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Evidence for Transition-State Stabilization by Serine-148 in the Catalytic Mechanism of Chloramphenicol Acetyltransferase†,‡

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ABSTRACT: The function of conserved Ser-148 of chloramphenicol acetyltransferase (CAT) has been investigated by site-directed mutagenesis. Modeling studies (P. C. E. Moody and A. G. W. Leslie, unpublished results) suggested that the hydroxyl group of Ser-148 could be involved in transition-state stabilization via a hydrogen bond to the oxyanion of the putative tetrahedral intermediate. Replacement of serine by alanine results in a mutant enzyme (Ala-148 CAT) with k_{cat} reduced 53-fold and only minor changes in K_m values for chloramphenicol and acetyl-CoA. The Ser-148 → Gly substitution gives rise to a mutant enzyme (Gly-148 CAT) with k_{cat} reduced only 10-fold. A water molecule may partially replace the hydrogen-bonding potential of Ser-148 in Gly-148 CAT. The three-dimensional structure of Ala-148 CAT at 2.34-Å resolution is isosteric with that of wild-type CAT with two exceptions: the absence of the Ser-148 hydroxyl group and the loss of one poorly ordered water molecule from the active site region. The results are consistent with a catalytic role for Ser-148 rather than a structural one and support the hypothesis that Ser-148 is involved in transition-state stabilization. Ser-148 has also been replaced with cysteine and asparagine; the Ser-148 → Cys mutation results in a 705-fold decrease in k_{cat} and the Ser-148 → Asn substitution in a 214-fold reduction in k_{cat}. Removing the hydrogen bond donor (Ser-148 → Ala or Gly) is less deleterious than replacing Ser-148 with alternative possible hydrogen bond donors (Ser-148 → Cys or Asn).

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28) catalyzes the inactivation by O-acetylation of chloramphenicol using acetyl coenzyme A (acetyl-CoA) as acetyl donor. The acetylated antibiotic cannot bind to bacterial ribosomes and consequently is inactive as an inhibitor of protein synthesis [reviewed by Shaw (1983)]. CAT variants have been isolated from both Gram-negative and Gram-positive bacteria where *cat* genes are commonly plasmid-borne (Shaw & Leslie, 1989). The nucleotide sequences of seven *cat* determinants are now known [reviewed by Shaw and Leslie (1989) and Murray et al. (1989)], and the deduced amino acid sequences show a marked degree of homology. It is likely that all CAT variants are trimeric enzymes of identical subunits of M_r 25 000 (Leslie et al., 1986; Harding et al., 1987).

The three-dimensional structure of the catalytically efficient type III variant of CAT (CAT_{III}) has recently been determined (Leslie et al., 1988). The gene encoding this variant (cat_{III}) was originally isolated from a naturally occurring, transmissible plasmid (R387). CAT_{III} is expressed at high levels (30–50% of the total soluble protein) in *Escherichia coli* harboring the cat_{III} gene inserted into appropriate expression vectors (Murray et al., 1988).

The structures of the binary complexes of CAT_{III} with chloramphenicol and CoA have been refined to 1.75 and 2.4 Å, respectively (Leslie et al., 1988; P. C. E. Moody and A. G. W. Leslie, unpublished results). The active site of CAT_{III} is located at the intersubunit interface such that there are three

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‡ Crystallographic coordinates of the Ser-148 → Ala mutant of chloramphenicol acetyltransferase have been submitted to the Brookhaven Protein Data Bank under the name 1CLA coordinates.
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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; TSE buffer, 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA; Tris, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MPD, 2-methyl-2,4-pentanediol; CM, chloramphenicol; CoA, coenzyme A.

