Structural Studies of Mutants of T4 Lysozyme That Alter Hydrophobic Stabilization*

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Multiple replacements at amino acid position 3 of bacteriophage T4 lysozyme have shown that the conformational stability of the protein is directly governed by the hydrophobicity of the residue substituted (Matsumura, M., Becktel, W. J., and Matthews, B. W. (1988) Nature 334, 406-410). Of the 13 mutant lysozymes made by site-directed mutagenesis, two variants, one with valine (I3V) and the other with tyrosine (I3Y), were crystallized and their structures solved. In this report we describe the crystal structures of these variants at 1.7 Å resolution. While the structure of the I3V mutant is essentially the same as that of wild-type lysozyme, the I3Y mutant has substantial changes in its structure. The most significant of these are that the side chain of the tyrosine is not accommodated within the interior of the protein and the amino-terminal poly peptide (residues 1-9) moves 0.6-1.1 Å relative to the wild-type structure. Using coordinates based on the wild-type and available mutant structures, solvent accessible surface area of residue 3 as well as the adjacent 9 residues in the folded form were calculated. The free energy of stabilization based on the transfer of these residues from a fully extended form to the interior to the folded protein was found to correlate well with the protein stability determined by thermodynamic analysis. The enhanced thermostability of the variant Ile-3 → Leu, relative to wild-type lysozyme, can also be rationalized by surface-area calculations based on a model-built structure.

Noncrystallization of most lysozyme variants at position 3 appears to be due to disruption of intermolecular contacts in the crystal. The Ile-3 → Val variant is closely isomorphous with wild-type and maintains the same crystal contacts. In the Ile-3 → Tyr variant, however, a new set of contacts is made in which direct protein-protein hydrogen bonds are replaced by protein-water-protein hydrogen bonds as well as a novel hydrogen bond involving the phenolic hydroxyl of the substituted tyrosine.

In phage T4 lysozyme, the isoleucine at amino acid position 3 (Ile-3) contributes to the major hydrophobic core of the COOH-terminal lobe and thereby helps link the NH2- and COOH-terminal domains. To investigate the role of Ile-3 in the stabilization of lysozyme, this residue was replaced with 13 other amino acids (7). Thermodynamic analyses showed that changes in the stability of the mutant lysozymes were directly related to the hydrophobicity of the substituted residue (7). Similar correlations between protein stability and residue hydrophobicity have been reported in a series of mutants of tryptophan synthase (12), kanamycin nucleotidyltransferase (13), and barnase (14).

In this paper, we report the details of the crystallographic analyses of the mutants in which Ile-3 is replaced by valine (I3V) and tyrosine (I3Y). Of the 13 non-native variants at residue 3, these were the only two that could be crystallized. The I3V variant illustrates how a mutant protein can be crystallized in spite of substantial changes in its structure. The contribution of hydrophobic interactions to the stability of lysozyme is discussed in light of the solvent accessible surface areas of the mutant and wild-type proteins.

EXPERIMENTAL PROCEDURES
Preparation and Crystallization of Mutant Lysozymes—The mutant T4 phage lysozymes with Trp, Phe, Tyr, Leu, Val, Met, Cys, Ala, Gly, Thr, Ser, Glu, and Asp at position 3 were constructed by oligonucleotide-directed mutagenesis as previously described (7). The wild-type and mutant proteins were expressed in Escherichia coli and purified (15, 16).

Crystallization of each of the mutant lysozymes was attempted by batch methods from concentrated phosphate solutions as used for wild-type and a number of other mutants (17-19). Typically, 20 or more different crystallization set-ups were made for each variant using a matrix of phosphate concentrations over the pH range 6.5-7.1. For the mutants I3C, I3A, and I3L, similar crystallization experiments were repeated using freshly prepared batches of protein. The above procedure has been sufficient to obtain crystals of many mutant lysozymes. In the present case, however, crystals were obtained only for I3V and I3Y, these crystals being isomorphous with wild-type lysozyme. In the present case no attempt was made to explore precipitants other than phosphate. Prior to x-ray photography, the crystals were equilibrated with a solution of 1.05 M K2HPO4, 1.26 M NaH2PO4, 0.23 M NaCl, 1.4 mM β-mercaptoethanol (pH 6.7).

