Elimination of a reactive thiol group from the active site of chloramphenicol acetyltransferase

Ann LEWENDON and William V. SHAW*

Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K.

1. The type III variant of chloramphenicol acetyltransferase (CAT_{III}) is resistant to inactivation by ionizable modifying reagents such as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and iodoacetate, whereas it is sensitive to inhibition by similar but uncharged reagents, including 4,4'-dithiodipyridine, methyl methanethiolsulphonate (MTS) and iodoacetamide. The target for these thiol-modifying reagents has been postulated to be Cys-31. This residue is situated within a part of the chloramphenicol-binding site formed largely from the side chains of hydrophobic amino acid residues, which might be expected to discriminate against the access of ionized ligands to Cys-31. 2. The substitution of Cys-31 by alanine, serine, threonine or methionine yields an enzyme that is resistant to inactivation by thiol-specific reagents. Replacement of Cys-31 by alanine, serine or threonine results in increased K_m values for chloramphenicol with only small changes in k_{cat} . In contrast, the Cys-31→Met substitution mainly affects k_{cat} values. Although the k_{cat} for chloramphenicol acetylation is decreased 13-fold compared with wild-type CAT, the k_{cat} for the acetyl-CoA hydrolysis reaction, which occurs in the absence of chloramphenicol, is increased 2.7-fold. 3. MTS modification of cysteine residues results in an adduct ($-\text{CH}_2-\text{S}-\text{CH}_3$) that is structurally similar to the side chain of a methionine residue ($-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_3$). The kinetic properties of MTS-modified CAT_{III} closely resemble those of [Met³¹]CAT.

INTRODUCTION

Chloramphenicol acetyltransferase (CAT, EC 2.3.1.28) catalyses the *O*-acetylation of chloramphenicol, using acetyl-CoA as acetyl donor. The acetylated antibiotic does not inhibit protein synthesis as it fails to bind to bacterial ribosomes (reviewed by Shaw, 1983).

Genes encoding CAT are commonly plasmid-borne, and CAT activity has been detected in a wide range of bacterial species. The amino acid sequences of seven CAT variants are known and show a marked degree of similarity to each other (Shaw & Leslie, 1989; Murray *et al.*, 1989). CAT is a trimeric enzyme of identical subunits of M_r 25000 (Leslie *et al.*, 1986; Harding *et al.*, 1987).

Three general classes of CAT variants have been isolated from Gram-negative organisms, designated types I, II and III (Shaw, 1983). This classification is based upon differences in immunological cross-reactivity, electrophoretic mobility and sensitivity to reagents that modify cysteine residues (Gaffney *et al.*, 1978). The type I variant of CAT, unlike types II and III, binds the steroidal antibiotic fusidic acid with high affinity, thereby conferring resistance against this antibiotic (Bennett & Shaw, 1983). The type II enzyme is exquisitely sensitive to inhibition by reagents that modify thiol groups, whereas types I and III are relatively insensitive (Gaffney *et al.*, 1978; Murray *et al.*, 1990). The three-dimensional structure of a type III CAT variant (CAT_{III}) has recently been determined. The gene for this catalytically efficient variant was isolated from plasmid R387 and can be expressed at high levels in *Escherichia coli* (Murray *et al.*, 1988). The structure of the CAT_{III}-chloramphenicol binary complex has been refined to 0.175 nm resolution, and that of the CAT_{III}-CoA complex to 0.24 nm (Leslie *et al.*, 1988).

CAT possesses three active sites per trimer, located equidistant from the threefold axis at the intersubunit interfaces. The

chloramphenicol-binding and CoA-binding sites are largely composed of residues from one subunit, whereas a conserved histidine residue (His-195) required for catalysis is supplied by the opposing subunit. The chloramphenicol-binding and CoA-binding sites together form a tunnel through the protein, which is occupied by solvent in the absence of ligands (Leslie *et al.*, 1988).

