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Acetyl Coenzyme A Binding by Chloramphenicol Acetyltransferase: Long-Range Electrostatic Determinants of Coenzyme A Recognition†

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ABSTRACT: The possible involvement of arginyl and lysyl side chains of chloramphenicol acetyltransferase (CAT) in binding coenzyme A (CoA) was studied by means of chemical modification, site-directed mutagenesis, variation in ionic strength, use of competitive inhibitors or substrate analogues, and X-ray crystallography. Unlike a number of enzymes, including citrate synthase, CAT does not employ specific ion pairs with the phosphoanionic centers of CoA to bind the acetyl donor, and arginyl residues play no role in recognition of the coenzyme. Although phenylglyoxal inactivates CAT reversibly, it does so by the formation of an unstable adduct with a thiol group, that of Cys-31 in the chloramphenicol binding site. The inhibitory effect of increasing ionic strength on k_{cat}/K_m(acetylCoA) can be explained by long-range electrostatic interactions between CoA and the ϵ-amino groups of Lys-54 and Lys-177, both of which are solvent-accessible. The ϵ-amino group of Lys-54 contributes 1.3 kcal·mol^{-1} to the binding of acetyl-CoA via interactions with both the 3′- and 5′-phosphoanions of CoA. Lys-177 contributes only 0.4 kcal·mol^{-1} to the productive binding of acetyl-CoA, mediated by long-range (~14 Å) interactions with the 5′-α- and -β-phosphoanions of CoA. The combined energetic contribution of Lys-54 and Lys-177 to acetyl-CoA binding (1.7 kcal·mol^{-1}) is less than that previously demonstrated (2.4 kcal·mol^{-1}) for a simple hydrophobic interaction between Tyr-178 and the adenine ring of CoA (Day & Shaw, 1992). In contrast to citrate synthase, the only other CoA binding enzyme for which high-resolution structural information is available, CAT recognizes CoA mainly by hydrophobic and polar (but uncharged) interactions.

Chloramphenicol acetyltransferase (CAT; EC 2.3.1.28)[1] is the enzyme responsible for high-level bacterial resistance to chloramphenicol (Shaw, 1983; Shaw & Leslie, 1991). The acetyl coenzyme A-dependent enzymic acetylation of chloramphenicol yields 3-acetylchloramphenicol which fails to bind to prokaryotic ribosomes (Shaw & Unowsky, 1968) and thus is devoid of antibiotic activity. Although CAT catalyzes the hydrolysis of acetyl-CoA in the absence of chloramphenicol, the thioesterase activity is 3 orders of magnitude lower than that of acetyl transfer to the antibiotic (Kleanthous & Shaw, 1984). Of more than a dozen naturally occurring CAT variants which have been described, only the type III enzyme has been studied in detail. The structure of CAT_{III}[2] is that of a homomeric trimer (3 × 25 kDa) with each of its three active sites lying deep in the clefts between subunits (Leslie et al., 1988; Leslie, 1990). The substrates approach the active site from opposite faces of the trimer to form a ternary complex, consistent with steady-state kinetic studies which revealed a sequential mechanism with a random order of addition of substrates (Kleanthous & Shaw, 1984).

Many enzymes which bind anionic substrates or cofactors have been shown to contain critical arginyl residues in their ligand binding sites (Riordan et al., 1977). Coenzyme A is such a cofactor, and on the basis of the results of chemical modification experiments with arginyl-specific reagents, it has been suggested that a number of enzymes bind CoA via ionic interactions with one or more arginyl residues (Mautner et al., 1981; Ramakrishna & Benjamin, 1981; Ragione et al.,

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM sodium chloride and 0.1 mM EDTA; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CoA, coenzyme A; AcCoA, acetyl-CoA; DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] Alignment of the amino acid sequences of seven CAT variants has resulted in a general numbering system which is used here. The numbering is related to the CAT_{III} linear sequence by subtracting 5 from residues 6-74 and 6 from residues 75-219 (Murray et al., 1988).

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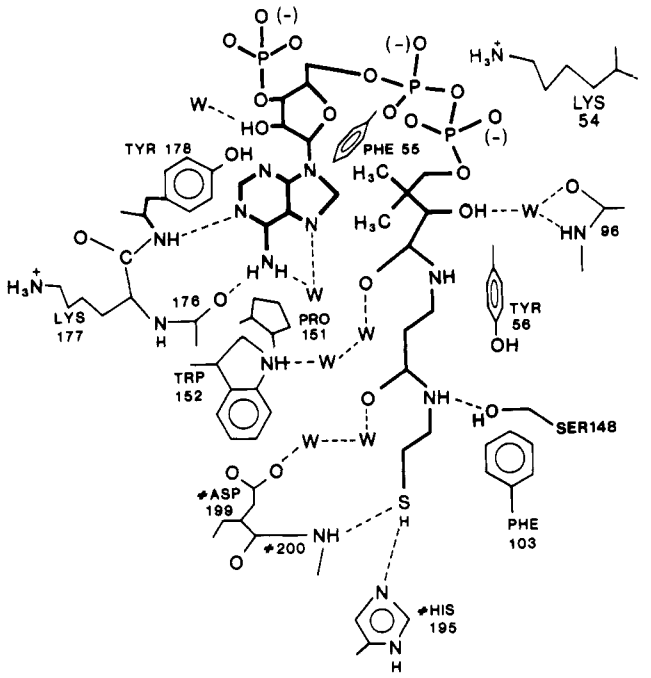

FIGURE 1: Schematic diagram of the CoA binding site of CAT_{III} (Leslie et al., 1988). Hydrogen bonds are indicated by dashed lines and ordered water molecules by W. The structure of CoA is shown in boldface. Residue names preceded by (#) are from the facing subunit. The main chain amide of residue 54, which is hydrogen-bonded to the C-2 hydroxyl of CoA via a bridging water molecule, has been omitted to simplify the positioning of the side chain of Lys-54.