Crystallographic Data Collection and Model Building—The oscillation method (20, 21) was used to collect three-dimensional x-ray data sets for the variants I3V and I3Y. Two crystals of each mutant lysozyme were used in the data collection and sufficed to provide 73-74% of the data to 1.8 Å resolution.

A difference electron density map with amplitudes (FmWild-type) and phases calculated from the refined wild-type model (19) was used for initial inspection of the structural changes associated with the amino acid substitution (Figs. 1 and 2). A map with coefficients (2FmWild-type - FmWild-type) and phases calculated as above was used to build a model of the mutant structure using the FRODO software package on an Evans and Sutherland PS300 interactive graphics system (22).

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1. K. Wilson, R. Faber, and B. W. Matthews, unpublished results.
Structural Refinement—The structure of each mutant lysozyme was refined using the TNT package (23). The coordinates of wild-type lysozyme were used as the starting point with the mutant side chain placed initially from inspection of a \((2F_{\text{obs}} - F_{\text{calc}})\) electron density map. In early stages of refinement, the starting model was allowed to relax to conform to the observed mutant crystallographic data. Stereoechemical restraints were then applied to achieve the desired agreement with ideal bond lengths and angles. The refinement statistics are summarized in Table I.

Calculation of Solvent Accessibility—Solvent accessible surface area was calculated by the method of Lee and Richards (24) using the program ACCESS. A rolling water molecule of radius 1.4 Å was used as a probe to determine the area of the van der Waals surface of each atom. Theoretical extended forms of wild-type and mutant lysozymes were obtained by using the program FRODO (22) and setting \(\phi = 135^\circ\) and \(\psi = 135^\circ\) (25).

RESULTS AND DISCUSSION

Structures of Mutant Lysozymes

**Ile-3 \(\rightarrow\) Val**—The structural changes in the mutant lysozymes were initially visualized by calculating maps showing the difference in electron density between the mutant and wild-type structure. Fig. 1A shows the difference in electron density between the I3V (Ile-3 \(\rightarrow\) Val) mutant and wild-type lysozyme. The negative difference density near atom CD1 of the side chain of Ile-3 in wild-type lysozyme indicates the deletion of this atom, confirming that Ile-3 is replaced by valine. Adjacent to the negative density, a smaller positive feature can be seen, suggesting that the side chain of valine in the mutant lysozyme slightly rotates around its CA-CB bond relative to the wild-type isoleucine. In addition to the difference density at residue 3, there is a large positive peak close to the sulfur atom of Cys-97. From experience with different wild-type and mutant crystallographic lysozyme data sets, it is interpreted that this feature is due to disulfide bond formation between Cys-97 and \(\beta\)-mercaptoethanol, a component of the “mother liquor” in which the crystals were grown and equilibrated prior to data collection (see “Experimental Procedures”). Further away from the site of the substitution, the electron density difference map is featureless, indicating that the I3V mutant structure is very similar to wild-type. The crystallographic thermal factors of the atoms in the vicinity of residue 3 are also very similar in the wild-type and I3V mutant structures.

**Ile-3 \(\rightarrow\) Tyr**—In contrast to the I3V mutant, the replacement of Ile-3 with Tyr is associated with substantial changes in the three-dimensional structure. The difference map (Fig. 2A) has a large, positive, disk-shaped feature at residue 3, which corresponds to the side chain of the introduced tyrosine.
FIG. 2. A, electron density difference map for 13Y mutant lysozyme minus wild-type lysozyme. Coefficients, resolution, contour levels of positive and negative density, and conventions are as in Fig. 1A. B, superposition of the refined structures of 13Y (open bonds) and wild-type lysozyme (solid bonds).

Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Wild-type</th>
<th>13V</th>
<th>13Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell dimensions</td>
<td>61.2</td>
<td>61.1</td>
<td>61.0</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>19,200</td>
<td>14,393</td>
<td>14,230</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.7</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>9.1</td>
<td>6.6</td>
<td>7.4</td>
</tr>
<tr>
<td>R (%)</td>
<td>16.7</td>
<td>15.7</td>
<td>15.7</td>
</tr>
<tr>
<td>Δbond (Å)</td>
<td>0.018</td>
<td>0.016</td>
<td>0.017</td>
</tr>
<tr>
<td>Δangle (°)</td>
<td>2.2</td>
<td>2.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Clearly, the side chain is extending “outward” toward the surface of the molecule. In so doing two solvent molecules, 197 and 240, are displaced, as indicated by negative density at these sites. In addition there are many positive and negative features that extend along the backbone from residue 1 to 10 indicating movement of the whole NH2-terminal helix. On the other hand, there is little difference density close to the helix opposite the mutation site (residues 93–106), suggesting that this helix remains essentially fixed.

For this mutant the starting model for crystallographic refinement was built from the wild-type structure by adjusting the configuration of side-chain Tyr-3 as well as the polypeptide backbone of the NH2-terminal helix to fit the (2Fobs - Fcalc) electron density map. The refinement statistics are included in Table I.

As was the case with the 13V mutant, the refined structure of 13Y was rotated and translated to optimize agreement with wild-type lysozyme. The transformation is

\[
X' = 0.99995X + 0.00076Y + 0.01045Z + 0.127
\]

\[
Y' = -0.00067X + 0.99995Y + 0.00948Y + 0.186
\]

\[
Z' = -0.01045X + 0.00947Y + 0.99990Z + 0.249
\]

The root mean square difference in coordinates for backbone atoms is 0.17 Å. Fig. 2B shows the superposition of the structure of 13Y mutant on wild-type lysozyme. The most remarkable feature is that the side chain of Tyr-3 is not accommodated within the interior of protein. Rather, the bulky side chain rotates ~128° so that it is largely exposed to solvent. This configuration of the side chain of Tyr-3 is obviously preferred in terms of protein stability because model building shows that the bulky tyrosine side chain is much too large to be accommodated in the cavity created by the removal of the isoleucine side chain.

The complete removal of the side chain of Ile-3 from the interior of protein would tend to create a cavity adjacent to the hydrophobic core. This is similar to the situation that occurs in the 13Y mutant. It appears that structure of the 13Y mutant rearranges in two ways to partially fill this cavity. First, the cavity is occupied by a bound water molecule (Sol 603 in Fig. 2B) not present in wild-type lysozyme. Secondly, the α-, β-, and γ-carbon atoms of Tyr-3 move 0.7–1.7 Å into the hydrophobic cavity relative to their counterparts in the wild-type structure. In apparent association with these movements, the NH2-terminal polypeptide (residues 1–9) shifts from 0.6 Å to 1.1 Å toward the interior of the protein to improve the packing.

Although the bound water molecule in the 13Y structure occupies an essentially hydrophobic cavity, it nevertheless achieves at least three potential hydrogen bonds, one to the...
participate in normal a-helical hydrogen bonding. In the carbonyl oxygens of a-helices (ZS).

The unit cell dimensions and any other global differences (see text).

tant structures were rotated and translated to eliminate changes in bonding of the water molecule is reminiscent of the crystal mutant; however, these oxygens can accept a second hydrogen bond from the bound water molecule. This hydrogen bond is between Asp OD2 and Tyr 88 OH via a new water molecule (Fig. 2B). Cys-97 and Tyr-3 are both located within a-helices. In the vicinity of residue 3 are indicated by bars. A, I3V mutant; B, I3Y mutant.