The details of the CAT_{III} structure explain data from steady-state kinetic analyses that indicated that CAT_{III} follows a ternary-complex mechanism with random-order binding of substrates (Kleanthous & Shaw, 1984). The importance of His-195 in the catalytic mechanism of CAT was inferred from chemical-modification studies in which an active-site-directed reagent [3-(bromoacetyl)chloramphenicol] modified only the N-3 position of His-195 (Kleanthous *et al.*, 1985). In the crystal structure of the CAT_{III}-chloramphenicol complex the N-3 atom of His-195 is hydrogen-bonded to the 3-hydroxy group of chloramphenicol, which is the site of acetylation. The imidazole N-3 atom of His-195 is presumed to act as a general base during the CAT reaction, abstracting a proton from the primary hydroxy group of chloramphenicol and promoting nucleophilic attack at the thioester carbonyl group of acetyl-CoA. In the absence of chloramphenicol, CAT catalyses the hydrolysis of acetyl-CoA at a rate approx. 10⁴-fold lower than the rate of acetylation of chloramphenicol.

Early chemical-modification studies also demonstrated that some CAT variants are remarkably susceptible to inactivation by reagents that modify thiol groups (Zaidenzaig *et al.*, 1979). Since chloramphenicol protects against such inhibition, it was postulated that the reactive cysteine residue might be located at or near the active site of CAT. Examination of the structure of CAT_{III} indicates that Cys-31 is a prime candidate for the reactive thiol group, located as it is within the chloramphenicol-binding

Abbreviations used: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of CAT; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTDP, 4,4'-dithiodipyridine; MMTS, methyl methanethiolsulphonate; TSE buffer, 50 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl and 0.1 mM-EDTA.

* To whom correspondence should be addressed.

Fig. 1. Stereoview of the residues forming the chloramphenicol-binding pocket of CAT

Chloramphenicol is shown in **boldface** and residue names preceded by * belong to an adjacent subunit. Large circles represent ordered water molecules, and broken lines represent predicted hydrogen bonds.

pocket close to His-195 (Fig. 1). Cys-31 is present in most of the known CAT sequences; however, its substitution by threonine occurs in two staphylococcal CAT variants that are resistant to thiol-modifying reagents (Shaw, 1983). Although Cys-31 is quite strongly conserved, it is clearly not essential for catalytic activity.

It is now known that replacement of structurally important residues in CAT_{III} by site-directed mutagenesis can lead to large changes in the sensitivity of the enzyme to thiol-modifying reagents. For example, removal of a buried salt bridge (Asp-199...Arg-18) close to the active site of CAT_{III} (Asp-199→Ala or Arg-18→Val) results in 110-fold or 170-fold increase respectively in the rates of modification by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Lewendon *et al.*, 1988). In addition to their importance in understanding structure-function relationships, such observations have important practical implications, since the standard CAT assay for steady-state kinetic analysis relies on the reaction of the product, CoA, with DTNB (Shaw, 1975). Consequently, increasing the sensitivity of CAT to DTNB could compromise kinetic measurements.

Zaidenzaig & Shaw (1978) demonstrated that type I CAT was susceptible to inhibition by uncharged reagents but resistant to anionic reagents. Corney (1983) extended this work to CAT_{III}, and showed that iodoacetamide inactivation of CAT_{III} resulted almost exclusively in modification of histidine rather than cysteine residues. We report here the susceptibility of CAT_{III} to three reagents that specifically modify thiol groups, namely DTNB, 4,4'-dithiodipyridine (DTDP) and methylmethanethiolsulphonate (MMTS), in addition to iodoacetamide and iodoacetate. We have replaced Cys-31 with alanine, serine, threonine or methionine in an attempt to produce an enzyme that is resistant to thiol-modifying reagents but presents broadly similar kinetic properties to those of wild-type CAT_{III}. In addition, the kinetic properties of [Met³¹]CAT are compared with those of wild-type CAT that had been fully modified by MMTS. This reagent modifies cysteine residues to produce an adduct of similar structure to a methionine residue (Smith *et al.*, 1975). Since the kinetic properties of MMTS-modified wild-type CAT closely resemble those of [Met³¹]CAT, it is likely that specific chemical modification and site-directed mutagenesis produce comparable alterations in the structure of CAT_{III}, resulting in similarities in the functional properties of the two altered enzymes.

