Substitutions in the Active Site of Chloramphenicol Acetyltransferase: Role of a Conserved Aspartate†

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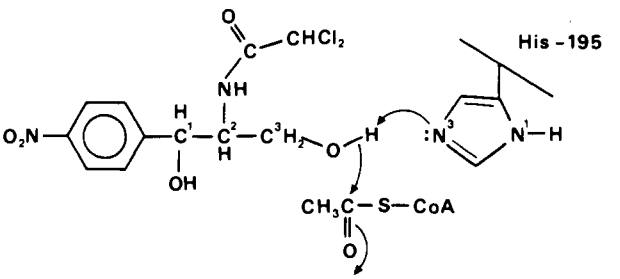
ABSTRACT: The role of conserved Asp-199 in chloramphenicol acetyltransferase (CAT) has been investigated by site-directed mutagenesis. Substitution of Asp-199 by alanine results in a thermolabile mutant enzyme (Ala-199 CAT) with reduced k_{cat} (13-fold) but similar K_m values to wild type CAT. Replacement by asparagine gives rise to a thermostable mutant enzyme (Asn-199 CAT) with much reduced k_{cat} (1500-fold). Furthermore, Asn-199 CAT shows anomalous inactivation kinetics with the affinity reagent 3-(bromoacetyl)chloramphenicol. These results favor a structural role for Asp-199 rather than a catalytic one, in keeping with crystallographic evidence for involvement of Asp-199 in a tight salt bridge with Arg-18. Replacement of Arg-18 by valine results in a mutant enzyme (Val-18 CAT) with similar properties to Ala-199 CAT. The catalytic imidazole of His-195 appears to be conformationally constrained by hydrogen bonding between N^1–H and the carbonyl oxygen of the same residue and by ring stacking with Tyr-25.

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28) catalyzes the O-acetylation of chloramphenicol using acetyl coenzyme A (acetyl-CoA) as acyl donor (Shaw, 1983). Whereas chloramphenicol binds to the 50S subunit of bacterial ribosomes and inhibits peptidyl transferase activity, the acetylated antibiotic is inactive as an inhibitor of protein synthesis (Shaw & Unowsky, 1968). Three plasmid-specified natural variants of CAT (types I–III) have been detected in Gram-negative organisms (Foster & Shaw, 1973; Gaffney et al., 1978). It is likely that all CAT variants are trimeric enzymes of M_r 25 000 (Leslie et al., 1986; Harding et al., 1987).

Recent studies have concentrated on the catalytically efficient type III CAT (CAT_{III}), a natural variant encoded by plasmid R387 and purified from *Escherichia coli*. Steady-state kinetic analysis showed that the reaction proceeds by a ternary complex mechanism wherein the substrates appear to bind independently (Kleanthous & Shaw, 1984). An active site directed inhibitor of CAT, 3-(bromoacetyl)chloramphenicol, inactivates CAT_{III} by stoichiometric modification of a unique histidine residue (His-195[2]). This residue is alkylated solely at the N^3-position of the imidazole ring, a result that suggests the presence of a preferred or uniquely accessible tautomeric form of the His-195 imidazole group within the active site of CAT (Kleanthous et al., 1985).

It has been proposed that His-195 of CAT functions as a general base during catalysis (Kleanthous et al., 1985), the N^3 atom of the imidazole ring abstracting a proton from the primary hydroxyl of chloramphenicol (or from water in the absence of chloramphenicol) thereby promoting attack at the thioester of acetyl-CoA (see Scheme I). Such a mechanism would result in the formation of a tetrahedral intermediate, which would collapse to yield the 3-acetoxy ester of chloramphenicol and coenzyme A (acetate and coenzyme A in the hydrolytic reaction).

