The role of residues outside the active site: structural basis for function of C191 mutants of Escherichia coli aspartate aminotransferase

Constance J. Jeffery, Lisa M. Gloss, Gregory A. Petsko and Dagmar Ringë

Rosenstiel Basic Medical Sciences Research Center, MS029, Brandeis University, Waltham, MA 02454-9110 and Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA 94720, USA

In previous kinetic studies of Escherichia coli aspartate aminotransferase, it was determined that some substitutions of conserved cysteine 191, which is located outside of the active site, altered the kinetic parameters of the enzyme (Gloss, L.M., Spencer, D.E. and Kirsch, J.F., 1996, Protein Struct. Funct. Genet., 24, 195–208). The mutations resulted in an alkaline shift of 0.6–0.8 pH units for the pKₐ of the internal aldimine between the PLP cofactor and Lys258. The change in the pKₐ affected the pH dependence of the kₐ following the cofactor (aspartate) values for the mutant enzymes. To help understand these observations, crystal structures of five mutant forms of E. coli aspartate aminotransferase (the maleate complexes of C191S, C191F, C191Y and C191W, and C191S without maleate) were determined at about 2 Å resolution in the presence of the pyridoxal phosphate cofactor. The overall three-dimensional fold of each mutant enzyme is the same as that of the wild-type protein, but there is a rotation of the mutated side chain around its Cα–Cβ bond. This side chain rotation results in a change in the pattern of hydrogen bonding connecting the mutated residue and the protonated Schiff base of the cofactor, which could account for the altered pKₐ of the Schiff base imine nitrogen that was reported previously. These results demonstrate how residues outside the active site can be important in helping determine the subtleties of the active site amino acid geometries and interactions and how mutations outside the active site can have effects on catalysis. In addition, these results help explain the surprising result previously reported that, for some mutant proteins, replacement of a buried cysteine with an aromatic side chain did not destabilize the protein fold. Instead, rotation around the Cα–Cβ bond allowed each large aromatic side chain to become buried in a nearby pocket without large changes in the enzyme’s backbone geometry.

Keywords: aspartate aminotransferase/conserved cysteine/crystal structure/maleate/pyridoxal phosphate

Introduction

L-Aspartate aminotransferase (L-Asp-AT, EC 2.6.1.1) is a pyridoxal phosphate-dependent enzyme that catalyzes the reversible transamination reaction

\[ \text{L-aspartate} + 2\text{-oxoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{L-glutamate}. \]

It is a key metabolic enzyme that plays important roles in amino acid and carbohydrate metabolism, ureogenesis and the transfer of reducing equivalents into the mitochondria and chloroplasts via the malate/aspartate shuttle. During catalysis, the enzyme goes through several intermediate steps and alternates between a PLP [pyridoxal phosphate] form, which contains the cofactor covalently bound to active site lysine residue 258 via a Schiff base linkage, and a PMP [pyridoxamine phosphate] form of the enzyme. Extensive studies of the structure and function of L-Asp-AT have made it the prototype of PLP-containing enzymes. An understanding of the enzyme’s complex ping-pong bi-bi reaction mechanism and the contributions of many of the catalytic residues and the PLP cofactor has emerged from extensive mutagenic and kinetics experiments (summarized in Kirsch et al., 1984; Arnone et al., 1985; Jansonius and Vincent, 1987; Hayashi et al., 1990; Kirsch et al., 1990, 1995b, Goldberg and Kirsch, 1996; Hayashi and Kagamiyama, 1997 and references therein) and from the crystal structures from several organisms. Crystal structures are available for E. coli L-Asp-AT (Smith et al., 1989; Kamitori et al., 1990; Jager et al., 1994; Okamoto et al., 1994), the mitochondrial and cytosolic isozymes from chicken (Ford et al., 1980; Borisov et al., 1985; Harutyunyan et al., 1985; McPhalen et al., 1992; Malashkevich et al., 1995), the cytosolic isozyme from pig (Arnone et al., 1985) and the cytosolic isozyme from yeast (Jeffery et al., 1998). The E. coli enzyme has been the subject of the greatest number of crystallographic and mutagenesis experiments, owing in part to the ease with which it can be expressed and manipulated genetically. Structures of the wild-type E. coli enzyme have been determined at high resolution in both ‘closed’ (inhibitor-bound) and ‘open’ (without inhibitor) forms (Jager et al., 1994). Site-directed mutagenesis combined with crystallographic analysis has probed the roles of the essential Lys258, the Tyr225 that makes a hydrophobic bond to the imine nitrogen of the PLP cofactor, an aspartic acid (Asp222) located near the pyridine nitrogen of PLP, and two arginines, Arg386 and Arg292, that flank the substrate binding pocket. These studies have led to a structural description of the L-Asp-AT mechanism in which Lys258 functions as the acid/base group to transfer a proton from the amino acid substrate to the cofactor to form the internal aldimine; Arg292 and Arg386 form salt-bridges to the side chain and alpha carboxylates, respectively, of the substrate, thus providing specificity for negatively-charged amino acids. Tyr225 stabilizes the kinetically competent form of the bound PLP group, and Asp222 assists in the cofactor’s function as an electron sink by forming a salt-bridge to the pyridine nitrogen of PLP.