1983). Furthermore, the structure of the complex of citrate synthase and CoA reveals the presence of salt bridges between each of the phosphoanions of CoA and the guanidinium groups of arginyl residues (Remington et al., 1982). Preliminary chemical modification studies of CAT_{III} showed that treatment with diketone reagents is accompanied by a rapid loss of activity, suggesting that arginyl residues might be important for CoA binding to CAT (Corney, 1983). However, the 2 arginyl residues (Arg-18 and Arg-74) which are conserved in 12 known CAT sequences (Shaw & Leslie, 1991) have been shown, by both site-directed mutagenesis (Lewendon et al., 1988) and structural studies (Leslie et al., 1988; Gibbs et al., 1990), to play structural roles via intrasubunit ion pairs with aspartyl residues.

Unlike that of citrate synthase, the structure of the CAT–CoA binary complex (Leslie et al., 1988) reveals that on binding to CAT the charged phosphoanionic groups of CoA remain solvent-accessible and do not interact directly with arginyl side chains. Nonetheless, the CoA binding site of CAT_{III} does contain two well-conserved lysyl residues, Lys-54 and Lys-177.

Lysine-54 is conserved in 11 of the 12 known CAT sequences and is positioned at the amino-terminal end of α-helix 3. The neighboring residues, Phe-55 and Tyr-56, form hydrophobic interactions with the adenine and pantetheine moieties of CoA, respectively (Figure 1). The main chain amide of residue 54 forms a hydrogen bond to the C-2 hydroxyl of the ribose moiety of CoA via a bridging water molecule. Although the side chain of Lys-54 appears to be disordered in the crystal structure of the CAT–CoA binary complex, modeling studies suggest that its ϵ-amino group could form a salt bridge to either of the 5′-phosphoanions of CoA.

Lysine-177, which is a basic residue in all known CAT sequences, is part of a β-bulge (also including Tyr-178 and Leu-187; Leslie, 1990) which accommodates the adenine ring of CoA. The main chain carbonyl of Ala-176 and the main chain amide of Tyr-178 are hydrogen bonded to N-10 and N-1 of the adenine ring, respectively (Figure 1). The ϵ-amino group of Lys-177 is 11.8, 13.5, and 15.1 Å from the 3′-, 5′-α-, and 5′-β-phosphates of CoA, respectively, and is thus too far away to form direct ion pairs with any of the phosphate groups of CoA.

Electrostatic interactions between charged residues up to 13 Å apart have been shown to have significant effects on catalysis and substrate binding in subtilisin (Russell & Fersht, 1987; Russell et al., 1987) and metal ion binding to calbindin D_{9K} (Linse et al., 1988, 1991). The substrate specificity can be changed in a predictable manner (Wells et al., 1987) by changing such interactions by site-directed mutagenesis or by varying the charge of the substrate. The present study aimed to examine the magnitude and likely nature of electrostatic interactions of CoA with CAT_{III} by using chemical modification, substrate analogues, competitive inhibitors, and directed mutagenesis.

Experimental Procedures

Assay of CAT Activity. Enzyme activity was measured at 25 °C as described by Lewendon et al. (1988). 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 CoA release measured spectrophotometrically at 412 nm. A unit of enzyme activity is defined as the amount converting 1 μmol of substrate to product per minute. For steady-state kinetic analysis, the concentrations of both substrates were varied in the standard mixture and kinetic constants determined as described by Kleanthous and Shaw (1984). Specificity constants (k_{cat}/K_m) for acetyl-CoA and acetyl-3′-dephospho-CoA were also estimated using the equation $v_i = (V_{max}/K_m)$[acetyl donor] by assaying in the presence of excess chloramphenicol (200 μM, 50 × K_d), under conditions where the concentration of the acetyl donor was well below its K_m in each case.

Hydrolysis of Acetyl-CoA. Rates of acetyl-CoA hydrolysis were determined under the conditions described above except that chloramphenicol was omitted from the reaction mixture. Enzyme concentrations approximately 1000-fold greater than those used for the standard transacetylation reaction were required to monitor the hydrolysis of acetyl-CoA. Acetyl-CoA was prepared as described by Simon and Shemin (1953).

Purification of CAT. Wild-type and mutant CAT proteins were purified from *Escherichia coli* extracts by affinity chromatography on chloramphenicol–Sepharose as described previously (Lewendon et al., 1988). Purified enzymes gave single bands of identical mobility on SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The concentration of purified CAT was determined from the absorbance at 280 nm ($\epsilon^{0.1\%} = 1.31$) or by the method of Lowry (1951) using bovine serum albumin as standard and reference CAT_{III} determined by amino acid analysis.

Site-Directed Mutagenesis and Expression of CAT. Oligonucleotide-directed mutagenesis was performed using the deoxyuridine selection protocol with *Escherichia coli* strain RZ1032 (Künkel et al., 1987). The presence of the desired mutation and the absence of secondary mutations were confirmed by nucleotide sequencing 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).

Chemical Modification of CAT. (*i*) *Phenylglyoxal.* Modification reactions were carried out in 45 mM HEPES buffer (pH 8.0) at 25 °C. The extent of inactivation was

monitored by removal of aliquots at timed intervals followed by appropriate dilution into TSE buffer and immediate assay. For protection experiments, enzyme was preincubated with either 1 mM acetyl-CoA or 0.1 mM chloramphenicol for 10 min at 25 °C. Enzyme concentrations varied in the range 1.5–4.0 μM when inhibition of the transacetylation activity was monitored, and 45 μM was used to monitor the effects of chemical modification on the intrinsic hydrolytic activity of CAT.

(*ii*) *Ethyl Acetimidate.* Reactions were carried out as described by Packman and Shaw (1981) in 0.2 M triethanolamine, pH 8.5, and 1 mM 2-mercaptoethanol. Stock solutions of ethyl acetimidate (1 M) were prepared immediately before use in the same buffer containing 1 equiv of NaOH. The reactions were initiated by the addition of reagent, and loss of activity was followed by dilution of timed aliquots into TSE buffer and assay under standard conditions.

