Transition State Stabilization by Chloramphenicol Acetyltransferase

ROLE OF A WATER MOLECULE BOUND TO THREONINE 174*

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The structure of the type III variant of chloramphenicol acetyltransferase reveals that Thr-174, a conserved residue, is hydrogen-bonded to a bound water molecule (water 252). Modeling studies (P. C. E. Moody and A. G. W. Leslie, unpublished data) suggested that water 252 could play a part in transition state stabilization via a hydrogen bond to the oxyanion of the putative tetrahedral intermediate. In addition, water 252 is one of three bound water molecules hydrogen-bonded to the 1-hydroxyl group of chloramphenicol in the chloramphenicol acetyltransferase-chloramphenicol binary complex. A combination of site-directed mutagenesis and the use of an alternative substrate has allowed the quantification of the energetic contribution of each of the interactions made by water 252 to catalysis. Thr-174 was replaced by alanine, valine, and isoleucine, each substitution removing the hydroxyl group hydrogen-bonded to water 252. Steady-state kinetic analysis of the mutant enzymes was carried out using both chloramphenicol and 1-deoxychloramphenicol as acetyl acceptors. The substitutions at Thr-174 result in a fall in $k_{cat}$ and in decreased affinities for each acetyl acceptor in the binary complexes and also in the ternary complexes with acetyl-CoA. From the calculated free energies in the transition state, the hydrogen bond between water 252 and the oxyanion of the tetrahedral intermediate can be estimated to contribute 0.9 kcal mol$^{-1}$ toward transition state stabilization, whereas the free energy of the hydrogen bonds between the 1-hydroxyl of chloramphenicol and three bound water molecules provides 1.6 kcal mol$^{-1}$.

It is generally accepted that bound water molecules identified in highly refined X-ray structures of enzymes may play important roles in substrate binding and catalysis. Confirmation of the functional importance of such water molecules has tended to come from the conservation of bound water either in different crystal forms of an enzyme or between the structures of homologous enzymes (Finner-Moore et al., 1992). However, the proposed function of a bound water molecule is often difficult to verify since the removal or replacement of individual water molecules cannot be easily achieved. Although the use of site-directed mutagenesis and substrate analogues has allowed the quantitative assessment of the contribution of functional enzyme groups to substrate binding and catalysis, this approach has not been used to examine the role of bound water molecules. An example of a water molecule amenable to this type of analysis occurs with chloramphenicol acetyltransferase, wherein one of the hydrogen bonds between the enzyme and the substrate chloramphenicol is via a bridging water molecule. Consequently, the role of this water molecule can be investigated by alteration of enzymes or substrate.

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 fails to bind to bacterial ribosomes and consequently is inactive as an inhibitor of protein synthesis (reviewed by Shaw (1983) and Shaw and Leslie (1991)). Of the many variants of CAT found in nature, only the type III variant (CAT_{III} (Murray et al., 1988) has been intensively studied, both structurally and mechanistically. Steady-state kinetic studies showed that CAT_{III} follows a ternary complex mechanism with random order addition of substrates (Kleanthous and Shaw, 1984). Chemical modification with an active site-directed reagent, 3-(bromoacetyl)chloramphenicol, implicated a histidyl residue (His-195)$^2$ in the catalytic mechanism (Kleanthous et al., 1985).

CAT_{III} is a trimer of identical subunits with an $M$, of 25,000 (Leslie et al., 1986; Harding et al., 1987). The structures of the binary complexes of CAT_{III} with chloramphenicol and with CoA have been determined to 1.75- and 2.4-Å resolution, respectively. The three active sites of CAT are located at each inter-subunit interface, such that the catalytically essential imidazole ring of His-195 is supplied from one subunit, whereas the amino acid residues participating in substrate binding are mainly supplied from the opposing subunit. The substrates approach each active site from opposite ends of a tunnel (~25 Å long) that extends through the protein (Leslie et al., 1988; Leslie, 1990).

A mechanism has been proposed for CAT wherein His-195 acts as a general base, abstracting a proton from the 3-hydroxyl of chloramphenicol, thus promoting nucleophilic attack on the thioester of acetyl-CoA. The 3-hydroxyl of chloramphenicol is correctly positioned to hydrogen bond to N$^2$ of His-195 (Leslie et al., 1988). This mechanism predicts the formation of a charged (oxyanion) tetrahedral intermediate (Scheme I) that collapses to give the products CoA and 3-acetylchloramphenicol.

