Replacement of Catalytic Histidine-195 of Chloramphenicol Acetyltransferase: Evidence for a General Base Role for Glutamate†

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ABSTRACT: The imidazole $N^{\epsilon 2}$ of His-195 plays an essential part in the proposed general base mechanism of chloramphenicol acetyltransferase (CAT), hydrogen bonding to and abstracting a proton from the primary hydroxyl group of chloramphenicol. Replacement of His-195 by alanine or glutamine results in apparent decreases in k_{cat} of (9×10^5)- and (3×10^5)-fold, respectively, whereas K_m values for both substrates (chloramphenicol and acetyl-CoA) are similar to those of wild-type CAT. The structure of Gln-195 CAT has been solved at 2.5-Å resolution and is largely isosteric with that of wild-type CAT. Substitution of His-195 by glutamate resulted in a (5×10^4)-fold decrease in k_{cat} together with a 3-fold increase in the K_m for chloramphenicol. Direct determination of binding constants for both substrates demonstrated that these substitutions result in only small decreases in the affinity of CAT for acetyl-CoA (K_d values increased 2- to 3-fold), whereas chloramphenicol K_d values are elevated 26-, 20-, and 53-fold for Ala-195 CAT, Gln-195 CAT, and Glu-195 CAT, respectively. The pH dependence of k_{cat}/K_m yields apparent pK_a values of 6.5 and 6.7 for Ala-195 CAT and Gln-195 CAT, respectively, which are very similar to that (6.6) determined for the ionization of His-195 in wild-type CAT. In contrast, the pH dependence of k_{cat}/K_m for Glu-195 CAT (pK_a = 8.3) is very different from that of wild-type CAT. Treatment with the affinity reagent 3-(bromoacetyl)chloramphenicol results in the rapid and complete inhibition of Ala-195 CAT, Gln-195 CAT, and wild-type enzyme whereas Glu-195 CAT retains 80% of its original activity. The results suggest that the activity detected in Ala-195 CAT and Gln-195 CAT arises from traces of wild-type CAT due to misincorporation of histidine. Since the properties of Glu-195 CAT cannot be explained by histidine misincorporation alone, it is likely that Glu-195 can replace His-195 as a general base, albeit inefficiently, in the mechanism of CAT. The anomalously high apparent pK_a of Glu-195 probably reflects the nonpolar environment of residue-195 in CAT.

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28) catalyzes the 3-O-acetylation of chloramphenicol using acetyl-CoA as acyl donor. The acetylated antibiotic does not inhibit protein synthesis as it fails to bind to bacterial ribosomes [reviewed by Shaw (1983) and Shaw and Leslie (1991)]. Of the many variants of CAT which have been described, the type III enzyme (CAT_{III}) has been studied in the greatest detail since the structures of the binary complexes of CAT_{III} with chloramphenicol and CoA have been determined at 1.75- and 2.4-Å resolution, respectively (Leslie *et al.*, 1988; Leslie, 1990). CAT_{III} is a trimer of identical subunits of M_r 25 000 with three active sites per trimer located at each of the intersubunit interfaces. Chloramphenicol and CoA approach each active site from opposite faces of the protein via a tunnel that is formed from the binding sites of the two substrates. The structural separation of the substrate binding sites explains the results of steady-state kinetic studies which demonstrated that CAT_{III} follows a sequential (ternary complex) mechanism with rapid-equilibrium and random-order addition of substrates (Kleanthous & Shaw, 1984; Ellis *et al.*, 1991). In the absence of chloramphenicol, CAT hydrolyzes acetyl-CoA at approximately 0.01% of the rate observed for acetylation of chloramphenicol (Kleanthous & Shaw, 1984).

Although the chloramphenicol and CoA binding sites are largely composed of residues from a single subunit, an essential histidyl residue (His-195) is supplied by the opposing subunit. A catalytic role for His-195 was first inferred from chemical modification studies with 3-(bromoacetyl)chloramphenicol, an active-site-directed inhibitor of CAT, which inactivates the enzyme by specific and stoichiometric alkylation at $N^{\epsilon 2}$ of His-195 (Kleanthous *et al.*, 1985). On the basis of this result, Kleanthous *et al.* (1985) proposed a mechanism for CAT in which $N^{\epsilon 2}$ of His-195 acts as a general base to deprotonate the 3-hydroxyl group of chloramphenicol, thereby promoting nucleophilic attack at the carbonyl of the thioester of acetyl-CoA (Scheme 1). Modeling studies (P. C. E. Moody and A. G. W. Leslie, unpublished results) and analysis by site-directed mutagenesis (Lewendon *et al.*, 1990; Lewendon

