Properties of Hybrid Active Sites in Oligomeric Proteins: Kinetic and Ligand Binding Studies with Chloramphenicol Acetyltransferase Trimers†

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ABSTRACT: Alteration of the charge of surface lysyl residues of chloramphenicol acetyltransferase (CAT) by site-directed mutagenesis was used to increase the charge difference between the subunits of two naturally occurring enzyme variants (CAT_I and CAT_{III}). The introduced charge change greatly facilitates the purification of CAT_I/CAT_{III} and CAT_{III}/CAT_{III} hybrid trimers by ion-exchange chromatography. Hybrids containing only one functional active site per trimer were generated *in vitro* by reversible denaturation of mixtures of "active" subunits (retention of a catalytic histidine at position 195) and "inactive" subunits (with alanine replacing histidine 195). Such hybrids were used (1) to demonstrate that the previously observed novel binding of a steroidal antibiotic (fusidic acid) by CAT_I involves amino acid residues at each subunit interface and (2) to identify specific residues contributing to such interactions. A pre-steady-state kinetic characterization of homotrimers containing the H195A substitution also revealed that fusidate binding to CAT_I may, like chloramphenicol binding, involve a hydrogen bond with the catalytic histidine residue. In addition, confirmation of the fact that His-195 interacts with chloramphenicol in CAT_I as well as in CAT_{III} makes it likely that it is essential for the catalytic mechanism of all naturally occurring variants of CAT, as first suggested by structural evidence for the type III enzyme (Leslie, 1990).

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28), the enzyme responsible for high-level bacterial resistance to chloramphenicol, inactivates chloramphenicol by acetyl CoA dependent O-acetylation (Shaw, 1967). The reaction product, 3-acetylchloramphenicol, does not bind to bacterial ribosomes and is thus devoid of antibacterial activity (Shaw & Unowsky, 1968).

Steady-state kinetic analysis of the type III enzyme (CAT_{III}) has shown that the reaction proceeds by a ternary complex mechanism with a random order of addition of substrates (Kleanthous *et al.*, 1985). Histidine 195[2] acts as a catalytic base, abstracting a proton from the 3-hydroxyl of chloramphenicol and thus promoting nucleophilic attack on the thioester carbonyl of acetyl CoA to yield a tetrahedral oxyanion intermediate [reviewed in Shaw and Leslie (1991)]. Replacement of His-195 in CAT_{III} by site-directed mutagenesis results in a more than 10^7-fold decrease in catalytic activity (Lewendon *et al.*, 1994).

The structures of the binary complexes of CAT_{III} with chloramphenicol and CoA have been solved at 0.175- and 0.24-nm resolution, respectively (Leslie, 1990; Leslie *et al.*, 1988). CAT is a trimer of identical (25 kDa) subunits with each of its three active sites lying at an interface between subunits. Binding of acetyl CoA predominantly involves interactions with only one subunit, with the catalytic imidazole of His-195 arising from the facing subunit. Chloramphenicol binds in a cleft between adjacent subunits and is involved in significant interactions with both of them (Figure 1).

The residues involved in binding of acetyl CoA are highly conserved in the "family" of known CAT sequences (Shaw & Leslie, 1991), whereas the chloramphenicol binding site is somewhat more variable. Of the 17 residues involved in direct interactions of CAT_{III} with chloramphenicol, only 5 are strictly conserved.

Type I CAT (CAT_I), unlike all other known natural variants, binds novel ligands of two chemical classes: the triphenyl methane dyes, such as crystal violet, and steroidal compounds related to fusidic acid (Bennett & Shaw, 1983). In each case binding is competitive with respect to chloramphenicol. Indeed, when expressed in *Escherichia coli* DB10, a fusidate-sensitive mutant strain which is permeable to the antibiotic, CAT_I confers resistance to fusidic acid, an inhibitor of bacterial protein synthesis that is structurally and mechanistically unrelated to chloramphenicol (Bennett & Shaw, 1983). Since crystallization trials with CAT_I have failed to yield crystals suitable for X-ray diffraction studies, a structural explanation of the mode of binding of such compounds to CAT_I has not been possible.

CAT_I and CAT_{III} have similar affinities for chloramphenicol ($K_m \sim 12$ μM), although the residues involved in

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CAT_I, type I variant of CAT; CAT_{III}, type III variant of CAT; CoA, coenzyme A; ATP, adenosine triphosphate; SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; TSE, 50 μM Tris-HCl, pH 7.5, 100 μM sodium chloride, and 0.1 μM EDTA; TME, 50 μM Tris-HCl, pH 7.5, 0.1 μM 2-mercaptoethanol, and 0.1 μM EDTA.

[2] Alignment of the amino acid sequences of CAT variants has resulted in a general numbering system. The numbering is related to the CAT_I linear sequence by subtracting 1 from residues 80−221, and to the CAT_{III} linear sequence by subtracting 5 from residues 6−79 and 6 from residues 80−219.