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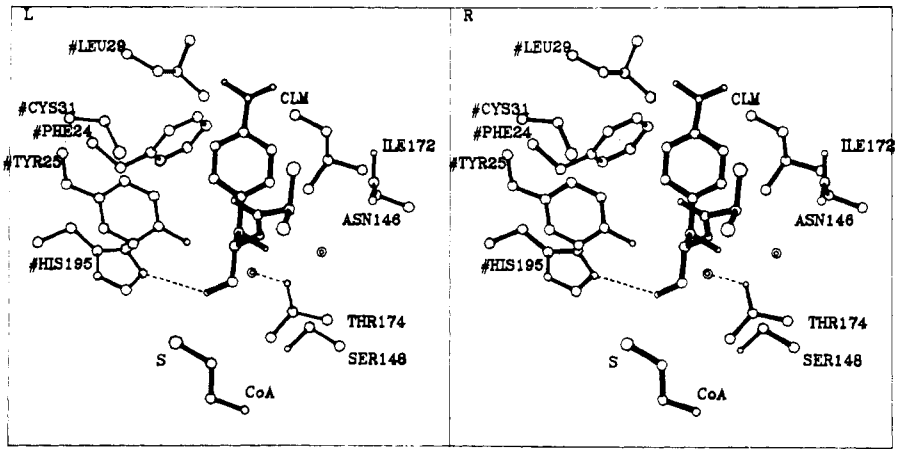


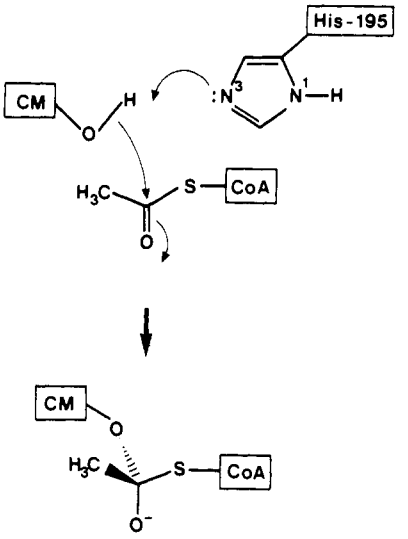
FIGURE 1: Stereoview of the active site showing the position of Ser-148 relative to the essential histidine residue (His-195), chloramphenicol (CLM), and coenzyme A (CoA). For clarity, only the terminal four atoms of CoA are illustrated. Residue names preceded by # belong to an adjacent subunit. Double circles indicate ordered water molecules, and dashed lines represent predicted hydrogen bonds.

active sites per trimer. One subunit provides most of the residues that form the substrate binding sites whereas the opposing subunit supplies a catalytically important histidine residue. A catalytic role for the imidazole moiety of His-195[2] was indicated from the results of studies with an active site directed inhibitor of CAT, 3-(bromoacetyl)chloramphenicol, which inactivates by stoichiometric modification of the N^3 atom of His-195 (Kleanthous et al., 1985). The chloramphenicol and acetyl-CoA binding sites together form a remarkable tunnel (approximately 25 Å long) that extends from one side of the molecule to the other. Chloramphenicol and acetyl-CoA approach the active site from opposite ends of the tunnel to bring the 3-hydroxyl of chloramphenicol and the S atom of CoA within 2.8 and 3.3 Å, respectively, of the N^3 atom of His-195 (Leslie et al., 1988). This structural information rationalizes data from steady-state kinetic analysis which suggested a mechanism for CAT_{III} involving a ternary complex with both substrates binding independently (Kleanthous & Shaw, 1984).

A mechanism has been proposed for CAT wherein His-195 acts as a general base, abstracting a proton from the 3-hydroxyl of chloramphenicol, thereby promoting nucleophilic attack at the carbonyl of the thioester of acetyl-CoA (Kleanthous et al., 1985). The primary hydroxyl of chloramphenicol in the CAT/chloramphenicol binary complex is correctly positioned to hydrogen bond to N^3 of His-195 (Leslie et al., 1988). Such a mechanism predicts the formation of a charged (oxyanion) tetrahedral intermediate (Scheme I) which would collapse to give the products 3-acetylchloramphenicol and CoA. In the absence of chloramphenicol, CAT catalyzes the hydrolysis of acetyl-CoA (albeit at a rate approximately 10000-fold lower than that for acetyl transfer to chloramphenicol) where water acts as the nucleophile in place of chloramphenicol (Kleanthous & Shaw, 1984).