MATERIALS AND METHODS

Materials

Reagents for chemical modification were obtained from the following sources: MMTS from Aldrich Chemical Co., DTNB, DTDP and iodoacetamide from Sigma Chemical Co., and iodoacetate from BDH Chemicals. Acetyl-CoA was prepared as described by Simon & Shemin (1953).

Assay of CAT activity

CAT activity was assayed spectrophotometrically at 25 °C. A modification of the method of Shaw (1975) was used to measure rates of chloramphenicol acetylation. The standard assay mixture contained TSE buffer, pH 7.5, 1 mM-DTNB, 0.1 mM-chloramphenicol and 0.4 mM-acetyl-CoA. The reaction was initiated by the addition of enzyme, and the rate of formation of CoA was monitored by its reaction with DTNB and liberation of the thionitrobenzoate dianion ($\lambda_{\max}$, 412 nm).

Rates of acetyl-CoA hydrolysis were determined exactly as above except that chloramphenicol was omitted from the reaction mixture. The amount of enzyme used in such assays of thioesterase activity (0.1–10 units) was much greater than that employed in the standard assay of chloramphenicol acetylation (0.004 unit). One unit of enzyme activity is defined as the amount converting 1 μ mol of substrate into product/min.

The concentrations of chloramphenicol and acetyl-CoA were varied in the standard assay mixture during kinetic analysis. Linear initial rates for steady-state kinetic analyses were measured over times during which less than 15% depletion of substrates occurred. Kinetic parameters were determined from linear slope and intercept replots from manually drawn double-reciprocal plots (Kleanthous & Shaw, 1984).

Site-directed mutagenesis and expression of CAT

Oligonucleotide-directed mismatch mutagenesis was performed by using the deoxyuridine selection procedure with the *dut ung E. coli* strain RZ1032 (Kunkel *et al.*, 1987). The oligonucleotides were 5'-AAAACCAGATGGTAAA (one mismatch, Cys-31→Ser), 5'-AGCTAAAACCAGCTGGTAAACGA (two mismatches, Cys-31→Ala), 5'-AGCTAAAACCAGT-TGGTAAACGA (two mismatches, Cys-31→Thr) and 5'-AGCTAAAACCCATTGGTAAACGA (three mismatches,

Cys-31→Met). In each case the presence of the desired mutation and the absence of second-site mutations was confirmed by determination of the DNA sequence spanning the entire *cat* coding and 5' non-coding regions. Mutant *cat* determinants were over-expressed in *E. coli* after transfer to pUC18 (Murray *et al.*, 1988).

Purification of CAT

Purification of wild-type and mutant CAT from *E. coli* extracts was carried out by affinity chromatography on chloramphenicol-Sepharose as previously described (Lewendon *et al.*, 1988). The purity of enzyme preparations was assessed by SDS/PAGE (Laemmli, 1970).

The concentration of purified CAT was calculated from the $A_{280}^{1\%} = 13.1$ or by the method of Lowry *et al.* (1951) with wild-type CAT_{III} as standard.

Chemical modification of CAT

The time courses of chemical modification were monitored at 25 °C in TSE buffer, pH 7.5. Stock solutions of iodoacetate, iodoacetamide, DTNB and MMTS were made up in TSE buffer and the pH was adjusted to 7.5 if necessary. These reagents were added to CAT in TSE Buffer, pH 7.5, at zero time. DTDP was added to incubations in acetonitrile, the final concentration of which was 0.95 M. This concentration of acetonitrile had no effect on CAT activity over the duration of incubations. At appropriate time intervals, samples were withdrawn, diluted in TSE buffer and assayed for CAT activity.