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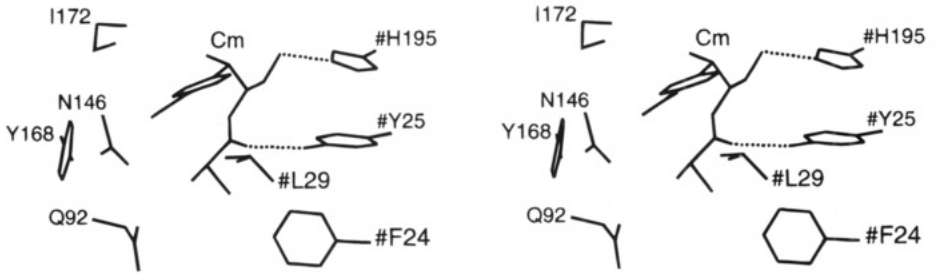

FIGURE 1: Stereoview of the chloramphenicol (Cm) binding site of CAT_{III}. Residues indicated by # belong to the adjacent subunit of the trimer. Dashed lines represent deduced hydrogen bonds.

Table 1: Amino Acid Residues Known To Interact with Chloramphenicol in the CAT_{III}/Chloramphenicol Binary Complex (Leslie, 1990) and Their Counterparts in CATs[a]

residue	CAT_I	CAT_{III}
24	*A*	*F*
25	*F*	*Y*
29	*A*	*L*
31	C	C
92	*C*	*Q*
94	T	T
103	F	F
105	*S*	*A*
135	F	F
146	*F*	*N*
148	S	S
160	L	L
162	V	V
168	*F*	*Y*
172	*V*	*I*
174	T	T
195	H	H

[a] Residues which differ between CAT_I and CAT_{III} are shown in italics.

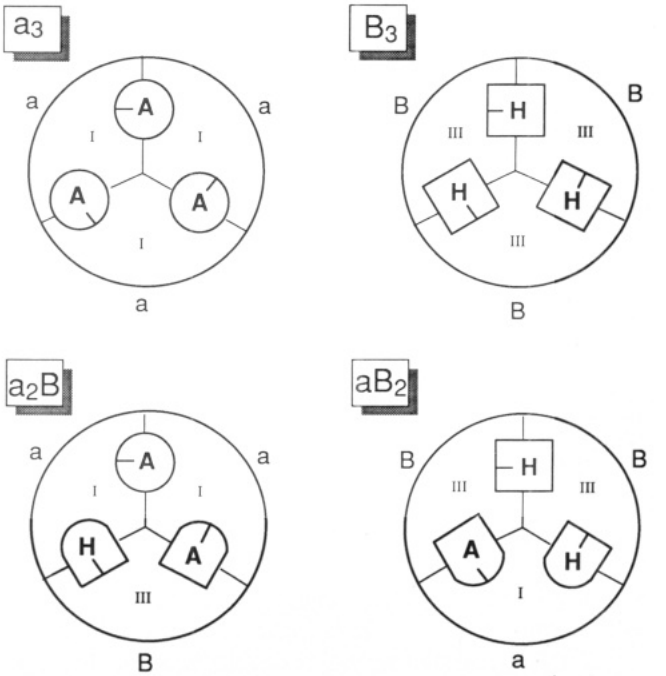

FIGURE 2: Schematic representation of homotrimers and some hybrid trimers of CAT, illustrating the four possible species formed on refolding mixtures of parental trimers a_3 (inactive CAT_I) and B_3 (active CAT_{III}). Residue 195 is indicated by H (histidine) or A (alanine). The a_2B hybrid contains only one catalytic substrate binding site per trimer, that formed from His-195 of CAT_{III}. The curved surfaces indicate the contribution of amino acid residues from the CAT_I framework to the active site, and the rectangular surfaces those of CAT_{III} origin.

interactions with chloramphenicol differ significantly between the two variants. For example, 8 of the 17 residues of CAT_{III} which interact with chloramphenicol are different in CAT_I (Table 1). The two natural variants have, however, very different affinities for fusidate ($K_i = 1.5\ \mu M$ for CAT_I and $278\mu M$ for CAT_{III}; I. A. Murray, P. A. Cann, P. J. Day, J. P. Derrick, A. G. W. Leslie, and W. V. Shaw, in preparation). Since fusidate binds competitively with respect to chloramphenicol, it seemed likely to occupy at least part of the chloramphenicol binding site in each case.

Elucidation of the likely residues involved in fusidate binding was approached in two ways. The most direct involved site-directed substitution of residues in the known chloramphenicol binding site of CAT_{III}, in particular those which differ between CAT_I and CAT_{III}, to introduce high-affinity fusidate binding into the CAT_{III} framework and facilitate X-ray diffraction studies of the binary complex (A. Murray, P. A. Cann, P. J. Day, J. P. Derrick, A. G. W. Leslie, and W. V. Shaw, in preparation). A complementary approach involved the construction of two classes of heteromeric trimers (Figure 2), one composed of a single type I subunit and two type III subunits (AB_2) and the other consisting of two type I subunits and one type III subunit (A_2B).

The contribution of subunit interfaces to ligand binding and catalysis has been investigated by the use of hybrid enzymes for a number of multimeric systems (Larimer *et al.*, 1987; Graddis *et al.*, 1988; Distefano *et al.*, 1990). A frequent constraint in experimental design is, however, the separation of a hybrid species from the parental counterparts, requiring resort to naturally occurring isozymes with favorable properties or the use of reversible chemical modification of one of the parental species (Robey & Schachman, 1985; Wente & Schachman, 1987).

More recently Perham and co-workers have proposed that the addition of a pentaarginyl sequence to the N-terminus of proteins can be used as a general method to introduce charge differences between parental multimers with little or no effect on catalytic function (Deonarian *et al.*, 1992a), an approach which confirmed the importance of inter-subunit interactions in the mechanism of glutathione reductase (Deonarian *et al.*, 1992b).

