\Big\downarrow \text{pH>8}$$

$$R{-}NH{-}\overset{\displaystyle \overset{+}{N}H_2}{\underset{\|}{C}}{-}R' \quad + \quad R''OH$$
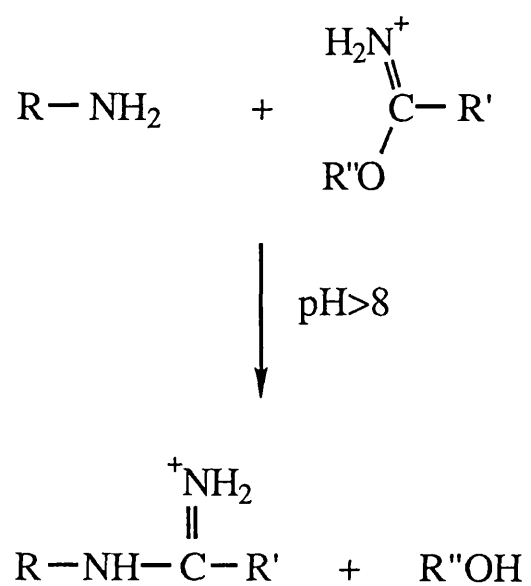
Figure 4.2. The time course of inactivation of CAT_{III} (1.5μM) with ethyl acetimidate (100mM) in 0.2M TEA buffer (pH 8.5).

Inactivations were carried out at 25°C as described (2.11) in the absence of substrates (▲) or in the absence of reagent (Δ). The presence of either substrate did not alter the inactivation curve.
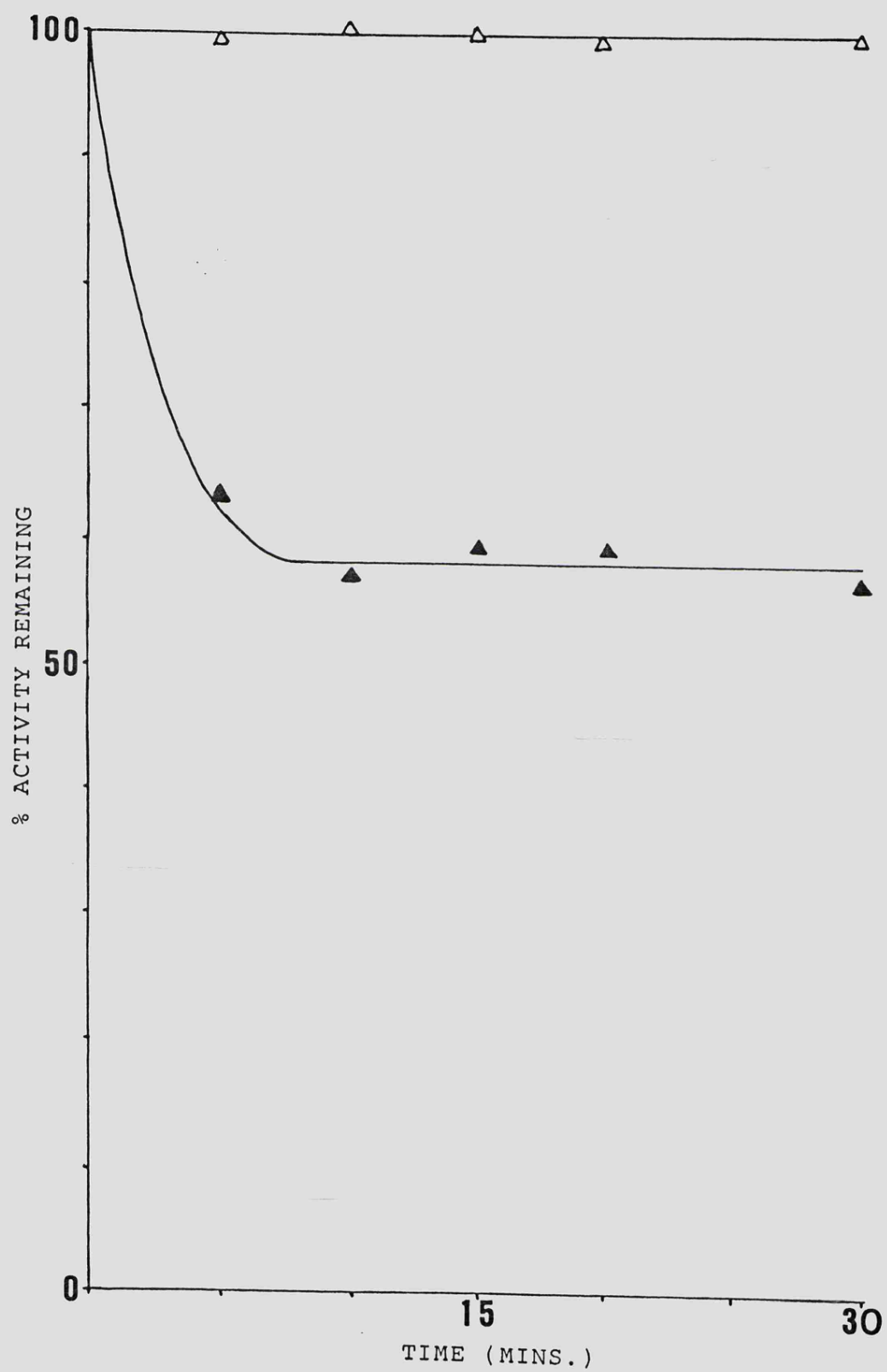
Figure 4.3. Labelling of lysyl residues, protected from modification by ethyl acetimidate, with ethyl [1-^{14}C] acetimidate on denaturation.

CAT$_{III}$ (3.0mg) was modified with ethyl acetimidate (100mM) for 1 hour at 25°C in the presence of acetyl CoA (1mM), chloramphenicol (0.2mM) or in the absence of substrates. A further addition of reagent was made and reaction allowed to proceed for a further hour. The reaction was quenched by addition of a 2-fold excess of 0.5M ammonium acetate (pH 5.5) and after extensive dialysis against water and lyophilisation, modified protein was resuspended in 1ml TEA buffer (pH 8.5) containing 8M guanidine hydrochloride. Ethyl [1-^{14}C] acetimidate (4.3x10^5 dpm/μmol) was added to 0.1M and reaction allowed to proceed for 1 hour at 25°C. Reaction was quenched as above, reagents removed by dialysis against water and the samples lyophilised. Radiolabelled protein was resuspended in 1ml 0.5% (w/v) ammonium bicarbonate and digested with chymotrypsin (1% w/w) for 4 hours at 37°C (2.12). After reduction and carboxymethylation (2.12) the samples were filtered and 10% of each loaded onto a C18 FPLC reverse phase column. Peptides were eluted with an acetonitrile gradient, 1ml fractions collected and the fractions assayed for ^{14}C radioactivity. Elution profiles of fractions containing ^{14}C radioactivity for enzyme modified in the presence of chloramphenicol (C), acetyl CoA (B) or in the absence of substrates (A) are shown in figure 4.3.
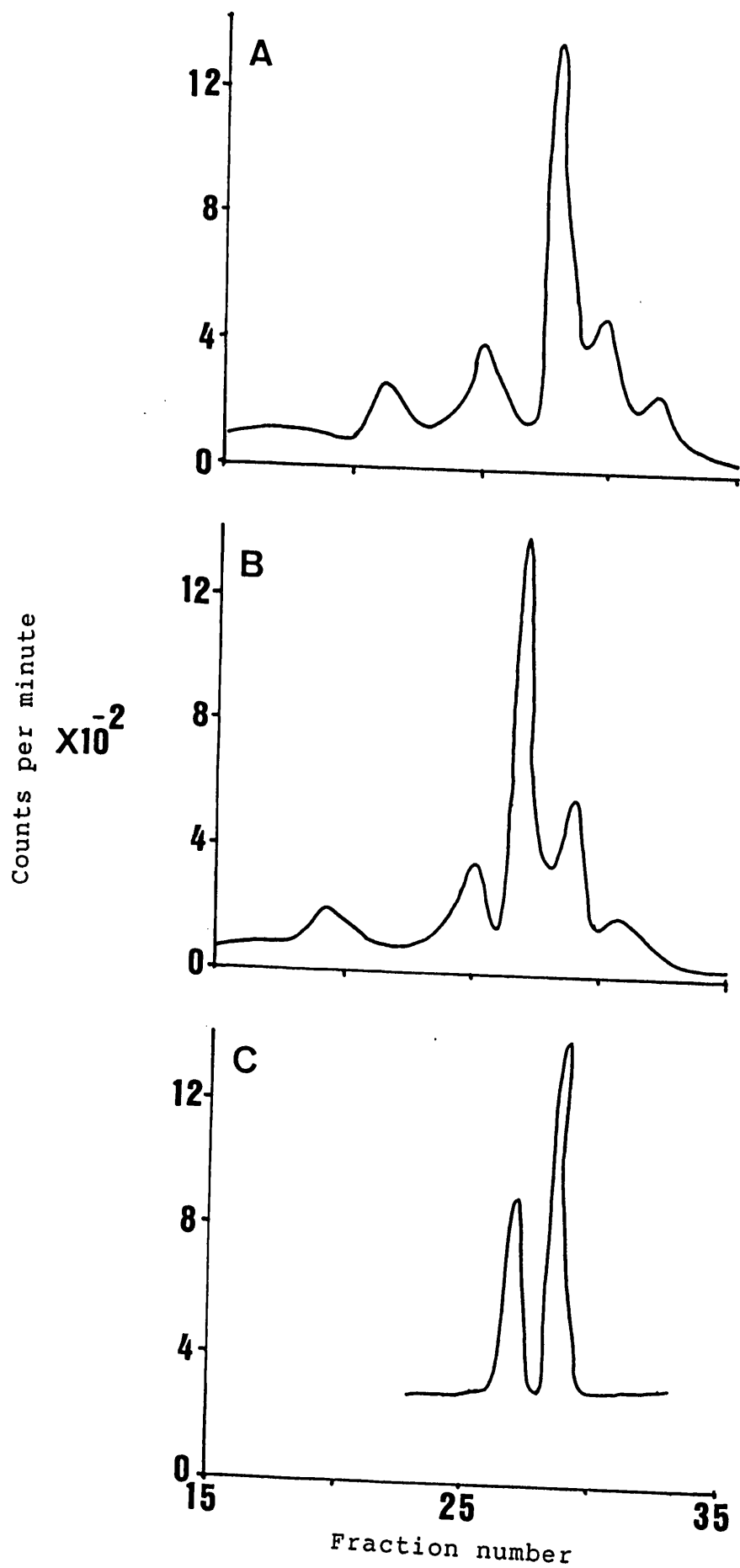
Figure 4.4. Polyacylamide gel electrophoresis of mutant chloramphenicol acetyltransferases.

A. Comparison of the subunit molecular weights of mutant CATs. A 12% denaturing (SDS) gel is illustrated.

Lane	sample
1,4 and 8	molecular weight markers
2	10μg wild type CAT
3	10μg K177E CAT
5	10μg K177A CAT
6	10μg K177Q CAT
7	10μg K54Q CAT

molecular weight markers: Bovine serum albumin, 66kDa

Trypsinogen, 24kDa

β-lactoglobulin, 18kDa

lysozyme, 14kDa.

B. Comparison of the surface charge of mutant CATs by native PAGE. A 6% gel is illustrated.

Lane	sample
1 and 6	10μg wild type CAT
2	10μg K177E CAT
3	10μg K177Q CAT
4	10μg K177A CAT
5	10μg K54Q CAT

A

1 2 3 4 5 6 7 8

B

1 2 3 4 5 6

Figure 4.5. The effect of ionic strength on the specificity constant (k_{cat}/K_m) for the hydrolysis of acetyl CoA by wild type and mutant chloramphenicol acetyltransferases.

Steady-state kinetic parameters were determined as described (2.6) in TSE buffer (pH 7.5), but S, the NaCl concentration, was varied over the range 0-1M.

```
            Key:  wild type  ▲
                  K177A       □
                  K177Q       O
                  K177E       ●
                  K54Q        ■
```

Figure 4.6. The time course of inactivation of wild type and mutant chloramphenicol acetyltransferases with 100mM ethyl acetimidate.

Inactivations were carried out as described (2.11) in the absence of substrates. All enzymes retained ~100% activity in the absence of reagent.

Key: wild type ▲

    K177A   □

    K177Q   ●

    K177E   ■

    K54Q   ○
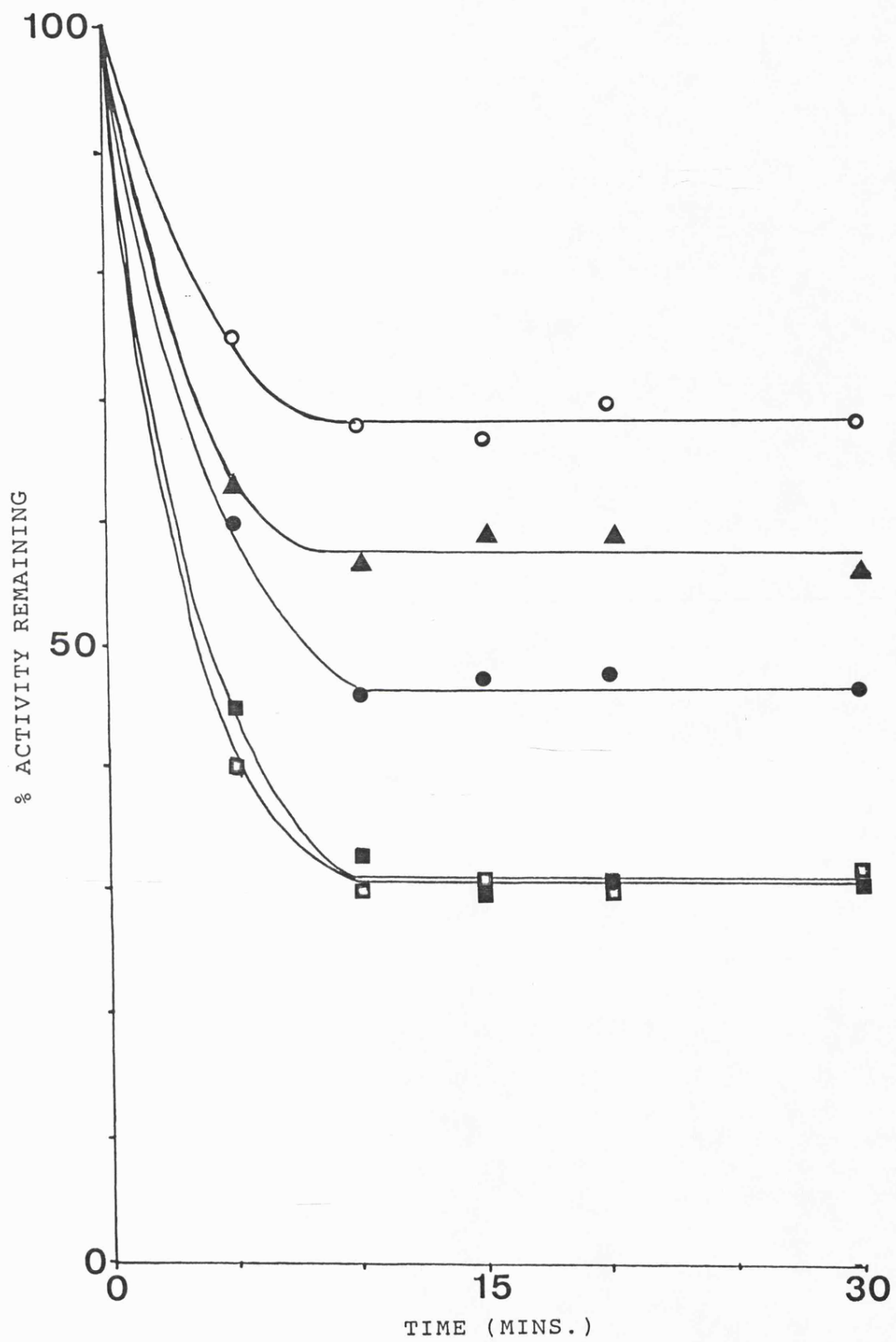
Table 4.1. Apparent steady-state kinetic parameters of CAT_{III} modified with ethyl acetimidate.

CAT_{III} (1.5μM) was modified with ethyl acetimidate (100mM) as described (2.11) for 1 hour at 25°C. The reaction was quenched by dilution into TSE pH 7.5 containing 1mg/ml BSA, and steady-state kinetic analysis carried out as described (2.6) using a fixed concentration of chloramphenicol (100μM).

Addition	$k_{cat}app(s^{-1})$	$K_m app(\mu M)$
None	485	81
Acetimidate (100mM)	538	456

Table 4.2. Incorporation of ^{14}C radioactivity on modification of denatured CAT with ethyl [1-^{14}C] acetimidate.

Enzyme (3mg) was modified with ethyl acetimidate (100mM) in the presence or absence of substrates, denatured, carboxymethylated and resuspended in TEA (pH8.5) containing 8M guanidine hydrochloride. Ethyl [1-^{14}C] acetimidate (100mM, 4.3×10^5 dpm/μmol) was added and reaction allowed to proceed for one hour. After removal of reagents by dialysis, incorporation of ^{14}C radioactivity was measured using a Tri-Carb 2000CA liquid scintillation analyser.

Addition	Incorporation of radioactivity (cpm)	number of Lys modified
None	2.1×10^5	1.2
1mM acetyl CoA	2.2×10^5	1.3
0.2mM Cm	1.5×10^5	0.9

Table 4.3. Steady-state kinetic parameters for wild type and mutant chloramphenicol acetyltransferases.

Enzyme	$k_{cat}(s^{-1})$	$K_m A(\mu M)$	$K_m B(\mu M)$	$K_d A(\mu M)$	$K_d B(\mu M)$
K54/K177	600	93	12	31	4
K54Q	680	894	21	244	6
K177Q	645	180	15	57	5
K177E	620	417	14	165	6
K177A	571	658	17	136	4

A = acetyl CoA, B = chloramphenicol

Table 4.4. Variation of the steady-state kinetic parameters for the hydrolysis of acetyl CoA of mutant chloramphenicol acetyltransferases with ionic strength.

Ionic strength (mM)	$k_{cat}(s^{-1})$	$K_m(\mu M)$	$k_{cat}/K_m(s^{-1}.M^{-1})$
a) K54/K177			
45	0.12	65	1846
135	0.13	66	1970
270	0.10	182	549
495	0.11	502	219
945	0.11	957	115
b) K54Q			
45	0.14	539	260
135	0.12	522	230
270	0.12	533	225
495	0.10	655	153
945	0.09	722	125
c) K177Q			
45	0.13	113	1150
135	0.10	89	1124
270	0.10	144	694
495	0.10	194	515
960	0.09	394	228
d) K177E			
45	0.13	340	382
135	0.13	250	520
270	0.12	340	353
495	0.13	376	346
945	0.09	557	162

Table 4.5. Inhibitor constants of CoA analogues for wild type and mutant chloramphenicol acetyltransferases.