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of CAT; Cm, chloramphenicol; TSE buffer, 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA; EDTA, ethylenediaminetetraacetic acid; MPD, 2-methyl-2,4-pentanediol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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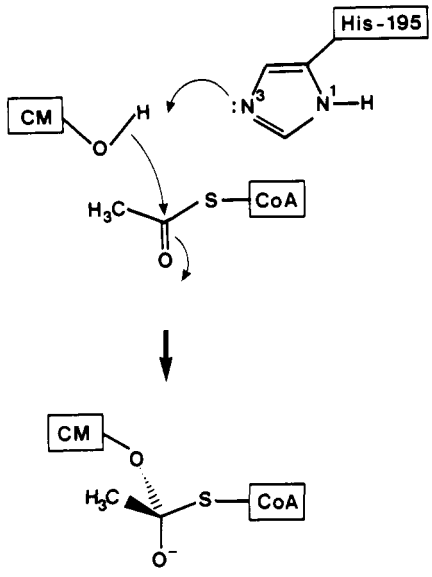
Scheme 1



& Shaw, 1993a) support the formation of a tetrahedral intermediate (Scheme 1) during the course of the reaction. Furthermore, the three-dimensional structure of CAT_{III} supports this mechanism since the active-site geometry is consistent with a hydrogen bond between the 3-hydroxyl of chloramphenicol and $N^{\epsilon 2}$ of His-195 in the CAT_{III}/chloramphenicol binary complex (Leslie, 1990). A study of the pH dependence of chemical modification of His-195 and k_{cat}/K_m has revealed that the pK_a of the catalytic imidazole $N^{\epsilon 2}$ is 6.6 (Lewendon & Shaw, 1993b), a value low enough to ensure that His-195 is an effective proton acceptor at physiological pH values.

In order to assess the quantitative importance of His-195 in the catalytic mechanism of CAT, we have replaced it with alanine and glutamine. Neither Ala-195 nor Gln-195 can accept a proton from the 3-hydroxyl of chloramphenicol, although the latter retains the potential for hydrogen bonding with the substrate. His-195 was also replaced by aspartate, glutamate, and tyrosine, each of which may accept a proton under appropriate conditions and could in principle substitute for the general base role of His-195.

Each enzyme substituted at His-195 retains a very low level of catalytic activity. Although the residual activity observed upon substitution of His-195 by aspartate, tyrosine, or glutamate might be the result of catalysis by the alternative general base, a different explanation must be sought for the residual activity of Ala-195 CAT and Gln-195 CAT. Three possible explanations were considered: (i) that an alternative base in the active sites of the substituted enzymes acts as a proton acceptor in place of His-195; (ii) that even in the absence of His-195, the juxtaposition of the substrates at the active site of CAT may bring about an enhancement of the rate of reaction over the uncatalyzed rate because of proximity and orientation effects; and (iii) that the residual CAT activity of the His-195-substituted enzymes may simply be due to errors in transcription and/or translation resulting in the misincorporation of the wild-type residue instead of that specified by the DNA sequence. In order to distinguish between these possibilities, the mutant enzymes were characterized by ligand binding studies, chemical modification with 3-(bromoacetyl)-chloramphenicol, and steady-state kinetic analysis, including an examination of the pH dependence of catalysis. In addition, the three-dimensional structure of Gln-195 CAT was determined at 2.5-Å resolution to ensure that structural changes are limited to the site of the substitution.

The results suggest that the residual activity observed for Ala-195 CAT and Gln-195 CAT is due to misincorporation of histidine at residue 195. Furthermore, neither aspartate nor tyrosine appears to be able to substitute for the general base role of His-195 since the activity detected in Asp-195 CAT and Tyr-195 CAT can also be attributed to histidine misincorporation. However, the His-195 → Glu substitution yields a protein which possesses acetyltransferase activity that cannot be wholly ascribed to misincorporation of histidine. The results suggest that the carboxylate of Glu-195 can function, albeit imperfectly, as an alternative to the $N^{\epsilon 2}$ of the imidazole of His-195. The pH dependence of k_{cat}/K_m for Glu-195 CAT yields an apparent pK_a of 8.3, a high value for a carboxylate group, which may be a consequence of the nonpolar environment of residue-195 in CAT.

EXPERIMENTAL PROCEDURES

Materials. Acetyl-CoA for fluorescence titrations was obtained from Pharmacia LKB. For other purposes it was prepared from CoA (Pharmacia LKB) as described by Simon and Shemin (1953). [^{14}C]Chloramphenicol and [^{3}H]acetyl-CoA were obtained from New England Nuclear and were diluted with unlabeled material prior to use.

The substrate analogue ethyl-S-CoA was prepared by the following procedure: CoA (200 mg, 240 μmol) was dissolved in 5 mL of 40 mM potassium bicarbonate containing 600 μmol of 2-mercaptoethanol, and the pH was adjusted to between 9 and 10 by addition of NaOH. The solution was protected from light, iodoethane (120 μL, 1500 μmol) was added, and the solution was stirred at room temperature for 1 h. Ethyl-S-CoA was evaporated to dryness, redissolved in water, and then desalted on a column of Sephadex G-10. The desalted ethyl-S-CoA was lyophilized and redissolved in TSE buffer.