Scheme I



Kleanthous et al. (1985) proposed that a hydrogen bond between a neighboring acidic residue and the N^1–H of the active site histidine might assist general-base catalysis and account for the stabilization of a preferred tautomeric form of this residue, a proposal based upon an attractive analogy with the mechanism of serine proteases. Hydrogen bonding between catalytic histidine residues and aspartate or glutamate carboxyl groups has been demonstrated by X-ray crystallography of chymotrypsin (Blow et al., 1969), phospholipase A_2 (Verheij et al., 1980), glutathione reductase (Pai & Schulz, 1983), and deoxyribonuclease I (Suck & Oefner, 1986). Modification of the catalytic histidine residues in chymotrypsin and phospholipase A_2 by methyl 4-nitrobenzenesulfonate resulted in specific methylation of the N^3- and N^1-positions, respectively (Nakagawa & Bender, 1970; Verheij et al., 1980). In contrast, modification of a catalytic histidine residue in D-amino acid oxidase by the same reagent resulted in both N^1- and N^3-methylhistidine, a result that suggests the absence of

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

[2] Alignment of the amino acid sequences of seven CAT variants has resulted in a general numbering system, which is used here. Arg-18, Tyr-25, Cys-31, His-195, and Asp-199 are residues 13, 20, 26, 189, and 193, respectively, in the primary sequence of type III CAT (Murray et al., 1988).

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tautomeric stabilization (Swenson et al., 1984).

The hypothesis that a glutamyl or aspartyl carboxylate is hydrogen bonded to N^1–H of His-195 in CAT predicts that removal of the charged side chain by mutagenesis should reduce the basicity of the N^3 atom of His-195. Removal of the carboxylate may also result in loss of the preferred tautomeric form of His-195, manifesting itself in the production of both N^1- and N^3-modified histidine after inactivation of the mutant enzyme with the affinity reagent 3-(bromoacetyl)-chloramphenicol.

In the absence of a three-dimensional structure for CAT_{III}, acidic amino acid residues were selected for mutation on the basis of their absolute conservation in the primary sequences of all seven CAT variants then available [Alton and Vapnek (1979); Shaw et al. (1979); Horinouchi and Weisblum (1982); Harwood et al. (1983); Charles et al. (1985); Shaw et al. (1985); Murray et al. (1988); I. A. Murray, J. A. Gil, D. A. Hopwood, and W. V. Shaw, unpublished results]. Asp-40 and Asp-199 are the only absolutely conserved acidic residues, and each was replaced by asparagine. The characterization of the resultant mutant enzymes is described as are studies of additional CAT mutants designed to further an understanding of the roles of the conserved aspartyl residues.

Experimental Procedures

Materials

3-(Bromoacetyl)chloramphenicol and 3-(bromo[^{14}C]-acetyl)chloramphenicol were prepared as described by Kleanthous et al. (1985). Acetyl-CoA was prepared by the method of Simon and Shemin (1953) and purified as described by Kleanthous and Shaw (1984).

Methods

Site-Directed Mutagenesis and Expression of CAT. Oligonucleotide-directed mismatch mutagenesis was performed by the "dual-primer" method of Zoller and Smith (1984). Oligonucleotides were synthesized by means of solid-phase phosphotriester chemistry on 3MM paper supports (Brenner & Shaw, 1985). Mutant *cat* determinants were overexpressed in *E. coli* following transfer to plasmid pUC18 (Murray et al., 1988). Yields varied between 300 and 500 mg of CAT per liter of stationary-phase cultures of *E. coli* harboring the appropriate plasmids.

Purification of CAT. Purification of wild type CAT from *E. coli* extracts was carried out by a two-step procedure (Packman & Shaw, 1981). *E. coli* cells were broken by sonication, and cell debris was removed by centrifugation. The resulting extracts were heated at 65 °C for 10 min, and precipitated protein was removed by a second centrifugation. This heat step was omitted during purification of mutant enzymes when *E. coli* extracts were taken directly to the next step in the purification procedure, affinity chromatography on chloramphenicol–Sepharose. Identical conditions were employed for affinity chromatography of wild type and mutant enzymes, namely, elution by 5 mM chloramphenicol in the presence of 0.3 M NaCl (Zaidenzaig & Shaw, 1976). The purity of each enzyme preparation was assessed by SDS–polyacrylamide gel electrophoresis wherein all CAT mutants produced single bands identical in mobility with that of wild type CAT.

Protein estimations were made by the method of Lowry et al. (1951), using bovine serum albumin as standard, and were directly related to the true CAT concentration (derived from amino acid analysis of reference samples) by a correction factor.

Assay of CAT Activity. CAT activity was assayed spectrophotometrically at 25 °C (Shaw, 1975). The standard assay mixture contained TSE buffer, pH 7.5, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mM acetyl-CoA, and 0.1 mM chloramphenicol. The reaction was initiated by addition of enzyme and was monitored at 412 nm. The measurement of initial velocities for steady-state kinetic analysis was as described by Kleanthous and Shaw (1984). Kinetic parameters were calculated from linear intercept and slope replots from manually drawn double-reciprocal plots.