All analogues are competitive with respect to acetyl CoA. Steady-state kinetic analyses were performed as described (2.6) with the concentration of chloramphenicol fixed at 25μM.

K_i(mM)

Enzyme	ATP	5'AMP	3'AMP
K54/K177	1.0	5.4	4.6
K54Q	23.1	20.2	6.7
K177Q	1.9	12.8	4.3
K177E	3.7	19.3	5.3
K177A	3.7	20.9	9.6

Table 4.6. Apparent steady-state kinetic parameters of mutant chloramphenicol acetyltransferases modified with ethyl acetimidate.

Modification reactions were carried out as described (2.11) in the absence of substrates. Reaction was quenched by dilution into TSE (pH 7.5) containing 1mg/ml BSA and kinetic analyses performed as described (2.6) using a fixed concentration of chloramphenicol (100μM).

Addition		None		Acetimidate (100mM)	
Enzyme		$k_{cat}app(s^{-1})$	$K_{m}app(\mu M)$	$k_{cat}app(s^{-1})$	$K_{m}app(\mu M)$
K54/K177		485	81	538	456
K54Q		576	824	539	1458
K177Q		500	161	499	820
K177E		496	355	379	1880
K177A		468	477	511	2461

Chapter 5. Analogues of acetyl CoA.

5.1 Introduction.

Since many of the contacts of CoA with CAT involve protein main chain atoms such interactions can not be studied directly by protein engineering techniques. An alternative approach is to use substrate analogues in which one or more interacting groups have been removed. Analogues can be used in the form of alternative substrates, which allow the study of both binding and catalysis, or as inhibitors which give information about binding only. The main advantage of the use of substrate analogues, compared with changing the protein structure, is that the variety of chemical groups which can be substituted is well in excess of the 20 amino acids found in proteins. Also, unwelcome changes in structure and stability which can occur on amino acid substitution in proteins are much less likely to occur when using substrate analogues.

The development of methods for the chemical synthesis of CoA has allowed the synthesis of many CoA analogues such as guanoCoA, dethioCoA, dephosphoCoA and desaminoCoA (see Moffatt and Khorana, 1959, 1961; Baddiley and Thain, 1953). Such analogues have been used to determine the importance of specific groups to CoA binding for several enzymes (e.g. Choline acetyltransferase, Rossier, 1977; Currier and Mautner, 1977; carbon monoxide dehydrogenase, Raybuck et al., 1988; thiolase, Davis et al., 1987). In many cases the synthetic route to such analogues is tortuous and the published yields are poor.

5.2 Acetyl pantetheine.

In order to study substrate analogues in which the thioester moiety of CoA has been changed; for example, to an oxyester, a simpler but tight binding analogue of CoA would be a more attractive target for chemical synthesis. Pantetheine, a truncated derivative of CoA in which the adenosine 3' phosphate 5' diphosphate moiety (3-phosphoADP) has been removed, and substituted pantetheines are good substrates for a biosynthetic thiolase (Davis et al., 1987), although 4-phosphopantetheine is not an inhibitor of rat brain choline acetyltransferase (Rossier, 1977). To see if such an analogue of CoA could be used with the CAT system the kinetic parameters for acetyl pantetheine were determined.

Acetyl pantetheine (Table 5.1) is a very poor substrate for CAT. The rate observed in the standard CAT assay (containing $400\mu M$ acetyl donor) is more than three orders of magnitude lower than that for acetyl CoA. The K_m for acetyl pantetheine is more than 1500-fold greater than that for acetyl CoA and k_{cat} is reduced 13-fold (Table 5.2, fig. 5.1). Removal of the 3-phosphoADP moiety of CoA therefore, results in a 2×10^4-fold decrease in the specificity (k_{cat}/K_m) of the enzyme for the coenzyme. The 3-phosphoADP moiety of CoA contributes 2.9 kcal/mol and 4.3 kcal/mol to the binding of CoA to the free enzyme (K_d) and to the CAT/chloramphenicol binary complex (K_m) respectively. Its removal also reduces transition-state stabilisation by 6.2 kcal/mol. Thus, assuming that the enzyme catalysed reactions, using acetyl CoA and acetyl pantetheine as the acetyl donor, have the same rate determining step, the 3-phosphoADP moiety of CoA appears

to contribute more binding energy for binding of the transition-state, than to binding to either the free enzyme or the CAT/chloramphenicol binary complex. The use of acetyl pantetheine as the acetyl donor also results in a 20-fold increase in the K_m for chloramphenicol, corresponding to a weakening in the binding of the chloramphenicol to the CAT/CoA binary complex of 1.8 kcal/mol. The K_d for chloramphenicol deduced from steady-state kinetics is only slightly increased (less than 2-fold) as expected, since the K_d is a function of binding to the free enzyme. For both substrates removal of the 3-phosphoADP moiety from CoA results in greatly reduced binding to the corresponding binary complex to form a ternary complex (K_m), with a much smaller effect on binding to the free enzyme to form a binary complex (K_d). Thus, a major function of the 3-phosphoADP moiety of CoA appears to be the correct formation of a productive ternary complex and stabilisation of the transition-state, although it clearly influences affinity for acetyl CoA in binary complex formation.

5.3 Substituted pantetheines.

In an attempt to improve the binding of acetyl pantetheine, several analogues substituted at the primary hydroxyl group have been made which could potentially retain the specific enzyme/substrate interactions found for CoA or introduce novel interactions (Table 5.1).

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5.3.1 4-phosphopantetheine.

Addition of a phosphate group to the primary hydroxyl group of pantetheine yields 4-phosphopantetheine (2.16) an intermediate in the biosynthesis of CoA (Hoagland and Novelli, 1954). The charged phosphate group might be expected to strengthen binding of pantetheine by introducing possible electrostatic interactions with Lys 54 and/or Lys 177 (Chapter 4). However, the presence of two negative charges, where only one would be present with CoA, could perturb binding.

Although the K_m for acetyl 4-phosphopantetheine is almost 5-fold lower than that for acetyl pantetheine, the K_m for chloramphenicol and k_{cat} are relatively unchanged (Table 5.2). In fact, the phosphate group of 4-phosphopantetheine contributes only 1.0 kcal/mol in apparent binding energy, consistent with the relatively small changes in ΔG_{app} observed for mutations which removed electrostatic interactions between the above lysyl residues and CoA (Chapter 4). This result suggests that the phosphate groups of 4-phosphopantetheine and CoA probably bind in a similar position.

5.3.2 11-O-succinyl pantetheine.

Succinyl groups are similar in length to diphosphate moieties, thus addition of a succinyl group to the primary hydroxyl group of pantetheine (2.15) might be expected to allow interactions normally associated with the 5' phospho-anion of CoA on binding. Furthermore, the flexibility of a

succinyl group might allow it to form novel interactions with Lys 54 as observed with the γ-phosphate of ATP (4.7).

Although the K_m for acetyl 11-O-succinyl pantetheine is 4.4-fold lower than that for acetyl pantetheine, it is still more than 300-fold greater than that for acetyl CoA (Table 5.2, fig. 5.2). As in the case of acetyl pantetheine, the K_m for chloramphenicol is greatly increased and k_{cat} is decreased. The corresponding 4-fold increase in k_{cat}/K_m for acetyl 11-O-succinyl pantetheine, as compared with acetyl pantetheine, yields a calculated contribution to binding for the charged succinyl group of 0.8 kcal/mol.

5.3.3 11-O-pivaloyl pantetheine.

Due to the conformation of the CoA binding site, when CoA is bound to CAT, the adenine moiety of CoA is in close proximity to the end of the pantetheine arm and is in fact in van der Waals' contact with the dimethyl group. Thus, the addition of a large, relatively hydrophobic group to the primary hydroxyl group of pantetheine could enhance binding by exploiting the hydrophobic adenine binding surface.

Such an analogue, acetyl 11-O-pivaloyl pantetheine (kindly provided by J.Williams, Department of Chemistry) gave a rate in the standard assay more than 600-fold lower than that observed with acetyl CoA as the acetyl donor, suggesting that the presence of a trimethylacetyl (pivaloyl) group does not significantly improve binding of pantetheine. Due to the low yield on synthesis and the very slow rate observed in the standard assay for acetyl 11-O-pivaloyl pantetheine the

kinetic parameters for this analogue were not determined.

5.4 A role for the 3-phosphoADP moiety of CoA.

5.4.1 In the absence of the 3-phosphoADP moiety of CoA, acetyl pantetheine may be able to bind in other, less catalytically favourable conformations. For example, the pantetheine arm of acetyl pantetheine could bind deeper into the CoA 'tunnel' such that the acetyl moiety interferes slightly with chloramphenicol binding, resulting in a higher than expected K_m for both substrates, and possibly also a decrease in k_{cat}. Alternative binding modes for acetyl pantetheine are not unreasonable because of the relatively nonspecific nature of many interactions between enzyme and the pantetheine arm of CoA. Most hydrogen bonds to the pantetheine arm involve one or more water molecules and the direct interaction between the side chain of Ser 148 and N71 is rather long. Also, removal of the 3-phosphoADP moiety of CoA removes part of the pantetheine binding site, the adenine ring normally being in van der Waals' contact with the dimethyl group. However, the changes in K_m observed on addition of charged groups to the primary hydroxyl group of pantetheine support the view that pantetheine does bind in a similar manner to the pantetheine arm of CoA.

5.4.2 In unpublished experiments, P.C.E.Moody and A.G.W.Leslie showed that addition of CoA to crystals of substrate free CAT or CAT/chloramphenicol binary complex results in rapid disintegration of the crystals, suggesting that binding of CoA to CAT might cause significant structural

changes in the enzyme. Such a change is most likely to be induced by the adenosine moiety of CoA as addition of ATP or 5' AMP also results in disintegration of such crystals. A further possibility is that addition of CoA causes crystal dissolution due to chelation of the cobalt ions which are essential for crystal integrity, rather than to CoA binding itself. Addition of excess cobalt ions may exclude this possibility. Addition of chloramphenicol to crystals of the CAT/CoA binary complex has no effect on crystal integrity, but subsequent structure determination showed that no chloramphenicol was bound (P.C.E.Moody and A.G.W.Leslie, unpublished results).

Since the structures of substrate-free CAT and both binary complexes are essentially superimposable it follows that CoA probably does not cause a significant conformational change on binding to CAT to form a binary complex. However, due to the difficulty in reproducing crystals of the CAT/CoA binary complex, and the lower resolution (0.24nm) of its structure, some caution is warranted. In order to show that a conformational change does occur on binding of CoA to CAT independent evidence (for example, from fluorescence studies) is required. Most important of all is structural data on the ternary complex itself.

5.5 Effect of 3'AMP on the kinetic parameters for acetyl pantetheine.

In order to investigate the possibility that adenine nucleotides might be able to substitute for the 3-phosphoADP moiety of CoA in the catalytic reaction, 3'AMP (5mM) was

added to assays containing 1mM acetyl donor. With acetyl CoA as the acetyl donor, the addition of 3'AMP results in a 25% decrease in activity, compared with a 50% increase in activity when acetyl pantetheine is the acetyl donor. The K_m for both acetyl pantetheine and chloramphenicol is reduced 2-3-fold, but k_{cat} is virtually unchanged (Table 5.2). Although 3'AMP appears to increase activity slightly, the adenosine and pantetheine moieties of CoA must be covalently connected for full activity.

5.6 Acetyl CoA analogues.

In order to solve the structure of the ternary complex, crystals of CAT with both substrates bound are required. However, catalysis must be prevented to stop turnover of substrates. This can be achieved by using either nonreactive substrate analogues or bisubstrate analogues. The latter are often very tight binding inhibitors and one such analogue, citrylthioether CoA, was used in the elucidation of the ternary complex structure of citrate synthase (Remington et al., 1982). Due to the nature of the active site of CAT, were substrates bind from opposite ends of a long 'tunnel', a bisubstrate analogue would be unlikely to bind, thus if crystals of the ternary complex are to be obtained an unreactive analogue of acetyl CoA is required.

5.6.1 Acetonyl CoA.

Acetonyl CoA, a thioether analogue of acetyl CoA, is a good inhibitor of citrate synthase, phosphotransacetylase and

carnitine acetyl transferase with values of K_i generally less than 2-fold greater than those of the K_m for acetyl CoA (Rubenstein and Dryer, 1980). Thus this compound, which is relatively simple to make, appeared suitable for studies with CAT.

Propionyl CoA is a good substrate for CAT (K_m = 167μM and k_{cat} = 709s^{-1}, Kleanthous and Shaw, 1984) suggesting that the active site can accommodate an extra methylene group without greatly reducing binding. Acetonyl CoA, synthesised as described (2.17), is an inhibitor of CAT and is competitive with respect to acetyl CoA. The calculated K_i for acetonyl CoA (Table 5.3) is more than 8-fold greater than the K_d for acetyl CoA determined from steady-state kinetic analysis suggesting that the position of the substrate carbonyl group has a significant effect on binding. Thus acetonyl CoA is probably not a suitable candidate for crystallographic studies.

5.6.2 2-Hydroxypropyl CoA.

An attractive feature of acetonyl CoA is the potential for chemical reduction of the carbonyl group to give a CoA analogue with tetrahedral geometry in the 'acyl' moiety, which could perhaps act as a transition-state analogue.

Reduction of acetonyl CoA with sodium borohydride (2.18) produces a mixture of epimers of 2-hydroxypropyl CoA. Due to the difficulty in separation of optical isomers the compound was used as the mixture for kinetic analysis. In fact both epimers may be able to form transition-state like hydrogen bonds with either His 195 or Ser 148 (fig. 5.3). The K_i for

2-hydroxypropyl CoA (Table 5.3) is 25-fold lower than that of the trigonal parent compound, acetonyl CoA, suggesting that the tetrahedral geometry does allow formation of transition state-like interactions. The low K_i also makes this compound potentially useful for crystallographic studies, possibly allowing the structure of pseudo-transition states of both the hydrolysis and transacetylation (in the presence of chloramphenicol) reactions to be solved, provided that suitable crystals can be obtained.

5.7 Discussion.

5.7.1 Truncated analogues of CoA, based on acetyl pantetheine, are very poor substrates for CAT. Such analogues appear to have low affinities and their use results in a large increase in the K_m value for chloramphenicol. It is likely that acetyl pantetheine either binds to CAT in a different fashion than does the pantetheine arm of CoA, or that it fails to induce a conformational change which may normally occur on CoA binding. The disintegration of CAT crystals on addition of CoA suggests that such a conformational change does occur. Although adenine nucleotides also cause disintegration of crystals, implicating direct involvement of the adenosine moiety of CoA in a conformational change, 3'AMP can not replace the 3-phosphoADP moiety of CoA in assays containing acetyl pantetheine as the acetyl donor. Thus, for full catalytic activity, the adenosine and pantetheine moieties must be covalently linked to each other.

Due to the high K_m values and potential problems with

alternative binding modes or conformational changes, pantetheine-based analogues are unsuitable as a tool to investigate the role of specific interactions between CoA and CAT. Thus, in order to study the role of, for example, the sulphur atom or of hydrogen bonds to the pantetheine arm of CoA, total synthesis of CoA analogues is required.

5.7.2 The active site of CAT can accommodate an extra substrate methylene group without greatly affecting binding (Kleanthous and Shaw, 1984). However, the position of the carbonyl group does seem to be important. Acetonyl CoA has a K_i more than 8-fold greater than the corresponding calculated K_d for acetyl CoA, but on reduction of its carbonyl group to produce 2-hydroxypropyl CoA, the K_i is reduced to only $9.6\mu M$. Enhanced binding of 2-hydroxypropyl CoA might be expected if the catalytic reaction proceeds via a tetrahedral intermediate, however due to the presence of an extra methylene group in this compound, the introduction of novel interactions can not be ruled out.

More easily interpreted data could be derived from binding studies with acetonyldethio CoA, an analogue in which the sulphur atom of acetyl CoA is replaced with a methylene group such that the 'acyl' chain length is not altered. Acetonyldethio CoA is a competitive inhibitor of citrate synthase (Stewart and Wieland, 1978) and a substrate for acetyl CoA carboxylase (Nikawa et al., 1978) suggesting that substitution of the sulphur atom with a methylene group does not significantly alter the conformation of 'acetyl CoA'. Reduction of this compound gives a mixture of epimers of 2-hydroxypropyldethio CoA, which might be expected to bind

tightly to CAT if a tetrahedral intermediate is important in the transacetylation reaction.

Unfortunately, synthesis of acetonyldethio CoA involves total CoA synthesis (Stewart and Wieland, 1978) and is, therefore, both difficult and time consuming. However, if 2-hydroxypropyldethio CoA does act as a transition-state analogue only small quantities would be required. Such analogues should prove useful for both kinetic and crystallographic studies.

Figure 5.1. Lineweaver–Burk plots for the acetylation of chloramphenicol by CAT_{III} using acetyl pantetheine as the acetyl donor.