Here we demonstrate the use of site-directed mutagenesis of nonconserved surface residues of CAT_{III} (Lys-14 and Lys-217) to establish a total difference between hybrid species of 5 charge units per monomer with only minor changes in kinetic properties. Heteromeric trimers generated from such subunits were used to identify the interface residues involved in binding of fusidate by CAT_I, a natural variant which has

thus far failed to yield crystals suitable for X-ray diffraction studies.

EXPERIMENTAL PROCEDURES

Purification of Wild-Type CAT_{III} and Its Catalytically Active Substituted Variants. Wild-type enzyme and CAT_{III} (K14E, K217E) were purified from *E. coli* extracts by affinity chromatography on chloramphenicol−Sepharose as described previously (Lewendon *et al.*, 1988). The purity of the enzyme preparations was assessed by SDS−polyacrylamide gel electrophoresis, and protein concentrations were determined from absorbance at 280 nm ($\epsilon^{0.1\%} = 1.31$).

CAT_I, CAT_I (H195A), and CAT_{III} (K14E, H195A, K217E). Cell-free extracts were clarified by centrifugation and passed over a DEAE-Sephacel column equilibrated with TME buffer. CAT was eluted with a linear (0−1 M) sodium chloride gradient. Peak CAT-containing fractions were pooled, dialyzed against TME, and passed over a Cibacron-Blue Sepharose 4B column. The column was washed with 1 L of TME and 1 L of TME + 1 mM ATP, after which CAT was eluted with TME containing 25 mM ATP. Peak fractions were pooled, concentrated by ultrafiltration (and washed exhaustively to remove the ATP), and stored at 4 °C.

Assay of CAT Activity. CAT activity was measured spectrophotometrically at 25 °C as described previously (Lewendon *et al.*, 1988). The standard assay contained 1 mM DTNB, 0.1 mM chloramphenicol, and 0.4 mM acetyl CoA in TSE buffer (pH 7.5). Reactions were initiated by the addition of enzyme, and the release of CoA was monitored spectrophotometrically at 412 nm. For steady-state kinetic analysis of homomeric and hybrid CAT variants the concentrations of both substrates were varied in the standard assay and the kinetic constants were calculated as described (Kleanthous & Shaw, 1984). One unit of CAT activity is defined as the amount of enzyme converting 1 μmol of substrate to product per minute at 25 °C. For inhibitor studies, the concentration of acetyl CoA was kept constant (0.4 mM) and the concentrations of chloramphenicol and fusidate were varied. In each instance the binding of fusidate to trimeric CAT species was competitive with respect to chloramphenicol.

Transient Kinetics. Off-rates for chloramphenicol and fusidate were determined as described previously (Ellis *et al.*, 1991b) using an Applied Photophysics stopped-flow apparatus. Binding of chloramphenicol results in a 20% quench of intrinsic protein fluorescence. Hence, pushing CAT with bound fusidate or with the *p*-cyano analogue of chloramphenicol (neither of which gives a quench) against an excess of chloramphenicol allows the dissociation rate for the two ligands to be determined indirectly by monitoring the fluorescence quench on binding of chloramphenicol (Ellis *et al.*, 1991a). Syringe A contained 4 μM CAT, with various concentrations of either sodium fusidate or *p*-cyanochloramphenicol in TSE (pH 7.5). Syringe B contained chloramphenicol in TSE (pH 7.5). The excitation wavelength was 295 nm.

Thermal Stability of CAT Variants. CAT (10 mg/mL in TSE) was incubated at 70 °C in 0.5 mL tubes containing a solution volume of 50 μL. Tubes were removed at various times and cooled rapidly on ice, and 10 μL was taken from each for measurement of CAT activity, after appropriate dilution in TSE buffer.

Site-Directed Mutagenesis and Expression of CAT. Oligonucleotide-directed mutagenesis was performed using the deoxyuridine selection protocol (Künkel *et al.*, 1987). The presence of the desired mutation and the absence of secondary mutations in each case were confirmed by nucleotide sequencing of the entire *cat*-coding and 5′-noncoding regions. Mutant *cat* genes were overexpressed in *E. coli* by cloning into pUC18 (Murray *et al.*, 1988).

Reversible Unfolding of CAT. CAT was unfolded by dilution to a final concentration of 0.1 mg/mL in 0.1 M sodium phosphate (pH 2.9) containing ultrapure urea (8M). After the mixture was stirred for 1 h at 25 °C, refolding was initiated by 10-fold dilution into 0.1M sodium phosphate (pH 7.5). The progress of refolding was monitored by removing 10 μL aliquots at timed intervals for CAT assays using the standard conditions. For production of hybrids by renaturation the unfolding step employed a range of input ratios of parental homotrimers. The total concentration of CAT in each experiment was 4 μM for the unfolding reaction and 0.4 μM for the refolding mixture.