Although the CAT mutants studied were observed to be more susceptible to inhibition by DTNB than the wild type enzyme, the concentrations of substrates in the assay were sufficient to prevent significant inactivation of the mutant enzymes. Linear initial rates were observed over times during which less than 20% depletion of substrates occurred.

Chemical Modification of CAT with 3-(Bromo[^{14}C]-acetyl)chloramphenicol. Wild type CAT, Asn-199 CAT, and Ala-199 CAT (1 mg, 40 nmol) were incubated in TSE buffer with a 2- to 5-fold excess of 3-(bromo[^{14}C]acetyl)chloramphenicol at room temperature for up to 1 h. When residual CAT activity had fallen to less than 5%, modified CAT was collected and subjected to acid hydrolysis and thin-layer electrophoresis as described by Kleanthous et al. (1985). After fluorescamine staining, the plate was cut into strips, which were counted for radioactivity.

Inactivation of CAT by Cysteine- and Histidine-Modifying Reagents. The time courses of chemical modification were measured at 25 °C in TSE buffer at pH 7.5. At appropriate time intervals, aliquots were withdrawn, diluted in TSE buffer if necessary, and assayed for CAT activity. 3-(Bromoacetyl)chloramphenicol, methyl 4-nitrobenzenesulfonate, and 4,4′-dithiodipyridine were added to incubations in acetonitrile, the final concentration of which was 0.95 M. Although without effect on the activity of wild type, Ala-199 CAT, or Asn-199 CAT, the activity of Val-18 CAT was increased ~2-fold by the addition of acetonitrile at this concentration.

(*i*) *3-(Bromoacetyl)chloramphenicol.* The final concentrations of CAT mutants in the incubations were Ala-199 CAT, 1 μM, Asn-199 CAT, 27 μM, and Val-18 CAT, 0.87 μM. The concentration of 3-(bromoacetyl)chloramphenicol in each case was such that an excess (2- to 300-fold) of reagent over enzyme monomers was present in each incubation.

(*ii*) *Methyl 4-Nitrobenzenesulfonate.* The concentration of Asn-199 CAT was 20 μM, and methyl 4-nitrobenzenesulfonate concentrations varied between 0.5 and 4.0 mM.

(*iii*) *5,5′-Dithiobis(2-nitrobenzoic acid).* The concentrations of enzymes were wild type CAT, 17.2 μM, Asn-199 CAT, 36.8 μM, Ala-199 CAT, 2.3 μM, and Val-18 CAT, 1.0 μM. DTNB was added to incubations in TSE buffer, pH 7.5, to give a final concentration of 0.9 mM.

(*iv*) *4,4′-Dithiodipyridine.* The concentrations of enzymes were wild type CAT and Asn-199 CAT, 20 μM, Ala-199 CAT, 22.4 μM, and Val-18 CAT, 1.0 μM. 4,4′-Dithiodipyridine was added to each incubation to give a final concentration of 0.2 mM.

Analysis of the Thermal Stability of CAT. Wild type and mutant enzymes (4.0 μM) in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, and 0.2 mM chloramphenicol were incubated in a water bath at 70 °C. At appropriate time intervals, samples were removed, diluted if necessary, and assayed for CAT activity.

Results and Discussion

Characterization of Asn-199 CAT. The conserved acidic amino acid residues Asp-40 and Asp-199 were candidates for the role of general base and for stabilization of the preferred tautomer of His-195. These residues were initially replaced

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

CAT	k_{cat} (s^{-1})	K_m (μM) chloramphenicol	K_m (μM) acetyl-CoA
wild type	599	11.6	93
Asp-40 → Asn	714	10.6	76
Asp-199 → Asn	0.28	46.5	314
Asp-199 → Ala	45	14.5	83
Arg-18 → Val	68	16.2	101

