The imidazole of His-195 plays an essential role in the proposed general base mechanism of chloramphenicol acetyltransferase (CAT). The structure of the binary complex of CAT<sub>II</sub> and chloramphenicol suggests that two unusual interactions might determine the conformation of the side chain of His-195: (i) an intr Residue hydrogen bond between its main chain carboxyl and the protonated N<sup>ε</sup> of the imidazole ring and (ii) face-to-face van der Waals contact between the His-195 imidazole group and the aromatic side chain of Tyr-25. Tyr-25 also makes a hydrogen bond, via its phenolic hydroxyl, to the carboxyl oxygen of the sub- strate chloramphenicol.

Replacement of Tyr-25 of CAT<sub>II</sub> by phenylalanine results in a modest increase in the K<sub>m</sub> for chloramphenicol (from 11.6 to 14.6 μM) and a 2-fold fall in k<sub>cat</sub> (599 to 258 s<sup>-1</sup>), indicative of a free energy contribution to transition state binding of 0.6 kcal mol<sup>-1</sup> for the hydrogen bond between Tyr-25 and chloramphenicol. In contrast, substitution of Tyr-25 by alanine yields an enzyme that is dramatically impaired in its ability to bind chloramphenicol (K<sub>m</sub> = 173 μM). As k<sub>cat</sub> for Ala-25 CAT is also reduced (130 s<sup>-1</sup>), the loss of the aryl group results in a 69-fold decrease in k<sub>cat</sub>/K<sub>m</sub>, corresponding to a free energy contribution to binding and catalysis of 2.5 kcal mol<sup>-1</sup>.

In addition to the loss of the hydrogen bond between Tyr-25 and chloramphenicol, the loss of substrate affinity in Ala-25 CAT may be a direct consequence of reduced hydrophobicity of the chloramphenicol-binding site and/or the loss of critical constraints on the precise conformation of the catalytic imidazole. However, as with wild type CAT, inactivation of Ala-25 CAT by the affinity reagent 3-(bromoacetyl)chloramphenicol is accompanied by modification solely at N<sup>ε</sup> of His-195. Hence, the results demonstrate that tautomeric stabilization of the imidazole ring persists in the absence of van der Waals interactions with the side chain of Tyr-25, probably as a consequence of hydrogen bonding between the protonated N<sup>ε</sup> and the carboxyl oxygen of His-195.

Stabilization of the Imidazole Ring of His-195 at the Active Site of Chloramphenicol Acetyltransferase

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† Supported by the Protein Engineering Initiative of the Science and Engineering Research Council.

‡ To whom correspondence should be addressed.

1 The abbreviations used are: CAT, chloramphenicol acetyltransferase; CAT<sub>II</sub>, type I variant of CAT; CAT<sub>III</sub>, type III variant of CAT; cat, gene encoding CAT.

2 Alignment of the amino acid sequences of 10 CAT variants has resulted in the general numbering system that is used here. Arg-18, Tyr-25, Cys-31, and His-195 are residues 13, 20, 26, and 189, respectively, in the primary structure of CAT<sub>II</sub> (Murray et al., 1988).

Bacterial resistance to the antibiotic chloramphenicol (Fig. 1), an inhibitor of the peptidyltransferase activity of prokaryotic ribosomes, is commonly conferred by the enzyme chloramphenicol acetyltransferase (CAT; EC 2.3.1.28; Shaw, 1967) which catalyzes acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol. The product, 3-acetylchloramphenicol, fails to bind bacterial ribosomes and is devoid of antibacterial activity (Shaw and Unowsky, 1968).

Kinetic characterization of the catalytically efficient enterobacterial type III variant (CAT<sub>III</sub>) suggests that the reaction proceeds via a random order, rapid equilibrium, ternary complex mechanism wherein the N<sup>ε</sup> of the imidazole group of His-195 acts as a general base in the abstraction of a proton from the 3-hydroxyl of chloramphenicol, thereby promoting nucleophilic attack at the acetyl-CoA thioester (Kleanthous and Shaw, 1984; Kleanthous et al., 1985). Serine 148, another conserved residue, is thought to function in transition state stabilization via a hydrogen bond from its γ-hydroxyl to the oxygen of the tetrahedral intermediate in the acetyl transfer reaction (Lewendon et al., 1990).