Iodoacetate and iodoacetamide. The final concentration of CAT was 0.225 mg/ml (9 μ M monomers). Iodoacetate was added to give a final concentration of 0.1 M. Iodoacetamide concentrations were varied between 5 mM and 25 mM.

DTNB. The final concentration of wild-type CAT was 0.4 mg/ml (16 μ M). DTNB concentrations were varied between 0.25 mM and 2.0 mM. In experiments to assess the sensitivity of Cys-31-substituted CATs, the enzymes (0.225 mg/ml, 9 μ M) were incubated in a final concentration of 1 mM-DTNB for 30 h.

DTDP. The final concentration of wild-type CAT was 0.2 mg/ml (8 μ M). DTDP concentrations were varied between 0.2 mM and 4 mM. The Cys-31-substituted CATs (0.45 mg/ml, 18 μ M) were incubated in a final concentration of 0.2 mM-DTDP for 6 h.

MMTS. The final concentration of wild-type CAT was 0.09 mg/ml (3.6 μ M). MMTS concentrations were varied between 0.05 mM and 0.4 mM. The Cys-31-substituted CATs (0.225 mg/ml, 9 μ M) were incubated in a final concentration of 0.4 mM-MMTS for 60 min.

Re-activation of MMTS-modified CAT. Wild-type CAT (0.2 mg/ml, 8 μ M monomers) was treated with 0.4 mM-MMTS for 15 min at 25 °C in TSE buffer, pH 7.5. After this treatment, the CAT activity was 4.5 % of its initial value. Dithiothreitol was added to the MMTS-treated CAT to give a final concentration of 10 mM and this mixture was incubated at 25 °C for 5 h. Samples were withdrawn at intervals and assayed for CAT activity.

Preparative MMTS modification of wild-type and [Met³¹]CAT. Both wild-type CAT and [Met³¹]CAT (0.5 mg/ml, 20 μ M) were incubated in 0.5 mM-MMTS for 2 h at 25 °C. Excess reagent was removed by dialysis against TSE buffer, pH 7.5, for 24 h at 4 °C.

Preparative DTDP modification of wild-type CAT. Wild-type CAT (4.8 mg/ml, 192 μ M) was incubated in 3.6 mM-DTDP for 6 h at 25 °C. Excess reagent was removed by dialysis against TSE buffer, pH 7.5, for 24 h at 4 °C.

RESULTS AND DISCUSSION

Chemical modification of CAT_{III}

Iodoacetamide and iodoacetate. CAT_{III} activity is largely unaffected by treatment of the enzyme with iodoacetate; 0.1 M-iodoacetate produced only a 10% decrease in activity over 75 min. In contrast, iodoacetamide, its uncharged homologue, is an effective inhibitor of CAT_{III} (Table 1).

Table 1. Chemical modification of CAT_{III}

CAT_{III} was treated with the following reagents as described in the Materials and methods section: MMTS, iodoacetamide, DTNB and DTDP. The pseudo-first-order rate of inactivation (k_{app}) was determined at several different concentrations of each inhibitor. For iodoacetamide and MMTS, k_{app} was directly proportional to the concentration of inhibitor, and thus inhibition is a simple bimolecular reaction. For DTNB and DTDP, saturation of the rate of inactivation is observed at high concentrations of inhibitor. This implies that each of these reagents forms a reversible enzyme-inhibitor complex before modification (eqn. 1):

The following linear rate expression has been derived (eqn. 2):

$$t_{\frac{1}{2}} = 1/[I](t_{min} K_{inact.}) + t_{min} \quad (2)$$

where $K_{inact.}$ is $(k_{-1} + k_{+2})/k_{+1}$ and is analogous to the Michaelis constant for a substrate and where $t_{min.}$ is the finite inactivation half-time (Meloche, 1967). A plot of $t_{\frac{1}{2}}$ versus $1/[I]$ yields a straight line from which both $K_{inact.}$ and a pseudo-unimolecular rate of inactivation at infinitely high concentrations of inhibitor (k_i) can be calculated. The apparent second-order rate constant, $k_i/K_{inact.}$, is analogous to k_{cat}/K_m , the so-called specificity constant for catalysis. Abbreviation: N.A., not applicable.