Site-Directed-Mutagenesis and Expression of CAT. Oligonucleotide-directed mismatch mutagenesis was performed using the deoxyuridine selection protocol with the *dut ung Escherichia coli* strain RZ1032 (Künkel *et al.*, 1987). The His-195 codon (CAU) was replaced by GCU (His-195 → Ala), UAC (His-195 → Tyr), GAC (His-195 → Asp), GAG (His-195 → Glu), and CAG (His-195 → Gln). 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 CAT from *E. coli* extracts was performed by affinity chromatography on chloramphenicol–Sepharose (Lewendon *et al.*, 1988). Purification of enzymes substituted at His-195 was carried out by a three-step procedure consisting of ion-exchange chromatography (DEAE-Sephacel) followed by affinity chromatography on Procion-green Sepharose and Cibacron-blue Sepharose (Murray *et al.*, 1991). The purity of enzyme preparations was assessed by SDS–polyacrylamide gel electrophoresis wherein each substituted enzyme had the same mobility as wild-type CAT. The concentration of purified CAT was calculated from the $\epsilon^{1\%}_{280}$ of 13.1.

Assay of CAT Activity. (i) Standard Spectrophotometric Assay. The 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 CAT, and the progress of the reaction at 25 °C was monitored at 412 nm (Shaw, 1975). Rates of acetyl-

CoA hydrolysis were determined as described above except that chloramphenicol was omitted from the reaction mixture.

(ii) Determination of Kinetic Parameters. The concentrations of chloramphenicol and acetyl-CoA were varied in the standard assay mixture during kinetic analyses. A Uvikon 930 spectrophotometer (Kontron) equipped with an automatic cell changer was used for kinetic analysis of His-195-substituted enzymes. Assays (in quadruplicate) were performed over 6–15 min in batches of 10. The maximum concentration of CAT monomers in the assay mixture was 0.8 μM. Kinetic constants were derived from linear slope and intercept replots from manually drawn double-reciprocal plots (Kleanthous & Shaw, 1984).

(iii) Radiometric Assay. In order to examine the pH dependence of k_{cat}/K_m,[2] CAT activity was assayed by a radiometric method as described by Lewendon and Shaw (1993b) in 50 mM buffer containing 0.1 mM EDTA and a concentration of NaCl sufficient to adjust the final ionic strength of the buffer solution to 0.122 M. The following buffers were used over the pH ranges specified: Mes/NaOH, pH 5.1–6.7, Mops/NaOH, pH 6.7–7.5; Hepes/NaOH, pH 7.5–8.3; and Ches/NaOH, pH 8.3–10.3. Rates were determined in both buffers at pH values where the buffer ranges overlap.

Equilibrium Dialysis. Determination of dissociation constants for chloramphenicol was performed by equilibrium dialysis at 25 °C as described by Ellis *et al.* (1991). Ligand binding studies were carried out in the presence and absence of 1 mM ethyl-S-CoA to yield values for the ternary and binary dissociation constants. The concentration of monomers of each His-195-substituted enzyme varied between 120 and 180 μM, and the concentration of chloramphenicol was varied between 10 and 2000 μM. The mean concentrations of ligand present in each chamber were plotted directly as [bound] versus [free] and were analyzed using the data analysis program Enzfitter (Leatherbarrow, 1987) assuming a single ligand binding site.

Fluorimetric Titrations. Manual titrations of CAT (1 μM monomers) in TSE buffer at 25 °C were performed as described by Ellis *et al.* (1991). Data were plotted as fluorescence intensity versus [acetyl-CoA] or [ethyl-S-CoA] and fitted to a hyperbolic function using Enzfitter to calculate the dissociation constants.

Crystallization and Structure Determination of Gln-195 CAT. Single crystals were grown by microdialysis in small "Lucite" buttons. Each button contained 30 μL of protein (5 mg·ml^{-1}) in 10 mM Mes, pH 6.3, and was dialyzed against 4 mL of 2–4% 2-methyl-2,4-pentanediol (MPD), 10 mM Mes, pH 6.3, 1 mM chloramphenicol, 0.5 mM hexaamminecobalt-(III) chloride, and 5 mM 2-mercaptoethanol at 4 °C (Leslie *et al.*, 1986). Crystals were harvested in 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 = 124.1 Å (wild-type values a = 107.6 Å, c = 123.6 Å).

X-ray data were collected to 2.5-Å resolution from a single crystal (dimensions 0.4 × 0.2 × 0.2 mm) using an Enraf Nonius FAST diffractometer and CuKα radiation from a rotating anode source. The data were collected using images 0.1° in width and processed with MADNES (Messerschmidt & Pflugrath, 1987). The resulting data were scaled and merged using the CCP4 programs ABSURD, ROTOVATA, and AGROVATA. A total of 23 710 observations were merged to give 9485 independent reflections, representing 97% of the unique data. The crystallographic merging R factor[3] was 4.3% for all data to 2.5-Å resolution (10.3% in the highest resolution bin). The refined 1.75-Å resolution wild-type structure (Leslie, 1990) was used as a starting model for refinement of Gln-195 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 18.3%, which dropped to 13.1% after refinement, with good stereochemistry (Table 1).