The proposed tetrahedral intermediate has been modeled into the active site of CAT_{III} on the basis of the refined structures of the CAT_{III}-chloramphenicol and CAT_{III}-CoA binary complexes using a combination of computer graphics and model refinement. The modeled tetrahedral intermediate (Fig. 1), which has good stereochemistry and no unacceptable van der Waals contacts, has its 3-hydroxyl hydrogen-bonded to the imidazole ring of His-195. This hydrogen bond is 2.8 Å long, and the 3-hydroxyl of chloramphenicol is 3.4 Å away from the general base His-195. Hence, the proposed mechanism is consistent with the crystallographic data and with the chemical modification of His-195.

1. The abbreviations used are: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of CAT.
2. Alignment of the amino acid sequences of 11 CAT variants has resulted in the general numbering system used here. Ser-148, Thr-174, and His-195 are residues 142, 189, and 189, respectively, in the primary structure of CAT_{III}.
Bound Water in Transition State Stabilization by CAT

Experimental Procedures

Materials—Acetyl-CoA was prepared as described by Simon and Shenmin (1953). 1-Deoxychloramphenicol was prepared by J. A. Williams (Murray et al., 1991).

Site-directed Mutagenesis and Expression of CAT—Oligonucleotide-directed mismatch mutagenesis was performed using the deoxyuridine selection protocol with the dut ung E. coli strain RJ032 (Künkel et al., 1987). The oligonucleotides used were 5′-ATTTTGGCAT-TGCTATAATGGGT (Thr-174 → Ala; one mismatch), 5′-ATTTTGGCAT-TACTATAATGGGT (Thr-174 → Val; two mismatches), and 5′-ATTTTGGCATATTATAATGGGT (Thr-174 → Ile; one mismatch). The presence of the desired 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 and mutant CAT from E. coli extracts was carried out by affinity chromatography on chloramphenicol-Sepharose as previously described (Lewendon et al., 1988). The purity of enzyme preparations was assessed by SDS-polyacrylamide gel electrophoresis, where each mutant CAT produced a single band of mobility identical to that of wild-type CAT.

The concentration of purified CAT was calculated from ε198nm = 13.1. The method of Lowry et al. (1951) with wild-type CAT as standard.

Assay of CAT Activity—CAT activity was measured spectrophotometrically at 25°C by detection of reduced CoA (Shaw, 1975). The standard assay mixture comprised 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 0.1 mM EDTA, 1 mM 5,5′-dithio-bis-2-nitrobenzoic acid, 0.4 mM acetyl-CoA, and 0.1 mM chloramphenicol. The reaction was initiated by the addition of enzyme, and its progress was monitored at 412 nm. Chromamphenicol or 1-deoxychlamphenicol and acetyl-CoA were varied in the standard mixture during kinetic analysis. The measurement of initial rates for steady-state kinetic analysis was as described by Klembous and Shaw (1984). Kinetic coefficients in the initial rate equation (Equation 1).

\[ \frac{d[E]}{dt} = \frac{V_{\max} \cdot [S]}{K_m + [S]} \]

were calculated from linear slope and intercept replots from manually drawn double-reciprocal plots (Dalziel, 1957).

Results and Discussion

The role of bound water 252 in CAT was investigated by replacing conserved Thr-174 with alanine, valine, and isoleucine by site-directed mutagenesis. The steady-state kinetic parameters of the wild-type and mutant enzymes, with chloramphenicol or 1-deoxychlamphenicol as acyl acceptor, are shown in Tables I and II, respectively.