Ser-148 is one of only 27 absolutely conserved residues in the amino acid sequences of seven CAT variants. This residue is located in the active site region of CAT_{III} (Figure 1); the hydroxyl group of Ser-148 lies 4.3 Å from the 3-hydroxyl of chloramphenicol and 5.0 Å from the sulfur of CoA. The position of the Ser-148 side chain suggested two possible roles for its hydroxyl group: (i) binding the proposed tetrahedral intermediate, thus stabilizing the transition state, and/or (ii) binding the acetyl groups of acetyl-CoA or 3-acetylchloramphenicol (the structures of the binary complexes of CAT_{III} with each of the two acetylated ligands are not available).

Scheme I



The role of Ser-148 was investigated by replacing it with alanine, glycine, cysteine, and asparagine. Neither alanine nor glycine can serve as hydrogen bond donors whereas both cysteine and asparagine have the potential to act as alternative hydrogen bond donors. If Ser-148 acts as a hydrogen bond donor in binding the acetyl groups of acetyl-CoA and/or 3-acetylchloramphenicol, the binding of these ligands should be weaker in the Ser-148 → Ala and Ser-148 → Gly mutants as inferred from increases in the K_m values for these substrates. Alternatively, a role for the hydroxyl of Ser-148 in transition-state binding should be reflected mainly by a fall in k_{cat} (providing there is no change in the rate-determining step of the reaction).

An earlier analysis of the function of a conserved residue at the active site of CAT_{III} demonstrated a structural role for Asp-199 rather than a functional one (Lewendon et al., 1988). Subsequent crystallographic studies demonstrated unexpected and far-reaching structural changes caused by the substitution Asp-199 → Asn (M. R. Gibbs, P. C. E. Moody, and A. G. W. Leslie, unpublished results). In the present study, the determination of the structure of a mutant enzyme was therefore considered to be one of the prime objectives. The three-dimensional structure of the Ser-148 → Ala CAT has

[2] Alignment of the amino acid sequences of seven CAT variants has resulted in a general numbering system which is used here. Phe-33, Asn-146, Ser-148, Phe-158, Thr-174, Met-175, and His-195 are residues 28, 140, 142, 152, 168, 169, and 189, respectively, in the primary sequence of type III CAT (Murray et al., 1988).

been determined at 2.34-Å resolution and shown to be isosteric with that of wild-type CAT. A structural role for Ser-148 has been ruled out, and a catalytic role is indicated from the results of kinetic studies on the mutant enzymes.

Experimental Procedures

Site-Directed Mutagenesis and Expression of CAT. Oligonucleotide-directed mismatch mutagenesis was performed according to the deoxyuridine selection protocol with the *dut ung E. coli* strain RZ1032 (Künkel et al., 1987). The presence of the desired nucleotide substitution and the absence of second-site mutations were confirmed by determination of the nucleotide sequence of the DNA spanning the entire *cat* coding and 5′ noncoding regions. Mutant *cat* determinants were overexpressed in *E. coli* following 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-polyacrylamide gel electrophoresis where each CAT mutant produced single bands of identical mobility to that of wild-type CAT.

The concentration of purified CAT was calculated from the $\epsilon_{280}^{1\%} = 1.31$ or by the method of Lowry (1951), using wild-type CAT_{III} as standard.

Assay of CAT Activity. CAT activity was assayed spectrophotometrically at 25 °C. One unit of enzyme activity is defined as the amount converting 1 μmol of substrate to product per minute.

(*i*) *Forward Reaction.* A modification of the procedure used by Shaw (1975) was used to measure rates of chloramphenicol acetylation. The standard assay mixture contained TSE buffer, pH 7.5, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 mM chloramphenicol, and 0.4 mM acetyl-CoA. The reaction was initiated by addition of enzyme, and the formation of CoA was monitored at 412 nm. Chloramphenicol and acetyl-CoA concentrations were varied in the standard mixture during kinetic analysis.

(*ii*) *Acetyl-CoA Hydrolysis.* Rates of acetyl-CoA hydrolysis were determined exactly as for the forward reaction except that chloramphenicol was omitted from the reaction mixture. The amount of enzyme added to these assays was generally 1000-fold greater than that added to the forward reaction assays.