Table 1: Stereochemistry of the Refined Model of Gln-195 CAT

stereochemical refinement parameter	rms deviation from ideal values	refinement restraint weighting values
bond distances (Å)	0.020	0.02
angle distances (Å)	0.044	0.03
planar 1–4 distances (Å)	0.062	0.05
planes (Å)	0.019	0.02
chiral volumes (Å^3)	0.19	0.15
van der Waals contacts (Å)		
single torsion	0.16	0.20
multiple torsion	0.18	0.20

RESULTS AND DISCUSSION

Characterization of His-195-Substituted Enzymes. (i) Steady-State Kinetic Analysis and Substrate Binding Studies. As expected, substitution of His-195 results in a dramatic reduction in activity which appears to be due predominantly to a decrease in k_{cat}; the His-195 → Ala substitution results in a (9×10^5)-fold reduction in k_{cat} whereas the decreases observed for Tyr-195 CAT and Gln-195 CAT are somewhat smaller [(2×10^5)- and (3×10^5)-fold, respectively] and the k_{cat} for Glu-195 CAT is decreased only (5×10^4)-fold (Table 2). The K_m values for chloramphenicol and acetyl-CoA are similar to those of wild-type CAT except in the case of Glu-195 CAT where the K_m for chloramphenicol is increased 3-fold. The steady-state kinetic parameters for Asp-195 CAT could not be determined due to nonlinearity of rates.

CAT_{III} follows a rapid-equilibrium random-order kinetic mechanism wherein K_d and K_m values derived for both substrates from steady-state kinetic analysis are equivalent, respectively, to the dissociation constants from the binary and ternary complexes and are in good agreement with the directly measured dissociation constants (Kleanthous & Shaw, 1984; Ellis *et al.*, 1991). K_d values for acetyl-CoA and ethyl-S-CoA [a competitive inhibitor (K_i = 103 μM) with respect to acetyl-CoA; P. J. Day, unpublished results] for the His-195-substituted enzymes were determined by fluorimetric titration,

[2] Values of k_{cat}/K_m were derived from the following expression which applies when [chloramphenicol] < K_m.

$$\text{rate} = [\text{CAT}][\text{chloramphenicol}](k_{cat}/K_m)$$

[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 weighed 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.

Table 2: Kinetic Parameters for Wild-Type and Mutant Chloramphenicol Acetyltransferases[a]

CAT	k_{cat} (s^{-1})	chloramphenicol		acetyl-CoA	
		K_m (μM)	K_d (μM)	K_m (μM)	K_d (μM)
wild-type	600[b]	11.6[b]	4[c]	93[b]	28[c]
Ala-195	$6.5 \times 10^{-4} \pm 0.7 \times 10^{-4}$	6.6 ± 0.8	1.6 ± 0.1	40 ± 11	10 ± 2
Gln-195	$1.8 \times 10^{-3} \pm 0.1 \times 10^{-3}$	11.4 ± 0.2	2.0 ± 0.2	55 ± 12	10 ± 2
Tyr-195	$3.1 \times 10^{-3} \pm 0.5 \times 10^{-4}$	14.0 ± 5.0	2.3 ± 0.8	95 ± 18	18 ± 0.6
Glu-195	$1.2 \times 10^{-2} \pm 0.3 \times 10^{-2}$	32.6 ± 9.3	21.2 ± 5.9	92 ± 15	70 ± 8

[a] Kinetic parameters are the mean of at least three determinations performed as described under Experimental Procedures and are given ±SE. [b] Taken from Lewendon *et al.* (1988). [c] Taken from Ellis *et al.* (1991).

Table 3: Dissociation Constants for Wild-Type and Mutant Chloramphenicol Acetyltransferases[a]

CAT	K_d for acetyl-CoA (μM)	K_d for ethyl-S-CoA (μM)	K_d for chloramphenicol	
			no addition (μM)	+1 mM ethyl-S-CoA (μM)
wild-type[b]	28	12	4	12
Ala-195	52 ± 12	23 ± 8	106 ± 6	98 ± 10
Gln-195	79 ± 27	69 ± 11	82 ± 10	95 ± 9
Glu-195	58 ± 14	51 ± 11	211 ± 58	275 ± 71

[a] K_d values were determined by equilibrium dialysis in the case of chloramphenicol, and by fluorimetric titration in the cases of acetyl-CoA and ethyl-S-CoA, as described under Experimental Procedures. All K_d values are the mean of at least two determinations and are given ±SE. [b] K_d values for wild-type CAT are taken from Ellis *et al.* (1991).

and K_d values for chloramphenicol in the presence and absence of ethyl-S-CoA were determined by equilibrium dialysis (Ellis *et al.*, 1991). The substitutions at His-195 result in slightly reduced affinity for acetyl-CoA or ethyl-S-CoA in the binary complex (Table 3). However, the affinity of each of the His-195-substituted enzymes for chloramphenicol is greatly decreased compared to wild-type (Table 3), a result expected from structural considerations. Compared to wild-type enzyme, the affinities of Gln-195 CAT, Ala-195 CAT, and Glu-195 CAT for chloramphenicol in their binary complexes are decreased 20-, 26-, and 53-fold, respectively. In the case of wild-type CAT, the affinity of the enzyme for each substrate is diminished upon binding of the other substrate (or its analogue), an effect described as negative cooperativity (Ellis *et al.*, 1991). The substituted enzymes differ from wild-type CAT in that they possess the same affinity for chloramphenicol in both the binary and ternary complexes since the affinity of the substituted enzymes for chloramphenicol is unaltered in the presence of ethyl-S-CoA (Table 3).