Preparation of 3'-Dephospho-CoA. CoA (200 μmol) was incubated with alkaline phosphatase (200 units, Boehringer Mannheim) in 50 mM Tris-HCl, pH 8.5, 1 mM magnesium chloride, and 0.05 mM zinc chloride at 25 °C. The progress of the reaction was monitored by elution of samples from a DEAE-Sephadex column with a TEAB gradient (pH 7.5). After dilution with 20 mM TEAB (pH 7.5) containing 200 μmol of dithiothreitol, the reaction mixture was applied to a DEAE-Sephadex column preequilibrated with 20 mM TEAB, and the products were eluted with a 1-L 20–700 mM TEAB gradient. Peak fractions (by A_{260nm}) which also contained free thiols were pooled and repeatedly evaporated to dryness in the presence of methanol to remove the TEAB. Purified product was acetylated with acetic anhydride (Simon & Shemin, 1953), desalted on a Sephadex G10 column, and stored at −20 °C.

Crystallization of Ala-177 CAT and Determination of Its Structure. Crystals of Ala-177 CAT were grown by microdialysis. Protein (5 mg/mL) in 10 mM MES, pH 6.3, was dialyzed against 4 mL of 4% (v/v) 2-methyl-2,4-pentanediol (MPD), 10 mM MES, pH 6.3, 1 mM chloramphenicol, and 0.5 mM hexaminecobalt(III) chloride at 4 °C (Leslie et al., 1986). Crystals were harvested into the same solution as the dialysate but containing 8% MPD. The crystals were isomorphous with those of the wild-type enzyme, space group *R*32, with cell dimensions a = 107.6 Å, c = 123.3 Å (wild-type values are a = 107.6 Å, c = 123.6 Å.

Crystallographic data were collected to 2.2-Å resolution from a single crystal (dimensions 0.8 × 0.4 × 0.4 mm) on an Arndt–Wonacott oscillation camera on the Wiggler line at the SERC synchrotron radiation source at Daresbury, U.K., using radiation of wavelength 0.895 Å. The crystal was cooled to 4 °C to minimize radiation damage. Films were processed using the MOSFLM suite of programs, and the resulting data were scaled and merged using the CCP4 programs ROTAVATA and AGROVATA. A total of 29 454 observations were merged to give 12 315 independent reflections, representing 86% of the unique data. The crystallographic merging R factor[3] was 0.031 for all data to 2.2-Å resolution. The refined 1.75-Å resolution wild-type structure (Leslie, 1990) was used as a starting model for refinement of Ala-177 CAT. The mutant structure was refined using the restrained least-squares structure factor refinement program PROLSQ (Hendrickson & Konnert, 1980). Manual rebuilding was performed using the interactive graphics program FRODO (Jones, 1978). The atomic model gave an initial R factor[4] of 0.194 which dropped to 0.147 after refinement, with good stereochemistry (rms deviation in bond lengths 0.020 Å, in bond angles 3.0°). The structure of Ala-177 CAT is isosteric with the wild-type enzyme, with the exception of the mutated residue. The rms deviation in all protein atomic coordinates is 0.14 Å, which is comparable with the accuracy of the coordinates.

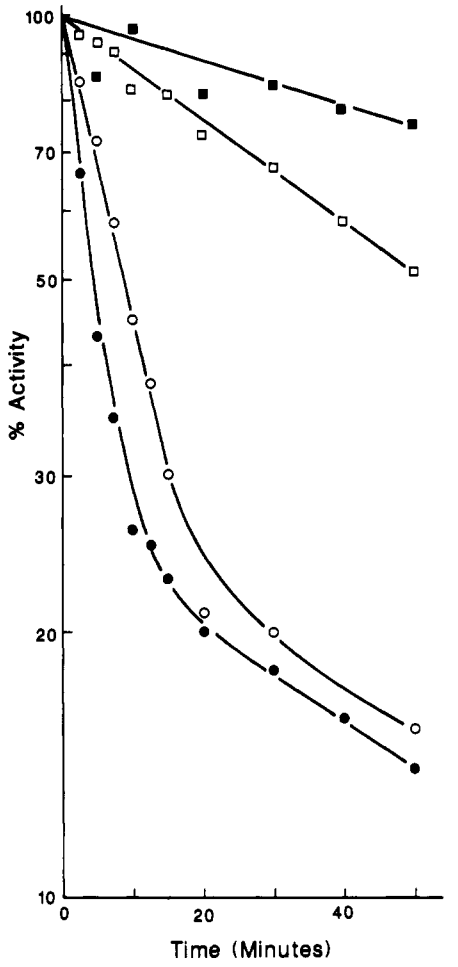

FIGURE 2: Chemical modification of CAT by phenylglyoxal. Ala-31 CAT [(■) 4.0 μM] and wild-type CAT_{III} (1.5 μM) were treated with 10 mM phenylglyoxal in 45 mM HEPES, pH 8 at 25 °C, in the presence of 0.1 mM chloramphenicol (□) or 1 mM acetyl-CoA (○) or in the absence of substrates (●) as described under Experimental Procedures.

RESULTS AND DISCUSSION

Chemical Modification of Arginyl Residues. Although the results of preliminary chemical modification studies, using 2,3-butanedione, were compatible with a direct role for arginyl residues in the binding of CoA to CAT_{III} (Corney, 1983), the structure of the CAT-CoA binary complex suggested that such cannot be the case (Leslie et al., 1988). To elucidate a possible role for arginyl residues in CoA binding, further chemical modification experiments were performed using the arginyl-

[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 refinement is 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.