Each of the three substitutions at Thr-174 affects chloramphenicol binding as both the \( K_m \) and \( K_d \) values for chloramphenicol are increased in the mutant enzymes (Table I). All three substitutions have similar effects on chloramphenicol binding by catalase; increases in \( K_m \) values of between 10- and 13-fold reflect decreases in the affinity in the binary complex, whereas the \( K_m \) values (measures of the affinity of the CAT-acetyl-CoA complex for chloramphenicol) are increased 4-fold for Ala-174 CAT and Val-174 CAT and 8-fold for Ile-174 CAT. Although the \( K_m \) values for acetyl-CoA are increased 2-fold for Val-174 CAT and 4-fold for Ala-174 CAT, suggesting that disruption of the water structure around residue 174 may decrease the affinity of CAT for acetyl-CoA in the binary complex, the acetyl-CoA \( K_m \) values for each substituted enzyme are similar to that of the wild-type enzyme, indicating that the affinity of CAT for acetyl-CoA in the ternary complex is unaffected by the substitutions. In addition, small decreases in \( k_{cat} \) are observed: 2.2-fold for both Ala-174 CAT and Ile-174 CAT and 4.2-fold for Val-174 CAT.

Previous studies have shown that the affinity of wild-type

\[ \text{Scheme I. CM, chloramphenicol.} \]
The effects of the substitutions at Thr-174 on the kinetic properties of CAT are somewhat different from those previously observed upon replacement of Ser-148. For example, the Ser-148 → Ala substitution resulted in a 53-fold decrease in $k_{cat}$, with little change in the $K_m$ values for either substrate (Lewen-

don et al., 1990). The substitutions at Thr-174, however, result in very much smaller effects on $k_{cat}$, a result which suggests that the proposed hydrogen bond between the oxyanion of the tetrahedral intermediate and water 252 is less important in transition state stabilization.

The energetic consequences of the substitutions at Thr-174 can be determined from Equation 2,

$$\Delta G_{\text{trap}} = -RT \ln \left( \frac{k_{cat}(A)/K_m(A)}{k_{cat}(B)/K_m(B)} \right)_{\text{mutant}}$$

(Eq. 2)

where $A$ is the acetyl acceptor and $B$ is acetyl-CoA, which applies to interactions in the transition state. Equation 2 can also be represented in terms of Dalziel (1957) coefficients (used in Tables I and II) in the form of Equation 3.

$$\Delta G_{\text{trap}} = -RT \ln \left( \frac{1/\phi_{AB}}{1/\phi_{AB}} \right)_{\text{wild-type}}$$

(Eq. 3)

The $\Delta G_{\text{trap}}$ values for Ala-174 CAT, Val-174 CAT, and Ile-174 CAT calculated using Equation 3 are 2.2, 2.3, and 2.0 kcal mol$^{-1}$, respectively. A portion of this difference in free energy between mutant and wild-type enzymes must be attributed to a decrease in transition state stabilization due to the loss of the

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**Table I**

Kinetic coefficients for wild-type and mutant chloramphenicol acetyltransferases with chloramphenicol acting as acetyl acceptor

<table>
<thead>
<tr>
<th>CAT</th>
<th>$1/\phi_0$</th>
<th>$K_{cat}$</th>
<th>$K_d$ (CM)</th>
<th>$K_d$ (acetyl-CoA)</th>
<th>$K_{cat}/K_d$ (CM)</th>
<th>$K_{cat}/K_d$ (acetyl-CoA)</th>
<th>$\phi_{AB}$</th>
</tr>
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<tr>
<td></td>
<td>$s^{-1}$</td>
<td>$\mu M$</td>
<td>$\mu M$</td>
<td>$\mu M$</td>
<td>$\mu M^2$</td>
<td>$\mu M^2$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>Wild-type</td>
<td>599 ± 40</td>
<td>11.6 ± 1.3</td>
<td>3.4 ± 0.8</td>
<td>93 ± 4</td>
<td>30 ± 4</td>
<td>9.54 ± 0.02</td>
<td>1.3</td>
</tr>
<tr>
<td>Ala-174</td>
<td>276 ± 16</td>
<td>46.1 ± 8.1</td>
<td>41.9 ± 7.9</td>
<td>153 ± 14</td>
<td>143 ± 30</td>
<td>23.0 ± 4</td>
<td>7.9</td>
</tr>
<tr>
<td>Val-174</td>
<td>144 ± 19</td>
<td>54.3 ± 9.8</td>
<td>53.2 ± 6.3</td>
<td>79 ± 20</td>
<td>72 ± 7</td>
<td>26.8 ± 1.4</td>
<td>10.5</td>
</tr>
<tr>
<td>Ile-174</td>
<td>269 ± 48</td>
<td>98.6 ± 17.6</td>
<td>46.6 ± 10.5</td>
<td>100 ± 11</td>
<td>47 ± 8</td>
<td>17.8 ± 3.8</td>
<td>14.3</td>
</tr>
</tbody>
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**Table II**