A comparison of the K_d values in Table 3 with those derived from steady-state kinetic analysis (Table 2) implies that the catalytically active species of each of the His-195-substituted enzymes has a greater affinity for chloramphenicol than does the bulk protein. In fact, the results suggest that the latter does not contribute significantly to catalysis, and given the similarity of the K_m values of the substituted enzymes to those of wild-type CAT, it seems likely that the rates of acetyl transfer observed for these mutant enzymes are due to small amounts of misincorporated histidine at residue-195.

(ii) Acetyl-CoA Hydrolysis. In the absence of chloramphenicol, wild-type CAT hydrolyzes acetyl-CoA at a rate of 5.3×10^{-2} s^{-1}, approximately 0.01% of that observed for chloramphenicol acetylation (Kleanthous & Shaw, 1984; Lewendon *et al.*, 1990). Although hydrolysis of acetyl-CoA by Ala-195 CAT or Gln-195 CAT could not be detected, Glu-195 CAT hydrolyzes acetyl-CoA at approximately 1.5% ($k_{cat} = 1.8 \times 10^{-4}$ s^{-1}) of the rate at which it catalyzes acetylation of chloramphenicol. If all the catalytic activity of Glu-195 CAT is due to the presence of misincorporated His-195, the ratio of the rates of acetyl-CoA hydrolysis to chloramphenicol acetylation should be the same for Glu-195 CAT as for wild-type enzyme. Since this ratio is increased 150-fold in Glu-195 CAT, it is likely that Glu-195 acts as a general base in the thioesterase reaction mechanism.

(iii) pH Dependence of Catalysis. Previous studies on wild-type CAT established that k_{cat}/K_m increases with pH over the range 5.1–8.3, yielding a pK_a value of 6.6 which is attributed to His-195 (Lewendon & Shaw, 1993b). Furthermore, k_{cat}/K_m is reduced above pH 8.5 (Figure 1), an effect likely to be due to instability of the enzyme at high pH. The pH dependence of k_{cat}/K_m for Ala-195 CAT, Gln-195 CAT, and Glu-195 CAT was examined since such pH profiles should be the same as that of wild-type CAT if the activity detected in the substituted enzymes results from misincorporation of histidine at residue 195.

The pH dependence of k_{cat}/K_m for Gln-195 CAT is almost identical to that of wild-type enzyme over the entire pH range (Figure 1). Moreover, the pH profile of Ala-195 CAT is very similar except that k_{cat}/K_m increases above pH 9.1. The fact that the pK_a values of 6.5 and 6.7 derived from the pH dependence of k_{cat}/K_m over the range 5.1–8.3 for Ala-195 CAT and Gln-195 CAT, respectively, approximate to that of wild-type supports the proposal that the activity detected in Ala-195 CAT and Gln-195 CAT is due to misincorporated His-195.

The observation that the catalytic activity of Ala-195 CAT increases above pH 9.1 suggests that another base can accept a proton from chloramphenicol at high pH. Craik *et al.* (1987) observed a similar rate enhancement at high pH in a mutant trypsin (Asp-102 → Asn) and suggested that the probable explanation was the participation of hydroxide ion or another titratable base in the catalytic mechanism of the mutant enzyme. In the case of CAT, the fact that an enhancement of catalytic activity at high pH is observed for Ala-195 CAT but not Gln-195 CAT suggests that access of the alternative base to the 3-hydroxyl group of chloramphenicol is prevented by the bulky glutamine side chain.

The pH dependence of k_{cat}/K_m for Glu-195 CAT is strikingly different from that of wild-type CAT (Figure 1). Activity increases with pH, yielding a pK_a value of approximately 8.3 (calculated from data for the pH range 5.1–9.1) which can be tentatively assigned to the carboxylate of Glu-195. As in the case of wild-type CAT, the activity of Glu-195 CAT decreases above pH 9. Although it is not possible to exclude rigorously the participation of an alternative base such as hydroxide ion in the chemical mechanism of Glu-195 CAT, access of the solvent-derived base to the 3-hydroxyl of chloramphenicol in the active site of Glu-195 CAT would not be expected to be significantly easier or more likely than with Gln-195 CAT. Furthermore, were hydroxide to be directly involved in catalysis, the pH profile of Glu-195 CAT would then be expected to follow that of Ala-195 CAT above pH 9 rather than that seen with wild-type and Gln-195 CAT.