Reagent	Bimolecular rate constant ($M^{-1} \cdot s^{-1}$)	Pseudo-unimolecular rate constant (k_i) (s^{-1})	$K_{inact.}$ (mM)	$k_i/K_{inact.}$ ($M^{-1} \cdot s^{-1}$)
Iodoacetamide	0.020	N.A.	N.A.	N.A.
MMTS	19.6	N.A.	N.A.	N.A.
DTNB	N.A.	2.7×10^{-4}	4.5	0.06
DTDP	N.A.	1.8×10^{-3}	0.62	2.90

DTNB and DTDP. Under the conditions of modification (at pH 7.5), the anionic form of DTNB and the uncharged form of DTDP are the predominant species present. Again, CAT_{III} is more resistant to the charged reagent than to the uncharged one (Table 1). Since the inactivation of CAT by both disulphides shows rate saturation with increasing concentration of inhibitor, it follows that both these reagents bind to a site on the enzyme before the chemical-modification step. Protection against inactivation can be observed when chloramphenicol is present during the inactivation (results not shown). A comparison of k_i/K_{inact} values (analogous to k_{cat}/K_m) indicates that DTDP is a 48-fold better inhibitor of CAT_{III} than is DTNB (Table 1).

MMTS. MMTS modifies cysteine residues to produce the side-chain adduct $\text{—CH}_2\text{—S—S—CH}_3$ (Smith *et al.*, 1975). Treatment of CAT_{III} with MMTS results in rapid inhibition of activity with no evidence of MMTS binding to CAT before inactivation (Table 1). Again, chloramphenicol provides protection against inactivation. MMTS inactivation of CAT can be completely reversed by treatment of the modified enzyme with an excess of dithiothreitol. MMTS treatment does not totally inhibit CAT, since 4–5% of the initial activity remains after extensive modification, and further addition of MMTS does not result in an additional decrease in activity. This contrasts with inactivation by DTDP, where approx. 0.5% of the initial activity can be detected after modification.

The resistance of CAT_{III} to modification by charged reagents is entirely consistent with the hypothesis that the sites of action for these reagents (His-195 for iodoacetate and Cys-31 for DTNB) both lie within the chloramphenicol-binding pocket (Fig. 1). This pocket is amphiphilic, reflecting the nature of the substrate (Leslie *et al.*, 1988). Cys-31, and to a lesser extent His-195, are located within the more hydrophobic region of the chloramphenicol-binding site, which would tend to discriminate against the access of hydrophilic and charged reagents to these residues. The pre-modification binding of DTNB and DTDP is explained most economically by the fact that both of these reagents contain aromatic rings, which may fit into that part of the binding site normally occupied by the *p*-nitrophenol moiety of chloramphenicol.

Substitution of Cys-31 by alanine, serine, threonine or methionine

Replacement of Cys-31 was accomplished by means of site-directed mutagenesis as described in the Materials and methods section. The mutant enzymes were purified by affinity chromatography on chloramphenicol–Sephacrose. Each CAT mutant was homogeneous as judged by SDS/PAGE, producing a single band of mobility identical with that of wild-type CAT.

The steady-state kinetic properties of wild-type and mutant enzymes are shown in Table 2.

Cys-31→Ala, Cys-31→Ser or Cys-31→Thr. The Cys-31→Ser substitution is the only one of those examined that does not produce a substantial decrease in catalytic activity, although it does result in a 3.6-fold increase in the K_m value for chloramphenicol. Replacement of Cys-31 by alanine or threonine also results in modest increases in the K_m values for chloramphenicol, but here the k_{cat} values are decreased, 2.4-fold for [Ala³¹]CAT and 1.9-fold for [Thr³¹]CAT. Substitution of Cys-31 by alanine, serine or threonine does not appear to affect acetyl-CoA binding, since the K_m values for acetyl-CoA for chloramphenicol acetylation and for acetyl-CoA hydrolysis are not significantly altered in the mutant enzymes. The changes in k_{cat} values for acetyl-CoA hydrolysis parallel those observed for the k_{cat} values for chloramphenicol acetylation.