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

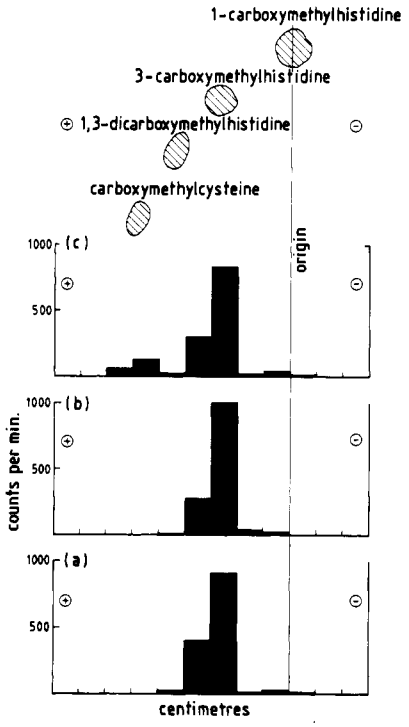

FIGURE 1: High-voltage electrophoresis of acid hydrolysates of chloramphenicol acetyltransferases modified with 3-(bromo[^{14}C]-acetyl)chloramphenicol: (a) wild type, (b) Asn-199 CAT, and (c) Ala-199 CAT. A sample (10 nmol) of each hydrolysate and carboxymethylated amino acid standards (25 nmol) were subjected to electrophoresis at pH 6.5 on a silica plate. The plate was stained with fluorescamine, the tracks were cut into strips, and radioactivity was located by liquid scintillation counting of the strips.

by asparagine to remove charge without greatly changing the size of the side chain. The steady-state kinetic properties of the wild type and mutant enzymes are summarized in Table I. Since the Asp-40 → Asn substitution results in an enzyme (Asn-40 CAT) with very similar kinetic properties to wild type CAT, this mutant was not studied further. In contrast, the Asp-199 → Asn mutation produced an enzyme (Asn-199 CAT) with greatly decreased catalytic activity due mainly to a ca. 1500-fold decrease in k_{cat} and to small increases in K_m values for both substrates. Such a striking decrease in k_{cat} seemed in keeping with the notion that Asp-199 acts in concert with His-195 to increase the nucleophilicity of the primary hydroxyl group of chloramphenicol. Studies by Bender and co-workers have suggested surprisingly large rate accelerations on introducing a carboxylate to assist imidazole in a model of the chymotrypsin reaction (D'Souza et al., 1985; Bender, 1987).

To test whether Asn-199 CAT had lost some of its preference for N^3-alkylation of His-195, wild type and Asn-199 CAT were each modified with 3-(bromo[^{14}C]acetyl)chloramphenicol and the resulting labeled amino acids were identified by thin-layer electrophoresis after acid hydrolysis of the

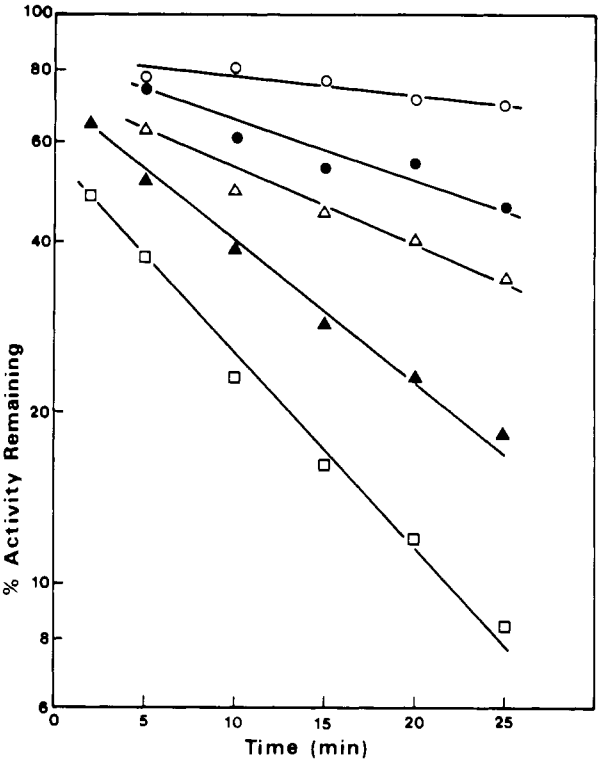

FIGURE 2: Kinetics of inactivation of Asn-199 CAT by 3-(bromoacetyl)chloramphenicol at the following concentrations: (○) 27, (●) 67, (△) 134, (▲) 268, and (□) 536 μM. Conditions were as described under Experimental Procedures. Initial CAT activity in all incubations was 100% before addition of 3-(bromoacetyl)chloramphenicol in acetonitrile. Addition of acetonitrile alone did not result in a decrease in CAT activity over the incubation period (not shown).

protein, a procedure that converts the modified amino acids to their ^{14}C-carboxymethylated derivatives (Kleanthous et al., 1985). In both cases, ^{14}C label was detected solely as 3-(carboxymethyl)histidine, and no radioactivity corresponding to 1-(carboxymethyl)histidine could be detected (Figure 1). Although this result rules out a His-195/Asp-199 interaction as the cause of the selective modification of N^3 of His-195, it does not rule out the possibility that Asp-199 assists His-195 in its role as general base.