(*iii*) *Reverse Reaction.* Initial rates for acetylation of CoA by 3-acetylchloramphenicol were measured at 340 nm by coupling the production of acetyl-CoA to the citrate synthase and malate dehydrogenase reactions as described by Kleanthous and Shaw (1984). The assay mixture contained TSE buffer, pH 7.5, 5 mM L-malate, 1.5 mM NADH, 0.11 mM NAD, 2 units of malate dehydrogenase, 4 units of citrate synthase, 3-acetylchloramphenicol, and CoA. The reaction was initiated by the addition of 3-acetylchloramphenicol.

(*iv*) *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).

Crystallization of Ala-148 CAT and Determination of Its Structure. Single crystals were grown by microdialysis using small Lucite "buttons". Each button contained 30 μL of protein (5 mg/mL) in 10 mM MES, pH 6.3, and was dialyzed against 4 mL of 4% 2-methyl-2,4-pentandiol (MPD), 10 mM MES, pH 6.3, 1 mM chloramphenicol, 0.5 mM hexaamminecobalt(III) chloride at 4 °C (Leslie et al., 1986). Crystals grew in 8 weeks, the largest crystal having dimensions 800 × 400 × 400 μm³. This large crystal was harvested into fresh solution identical with the dialysate but containing 8% MPD. The crystals were isomorphous with those of the wild-type enzyme, with unit cell parameters very similar to the wild-type values: space group $R32$, $a = 107.6$ Å, $c = 123.6$ Å for wild type and $a = 107.3$ Å, $c = 123.3$ Å for Ala-148 CAT (Leslie et al., 1988).

Crystallographic data were collected to 2.34-Å resolution on an Arndt–Wonacott oscillation camera on the Wiggler line at the SERC synchrotron radiation source at Daresbury, U.K., using radiation of wavelength 0.89 Å. The crystal was cooled to 4 °C to reduce radiation damage. Data were processed by using the MOSFLM program suite. The crystallographic merging R factor was 3.3% for all data.[3] The refined 1.75-Å resolution wild-type structure (Leslie et al., 1988) was used as a starting model for refinement of Ala-148 CAT. The mutant structure was refined by using a restrained least-squares structure factor refinement program (Hendrickson & Konnert, 1980). Manual rebuilding was performed by using the interactive graphics program FRODO (Jones, 1978). In all, 19 cycles of refinement were performed, giving a final model with good stereochemistry and an overall conventional crystallographic R factor of 17.2%[4] (Table I).

Table I: Stereochemistry of Ala-148 CAT

stereochemical refinement parameter	rms deviation from ideal values	refinement restraint weighting values
bond distances (Å)	0.019	0.02
angle distances (Å)	0.043	0.03
planar 1–4 distances (Å)	0.056	0.05
planes (Å)	0.02	0.02
chiral volumes (Å³)	0.17	0.15
van der Waals		
single torsion (Å)	0.17	0.2
multiple torsion (Å)	0.23	0.2

Table II: Kinetic Parameters for Acetylation of Chloramphenicol by Wild-Type and Mutant Chloramphenicol Acetyltransferases[a]

CAT	k_{cat} (s^{-1})	K_m (μM)	
		chloramphenicol	acetyl-CoA
wild type (Ser-148)	599	11.6	93.4
Ser-148 → Ala	11.2	5.2	121
Ser-148 → Gly	59.3	17.6	135
Ser-148 → Cys	0.85	13.6	192
Ser-148 → Asn	2.8	20.9	156

[a] Kinetic parameters are the mean of at least two determinations performed as described under Experimental Procedures.

Results and Discussion

The role of conserved Ser-148 in CAT_{III} was investigated by replacing serine with alanine, glycine, cysteine, and as-

[3] The merging R factor (R_{merge}) is defined as

$$R_{merge} = \sum\sum|I(h)_i - \langle I(h)\rangle|/\sum\sum I(h)_i$$

where $I(h)_i$ is the scaled intensity for the reflection h from the ith film, $\langle I(h)\rangle$ is the weighted mean of all observations of reflection h, and the summation includes all observations.