The roles of the five cysteines found in the primary structure of L-Asp-AT from E. coli were examined by mutagenesis (Gloss et al., 1992). Only Cys191 is conserved in all of the closely related transaminase sequences (>25% sequence identity, including L-Asp-AT and E. coli tyrosine aminotransferase). A residue that is conserved in eubacteria, yeast, birds and mammals is likely to play an important role in catalysis, folding or stability, and mutation of this residue is likely to
a position 191 (Cys to Phe, Tyr, Trp, Arg and Gly, as well as Ser) Coomassie-stained SDS polyacrylamide gel. The best crystals hypothesized (Gloss et al., 1996). All of the L-Asp-AT variants diffusion method in 24 well Lindbro dishes with siliconized cover slips. Hanging drops contained 3 μl reservoir solution. The final crystallization conditions contained 6–13 mg/ml l-Asp-AT (7, 13, 6 and 9 mg/ml for the C191S, C191F, C191Y and C191W mutants, respectively), 20 mM potassium phosphate buffer, pH 7.5, 10 μM PLP and cofactor and Lys258. For the wild-type and C191A enzyme, this pKₐ is approximately 7, whereas these four mutations resulted in an alkaline shift of 0.6–0.8 pH units for this pKₐ. This pKₐ value is reflected in the pH-dependence of kₐ/kₘ for aspartate of the enzymes (Kiick and Cook, 1983; Gloss and Kirsch, 1995a). At physiological pH, the kₐ/kₘ Asp values of wild type and C191S are very similar; the values for C191F, C191Y and C191W are 2.4–4-fold lower than that of wild type. This difference in the kₐ/kₘ Asp values is smaller at the optimal pH values of each enzyme, pH 8.2–8.6 (Gloss et al., 1996). It was also noted that replacing a buried cysteine with a large aromatic side chain (C191F, Y, W) had only a marginal effect on protein stability (0.3–0.6 kcal/mol decrease).

Cys191 is not located in the active site and does not interact directly with the substrate or PLP cofactor (Figure 1A). It does however form a hydrogen bond to the backbone carbonyl of Tyr225, whose side chain forms an important hydrogen bond to O₃' of the cofactor (Figure 1B), and whose mutation strongly affects the internal aldimine pKₐ and enzyme activity (Goldberg et al., 1991; Goldberg and Kirsch, 1996; Park et al., 1997). The hydrogen-bonding properties of Ser and Cys are sufficiently different to perhaps propagate a perturbation at position 191 to the hydrogen bond between Tyr225 and the PLP O₃’. Crystallographic evidence is required to test this possibility. In addition, from the crystal structure of the wild-type enzyme, it is not clear how a large aromatic side chain would be accommodated into the site of Cys191 without affecting the stability of the enzyme. We report here the crystal structures of the C191S, C191F, C191Y and C191W mutants and describe how changes in the protein structure around residue 191 account for the observed change in the kinetic activity, the shift in pKₐ and the lack of a large change in protein stability.