The structures of the binary complexes of CAT<sub>III</sub> with chloramphenicol and with CoA have been solved at resolutions of 1.75 and 2.4 Å, respectively (Leslie, 1990; Leslie et al., 1988). The enzyme is a trimer with active sites lying at each of the three interfaces between adjacent subunits. The majority of the residues forming the chloramphenicol-binding site are contributed by one subunit, whereas the imidazole of His-195 is provided by the adjacent monomer.

The affinity reagent 3-(bromoacetyl)chloramphenicol, an analog of the product of the CAT reaction, is known to inactivate CAT<sub>II</sub> by chemical modification of the imidazole of His-195 (Kleanthous et al., 1985). Hydrolysis of modified CAT yields 3-carboxymethylhistidine as the sole product, indicating that modification is targeted solely at N<sup>ε</sup> of His-195. Inspection of the structure of the CAT-chloramphenicol binary complex (Leslie, 1980) suggests two factors that might contribute to the apparent tautomeric stabilization of the imidazole of His-195. First, the imidazole ring is involved in face-to-face van der Waals contact with the aryl side chain of tyrosine 25 (Fig. 2), a conserved residue in all CAT variants with the exception of the type I "class," where it is replaced by phenylalanine (Shaw et al., 1979; Charles et al., 1985). Since ring-stacking interactions involving histidyl side chains occur relatively infrequently in protein structures, as compared with energetically more favorable "edge-to-face" contacts (Burley and Petsko, 1986), the conservation of this feature in the active site of CAT might reflect an important mechanistic or structural constraint. Clearly, such an interaction with the side chain of Tyr-25 could orient N<sup>ε</sup> of His-195.
195 with respect to the 3-hydroxyl of the substrate by restricting movement of the imidazole ring and thus account for its unique reactivity with the affinity reagent. A second possible constraint on the position and function of the imidazole of His-195 is that of an internal hydrogen bond between N* and the carbonyl oxygen of the same residue (Fig. 2), an interaction that is unique among proteins within the Brookhaven database and that could determine the apparent tautomeric stabilization. This hydrogen bond results in short (3.1 Å) contacts between the two atoms and is achieved at the expense of energetically unfavorable torsion angles for the His-195 side chain (Leslie, 1990). The strained conformation of the latter may be further stabilized by its interaction with Tyr-25.

In addition to its interaction with His-195, the side chain of Tyr-25 may also contribute to the binding of the acetyl acceptor via a hydrogen bond from its phenolic hydroxyl to the carbonyl oxygen of the dichloroacetamido moiety of chloramphenicol (Figs. 1 and 2), one of only three hydrogen bonds between enzyme and substrate that occur in an otherwise predominantly hydrophobic binding site.

We have assessed the role of the hydrogen bond and ring-stacking interactions of Tyr-25 in the acetyl transfer reaction catalyzed by CAT by making use of site-directed mutagenesis to produce enzymes that contain phenylalanine or alanine at this position. The results of steady state kinetic analyses suggest that the conservation of an aromatic group at residue 25 of CAT arises from a major contribution of hydrophobic effects to substrate binding, with only a minor component due to the hydrogen bond between chloramphenicol and Tyr-25. Furthermore, as tautomeric stabilization of the imidazole ring of His-195 is maintained in Ala-25 CAT, it appears that the principal determinant of the unique reactivity of N* of the catalytic residue of CAT cannot be ring-stacking with Tyr-25 and is likely to be a consequence of the unusual intraresidue hydrogen bond between protonated N* and the carbonyl oxygen of His-195.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis and Expression of CAT*

Oligonucleotide-directed mismatch mutagenesis was performed according to the deoxyuridine selection protocol with the dut ung *Escherichia coli* strain RZ1032 (Kunkel et al., 1987). The presence of the desired nucleotide substitutions and the absence of second site mutations were confirmed by determination of the nucleotide sequence of the DNA spanning the *cat* coding sequence and the adjoining 5'noncoding region. Mutant and wild type *cat* determinants were overexpressed in *E. coli* following transfer to plasmid pUC18 (Murray et al., 1988).