In an attempt to assess the intrinsic reactivity of His-195 in the mutant protein, the kinetics of inactivation by the affinity reagent were examined. The inactivation of Asn-199 CAT by 3-(bromoacetyl)chloramphenicol showed biphasic kinetics (Figure 2). The initial inactivation occurred at a rate too fast to measure by manual means but was complete within 30 s, after which a considerably slower (0.002 s^{-1}) pseudo-first-order phase could be detected.

Methyl 4-nitrobenzenesulfonate also inactivates CAT_{III} by stoichiometric modification of the N^3-position of a histidyl residue (Corney, 1983). The modified residue has not been identified but is probably His-195, given the structural similarity between methyl 4-nitrobenzenesulfonate and chloramphenicol and that the former acts as an affinity reagent. Modification of Asn-199 CAT by methyl 4-nitrobenzenesulfonate also showed biphasic kinetics of inactivation (data not shown). In contrast, inactivation of Asn-199 CAT by the thiol-specific reagents DTNB and 4,4′-dithiodipyridine showed no anomalies and appeared to follow pseudo-first-order kinetics (see below).

The above results could be accommodated by assuming alternative geometries of the active site around the catalytic imidazole of Asn-199 CAT arising from the novel hydrogen-bonding interactions available to Asn-199. In contrast to

Asp-199 in wild type CAT, Asn-199 can serve as both a hydrogen bond donor and acceptor. During the course of this study a similar anomaly has been noted with the Asp-102 → Asn mutation of the Ser/His/Asp catalytic triad of trypsin. In the latter case, X-ray crystallography has demonstrated that the normal hydrogen-bonded network is reversed in the mutant Asn-102 trypsin (Craik et al., 1987; Sprang et al., 1987).

Characterization of Ala-199 CAT. Ala-199 CAT was constructed to address the problems associated with Asn-199 CAT. The steady-state kinetic parameters of Ala-199 CAT are summarized in Table I. The Asp-199 → Ala mutation leads to a 13-fold reduction in k_{cat} with K_m values for both substrates that are not significantly different from those of wild type CAT. The fact that substitution of an amide group for the carboxylate at position 199 (Asn-199 CAT) results in a far greater decrease in catalytic activity than does the removal of the carboxylate (Ala-199 CAT) provides the most direct evidence for the introduction of novel and deleterious interactions at the active site of Asn-199 CAT.

Inactivation of Ala-199 CAT by 3-(bromoacetyl)chloramphenicol followed pseudo-first-order kinetics with a rate constant of 0.01 s^{-1} and resulted in 3-(carboxymethyl)histidine with no detectable 1-(carboxymethyl)histidine (Figure 1). The extreme rapidity of inactivation of wild type CAT by 3-(bromoacetyl)chloramphenicol precluded the measurement of a pseudo-first-order rate constant. Consequently, Kleanthous et al. (1985) reported an apparent bimolecular rate constant of 30 × 10^3 M^{-1} s^{-1}. Based upon an estimated apparent biomolecular rate constant [from plots of apparent rate constant vs [3-(bromoacetyl)chloramphenicol] at low concentrations of inhibitor], it appears that the rate of inactivation of Ala-199 CAT is reduced ca. 200- to 400-fold compared to wild type CAT. The decrease in reactivity toward 3-(bromoacetyl)chloramphenicol is considerably greater than the 13-fold decrease in k_{cat} and seems unlikely to arise solely from decreased nucleophilicity of His-195. It was not possible to determine the nucleophilicity of His-195 in wild type and mutant enzymes with nonspecific reagents (e.g., iodoacetamide), since these are known to inactivate CAT by modification of cysteine in addition to the catalytic imidazole (Corney, 1983). In the case of Ala-199 CAT, inactivation via alkylation of cysteine is detectable (at low levels) in the case of the affinity reagent. Modification by 3-(bromo[^{14}C]-acetyl)chloramphenicol results in limited incorporation of ^{14}C label into *S*-(carboxymethyl)cysteine (~15% of total incorporated). The above result is compatible with greater movement of bound ligand and/or active site residues in Ala-199 CAT, allowing the affinity reagent access to a cysteine residue (probably Cys-31, see below) that is less accessible in wild type CAT. Such increased flexibility at the catalytic center might itself account for the observed reduction in k_{cat}.