Assays were performed as described (2.5) at 25°C over a range of chloramphenicol concentrations (5, 10, 20 and 100μM) for each of four fixed acetyl pantetheine concentrations a) 0.25mM b) 0.5mM c) 1mM and d) 4mM. Reciprocal initial rates were plotted against reciprocal chloramphenicol concentration for each concentration of acetyl pantetheine (A) and steady-state kinetic parameters calculated from intercept and slope replots (B). Data was also plotted as reciprocal rates against reciprocal acetyl CoA concentration for each chloramphenicol concentration (not shown).
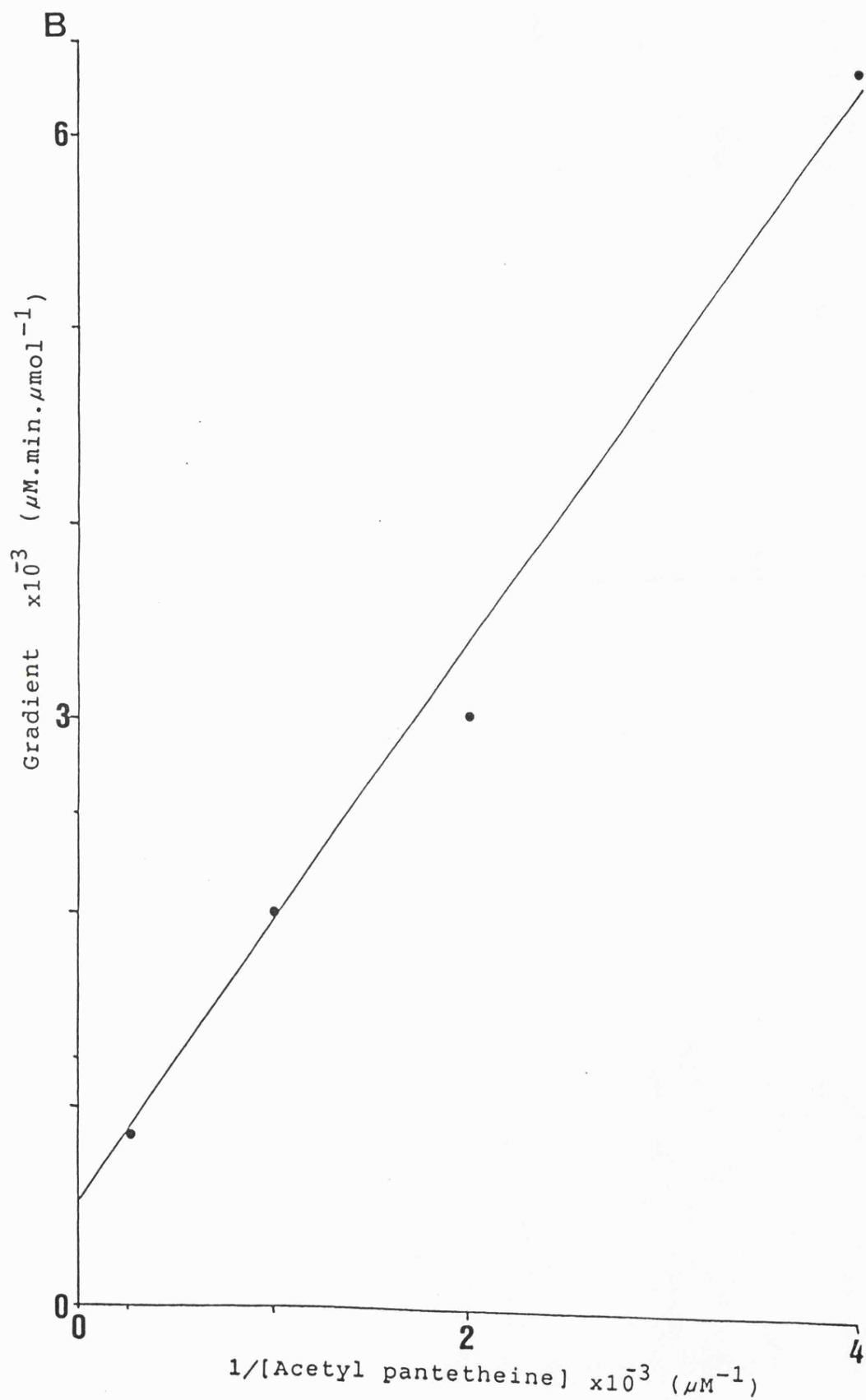
A

$1/V_i \times 10^{-3} \; (\mu mol/min.^{-1})$

25

15

5

0

a

b

c

d

0   0·1   0·2
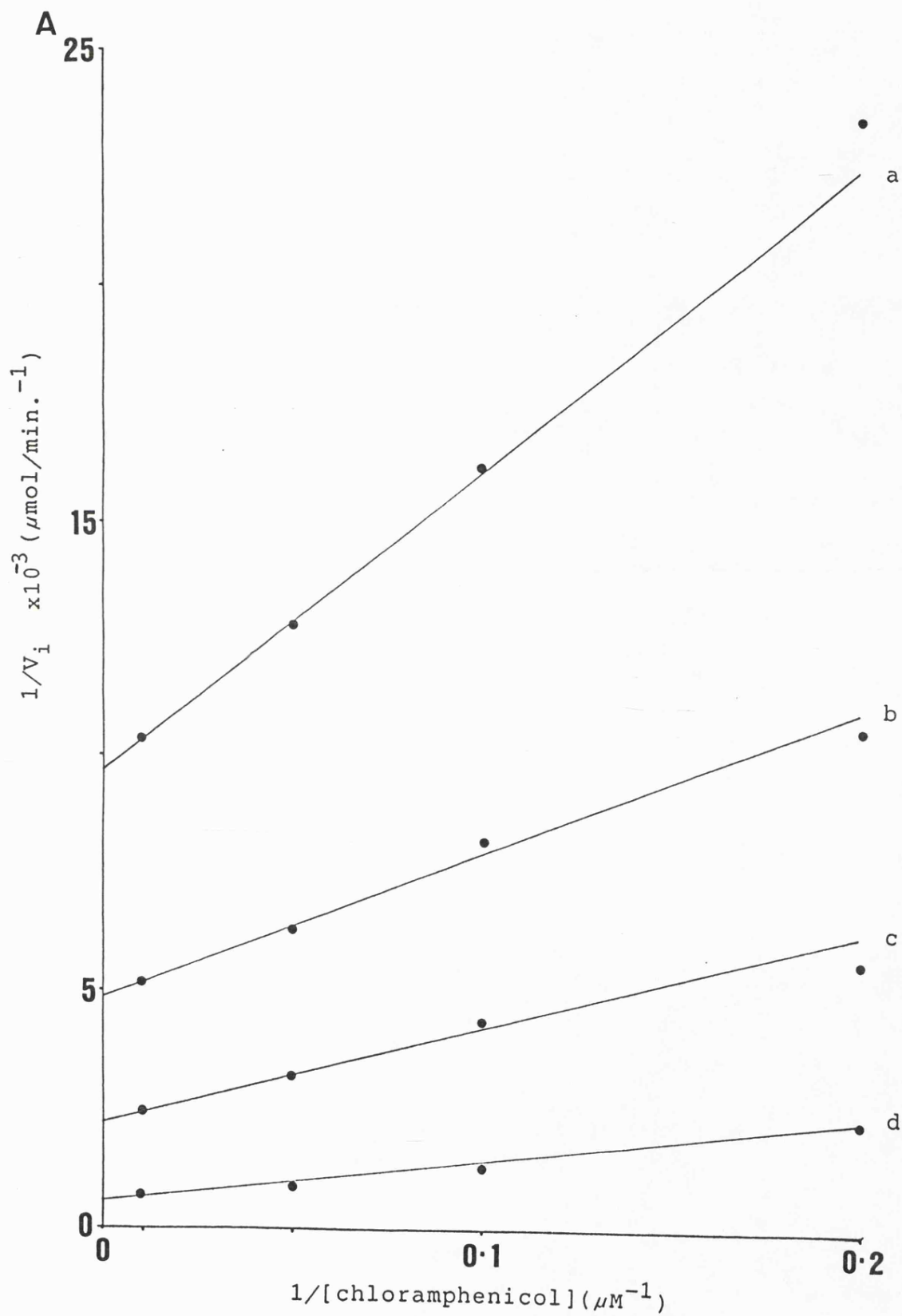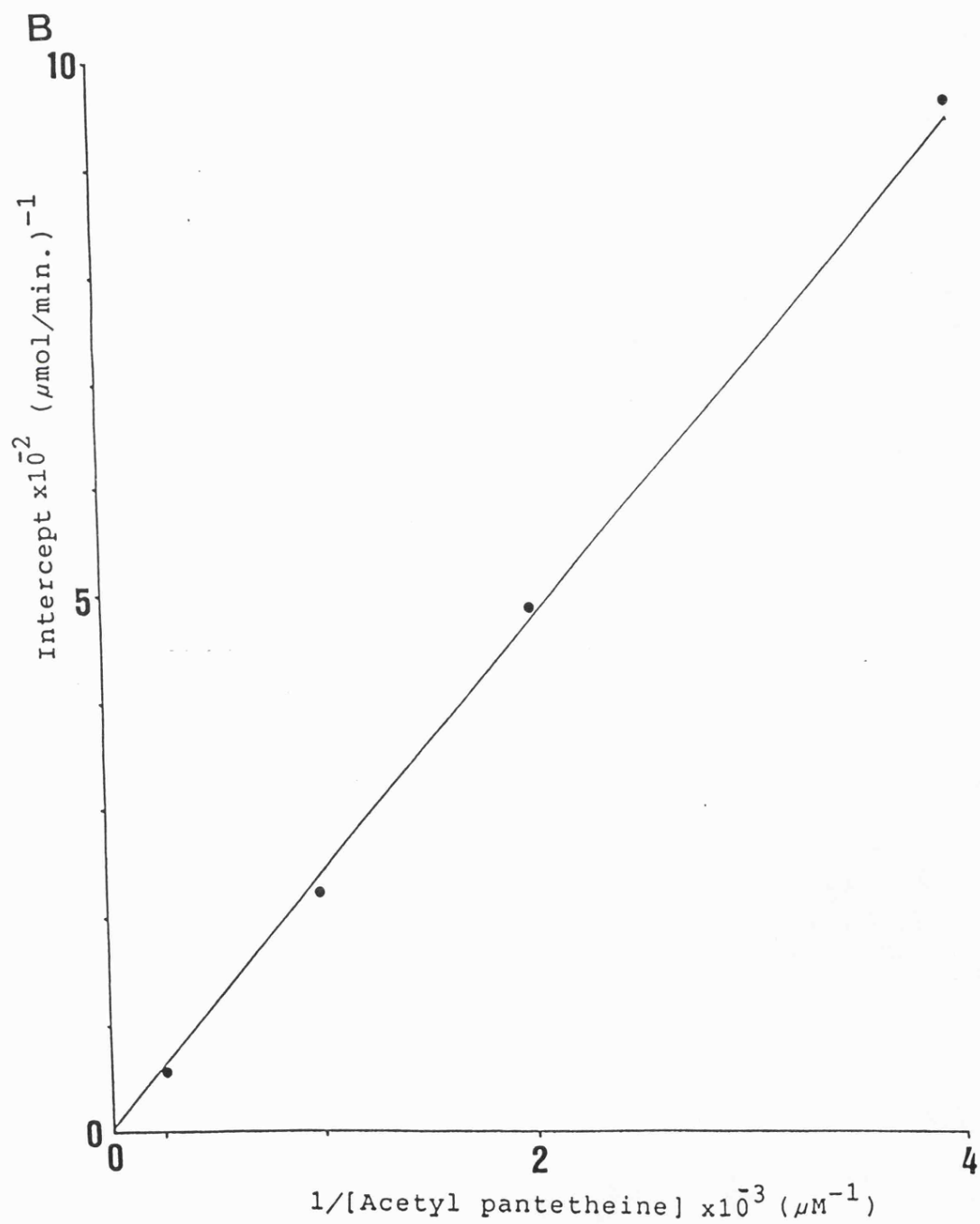
$1/[chloramphenicol](\mu M^{-1})$

Figure 5.2. Lineweaver-Burk plots for the acetylation of chloramphenicol by CAT_{III} using acetyl 11-0-succinyl pantetheine as the acetyl donor.

Assays were performed as described (2.5) at 25°C over a range of chloramphenicol concentrations (5, 10, 20 and 100μM) for each of eight fixed acetyl 11-0-succinyl pantetheine concentrations a) 0.125mM b) 0.16mM c) 0.2mM d) 0.25mM e) 0.33mM f) 0.5mM g) 1mM h) 1.4mM. Reciprocal initial rates were plotted against reciprocal chloramphenicol concentration for each concentration of acetyl 11-0-succinyl pantetheine (A) and steady-state kinetic parameters calculated from intercept and slope replots (B). Data was also plotted as reciprocal rates against reciprocal acetyl pantetheine concentration for each chloramphenicol concentration and kinetic parameters calculated from secondary plots (not shown).
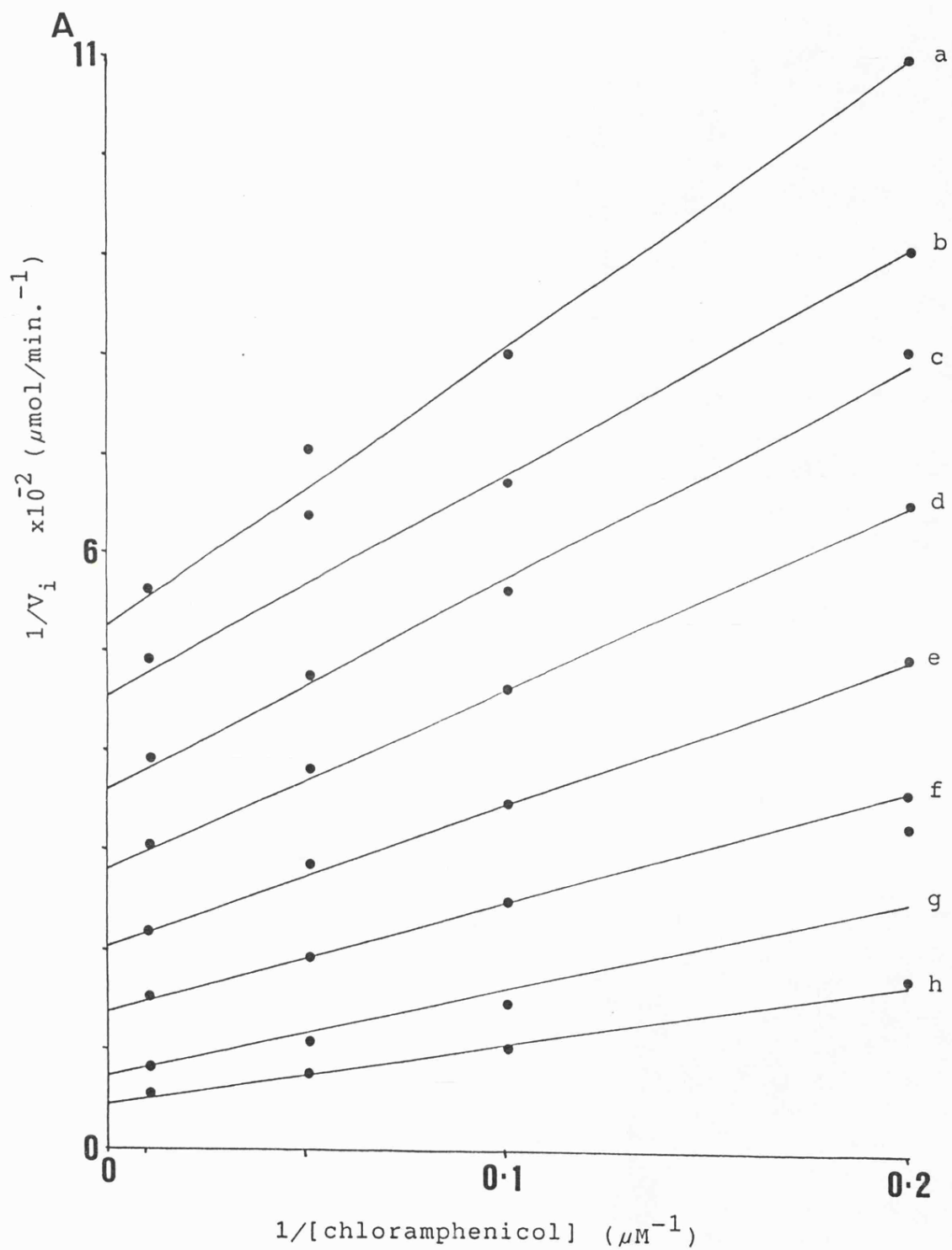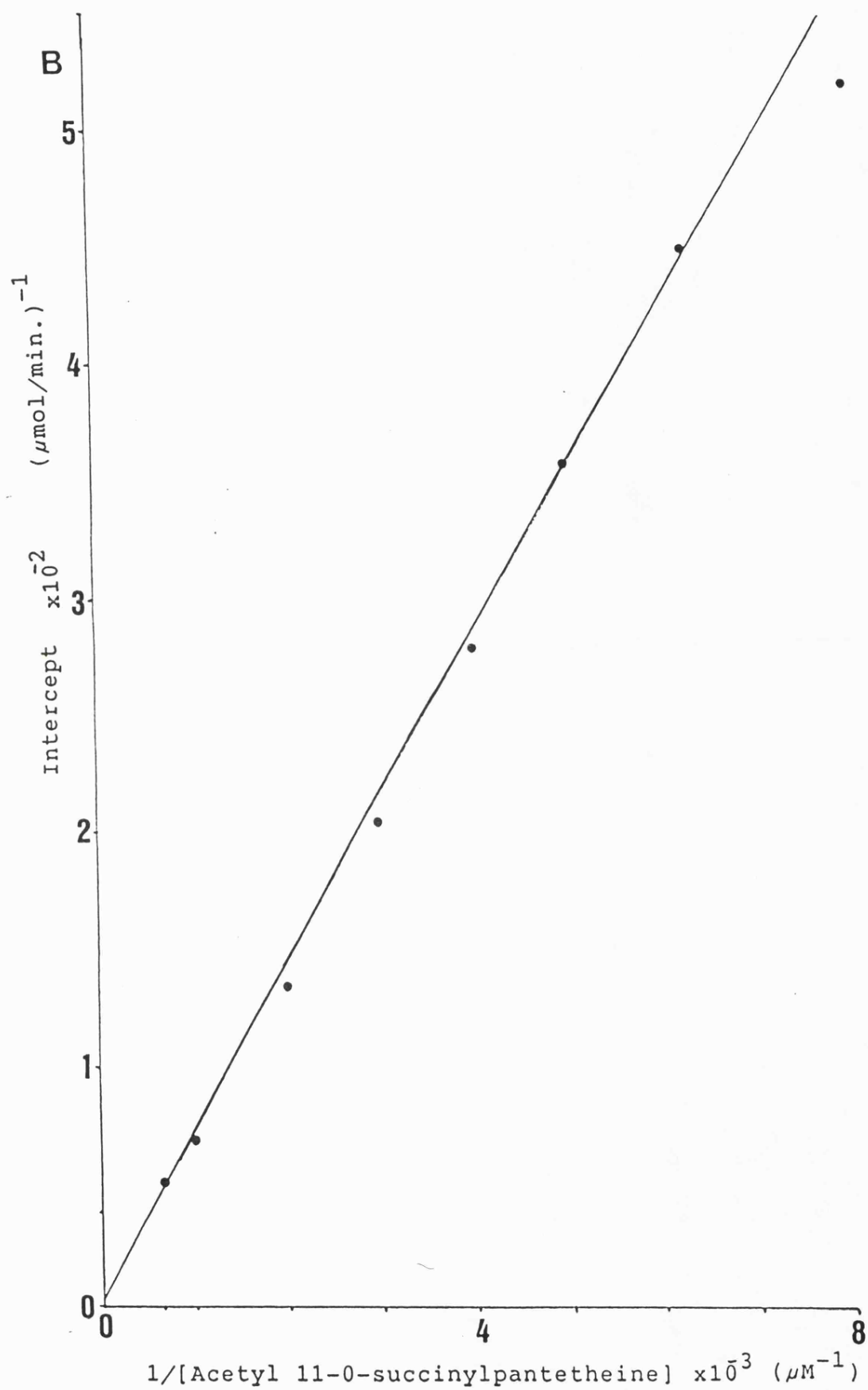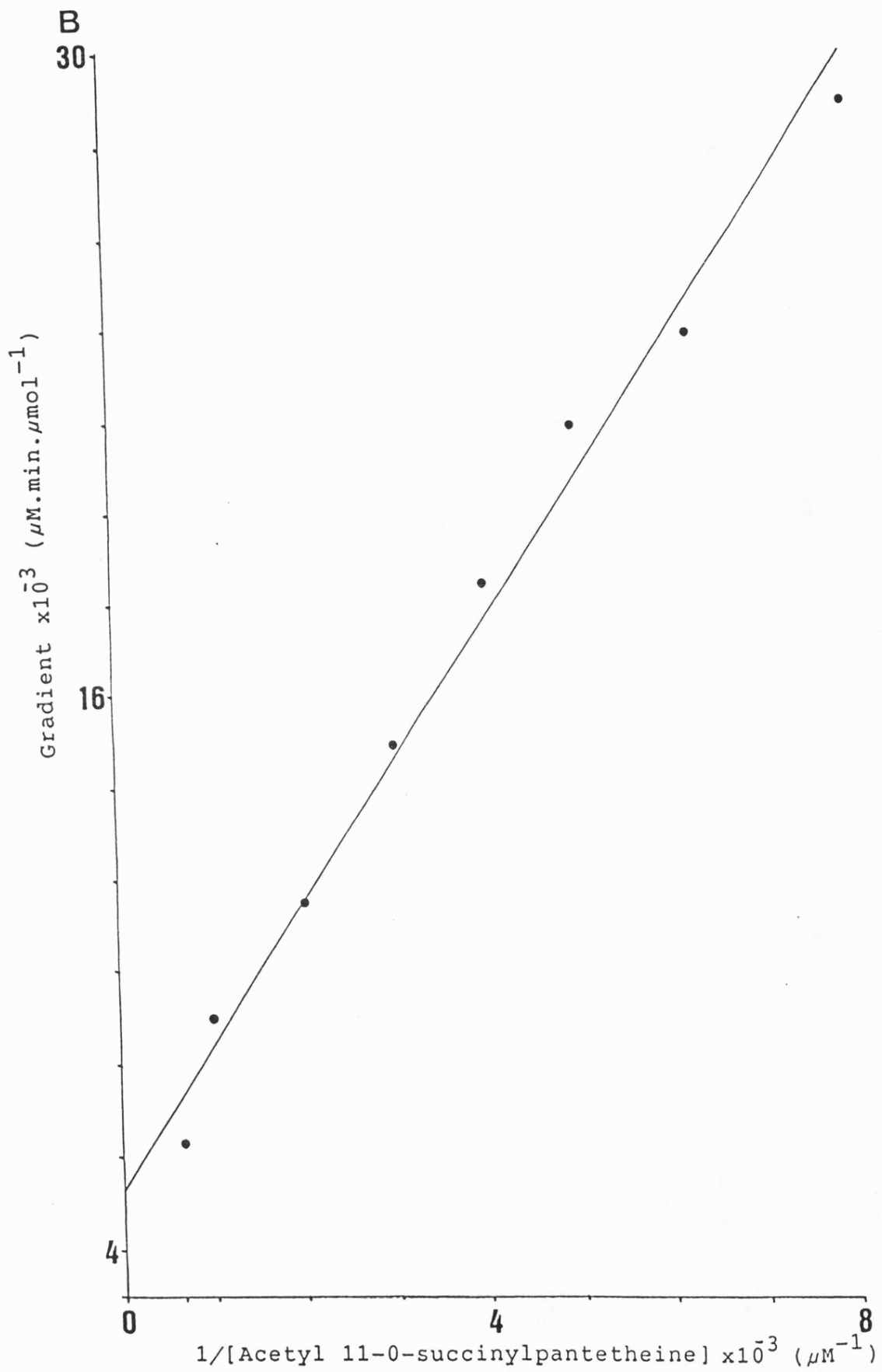
Figure 5.3. Comparison of the structure of the tetrahedral intermediate proposed for the reaction of acetyl CoA with chloramphenicol with that of 2-hydroxpropyl CoA

