The pK_a of the catalytic histidine residue of chloramphenicol acetyltransferase

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A catalytically essential histidine residue (His-195) of chloramphenicol acetyltransferase (CAT) acts as a general base in catalysis, abstracting a proton from the primary hydroxy group of chloramphenicol. The pK_a of His-195 has been determined from the pH-dependence of chemical modification. Both methyl 4-nitrobenzenesulphonate and iodoacetamide inactivate CAT by irreversible modification of His-195. The kinetics of inactivation by methyl 4-nitrobenzenesulphonate are pseudo-first-order, and the pH-dependence of inactivation yields a pK_a value of 6.60. Iodoacetamide inactivation proceeds with second-order kin-

etics and a pK_a value of 6.80. An alternative site of modification at the active site of CAT is the thiol group of Cys-31, a residue which has no catalytic role. On replacement of Cys-31 with alanine (Ala-31 CAT), the pH-dependence of iodoacetamide inactivation gives a pK_a value of 6.66. The pK_a values derived from chemical-modification experiments directed at His-195 are in agreement with the pK_a values of 6.62 and 6.61 determined for wild-type and Ala-31 CAT respectively from the pH-dependence of k_{cat}/K_m .

INTRODUCTION

Bacterial resistance to chloramphenicol is commonly associated with the presence of chloramphenicol acetyltransferase (CAT; EC 2.3.1.28). CAT inactivates the antibiotic by O-acetylation using acetyl-CoA as acetyl donor. Once acetylated, chloramphenicol is inactive as an inhibitor of protein synthesis (reviewed by Shaw, 1983; Shaw and Leslie, 1991). CAT is a trimeric enzyme with identical subunits of M_r 25000 (Leslie et al., 1986; Harding et al., 1987). The amino acid sequences of twelve CAT variants show a significant similarity (Shaw and Leslie, 1991).

Of the many variants of CAT which have been described, only the structure of the type III variant (CAT_{III}) is known (Leslie et al., 1988; Leslie, 1990). The structures of the binary complexes of CAT_{III} with chloramphenicol and CoA have been determined at 0.175 nm and 0.24 nm resolution respectively. There are three active sites per trimer, located at the intersubunit interfaces. The chloramphenicol- and CoA-binding sites together form a tunnel (0.25 nm long) through the protein, and the substrates approach the active site from opposite ends of this tunnel.

In the CAT-chloramphenicol binary complex the primary (C-3) hydroxy group of chloramphenicol, which is the site of acetylation, is hydrogen-bonded to N-3 of His-195. The latter is absolutely conserved in all of the amino acid sequences of CAT variants and is subject to stoichiometric modification (at N-3) by the active-site-directed inhibitor 3-(bromoacetyl)chloramphenicol (Kleanthous et al., 1985). The latter observation suggested the presence of a preferred tautomeric form of His-195 in the active site of CAT_{III}, probably arising from an unusual hydrogen-bonding interaction between N-1 of His-195 and the backbone carbonyl group of the same residue (Leslie, 1990; Murray et al., 1991).

Kleanthous et al. (1985) proposed that N-3 of His-195 acted as a general base during catalysis, abstracting a proton from the primary hydroxy group of chloramphenicol, thereby facilitating nucleophilic attack on the thioester of acetyl-CoA. CAT is

believed to follow the mechanism shown in Scheme 1, which proceeds via a tetrahedral intermediate. Modelling studies (P. C. E. Moody and A. G. W. Leslie, unpublished work) and analysis by site-directed mutagenesis (Lewendon et al., 1990) have supported the existence of such an intermediate.