carbol oxygen of Cys-97 (2.8 Å), one to the carbonyl oxygen of Tyr-3 (3.3 Å), and one to the sulfur of Met-6 (2.8 Å) (Fig. 2B). Cys-97 and Tyr-3 are both located within α-helices. In the wild-type structure the carbonyl oxygens of these residues participate in normal α-helical hydrogen bonding. In the mutant structure, however, these oxygens can accept a second hydrogen bond from the bound water molecule. This hydrogen bonding of the water molecule is reminiscent of the crystal structures of the apolar helical peptides studied by Karle and Balam and their co-workers (28). Here also water molecules in otherwise strictly hydrophobic environments satisfy their hydrogen-bonding potential by forming hydrogen bonds to the carbonyl oxygens of α-helices (28).

Fig. 3 illustrates the coordinate shifts of the α-carbon atoms of the two mutant lysozymes relative to the wild-type structure. The difference between the two mutants is striking. In the case of I3V the changes are within experimental error, whereas the I3Y mutant shows significant and extensive perturbations of the structure. In addition to the movement of NH2-terminal helix (residues 3–10), another helix (residues 60–79) adjacent to position 3 shifts ~0.6 Å. It appears that

this movement is due to the bulky side chain of Tyr-3 which pushes the helix away. In contrast to the movements of these two helices, a third helix (residues 93–106) that is located on the opposite side of the cavity does not show any significant shift of coordinates. The reason may simply be that the helix (residues 93–106) is the most buried and rigid part of the T4 lysozyme structure.

The crystallographic thermal factors of the atoms in the vicinity of residue 3 in the I3Y mutant structure are very similar to those in the wild-type structure, except for several atoms of Met-6. The thermal factors of SD (23.7 Å²), CG (21.4 Å²), and CB (15.3 Å²) of Met-6 in the I3Y mutant are higher than those of SD (12.6 Å²), CG (7.4 Å²), and CB (6.6 Å²) in the wild-type structure. The side chain of Met-6 in the mutant structure is in contrast with the newly bound water molecule and the increase of thermal motion may reflect looser packing in the I3Y structure. The crystallographic thermal factor of the bound water molecule (41 Å²) is slightly below the average for ordered solvent in T4 lysozyme structure (~45 Å²).

**Intermolecular Contacts in I3Y Mutant Crystal**

Although all of the 13 mutant proteins were subjected to crystallization experiments, these attempts were successful only for the two mutant lysozymes, I3V and I3Y. The apparent reason is that changes at position 3 can disturb intermolecular contacts with a symmetry-related molecule in the crystal. As shown in Table II, residues Met-1, Asn-2, Phe-4, Asp-72, Tyr-88, and Arg-96, which are in the vicinity of position 3, participate in extensive intermolecular contacts. In addition, residue 3 is very close to a crystallographic 2-fold axis (Fig. 4). Therefore it is not surprising that mutations at this site can disrupt the crystallization of the protein.

As described in the previous section, the Ile-3 → Tyr mutation causes large changes in the structure of lysozyme. Nonetheless, isomorphous crystals were obtained with this mutant protein. Table II compares the intermolecular contacts of the wild-type and I3Y mutant lysozymes in the vicinity of residue 3. It is clear from the table that in the I3Y mutant two hydrogen bonds (Met 1 N-Glu 64 OEl  and Asp 72 OD1-Arg 96 NH1) are lost or weakened. However, the I3Y mutant gains other interactions as a result of its structural change. For example, the I3Y mutant has two new hydrogen bonds between Asp OD2 and Tyr 88 OH via a new water molecule (Solvents 600 and 601). The most remarkable feature is that the mutant protein provides an additional, strong intermolecular hydrogen bond (2.7 Å) between the phenolic hydroxyl groups of a pair of symmetry-related Tyr-3 residues in the crystal (also see Fig. 4). Moreover, the phenol ring of Tyr-3 has extensive van der Waals contacts with its 2-fold related symmetry mate. These extra interactions provided by the side chain of Tyr-3 must facilitate the crystallization of this mutant protein.