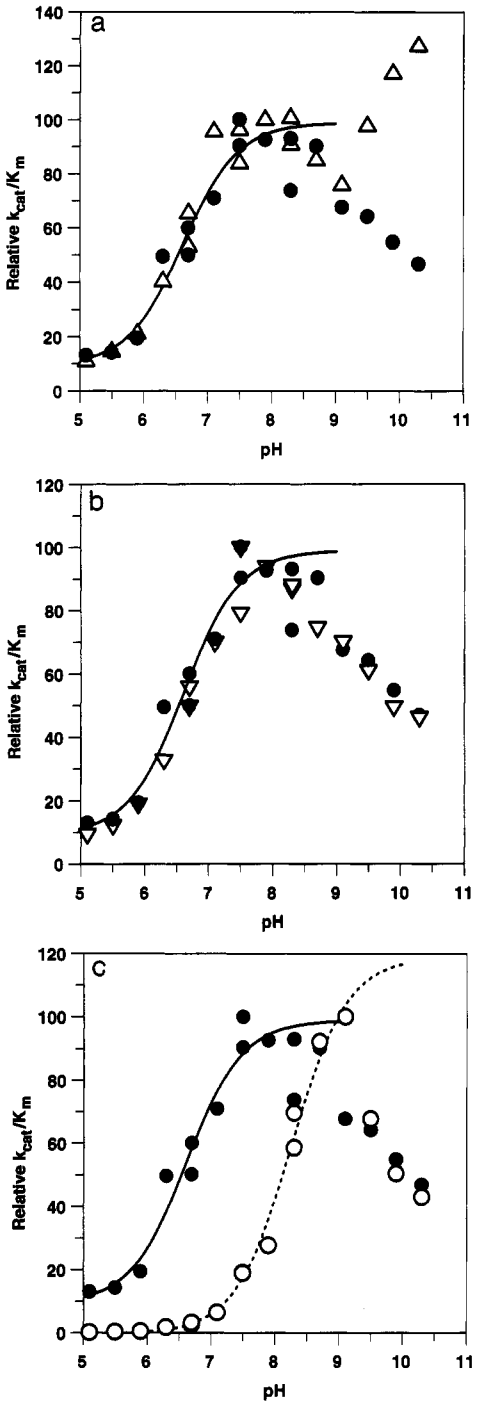


FIGURE 1: pH dependence of k_{cat}/K_m for wild-type and mutant chloramphenicol acetyltransferases. Values of k_{cat}/K_m are plotted relative to the maximum value in each case, with the exception of Ala-195 CAT where the k_{cat}/K_m in Hepes buffer pH 7.5 (55.4 $s^{-1}\cdot M^{-1}$), is taken as 100. The maximum values for wild-type and Gln-195 CAT both occur in Hepes buffer, pH 7.5, and are 3.76×10^7 $s^{-1}\cdot M^{-1}$ and 1.60×10^2 $s^{-1}\cdot M^{-1}$ respectively, whereas that for Glu-195 CAT is 4.94×10^3 $s^{-1}\cdot M^{-1}$ and occurs in Ches buffer, pH 9.1. The pH dependences of k_{cat}/K_m for each of Ala-195 CAT (Δ), Gln-195 CAT (▽) and Glu-195 CAT (○) are shown as pairwise comparisons with that of wild-type CAT (●) in (a), (b), and (c), respectively. The best-fit theoretical curves shown are fitted to the data for wild-type CAT (—) between pH 5.1 and 8.3 and Glu-195 CAT (---) between pH 5.1 and 9.1 and give pK_a values of 6.62 and 8.26, respectively.

The pK_a value of 8.3 is elevated by approximately 4 pH units above the pK_a of a solvated carboxylate side chain. Examples of carboxylates with highly perturbed pK_a values in other proteins are well-known. A catalytic aspartyl residue (Asp-26) in *E. coli* dihydrofolate reductase has a pK_a of 6.5 in wild-type enzyme which is elevated to 8.4 in the Arg-44 → Leu substituted variant (Adams *et al.*, 1989). Another example is Asp-26 of thioredoxin where a pK_a value of 7.5 has been reported (Langsetmo *et al.*, 1991). Like Glu-195 in CAT, such carboxylate groups are buried in the interior of the protein and the raised pK_a reflects the energetic cost of burying an unpaired charge.

(iv) 3-(Bromoacetyl)chloramphenicol Inhibition. 3-(Bromoacetyl)chloramphenicol is a potent inhibitor of wild-type CAT_{III}, reacting rapidly and specifically with $N^{\epsilon 2}$ of His-195 such that a 1.5-fold molar excess of reagent over active sites results in complete inhibition of enzyme activity (Kleanthous *et al.*, 1985). Wild-type CAT and each mutant enzyme were treated with a 2-fold excess of 3-(bromoacetyl)chloramphenicol. After a 1-min incubation, wild-type CAT, Ala-195 CAT, Gln-195 CAT, Asp-195 CAT, and Tyr-195 CAT were each completely inhibited whereas Glu-195 CAT retained approximately 80% of its original activity. Previous studies have demonstrated that Cys-31, a residue in the chloramphenicol binding site, can be modified by 3-(bromoacetyl)chloramphenicol in substituted enzymes where the reactivity of the $N^{\epsilon 2}$ of His-195 is reduced (Lewendon *et al.*, 1988; Murray *et al.*, 1991), resulting in decreased catalytic activity, probably because of steric interference (Lewendon & Shaw, 1990). However, after treatment of the double mutants Ala-31/Ala-195 CAT and Ala-31/Glu-195 CAT with 3-(bromoacetyl)-chloramphenicol the extent of inactivation observed was indistinguishable from that observed with the single mutants. Consequently, the incidental modification of Cys-31 cannot be a factor in the inhibition observed upon treatment of the His-195-substituted enzymes by 3-(bromoacetyl)chloramphenicol.