Materials and methods

Protein crystallization

Mutant E. coli l-Asp AT was prepared as described previously (Gloss et al., 1996) and appeared as a single band on a Coomassie-stained SDS polyacrylamide gel. The best crystals were obtained at room temperature by the hanging-drop vapor diffusion method in 24 well Lindbro dishes with siliconized cover slips. Hanging drops contained 3 μl protein solution and 3 μl reservoir solution, and were allowed to equilibrate against 1 ml reservoir solution. The final crystallization conditions contained 6–13 mg/ml l-Asp-AT (7, 13, 6 and 9 mg/ml for the C191S, C191F, C191Y and C191W mutants, respectively), 20 mM potassium phosphate buffer, pH 7.5, 10 μM PLP and

![Fig. 1. Schematic diagrams indicating the locations of key residues and hydrogen bonds. (A) An aspartate aminotransferase dimer indicating the locations of residues 191, 225 and the PLP cofactor. One subunit is shown in grey, and the second in white. The PLP cofactor, the maleate bound in the active site, several active site residues and residue 191 (shown here as in the C191F mutant) are indicated by ball-and-stick models. The maleate, PLP and active site residues are grey. Residue 191 is black. This figure and Figures 2, 3 and 4 were prepared with MOLSCRIPT (Kraulis, 1991). (B) Schematic diagram of interactions between Tyr225 and the PLP cofactor in the active site. The strength of the hydrogen bond between the Tyr225 hydroxyl group and the PLP O₃' affects the pKₐ of the PLP imine nitrogen. affect structure and/or function adversely. However, the two-base mutation, C191A, yielded an active and stable enzyme, with properties very similar to those of the wild-type enzyme. The single base mutation, C191S, was also active and stable, but exhibited some striking kinetic differences from the wild-type enzyme. The hypothesis was advanced that Cys191 was conserved, not because it is essential, but because there is no neutral mutational corridor to the phenotypically similar enzyme, Ala191 (Gloss et al., 1992).

In a subsequent study, all possible single base mutations at position 191 (Cys to Phe, Tyr, Trp, Arg and Gly, as well as Ser) were made and characterized to test further this evolutionary hypothesis (Gloss et al., 1996). All of the l-Asp-AT variants are active and stable, but have significant decreases in some kinetic or stability parameters, relative to wild type or C191A, supporting the evolutionary hypothesis (Table I). The most striking difference observed in the set of single base mutations was that four mutations, C191S, C191F, C191Y and C191W, affected the pKₐ of the internal aldimine between the PLP

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild type</th>
<th>C191S</th>
<th>C191F</th>
<th>C191Y</th>
<th>C191W</th>
</tr>
</thead>
<tbody>
<tr>
<td>kₐ/kₘ l-Asp (M⁻¹s⁻¹)</td>
<td>90 800</td>
<td>107 000</td>
<td>23 400</td>
<td>37 400</td>
<td>28 700</td>
</tr>
<tr>
<td>pKₐ of internal aldimine</td>
<td>6.95</td>
<td>7.64</td>
<td>7.57</td>
<td>7.76</td>
<td>7.72</td>
</tr>
<tr>
<td>ΔG°(f) (kcal/mol)</td>
<td>10.0</td>
<td>8.1</td>
<td>8.4</td>
<td>7.7</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Data are reproduced from Gloss et al. (1996). Conditions: pH 7.5, 25°C. Values are derived from the steady-state kinetics with the l-Asp and α-KG substrate pair. Determined by spectrophotometric titration. Urea denaturation parameters determined by enzyme activity. Values represent a standard state of 0.7 μM monomer.
Structures of L-Asp-AT Cys191 mutants

Fig. 2. Proximal stereo diagram showing positions of side chain and backbone atoms for the PLP cofactor, residues 191 and 225 and nearby residues. The PLP cofactors of the C191S (dark grey), C191F (medium grey), C191Y (light grey), C191W (white) and wild-type (black, PDB entry 1ASM) structures were superposed using the program O (Jones, 1985; Jones et al., 1991).