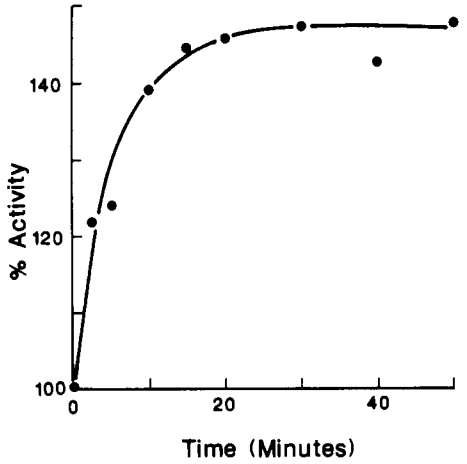

FIGURE 3: Effect of treatment with phenylglyoxal on the intrinsic hydrolytic activity of CAT_{III}. Wild-type CAT_{III} (45 μM) was treated with 10 mM phenylglyoxal as described under Experimental Procedures. Aliquots were removed at timed intervals, diluted as appropriate into TSE buffer, and immediately assayed for acetyl-CoA hydrolysis activity.

specific reagent phenylglyoxal. As found in earlier experiments with 2,3-butanedione, treatment of CAT with phenylglyoxal resulted in a biphasic loss of enzyme activity (Figure 2). The extent of inactivation during the rapid phase of activity loss was observed to be dependent on the initial inhibitor concentration, and the adduct formed is unstable, such that the removal of excess reagent by dilution results in a gradual recovery ($t_{1/2}$ = 10 min) of activity (Day, 1990). Although preincubation with either chloramphenicol or acetyl-CoA provides good protection against inactivation by 2,3-butanedione (Corney, 1983), only chloramphenicol does so against phenylglyoxal (Figure 2). Furthermore, since modification of CAT with phenylglyoxal is accompanied by a 50% increase in the intrinsic thioesterase activity (Figure 3), the loss of transacetylation activity on modification with the same reagent is unlikely to be due to modification of a residue involved directly in catalysis or acetyl-CoA binding.

The structural similarity between phenylglyoxal and chloramphenicol suggests that inactivation by phenylglyoxal could be due to modification of a residue in or close to the chloramphenicol binding site, the most likely candidate being the reactive cysteinyl residue Cys-31. Modification of Cys-31 with methyl methanethiolsulfonate results in a 95% reduction in transacetylation activity whereas the acetyl-CoA hydrolysis activity is enhanced (Lewendon & Shaw, 1990) as observed on modification of CAT_{III} by phenylglyoxal. Although generally a highly specific reagent for arginyl residues, phenylglyoxal can react, usually more slowly, with both cysteinyl and lysyl residues (Takahashi, 1977a,b). Substitution of Cys-31 with alanine (Ala-31 CAT) renders CAT_{III} insensitive to thiol-specific reagents (Lewendon & Shaw, 1990) and also abolishes the rapid phase of inactivation by phenylglyoxal (Figure 2), confirming the likelihood that loss of CAT activity on modification with phenylglyoxal is due to modification of Cys-31 rather than a critical arginyl residue.

The slow phase of inactivation is not prevented by the presence of either substrate and therefore seems likely to be due to indirect effects resulting from the modification of one or more of the eight arginyl residues (per subunit) which are, to varying degrees, solvent-accessible (Leslie et al., 1988).

Chemical Modification of Lysyl Residues. Treatment of CAT_{III}, which contains 12 lysyl residues per subunit, with methyl acetimidate results in a 40% decrease in enzyme activity and the amidination of all ε-amino groups except that

Table I: Steady-State Kinetic Parameters for Native and Chemically Modified CAT_{III}[a]

treatment	k_{cat}^{app} (s^{-1})	K_m^{app}(acetyl-CoA) (μM)
none	485	81
0.1 M ethyl acetimidate	538	456

[a] Wild-type CAT_{III} was treated with ethyl acetimidate as described under Experimental Procedures. Steady-state kinetic parameters were determined as described, except that the concentration of chloramphenicol was constant at 100 μM (25 × K_d). Kinetic parameters are the mean of at least two determinations performed as described under Experimental Procedures.

Table II: Steady-State Kinetic Parameters for Wild-Type and Mutant CAT Variants[a]

variant	k_{cat} (s^{-1})	K_m (μM) chloramphenicol	K_m (μM) acetyl-CoA
wild-type CAT_{III}	600	12.0	93
Gln-54 CAT	680	20.9	894
Glu-54 CAT	683	24.7	17100
Gln-177 CAT	645	15.2	180
Glu-177 CAT	620	13.9	417
Ala-177 CAT	571	16.9	658

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

of Lys-38 (Packman & Shaw, 1981), which is involved in an intrasubunit ion pair (Leslie, 1990). Neither chloramphenicol nor acetyl-CoA protects against loss of activity nor reduces the number of lysyl residues modified (Day, 1990). Lack of protection by acetyl-CoA against loss of activity suggests that any ion pair formed between CoA and Lys-54 must be solvent-exposed, such that Lys-54 remains accessible to the reagent in the binary complex, or that the rate of modification of Lys-54 is exceptionally fast. Fully modified enzyme has steady-state kinetic parameters which are essentially unchanged from those of the native enzyme, except for a 5-fold increase in the K_m for acetyl-CoA (Table I). Whereas modification of a lysyl residue with a monofunctional imido ester results in an increase in its side chain size, the positive charge is retained. In contrast, modification of the ε-amino group of a lysyl side chain by a dicarboxylic acid anhydride results in charge reversal. Indeed, earlier experiments demonstrated that treatment of CAT_{III} with citraconic anhydride results in a rapid and virtually complete loss of enzymic activity in the absence of evidence of subunit dissociation (Packman & Shaw, 1981). Loss of activity cannot be due to esterification of Cys-31 since treatment of Ala-31 CAT with citraconic anhydride also leads to almost complete inactivation (P.J. Day, unpublished results). Such chemical modification studies suggest that one or more lysyl residues are important, although not essential, for enzyme activity, possibly via interactions with the anionic centers of CoA.