The pK_a of the catalytically important imidazole N-3 of His-195 was determined from the pH-dependence of chemical modification of CAT_{III} by two different reagents, methyl 4-nitrobenzenesulphonate and iodoacetamide. The use of 3-(bromoacetyl)chloramphenicol is precluded by virtue of its extremely rapid inactivation of CAT_{III} at pH 7.5 ($t_{1/2} = 2$ min at 240 nM reagent), such that saturation of the rate of inactivation cannot be observed (Kleanthous et al., 1985). Methyl 4-nitrobenzenesulphonate inactivates a number of enzymes by methylation of catalytically essential histidine residues, e.g. chymotrypsin (Nakagawa and Bender, 1970), phospholipase A₂ (Verheij et al., 1980) and D-amino acid oxidase (Swenson et al., 1984). Corney (1983) showed that methyl 4-nitrobenzenesulphonate is an active-site-directed inhibitor of CAT_{III} and that inactivation occurs by stoichiometric modification of the N-3 position of a single histidine residue. This type of modification is strongly reminiscent of the inhibition of CAT_{III} by 3-(bromoacetyl)chloramphenicol, where the sole site of modification is the N-3 atom of His-195 (Kleanthous et al., 1985). Given the strong structural similarity of methyl 4-nitrobenzenesulphonate to the substrate chloramphenicol (Figure 1), it is extremely likely that the modified residue is His-195. Consequently, the pH-dependence of inactivation of CAT_{III} by this reagent has been examined. The second reagent used was iodoacetamide, which inactivates CAT_{III} by a second-order process, in contrast with methyl 4-nitrobenzenesulphonate, where inactivation is pseudo-first-order in reagent. Modification of CAT_{III} by iodo-[¹⁴C]acetamide, followed by isolation and sequencing of labelled peptides, showed that only two residues were modified, His-195 and Cys-31 (Corney, 1983). Cys-31 is a partially conserved residue among CAT variants and it is located in the chloramphenicol-binding site such that its sulphur atom is 0.52 nm

Scheme 1 Proposed mechanism of CAT

Abbreviation used: CM, chloramphenicol.

Figure 1 Structures of chloramphenicol (a) and methyl 4-nitrobenzenesulphonate (b)

from N-3 of His-195 (Leslie, 1990). The pH-dependence of iodoacetamide inactivation was examined both in wild-type CAT_{III} and in a mutant enzyme where Cys-31 has been replaced by alanine (Ala-31 CAT; Lewendon and Shaw, 1990). The pK_a values obtained from chemical-modification studies agree well with the values determined from the pH-dependence of $k_{cat.}/K_m$ for wild-type and Ala-31 CAT.

EXPERIMENTAL

Materials

Iodoacetamide was obtained from Sigma Chemical Co., and methyl 4-nitrobenzenesulphonate was from Aldrich Chemical Co. Acetyl-CoA was prepared as described by Simon and Shemin (1953). [3H]Acetyl-CoA was obtained from du Pont (U.K.) and was diluted with unlabelled acetyl-CoA prior to use.

Purification of CAT

Purification of wild-type and Ala-31 CAT from *Escherichia coli* extracts was carried out by affinity chromatography on chloramphenicol-Sepharose (Lewendon et al., 1988). The purity of enzyme preparations was assessed by SDS/PAGE, wherein both enzymes produced single bands of identical mobility.

The concentration of purified CAT_{III} was calculated from $\epsilon_{280}^{1\%}$ of 13.1 or by the method of Lowry et al. (1951), using wild-type CAT_{III} as standard.

Assay of CAT activity

CAT activity was assayed spectrophotometrically at 25 °C (Shaw, 1975). The standard assay mixture contained 50 mM Tris/HCl buffer, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 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 was monitored at 412 nm.

In order to examine the pH-dependence of $k_{cat.}/K_m$, CAT activity at low chloramphenicol concentrations was measured by a radiometric method at 25 °C 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 specified pH ranges: Mes/NaOH, pH 5.1–6.7; Mops/NaOH, pH 6.7–7.5; Hepes/NaOH, pH 7.5–8.3. The value of $k_{cat.}/K_m$ was determined in both buffers at pH values where the buffer ranges overlap. The assay mixture contained 1 mM [3H]acetyl-CoA (0.023 MBq), 5 μ M chloramphenicol and the appropriate buffer in a final volume of 600 μ l. Assays were initiated by the addition of CAT. Aliquots (100 μ l) were removed at intervals and extracted with 600 μ l of ethyl acetate, which selectively removes both chloramphenicol and acetylchloramphenicol from the aqueous phase. The mixture was centrifuged for 1 min, then 400 μ l of the ethyl acetate phase was removed for liquid-scintillation counting. Control incubations were performed concurrently at each pH value in the absence of CAT in order to correct for any blank rate.