Table II. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Mutant (± maleate)</th>
<th>C191S (+)</th>
<th>C191F (+)</th>
<th>C191Y (+)</th>
<th>C191W (+)</th>
<th>C191S (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit cell a</td>
<td>157.13</td>
<td>157.30</td>
<td>157.18</td>
<td>157.45</td>
<td>156.27</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>86.69</td>
<td>85.10</td>
<td>85.40</td>
<td>85.05</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>79.87</td>
<td>78.37</td>
<td>78.51</td>
<td>78.10</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>2.45</td>
<td>1.90</td>
<td>2.20</td>
<td>1.90</td>
<td>2.49</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>17.70</td>
<td>40.92</td>
<td>34.52</td>
<td>38.21</td>
<td>19.53</td>
</tr>
<tr>
<td>% completeness</td>
<td>86.69</td>
<td>90.00</td>
<td>80.00</td>
<td>80.00</td>
<td>92.38</td>
</tr>
<tr>
<td>R_{merge}</td>
<td>5.9</td>
<td>3.8</td>
<td>6.0</td>
<td>4.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution range</td>
<td>10.0–2.45</td>
<td>10.0–1.90</td>
<td>10.0–2.20</td>
<td>10.0–1.90</td>
<td>10.0–2.4</td>
</tr>
<tr>
<td>Final R-factor</td>
<td>20.7</td>
<td>18.8</td>
<td>18.5</td>
<td>18.9</td>
<td>21.9</td>
</tr>
<tr>
<td>Restraints (r.m.s.) observed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bond lengths</td>
<td>0.018</td>
<td>0.015</td>
<td>0.015</td>
<td>0.020</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>3.523</td>
<td>2.927</td>
<td>3.064</td>
<td>3.431</td>
<td>2.036</td>
</tr>
<tr>
<td>dihedral angles</td>
<td>1.793</td>
<td>1.265</td>
<td>1.502</td>
<td>2.258</td>
<td>1.828</td>
</tr>
<tr>
<td>improper angles</td>
<td>39.54</td>
<td>22.97</td>
<td>22.18</td>
<td>23.16</td>
<td>21.36</td>
</tr>
<tr>
<td>Average B-factor</td>
<td>46.48</td>
<td>28.37</td>
<td>28.00</td>
<td>28.33</td>
<td>22.87</td>
</tr>
<tr>
<td>main chain</td>
<td>37.75</td>
<td>36.40</td>
<td>28.03</td>
<td>32.05</td>
<td>31.94</td>
</tr>
<tr>
<td>side chain</td>
<td>3084</td>
<td>3089</td>
<td>3090</td>
<td>3092</td>
<td>3084</td>
</tr>
<tr>
<td>water</td>
<td>26.00</td>
<td>144.00</td>
<td>80.00</td>
<td>117.00</td>
<td>120.00</td>
</tr>
<tr>
<td>Protein atoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Waters             | 5 mM EDTA in the protein solution. The reservoir buffers contained 40–54% ammonium sulfate and 20 mM potassium phosphate buffer, pH 7.5. Some crystals (for the C191S + maleate and the C191F, C191Y and C191W structures) were grown in the presence of the competitive inhibitor maleate (5 mM). All the crystals were bright yellow due to the presence of the PLP cofactor. Crystals used for data collection were rectangular rods approximately 0.3 mm in diameter and between 0.5 and 1.0 mm in length.