Substitution of Conserved Lysyl Residues. To discern whether the charged side chains of residues 54 and 177, the conserved lysyl residues closest to the CoA binding site, are important for the binding of CoA to CAT, a number of substitutions were made to alter their charge. Each CAT variant described in Table II gave yields similar to the wild-type enzyme after purification by affinity chromatography, and the expected changes in charge were confirmed by polyacrylamide gel electrophoresis under nondenaturing conditions (data not shown).

Properties of CAT Variants with Substitutions for Lys-54. Replacement of the lysyl side chain of residue 54 with that of glutamine results in a 10-fold increase in the K_m for acetyl-CoA, with only small changes in the other steady-state

Table III: Steady-State Kinetic Parameters for Acetyl-CoA Hydrolysis by Wild-Type and Mutant CAT Variants[a]

variant	k_{cat} (s^{-1})	K_m (acetyl-CoA) (μM)	k_{cat}/K_m (acetyl-CoA) ($M^{-1}\cdot s^{-1}$)
wild-type CAT_{III}	0.13	66	1970
Gln-54 CAT	0.12	522	230
Glu-54 CAT	ND[c]	ND	16.3[b]
Gln-177 CAT	0.10	89	1124
Glu-177 CAT	0.13	250	520
Ala-177 CAT	0.14	439	319

[a] Kinetic parameters are the mean of at least two determinations performed as described under Experimental Procedures. [b] Estimated under conditions where the acetyl-CoA concentration was well below its K_m value using the equation $v_i = (V_{max}/K_m)$[acetyl-CoA]. [c] ND, not determined.

kinetic parameters (Table II), suggesting that the charge of residue 54 is important, though not essential, for CoA binding. A further estimate of the contribution of Lys-54 to CoA binding can be obtained from the effect of the substitution on the thioesterase activity of CAT (Table III). The decrease in the specificity constant (k_{cat}/K_m) for the hydrolysis of acetyl-CoA (9-fold) resulting from the Gln-54 substitution is in good agreement with the corresponding parameter for the transacetylation reaction. The energetic contribution to acetyl-CoA binding of the charged lysyl group can be estimated from such data (Wilkinson et al., 1983; Wells & Fersht, 1986)[5] to be 1.3 kcal·mol^{-1}, smaller than might be expected for an ion pair (Fersht et al., 1985). However, although substitution with glutamine removes the potential for charge–charge interactions, the possibility for a charged hydrogen bond between CoA and residue 54 is retained. Hence, such a substitution might be expected to underestimate the strength of the interaction.

Reversal of the charge of residue 54 has a profound effect on the affinity of CAT for acetyl-CoA. Binding of acetyl-CoA to the free enzyme (K_d) and CAT–chloramphenicol complex (K_m) is reduced almost 200-fold with only small increases in k_{cat} and the K_m for chloramphenicol (Table II). The specificity constant (k_{cat}/K_m) for the hydrolysis of acetyl-CoA (Table III) is also greatly reduced (120-fold) for Glu-54 CAT.

Properties of CAT Variants with Substitutions for Lys-177. The most significant discernible effect on removal of the positive charge from the side chain of residue 177 is to increase the K_m for acetyl-CoA (Table II). The essentially unchanged values for k_{cat} and K_m for chloramphenicol support the view that the effects for acetyl-CoA are not the results of more global structural changes. Such a conclusion is also supported by the crystallographic data for Ala-177 CAT. Except for the replacement of the Lys-177 side chain by a methyl group, the refined structure of the Ala-177 CAT–chloramphenicol binary complex at 2.2-Å resolution appears indistinguishable from that of wild-type CAT and the wild-type CAT–CoA binary complex (see Experimental Procedures).

Substitution of Lys-177 by glutamine (Gln-177 CAT) is accompanied by a much smaller change in the K_m for acetyl-CoA than that observed for the corresponding replacement at residue 54 (Gln-54 CAT), the most plausible explanation

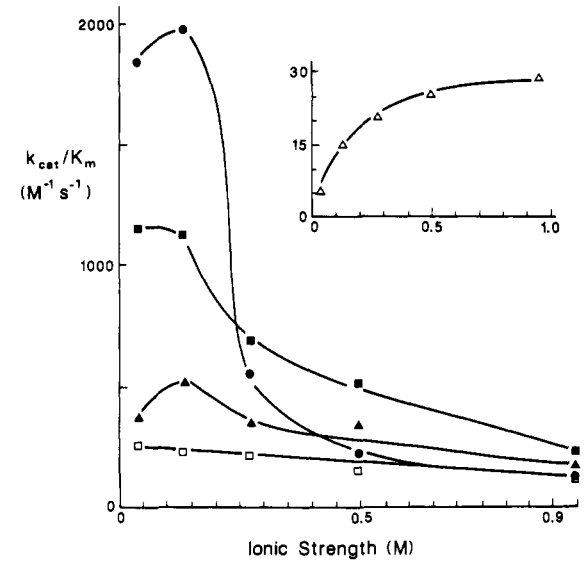

FIGURE 4: Effect of ionic strength on the acetyl-CoA hydrolysis activity of CAT. Steady-state kinetic parameters for the hydrolysis of acetyl-CoA were determined for wild-type CAT_{III} (●), Gln-177 CAT (■), Glu-177 CAT (▲), and Gln-54 CAT (□) at various ionic strengths, and the specificity constant (k_{cat}/K_m) was plotted against ionic strength. The inset shows similar data for Glu-54 CAT (△) measured as described under Experimental Procedures.

being the relative distances between the ε-amino groups and the nearest phosphoanion center of acetyl-CoA in each case. The small contribution (0.4 kcal·mol^{-1}) to acetyl-CoA binding of the protonated amino group of Lys-177, calculated from steady-state kinetic data, is unlikely to be due to a direct ion pair interaction, in agreement with the observed structure. Charge reversal associated with Glu-177 CAT is accompanied by a larger increase in the K_m for acetyl-CoA than that for Gln-177 CAT, in keeping with the long-range electrostatic role proposed for Lys-177. The effect of changing the charge of the side chain of residue 177 is approximately additive, in that neutralization of the positive charge of Lys-177 (Gln-177 CAT) results in a 0.4 kcal·mol^{-1} decrease in the apparent free energy of binding of acetyl-CoA and charge reversal (Glu-177 CAT) results in a further 0.4 kcal·mol^{-1} decrease in apparent binding energy. Such arguments also apply to the consequences of substitutions for Lys-54, although the additivity is less precise. As seen for Gln-54 CAT, similar changes in steady-state kinetic parameters are observed for both thioesterase and transacetylation activities on substitution of Lys-177 (Tables II and III).