Chemical modification of CAT

Time courses of chemical modification were monitored at 25 °C in the buffers described above, and, in addition, Ches/NaOH buffers were used over the pH range 8.3–9.5. CAT_{III} was dialysed extensively against the appropriate buffer before chemical modification.

Methyl 4-nitrobenzenesulphonate

Inactivation of CAT_{III} by methyl 4-nitrobenzenesulphonate was examined over the pH ranges 5.1–7.9. At each pH, the rate of inactivation ($k_{obs.}$) was determined at five different reagent concentrations. The final concentration of CAT_{III} in the incubations was 0.48 mg/ml (19.2 μ M monomers). Methyl 4-nitrobenzenesulphonate concentrations were varied over the range 0.5–4.0 mM. The limited solubility of the reagent (Nakagawa and Bender, 1970) precluded the use of concentrations greater than 4 mM. The reagent was added to incubations in acetonitrile, the final concentration of which was 0.95 M. Incubation of CAT in 0.95 M acetonitrile had no effect on CAT activity over the duration of inactivation-rate determinations (typically between 25 and 50 min.). Addition of acetonitrile to buffers had no significant effect on pH-meter readings.

Iodoacetamide

Iodoacetamide inactivation of wild-type CAT and Ala-31 CAT was studied over the pH range 5.1–9.5. At each pH the rate of inactivation was measured at a fixed concentration of 100 mM iodoacetamide. The final concentration of CAT_{III} in the incubations was 0.2 mg/ml (8 μM monomers). Iodoacetamide was added to CAT as a solution in the appropriate buffer adjusted to the pH being examined.

pH-rate profiles

The pH-rate profiles were fitted to theoretical ionization curves by non-linear regression analysis using the data-analysis program Enzfitter (Leatherbarrow, 1987).

RESULTS AND DISCUSSION

pH-dependence of methyl 4-nitrobenzenesulphonate inhibition of CAT

Inhibition of CAT_{III} by methyl 4-nitrobenzenesulphonate was examined over the pH ranges 5.1–7.9. The inhibition followed pseudo-first-order kinetics with saturation of the rate of inactivation observed at all pH values, indicating that methyl 4-nitrobenzenesulphonate binds reversibly to CAT_{III} prior to inactivation (eqn. 1; Kitz and Wilson, 1962):

The half-times ($t_{1/2}$) of inactivation at five different reagent concentrations were determined at each pH. The following linear rate expression has been derived (eqn. 2) where $K_{\text{inact.}}$ is $(k_{-1} + k_{+2})/k_{+1}$ and is analogous to the Michaelis constant for a substrate and where $t_{\text{min.}}$ is the minimum inactivation half-time at infinitely high inhibitor concentration (Meloche, 1967):

$$t_{1/2} = 1/[I] (t_{\text{min.}} \cdot K_{\text{inact.}}) + t_{\text{min.}} \quad (2)$$

A plot of $t_{1/2}$ against $1/[\text{inhibitor}]$ yields a straight line from which both $K_{\text{inact.}}$ and pseudo-first-order rate of inactivation at infinitely high concentrations of inhibitor (equivalent to k_{+2}) can be calculated.