Data collection

Single crystals were mounted in thin-walled quartz capillary tubes. X-Ray diffraction data were collected at 4°C with an oscillation range of 1° per frame with 30 min exposures, using a 0.3 mm collimator on a R-Axis IIC imaging plate system. CuKα radiation (λ = 1.54 Å) was provided by a Rigaku RU200-HB rotating anode generator operated at 50 kV and 145 mA. For the C191F, C191Y and C191W structures and the C191S structure with maleate, still photos were taken and used in indexing to determine unit cell parameters, and the data frames were integrated, scaled and merged with standard R-Axis software (Wonacott, 1980; Higashi, 1990). For the C191S wild-type structure, a full dataset with 1° exposure was collected and processed with the program O (Jones, 1985; Jones et al., 1991).

Table III. Interatomic distances in structures without bound inhibitor

<table>
<thead>
<tr>
<th></th>
<th>Wild type⁴</th>
<th>C191S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y225OH → PLPN_{ζ}</td>
<td>2.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Y225OH → PLPO-3'</td>
<td>2.5</td>
<td>2.9</td>
</tr>
<tr>
<td>1915SH/OH → Y225O</td>
<td>3.1</td>
<td>3.9</td>
</tr>
</tbody>
</table>

⁴Wild type is PDB entry 1AAW.
structure without maleate, 1° oscillation photos were taken and used in indexing to determine unit cell parameters, and the data frames were integrated, scaled and merged with DENZO (Otwonowski and Minor, 1997). The symmetry of each diffraction pattern in the photos used for indexing was consistent with the orthorhombic space group C2221.

Data reduction and model refinement

C191S mutant with maleate

A native data set was obtained at a crystal to detector distance of 130 mm. The Rmerge was 5.9% on intensities for all reflections (Table II). The final merged data set contained 17 708 unique reflections with I > 0, corresponding to 86% completeness in a resolution range of 10.0–2.45 Å. The protein coordinates from an aspartate aminotransferase structure, from which the water molecule coordinates were removed, were used as the initial model (PDB entry 1ASA; Smith et al., 1991; Otwinowski and Minor, 1997). The model was refined with XPLOR (Brünger et al., 1989; Brünger, 1990, 1996) against all reflections between 10.0 and 2.45 Å resolution. The final model has an R-factor of 20.7% and contains 26 water molecules.

Mutual rebuilding for this mutant structure and for the C191F, C191Y and C191W structures was begun using FRODO (Jones, 1978, 1985) on an Evans and Sutherland Workstation and continued using O (Jones et al., 1991; Collaborative Computational Project, Number 4, 1994) on a Silicon Graphics Workstation. After the first round of XPLOR refinement for each of these structures, water molecules were placed, initially using the program WATERHUNTER and, in later rounds of refinement, manually using a difference Fourier map with the coefficients |Fobs − Fcal| and a 2.0 σ cut-off.

C191F mutant with maleate

A native data set was obtained at a crystal to detector distance of 100 mm. The Rmerge was 3.8% on intensities for all reflections (Table II). The final merged data set contained 40 823 unique reflections with I > 0, corresponding to 90% completeness in a resolution range of 10.0–1.9 Å. The protein coordinates from a partially refined C191S with maleate structure were used as the initial model. The model was refined with XPLOR against all reflections between 10.0 and 1.90 Å resolution. The final model has an R-factor of 18.8% and contains 144 water molecules.

C191Y mutant with maleate

A native data set was obtained at a crystal to detector distance of 80 mm. The Rmerge was 6.0% on intensities for all reflections (Table II). The final merged data set contained 34 520 unique reflections with I > 0, corresponding to 80% completeness in a resolution range of 10.0–2.2 Å. The protein coordinates from a partially refined C191S with maleate structure were used as the initial model. The model was refined with XPLOR against all reflections between 10.0 and 2.20 Å resolution. The final model has an R-factor of 18.5% and contains 80 water molecules.