The greater than predicted effect of substituting Lys-177 with alanine (Ala-177 CAT) on the binding of acetyl-CoA by CAT may be explicable in part by the observation that in the CAT–CoA binary complex the Cγ of the side chain of Lys-177 is in van der Waals' contact with C-2 of the adenine ring of CoA (Leslie et al., 1988). Hence, replacement of the lysyl side chain at residue 177 with a methyl group may result in the loss of a small but significant hydrophobic interaction.

Effect of Ionic Strength on Acetyl-CoA Binding. One of the hallmarks of electrostatic interactions of any kind is their sensitivity to increases in ambient ionic strength and corresponding changes in the dielectric constant of bulk solvent. Accordingly, the effect of changes in ionic strength on the specificity constant (k_{cat}/K_m) for a given substrate is a useful guide to the presence of electrostatic interactions in the transition state. It follows that site-directed changes to the structure of an enzyme, aimed at removing a partner in such a proposed interaction, should be accompanied by a reduction in the sensitivity of k_{cat}/K_m to increases in ionic strength.

[5] The energetic consequences of amino acid substitutions were determined from the expression

$$\Delta G = -RT \ln \{[k_{cat}/K_m(\text{AcCoA})K_d(\text{Cm})]^{mt}/[k_{cat}/K_m(\text{AcCoA})K_d(\text{Cm})]^{wt}\}$$

which relates to interactions in the transition-state complex (mt, substituted enzyme; wt, wild-type enzyme; Cm, chloramphenicol).

Table IV: Inhibitor Constants of Adenine Nucleotides for Wild-Type and Mutant CAT Variants[a]

variant	K_i (mM) ATP	5'AMP	3'AMP
wild-type CAT_{III}	1.0	5.4	4.6
Gln-54 CAT	23.1	20.2	6.7
Gln-177 CAT	1.9	12.8	4.3
Glu-177 CAT	3.7	19.3	5.3
Ala-177 CAT	3.7	20.9	9.6

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

Table V: Steady-State Kinetic Parameters for Wild-Type and Mutant CAT Variants with Acetyl-3'-dephospho-CoA as the Acetyl Donor[a]

variant	k_{cat} (s^{-1})	K_m (μM) chloramphenicol	acetyl-3'-dephospho-CoA	k_{cat}/K_m[b] ($M^{-1}\cdot s^{-1} \times 10^{-5}$)
wild-type CAT_{III}	550	24.2	671	8.20
Gln-54 CAT	603	30.9	1978	3.00
Glu-54 CAT	ND[d]	ND	ND	0.26[c]
Gln-177 CAT	579	29.0	1029	5.63
Glu-177 CAT	ND	ND	ND	3.40[c]

[a] Kinetic parameters are the mean of at least two determinations performed as described under Experimental Procedures. [b] Calculated using the K_m for acetyl-3'-dephospho-CoA. [c] Estimated under conditions of saturating chloramphenicol (200 μM) with the acetyl-CoA concentration well below its K_m value as described. [d] ND, not determined.

Figure 4 summarizes the results of such experiments with wild-type and mutant variants of CAT. The steady-state kinetic data illustrated are for the thioesterase activity, which occurs in the absence of chloramphenicol and takes place at the same catalytic center used for the acetyl-transfer reaction (Kleanthous & Shaw, 1984). Study of the binary complex simplifies the kinetic analysis but, more importantly, avoids complications arising from possible effects of variations in ionic strength on the binding of chloramphenicol. As predicted, an increase in ionic strength from 135 to 945 mM leads to a striking decrease (17-fold) in k_{cat}/K_m with wild-type CAT for acetyl-CoA hydrolysis, due almost exclusively to an increase in the K_m for acetyl-CoA. Substitutions for Lys-54 and Lys-177 yield enzymes for which k_{cat}/K_m is generally less affected by changes in ionic strength. The specificity constant for Gln-54 CAT decreases less than 2-fold as the ionic strength is increased from 135 to 945 mM, whereas that for Gln-177 CAT decreases 5-fold, reflecting the relative strengths of the interactions between acetyl-CoA and the ϵ-amino groups of these two lysyl residues.

Charge reversal, associated with the Glu-177 substitution, causes a further reduction in the sensitivity of k_{cat}/K_m to changes in ionic strength over that observed with Gln-177 CAT. At high ionic strength, the charge repulsion between the introduced carboxylate of Glu-177 and the phosphoanions of CoA is greatly reduced, but is insufficient to fully compensate for the salting-out of other and more favorable interactions, presumably between Lys-54 and CoA. As a result, k_{cat}/K_m for Glu-177 CAT remains inversely related to ionic strength. In contrast, Glu-54 CAT is more active at higher ionic strengths, with k_{cat}/K_m increasing almost 5-fold over the range where that of the wild-type enzyme is reduced 17-fold (Figure 4). At high ionic strength (945 mM), Glu-54 CAT is only 4-fold less active than the wild-type enzyme compared with the 120-fold lower activity observed under standard conditions.