Binding of methyl 4-nitrobenzenesulphonate by CAT_{III}, as measured by $K_{\text{inact.}}$, does not appear to be pH-dependent, and the average value of $K_{\text{inact.}}$ (over the entire pH range) is 5.3 ± 1.3 mM. However, the rate of inactivation is pH-dependent. The observed rate of inactivation ($k_{\text{obs.}}$) can be represented by eqn. (3), where K_A is the ionization constant for the modified group:

$$k_{\text{obs.}} = \frac{k_{+2} [\text{inhibitor}]}{K_{\text{inact.}} (1 + [\text{H}^+]/K_A) + [\text{inhibitor}]} \quad (3)$$

Since the equation is similar in form to that of Michaelis and Menten, a plot of $k_{+2}/K_{\text{inact.}}$ should vary with pH in the same manner as a plot of $k_{\text{cat.}}/K_m$, and thus reflect the pK_a of the free enzyme (Tipton and Dixon, 1979). The variation of $k_{+2}/K_{\text{inact.}}$ with pH, which is shown in Figure 2, yields a pK_a value of 6.60. This value is within the range that is described as typical for a histidine residue, and the ionizing group can be tentatively identified as His-195.

Iodoacetamide inhibition of wild-type and Ala-31 CAT

Although iodoacetamide inhibition of CAT_{III} mainly involves alkylation at N-3 of His-195, a small amount of modified Cys-31

can be detected (Corney, 1983). In order to eliminate the possibility of modification at this secondary site, the pH-dependence of iodoacetamide inactivation of Ala-31 CAT was examined, since the Cys 31 → Ala substitution results in an enzyme with very similar properties to wild-type CAT (Lewendon and Shaw, 1990). Unlike methyl 4-nitrobenzenesulphonate, iodoacetamide inactivation of CAT_{III} proceeds via simple second-order kinetics (eqn. 4), where there is no preliminary binding step and $k_{\text{obs.}}$ is proportional to the concentration of inhibitor:

Figure 2 Effect of pH on the inactivation of CAT_{III} by methyl 4-nitrobenzenesulphonate

Values of $k_{+2}/K_{\text{inact.}}$ were calculated as described in the text. The line describes a best-fit theoretical titration curve with a pK_a of 6.60 ± 0.12 .

Figure 3 Effect of pH on the inactivation of wild-type and Ala-31 CAT by iodoacetamide

The inactivation rate ($k_{\text{obs.}}$) at 100 mM iodoacetamide was determined as described in the text for wild-type CAT (●) and Ala-31 CAT (▲). Rates are plotted relative to the rate of inactivation at pH 7.9. The values of $k_{\text{obs.}}$ are $1.26 \times 10^{-3} \text{ s}^{-1}$ and $2.36 \times 10^{-3} \text{ s}^{-1}$ for wild-type and Ala-31 CAT respectively. The lines are theoretical titration curves with pK_a values of 6.66 ± 0.06 for wild-type CAT (—) and 6.80 ± 0.09 for Ala-31 CAT (-----).

Figure 4 Effect of pH on k_{cat}/K_m

Values of k_{cat}/K_m for wild-type (●) and Ala-31 CAT (▲) were determined from eqn. (6) and are plotted relative to the maximum value in each case ($3.76 \times 10^{-7} \text{ s}^{-1} \cdot \text{M}^{-1}$ for wild-type and $8.92 \times 10^{-6} \text{ s}^{-1} \cdot \text{M}^{-1}$ for Ala-31 CAT). The lines describe a best-fit theoretical curves with pK_a values of 6.62 ± 0.13 for wild-type (—) and 6.61 ± 0.12 (-----) for Ala-31 CAT.

In this case, $k_{\text{obs.}}$ can be represented by eqn. (5), and the value of K_a can be determined from the effect of pH on $k_{\text{obs.}}$:

$$k_{\text{obs.}} = \frac{k_{+1} \cdot [\text{inhibitor}]}{1 + [\text{H}^+]/K_a} \quad (5)$$

The variation of $k_{\text{obs.}}$ with pH was measured at a fixed concentration of 100 mM iodoacetamide for both wild-type and Ala-31 CAT (Figure 3). The pK_a value derived for wild-type CAT is 6.80 and that for Ala-31 CAT is 6.66. Both values agree reasonably well with each other and with the pK_a value determined from the pH-dependence of methyl 4-nitrobenzenesulphonate inhibition.