*Purification of Wild Type and Phe-25 CAT*

Purification of wild type and Phe-25 CAT from *E. coli* extracts was carried out using affinity chromatography on chloramphenicol-Sepharose, as previously described (Lewendon et al., 1988).

*Purification of Ala-25 CAT*

Cell-free extracts were prepared by sonication of *E. coli* cells suspended in 50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol (TME buffer) followed by centrifugation.

**FIG. 1.** Acetyl transfer between acetyl-CoA and chloramphenicol catalyzed by CAT. The acetyl group is transferred from acetyl-S-CoA (AcSCoA) to the primary hydroxyl (3-OH) of the substrate, producing CoA (HSCoA) and 3-acetylchloramphenicol, which is unable to bind to prokaryotic ribosomes and is, therefore, devoid of antibiotic activity.

**FIG. 2.** Stereoview of the chloramphenicol-binding site of CAT showing the co-planar stacking of the side chains of Tyr-25 and His-195. Atoms are represented by open circles of increasing size in the order carbon, nitrogen, oxygen, sulfur, and chlorine. Hydrogen bonds are indicated by broken lines. The main chain atoms are represented in the case of His-195 in order to show the intramolecular hydrogen bond between the carbonyl oxygen and N* of the imidazole ring and also the hydrogen bond made by the same carbonyl to the guanidinium side chain of Arg-18. Cm, chloramphenicol.
Ala-25 CAT was purified by ion exchange chromatography and dye-ligand chromatography as follows.

Preparation of Procion Green-Sepharose and Cibacron Blue-Sepharose—Procion green-Sepharose and Cibacron blue-Sepharose were prepared by coupling Procion green HE-4B and Cibacron blue 3GA to Sepharose CL-4B as described by Lowe and Pearson (1984).

Ion Exchange Chromatography—The cell-free extract was loaded onto a column (160 ml) of DEAE-Sepharose equilibrated in TME buffer. After the column was washed with TME buffer (1 liter), CAT was eluted using a linear gradient of NaCl (0-1 M) in TME. Fractions containing CAT activity were pooled and dialyzed against TME (2 liters).

Procion Green-Sepharose Chromatography—The dialyzed pool resulting from the ion exchange chromatography was loaded onto a column (220 ml) of Procion-green-Sepharose equilibrated in TME. After the column was washed with TME (500 ml), CAT activity was eluted using 1 M NaCl in TME. Fractions containing CAT were pooled and dialyzed against TME as previously.

Cibacron Blue-Sepharose Chromatography—The dialyzed pool resulting from the Procion green-Sepharose chromatography was loaded onto a column (220 ml) of Cibacron blue-Sepharose equilibrated in TME, and the column was washed with TME (1 liter). CAT activity was eluted using 2 mM ATP in TME, pH 7.5. Fractions containing CAT were pooled and dialyzed against TME (2 x 2 liters), concentrated by ultrafiltration, and dialyzed against TME (1 liter) prior to storage at -20 °C.

The final purity of each enzyme preparation was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis wherein each sample produced single protein bands of identical relative mobility.

Assay of CAT Activity

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

Forward Reaction—The procedure described by Lewendon et al. (1990) was used to measure rates of acetylation of chloramphenicol. The standard assay contained 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA (TSE buffer), 1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM chloramphenicol, and 0.4 mM acetyl-CoA. The reaction was initiated by addition of enzyme, and the formation of CoA was monitored at 412 nm. Concentrations of acetyl-CoA and chloramphenicol were varied in the standard assay during kinetic analyses.

Kinetic Analysis—Linear initial rates for steady state kinetic analyses were measured in triplicate over times during which less than 15% depletion of substrates occurred. Kinetic parameters were determined from linear slope and intercept replots from manually drawn double-reciprocal plots (Klethous and Shaw, 1984).