Purification of Hybrid Trimers. Mixtures of CAT trimers resulting from the above unfolding/refolding procedure were separated by ion-exchange chromatography. The dilute refolding mixtures (typically 100 mL containing 1 mg of protein) were concentrated to 3−4 mL by ultrafiltration, transferred to 10 mM Tris-HCl buffer (pH 7.5), and loaded onto a MonoQ 5/5 anion-exchange column (Pharmacia) which had been preequilibrated with 10 mM Tris-HCl (pH 7.5). Protein was eluted with a 40-mL linear gradient (0−400 mM) of sodium chloride. Purity was estimated by electrophoresis on non-denaturing discontinuous alkaline polyacrylamide gels (Figure 3).

RESULTS AND DISCUSSION

Mutations Introduced into CAT_{III} to Distinguish between Hybrid Species. Previous purification of CAT_I/CAT_{III} hybrids formed *in vivo* relied on the subunit net charge difference (1) between these natural CAT variants (Packman & Shaw, 1981). In order to increase the net charge difference and hence facilitate purification of hybrid (CAT_I/CAT_{III}) species by ion-exchange chromatography, substitutions were made in CAT_{III} for two nonconserved surface basic residues (Lys-14 and Lys-217). The side chains of both, each of which is disordered in the crystal structure (Leslie, 1990), were changed to those of glutamyl residues to yield a net charge difference of 5 per monomer (15 for the homotrimer). As predicted, the resulting purified protein (designated CAT_{III} [K14E, K217E]) has steady-state kinetic parameters very similar to those of the wild-type enzyme (Table 2). The slightly elevated K_m for acetyl CoA may be due to long-range electrostatic repulsion between the introduced carboxylate groups and the phosphoryl groups of CoA, a phenomenon noted on substitution of two other surface lysyl residues in CAT_{III} (Day *et al.*, 1992). Nonetheless, the charge inversions introduced by the two substitutions have no significant effect on the off-rates for the acetyl acceptor, chloramphenicol, or for the inhibitor, fusidate (Table 3).

The analysis of a CAT trimer possessing different structural and functional active sites by steady-state kinetic methods is probably not feasible. The strategy employed for the experiments to be described simplifies the interpretation of the kinetic data, including the effects of inhibitors

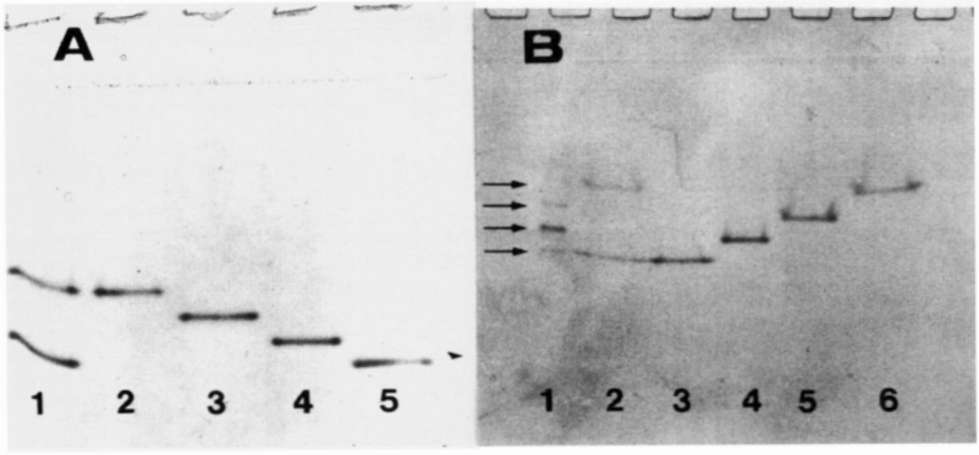

FIGURE 3: Non-denaturing polyacrylamide gel electrophoresis of purified CAT variants and hybrids. Gels were stained using a silver stain kit (Bio-Rad). Lanes in both panels are numbered left to right. (A) Lane 1, a mixture of CAT_{III} and b_3 [CAT_{III} (K14E, H195A, K217E)]; lane 2, wild-type CAT_{III} homotrimer; lane 3, [$(CAT_{III})_2(b)$] hybrid; lane 4, [$(CAT_{III})(b)_2$] hybrid; lane 5, b_3 homotrimer. (B) Lane 1, unpurified refolding products from a mixture of CAT_I (A_3) and [CAT_{III} (K14E, H195A, K217E)] (b_3); lane 2, a mixture of reference A_3 and b_3 homotrimers; lanes 3−6, purified CAT trimers from refolding experiment; lane 3, b_3 homotrimer; lane 4, Ab_2 hybrid; lane 5, A_2b hybrid; lane 6, A_3 homotrimer.

Table 2: Steady-State Kinetic Parameters of CAT Variants[a]

variant	acetyl CoA K_m (μM)	CM[b] K_m (μM)	k_{cat} (s^{-1})	fusidate k_i (μM)
CAT_{III}	93	12	600	278
CAT_{III} (K14E, K217E)	166	17	523	ND
[CAT_I (H195A)]2[CATIII (K14E, K217E)]	72	18	85	213
[CAT_I][CATIII (K14E, H195A, K217E)]$_2$	107	20	303	56
CAT_I	55	11	97	1.5
CAT_{III} (F24A, Y25F, L29A)	95	23	176	70
CAT_{III} (Q92C, N146F, Y169F, I172V)[c]	165	20	377	5.4

[a] Kinetic parameters are the mean of a least two determinations performed as described in Experimental Procedures. The standard error for each value of k_{cat} was less than 5% and less than 10% for determinations of K_m (acetyl CoA or CM) and K_i (fusidate). [b] Chloramphenicol. ND Not determined. [c] Data from Murray *et al.* (in preparation).