[4] Progress of the structure refinement was monitored by a reliability index, R, defined as

$$R = \sum|F_{obs} - F_{calc}|/\sum F_{obs}$$

where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

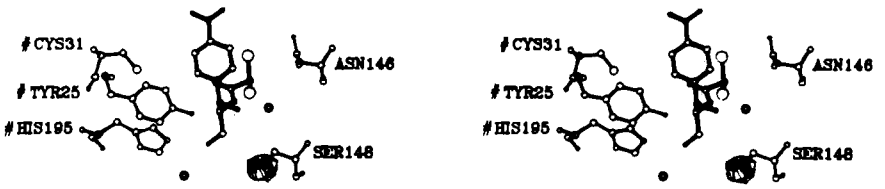


FIGURE 2: Difference Fourier map for Ala-148 CAT minus wild-type CAT superimposed on the wild-type structure to show the loss of the Ser-148 hydroxyl group. Water molecules are represented as double circles. Chloramphenicol is shown in boldface. Residue names preceded by # belong to an adjacent subunit. The map was contoured at $\pm 10\sigma$ (0.7 e/Å^3). Only negative density is illustrated.

Table III: Kinetic Parameters for Hydrolysis of Acetyl-CoA by Wild-Type and Mutant Chloramphenicol Acetyltransferases[a]

	$10^3 k_{cat}$ (s^{-1})	K_m for acetyl-CoA (μM)
wild type (Ser-148)	53	44
Ser-148 → Ala	1.5	139
Ser-148 → Gly	3.7	178
Ser-148 → Cys	1.0	38
Ser-148 → Asn	0.44	40

[a] Kinetic parameters are the mean of two determinations as described under Experimental Procedures.

paragine by use of site-directed mutagenesis. The steady-state kinetic properties of the wild-type and mutant enzymes for the acetylation of chloramphenicol and the hydrolysis of acetyl-CoA catalyzed by CAT in the absence of chloramphenicol are shown in Tables II and III, respectively.

Characterization of Ala-148 CAT and Gly-148 CAT. The most conservative substitution is that involving removal of hydrogen-bonding potential by deletion of the hydroxyl group. The Ser-148 → Ala substitution results in a mutant enzyme (Ala-148 CAT) with k_{cat} values reduced 53-fold for the acetylation of chloramphenicol (Table II) and 35-fold for the hydrolysis of acetyl-CoA (Table III). The Ser-148 → Gly substitution also removes hydrogen-bonding potential but at the expense of the entire hydroxymethyl side chain. In the case of Gly-148 CAT, the k_{cat} values for chloramphenicol acetylation and acetyl-CoA hydrolysis reactions were decreased only 10- and 14-fold, respectively.

Although replacement of Ser-148 by alanine or glycine results in substantial decreases in k_{cat}, only minor changes in substrate binding occur in both mutant enzymes. However, the increased K_m values for acetyl-CoA in the hydrolysis reaction (4-fold for Gly-148 CAT and 3-fold for Ala-148 CAT) suggest that the hydroxyl of Ser-148 may hydrogen bond to acetyl-CoA when it binds to the free enzyme. The affinity of CAT_{III} for acetyl-CoA in the ternary complex (as measured by K_m values for the chloramphenicol acetylation reaction) is largely unaffected. The K_m values for 3-acetylchloramphenicol and CoA in the reverse reaction are not significantly altered in Ala-148 CAT or Gly-148 CAT (results not shown).

The above results are consistent with the hypothesis that the hydroxyl of Ser-148 acts as a hydrogen bond donor to stabilize the transition state, thereby promoting rate enhancement. The further deletion of a methyl group in going from Ala-148 CAT to Gly-148 CAT results in an increase in catalytic activity which is largely due to an increase in k_{cat}. A plausible explanation is that the larger cavity in Gly-148 CAT, as compared to Ala-148 CAT, admits a water molecule which can replace the hydroxyl of Ser-148 as a hydrogen bond donor and can contribute to the stabilization of the transition state.