Kinetic coefficients for wild-type and mutant chloramphenicol acetyltransferases with 1-deoxychloramphenicol acting as acetyl acceptor

<table>
<thead>
<tr>
<th>CAT</th>
<th>$1/\phi_0$</th>
<th>$K_{cat}$</th>
<th>$K_d$ (1-deoxyCM)</th>
<th>$K_d$ (acetyl-CoA)</th>
<th>$K_{cat}/K_d$ (1-deoxyCM)</th>
<th>$K_{cat}/K_d$ (acetyl-CoA)</th>
<th>$\phi_{AB}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$s^{-1}$</td>
<td>$\mu M$</td>
<td>$\mu M$</td>
<td>$\mu M$</td>
<td>$\mu M^2$</td>
<td>$\mu M^2$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>Wild-type</td>
<td>311 ± 30</td>
<td>22 ± 2</td>
<td>18 ± 4</td>
<td>151 ± 13</td>
<td>119 ± 38</td>
<td>8.8 ± 1.9</td>
<td>11.6</td>
</tr>
<tr>
<td>Ala-174</td>
<td>198 ± 21</td>
<td>46 ± 12</td>
<td>50 ± 13</td>
<td>166 ± 46</td>
<td>178 ± 30</td>
<td>39.9 ± 10.9</td>
<td>10.9</td>
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<tr>
<td>Val-174</td>
<td>86 ± 9</td>
<td>78 ± 6</td>
<td>65 ± 14</td>
<td>152 ± 26</td>
<td>122 ± 38</td>
<td>109.3 ± 28.8</td>
<td>10.3</td>
</tr>
<tr>
<td>Ile-174</td>
<td>132 ± 7</td>
<td>64 ± 17</td>
<td>59 ± 10</td>
<td>124 ± 31</td>
<td>100 ± 17</td>
<td>47.2 ± 8.2</td>
<td>11.5</td>
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hydrogen bond between the 1-hydroxyl of chloramphenicol and the enzyme. The absence of the postulated hydrogen bond of the Thr-174/water 252 couple with the oxyanion of the tetrahedral intermediate would also contribute toward the observed $\Delta G_{\text{app}}$.

To separate the effects of the substitutions at Thr-174 on two of the possible hydrogen bonds of the Thr-174/water 252 couple, the acetylation of an alternative substrate (1-deoxychloramphenicol) was examined. Although water 252 cannot hydrogen bond to the acetyl acceptor since the latter lacks the 1-hydroxyl group, hydrogen bonding between water 252 and the oxyanion is still possible.

As has been observed previously (Murray et al., 1991), 1-deoxychloramphenicol is a poorer substrate than chloramphenicol. A comparison of the kinetic parameters for wild-type CAT with 1-deoxychloramphenicol replacing chloramphenicol shows that the $k_{\text{cat}}$ is decreased 2-fold, the $K_m$ value for the acetyl acceptor is increased ~2-fold, and the $K_s$ value is increased 5-fold (Tables I and II).

The apparent binding energy ($\Delta G_{\text{app}}$) of the 1-hydroxyl group in the transition state can be estimated from Equation 4,

$$\Delta G_{\text{app}} = -RT \ln \left( \frac{1}{\phi_{\text{ap}}} \right)$$

where 1-deoxyCM stands for 1-deoxychloramphenicol and CM represents chloramphenicol. The calculated value of $\Delta G_{\text{app}}$ is 1.6 kcal mol$^{-1}$, which represents the energetic contribution to catalysis in the transition state of the hydrogen bonds between the 1-hydroxyl of chloramphenicol and the bound water molecules 249, 252, and 360.