Chemical Modification of Ala-25 CAT with 3-(Bromo[14C]acetyl)chloramphenicol

3-(Bromo[14C]acetyl)chloramphenicol was prepared as described by Klethous et al. (1985), with a specific activity of 0.45 mCi mmol⁻¹. Ala-25 CAT (1 mg, 40 nmol) was incubated in TSE buffer, pH 7.5, in the presence of a 3-fold excess of 3-(bromo[14C]acet)-1-chloramphenicol for 30 minutes at room temperature, after which period approximately 20% of the original CAT activity remained. 2-Mercaptoethanol (2.5 µmol) was added to remove excess reagent; the modified protein was collected and subjected to acid hydrolysis. The hydrolysis products were resolved by thin layer electrophoresis as described previously (Klethous et al., 1985). Following staining with fluorescamine, the plate was cut into strips and analyzed by scintillation counting to identify modified amino acid residues.

RESULTS AND DISCUSSION

The role of Tyr-25 in CAT₂₅ was investigated by replacing tyrosine with phenylalanine and alanine using site-directed mutagenesis. In the case of Ala-25 CAT, it was not possible to purify the enzyme by the standard one-step purification by affinity chromatography using chloramphenicol-Sepharose (Lewendon et al., 1988). An alternative purification strategy was devised, one which exploits the affinity of nucleotide-binding enzymes for dye-ligands such as Procion green and Cibacron blue and which was used to purify Ala-25 CAT to apparent homogeneity. The steady state kinetic parameters determined for the acetyl transfer reaction catalyzed by wild type and mutant enzymes are summarized in Table I.

Characterization of Phe-25 CAT—Elimination of the hydrogen bonding potential of Tyr-25 by deletion of its hydroxyl group results in a minor increase in the $k_{cat}$ for chloramphenicol (from 11.6 to 14.6 µM) and a small fall in $k_{cat}$ (599 to 258 s⁻¹). The binding of acetyl-CoA appears to be unaffected by the nature of the side chain at position 25. Calculations using the ratio of specificity constants ($k_{cat}/K_m$) indicate a difference in free energy changes between the reactions catalyzed by Phe-25 CAT and wild type enzyme of ~0.6 kcal mol⁻¹, a value that is on the low side of the range (0.5-1.8 kcal mol⁻¹) of the binding energy contributions expected for a hydrogen bond between an uncharged donor and acceptor (Fersht, 1988).

Therefore, it appears that the hydroxyl of Tyr-25 is not a major determinant of specificity in the reaction catalyzed by CAT₂₅. Consistent with such a conclusion are the observations that the $K_m$ for chloramphenicol of CAT₁ (which contains phenylalanine at residue 25) is almost identical with that of CAT₂₅ and that the reciprocal substitution (Phe-25 → Tyr) does not enhance the catalytic competence of the type I variant.

Characterization of Ala-25 CAT—Elimination of the aromatic moiety of Tyr-25 by the substitution Tyr → Ala yielded an enzyme that failed to bind with high affinity to chloramphenicol-Sepharose. The steady state kinetic parameters observed for Ala-25 CAT confirmed a reduced affinity for chloramphenicol ($K_m$ elevated 15-fold to 173 µM) in concert with a relatively minor decrease in $k_{cat}$ from 599 to 130 s⁻¹. As was observed with Phe-25 CAT, the apparent affinity for acetyl-CoA is unchanged, indicating that the general architecture of the active site is unaffected by the residue substitution. The 69-fold reduction in $k_{cat}/K_m$ for Ala-25 CAT corresponds to a free energy contribution to binding and catalysis for the aromatic moiety of Tyr-25 of 2.5 kcal mol⁻¹.

In addition to the loss of the hydrogen bond to the carbonyl oxygen of chloramphenicol (cf. Phe-25 CAT), the reduction in apparent affinity for chloramphenicol due to loss of the aromatic side chain may simply reflect reduced hydrophobicity of the binding site in Ala-25 CAT. A study of the effectiveness of inhibition of CAT by substrate analogs has shown that chloramphenicol binding is dominated by hydrophobic effects (Cullis et al., 1991). Furthermore, mutations replacing aromatic or aliphatic side chains within the chloramphenicol-binding site with less hydrophobic residues generally result in reduced affinity for the acetyl acceptor (Day, 1990; Murray et al., 1991).