Wild type CAT_{III} is generally resistant to inactivation by negatively charged thiol-modifying reagents, e.g., DTNB, but is sensitive to uncharged compounds, such as 4,4′-dithiodipyridine (Zaidenzaig et al., 1979; Corney, 1983). Inactivation occurs via modification of a thiol adjacent to the active site, probably that of Cys-31, as substitution of this residue by serine eliminates susceptibility to inactivation by 4,4′-dithiodipyridine (A. Lewendon, unpublished results). On circumstantial grounds it was proposed that one or more acidic residues located close to the active site might account for the discrimination against anionic reagents (Zaidenzaig & Shaw, 1978; Corney, 1983). The detection of a modest degree of alkylation of cysteine by 3-(bromoacetyl)chloramphenicol prompted the study of the inactivation of the mutant enzymes by thiol-specific reagents. Both Ala-199 CAT and Asn-199 CAT showed enhanced sensitivity to inactivation by DTNB and, to a lesser extent, by 4,4′-dithiodipyridine (Table II). Although an electrostatic argument for the increased rate of inactivation by DTNB appears attractive, it may be a simplistic one, since an additional mutant CAT (Arg-18 → Val) also shows a similarly increased sensitivity to DTNB and, in this case, the negative charge at Asp-199 remains (see below).

Table II: Inhibition of Chloramphenicol Acetyltransferases by Thiol-Modifying Reagents[a]

CAT	$t_{1/2}$ (min) for inactivation by 0.9 mM DTNB	$t_{1/2}$ (min) for inactivation by 0.2 mM 4,4′-dithiodipyridine
wild type	>400	28
Asp-199 → Asn	18	2.6
Asp-199 → Ala	3.5	2.2
Arg-18 → Val	2.4	1.2

[a] Half-times ($t_{1/2}$) for inactivation by DTNB and 4,4′-dithiodipyridine were determined as described under Experimental Procedures.

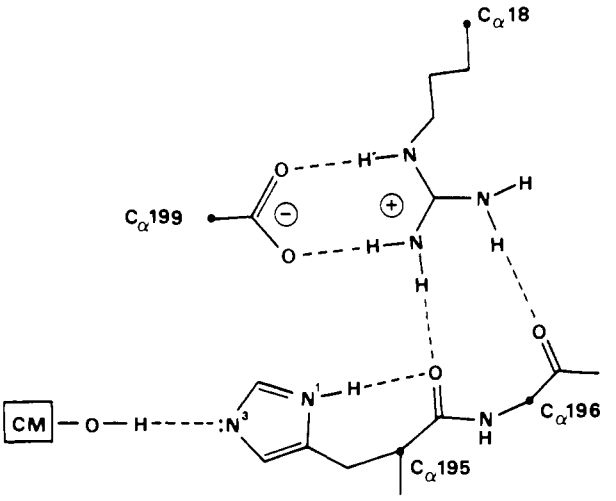

FIGURE 3: Hydrogen-bonding interactions of Arg-18, His-195, and Asp-199. This represents a part of the network of hydrogen bonds around the active site of CAT. Only the primary hydroxyl of chloramphenicol (CM) is shown.