**Solvent Accessible Surface Area and Protein Stability**

In the previous report (7), it was shown that the conformational stability of mutant T4 lysozymes substituted by 13 different amino acid residues at position 3 is highly correlated with a number of proposed hydrophobicity-scales, such as water-ethanol transfer energy of amino acid residues, except for mutant proteins with Phe, Tyr, and Trp. The reason why the tyrosine mutant, and presumably also phenylalanine and tryptophan, are exceptions is apparent from the observed structure of the I3Y mutant. In this case, the side chain of the tyrosine protrudes into the solvent and remains partly

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2 I. Karle, personal communication.
solvent accessible surface area of protein in a quantitative manner. Since the origin of hydrophobic interactions of a nonpolar atom is thought to arise from the entropy of ordered water molecules that surround it in aqueous solution (29-31), the hydrophobic energy of such an atom should be proportional to its interface area (32-34). Thus, it is possible to estimate the net contribution of hydrophobic energy to the stabilization of a protein from the difference of solvent accessible surface area between the folded (native) and unfolded (denatured) states. While the unfolded state of the protein cannot be accurately modeled, comparisons to a defined reference state, such as a fully extended polypeptide, can be made.

Table III lists the surface areas exposed to solvent for residues Ile-3 and Val-3 in the folded and fully extended structures of the wild-type and 13V mutant, respectively. Also included in the table are the corresponding values for residues alanine and glycine at position 3 in the presumed structures of I3A and I3G mutant lysozymes. In the previous report (7), the free energies of hydrophobic stabilization were calculated by using the coordinates of wild-type lysozyme and deleting the appropriate residue 3 side-chain atoms. In this study, however, the coordinates of the two hypothetical structures were obtained using the coordinates of the 13Y mutant structure. Since the mutations Ile→Ala and Ile→Gly probably create a cavity similar to that seen in the 13Y mutant structure (see Fig. 2), it seems to be more appropriate to infer the structures of I3A and I3G mutant from the 13Y mutant. As shown in Table III, the estimated energies agree very well with the thermodynamically observed difference of stability between the wild-type and mutant lysozymes. The corresponding values deduced from the wild-type structure are also given in Table IV in parentheses. It clearly makes little difference whether the hypothetical structures of I3A and I3G come from wild-type lysozyme or the 13Y mutant. This suggests that the solvent accessible surface area of a buried residue is insensitive to localized changes in three-dimensional structure.

Table III also includes the solvent accessibility values for the 13L and 13Y mutant lysozymes. The 13L mutant has increased thermal stability relative to wild-type lysozyme, and it is therefore of interest to understand why this is the case. Because the 13L mutant protein could not be crystallized, its hypothetical structure was inferred from model building, based on the wild-type structure. In wild-type lysozyme, C2G is the only atom of the side chain of Ile-3 that is partly exposed to solvent. Model building indicates that in the 13L mutant the complete side chain can be located within the interior of the protein without significant steric hindrance. This is also reflected in the calculated decrease in solvent accessibility of the folded form of the 13L relative to wild-type lysozyme, suggesting that the enhanced thermostability of 13L is due at least in part to increased hydrophobic interactions. In the case of the 13Y mutant, however, the calculated hydrophobic energy does not agree with the observed stabilization. The observed free energy of stabilization is about 2 kcal mol⁻¹ less than that expected from the hydrophobic effect alone. Although the
Fig. 4. Stereo view illustrating the intermolecular crystal contact region in the vicinity of residue 3. One lysozyme molecule is drawn with solid bonds, the other with open bonds. The view is along the 2-fold axis of symmetry which passes through the center of each figure. A, wild-type lysozyme; B, I3Y mutant.

exact reasons for this discrepancy are uncertain, it seems that the relatively large perturbation of the I3Y structure, which includes the trapping of a water molecule within the interior of the protein, might offset the stabilization energy due to the hydrophobic interactions.