Specificity of Electrostatic Interactions between CAT and Acetyl-CoA. A number of analogues or constituent units of CoA, with phosphate groups at different sites, were used to determine which of the anionic centers of acetyl-CoA is most likely to interact with either Lys-54 or Lys-177. Although both adenosine 5'-monophosphate (5'AMP) and adenosine 3'-monophosphate (3'AMP) are competitive inhibitors with respect to acetyl-CoA, neither is as effective as adenosine 5'-triphosphate (ATP). The data in Table IV also show that the K_i values for 3'AMP are approximately the same for wild-type CAT_{III}, Gln-177 CAT, and Glu-177 CAT, suggesting that Lys-177 does not interact significantly with the 3'-phosphate of CoA. The higher than expected K_i value for Ala-177 CAT again suggests, as noted above, that the K177A substitution perturbs interactions other than purely electrostatic ones. The modest increase in the K_i for 3'AMP seen with Gln-54 CAT supports the view that the side chain of Lys-54 forms an electrostatic interaction with the 3'-phosphate of CoA but the effect is much smaller than the corresponding increase (10-fold) in the K_m for acetyl-CoA. Taken together, the data suggest that the 3'-phosphate is not the only determinant of the interaction between acetyl-CoA and Lys-54.

The K_i values for 5'AMP and ATP (Table IV) follow a pattern similar to that of the K_m for acetyl-CoA (Table II) for substitutions of Lys-177. Although the K_i values for Ala-177 CAT are higher in each case than predicted on the basis of changes in charge alone, a conclusion which emerges clearly is that Lys-177 must make a weak electrostatic interaction with the phosphodiester backbone of CoA. The kinetic data for Gln-54 CAT also favor a similar conclusion for the interaction of Lys-54 with CoA. Although the Gln-54 substitution shows only a 4-fold increase in the K_i for 5'AMP (against a 10-fold increase in the K_m for acetyl-CoA), the increase in the K_i for ATP is substantially greater than expected (23-fold), suggesting that in the wild-type enzyme Lys-54 makes novel and energetically important contacts with the long triphosphate arm of ATP.

Finally, adenosine, which differs from 3'AMP and 5'AMP only by the absence of a phosphate group, fails to bind to CAT competitively with respect to acetyl-CoA, suggesting that the charged moieties of CoA are important for recognition. Adenosine is, nonetheless a noncovalent inhibitor of CAT, which binds competitively with respect to chloramphenicol ($K_i \sim 5.1$ mM).

Acetyl-3'-dephospho-CoA. In order to determine whether the 3'-phosphate moiety of CoA is important for binding to CAT, 3'-dephospho-CoA was prepared by alkaline phosphatase treatment of CoA as described. Using the wild-type enzyme, the loss of the 3'-phosphate group results in a 7-fold decrease in the affinity of CAT_{III} for the acetyl donor (Table V), corresponding to a 1.2 kcal·mol^{-1} decrease in the apparent free energy of binding. However, a similar increase in k_{cat}/K_m is observed with both Gln-177 CAT and Glu-177 CAT when 3'-dephospho-CoA replaces CoA (Table V). These results suggest that the residue(s) which interact(s) with the 3'-phosphate of CoA is (are) retained in both mutant enzymes, in agreement with the results of inhibitor studies (see above). Although a difference in the preferred conformers of CoA and dephospho-CoA in solution cannot be ruled out as an explanation for the diminished binding affinity of the latter, such an explanation seems unlikely in light of the results of NMR studies (Lee & Sarma, 1974, 1975).

A change in the charge on the side chain of residue 54 has differential effects on the binding of acetyl-CoA and acetyl-3'-dephospho-CoA. The affinity of CAT for acetyl-CoA is impaired to a greater extent than that for acetyl-3'-de-

phospho-CoA such that with Glu-54 CAT k_{cat}/K_m is decreased more than 160-fold compared to the wild-type enzyme when acetyl-CoA is the acetyl donor, whereas that for acetyl-3′-dephospho-CoA is decreased only 31-fold. Thus, it appears that Lys-54 makes a significant contribution to the interaction of CAT with the 3′-phosphate of CoA. In summary, Lys-54 appears to be involved in electrostatic interactions with both the 3′-phosphate and 5′-phosphoanions of CoA, and its presence contributes 1.3 and 0.6 kcal·mol^{-1}, respectively, to the apparent free energy of binding of acetyl-CoA and acetyl-3′-dephospho-CoA.

Conclusions

The results of the present study confirm and extend conclusions derived from the structure of the CAT–CoA binary complex. The latter reveals that there are no ion pairs involving the negatively charged moieties of CoA and positively charged side chains of the enzyme and that the phosphoanionic groups of CoA remain solvent-accessible on binding to CAT.

Arginyl residues, which are often involved in binding substrates which contain phosphoryl moieties (Riordan et al., 1977), are not important for the binding of CoA by CAT_{III}. Reversible inactivation of CAT by phenylglyoxal is probably due to binding of the inhibitor at the chloramphenicol binding site and formation of a relatively unstable adduct with the thiol group of Cys-31, rather than to modification of a critical arginyl residue.

Substitution of two well-conserved lysyl residues (Lys-54 and Lys-177) close to the CoA binding site results solely in changes in the binding constants for acetyl-CoA. Although Lys-177 is involved in long-range electrostatic interactions with the 5′-α- and 5′-β-phosphoanions of CoA, they contribute only 0.4 kcal·mol^{-1} to the apparent free energy of binding of acetyl-CoA. The magnitude of the contribution of Lys-177 to CoA binding from experiment compares favorably with that calculated from electrostatic theory (J. Warwicker, personal communication).