Alternatively, loss of a ring-stacking interaction might permit free rotation of the imidazole ring of His-195 such that formation of the critical hydrogen bond between N⁵ and the 3-hydroxyl of chloramphenicol is impaired. Since an estimate of $AG_{soc}$ for this hydrogen bond is 1.5 kcal mol⁻¹ (and the calculated value of $AG_{soc}$ is 2.7 kcal mol⁻¹) (Cullis et al., 1991), it is apparent that an indirect effect of the deletion of the aromatic group could make a significant contribution to the energetic penalty incurred by Ala-25 CAT.

In an attempt to distinguish between direct effects (hydrophobicity) and indirect consequences (mediated by the imidazole of His-195) of the Tyr-25 → Ala substitution, we have investigated the tautomeric specificity of modification of His-195 by the affinity reagent 3-(bromoacetyl)chloramphenicol. Wild type CAT is modified solely at N⁵ of His-195 (Klethous et al., 1985), and this specificity is common to a number of site-directed mutants of the enzyme with residue substitutions in, ¹ I. A. Murray and A. Lewendon, unpublished experiments.
or around, the chloramphenicol-binding site (Lewendon et al., 1988). However, free rotation of the imidazole of His-195, as a consequence of loss of ring-stacking with Tyr-25, might be expected to render N'2 accessible to the reactive electrophile of the reagent. However, analysis of the acid hydrolysis products of Ala-25 CAT modified with 3-(bromo[U]acetyl)chloramphenicol. Carboxymethylated amino acid standard compounds (25 nmol) were run separately but were represented as if combined within a single lane of the electrophoresis plate. 1, carboxymethylcysteine; 2, 1,3-dicarboxymethylhistidine; 3, 3-carboxymethylhistidine; and 4, 1-carboxymethylhistidine.

FIG. 3. High voltage electrophoresis of acid hydrolysate of Ala-25 CAT modified with 3-(bromo[U]acetyl)chloramphenicol. Carboxymethylated amino acid standard compounds (25 nmol) were run separately but are represented as if combined within a single lane of the electrophoresis plate, 1, carboxymethylcysteine; 2, 1,3-dicarboxymethylhistidine; 3, 3-carboxymethylhistidine; and 4, 1-carboxymethylhistidine.

TABLE I

<table>
<thead>
<tr>
<th>CAT</th>
<th>kcat</th>
<th>Km Chloramphenicol</th>
<th>Acetyl-CoA</th>
<th>kcat/Km CH3</th>
<th>kcat/Km Acetyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type*</td>
<td>5.09</td>
<td>11.6</td>
<td>93.4</td>
<td>5.2 x 10^7</td>
<td></td>
</tr>
<tr>
<td>Tyr-25 → Phe</td>
<td>258</td>
<td>14.6</td>
<td>98.1</td>
<td>1.8 x 10^7</td>
<td></td>
</tr>
<tr>
<td>Tyr-25 → Ala</td>
<td>130</td>
<td>173</td>
<td>101.4</td>
<td>7.5 x 10^6</td>
<td></td>
</tr>
</tbody>
</table>

*Kinetic parameters of wild type CAT are taken from Lewendon et al. (1988).

The carbonyl of His-195 forms an additional interaction with one of the charged guanidinium nitrogens of the conserved residue Arg-18 (Fig. 2), which in turn makes a salt bridge with the carboxylate of conserved Asp-199 (Lewendon et al., 1988). The conservation of this network of interactions within the highly divergent CAT "family" provides further circumstantial evidence that the unique intrasubunit hydrogen bond of His-195 is the principal determinant of the unusual conformation and reactivity of this catalytically essential group.

Acknowledgment—We thank Dr. Andrew Leslie of the Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom, for the provision of Fig. 2.

REFERENCES

Fersht, A. R. (1985) Biochemistry 24, 2077
Kleanthous, C., Shaw, W. V. & Cullis, P. M. (1985) Biochemistry 24, 5307

4 R. M. Crowl, personal communication.