Structure of Ala-148 CAT. The strongest feature in the initial difference electron density map between the wild-type enzyme and Ala-148 CAT is a large negative peak (−0.98 e/Å^3) resulting from the loss of the Ser-148 hydroxyl group (Figure 2). The only other significant peaks in this initial difference electron density map are a cluster of negative peaks (−0.46 to −0.51 e/Å^3) around the chloramphenicol molecule. Given the good electron density for chloramphenicol at the end of refinement, this indicates that it has a slightly lower occupancy than in the wild-type structure. There were also a number of features associated with residues known to adopt multiple conformations in the wild-type structure. Asn-146 and Met-175 were two such residues, and were both modeled in the wild-type conformation.

The structure of the Ala-148 enzyme is isosteric with the high-resolution CAT/chloramphenicol binary complex structure (Leslie et al., 1988), with the exception of the mutated residue. The root mean square (rms) deviation in atomic positions is only 0.18 Å (that for all main-chain and side-chain atoms is 0.13 and 0.21 Å, respectively).

The solvent structure in the active site of Ala-148 CAT is also identical with that of the CAT/chloramphenicol binary complex, with the exception of one water (water 403) that is absent in this mutant. Water 403 lies close to the site of acetylation of chloramphenicol. In the 1.75-Å resolution CAT/chloramphenicol structure, water 403 appears at the noise level of the electron density map, with a temperature factor of 78 Å^3. Such a high value for an internal water molecule indicates that it is not well-ordered; therefore, its loss cannot be considered significant. As the structure of Ala-148 CAT is identical with that of wild type (with the exceptions discussed above), a solely structural role for Ser-148 can be eliminated.

Ser-148 → Cys. Thiols are poorer hydrogen bond donors than hydroxyl groups (Weiner et al., 1984). Consequently, Cys-148 might be expected to provide less transition-state stabilization than Ser-148. In addition, this substitution is not strictly conservative: the van der Waals radius of a sulfur atom is 1.9 Å compared to 1.7 Å for an oxygen atom (McCammon et al., 1979), and more importantly, there is a large change in bond length on going from C–O (1.42 Å) to C–S (1.81 Å) which will disrupt hydrogen bond geometry.

The Ser-148 → Cys substitution resulted in a mutant enzyme (Cys-148 CAT) with a dramatic decrease (705-fold) in k_{cat} for acetyl transfer to chloramphenicol and only small increases in substrate K_m values. In contrast to the results with Ala-148 CAT and Gly-148 CAT, the extent of the decrease in k_{cat} for acetyl transfer is not parelleled in the acetyl-CoA hydrolysis reaction. In the latter case, k_{cat} is decreased only 53-fold and the K_m for acetyl-CoA is unchanged. Cysteine appears to be a better functional substitute for Ser-148 in the hydrolysis of acetyl-CoA than in the acetylation of chloramphenicol. It follows that cysteine may be able to form a better hydrogen bond to both acetyl-CoA and the tetrahedral intermediate in the hydrolysis reaction than in the chloramphenicol acetylation reaction. The relatively poor performance of Cys-148 CAT in the latter reaction may be explicable on steric grounds. The differing geometry of the