pH-dependence of k_{cat}/K_m

CAT activity was assayed by a radiometric method over the pH range 5.1–8.3. The value of k_{cat}/K_m was calculated from measurements of the rate of reaction (v) at low chloramphenicol concentrations by using eqn. (6):

$$v = k_{\text{cat}}/K_m [\text{CAT}] \cdot [\text{chloramphenicol}] \quad (6)$$

Figure 4 shows that k_{cat}/K_m decreases with pH as an ionizable group with an apparent pK_a of 6.62 for wild-type CAT and 6.61 for Ala-31 CAT is protonated. As the pH profile of k_{cat}/K_m shows the pK_a values of the free enzyme and free substrate (Tipton and Dixon, 1979), and chloramphenicol does not contain any groups that are ionizable over the pH range studied, the pK_a of 6.62 must be that of an ionizable group in CAT_{III}. These values agree well with those derived from the pH-dependence of chemical modification.

Conclusions

There is good agreement between the apparent pK_a values determined for His-195 from kinetic analysis and from chemical-

modification studies. The only anomalous value is that derived from iodoacetamide inactivation of wild-type CAT. As discussed above, iodoacetamide treatment of wild-type CAT results in modification of Cys-31 in addition to His-195 (Corney, 1983). Since modification of Cys-31 by a variety of reagents is accompanied by a loss of catalytic activity (Lewendon and Shaw, 1990), modification at this thiol, which must also be pH-dependent, can be expected to contribute towards the apparent pK_a derived from the rate of inactivation of wild-type CAT_{III}. The studies of the pH-dependence of k_{cat}/K_m clearly indicate that the Cys-31 → Ala substitution does not affect the pK_a of His-195.

Although apparent pK_a values determined from the pH-dependence of kinetic parameters and inactivation rates may not be equivalent to the microscopic pK_a of the group under study (reviewed by Knowles, 1976), in this case the close agreement between measurements made by both methods supports the view that the pK_a of the catalytic base of CAT_{III} is approx. 6.6. Using n.m.r. techniques, Derrick et al. (1991) attempted to determine directly the microscopic pK_a of His-195 by titrating the histidine residues of CAT_{III}. However, a signal for His-195 was not among the six C-2 proton resonances that were observed over the pH range 6–8. The authors concluded that the proton resonance of His-195 was too broad to detect, probably due to the restrained mobility imposed on the imidazole ring of His-195 by its coplanarity with the aromatic ring of Tyr-25 (Leslie, 1990).

From structural considerations, it is surprising that the pK_a of His-195 is so little perturbed from that of imidazole in solution. As discussed above, the imidazole ring of His-195 is involved in a face-to-face stacking interaction with the aromatic ring of Tyr-25 (Leslie, 1990), although the other face of the imidazole ring is relatively solvent accessible. Recently, Loewenthal et al. (1992) have reported that an interaction between an aromatic residue (tryptophan or tyrosine) and a histidine residue increases the pK_a of the latter by approx. 0.5 pH unit. However, Tyr-25 appears to have little effect on the protonation state of His-195, since the pH-dependence of k_{cat}/K_m for Tyr-25 → Ile CAT yielded a pK_a value of 6.69 ± 0.13 (results not shown). A change in the conformation of His-195 upon protonation cannot be ruled out.

The pK_a of 6.6 determined for His-195 implies that, at neutral pH, a considerable fraction of the N-3 atoms of His-195 will be in the active unprotonated form and able to accept a proton from chloramphenicol, as shown in Scheme 1. His-195 of CAT has been compared with His-57 of the catalytic triad (Ser-195/His-57/Asp-102) of the serine proteinases (Shaw et al., 1988), since upon formation of the acyl-enzyme intermediate the N-3 atom of His-57 accepts a proton from the hydroxy group of Ser-195, much as His-195 of the CAT does from the 3-hydroxy of chloramphenicol. In fact, the pK_a value of His-57 in both chymotrypsin and trypsin is approx. 6.8 (Fersht, 1985; Craik et al., 1987), similar to that of His-195 in CAT_{III}.

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