Cys-31→Met. The Cys-31→Met substitution results in an enzyme with kinetic properties that are somewhat different from those noted above. Here the K_m for chloramphenicol is unchanged whereas k_{cat} is decreased 13-fold and the K_m for acetyl-CoA is decreased 2.6-fold. Moreover, the k_{cat} for [Met³¹]CAT in the acetyl-CoA hydrolysis reaction is increased 2.7-fold over the wild-type k_{cat} . In fact, the Cys-31→Met substitution alters the specificity of CAT such that the ratio of the specificity constant (k_{cat}/K_m for acetyl-CoA) for transacetylation to that for acetyl-CoA hydrolysis, which is 8033 for wild-type CAT, is decreased to 670 for [Met³¹]CAT. The increase in acetyl-CoA hydrolysis activity could be due to a specific and favourable interaction between the side chain of Met-31 and either the acyl moiety of acetyl-CoA or the water molecule required for the hydrolysis reaction. The latter is perhaps more likely, as there is no evidence for an interaction between acetyl-CoA and Met-31 in the K_m values for acetyl-CoA in the hydrolysis reaction.

The increases in the K_m values for chloramphenicol observed for the alanine, serine and threonine replacements are readily explicable, as the site of mutagenesis is within the chloramphenicol-binding pocket. Although the side chain of Cys-31 is located within the chloramphenicol-binding site, it is not in van der Waal's contact with chloramphenicol. In fact, there is only a 0.5 nm³ change in the solvent-accessible area of Cys-31 on chloramphenicol binding (A. G. W. Leslie, unpublished work). The closest approaches of Cys-31 to chloramphenicol are the 0.414 nm and 0.420 nm that separate the γ -sulphur atom of this residue from two of the ring carbon atoms of chloramphenicol. The data presented in Table 3 suggest that the hydrophobicity of the side chain of residue 31 has a greater effect on chloramphenicol binding than does the volume of the residue.

Table 2. Kinetic parameters for wild-type and mutant CATs

Kinetic parameters are the means of at least two determinations performed as described in the Materials and methods section.

CAT	Chloramphenicol acetylation			Acetyl-CoA hydrolysis	
	k_{cat} (s ⁻¹)	K_m for chloramphenicol (μM)	K_m for acetyl-CoA (μM)	k_{cat} (s ⁻¹)	K_m for acetyl-CoA (μM)
Wild-type CAT	599	11.6	93.4	0.083	104
[Ser ³¹]CAT	818	41.4	104	0.089	96
[Ala ³¹]CAT	253	22.4	73.6	0.049	80
[Thr ³¹]CAT	310	34.2	69.8	0.045	73
[Met ³¹]CAT	46	12.0	36.0	0.226	118

Table 3. Hydrophobicity, volume and K_m values for chloramphenicol for cysteine and the amino acid residues replacing Cys-31 in CAT_{III}

The volume given is the mean volume of a residue buried in a protein where the volume of a glycine residue is 0.066 nm³ (Chothia, 1984). The hydrophobicity of the amino acid residue is taken as the free energy of transfer from water to ethanol (Nozaki & Tanford, 1971; Levitt, 1976). The K_m values for chloramphenicol are taken from Table 2.

Amino acid residue	Volume (nm ³)	Hydrophobicity (kJ/mol)	K_m for chloramphenicol (μ M)
Met	0.171	-6.3	12.0
Cys	0.118	-4.8	11.6
Ala	0.092	-2.4	22.4
Thr	0.122	-1.9	34.2
Ser	0.099	1.5	41.4

Generally, the K_m for chloramphenicol tends to increase as the hydrophobicity of the side chain of residue 31 decreases. Substitutions at residue 31 may alter the state of hydration of the chloramphenicol-binding site, thus affecting the affinity of CAT for this ligand.