The fact that the side chain of Lys-54 is disordered in the crystal structure of the CAT–CoA binary complex suggests that it does not form an ion pair with any of the phosphoanions of CoA. Nonetheless, substitution of Lys-54 results in a significant decrease in the affinity of CAT_{III} for CoA. The presence of its ϵ-amino group contributes 1.3 kcal·mol^{-1} to the binding of acetyl-CoA, significantly greater than that of Lys-177, but less than expected for an ion pair interaction (>3 kcal·mol^{-1}; Fersht et al., 1985). The contribution of Lys-54 to acetyl-CoA binding is partitioned (a) between the interactions of CAT with the 5′-α- and 5′-β-phosphoanions of CoA (0.6 kcal·mol^{-1}; calculated from the effects of removing the 3′-phosphoryl moiety from CoA on the steady-state kinetic parameters of wild-type CAT_{III} and Gln-54 CAT) and (b) with the 3′-phosphate group (0.7 kcal·mol^{-1}; calculated from the effect of substitution of Lys-54 on the binding of acetyl-3′-dephospho-CoA).

Assuming that the effects of substitutions of residues 54 and 177 act independently, the total energetic contribution of their basic side chains to the binding of acetyl-CoA ($\sim$1.7 kcal·mol^{-1}) is less than that for a single hydrophobic interaction between Tyr-178 and the adenine ring of CoA (2.4 kcal·mol^{-1}; Day & Shaw, 1992). The binding of CoA to CAT_{III} involves no ion pairs, a few long-range electrostatic interactions, and only five direct hydrogen bonds. Thus, although CoA is a highly polar substrate, apolar interactions make by far the greatest energetic contribution to its binding by CAT_{III}. The results are in striking contrast to the case of citrate synthase wherein the recognition and binding of CoA involve close-range and very precise ionic interactions, as well as hydrophobic interactions and hydrogen bonds. Thus, CAT and citrate synthase probably represent extremes in a spectrum of polar and nonpolar motifs which are able to bind this important coenzyme.

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Conserved Residues Flanking the Thiol/Disulfide Centers of Protein Disulfide Isomerase Are Not Essential for Catalysis of Thiol/Disulfide Exchange†

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ABSTRACT: Protein disulfide isomerase (PDI) catalyzes the oxidative folding of proteins containing disulfide bonds by increasing the rate of disulfide bond rearrangements which normally occur during the folding process. The amino acid sequences of the N- and C-terminal redox active sites (PWCGHCK) in PDI are completely conserved from yeast to man and display considerable identity with the redox-active center of thioredoxin (EWCGPCK). Available data indicate that the two thiol/disulfide centers of PDI can function independently in the isomerase reaction and that the cysteine residues in each active site are essential for catalysis. To evaluate the role of residues flanking the active-site cysteines of PDI in function, a variety of mutations were introduced into the N-terminal active site of PDI within the context of both a functional C-terminal active site and an inactive C-terminal active site in which serine residues replaced C379 and C382. Replacement of non-cysteine residues (W34 to Ser, G36 to Ala, and K39 to Arg) resulted in only a modest reduction in catalytic activity in both the oxidative refolding of RNase A and the reduction of insulin (10–27%), independent of the status of the C-terminal active site. A somewhat larger effect was observed with the H37P mutation where ~80% of the activity attributable to the N-terminal domain (~40%) was lost. However, the H37P mutant N-terminal site expressed within the context of an inactive C-terminal domain exhibits 30% activity, approximately 70% of the activity of the N-terminal site alone. While this mutation mimics the active site of thioredoxin, this PDI mutant is several orders of magnitude more active than thioredoxin in catalysis of the oxidative folding of RNase. The strict sequence conservation of residues flanking the active-site cysteines is surprising in light of very modest changes in catalytic properties observed with mutation. This suggests the possibility that the strict conservation of this sequence through evolution reflects an additional function for PDI.

Protein disulfide isomerase (PDI)[1] is a M_r 56 300 protein found in the lumen of the endoplasmic reticulum (Lambert & Freedman, 1983). The finding that purified PDI can both catalyze reduction of disulfide bonds and enhance the rate of oxidative renaturation of disulfide bond containing proteins (Anfinsen & Scheraga, 1975; Freedman, 1984; Morin & Dixon, 1985) has led to the proposal that the enzyme functions to assist oxidative refolding of proteins during translation and/or translocation. Experiments of Bulleid and Freedman (1988) in which the oxidative folding capacity of a reconstituted in vitro translation/translocation system was shown to depend on the presence of PDI also support a role for PDI in oxidative folding in the endoplasmic reticulum. Somewhat paradoxically, PDI has also been implicated in a number of other cellular processes (Yamauchi et al., 1987; Cheng et al., 1987; Boado et al., 1988; Geetha-Habib et al., 1988; Obata et al., 1988; Wetterau et al., 1990). In particular, PDI has been identified as the β-subunit of prolyl hydroxylase, an enzyme involved in collagen biosynthesis (Pihlajaniemi et al., 1987).

The complete sequence of rat PDI cDNA (Edman et al., 1985) revealed several intriguing features of the protein. First, PDI contains two sets of internally homologous domains: residues 1–100 display 37% sequence identity with residues 340–440, and residues 150–245 show over 55% identity with residues 250–345. Second, a segment of sequence found in both the N-terminal and C-terminal domains (PWCGHCK) displays extensive sequence identity with the thioredoxin active center (EWCGPCK). This finding suggested that the catalytic centers of PDI are contained within this thiol/disulfide-containing segments. Sequencing PDI cDNAs from a number of different species ranging from *Saccharomyces cerevisiae* (Scherens et al., 1991) to human (Morris & Varandani, 1988) has shown that these thiol/disulfide centers are completely conserved through evolution. Notably, while yeast and human PDI display only 29% sequence identity overall, the region containing the thiol/disulfide center is absolutely conserved. Interestingly, this sequence motif has also been found in proteins such as form I phosphoinositide-specific phospholipase

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[1] Abbreviations: PDI, protein disulfide isomerase; GSH, glutathione; GSSG, glutathione disulfide; RNase A, ribonuclease A; DTT, dithiothreitol; cCMP, cytidine cyclic 3′,5′-monophosphate; CMP, cytidine monophosphate.

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