Acetyl CoA   Acetonyl CoA

$CH_3COSCoA$ CH_3COCH_2SCoA
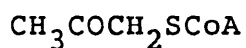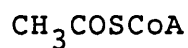
Tetrahedral intermediate (THI)   2-Hydroxypropyl CoA epimers

$$\overset{HO\ \ H}{\underset{|}{\diagdown\ /}}$$
$$CH_3-C-CH_2SCoA$$

$$\overset{RO\ \ O^-}{\underset{|}{\diagdown\ /}}$$
$$CH_3-C-SCoA$$

R = chloramphenicol

$$\overset{H\ \ OH}{\underset{|}{\diagdown\ /}}$$
$$CH_3-C-CH_2SCoA$$
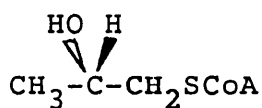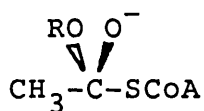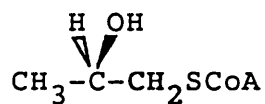
Proposed interactions of the THI

$$CH_3-C-SCoA$$
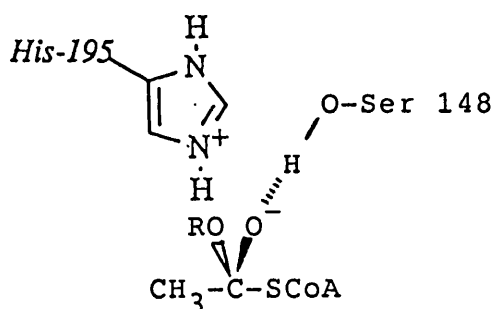
Table 5.1. The structures of pantetheine based analogues of acetyl CoA.

$CH_3COSCH_2CH_2NHCOCH_2CH_2NHCOCH(OH)C(CH_3)_2CH_2OR$

Analogue	R
Acetyl pantetheine	-H
Acetyl 4-phosphopantetheine	$-PO_3^-$
Acetyl 11-0-succinylpantetheine	$-COCH_2CH_2COO^-$
Acetyl 11-0-pivaloylpantetheine	$-COC(CH_3)_3$

Table 5.2. The steady-state kinetic parameters for alternative acyl donors.

Due to the presence of small amounts of hydrolysed material (<0.5%) in all preparations of acetyl pantetheine and derivatives, the maximum substrate concentration which could be used was only 10mM.

A = acyl donor

B = chloramphenicol

Acyl donor (A)	$k_{cat}(s^{-1})$	$K_m A(mM)$	$K_m B(\mu M)$	$K_d A(mM)$	$K_d B(\mu M)$
Acetyl CoA	600	0.093	12	0.031	4
Acetyl pantetheine	46	143	243	3.9	7
Acetyl 4-Phosphopantetheine	49	30	175	1.1	6
Acetyl 11-0-Succinylpantetheine	42	32	189	1.1	7
Acetyl pantetheine (+5mM 3'AMP)	44	74	72	7.3	7

Table 5.3. Inhibitor constants for analogues of acetyl CoA and comparison with binding constants for acetyl CoA.

Analogue		$K_i (\mu M)$	$K_m (\mu M)$	$K_d (\mu M)$
Acetyl CoA	$[CH_3COSCoA]$	–	93	31
Acetonyl CoA	$[CH_3COCH_2SCoA]$	248	–	–
2-Hydroxypropyl CoA	$[CH_3CH(OH)CH_2SCoA]$	9.6	–	–

Chapter 6. The role of hydrophobic interactions in the binding of acetyl CoA to CAT_{III}.

6.1. Introduction.

Hydrophobicity can be described as the tendency for non-polar compounds to transfer from aqueous solution to a non-polar phase such as a liquid hydrocarbon. The presence of a hydrophobic compound in an aqueous system results in local ordering of water molecules, thus promoting a loss in entropy of solvent water. The driving force for the sequestration of hydrophobic amino acid side chains to the interior of proteins is thought to be the gain in entropy of solvent water coincident with protein folding rather than the affinity of hydrophobic side chains for each other. An additional factor is the effect of increased dispersion interactions (van der Waals' forces) in a folded protein.

A measure of the hydrophobicity of a compound can be gained from its partition coefficient in aqueous/organic phases of two phase systems or from its relative solubility in a range of solvents. Cyclohexane, n-octanol and ethanol are often used as the organic phase for the determination of such parameters (Radzicka and Wolfenden, 1987; Nozaki and Tanford, 1971; Hansch and Coats, 1970). The hydrophobicity of amino acid side chains (relative to glycine, R=H) has been found to correlate well with the distribution of amino acids in protein structures and also with the calculated solvent accessible surface area of the residue (X) side chain in the extended form of the tripeptide Gly-X-Gly (Miller et al., 1987; Chothia, 1976). From such studies the magnitude of the hydrophobic effect has been estimated to be 20-30 cal/mol/Å^2 (Chothia, 1975; Eisenberg and McLachlan, 1986; Ooi et al., 1987; Reynolds et al., 1974).

The role of the hydrophobic effect in protein folding and stability has been widely studied (Garvey and Matthews, 1988; Matsumura et al., 1988a/b; Yutani et al., 1987). Often the residue in question has been identified by the isolation of thermolabile mutants and studied by replacement with all possible amino acids. For several proteins changes in stability on mutation were found to correlate well with the hydrophobicity of the amino acid (Kellis et al., 1989; Matsumura et al., 1988a/b; Yutani et al., 1987). Another approach to quantitating the hydrophobic effect is the selection of residues which are involved in interactions between secondary structures. By introducing subtractive mutations individually at several residues, an average contribution to protein stability of 1.0-1.5 kcal/mol per methylene group is observed (Kellis et al., 1988, 1989).

Estell et al. (1986) showed that similar approaches could be used to investigate the role of hydrophobic interactions in substrate binding. By changing the size and hydrophobicity of the P1 substrate binding site of subtilisin the specificity (k_{cat}/K_m) of the enzyme can be tailored to favour specific substrates. For example, substitution of Gly 166 with Ile results in a protease that is 16-fold more efficient with small (Val and Ala) P1 substrates, but is more than 1000-fold less efficient with Tyr substrates. Furthermore, the free energy of binding of synthetic peptides to the wild type enzyme (calculated from $ln[1/K_m]$ and $ln[k_{cat}/K_m]$) was found to be linearly related to the hydrophobicity of the P1 substrate.

The CoA binding site of CAT consists of the side chains of several hydrophobic residues, thus hydrophobic

interactions are likely to play a major role in CoA binding (fig. 1.9). To investigate the magnitude of such interactions in CoA binding to CAT mutations which change the hydrophobicity of specific amino acid side chains were made at two sites, one involving interactions with the adenosine moiety of CoA and the other involving interactions with the pantetheine chain.

6.2 Tyrosine 178.

Tyr 178, a residue near to the protein surface, is either Tyr or Phe in 10 of the 11 known CAT sequences. The main chain amide of Tyr 178 forms a hydrogen bond with N1 of the adenine ring of CoA and the tyrosyl side chain forms a major part of the adenine binding pocket (fig. 6.1). The side chain aromatic ring is almost coplanar with the adenine ring of CoA and forms a staggered stacking interaction with it. The two ring systems are separated by 0.3-0.4nm. Staggered stacking of aromatic rings is favoured to full stacking to allow maximum overlap of the partial charges generated in aromatic ring systems (Singh and Thornton, 1985; Burley and Petsko, 1985). The hydroxyl group of Tyr 178 appears to make no direct interactions with CoA.

The tyrosyl side chain of residue 178 is also in van der Waals contact with the phenyl ring of Phe 55, a non-conserved residue which also forms part of the adenine binding pocket. The orientation of the two rings is one which is commonly seen for intramolecular aromatic/aromatic interactions in proteins (see fig. 6.1).

6.3 Interactions of Tyr 178 with CoA.

To investigate the role of Tyr 178 in binding of CoA a series of substitutions were made which remove the hydroxyl group (Y178F) or change the size and relative hydrophobicity of the residue side chain (Y178I,Y178V and Y178A).

Tyr 178→Phe.

The structure of the wild type CAT/CoA binary complex shows that the hydroxyl group of Tyr 178 does not contribute significantly to CoA binding. Not surprisingly, the substitution Y178F does not significantly alter the kinetic parameters of CAT (Table 6.1).

Tyr 178→Ala.

Complete removal of the aromatic ring of Tyr 178 (Y178A) results in a 20-fold increase in the K_m for acetyl CoA with a small decrease in k_{cat} (Table 6.1). The specificity constant (k_{cat}/K_m) for acetyl CoA is reduced almost 40-fold, yielding a calculated contribution to the binding of acetyl CoA, for the aromatic ring of residue 178, of 2.2 kcal/mol (Table 6.2), almost 24% of the total apparent free energy of binding. This change in ΔG_{app} is however, considerably smaller than the changes in ΔG(unfolding) observed for subtractive mutations of hydrophobic residues in the hydrophobic core of proteins (Kellis et al., 1988, 1989). A smaller change in ΔG for subtractive mutations in substrate binding sites is not unexpected because such substitutions

usually result in the removal of a single binding surface, but substitutions in the closely-packed protein core result in the removal of interactions with several surfaces.

In the phosphofructokinase of E. coli, Tyr 55 is stacked with the purine ring of the activator GDP (or ADP) in a manner similar to that of Tyr 178 with CoA in CAT. Removal of this interaction by site-directed mutagenesis (Y55G) results in only a 5.5-fold increase in K_d, corresponding to a contribution to activator binding of just 1.0 kcal/mol (Lau et al., 1986), only half that observed for the Y178A substitution in CAT.

6.4 Effect of hydrophobicity on CoA binding.

The K_m (and K_d) for acetyl CoA increase as the size and hydrophobicity of the side chain of residue 178 is decreased. Substitution of Tyr 178 with Ile (Y178I) results in an almost 7-fold increase in K_m for acetyl CoA and the Y178V substitution yields a 19-fold increase, with corresponding contributions to binding of 1.1 kcal/mol and 2.0 kcal/mol respectively (Table 6.2). The kinetic parameters of the Y178V and Y178A mutants are very similar, suggesting that the isopropyl group of valine may form only limited interactions with CoA. Both of these mutations also result in a small decrease in k_{cat}.

For substitutions of residues involved solely in hydrophobic interactions with substrate, the apparent binding energy (ΔG_{app}) might be expected to be linearly related to the hydrophobicity of the residue side chain. Plots of ΔG_{app} calculated from k_{cat}/K_m and $1/K_d$, as a function of

hydrophobicity for the Tyr 178 mutants show a linear relationship with a gradient of 1.2, as expected if the interactions between Tyr 178 and CoA are predominantly hydrophobic in nature (fig. 6.2). A gradient greater than unity also suggests that the environment of Tyr 178 is more hydrophobic than ethanol and dioxane, the solvents used for the determination of amino acid hydrophobicities (Nozaki and Tanford, 1971).

6.5 Tyrosine 56.

Tyr 56 is one of only 22 residues absolutely conserved in all 11 known CAT sequences. It lies at the amino terminal end of α-helix 3 and its side chain forms a major part of the CoA 'tunnel' (see fig. 1.9). Tyr 56 interacts with the pantetheine arm of CoA, the tyrosyl ring centre being between 0.35nm and 0.50nm from O52, C44, C45 and C53 of CoA (fig. 6.3). The phenolic hydroxyl group appears not to be involved in interactions with CoA, but probably has a structural function. The hydroxyl group can potentially form hydrogen bonds with both the main chain carbonyl oxygen and amide group of Thr 94, a well conserved residue which forms part of both the chloramphenicol and CoA binding sites. Conservation of these hydrogen bonds suggests that they may be important for the architecture of the CoA binding tunnel, for protein stability, or for both functions. The aromatic side chain of residue 56 is part of a cluster of aromatic groups, which includes the well conserved residues Tyr 121 and Phe 93, again suggesting a role for Tyr 56 in stabilisation of the folded protein structure (fig. 6.3).

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6.6 Interaction of Tyr 56 with CoA.

The role of Tyr 56 in the binding of CoA was investigated by making the same amino acid substitutions as described for Tyr 178 (6.3) in order to allow a direct comparison of the effects of replacements at both sites.

Tyr 56→Phe.

As predicted from the structure of the CAT/CoA binary complex removal of the conserved hydroxyl group from Tyr 56 (Y56F) did not significantly alter the K_m for acetyl CoA or that for chloramphenicol. Nonetheless, k_{cat} is slightly reduced (Table 6.3).

Effect of residue 56 side chain hydrophobicity on CoA binding.

Reduction in the size and hydrophobicity of residue 56 side chain results in a small reduction in k_{cat} and a small increase in the K_m for acetyl CoA. The K_m for chloramphenicol was relatively unchanged in each case (Table 6.3). Removal of the aromatic ring (Y56A) results in a 7.5-fold decrease in specificity (k_{cat}/K_m), with a corresponding decrease in binding energy of 1.2 kcal/mol (Table 6.4). The decrease in specificity is due equally to changes in k_{cat} and K_m, unlike the case for substitutions at Tyr 178, where changes in K_m are the dominant factor. Also, for the substitutions at residue 56 the K_m for acetyl CoA does not increase steadily as the hydrophobicity of the residue side chain is decreased.

Indeed, the highest K_m is that observed for the Y56I substitution. The substitutions Y56V and Y56A yield enzymes with very similar kinetic parameters as observed for the corresponding substitutions at Tyr 178.

Plots of ΔG_{app} calculated for changes in k_{cat}/K_m and $1/K_d$ against side chain hydrophobicity are shown in figure 6.4. The plots are linear, confirming that there is a relationship between the hydrophobicity of the side chain of residue 56 and the binding of acetyl CoA. The gradients of the least squares regression lines are 0.76 and 0.68 respectively, suggesting that the environment of Tyr 56 is less hydrophobic than ethanol.

The substitutions Y56I and Y56V introduce side chains which, unlike that of tyrosine are branched at the β-carbon. Modelling of such substitutions into the CAT/CoA binary complex structure shows that β-branched side chains may introduce a steric clash between the γ-methyl group of the side chain and O36 of CoA, with the expectation that the K_m (and K_d) for the Y56I and Y56V mutants could be larger than would be predicted from hydrophobicity considerations alone. As a consequence, further substitutions by amino acids which are not branched at the β-carbon (e.g. leucine) need to be made to define more precisely the relationship between side chain hydrophobicity per se and the kinetic parameters for CoA binding for mutants at Tyr 56.

6.7 Thermostability of mutant CAT proteins.

Wild type CAT_{III} is very resistant to thermal inactivation, losing little activity on incubation at 70°C

for more than one hour (Lewendon et al., 1988). Incubation at 80°C results in rapid inactivation with only 50% activity remaining after 5 minutes (fig. 6.5). This loss of activity is not reversible on cooling to room temperature.

All of the mutant enzymes tested (Y56F/A, Y178F/A) are less stable than wild type at 80°C and this property is particularly notable for substitutions of residue 56 (fig. 6.6). The changes in stability are, nonetheless, rather small suggesting that Tyr 56 and Tyr 178 play only a minor role in the stabilisation of protein structure.