Three of the above substitutions result in side-chain replacements that are approximately the same volume (threonine) or smaller (serine or alanine) than a cysteine side chain. In contrast, the Cys-31→Met substitution is accompanied by a large increase in side-chain volume. Since the K_m value for this substrate is unchanged in [Met³¹]CAT, it is possible that the side chain of Met-31 can adopt a conformation that does not hinder chloramphenicol binding. Alternatively, the conformation of chloramphenicol bound to [Met³¹]CAT may be different from that observed in the binary complex of wild-type CAT and chloramphenicol. Such an alternative binding mode would be of approximately equal affinity but might disrupt the hydrogen-bonding interaction between the 3-hydroxy group of chloramphenicol and N-3 atom of His-195, thus providing a rationale for the large decreases in k_{cat} resulting from the Cys-31→Met substitution.

Each of the enzymes with substitutions for cysteine at residue 31 is resistant to inactivation by DTNB, DTDP and MMTS, whereas all are inactivated by iodoacetamide with bimolecular rate constants that are at least as great as that for wild-type CAT (Table 4). In such instances, iodoacetamide can inactivate CAT only by modifying His-195, whereas in wild-type CAT Cys-31 is

Table 4. Chemical modification of wild-type and mutant CATs

Bimolecular rate constants for iodoacetamide inactivation and the percentages of activity remaining after extensive treatment of wild-type and mutant enzymes with 0.4 mM-MMTS, 0.2 mM-DTDP and 1 mM-DTNB were determined as described in the Materials and methods section.

CAT	Bimolecular rate constant (M ⁻¹ ·s ⁻¹)	Activity remaining after extensive modification (% of initial activity)		
	Iodoacetamide	MMTS	DTNB	DTDP
Wild-type CAT	0.020	4.5	< 1	< 1
[Ala ³¹]CAT	0.034	97	98	98
[Met ³¹]CAT	0.030	95	95	102
[Ser ³¹]CAT	0.020	91	103	92
[Thr ³¹]CAT	0.037	103	98	91

an alternative target for modification. With the removal of the thiol group at residue 31 a competing reaction, which may not completely inactivate the enzyme, has been eliminated.

Kinetic properties of MMTS-modified wild-type and [Met³¹]CAT

The substitution of Cys-31 by methionine results in an enzyme that may be structurally similar to that produced on modification of CAT_{III} by MMTS. The inactivation of CAT by this reagent must be due to modification of Cys-31, as enzyme with the Cys-31→Met substitution is resistant to inactivation by MMTS (Table 4). We have therefore compared the kinetic properties of MMTS-modified wild-type CAT with those of similarly treated [Met³¹]CAT. As noted above, MMTS treatment of wild-type CAT yields an enzyme with 4–5% residual activity, whereas MMTS treatment of [Met³¹]CAT causes no significant decrease in catalytic activity. After removal of excess reagent by dialysis there was no re-activation of wild-type CAT. A similar procedure was followed to produce DTDP-modified wild-type CAT. Here the enzyme was modified until only 0.8% of the initial activity remained.

The kinetic properties of the MMTS-treated enzymes are shown in Table 5. MMTS treatment of wild-type CAT results in a 2-fold increase in the K_m for chloramphenicol, whereas the K_m for acetyl-CoA is decreased 1.8-fold. However, the major consequence of MMTS modification of wild-type CAT is a large (28-fold) decrease in k_{cat} . MMTS treatment of [Met³¹]CAT results in

Table 5. Kinetic parameters for MMTS-modified and DTDP-modified CATs

Wild-type CAT was treated with MMTS and DTDP and [Met³¹]CAT was treated with MMTS as described in the Materials and methods section. The kinetic parameters shown are the means of at least two determinations.