In the above case, we considered the solvent accessible surface area of only residue 3. However, replacement of this residue may also change the solvent accessible surface areas of adjacent residues in the three-dimensional structure. We therefore extended the calculations to estimate the total change in the solvent accessible surface area of residue 3 plus the 9 surrounding residues. Table IV shows the solvent accessible surface area of each of these residues in the folded (A) and hypothetical extended (A0) form. The surface areas of Asn-2 and Phe-4 in the extended form increase as residue 3 becomes smaller because the deletion of atom(s) of the side chain of Ile-3 makes it possible for water molecules to access residues 2 and 4. The calculated solvent accessible surface areas for the other residues are constant since these residues are far from the residue 3 in the extended model. As for the surface areas in the folded form, slight increases of the area of exposure to solvent are observed for the 9 surrounding residues when Ile-3 is replaced with Val. Also given in Table IV are the corresponding values for alanine and glycine, using coordinates inferred from the I3Y mutant structure. The results inferred from the wild type structure are also shown in parentheses. Table V summarizes the total changes in solvent accessible surface area of residue 3 and the surrounding residues as well as the corresponding change in the free energy of stabilization. On the whole, the results are very similar to those in Table III. The calculated energies inferred from the I3Y mutant structure for Ile, Val, and Ala are uniformly smaller by 0.4 kcal/mol than those inferred from the wild-type structure. This difference comes primarily from the variance in the surface area of the folded form of the Gly-3 mutant.

Conclusions

To date, over 100 mutant lysozymes have been obtained by random and site-directed mutagenesis. Approximately 80% of
TABLE III
Estimation of the hydrophobic free energy of stabilization of T4 lysozyme due to the transfer of residue 3 from a fully extended form to the interior of the folded protein

A$_0$ is the calculated solvent accessible surface area (24) of the residue in a hypothetical fully extended polypeptide with the amino-acid sequence of T4 lysozyme. A is the solvent accessible surface area of residue 3 in the folded protein. The difference in solvent exposure (A$_0$ - A) between the extended (A$_0$) and folded (A) state is taken as an estimate of the area that is buried when lysozyme folds. 

<table>
<thead>
<tr>
<th>Amino acid residue at position 3</th>
<th>A$_0$</th>
<th>A</th>
<th>A$_0$ - A</th>
<th>(A$<em>0$ - A)$</em>{gly}$</th>
<th>$\Delta$G$_{mac}$</th>
<th>$\Delta$G$_{obs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile</td>
<td>151.6</td>
<td>20.5</td>
<td>131.1</td>
<td>86.9</td>
<td>2.1</td>
<td>1.8 - 2.1</td>
</tr>
<tr>
<td>Val</td>
<td>128.7</td>
<td>17.6</td>
<td>111.1</td>
<td>66.9</td>
<td>1.6</td>
<td>1.2 - 1.7</td>
</tr>
<tr>
<td>Ala</td>
<td>86.5</td>
<td>11.0</td>
<td>75.5</td>
<td>31.3</td>
<td>0.8</td>
<td>0.7 - 1.4</td>
</tr>
<tr>
<td>Gly</td>
<td>55.3</td>
<td>11.1</td>
<td>44.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 - 0.0</td>
</tr>
<tr>
<td>Leu</td>
<td>155.3</td>
<td>14.3</td>
<td>141.0</td>
<td>151.4</td>
<td>1.7</td>
<td>-0.2 - -0.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>178.8</td>
<td>69.2</td>
<td>109.6</td>
<td>69.9</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

TABLE IV
Total changes in solvent accessible surface area of residue 3 and the surrounding 9 residues in the lysozyme structure