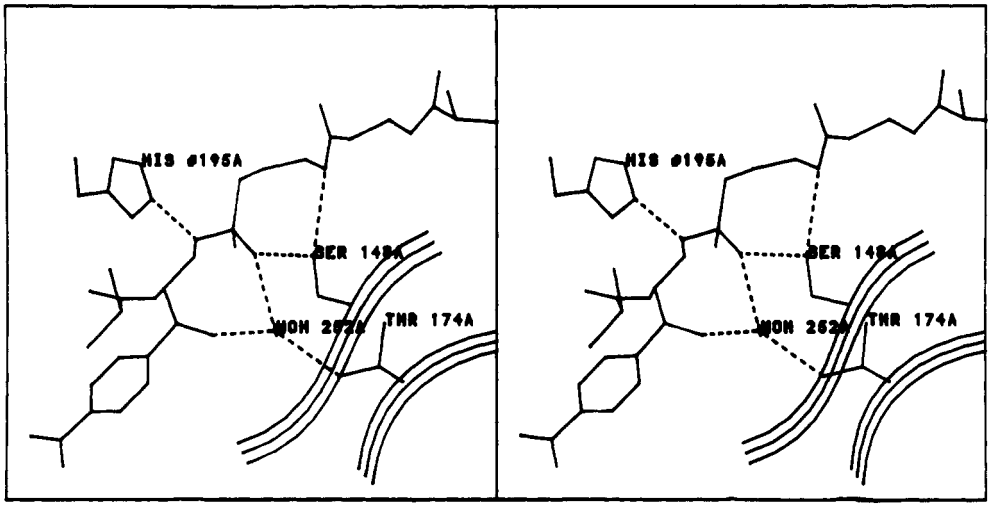


FIGURE 3: Stereoview of the modeled tetrahedral intermediate in the active site of CAT (P. C. E. Moody and A. G. W. Leslie, unpublished results). Only a portion of the CoA component of the intermediate is shown. Dashed lines represent predicted hydrogen bonds. Parallel lines indicate the position of Cα backbone.

cysteine side chain may be accommodated in the hydrolysis reaction because of the small size of the attacking nucleophile (water) but not in the acetylation reaction when there is a need to bind chloramphenicol precisely for effective nucleophilic attack by its 3-O atom at the carbonyl of the thioester.

Ser-148 → Asn. The tetrahedral intermediate proposed for the reaction catalyzed by CAT is formally similar to the oxyanion intermediate formed during the reaction catalyzed by the serine proteases. In the case of subtilisin, the oxyanion is stabilized by hydrogen bonding to the amide side chain of Asn-155 (Robertus et al., 1972). The Ser-148 → Asn replacement in CAT was made in an attempt to introduce a similar stabilizing interaction. The Ser-148 → Asn substitution gave rise, however, to a mutant enzyme (Asn-148 CAT) with a 214-fold reduction in k_{cat} for acetylation of chloramphenicol and a 120-fold decrease in k_{cat} for acetyl-CoA hydrolysis. The K_m for acetyl-CoA in the hydrolysis reaction is unchanged whereas there are small increases in the substrate K_m values for the acetylation of chloramphenicol. As in the case of Cys-148 CAT, and probably for similar reasons, the alternative hydrogen bond donor (the amide -NH of Asn-148) appears to function better in the acetyl-CoA hydrolysis reaction than in the acetylation of chloramphenicol.

CONCLUSIONS

The determination of the structure of Ala-148 CAT has ruled out a solely structural role for Ser-148. The results of steady-state kinetic studies on the mutant enzymes are consistent with the hypothesis that the hydroxyl group of Ser-148 plays a part in transition-state binding and stabilization. The proposed tetrahedral intermediate was modeled on the basis of the refined structures of the CAT/chloramphenicol and CAT/CoA binary complexes, by using a combination of computer graphics and model refinement (P. C. E. Moody and A. G. W. Leslie, unpublished results). In the initial model the methyl group (acetyl C2) was oriented toward the phenyl rings of Phe-33 and Phe-158, such that the oxyanion was within hydrogen-bonding distance of the hydroxyl of Ser-148. Refinement led to a very plausible intermediate with good stereochemistry and no unacceptable van der Waals contacts between the enzyme and the ligand.

The modeled tetrahedral intermediate (Figure 3) shows the oxyanion stabilized by two hydrogen-bonding interactions: with the hydroxyl group of Ser-148 and with an ordered water molecule (water 252). This water molecule is in turn hydrogen bonded to the hydroxyl group of Thr-174, which is also an absolutely conserved residue.

The model also predicts a hydrogen bond between the Ser-148 hydroxyl and the N atom (N-71) of the β-mercaptoethylamine component of CoA (Figure 3). This is consistent with the results from acetyl-CoA hydrolysis kinetics, which suggested an interaction between hydrogen bond donors at position 148 and acetyl-CoA.