6.8 Discussion.

Much of the CoA binding site consists of conserved hydrophobic residues. Two such residues (Tyr 56 and Tyr 178) interact with very different parts of the CoA molecule and consequently the substitution of each of these has quite different effects on the steady-state kinetic parameters of CAT. Mutations at residue 178, which interacts with the adenine moiety of CoA, result in large increases in the K_m for acetyl CoA. Removal of the aromatic side chain (Y178A) results in a 20-fold increase in K_m with only a small decrease in k_{cat}, and weakens binding of the transition-state by 2.2 kcal/mol. In contrast, replacing the phenolic side chain of Tyr 56 with a methyl group (Y56A) causes only small changes in both the K_m for acetyl CoA and k_{cat}, and weakens transition-state binding by 1.4 kcal/mol. Thus, Tyr 178 is predominantly, and importantly, involved in substrate binding, whilst Tyr 56 appears to play a smaller role in both binding and catalysis.

The effects of substitutions of Tyr 56 and Tyr 178 on acetyl CoA binding are due to changes in the hydrophobicity of the residue side chain. The log of the kinetic parameters for the series of mutations at residue 56 and 178 is linearly related to the side chain hydrophobicity, suggesting that this is the major factor affecting substrate binding for both residues. More mutations need to be made at both sites to confirm this relationship however, as such plots often show significant scatter (e.g. see Matsumura et al., 1988a/b; Yutani et al., 1987).

Tyr 56 is intimately involved not only in interactions between enzyme and substrate, but also those between enzyme secondary structures. It is therefore not surprising that Tyr 56 is conserved in all known CAT variants. However, contrary to expectation removal of these interactions by mutagenesis appears to have only a modest effect on both acetyl CoA binding and thermostability. As Tyr 56 is solvent accessible in the absence of CoA, water molecules may be able to occupy any cavity produced on mutation and replace the hydrogen bonds normally associated with the phenolic hydroxyl group, hence minimising changes in stability. The yields of mutant proteins (Table 6.5) are comparable with that for the wild type enzyme, suggesting that the stability of the mutants is not significantly altered in vivo.

<u>Figure</u> <u>6.1.</u> <u>Interactions</u> <u>of</u> <u>Tyr</u> <u>178.</u>

A. Residues within 0.75nm of the γ-carbon atom of Tyr 178.
Predicted hydrogen bonds are depicted as dashed lines.

B. Van der Waals' radii of Tyr 178, Phe 55 and CoA.

A

PHE 55
TYR 178
CoA
PRO 151

B

TYR178

PHE55

CoA

Figure 6.2. The relationship between the apparent free energy
of binding of acetyl CoA and the hydrophobicity of the side
chain of residue 178.

Apparent binding energies were calculated from kcat/K_m ($\bullet$)
and 1/K_d (O) and the free energies of transfer from aqueous
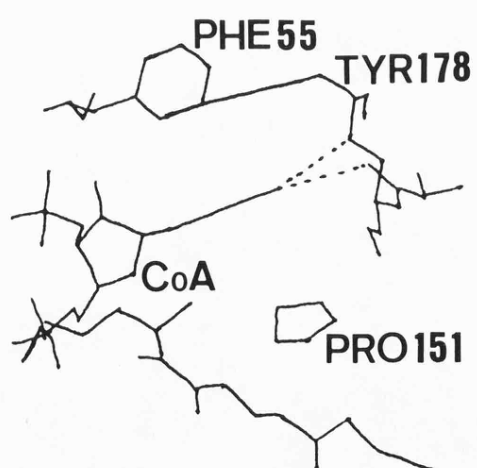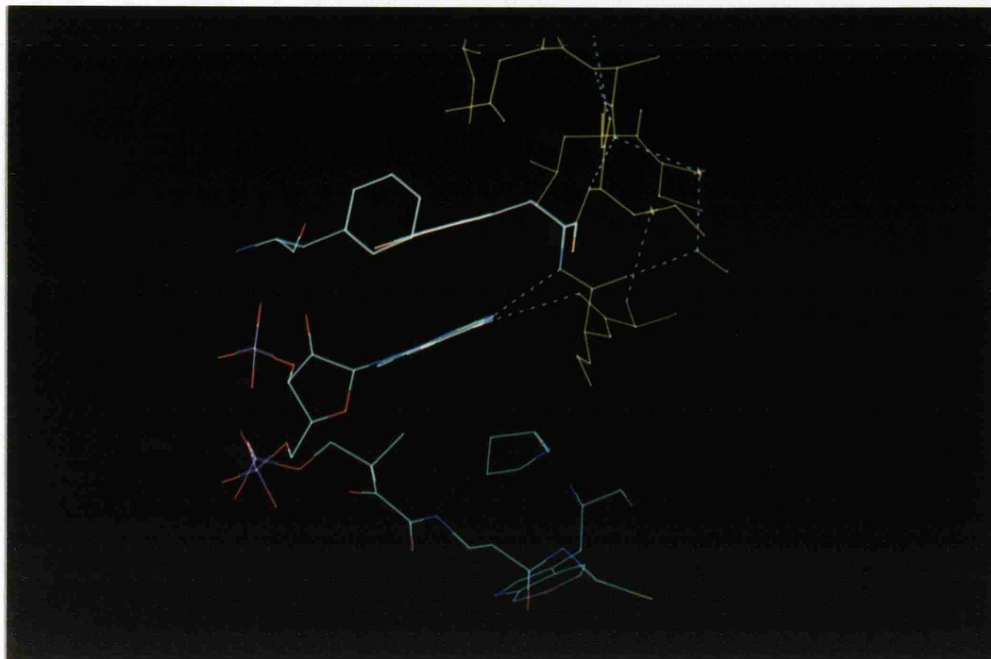solution to ethanol (ΔG[transfer]) taken from Nozaki and
Tanford (1971).

Figure 6.3. Interactions of Tyr 56.

A. Residues within 0.75nm of the γ-carbon atom of Tyr 56.
Predicted hydrogen bonds are depicted as dashed lines.

B. Van der Waals' radii of Tyr 56 and CoA.

A

CoA

THR94

PHE

TYR56

TYR121

B

CoA

TYR56

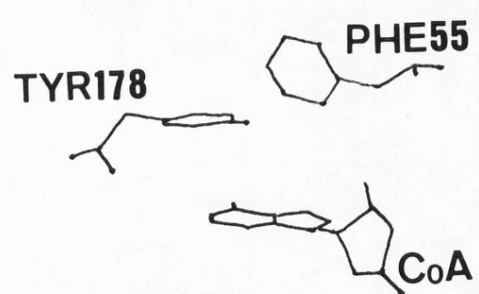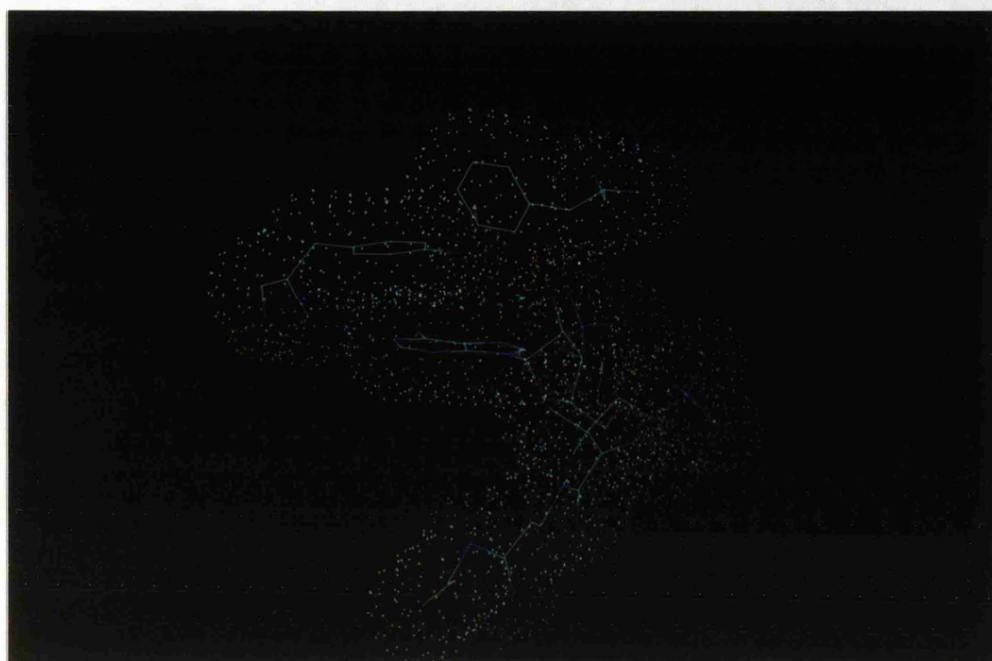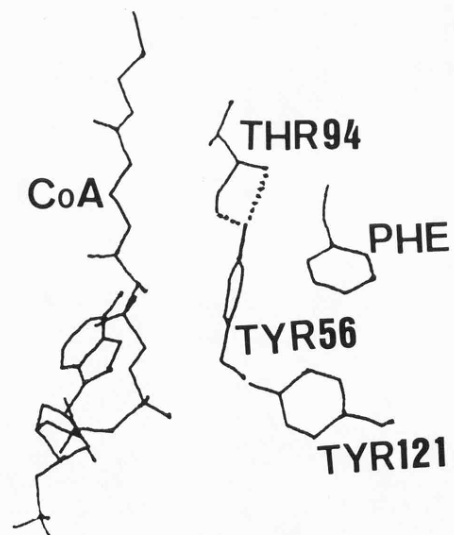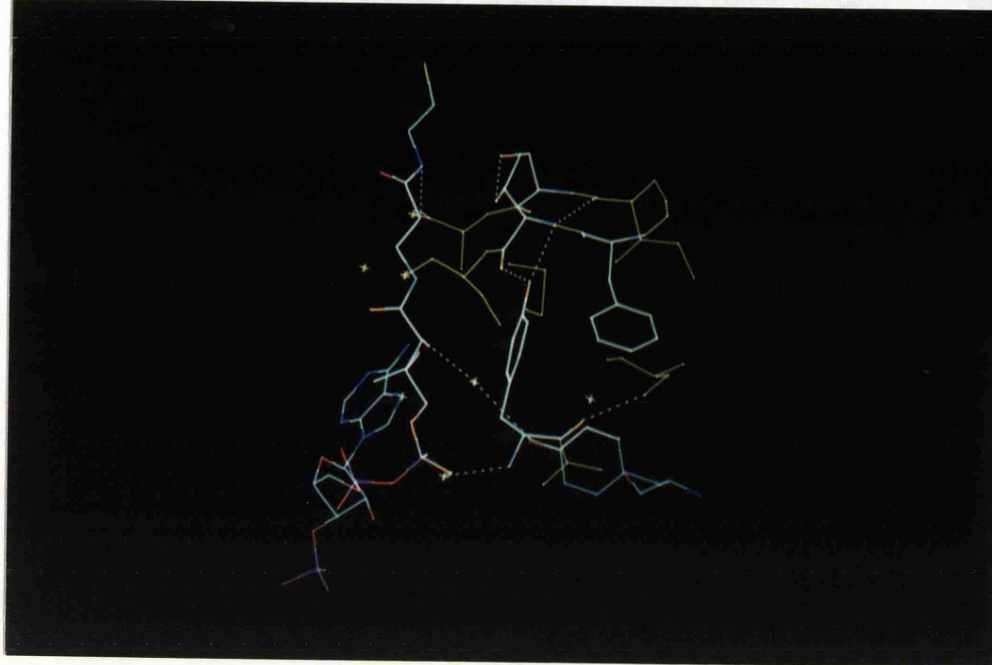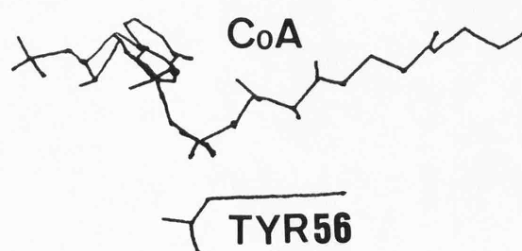**Figure 6.4.** Relationship between apparent free energy of binding of acetyl CoA and the hydrophobicity of the side chain of residue 56.

Apparent binding energies were calculated from $kcat/K_m$ (●) and $1/K_d$ (○) and the free energies of transfer from aqueous solution to ethanol ($\Delta G[transfer]$) taken from Nozaki and Tanford (1971).
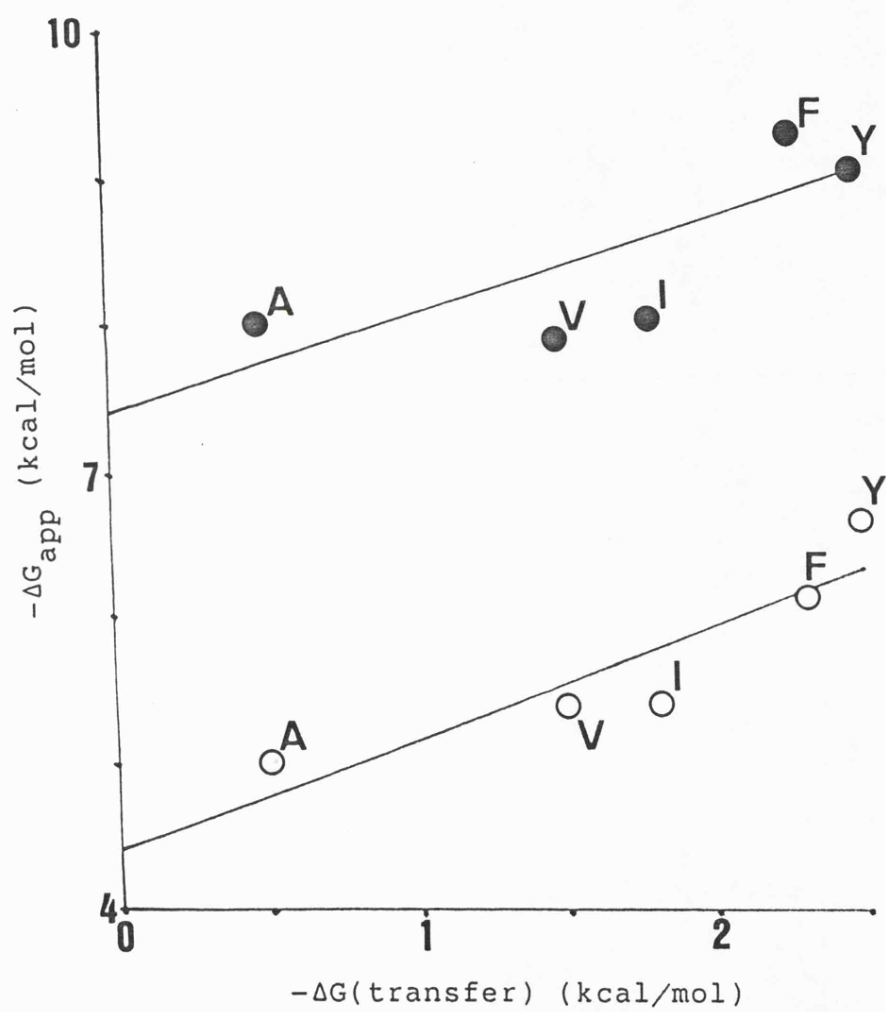
<u>Figure</u> <u>6.5.</u> <u>The</u> <u>time</u> <u>course</u> <u>of</u> <u>inactivation</u> <u>of</u> <u>wild</u> <u>type</u> <u>and</u>
<u>mutant</u> <u>chloramphenicol</u> <u>acetyltransferases</u> <u>on</u> <u>incubation</u> <u>at</u>
<u>80°C.</u>

Enzyme (0.3μM) was incubated in TSE at 80°C as 50μl
aliquots in 0.5ml Eppendorf tubes. A series of tubes were
incubated and one removed at timed intervals. A sample was
diluted if necessary and assayed for CAT activity (2.5).

Key:wild type ●

    Y178F   O

    Y178A   ■

    Y56F   ▲

    Y56A   △
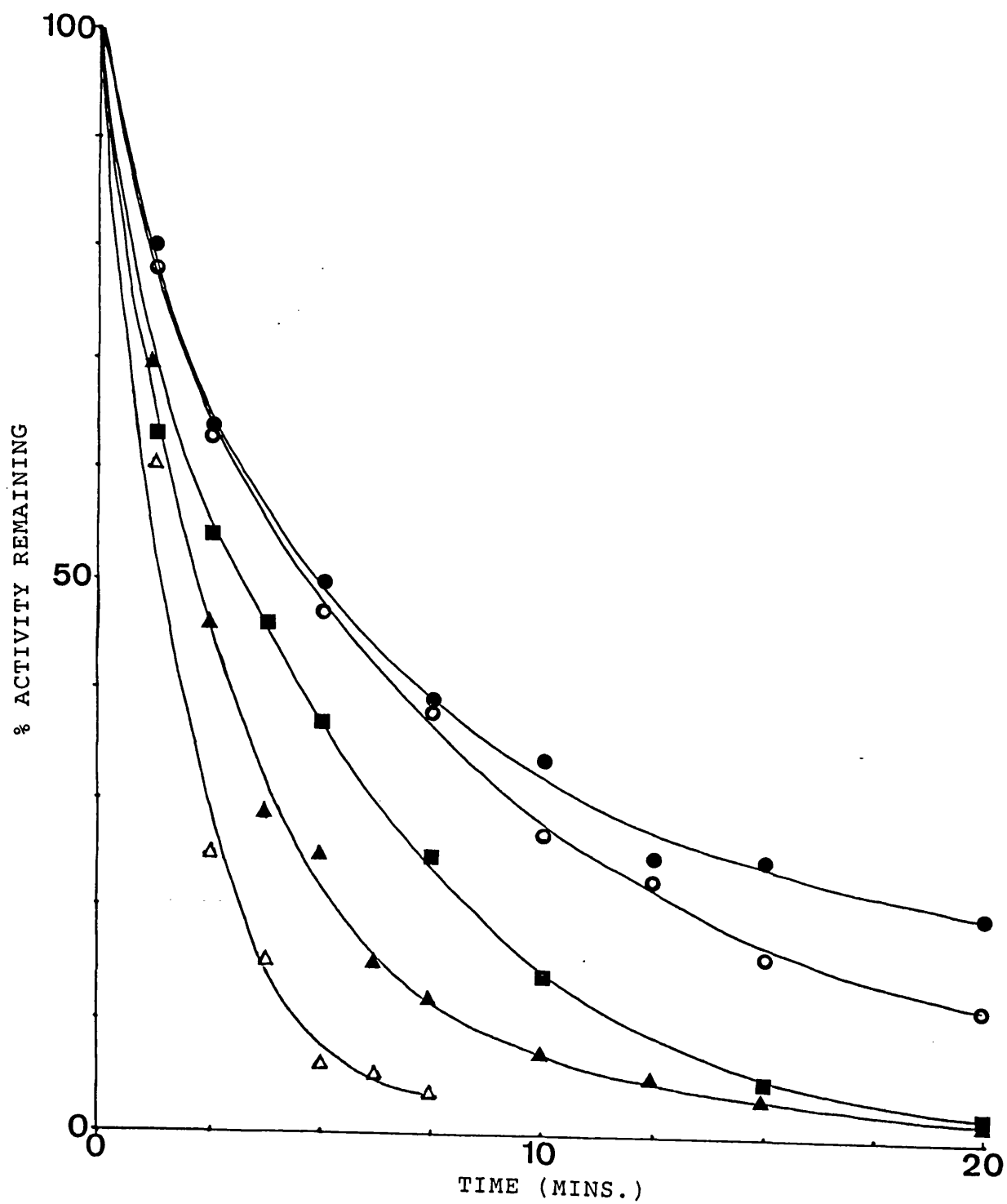
Table 6.1. Steady-state kinetic parameters of mutant chloramphenicol acetyltransferases.