Table 3: Pre-Steady-State Kinetic Parameters of CAT Variants: Rate Constants for the Loss of Chloramphenicol and Fusidate from Binary Complexes[a]

variant	chloramphenicol (s^{-1})	fusidate (s^{-1})
CAT_{III}	186	184
CAT_{III} (K14E, K217E)	183	190
CAT_{III} (K14E, H195A, K217E)	*b*	241
CAT_{III} (H195A)	*b*	190
CAT_I	138	18.3, 7.5
CAT_I (H195A)	*b*	133, 11.3

[a] Measured rates are the results of duplicate determinations with a standard error of less than 10% for each. [b] The rate was too fast (>600 s^{-1}) to measure at 25 °C.

such as fusidate, by limiting the number of functional active sites in each trimer via removal of the catalytic imidazole of histidine-195. The substitution required to abolish CAT activity (H195A) was introduced into both CAT_I and CAT_{III} [K14E, K217E], having been shown to diminish affinity for chloramphenicol as well as delete acyl transfer function (Lewendon *et al.*, 1994). Using such inactive trimeric proteins as starting materials, it was possibly by reversible denaturation to construct CAT hybrids containing only one "active" site per trimer.

The effects of removing the imidazole of His-195 from CAT_I or CAT_{III} on the off-rates for chloramphenicol and fusidate are shown in Table 3. The value of k_{off} for chloramphenicol is increased such that it becomes too fast to measure (>600 s^{-1}) for both CAT_I and the charge-modified CAT_{III} variant. The result for CAT_{III} is consistent with a predicted loss of the hydrogen bond between $N^{\epsilon 2}$ of His-195 and the 3-hydroxyl of chloramphenicol, observed in the crystal structure of CAT_{III} (Leslie, 1990). The demonstration of a similar effect for CAT_I supports the view that such a hydrogen bond, a prerequisite for the deprotonation step in the proposed catalytic cycle, must also be present in the CAT_I/chloramphenicol complex.

The off-rate for fusidate with CAT_{III} displays single-exponential kinetics and is unaffected by the presence or absence of His-195 (Table 3). In contrast, CAT_I displays biphasic kinetics, the slow rate being unaffected by changes at residue 195, whereas the faster rate is increased more than 7-fold by the H195A substitution (Table 3). Such observations suggest a likely contribution to the difference in affinity of CAT_I and CAT_{III} for fusidate, namely, that His-195 interacts with bound fusidate only in the type I enzyme. [ΔG = 1.1 kcal/mol, assuming no change in the fusidate on-rate].

CAT_{III}/CAT_{III} Mutant Hybrids. Although hybrids can be produced *in vivo* by coexpression of different CAT variants on compatible plasmids, such species are formed in small amounts and are often difficult to purify (Packman, 1978). Production of hybrids *in vitro* requires the complete unfolding of CAT trimers followed by renaturation after rapid removal of denaturant by dilution (P. J. Day and W. V. Shaw, in preparation). The refolding of equimolar mixtures of CAT_{III} and CAT_{III} (K14E, H195A, K217E) after rapid dilution from 8M urea at pH 2.8 (see Experimental Procedures) regularly yielded 60−70% of the starting material as soluble trimers, and the expected four CAT species that were produced

Table 4: Steady-State Kinetic Parameters of Wild-Type CAT_{III} and Hybrids with One or Two Functional Active Sites[a]

CAT_{III} variant	acetyl CoA K_m (μM)	CM[b] K_m (μM)	k_{cat} (s^{-1})	sp act.[c] obs (units/mg)	sp act.[d] expected (units/mg)
CAT_{III}	93	12	600	753	747
$[CAT_{III}]_2[CAT_{III}$ (K14E, H195A, K217A)]	143	16	485	504	498
$[CAT_{III}]$ $[CAT_{III}$ (K14E, H195A, K217A)$]_2$	198	20	492	263	249
CAT_{III} (K14E, H195A, K217A)				0	0

[a] Kinetic parameters are the mean of at least two determinations performed as described in Experimental Procedures. The standard errors were as described for the data in Table 2. [b] Chloramphenicol. [c] Specific activities calculated using standard assay conditions described in Experimental Procedures. [d] Expected values refer to those predicted to arise for CAT_{III} variants with one, two, or three active (H195) subunits.

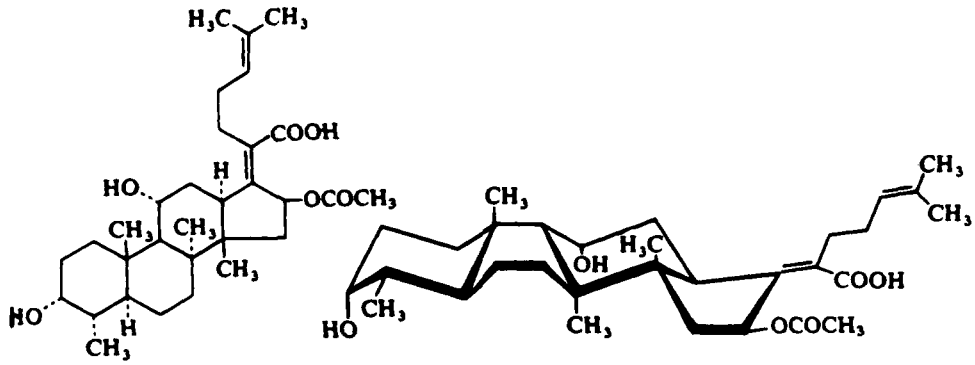

FIGURE 4: Structure of fusidic acid.