<table>
<thead>
<tr>
<th>Residues</th>
<th>A$_0$</th>
<th>A</th>
<th>A$_0$ - A</th>
<th>(A$<em>0$ - A)$</em>{gly}$</th>
<th>$\Delta$G$_{mac}$</th>
<th>$\Delta$G$_{obs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn-2</td>
<td>139.0</td>
<td>139.3</td>
<td>142.0</td>
<td>149.0</td>
<td>66.7</td>
<td>69.2</td>
</tr>
<tr>
<td>Residue 3</td>
<td>151.6</td>
<td>128.7</td>
<td>86.5</td>
<td>55.3</td>
<td>20.5</td>
<td>14.8</td>
</tr>
<tr>
<td>Phe-4</td>
<td>178.2</td>
<td>192.8</td>
<td>199.4</td>
<td>(191.4)</td>
<td>77.3</td>
<td>87.7</td>
</tr>
<tr>
<td>Met-6</td>
<td>154.5</td>
<td>154.5</td>
<td>155.0</td>
<td>142.3</td>
<td>66.7</td>
<td>69.2</td>
</tr>
<tr>
<td>Val-71</td>
<td>127.7</td>
<td>127.7</td>
<td>34.9</td>
<td>34.8</td>
<td>34.9</td>
<td>34.8</td>
</tr>
<tr>
<td>Val-75</td>
<td>143.5</td>
<td>143.5</td>
<td>34.9</td>
<td>34.8</td>
<td>34.9</td>
<td>34.8</td>
</tr>
<tr>
<td>Cys-97</td>
<td>112.1</td>
<td>112.1</td>
<td>34.9</td>
<td>34.8</td>
<td>34.9</td>
<td>34.8</td>
</tr>
<tr>
<td>Ile-100</td>
<td>151.4</td>
<td>151.4</td>
<td>9.5</td>
<td>8.7</td>
<td>7.5</td>
<td>9.4</td>
</tr>
<tr>
<td>Asn-101</td>
<td>132.2</td>
<td>132.2</td>
<td>9.5</td>
<td>8.7</td>
<td>7.5</td>
<td>9.4</td>
</tr>
<tr>
<td>Total</td>
<td>1445</td>
<td>1423</td>
<td>1398</td>
<td>1380</td>
<td>229</td>
<td>223</td>
</tr>
</tbody>
</table>

these have been crystallized isomorphously with wild-type lysozyme and over 50 mutant structures studied at high resolution (35). Usually such mutant structures are very similar to wild type except in the immediate vicinity of the mutation site. The I3V mutant obtained in this study can be taken as a typical example. In the case of the I3Y mutant, however, the structural changes are not restricted to the immediate vicinity of residue 3. Associated with rotation of the side chain of the tyrosine, the N-terminal polypeptide (residues 1-9) shifts from 0.6 to 1.1 Å, and the backbone atoms of the adjacent helix (residues 60-79) also move up to 0.7 Å. These structural shifts are more extensive than has been seen in
The mutant lysozyme. The single salt bridge Asp-72..Arg-96 plays a role in stabilizing the protein by interacting with residues around the symmetry axis. Consequently, the general character of the interface is essentially maintained in the crystal structure of the mutant lysozyme. The single salt bridge Asp-72..Arg-96 across the interface seems to be weakened somewhat in the mutant structure (Table II), but the fact that the lysozyme variant in which Arg-96 is replaced with histidine can be crystallized (36) shows that this salt bridge is not a critical interaction. On the other hand residues 157 and 86 are not directly involved in intermolecular contacts, nor are they close to a symmetry axis. This suggests that amino acid substitutions remote from crystal contacts do not inhibit crystallization (35). Substitutions that alter intermolecular contacts, however, may hinder either initiation or maintenance of the crystal lattice. The present study shows that the free energy of hydrophobic interactions estimated from the difference between the solvent accessible surface areas of residues in a fully extended model and the actual protein structure agrees with the observed free energy changes in overall stability of the protein. It should be pointed out, however, that the proportionality constant (24 cal/A²/mol) used here is dependent upon the nature of the organic solvent involved in the transfer process (37). In addition, whether van der Waals interactions in organic solvent are similar to those in protein interior is an arguable point (32). Nonetheless, our results show that a major component of the energy of the solvent-protein interaction can be understood by a simple semiempirical approach.

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