Enzyme	$k_{cat}(s^{-1})$	$K_mA(\mu M)$	$K_mB(\mu M)$	$K_dA(\mu M)$	$K_dB(\mu M)$
Y178	600	93	12	31	4
Y178F	625	110	15	18	2
Y178I	609	622	20	148	5
Y178V	359	1778	18	581	6
Y178A	281	1836	15	960	8

A = acetyl CoA, B = chloramphenicol

Table 6.2. The calculated changes in the apparent free energy of binding of acetyl CoA on substitution of Tyr 178.

$$\Delta G_{app}1 = -RT\ln([kcat/K_m]'/[kcat/K_m])$$

$$\Delta G_{app}2 = -RT\ln(K_d/K_d')$$

$[kcat/K_m]$, K_d specificity constant and dissociation constant for acetyl CoA of wild type CAT.

$[kcat/K_m]'$, K_d' specificity constant and dissociation constant for acetyl CoA of the mutant enzyme.

Enzyme	$\Delta G_{app}1(kcal/mol)$	$\Delta G_{app}2(kcal/mol)$
Y178F	<0.1	−0.3
Y178I	1.2	0.9
Y178V	2.0	1.7
Y178A	2.2	2.0

Table 6.3. Steady-state kinetic parameters of mutant chloramphenicol acetyltransferases.

Enzyme	$k_{cat}(s^{-1})$	$K_mA(\mu M)$	$K_mB(\mu M)$	$K_dA(\mu M)$	$K_dB(\mu M)$
Y56	600	93	12	31	4
Y56F	404	91	13	12	2
Y56I	264	305	11	102	4
Y56V	166	249	14	110	6
Y56A	221	256	10	201	8

A = acetyl CoA, B = chloramphenicol

Table 6.4. The calculated changes in the apparent free energy of binding of acetyl CoA on substitution of Tyr 56.

$$\Delta G_{app}1 = -RT\ln([kcat/K_m]'/[kcat/K_m])$$

$$\Delta G_{app}2 = -RT\ln(K_d/K_d')$$

$[kcat/K_m]$, K_d specificity constant and dissociation constant for acetyl CoA wild type CAT.

$[kcat/K_m]'$, K_d' specificity constant and dissociation constant for acetyl CoA of the mutant enzyme.

Enzyme	$\Delta G_{app}1(kcal/mol)$	$\Delta G_{app}2(kcal/mol)$
Y56F	0.2	−0.6
Y56I	1.2	0.7
Y56V	1.4	0.7
Y56A	1.2	1.1

Table 6.5. The yields and specific activities of purified chloramphenicol acetyltransferase mutants.

Enzymes were purified as described (2.3) from 500ml cultures of E. coli JM101 bearing plasmid pUC18:III:ME1 containing an appropriately mutated cat_{III} gene.

1 unit = the amount of enzyme required to convert 1μmol substrate to product per minute.

Enzyme	specific activity (U/mg)	yield[*] (mg)
Y56,Y178 (wt)	895	126
Y56F	601	45
Y56I	263	122
Y56V	211	165
Y56A	283	138
Y178F	881	135
Y178I	580	68
Y178V	147	130
Y178A	114	154

* purified CAT from a 500ml culture (see 2.3).

Chapter 7. The role of Phe 33 in acetyl CoA
binding and in catalysis.

7.1 Introduction.

As the structure of the binary complex of CAT and acetyl
CoA is not known, the acyl binding site of CAT can only be
deduced from modelling studies. Such studies have implicated
three highly conserved hydrophobic residues, Phe 33, Phe 158
and Leu 160 in binding of the methyl group of the proposed
tetrahedral transition-state intermediate and also in
interactions with the methyl group of the trigonal substrate
acetyl group (A.G.W.Leslie, unpublished results). Phe 158 and
Leu 160 are provided by the subunit to which both substrates
are predominantly bound, whereas Phe 33 arises from the
facing subunit, which also provides the catalytic imidazole
of His 195 (fig. 7.1). The side chain phenyl rings of Phe 33
and Phe 158 are in van der Waals' contact with each other and
thus form an inter-subunit aromatic/aromatic interaction
which may be important for trimer stabilisation.

As the carbon chain length of the CoA-linked acyl group
is increased, the corresponding K_m value does so as well,
such that butyryl CoA is a relatively poor substrate for CAT
(k_{cat}/K_m reduced 7-fold, Kleanthous and Shaw, 1984). From the
modelled transition-state structure it is not at all obvious
where a butyryl group could bind, suggesting that one or more
of the proposed 'acetyl binding' residues might need to move
to accommodate larger acyl groups.

To investigate the acetyl binding site two substitutions
of residue 33 have been made. The substitution F33A was
predicted to remove all interactions between the substrate
methyl group and the side chain of residue 33. This
substitution might also facilitate binding of larger acyl

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groups by creating a larger acyl binding cavity. In contrast the substitution F33W introduces a larger side chain and should reduce the volume of the binding site, thus excluding larger acyl groups.

7.2 Substitutions of Phe 33.

7.2.1 F33A.

The substitution F33A results in a 2.5-fold increase in the K_m for acetyl CoA and a 2.7-fold decrease in k_{cat}, consistent with the proposal that Phe 33 side chain not only makes a contribution to binding of the acetyl group of acetyl CoA, but is also involved in transition-state stabilisation (Table 7.1). Binding of acetyl CoA is weakened by 0.5 kcal/mol, that of chloramphenicol by 0.4 kcal/mol, whilst transition-state stabilisation is reduced by 1.8 kcal/mol, suggesting that stabilisation of the transition-state is the major function of Phe 33. As Phe 33 can not make any direct interactions with chloramphenicol, the apparent decrease in affinity for chloramphenicol could be due to disruption of the active site on removal of the phenyl ring. A further possibility is that the increase in the K_m (and K_d) for chloramphenicol results from the decrease in hydrophobicity of the active site on substitution of Phe 33 with Alanine.

7.2.2 F33W.

Although substitution of Phe 33 with Trp (F33W) has no effect on the specificity constant for acetyl CoA, both k_{cat}

and the K_m (acetyl CoA) are reduced 4.5-fold (Table 7.1).
Thus, whereas acetyl CoA binds more tightly to the F33W
enzyme than to wild type, tighter binding is achieved only at
the expense of turnover. The decrease in k_{cat} could simply be
due to increased stabilisation of the acetyl CoA binary
complex (or the ternary complex) rather than the
transition-state. Alternatively, the increase in side chain
size may be important. The large indole side chain of Trp 33
may introduce steric constraints into the active site, either
by clashing with the tetrahedral transition-state or by
resulting in a conformational change in the active site.
Since substitutions which introduce larger side chains are
more likely to cause significant conformational changes, it
is essential that structure determinations for such mutant
enzymes are carried out. The substitution L160F, which also
introduces a larger side chain into the active site of CAT
yields an enzyme which is isostructural with the wild type,
suggesting that the active site of CAT may be relatively
flexible and can accommodate some increase in side chain
sizes (A.G.W.Leslie, unpublished results).

7.2.3 Since the F33W substitution results in decreased K_m
values for both substrates, it seems likely that, as noted
with the F33A substitution, the hydrophobicity of the side
chain of residue 33 may influence binding of both substrates.
As discussed in chapter 6, for mutant CAT variants in which a
hydrophobic interaction is altered, plots of ΔG_{app} against
the free energy of transfer of the side chain from an aqueous
to an organic phase for such substitutions should be linear.
Plots of ΔG_{app} calculated from k_{cat}/K_m, $1/K_m$ and $1/K_d$ for

substitutions of Phe 33 against the free energy of transfer
of the side chain from aqueous solution to ethanol, are
linear for both substrates (fig. 7.2) suggesting that the
hydrophobicity of residue 33 is important in binding of both
the acetyl group of acetyl CoA and, more surprisingly,
chloramphenicol.

7.3 Butyryl CoA.

7.3.1 Wild type CAT$_{III}$.

Butyryl CoA is a relatively poor substrate for CAT
(Kleanthous and Shaw, 1984). Although the K_m for butyryl CoA
is only 2.5-fold greater than that for acetyl CoA, k_{cat} is
also reduced by almost 3-fold (Table 7.2). Since the K_m for
chloramphenicol is also increased (albeit only 2-fold) it
seems likely that binding of butyryl CoA may interfere with
chloramphenicol binding. The decrease in k_{cat} was proposed to
result from a change in the rate-determining step, perhaps
due to disruption of the active site by the bulky butyryl
group (Kleanthous and Shaw, 1984).

7.3.2 F33A.

Introduction of a cavity in the acyl binding site might
be expected to improve butyryl CoA as a substrate. The F33A
substitution produces such a cavity, yielding an enzyme for
which butyryl CoA is a better substrate than acetyl CoA.
However, the changes in kinetic parameters are small, K_m for
butyryl CoA being only ~20% lower than that for acetyl CoA,

and k_{cat} increased only 1.3-fold. As observed with the wild type enzyme, the K_m for chloramphenicol is increased when butyryl CoA is the acyl donor.

The kinetic parameters of the wild type and F33A enzymes are very similar, suggesting that the acyl moiety of butyryl CoA does not bind in the cavity produced by removing the phenyl ring from residue 33 side chain. The small increase in k_{cat} could be due to increased flexibility of the F33A active site, allowing nearby residues to move to accommodate the butyryl group. The use of larger substrates (i.e. with larger acyl groups) would be useful to show whether the acyl chain of acyl CoA can bind in the cavity created by removing the aromatic ring of Phe 33.

7.3.3 F33W.

The K_m for butyryl CoA is more than 5-fold larger than that for acetyl CoA for the F33W mutant, whilst k_{cat} is relatively unchanged. As with acetyl CoA, the specificity constant (k_{cat}/K_m) for butyryl CoA of the F33W mutant approximates to that of wild type CAT even though the changes in kinetic parameters are very different (Table 7.2). Whereas with wild type CAT, k_{cat} decreases almost 3-fold with butyryl CoA as substrate, for F33W CAT no such decrease is observed. Such a result suggests that the two enzymes could have different rate-determining steps and that that for F33W CAT may be independent of the nature of the acyl donor.

As observed with both wild type and F33A CAT, the K_m for chloramphenicol for F33W CAT also increases when butyryl CoA is the acyl donor. It therefore seems likely that the larger

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acyl group may partially block the chloramphenicol binding site, and so binding of either substrate to form a ternary complex would be perturbed.

7.4 The kinetics of acyl CoA hydrolysis.

In the absence of chloramphenicol CAT can catalyse the hydrolysis of acyl CoAs (Zaidenzaig and Shaw, 1978). To investigate the possibility that the high K_m for butyryl CoA was mainly due to the presence of chloramphenicol, the kinetic parameters for the hydrolysis of butyryl CoA were determined.

The K_m values for acetyl and butyryl CoA are very similar for the hydrolytic reaction, suggesting that the large acyl group of butyryl CoA does interfere with chloramphenicol binding on formation of the ternary complex. However, the decrease in k_{cat}/K_m on changing acyl donor is the same for both the hydrolytic and transacylation reactions. The K_m values for the hydrolysis of acetyl CoA and butyryl CoA are also similar for F33W CAT, and as observed for the transacylation reaction, the K_m values are much smaller than those for wild type CAT (Table 7.3).

7.5 Discussion.

7.5.1 Substitutions of residue 33 show that the phenyl ring of Phe 33 makes a significant contribution to acetyl CoA binding in wild type CAT. Modelling studies predicted that Phe 33 is in van der Waals' contact with the methyl group of the acetyl moiety of both substrate and transition-state.

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Plots of ΔG_{app} against the free energy of transfer of residue side chains from aqueous solution to ethanol for the Phe 33 mutants confirm that Phe 33 is probably involved in such a hydrophobic interaction with acetyl CoA. Also the hydrophobicity of the side chain of residue 33 affects the binding of chloramphenicol in a similar manner. An analogous situation arises for substitutions of Leu 160, another residue implicated in forming part of the acetyl binding site. Substitution of Leu 160 with a more hydrophobic residue (L160F) results in decreased K_m values for both substrates, whereas substitution with a more hydrophilic residue (L160Q) results in increases in substrate K_m values (I.A.Murray, unpublished results). However, although the structure of L160F CAT is identical to wild type CAT (except at the site of mutation) chloramphenicol binds in a significantly differently position such that the 3-hydroxyl group is further from N3-His 195 accounting for the marked decrease in k_{cat} observed for this mutant (A.G.W.Leslie, unpublished results).

7.5.2 Should the F33W mutant prove to be isostructural with wild type CAT (as is the case for L160F), it may be useful as a tool for the study of the unfolding of CAT or dissociation of trimers. As Phe 33 interacts with Phe 158, a residue on the facing subunit, on dissociation of trimers to monomers the environment of Phe 33 will change from hydrophobic to aqueous. Such changes in environment result in large changes in the fluorescence emission spectra of tryptophan residues. Thus F33W CAT could provide a fluorescence signal to distinguish between monomers and trimers and therefore allow

trimer stability to be investigated by mutagenesis to impair
inter-subunit interactions.

Figure 7.1. Interactions of Phe 33 with the modelled transition-state intermediate.

For clarity, chloramphenicol and CoA have been omitted. The dots represent the Van der Waals' radii of the residues and the dashed lines are predicted hydrogen bonds. Residue names followed by ● belong to an adjacent subunit. (The transition-state was modelled by P.C.E.Moody and A.G.W.Leslie).
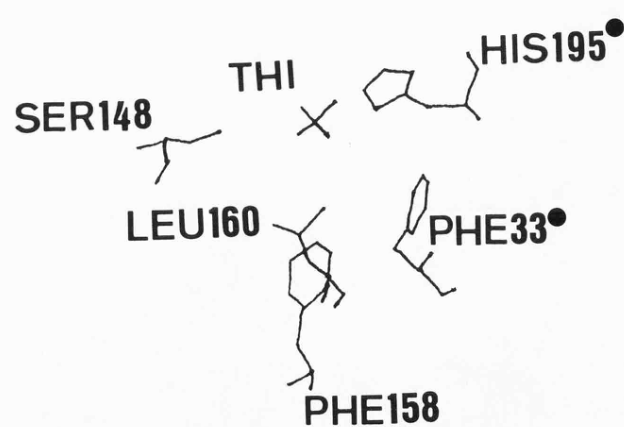
HIS195

THI

SER148

PHE33

PHE158

LEU160

SER148　THI　　　　　HIS195●

LEU160　　　　　PHE33●

PHE158
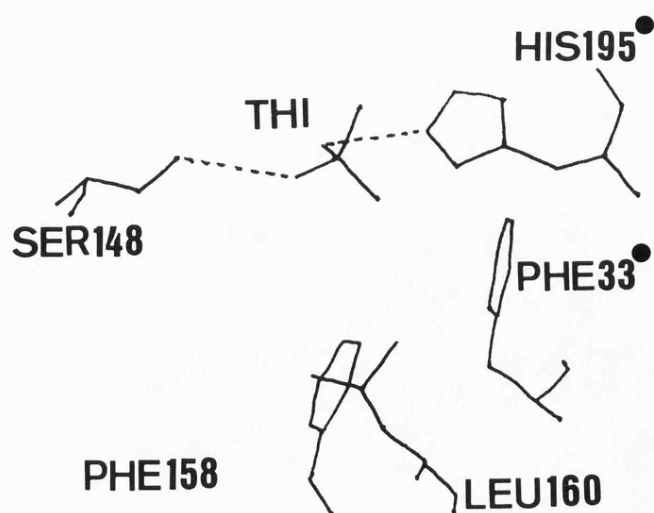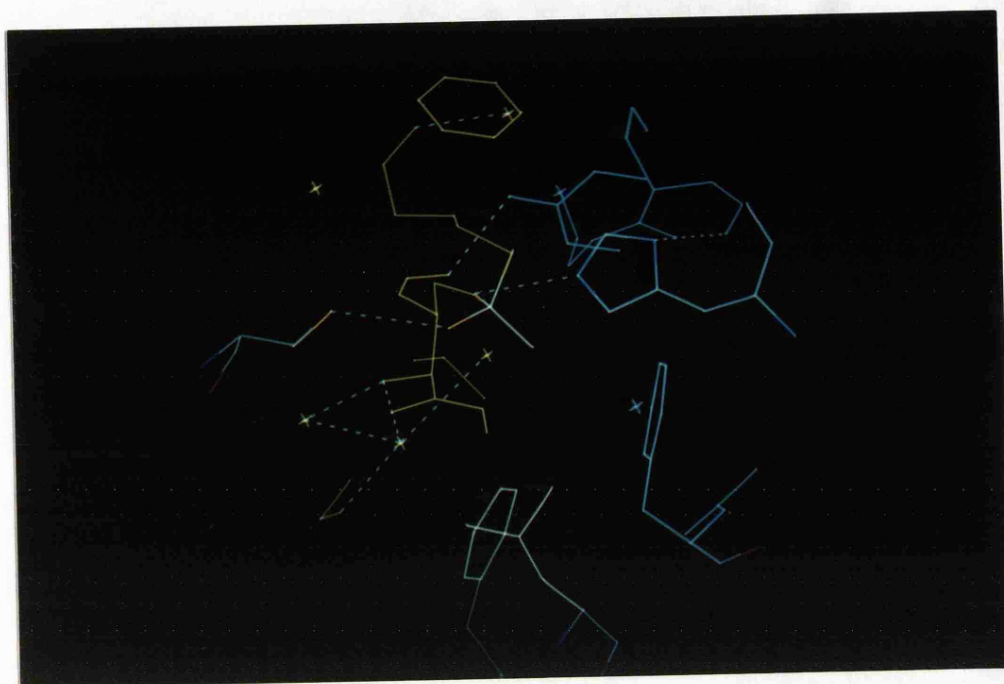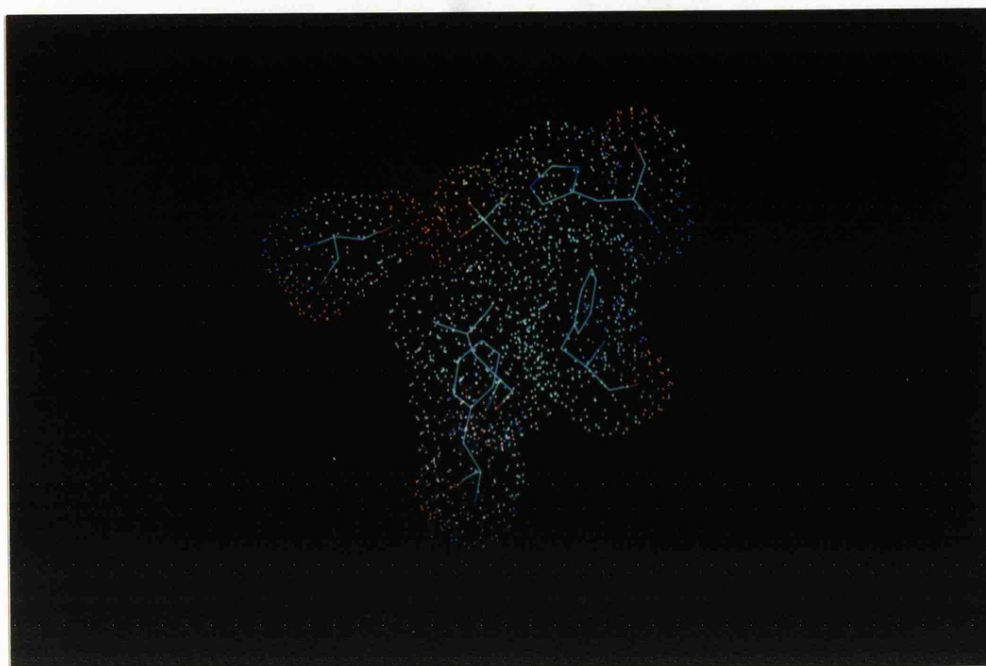
Figure 7.2. The relationship between the apparent free energy of binding of acetyl CoA and chloramphenicol with the hydrophobicity of the side chain of residue 33.