occurred in approximately the 1:3:3:1 binomial distribution expected for random association of subunits. To ensure that the unfolding/refolding process does not significantly alter the properties of CAT, the kinetic parameters of refolded CAT_{III} and CAT_{III} mutant hybrids were examined and found to closely match those expected for trimers containing three, two, one, or no functional active sites (Table 4). The assumption that the active sites act independently of each other had been validated in earlier studies with CAT_I/CAT_{III} hybrids prepared *in vivo* (Packman & Shaw, 1981).

CAT_I/CAT_{III} hybrids. On dilution from denaturing conditions, equimolar mixtures of CAT_I and CAT_{III} do not yield the expected binomial distribution of products, the major species being $(CAT_I)(CAT_{III})_2$. The nonrandom distribution may reflect the difference in stability of CAT_I and CAT_{III} subunits (Figure 5), as well as the pairwise amino acid differences between the subunit interfaces (Leslie, 1990). The yields of purified products are further distorted due to the consistently observed but variable precipitation of some CAT_I during purification (data not shown).

Overall yields for CAT_I/CAT_{III} mixtures are generally lower than for refolding CAT_{III}/CAT_{III} (mutant) mixtures. For CAT_I(H195A)/CAT_{III}(K14E, K217E) hybrids only ~35% of the initial activity (with ~40% of the initial protein) is recovered during refolding. In contrast, although 50−60% of the initial protein is recovered for CAT_I/CAT_{III}(K14E, H195A, K217E) mixtures, the yield of activity is more than double that present before the denaturation/refolding process, suggesting that hybrid trimers with their sole His-195 on the CAT_I subunit are convincingly more active than wild-type CAT_I itself (Table 2). Although a persuasive structural basis for this observation is not yet available, an explanation may lie with the intrinsically higher specific activity of CAT_{III} trimers (~6-fold greater than that of CAT_I), with the possibility that the presence of two (inactive) CAT_{III} subunits provides interfacial surfaces and structural constraints that improve the geometry necessary for optimal catalysis via the imidazole of His-195 (from the CAT_I subunit) or Ser-

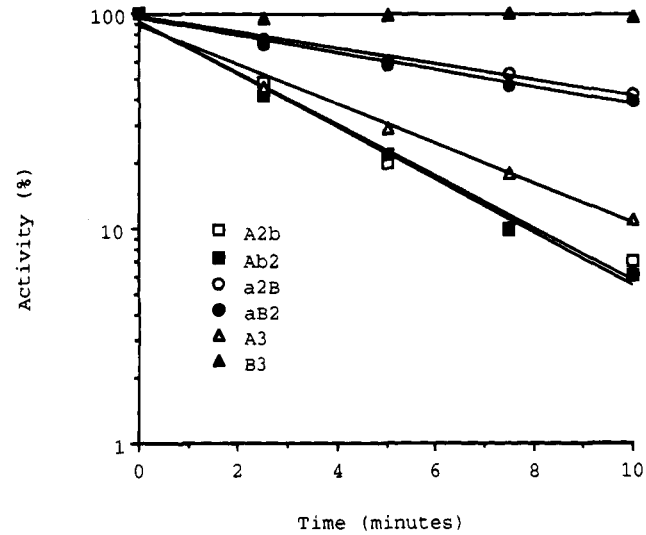

FIGURE 5: Thermal stability of parental CAT variants and hybrids. Each protein sample (10 μg/mL; 0.4 μM in CAT monomers) was incubated at 70 °C in 0.5 mL tubes. Samples were removed at timed intervals, and enzyme activity was measured by the standard CAT assay (see Experimental Procedures). The thermal stability of wild-type CAT_{III} is identical to that of B_3 [CAT_{III}(K14E, K217E)] (results not shown).

148 which stabilises the tetrahedral intermediate (Lewendon *et al.*, 1990).

$[CAT_I][CAT_{III}$ (K14E, H195A, K217E)$]_2$: The Ab_2 Hybrid Trimer.[3] The Ab_2 hybrid has a specific activity almost 2-fold higher than that of the parental CAT_I trimer, but it is inhibited by fusidate to a lesser degree (Table 5). The steady-state

[3] Abbreviations used to describe homotrimers and hybrid trimers. Use of upper case letters (A or B) indicates a subunit possessing His-195. Lowercase (a or b) refers to a subunit lacking the catalytic base by virtue of the substitution H195A. A_3, CAT_I; a_3, CAT_I(H195A); B_3, CAT_{III}(K14E, K217E); b_3, CAT_{III}(K14E, H195A, K217E); Ab_2, $[CAT_I]$ $[CAT_{III}$(K14E, H195A, K217E)$]_2$; A_2b, $[CAT_I]_2[CAT_{III}$(K14E, H195A, K217E)]; aB_2, $[CAT_I$(H195A)$][CAT_{III}$(K14E, K217E)$]_2$; a_2B, $[CAT_I$ (H195A)$]_2[CAT_{III}$(K14E, K217E)].