C191W mutant with maleate

A native data set was obtained at a crystal to detector distance of 80 mm. The Rmerge was 4.2% on intensities for all reflections (Table II). The final merged data set contained 38 214 unique reflections with I > 0, corresponding to 80% completeness in a resolution range of 10.0–1.90 Å. The protein coordinates from a partially refined C191S with maleate structure were used as the initial model. The model was refined with XPLOR against all reflections between 10.0 and 1.90 Å resolution. The final model has an R-factor of 18.9% and contains 117 water molecules.

C191S mutant without maleate

A native data set was obtained at a crystal to detector distance of 100 mm. The Rmerge was 7.2% on intensities for all reflections (Table II). The final merged data set contained 19 539 unique reflections with I > 0, corresponding to 92% completeness in a resolution range of 10.0–2.40 Å. The protein coordinates from a wild type E.coli L-Asp-AT structure were used as an initial model (PDB entry 1AAW; Almo et al., 1994). The model was refined with XPLOR against all reflections between 10.0 and 2.40 Å resolution. Water molecules were placed manually using a difference Fourier map with the coefficients |Fobs − Fcal| and a 2.0 σ cut-off. The final model has an R-factor of 21.9% and contains 120 water molecules.

Results and discussion

Five X-ray crystal structures were determined for mutant forms of E.coli aspartate aminotransferase in which the cysteine at position 191 was replaced with either serine, phenylalanine, tyrosine or tryptophan (the maleate complexes of C191S, C191F, C191Y and C191W, and C191S without maleate). All five structures were determined to between 1.90 and 2.45 Å resolution with final R-factors between 18.5 and 21.9% (Table II). Some bound water molecules were located and built into each structure, and the number of observed water molecules roughly corresponds to the high resolution limit of the data used in the refinement for the four structures that contain maleate. Each structure is a homodimer with one subunit of 45 kDa molecular weight per asymmetric unit, and the two subunits in the dimer are related by a crystallographic twofold axis. Each of the five final models has good geometry (Table II). There is one nonglycine residue in each of the closed structures that is found in a disallowed region of a phi–psi plot. This residue, Ser296, is also found in a strained conformation in other L-aspartate aminotransferase structures. The side chain of Ser296 has good electron density and forms a hydrogen bond with the side chain of active site residue Arg292. Ser296 helps position Arg292 so that it can form hydrogen bonds with the carboxylate oxygens of the maleate inhibitor.

Each of the mutant structures has the same overall fold as the wild-type protein (Jäger et al., 1994) with each subunit consisting of a large and a small domain and an N-terminal segment that extends over the other subunit in the dimer. Each of the five structures contains a PLP cofactor covalently bound in the active site of each monomer through a Schiff base linkage to the ε amino group of lysine 258. Four of the structures also contain a maleic acid inhibitor in the active site and are found to be in the ‘closed’ form of the protein (C191W, C191F, C191Y and C191S with maleate). The fifth structure does not contain a maleic acid inhibitor and is found to be in the ‘open’ form of the protein (C191S without maleate).

Differences between the wild-type protein and the mutant proteins

The general shape of the active site and the location of the active site residues relative to the maleic acid inhibitor and the PLP cofactor are largely unchanged compared with what is observed for wild-type L-Asp-AT. When the PLP cofactors in the structures are superposed, the nearby active site residues superpose closely (Figure 2). One exception is the distance between the side chain of Tyr225 and the O3’ of the PLP

108
Fig. 3. Proximal stereo view of the active site with possible hydrogen bonds between the PLP cofactor and nearby side chains indicated by dashed lines. The PLP cofactor and maleate inhibitor are shown with black bonds, and the active site residues are shown with white bonds. A section of the loop containing residue 191 is shown with grey bonds. (A) Wild-type *E.coli* aspartate aminotransferase with maleate. (B) C191S with maleate. (C) C191S without maleate.
Cofactor in the open forms of the C191S mutant and wild-type structures. In the wild-type enzyme, the side chain hydroxyl group of Tyr225 is in a position to make a good hydrogen bond (2.5 Å) with the O3' of the PLP cofactor. In the C191S mutant structures, this distance is increased by 0.4 Å (Table III).