The free energies of transfer from aqueous solution to ethanol or dioxane ($\Delta G(\text{transfer})$) were used as a measure of side chain hydrophobicity (values from Nozaki and Tanford, 1971). The apparent free energy of binding (ΔG_{app}) was calculated for the binary and ternary complexes using $\Delta G_{app} = -RT\ln K$, where $K = 1/[K_m \text{Cm} \times K_d \text{AcCoA}]$ for the ternary complex (▲), $1/K_d \text{Cm}$ for the CAT/Chloramphenicol binary complex (■) and $1/K_d \text{AcCoA}$ for the CAT/Acetyl CoA binary complex (●). N.B. $K_m \text{Cm} \times K_d \text{AcCoA} = K_m \text{AcCoA} \times K_d \text{Cm}$ (see appendices 1 and 2).

A = Alanine (F33A)

F = phenyalanine (Wild type)

W = tryptophan (F33W)
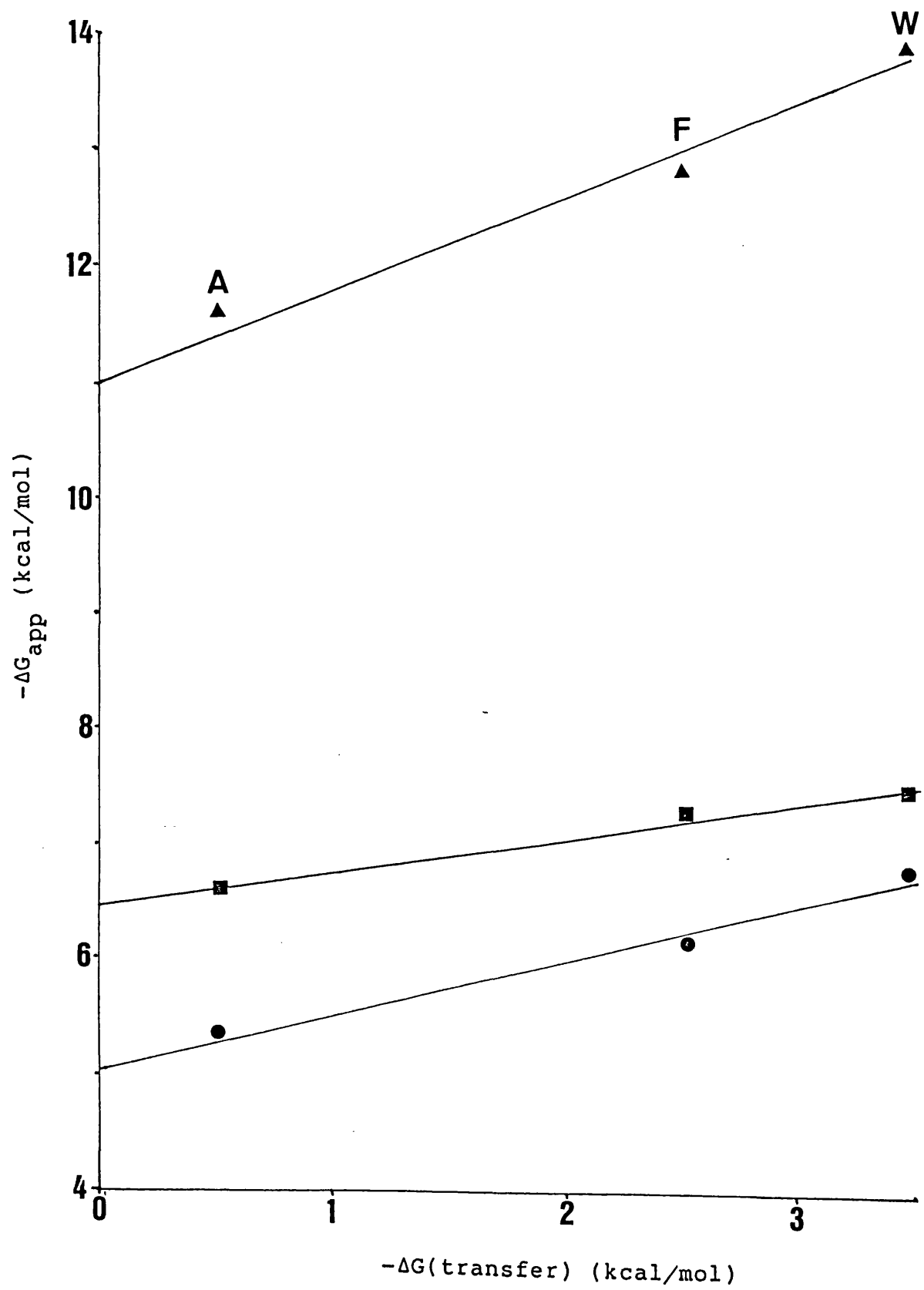
Table 7.1. Steady-state kinetic parameters for wild type and mutant chloramphenicol acetyltransferases.

Enzyme	$k_{cat}(s^{-1})$	$K_m A(\mu M)$	$K_m B(\mu M)$	$K_d A(\mu M)$	$K_d B(\mu M)$
F33	600	93	12	31	4
F33A	222	233	25	111	12
F33W	130	20	6	10	3

A = acetyl CoA, B = chloramphenicol

Table 7.2. Steady-state kinetic parameters for wild type and mutant chloramphenicol acetyltransferases using butyryl CoA as the acetyl donor.

Enzyme	$k_{cat}(s^{-1})$	$K_mA(\mu M)$	$K_mB(\mu M)$	$K_dA(\mu M)$	$K_dB(\mu M)$
F33	223	181	21	63	7
F33A	294	189	36	54	10
F33W	147	107	25	9	2

A = butyryl CoA, B = chloramphenicol

Table 7.3. Steady-state kinetic parameters for the hydrolysis of acyl CoAs by wild type and F33W CAT.

Enzyme	Acetyl CoA		Butyryl CoA	
	$k_{cat}(s^{-1})$	$K_m(\mu M)$	$k_{cat}(s^{-1})$	$K_m(\mu M)$
F33	0.13	66	0.027	74
F33W	0.048	29	0.014	24

Chapter 8. Discussion.

8.1 Discussion.

8.1.1 The role of electrostatic interactions in the binding of acetyl CoA to CAT.

It has been shown in earlier studies that CAT is rapidly inactivated by the arginyl specific reagent 2,3-butanedione (Corney, 1983). The structure of the citrate synthase/CoA complex shows that the phosphoanions of CoA are ion paired with the side chains of arginyl residues (Remington et al., 1982). It was therefore, proposed that similar interactions might also exist for CAT. Further circumstantial evidence was provided by two observations; the conservation of two arginyl residues (Arg 18 and Arg 74) in all known CAT sequences and the high K_m for acetyl 3-dephospho CoA.

In this study, the results of covalent modification studies with 2,3-butanedione and phenylglyoxal strongly suggest that arginyl residues are not directly involved in CoA binding to CAT_{III}. Although reaction with such reagents results in rapid inactivation of CAT, *both* substrates afford protection against loss of activity, suggesting either that modification of a residue at or near to the active site is responsible for inactivation or that binding of either substrate results in reduced reactivity of a residue at a remote, but structurally important site. Activation of the hydrolytic (thioesterase) activity on modification of CAT confirms that arginyl specific reagents do not modify a residue directly involved in acetyl CoA binding or in catalysis. Subsequent modification experiments with mutant enzymes (R18V and C31A) implied that inactivation of CAT by

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'arginyl specific' reagents is due to modification of a reactive, but nonessential cysteine residue (Cys 31) which lies close to the catalytic centre.

Covalent modification studies with ethyl acetimidate implicated lysyl residues in binding of CoA, but suggested that ion pair interactions with the enzyme are not involved. The high resolution structure of the CAT/CoA binary complex (Leslie et al., 1988) confirmed the absence of such ionic interactions with CoA and showed that both conserved arginyl residues play structural roles via intrasubunit ion pairs to aspartyl residues. Nonetheless, two lysyl residues were identified which are close to the CoA binding site and well conserved in known CAT sequences. The charged amino groups of these lysyl residues, Lys 54 and Lys 177, are however, between 0.6 and 1.2nm respectively from the nearest phosphoanions of CoA.

Removal of the positive charge from the side chains of Lys 54 and Lys 177 (K54Q and K177Q) results in an increase in the K_m for acetyl CoA, with corresponding 1.2 and 0.4kcal/mol decreases in the apparent free energy of binding for acetyl CoA. Since the K_m values for acetyl CoA for both mutant enzymes are less dependent on ionic strength than for wild type CAT, both lysyl residues may well be involved in long-range electrostatic interactions with CoA. Steady-state kinetic studies with inhibitors (competitive with respect to acetyl CoA) which possess only one phosphate group (3' and 5' AMP), have implicated Lys 54 and Lys 177 in interactions with the 5' phosphodiester moiety of CoA.

In summary, although CoA contains several groups which are negatively charged at physiological pH values, on binding to CAT, the coenzyme is not involved in ion pairs with positively charged groups on CAT, nor are the phosphoanions positioned to form favourable interactions with the dipole of an α-helix, as is often observed in nucleotide binding proteins (Wierenga et al., 1985).

Taken together, both kinetic studies with 3' and 5' AMP and structural considerations suggest that the 3' phosphate of CoA may not be important for binding to CAT. The K_m for acetyl 3-dephospho CoA is however, almost 10-fold greater than that for acetyl CoA. A possible solution to this apparent anomaly could be that simple adenosine derivatives (such as 3'AMP) are inappropriate models for CoA, since they may adopt conformations on binding to CAT which differ from that of the adenosine moiety of CoA. The 3'phosphate group may favour the preferred conformer of CoA for binding, or perhaps its presence allows the formation of other long-range electrostatic interactions with unidentified residues which play an indirect role in binding. The latter might be resolved by computational and modelling studies, and determination of the effects of substitutions of residues 54 and 177 on binding of 3-dephospho CoA may would be useful to confirm that 3' and 5' AMP are appropriate models for CoA.

8.1.2 The role of the hydrophobic effect in the binding of acetyl CoA to CAT.

The relatively minor role of direct electrostatic interactions in acetyl CoA binding to CAT prompted an

investigation into the importance of non-polar interactions in CoA binding. Three residues of CAT were chosen to investigate the role of the hydrophobic effect on binding of acetyl CoA; ones which provide side chains which interact with the adenine ring (Tyr 178), the pantetheine arm (Tyr 56) or the acetyl moiety (Phe 33) were investigated. Substitutions were made to remove the aromatic side chain of each of the three residues and a series of substitutions, which alter the hydrophobicity of the side chain, were also made at positions 56 and 178.

The major effect of substitutions for Tyr 178 is a large increase in the K_m values for acetyl CoA of each mutant protein, and such changes in affinity are directly related to the side chain hydrophobicities. Removal of the aromatic ring (Y178A) results in a 20-fold increase in K_m for acetyl CoA, corresponding to a change in ΔG_{app} of 2.2 kcal/ mol. Subtractive substitutions of residues 33 and 56 have similar effects to one another. The F33A and Y56A substitutions yield enzymes with increased K_m values for acetyl CoA and a decreased k_{cat}, with contributions to transition-state binding of 1.8 and 1.2 kcal/mol respectively. For all three residues changes in the steady-state kinetic parameters on substitution of the side chain are directly related to the hydrophobicity of that side chain. Furthermore, substitution of residue 33 also results in similar hydrophobicity-dependent changes in the K_m (and K_d) for chloramphenicol, reflecting the proximity of Phe 33 to both chloramphenicol and the acetyl group of acetyl CoA. Taken together, the results of these mutagenesis experiments demonstrate that the

hydrophobic effect plays a major role in the binding of acetyl CoA to CAT.

8.2 Future prospects.

8.2.1 Crystallographic studies.

Further structural information is of critical importance for a better understanding of both the binding of CoA and the mechanism of catalysis. A more highly refined structure of the CAT/CoA binary complex would be particularly useful to clarify interactions with CoA. However, since CoA is the product rather than the substrate for CAT in the forward reaction, it is the structure of the CAT/acetyl CoA binary complex which is required. Unfortunately, the hydrolytic activity of CAT precludes its crystallisation in the presence of acetyl CoA, thus an 'inert' analogue, such as acetonyldethio CoA, which has the sulphur atom replaced with a methylene group, must be used (Rubenstein and Dryer, 1980, Table 8.1). The structure of the CAT/acetyl CoA binary complex is important to define more precisely the position of the acetyl moiety, and to show if its presence results in any significant structural changes in the enzyme.

Binding constants for acetyl CoA can be measured directly by fluorescence spectroscopy by monitoring the enhancement of intrinsic fluorescence, contributed by Trp 152, on acetyl CoA binding (J.Ellis, C.R.Bagshaw and W.V.Shaw, unpublished results). Binding of CoA however, causes no such fluorescence enhancement, suggesting that the CAT/CoA and CAT/acetyl CoA binary complexes may differ

structurally in a subtle, but important way.

Due to its unreactivity, acetonyldethio CoA can also be used to estimate the dissociation constant of acetyl CoA in the presence of chloramphenicol by fluorescence methods, provided that its binding results in a similar fluorescence enhancement to that observed with acetyl CoA. Furthermore, crystallisation of CAT with both chloramphenicol and acetonyldethio CoA bound would allow the structure of a ternary complex to be solved.

It has been proposed that the reaction mechanism of CAT involves a tetrahedral oxyanion intermediate (Lewendon et al., 1990). Steady-state kinetic studies with 2-hydroxypropyl CoA and evidence from site-directed mutagenesis experiments (Lewendon et al., 1990), suggest that such a mechanism is likely to be correct. Confirmation of the tetrahedral intermediate from structural studies would be useful, not only to support the results of previous work, but also to identify other residues which may be important for transition-state stabilisation. For crystallisation of the CAT/acetyl CoA binary complex, a nonreactive acyl CoA analogue is required to avoid hydrolysis. A bisubstrate analogue is unlikely to bind to CAT as the 'tunnel' nature of the substrate binding sites imposes a requirement for independent binding of chloramphenicol and acetyl CoA. Reduction of acetonyldethio CoA with borohydride yields 2-hydroxypropyldethio CoA, a compound with tetrahedral geometry at the same position as the tetrahedral intermediate, but which lacks the charged oxyanion moiety. Crystallisation of CAT in the presence of both chloramphenicol and 2-hydroxypropyldethio CoA may allow the

structure of a pseudo transition-state to be determined (see fig. 5.3).

Before structures such as those desribed above can be determined by X-ray diffraction methods, the conditions required to obtain crystals of CAT with CoA (or an appropriate analogue) bound need to be defined and the analogues synthesised. Neither undertaking is trivial. Since acetyl pantetheine is such a poor substrate for CAT, and because of the possibility of alternative binding modes truncated analogues of CoA, there is no alternative to the total chemical synthesis of CoA analogues such as acetonyldethio CoA.

Attempts to reproduce crystals of the CAT/CoA binary complex (Leslie et al., 1988) to improve the resolution of that structure have so far been unsuccessful.

The determination of the structures of mutant enzymes would also be useful. Interpretation of the effects of amino acid substitutions on the steady-state kinetic parameters relies on a number of assumptions, one of which is that the only significant structural change is that at the site of substitution. Evidence that this is not always the case is easy to find. For example, the D199N substitution in CAT results in a dramatic decrease in k_{cat} with only small changes in the substrate K_m values, consistent with a proposed role for Asp 199 in tautomeric stabilisation of His 195. However, subsequent determination of the structures of the wild type and D199N enzymes revealed that Asp 199 is involved in a buried ion pair which determines and stabilises the architecture of the active site, but plays no role in

catalysis _per_ _se_. The D199N substitution results in major
disruption of the active site (which explains the decrease in
k_{cat} in the mutant enzyme) without significantly altering the
tautomeric stabilisation of the catalytic histidine residue
(Lewendon et al., 1988; Leslie et al., 1988; Gibbs, Moody and
Leslie, in preparation).

A further problem arises for mutations in substrate
binding sites. The structure determined needs to be that of
the relevant binary (and/or ternary) complex. The structure
of the K177A CAT/chloramphenicol binary complex suggests that
the mutation results in no structural changes apart from
removal of the lysyl side chain of residue 177. However,
steady-state kinetic studies suggest that this mutation also
alters interactions at the nearby binding site for the
adenine moiety of CoA.

8.2.2 Further directed mutagenesis.

The CoA binding site of CAT can be investigated further
by site-directed mutagenesis. Other residues involved in
binding of CoA which have not yet been studied include Phe
103, Pro 151 and Phe 158. Each is conserved in all known
sequences, forms intimate contacts with CoA and is part of
important secondary structures. Phe 103 forms part of the CoA
tunnel closest to the active site and interacts with the
cysteamine moiety of CoA (fig 1.9). Phe 103 may also interact
with the acetyl moiety of CoA, as does Phe 158. Phe 158 is in
van der Waals' contact with Phe 33, forming an inter-subunit
hydrophobic interaction. Together these two residues probably
define, to some degree, the size and shape of the 'acyl'

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binding site. Thus, by combining substitutions at residues 33 and 158 it may be possible to change the specificity of CAT for particular acyl groups.

It may also be possible to change the ratio of transacetylation and hydrolysis activities by introducing an enzyme hydroxyl group at the position normally occupied by the 3-hydroxyl group of chloramphenicol. Such structural alterations will require more extensive modelling to determine which substitutions might bring about the required changes in specificity.