Role of Asp-199. The three-dimensional structure of CAT_{III} at 0.175-nm resolution has been determined by X-ray crystallography during the course of this work, and the binding sites for chloramphenicol and CoA have been identified (Leslie et al., 1988). Examination of the structure reveals that in the crystal the side chain of Asp-199 is *not* hydrogen bonded to His-195. Instead, Asp-199 is involved in a salt bridge with the side chain of Arg-18. Like Asp-199, Arg-18 is absolutely conserved in all known CAT amino acid sequences. Thus, Asp-199 does not appear to have a direct role in either substrate binding or catalysis. The Asp-199/Arg-18 salt bridge forms an integral part of an extensive network of possible hydrogen bonds that maintains the geometry of the active site of CAT_{III} (Figure 3). Clearly, Ala-199 cannot form an ion pair with Arg-18, and this in itself, associated with changes in the preferred geometry of the catalytic center, may be sufficient to account for the 13-fold decrease in k_{cat}. To test the above hypothesis, a complementary mutation (Arg-18 → Val) was constructed giving the enzyme Val-18 CAT. The kinetic properties of Val-18 CAT are very similar to those of Ala-199 CAT in that k_{cat} is reduced 9-fold compared to wild type CAT with no significant changes in K_m values (Table I). Furthermore, the rate constant for inactivation by 3-(bro-

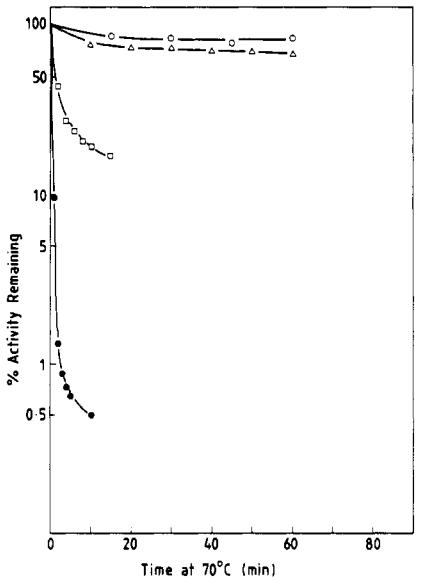

FIGURE 4: Time courses of thermal inactivation of chloramphenicol acetyltransferases: (○) wild type, (△) Asn-199 CAT, (□) Ala-199 CAT, and (●) Val-18 CAT. Conditions were as described under Experimental Procedures.

moacetyl)chloramphenicol (0.01 s^{-1}) and the increased sensitivity to thiol-modifying reagents (Table II) were similar to those noted for Ala-199 CAT. Collectively, the results provide strong support for the argument that the reduced k_{cat} of Ala-199 CAT arises from indirect effects on the geometry of the active site rather than via modification of the basicity of His-195.

The tertiary structure deduced from crystallographic studies has revealed that the tautomeric preference shown by the His-195 imidazole may be the result of stabilization by a hydrogen bond between N^1–H of the imidazole ring and the carbonyl oxygen of the same residue. There may also be a contribution from restriction in rotation (C_β–C_γ) of the imidazole ring associated with its coplanar association with Tyr-25. The conformation of the imidazole of His-195 in wild type CAT appears to be somewhat atypical of histidine residues in general (Leslie et al., 1988). Thus, the conformation of the catalytic imidazole may be particularly susceptible to local reorganization of the active site resulting from the substitution of neighboring side chains. Such a proposal seems particularly likely in the case of CAT where the active sites lie at the interface between subunits of the trimer such that the principal substrate binding contacts are comprised of residues from one subunit whereas the catalytic histidine is contributed by the opposing subunit.

Thermal Stability of the Mutant CAT Proteins. Both Ala-199 CAT and Val-18 CAT are strikingly sensitive to thermal inactivation. Whereas wild type CAT is stable to prolonged incubation at 70 °C, Ala-199 CAT and Val-18 CAT are rapidly and irreversibly inactivated at this temperature (Figure 4). Evidently, the absence of the Asp-199/Arg-18 salt bridge in Ala-199 CAT and Val-18 CAT results in decreased stability. Asn-199 CAT is comparatively thermostable, suggesting the presence of novel interactions that must compensate for the lack of the salt bridge. Moreover, the presence of the buried charges of unpaired Asp-199 or Arg-18 might be expected to destabilize the folded structures of the mutant enzymes.

Conclusions

Site-directed mutagenesis studies have ruled out a direct role for Asp-199 in catalysis. The observation of greatly increased thermolability on replacement of Asp-199 by Ala does, however, support a structural role for Asp-199 in CAT in solution consistent with the Asp-199/Arg-18 salt bridge observed in the protein crystal structure. The tautomeric stabilization of His-195 probably arises from two different effects: hydrogen bonding of the N^1–H to the carbonyl oxygen of the same residue and stacking of the imidazole ring of His-195 with the phenolic ring of Tyr-25. Finally, the strikingly large reduction in k_{cat} for Asn-199 CAT, as compared with the modest decrease for Ala-199 CAT, suggests that the amide of Asn-199 has introduced novel interactions with neighboring groups that are deleterious to catalysis. Evidently, asparagine may not represent a conservative substitution for aspartate, and the interpretation of data arising from mutant proteins containing such substitutions should be viewed with caution.