The other significant change in the mutant structures is found near the mutated position 191, which is not located in the active site of the enzyme. Cys191 is in a loop containing residues 189–196 that connects two β-strands in the β-sheet in the large domain. Another loop containing residues 220 through 225 is located between residue 191 and the PLP cofactor in the active site. In the wild-type structure (PDB accession numbers: 1AAW, Almo et al., 1994; 1ASM, Jager et al., 1994), the side chain of cysteine 191 is pointing toward the PLP cofactor and can make a hydrogen bond with the backbone carbonyl of Tyr225 (Figure 3A). In each of the mutant structures, residue 191 no longer makes this hydrogen bond, although the positions of the other residues in the loop containing 191 and the loop containing 225 are very similar in the wild type and these mutant structures (Figures 3 and 4).

While it makes sense that substitution of the cysteine with a large aromatic residue could prevent formation of the hydrogen bond between residue 191 and the backbone carbonyl of 225, the crystal structures help clarify why the serine side chain also does not form this bond. In both the open and closed C191S structures, the serine side chain is rotated around its Cα-Cβ bond so that its hydroxyl group is too far from the backbone carbonyl of Tyr225 to form a hydrogen bond (5.5 Å open; 3.9 Å closed). Instead, the serine side chain forms a hydrogen bond to water molecule 554. This water molecule is located in a pocket formed by the backbone nitrogens of residues 225 and 224, the backbone carbonyl oxygen of 191, and water molecule 514. The serine hydroxyl group can also make a hydrogen bond to the side chain of Asp236. It is possible that the rotation of the serine side chain in the closed form could also be due to the formation of similar hydrogen bonds, since the serine hydroxyl group is pointed in that direction, but a corresponding ordered water molecule was not observed, perhaps due to crystallographic disorder.

Since one of the C191S structures was solved in the absence of bound inhibitor, we can use it to help explain the observed shift in the pKα of the enzyme compared with the wild-type enzyme. The change in the hydrogen bonding pattern between residues 191 and 225 results in a shift of the side chain of active site residue Tyr225 so that its hydroxyl group moves away from the PLP oxygen (from 2.5 to 2.9 Å; Table III). The change in the normally close interaction of residue 225 with the PLP cofactor appears to explain the effect of the substitutions at Cys191 on the pKα of the internal aldimeine. The longer distance between the Tyr225 hydroxyl group and the PLP oxygen means that the Tyr225 side chain makes a weaker hydrogen bond with the oxygen. The weaker hydrogen bond then results in the 0.69 pH unit alkaline shift of the pKα of the internal aldimeine that has been reported previously (Table I) (Gloss et al., 1996). This is consistent with the results seen previously with a Tyr225 to Phe225 mutant enzyme (Goldberg et al., 1991) where the hydrogen bond between the Tyr225 side chain and the PLP oxygen is absent and there is a 1.3 pH unit alkaline shift in pKα.

**Conclusions**

This set of mutant enzymes provides an example of how catalysis can be altered by a single amino acid substitution at a position located outside of the active site and without changes in the overall structure or large decreases in the stability of the enzyme. It emphasizes the importance of amino acid residues near the active site that do not interact directly with the cofactor or substrate (or in this case, inhibitor). These residues may be important in helping to position precisely the functional groups in the active site, because, as is seen here, a change in the length of even one hydrogen bond between an active site residue and a cofactor can affect catalysis.

**Coordinates**

Atomic coordinates have been deposited in the Protein Data Bank with accession codes 5eaa, 1b4x, 1qir, 1qis and 1qi.

**Acknowledgements**

This work was supported by a Cystic Fibrosis Foundation Postdoctoral Fellowship to C.J.Jeffery and NSF grant #MCB9317373 awarded to D.Ringe. The work of L.M.Gloss was supported by NIH grant #GM35393 awarded to Jack F.Kirsch.

**References**


C.J.Jeffery et al.


Received May 7, 1999; revised November 15, 1999; accepted November 15, 1999