The results described above support the hypothesis that the acetyltransferase activity detected in Ala-195 CAT, Gln-195 CAT, Asp-195 CAT, and Tyr-195 CAT is due to misincorporation of histidine since the activity of each of the substituted enzymes is inactivated as readily as that of wild-type CAT by 3-(bromoacetyl)chloramphenicol. In contrast, Glu-195 CAT is relatively refractory to inactivation by stoichiometric amounts of the active site-directed reagent, suggesting (a) that the observed activity is not wholly due to misincorporated histidine and (b) that not less than 80% of the activity associated with Glu-195 CAT is due to catalysis promoted by Glu-195.

The Structure of Gln-195 CAT. With the exception of the mutated residue, and small changes in the side chains of Phe-33 and Ser-148, the structure of Gln-195 CAT is isosteric with that of wild-type enzyme. Whereas the aromatic ring of Phe-33 is merely shifted slightly (approximately 0.5 Å) from its wild-type position (Figure 2), the hydroxyl group of Ser-148 is disordered in Gln-195 CAT. Both of these residues play important roles in transition-state stabilization by CAT (Lewendon *et al.*, 1990; Day & Shaw, 1992). Moreover, chloramphenicol is absent from the active site of Gln-195 CAT despite its presence in the crystallization medium. Instead, the chloramphenicol binding pocket contains electron density which can be modeled as a single molecule of 2-mercaptoethanol. The latter forms no hydrogen bonds with any protein or solvent atoms and would presumably be displaced upon binding of chloramphenicol. In addition to the changes described above, three ordered water molecules (waters-276, -308 and -360) are absent from the active site of the mutant enzyme. Water-276 is located in the CoA binding site, but the other two water molecules are involved in bridging hydrogen bonds, between chloramphenicol and

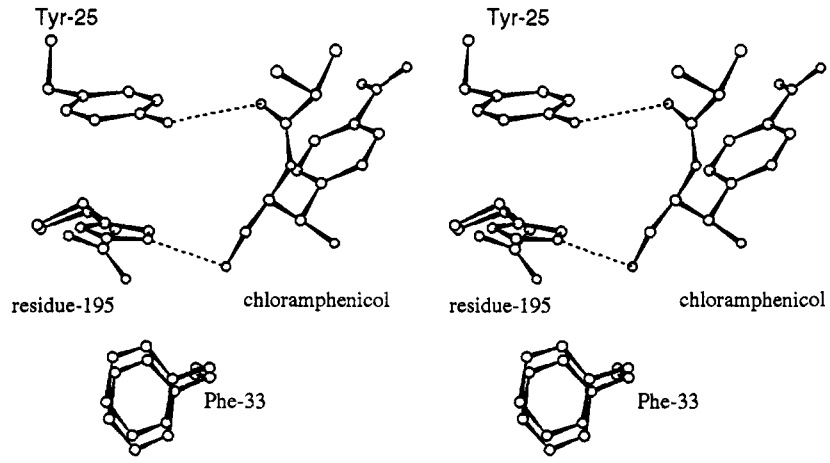


FIGURE 2: Stereoview showing the active sites of wild-type CAT and Gln-195 CAT superimposed, with residue 195 and Phe-33 shown from both molecules, chloramphenicol from wild-type CAT, and Tyr-25 from Gln-195 CAT. Possible hydrogen bonds are shown as broken lines.

two other water molecules in the case of water-360, and between chloramphenicol and the hydroxyl group of Ser-148 in the case of water-308.

The Gln-195 side chain adopts a conformation such that its amino group is within hydrogen bonding distance (3.06 Å) of the main-chain carbonyl of the same residue, an interaction reminiscent of that observed between $N^{\delta 1}$ and the backbone carbonyl oxygen of the same residue in His-195 of wild-type CAT. In addition, it is possible for the amino group of Gln-195 to hydrogen bond to one of the carboxylate oxygens of Asp-199, a residue that plays an important structural role in CAT_{III} (Lewendon *et al.*, 1988). When chloramphenicol is modeled into the active site of Gln-195 CAT using the coordinates from the binary complex of wild-type CAT with chloramphenicol (Leslie, 1990), the O^{ϵ} of Gln-195 is within hydrogen bonding distance (3.5 Å) of the 3-hydroxyl group of chloramphenicol.

Conclusions. Modeling studies suggested a somewhat long hydrogen bonding interaction between O^{ϵ} of Gln-195 and the 3-hydroxyl group of chloramphenicol. However, the affinity of Ala-195 CAT for chloramphenicol is similar to that of Gln-195 CAT (Table 3) despite the fact that Ala-195 cannot hydrogen bond to the substrate. This suggests either that the hydrogen bond between chloramphenicol and Gln-195 makes a negligible contribution toward chloramphenicol binding or that the 3-hydroxyl group of chloramphenicol is in fact involved in an energetically favorable hydrogen bonding interaction in Ala-195 CAT, perhaps with a water molecule from bulk solvent. The energetic cost (ΔG_{app})[5] of the His-195 → Gln substitution is a loss of 1.8 kcal·mol^{-1} of binding energy, a similar value to that (1.5 kcal·mol^{-1}) determined from a comparison of the binding constants of chloramphenicol and an analogue where the 3-hydroxyl group was replaced by a hydrogen atom (Cullis *et al.*, 1991).