Pro 151 interacts with the adenine ring of CoA forming the 'lower' surface of the adenine binding pocket, but is also part of a reverse turn. Hence, although substitution of Pro 151 might be expected to have a similar effect on CoA binding to substitution of Tyr 178, such a change could well lead to indirect and more extensive effects because of structural consequences arising from disruption of a turn which connects strands of an extensive β-sheet.

Together with Trp 152 and two less well conserved residues (Leu 150 and Thr 94), the residues discussed above determine the shape of the CoA 'tunnel' and hence the specificity for the pantetheine arm of CoA. In order to change the specificity of CAT to a 'simple' acyl donor extensive molecular modelling must be carried out to determine the surface 'shape' of potential acyl donors and this information used to deduce suitable substitutions which could be made to produce a good fit. The rather weak binding of acetyl pantetheine (K_m = 143mM) suggests that the introduction of specific interactions, such as hydrogen bonds and electrostatic interactions, also needs to be considered.

8.2.3 Analogues of acetyl CoA.

The importance of the thioester moiety of acetyl CoA in binding can be estimated using a number of substrate analogues (see Table 8.1). Dethio CoA and acetonyldethio CoA can be used to estimate the contribution of the sulphur atom to binding of CoA and acetyl CoA respectively, while ethyl CoA probes the role of the 'acyl' carbonyl group. Replacing the carbonyl group with another sulphur atom (CoA methyl disulphide) which is similar in size may also be interesting. In other systems (e.g. choline acetyltransferase) such CoA disulphides are found to bind very tightly (Currier and Mautner, 1977).

8.2.4 Transient kinetic studies.

The effects of substitutions (of either enzyme or substrate) on the kinetic parameters of the transacetylation reaction can be examined further using transient kinetics methods. Preliminary results suggest that it may prove possible to directly determine the substrate 'off' rates for several complexes by monitoring fluorescence changes which occur on substrate binding (due to Trp 152 with CoA binding, and Trp 86 with chloramphenicol binding) (J.Ellis, C.R.Bagshaw and W.V.Shaw, unpublished results). Transient kinetics would be particularly useful to determine the precise effects of amino acid substitutions (especially where k_{cat} is altered) and may allow the rate determining step for the reaction to be identified.

Table 8.1. The structures of CoA analogues.
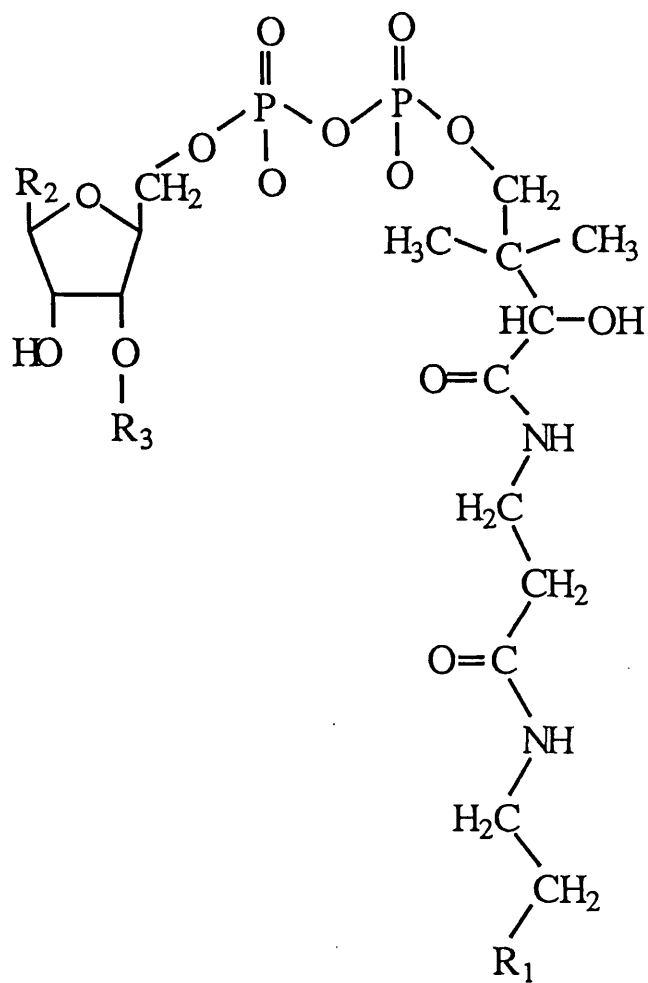
Compound	R$_1$	R$_2$	R$_3$	Comments
CoA	-SH	Adenine	Phosphate	reaction product
Dethio CoA	-H	Adenine	Phosphate	role of sulphur atom
Acetyl CoA	-SCOCH$_3$	Adenine	Phosphate	substrate
Ethyl CoA	-SCH$_2$CH$_3$	Adenine	Phosphate	role of carbonyl group
CoA methyldisulphide	-SSCH$_3$	Adenine	Phosphate	role of carbonyl group
Acetonyldethio CoA	-CH$_2$COCH$_3$	Adenine	Phosphate	role of sulphur/ crystallography
2-Hydroxypropyldethio CoA	-CH$_2$C*H(OH)CH$_3$	Adenine	Phosphate	transititon-state analogue
3-dephospho CoA	-SH	Adenine	-OH	electrostatic interactions
Guano-CoA	-SH	Guanine	Phosphate	base specificity
Desamino CoA	-SH	Inosine	Phosphate	base specificity

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Appendix 1.

Calculation of the steady-state kinetic parameters of an enzyme obeying a rapid equilibrium random order ternary complex mechanism from double reciprocal plots.

The enzymic acetylation of chloramphenicol (B) by CAT (E) using acetyl CoA (A) proceeds by the following scheme with a random order of addition of substrates and with free enzyme (E), the two binary complexes (EA and EB) and the ternary complex (EAB) in rapid equilibrium (Kleanthous and Shaw, 1984):
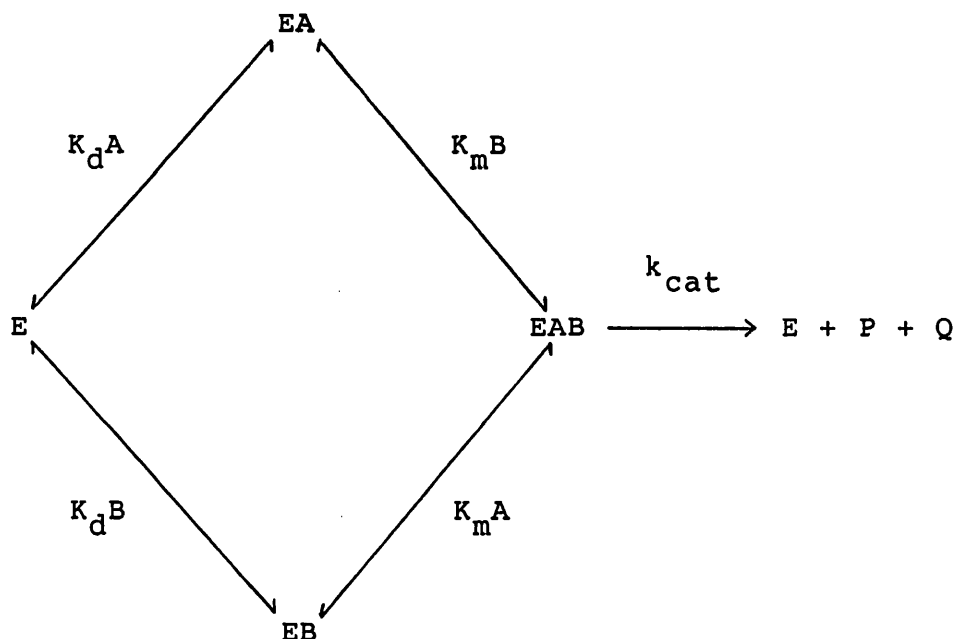
K_mA = Michaelis constant for substrate A.

K_mB = Michaelis constant for substrate B.

K_dA = Dissociation constant for dissociation of substrate A from binary complex EA, independent of nature of B.

K_dB = Dissociation constant for dissociation of substrate B

from binary complex EB, independent of nature of A.

The initial rate equation for a ternary complex mechanism with a random order of addition of substrates is as follows (Alberty, 1953):

$$V_i = \frac{V_{max}[A][B]}{K_m AB + K_m A[B] + K_m B[A] + [A][B]}$$

$K_m AB = K_m A \times K_d B = K_m B \times K_d A$

$V_{max} = k_{cat} x e_o$, e_o is the amount of enzyme in the assay in μmoles.

This equation can be rearranged into a form equivalent to the Michaelis-Menton equation for a single substrate reaction:

$$1/V_i = (1/V_{max} + K_m B/V_{max}[B]) + (K_m AB/V_{max}[B] + K_m A/V_{max})1/[A]$$

Double reciprocal plots of initial rate of reaction against substrate (A) concentration for each of four fixed concentrations of the second substrate (B, see section 2.6) should be linear with intercepts of $(1/V_{app} + K_m B_{app}/V_{app}[B])$ and gradients of $(K_m AB_{app}/V_{app}[B] + K_m A_{app}/V_{app})$ (fig. A1a). Secondary plots of intercepts and gradients against reciprocal concentration of substrate B are also linear (fig. A1b). Steady-state kinetic parameters are calculated from such plots as follows:

Secondary plot	Intercept	Gradient
Intercepts vs 1/[B]	$1/V_{max}$	$K_m B/V_{max}$
Gradients vs 1/[B]	$K_m A/V_{max}$	$K_m AB/V_{max}$

Figure A1a. Primary plots of reciprocal initial velocity (V_i) against reciprocal substrate concentration (A) for each of four concentrations of the second substrate (B).
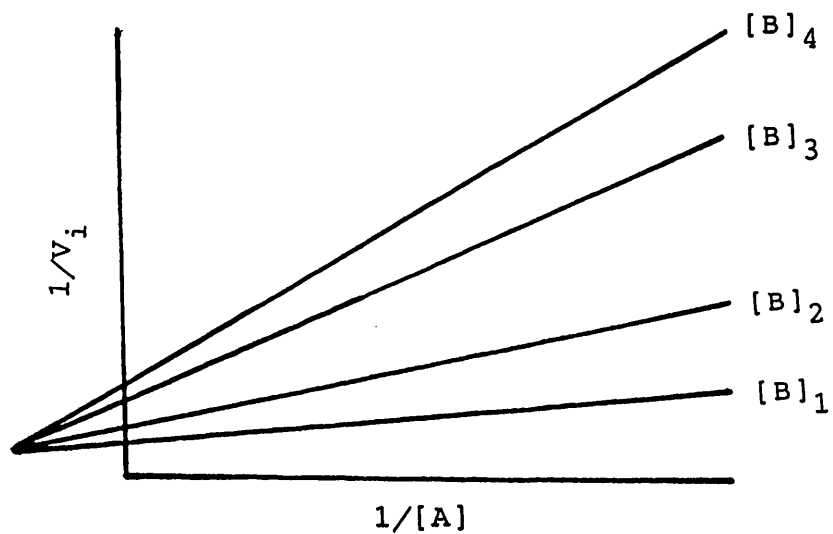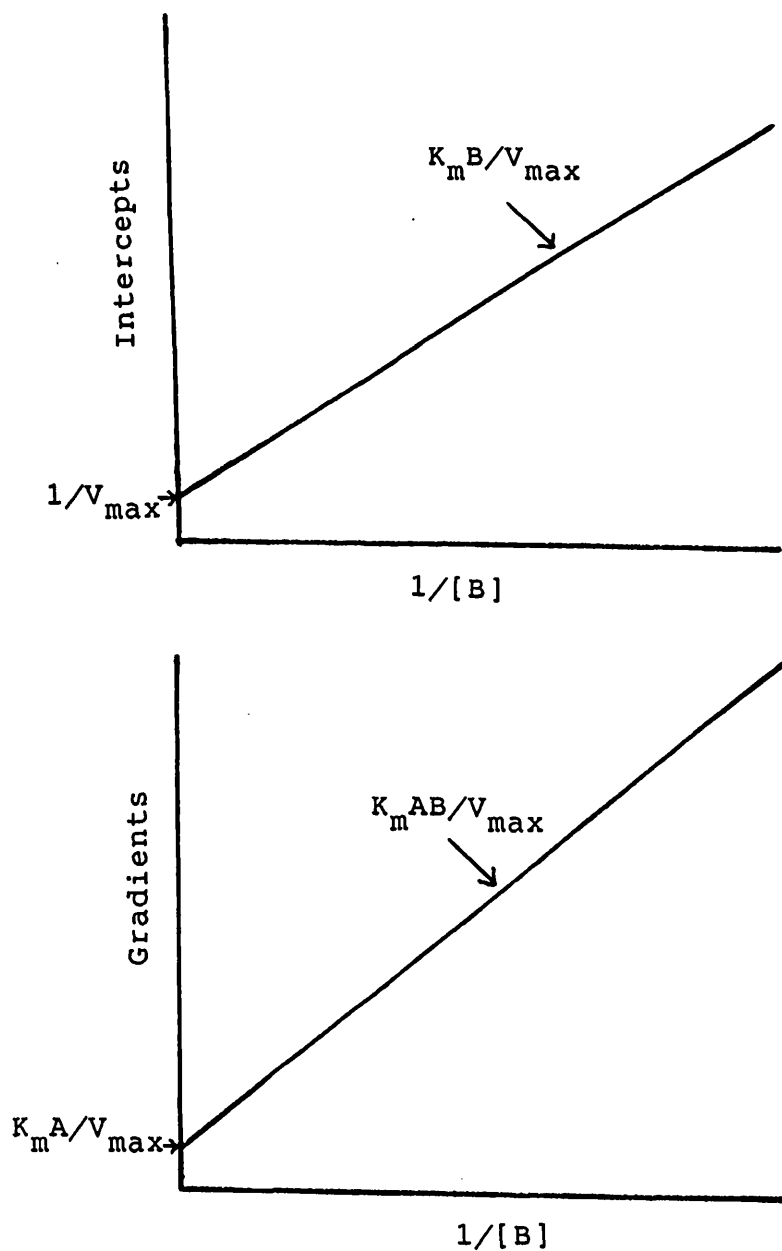
Figure A1b. Secondary replots of intercepts and gradients from double reciprocal plots (figure A1a) against reciprocal substrate concentration (B).

Steady-state kinetic parameters are calculated directly as shown.

Appendix 2.

Calculation of apparent free energy of binding from steady-state kinetic parameters.

The binding energy of the transition-state of an enzyme reaction is related to k_{cat}/K_m, calculated from steady-state kinetics, as shown in equation 1 (Fersht, 1985).

$$(1) \qquad RT\ln(k_{cat}/K_m) = RT\ln(kT/h) - \Delta G^{\ddagger} - \Delta G_s$$

R = gas constant, T = absolute temperature, k = Boltzmann's constant, h = Plank's constant.

$\Delta G^{\ddagger}$ = the chemical activation energy.

ΔG_s = the total binding energy of the enzyme and the substrate.

The contribution to transition-state binding (ΔG_R) of a particular group (R) on a substrate (SR) can be calculated from the ratio of the specificity constants (k_{cat}/K_m) for the two substrates, SR and S (where group R is removed), from equation 2, provided that (i) $\Delta G^{\ddagger}$ is not affected by the substitution and (ii) that group R is involved only in binding and is not involved in the chemical step.

$$(2) \qquad \Delta G_R = -RT\ln[(k_{cat}/K_m)_S/(k_{cat}/K_m)_{SR}]$$

Equation 2 can also be applied to binding of the same substrate to different enzyme forms (i.e. wild type/mutant or mutant1/mutant2). The contribution to binding of the enzyme

group (R) is then given by equation 3 (Wilkinson et al., 1983).

(3) $\Delta G_R = -RT\ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$

The binding energy contributed by group X of an enzyme, to binding of group Y of the substrate, compared with their interactions with solvent water (w, equation 4) is similarly

(4) $E-X.w + w.Y-S \longleftrightarrow E-X.Y-S + ww$

given by equation 5, where K_d is the (wild type) dissociation

(5) $\Delta G = -RT\ln(K_d/K_d')$

constant of the E-X.Y-S complex and K_d' is the (mutant) dissociation constant of the E/Y-S complex (Fersht, 1988). However, binding energies calculated in this way are only apparent binding energies (ΔG_{app}) and can seriously overestimate the true binding energy of the group (ΔG_{bind}), the discrepancy being especially large when the mutation leaves an charged acceptor or donor unpaired (Fersht, 1988). ΔG_{app} is a measure of specificity of binding and catalysis, and for enzyme reactions it is often better to use the ratio of the specificity constants (k_{cat}/K_m) to calculate ΔG_{app} to avoid complications from non-productive binding and induced fit mechanisms, which do not alter this parameter, but do change substrate K_d values.

Interpretation of values of ΔG_{app} calculated for the contribution to binding of specific groups relies principally

on three assumptions:

(i) The structures of the wild type and mutant enzymes are identical apart from at the site of mutation.

(ii) Both enzyme reactions proceed by the same mechanism.

(iii) The reactions catalysed by the wild type and mutant enzymes have the same rate determining step.

If any of the above assumptions are not valid, then equations (3) and (5) can not be used to calculate apparent binding energies, as the kinetic parameters used for such calculations may then contain different rate constants, and therefore, can not be directly compared. In many instances, ΔG_{app} is found to be a good approximation for ΔG_{bind}, especially when the cavity created by subtractive mutation is accessible to solvent water (Fersht, 1988). Mutations that introduce larger residues can not be analysed simply as above, but require extensive modelling studies or structure determination.

Multisubstrate enzyme reactions can be more complicated, depending on the effect of the mutation on the kinetic parameters of the enzyme. If the mutation only affects the binding of one substrate, then equations (3) and (5) apply as before. However, if the mutation has an effect on the binding of both (in a two substrate system) substrates (A and B) then the equations must be modified. For a random order ternary complex mechanism, such as that followed by CAT, the effect of a mutation on the binding of the transition-state is given by equation (6), where K_m and K_d are the dissociation constants for the ternary and binary complexes respectively, and $K_m A \times K_d B = K_m B \times K_d A$ (see appendix 1). It can be seen from equation (6) that if the binding of only one substrate is

(6) $\Delta G = -RT\ln(\{kcat/[K_m A x K_d B]\}_{mut}/\{kcat/K_m A x K_d B]\}_{wt})$

affected then equation (6) = equation (5). Similarly, changes in the binding energy of the ternary complex on mutation is again dependent on the effects on binding of both substrates. Equation (7) applies if only one substrate is affected, and equation (8) if both are affected.

(7) $\Delta G = -RT\ln([1/K_m]_{mut}/[1/K_m]_{wt})$

(8) $\Delta G = -RT\ln(\{1/[K_m A x K_d B]\}_{mut}/\{1/[K_m A x K_d B]\}_{wt})$