CAT	Treatment	Chloramphenicol acetylation			Acetyl-CoA hydrolysis	
		k_{cat} (s ⁻¹)	K_m for chloramphenicol (μ M)	K_m for acetyl-CoA (μ M)	k_{cat} (s ⁻¹)	K_m for acetyl-CoA (μ M)
Wild-type CAT	None	599	11.6	93.4	0.083	103.5
Wild-type CAT	MMTS	21.3	22.8	53.3	0.407	106.9
Wild-type CAT	DTDP	6.8	73.5	72.1	0.108	113.6
[Met ³¹]CAT	None	46	12.0	36.0	0.226	118.4
[Met ³¹]CAT	MMTS	51.4	15.3	54.6	0.315	100.3

small (less than 1.5-fold) increases in k_{cat} and K_m values; these changes may be the result of modification of [Met³¹]CAT at sites other than Cys-31. If such modification occurs, it has little effect on overall catalytic activity (Table 4).

In summary, the kinetic parameters of MMTS-treated wild-type CAT closely resemble those of similarly treated [Met³¹]CAT. This resemblance also extends to the kinetic parameters for the hydrolysis of acetyl-CoA wherein k_{cat} is increased in MMTS-treated wild-type CAT such that the value is similar to that of MMTS-treated [Met³¹]CAT.

In contrast, the kinetic parameters for DTDP-treated wild-type CAT for acetyl-CoA hydrolysis are almost identical with those of untreated CAT, whereas in the case of chloramphenicol acetylation k_{cat} is decreased 88-fold and the K_m for chloramphenicol is increased 6.3-fold compared with wild-type CAT (Table 5). This result is consistent with the presence of a bulky modifying group in the chloramphenicol-binding site.

CONCLUSIONS

Although studies of reactive thiol groups have made important contributions to the understanding of enzyme specificity and catalysis, it has not always been easy to draw firm conclusions from the results of chemical-modification studies. The advent of site-directed mutagenesis has simplified the task to some degree, but there remains the need to compare the two approaches to discern more clearly their respective advantages and limitations. The properties and location of a uniquely reactive thiol group (Cys-31) in CAT_{III} suggested that its study might provide a clearer view of the factors contributing to the loss of activity observed in some systems coincident with the modification or replacement of non-essential cysteine residues close to substrate-binding sites or catalytic centres.

The results described demonstrate that modification of Cys-31 is responsible for the inhibition of CAT activity resulting from treatment of CAT_{III} with DTNB, MMTS and DTDP. Substitution of Cys-31 by site-directed mutagenesis results in enzymes that are resistant to inactivation by each of the above reagents. The discrimination observed in the effectiveness of uncharged as compared with anionic reagents as inhibitors of CAT_{III} can be explained by noting that all of the reagents studied here are presumed to attack their target residues (Cys-31 or His-195) from within the hydrophobic portion of the chloramphenicol-binding pocket. Irrespective of whether the modification is preceded by a binding step (DTNB or DTDP) or not (MMTS or iodoacetamide), the non-polar nature of the environment of Cys-31 and His-195 probably discriminates against ionized ligands.

The kinetic properties of each of the Cys-31-substituted enzymes are significantly different from those of wild-type CAT. Each substitution affected k_{cat} , the K_m for chloramphenicol or both parameters. In fact, none of the substitutions provided an entirely satisfactory replacement for Cys-31.

It has been possible to mimic the consequences of the Cys-31→Met substitution by modification of CAT_{III} by MMTS. The unnatural amino acid residue ($R = -CH_2-S-S-CH_3$) produced by chemical modification with MMTS is very similar in volume and only approx. 0.05 nm longer than a methionine residue ($R = -CH_2-CH_2-S-CH_3$). This implies that the structure of [Met³¹]CAT is unlikely to differ from that of wild-type CAT except in the immediate environment of Met-31. This approach is feasible only when the protein of interest contains one uniquely reactive cysteine residue.

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