Taken together, the results described above strongly support the view that the acetyltransferase activity detected in preparations of Ala-195 CAT, Gln-195 CAT, Tyr-195 CAT, and Asp-195 CAT is due entirely to catalysis by enzyme molecules with misincorporated histidine at residue 195. Errors in the translation of mRNA in *E. coli* are commonly introduced from misreading of the first or third bases of a codon by a tRNA, although the frequency of errors depends on the nature and context of the codon [reviewed by Parker (1989)]. The level of activity of the substituted enzymes reflects the number of mismatches between the replacement codon and either of the two histidine codons: k_{cat} for Ala-195 CAT (2 mismatches) is lower than that for Gln-195 CAT or Tyr-195 CAT (1 mismatch). Since the glutamate codon contains two mismatches, it is likely to be misread less frequently than codons containing one mismatch; however, some part of the activity detected in preparations of Glu-195 CAT is presumably due to misincorporation of histidine.

The frequency of histidine misincorporation at residue 195 estimated from the ratios of k_{cat} for the wild-type and mutant enzymes ranges from 1.1×10^{-6} to 5.2×10^{-6}. These frequencies are comparable to that (2×10^{-5}) reported for the wild-type contamination of a His-57 → Arg mutant of trypsin (Corey & Craik, 1992), but low compared to the frequency of 1×10^{-3} reported for the misreading of a glycine codon as serine in a β-lactamase (Toth *et al.*, 1988).

The results provide evidence for the proposition that Glu-195, but not Asp-195, can act as a proton acceptor in the catalytic mechanism of CAT_{III}. Presumably, the additional methylene group of the glutamate side chain enables a carboxylate oxygen to approach the 3-hydroxyl of chloramphenicol closely enough to allow abstraction of a proton. The tyrosyl hydroxyl group suffers from a disadvantage as an enzymatic proton acceptor by virtue of its high pK_a (9.7) which implies that it can accept protons only at unphysiologically high pH. Nonetheless, even at pH 9.5, the activity of Tyr-195 CAT is not increased compared to its rate at pH 7.5. The most likely explanation for the failure of Tyr-195 to act as a proton acceptor is that its phenolate oxygen might be inappropriately positioned to accept a proton.

A comparison of k_{cat}/K_m values for wild-type and Glu-195 CAT at pH 7.5 suggests that Glu-195 CAT is (3.8×10^4)-fold less efficient than His-195 as a catalytic base. However, if the k_{cat}/K_m value for the pH at which Glu-195 CAT is most active (pH 9.1) is considered for the purposes of comparison, Glu-195 CAT is only (7.2×10^3)-fold less efficient than the wild-type enzyme at pH 7.5. The relatively poor catalytic activity of Glu-195 CAT may reflect the fact that neither of the carboxylate oxygens of Glu-195 is ideally positioned to hydrogen bond to the 3-hydroxyl of chloramphenicol, a consideration which might be addressed by determining the structure of the binary complex of Glu-195 CAT and chloramphenicol. However, given the fact that bound chloramphenicol is absent from crystals of Gln-195 CAT grown

[5] The energetic consequences of the substitutions at His-195 were determined from the expression:

$$\Delta G_{app} = -RT \ln[K_d(Cm)_{mutant}/K_d(Cm)_{wild\text{-}type}]$$

in the presence of antibiotic, it seems unlikely that it would be present in crystals of Glu-195 CAT, in which the affinity for chloramphenicol is further reduced. Furthermore, the unprotonated, active form of Glu-195 will be negatively charged, and introduction of a negative charge into the chloramphenicol binding site is known to have a deleterious effect on the affinity of CAT for chloramphenicol (A. Lewendon, unpublished results), an unsurprising observation since hydrophobic interactions make a major contribution to the binding of chloramphenicol (Leslie, 1990; Cullis *et al.*, 1991).

There have been a number of reports of enzymes that possess low levels of activity in the absence of a crucial catalytic residue. In the well-characterized example of subtilisin, replacement of the catalytic triad of serine, histidine, and aspartate by alanine led to the conclusion that these residues were responsible for a rate enhancement of 10^6-fold. Wild-type contamination was ruled out as a source of the residual activity, which was instead attributed to stabilization of the transition state by the altered enzyme (Carter & Wells, 1988, 1990). In contrast, the results described above suggest that all of the activity detected in preparations of Ala-195 CAT can be ascribed to contamination by wild-type monomers. This implies that His-195 confers a rate enhancement of more than the (9×10^5)-fold suggested from the reduction in k_{cat} resulting from the His-195 → Ala substitution. Treatment of Ala-195 CAT with 3-(bromoacetyl)chloramphenicol resulted in complete inhibition of activity although a residual rate of 5% of the initial rate would have been readily detectable. Consequently, a further factor of at least 20-fold can be combined with the decrease in k_{cat} for Ala-195 CAT to yield a minimum estimate of (2×10^7)-fold for the rate enhancement due to His-195 in CAT.

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