The effects of the substitutions at Thr-174 on the kinetic parameters for the acetylation of 1-deoxychloramphenicol parallel those described above for chloramphenicol acetylation, although the magnitude of the change in each case is not as great. The $k_{\text{cat}}$ values for the mutant enzymes are decreased 1.6-, 3.6-, and 2.4-fold, respectively, for the alanine, valine, and isoleucine substitutions. The $K_m$ values for acetyl-CoA are again essentially unaffected, whereas the $K_s$ values for 1-deoxychloramphenicol are increased 2.0-fold for Ala-174 CAT and 3.4- and 2.8-fold for Val-174 CAT and Ile-174 CAT, respectively. In fact, the $K_s$ values for chloramphenicol and 1-deoxychloramphenicol for the Thr-174-substituted enzymes are all remarkably similar, suggesting that there is little discrimination between chloramphenicol and the 1-deoxy analogue by each of the mutant enzymes. It is unlikely that water molecules from bulk solvent can replace water 252 in Ala-174 CAT since the kinetic properties of this enzyme are so similar to those of Val-174 CAT and Ile-174 CAT.

Using Equation 3, $\Delta G_{\text{app}}$ values of 0.9, 0.9, and 1.0 kcal mol$^{-1}$ were determined for Ala-174 CAT, Val-174 CAT, and Ile-174 CAT, respectively. Since 1-deoxychloramphenicol cannot hydrogen bond to bound water molecules 249, 252, and 360, this difference in free energy can be attributed to the loss of the postulated hydrogen bond between water 252 and the oxyanion of the tetrahedral intermediate. Consequently, the latter hydrogen bond must contribute ~0.9 kcal mol$^{-1}$ toward transition state stabilization.

As discussed above, the $\Delta G_{\text{app}}$ derived from the comparison of wild-type and Thr-174-substituted enzymes with chloramphenicol as acetyl acceptor describes the energetic consequences of the loss of two hydrogen bonds: one between water 252 and the oxyanion of the tetrahedral intermediate and another between water 252 and the 1-hydroxyl of chloramphenicol. Assuming that these interactions are additive, values of 1.3, 1.4, and 1.0 kcal mol$^{-1}$ can be derived for Ala-174 CAT, Val-174 CAT, and Ile-174 CAT, respectively, for the transition state stabilization provided by the hydrogen bond between water 252 and the 1-hydroxyl group. Since the hydrogen bonds formed between the latter and all three bound water molecules contribute 1.6 kcal mol$^{-1}$ toward transition state stabilization, it is evident that the hydrogen bond to the Thr-174/water 252 couple is the most important one in energetic terms.

In summary, evidence has been obtained for the existence of both of the hydrogen bonding interactions of the Thr-174/water 252 couple that were postulated on the basis of the modeled tetrahedral intermediate. The contribution toward transition state stabilization of the hydrogen bond between the Thr-174/water 252 couple and the oxyanion of the tetrahedral intermediate is on the order of 0.9 kcal mol$^{-1}$, whereas the hydrogen bonds between the 1-hydroxyl of chloramphenicol and the three bound water molecules 249, 252, and 360 contribute 1.6 kcal mol$^{-1}$ to stabilization of the transition state.

The hydrogen bond between the hydroxyl group of Ser-148 and the oxyanion was previously estimated to contribute at least 2.2 kcal mol$^{-1}$ toward transition state stabilization (Lewendon et al., 1990). The results described above show that a further contribution of 0.9 kcal mol$^{-1}$ is acquired from the
Thr-174/water 252 couple assuming that there has been no change in the rate-determining step of the reaction upon substitution at Thr-174. Together, Thr-174 and Ser-148 contribute 3.1 kcal mol\(^{-1}\) toward transition state stabilization via hydrogen bonding with the oxyanion of the tetrahedral intermediate. Further stabilization of the tetrahedral intermediate in wild-type CAT may occur from hydrogen bonding interactions between the oxyanion and bulk solvent water molecules.

Only in a minority of cases can the role of a bound water molecule be determined by alteration of the enzyme and/or the substrate, and just one other example of such an investigation has been reported. Quicho et al. (1989) examined the role of bound water molecules in L-arabinose-binding protein; x-ray crystallography of complexes of the binding protein with three ligands suggested that two bound water molecules were important in determining substrate specificity. In the favorable case of water 252 of CAT, altering both the enzyme and the substrate has furnished evidence in support of the proposed role of water 252 in transition state stabilization. Uniquely, this approach has allowed the quantitation of the energetic contribution of a bound water molecule to catalysis.

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REFERENCES