Table 5: Specific Activities of Homotrimers or Purified Hybrid Trimers and Effect of Fusidate on CAT Activity

CAT variant	trimer class	sp act. (units/mg)	effect of fusidate[b] (percent of control)
CAT_{III}	(B_3)[a]	747	63
CAT_{III} (K14E, K217E)	B_3	724	53
[CAT_I (H195A)][CATIII (K14E, K217E)]$_2$	aB_2	148	47
[CAT_I (H195A)]$_2$[CATIII (K14E, K217E)]	a_2B	82	53
CAT_I (H195A)	a_3	0	
CAT_I	A_3	120	2
[CAT_I]$_2$[CATIII (K14E, H195A, K217E)]	A_2b	192	23
[CAT_I][CATIII (K14E, H195A, K217E)]$_2$	Ab_2	207	16

[a] Wild-type CAT_{III} is structurally and functionally equivalent to B_3, the charge-substituted variant. [b] Residual CAT activity in the standard assay (see Experimental Prodecures) in the presence of fusidate at a final concentration of 1 mM. The inhibition is competitive with respect to chloramphenicol; K_i values for homomeric CAT_{III} and CAT_I trimers are 278 and 1.5 μM, respectively (see Table 2 and Results and Discussion).

kinetic parameters (Table 2) for the hybrid show that it behaves, as expected, like a composite of CAT_I and CAT_{III}, k_{cat} being midway between those of the two wild-type parental enzymes and K_i for fusidate being 5-fold lower than that for CAT_{III}. Nonetheless, the hybrid trimer, with only one CAT_I subunit, binds fusidate 30-fold less effectively than the corresponding parental CAT_I trimer. The principal changes in the only catalytically competent chloramphenicol binding site (via His-195 of CAT_I) by constructing such a hybrid are four "substitutions" in the opposing (CAT_{III}) subunit, the following departures from the CAT_I framework: C92Q, F146N, F168Y, and V172I (see Table 1 and Figure 1). Since it seemed likely that the side chains of these residues make significant interactions with bound fusidate in CAT_I, the Ab_2 hybrid was predicted to be functionally equivalent to the substitutions F24A, Y25F, and L29A in a CAT_{III} background, assuming that the position of His-195 remains unchanged *and* that only residues in the chloramphenicol binding site are involved in fusidate binding. In fact, when such substitutions are introduced into CAT_{III}, the resulting homotrimer has kinetic parameters gratifyingly similar to those of the Ab_2 hybrid (Table 2).

[CAT_I (H195A)]$_2$ [CAT_{III} (K14E,K217E)]: The a_2B Hybrid Trimer.[3] The specific activity of the a_2B hybrid is similar to that of CAT_I (A_3) and considerably lower than that of [CAT_{III} (K14E,K217E)] (B_3), the source of the sole catalytic histidine in this hybrid (Table 5). Indeed, the steady-state kinetic parameters for the hybrid are very similar to those for CAT_I (Table 2), both the k_{cat} and the K_m for acetyl CoA being lower than the corresponding values for CAT_{III}. Unlike CAT_I, however, the aB_2 hybrid has a relatively low apparent affinity for fusidate, being inhibited by a concentration of 1 mM only to the same extent (47%) as [CAT_{III} (K14E, K217E)] (see Table 5) and with a K_i (213 μM) similar to that for wild-type CAT_{III} (Table 2). [The data for inhibition by fusidate given in Table 5 are presented as percent inhibition at a single (high) concentration rather than as K_i values because the latter are not easily interpreted for those heteromeric trimers (A_2b and aB_2) wherein there are two *different* classes of active site, the latter of which is shown in Figure 2]. Thus, the a_2B CAT hybrid displays characteristics that are either CAT_I-like (enzyme activity) or [CAT_{III} (K14E, K217E)]-like (fusidate insensitivity), rather than intermediate between the two as seen for the Ab_2 hybrid (see above).

In effect, the formation of an a_2B hybrid trimer introduces the following "substitutions" [via the CAT_I(H195A) subunits] into the single CAT_{III} catalytic environment: Q92C, N146F, Y169F, and I172V (Table 1 and Figure 1). However, when these substitutions were introduced into CAT_{III} by genetic means, to create a *homo*trimeric recombinant enzyme, the resulting protein was observed to have kinetic parameters which are significantly different from those of the a_2B hybrid described here (I. A. Murray, P. A. Cann, P. J. Day, J. P. Derrick, A. G. W. Leslie, and W. V. Shaw, in preparation, Table 2). Although k_{cat} for the multiply substituted CAT_{III} homotrimer is only slightly reduced compared to that for the wild-type CAT_{III}, the mutant homotrimer binds fusidate with high affinity (~40-fold more tightly than the parental CAT_{III}). Thus, the mutant homotrimer (with three equivalent active sites) and the a_2B hybrid with a single catalytic center, are functionally quite different. A convincing explanation for this contrast is not readily apparent, but could be related to structural differences outside the chloramphenicol binding site, probably at heterologous subunit interfaces.