The difference in free energy changes between the reaction catalyzed by Ala-148 CAT and wild type can be calculated from k_{cat}/K_m values to be −2.2 kcal·mol^{-1}. Fersht and coworkers (Fersht, 1988) have suggested that 3–6 kcal·mol^{-1} is the expected range for the contribution of a hydrogen bond involving a charged donor or acceptor to the binding energy in protein–ligand interactions. Nonetheless, the value obtained for stabilization by any given hydrogen bond must be greatly affected by its immediate environment. With respect to CAT, there are several factors that should be taken into account in quantitating the contribution of the oxyanion/Ser-148 hydrogen bond to transiton-state stabilization. The model of the tetrahedral intermediate suggests that the transition state will still be stabilized by hydrogen bonding to water 252 in Ala-148 CAT. In addition, any substitution resulting in deletion of an amino acid side chain raises the possibility of water molecules substituting for the missing hydrogen bond donor or acceptor. It is likely that this effect occurs in Gly-148 CAT (see above), and it cannot be ruled out in Ala-148 CAT. As any such water molecules are unlikely to be ordered, the reorganization energy required to remove them from bulk solvent will reduce their energetic contribution toward stabilization of the transition state (Warshel et al., 1988). It should also be noted that the geometry of the hydrogen bond between the oxyanion and the Ser-148 hydroxyl may not be ideal (at 3.25 Å, the modeled hydrogen bond is rather long) and that the charge on the oxyanion may not develop fully in the tetrahedral intermediate during the course of the reaction.

This study underlines the need to make the most conservative changes possible in site-directed mutagenesis experiments. Both the Ser-148 → Ala and Ser-148 → Gly mutations remove hydrogen-bonding potential, but there is a 5-fold difference in catalytic activity between the resulting mutant enzymes. Replacing Ser-148 by alternative hydrogen bond donors drastically affects catalytic activity although it should be noted that structural changes due to novel interactions possible for Cys-148 and Asn-148 cannot be ruled out.

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Registry No. CAT, 9040-07-7; CM, 56-75-7; Ser, 56-45-1; Ala, 56-41-7; Gly, 56-40-6; Cys, 52-90-4; Asn, 70-47-3; acetyl-CoA, 72-89-9.

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Reactions of the NAD Radical with Higher Oxidation States of Horseradish Peroxidase†

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ABSTRACT: The reactions of the NAD radical ($NAD^{\bullet}$) with ferric horseradish peroxidase and with compounds I and II were investigated by pulse radiolysis. $NAD^{\bullet}$ reacted with the ferric enzyme and with compound I to form the ferrous enzyme and compound II with second-order rate constants of 8×10^8 and 1.5×10^8 M^{-1} s^{-1}, respectively, at pH 7.0. In contrast, no reaction of $NAD^{\bullet}$ with native compound II at pH 10.0 nor with diacetyldeutero-compound II at pH 5.0–8.0 could be detected. Other reducing species generated by pulse radiolysis, such as hydrated electron (e_{aq}^-), superoxide anion (O_2^-), and benzoate anion radical, could not reduce compound II of the enzyme to the ferric state, although the methylviologen radical reduced it. The results are discussed in relation to the mechanism of catalysis of the one-electron oxidation of substrates by peroxidase.

It has been established that in the presence of hydrogen peroxide, horseradish peroxidase catalyzes the one-electron oxidation of various bivalent redox molecules through the catalytic cycle (George, 1952; Chance, 1952; Yamazaki et al., 1960):

$$Fe^{3+} \xrightarrow{H_2O_2} \text{compound I} \underset{AH^{\bullet}}{\overset{AH_2}{\rightleftharpoons}} \text{compound II} \underset{AH^{\bullet}}{\overset{AH_2}{\rightleftharpoons}} Fe^{3+} \quad (1)$$

An important feature of this process is the formation of free radical intermediates, as clearly demonstrated by EPR techniques (Yamazaki et al., 1960; Yamazaki & Piette, 1963; Piette et al., 1964). The free radicals thus formed are very reactive and act as strong reductants or oxidants (Ohnishi et al., 1969). In the presence of suitable electron donors or acceptors, electron transfer occurs between the free radical and the added molecule (Yamazaki & Ohnishi, 1966; Ohnishi et al., 1969; Nakamura et al., 1985). Therefore, it might be expected that the free radicals would react directly with the peroxidase. If so, two-electron oxidation of the substrate would occur without formation of free radicals. However, no such reaction between substrate free radicals and enzyme has been detected, and the only radical decay mechanism observed has been dismutation or dimerization. Indeed, Nakamura et al. (1985) reported that the one-electron flux for hydroquinone oxidation by horseradish peroxidase is nearly 100%. One of the interesting questions to be answered is why the radical does

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