Acknowledgments

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Registry No. CAT, 9040-07-7; Asp, 56-84-8; Asn, 70-47-3; Ala, 56-41-7; Arg, 74-79-3; Val, 72-18-4; acetyl-CoA, 72-89-9; chloramphenicol, 56-75-7; 3-(bromoacetyl)chloramphenicol, 95610-68-7.

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Assignment of Histidine Resonances in the ^{1}H NMR (500 MHz) Spectrum of Subtilisin BPN′ Using Site-Directed Mutagenesis†

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ABSTRACT: A spin-echo pulse sequence has been used to resolve the six histidine C-2H protons in the 500-MHz NMR spectrum of subtilisin BPN′. Five of these residues have been substituted by site-directed mutagenesis, and this has enabled a complete assignment of these protons to be obtained. Analysis of the pH titration curves of these signals has provided microscopic pK_a's for the six histidines in this enzyme. The pK_a's of the histidine residues in subtilisin BPN′ have been compared with the values obtained for the histidines in the homologous enzyme from *Bacillus licheniformis* (subtilisin Carlsberg). Four of the five conserved histidines titrate with essentially identical pK_a's in the two enzymes. It therefore appears that the assignments made for these residues in subtilisin BPN′ can be transferred to subtilisin Carlsberg. On the basis of these assignments, the one histidine that titrates with a substantially different pK_a in the two enzymes can be assigned to histidine-238. This difference in pK_a has been attributed to a Trp to Lys substitution at position 241 in subtilisin Carlsberg.

Nuclear magnetic resonance spectroscopy is a powerful method for studying protein structure and function in solution (Jardetzky & Roberts, 1981). Interpretation of NMR data is, however, hindered in many cases by a lack of sequence-specific resonance assignments. For small proteins of less than 150 amino acids the introduction of methods based on 2-D NMR has largely overcome this problem (Wüthrich, 1986). Unfortunately, the application of these methods to larger proteins is limited by rapid spin–spin relaxation and broad resonances.

One promising new approach to the problem of resonance assignment in NMR spectroscopy is the use of site-directed mutagenesis (Prigodich et al., 1986; Clore et al., 1987). Naturally occurring protein variants have been used for resonance assignments for many years [reviewed in Jardetzky and Roberts (1981)], but this technique has been limited by the availability of suitable proteins. The advances in recombinant DNA techniques now allow the introduction of any desired mutation into proteins for which the cloned gene is available (Zoller & Smith, 1983). Provided that the mutation does not produce a large conformational change, the spectra of the mutant and wild-type proteins should be essentially identical, except for the absence of the peak of interest, making assignment straightforward.

We report the use of site-directed mutagenesis in the assignment of the histidine C-2H protons in the 500-MHz NMR spectrum of subtilisin BPN′, a bacterial serine protease containing 275 amino acids (Markland et al., 1971). This protein is currently being used as a model system in which to study electrostatic effects in proteins by protein engineering (Thomas et al., 1985; Russell et al., 1987; Russell & Fersht, 1987; Sternberg et al., 1987). In these studies, the effect of mutations on the kinetically determined pK_a of the active site histidine is being investigated. NMR can be used to determine the microscopic pK_a's of histidine residues in proteins by following the pH-dependent chemical shifts of the C-2H protons (Markley, 1975). By use of the assignments described here, the pK_a's of all the histidines in subtilisin BPN′ can be determined. This will enable the effects of mutations on the pK_a's of any of the histidines in this protein to be investigated.

Materials and Methods

Reagents were obtained from Sigma (London), Aldrich, Amersham International, and New England Nuclear. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

Mutagenesis and Protein Purification. The mutations His → Gln-17, His → Gln-39, His → Gln-67, His → Gln-226, and His → Gln-238 were constructed by site-directed mutagenesis of the subtilisin BPN′ gene cloned into M13mp9, with the primers 5′CTTGAGAT*TGCAGAGC3′, 5′AATCAGT*-

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