Thermostability of CAT_I/CAT_{III} Hybrids. CAT_{III} and [CAT_{III}(K14E, K217E)] do not lose activity on prolonged incubation at 70 °C. In contrast, CAT_I loses activity with a $t_{1/2}$ of 3 min (Figure 5). Not one of the hybrids has a dramatically reduced thermal stability, but the hybrids do fall into two distinct structure/stability catagories. Hybrids with one or two "active" [CAT_{III} (K14E, K217E)] subunits (aB_2, a_2B) have stabilities ($t_{1/2}$ ~ 8 min) intermediate between those of CAT_I and CAT_{III}, whereas those with "inactive" [CAT_{III} (K14E, H195A, K217E)] subunits (Ab_2 or A_2b) have stabilities ($t_{1/2}$ ~ 2 min) lower than that of CAT_I. Thus, although the presence of a single [CAT_{III} (K14E, K217E)] subunit appears sufficient to stabilize trimers containing a less stable CAT_I subunit, such stability enhancement is abolished on replacement of the CAT_{III} catalytic histidine by alanine. Precisely how the H195A substitution destabilizes the CAT_{III} (and CAT_I) subunits is not clear. Nor has an explanation been found for the intrinsic instability of the homotrimeric H195A variant of wild-type CAT_{III}, which is devoid of enzyme activity (Lewendon *et al.*, 1994) and analogous to the b_3 enzyme used in the present study.

CONCLUSIONS

The presence of a hydrogen bond between $N^{\epsilon 2}$ of His-195 and the primary hydroxyl of chloramphenicol (Figure 1) is required for the catalytic mechanism of CAT and is observed in the structure of the CAT_{III}/chloramphenicol binary complex, lying deep in the inter-subunit cleft that comprises each of the three active sites in trimers (Leslie *et al.*, 1988; Leslie, 1990). Pre-steady-state kinetic studies on CAT variants wherein His-195 is replaced by alanine show that such a hydrogen bond is likely to be present in the CAT_I/

chloramphenicol complex. Furthermore, the present results also implicate His-195 in the binding of fusidic acid to CAT_I and suggest the involvement of a hydrogen bond to a polar substituent of the steroid. A likely candidate for the hydrogen bond donor is the 3-α-hydroxyl of the A ring of fusidate (Figure 4) since the 3-β-hydroxyl and 3-oxo analogs are known to bind weakly to CAT_I (Bennett & Shaw, 1983). Such an interaction may also be absent in the natural (low affinity) CAT_{III}/fusidate complex since substitution of His-195 has no effect on fusidate binding to CAT_{III}. A working hypothesis is that fusidate has easier access to the "bottom" of the active site cleft of CAT_I than to that of CAT_{III}. The amino acid "substitutions" (relative to CAT_{III}) in the chloramphenicol binding site of CAT_I increase the volume and the nonpolar characteristics of the binding site (Table 1) and may thus facilitate a more favorable approximation of fusidate, in part by hydrophobic partitioning from bulk solvent, to the immediate environs of His-195.

The results for the Ab_2 hybrid also suggest that volume and hydrophobicity constraints are important for fusidate binding. Expansion of the binding site by introducing the changes F24A, Y25F, and L29A into a CAT_{III}-like active site enhances the binding of fusidate 5-fold, but it may not be sufficient to allow formation of the proposed hydrogen bond with His-195.

The a_2B hybrid had been predicted to bind fusidate more tightly than CAT_{III} on the grounds that it contains a partial CAT_I-like active site. Tighter binding of fusidate is also observed for the site-directed mutant of CAT_{III} which has the analogous substitutions in the chloramphenicol binding site (I. A. Murray, P. A. Cann, P. J. Day, J. P. Derrick, A. G. W. Leslie, and W. V. Shaw, in preparation). The large difference between observed and expected affinity for fusidate by the a_2B hybrid must be due to effects arising from incompatibility of residues outside the chloramphenicol binding site, which occurs neither in [CAT_{III} (Q92C, N146F, Y168F, I172V)] nor in the Ab_2 hybrid.

A slightly surprising feature of the CAT hybrids is the similarity of their affinities for chloramphenicol, given that its binding sites in the hybrids and the parental homotrimers are quite different. The productive binding of chloramphenicol, deduced from K_m measurements, which assess affinities in the ternary complex, is almost unchanged, and the principal kinetic differences occur only in k_{cat}. The latter effect can be rationalized by minor changes in the geometry and length of the H-bond between His-195 and the 3-hydroxyl group of the acetyl aceptor. For example, substitution of Leu-160 by Phe results in a 60-fold decrease in k_{cat}, whereas the binding of chloramphenicol is enhanced (Murray *et al.*, 1991). The structure of this mutant revealed a quite different binding mode for chloramphenicol and a large increase in the distance between the $N^{\epsilon 2}$ of His-195 and the 3-hydroxyl of chloramphenicol.

Finally, the present study demonstrates more generally the utility and convenience of using site-directed mutagenesis to introduce surface charge differences into subunits of multimeric proteins. Candidate residues for substitution can be confidently selected from a known tertiary structure or by alignment of amino acid sequences. The residues selected for charge change in CAT are solvent accessible, as judged from a high-resolution structure, and are highly variable in other known CAT sequences (Lys-14 is substituted by Ser, Asn, Glu, or Asp in other variants, and Lys-217 is substituted by Trp, Gly, Ile, Pro, Thr, Cys, or Val). Clearly the technique of surface charge "reversal" offers an attractive alternative to alteration of net charge by the addition of an N-terminal "tail" as suggested by the work of others (Deonarian *et al.*, 1992a). It seems likely that that a choice will be made in each system on the basis of available structural information and the likely stability of the alternative protein constructs.

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