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# Analysis of the Stability of Mutant Lysozymes at Position 15 Using X-Ray Crystallography

**Tadahiro Ohmura, Tadashi Ueda, Hiroyuki Motoshima, Tomohiro Tamura, and Taiji Imoto[1]**

*Graduate School of Pharmaceutical Sciences, Kyushu University 62, Fukuoka 812-82*

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**His 15 of hen lysozyme is located at the protein surface and is partly buried by the neighboring residues. The side chain of His 15 forms hydrogen bonds with surrounding residues and these hydrogen bonds are somewhat buried. A series of mutant lysozymes at the position 15 (Gly, Ala, Val, and Phe) was prepared, and their stabilities were analyzed by GdnHCl denaturation and X-ray crystallography. The mutants were less stable than the wild type at pH 5.5 and 35°C. In H15G and H15A, X-ray crystallography revealed two fixed water molecules at the mutated region, which formed similar hydrogen bonds to those in the wild type. On the other hand, it was suggested that the hydrogen bonds were disrupted and that several unfavorable van der Waals' contacts occurred in H15V and H15F. Therefore, we concluded that His 15 stabilized the lysozyme structure by forming hydrogen bonds and the best packing with the neighboring residues. Moreover, we found that the method of protein stabilization by increasing the hydrophobicity of an amino acid residue was not always effectively applicable, especially when the residue had formed a hydrogen bond.**

**Key words: hen lysozyme, hydrogen bond, hydrophobicity, protein stability, X-ray crystallography.**

A protein usually exists in equilibrium between folded and unfolded states. Its stability is determined by the difference in free energy between these states. Therefore, to stabilize a protein against reversible denaturation, the free energy change for the unfolding should be increased, by stabilizing the folded state by lowering its energy level, or by destabilizing the unfolded state by raising its energy level. Globular proteins usually consist of hydrophilic residues in the exterior surface and hydrophobic residues in the interior of the protein. The burial of hydrophobic residues is generally considered to be the major driving force in protein folding and to play an important role in maintaining the three-dimensional structure. One method of stabilizing protein is to increase hydrophobic interaction in the protein interior, and there are many reports on protein stabilization by increasing hydrophobicity (*1-4*). For example, the relationship between hydrophobicity and stability was elucidated in T4 lysozyme (*1*) and tryptophan synthase $\alpha$-subunit (*2*). Namely, it was shown that the stabilities of proteins increased linearly with increasing hydrophobicity at the mutated residue. However, mutations with aromatic residues such as Trp, Phe, and Tyr deviated from the relationship due to their bulkiness. This was elucidated by analyzing mutants using X-ray crystallography (*1*). Moreover, Malcolm *et al.* (*5*), Jackson *et al.* (*6*), and Buckle *et al.* (*7*) have demonstrated that the contribution of mutation to protein stability would not be easy to understand without elucidating its X-ray structure, because protein stability is often influenced by new interactions such as cavity-creation and unfavorable packing caused by structural changes. Therefore, X-ray crystallography is indispensable to elucidate the relationship between the structure and stability of a protein. However, the crystallization of a protein is a difficult task.

Hen egg white lysozyme, a 129-amino acid protein with four disulfide bonds, has three $\alpha$-helices and three antiparallel $\beta$-strands (Fig. 1). The helix consist of an A-helix (residues 4-15), a B-helix (residues 24-35), and a C-helix (residues 88-100). It is the first enzyme whose three-dimensional structure has been elucidated by X-ray crystallography (*9*), and its crystallization is comparatively easy. For these reasons, hen egg white lysozyme is the best target for research using X-ray crystallography. The His residue at position 15 of hen egg white lysozyme is located at the C-terminus of the A-helix and faces the N-terminus of the C-helix (Fig. 1). The N$\delta$1 and N$\varepsilon$2 atoms in His 15 form hydrogen bonds with the carbonyl-oxygen atom in Ala 11 and the O$\gamma$ atom in Thr 89, respectively, and these hydrogen bonds are somewhat buried. On the other hand, Shih and Kirsch (*10*) demonstrated that the Leu mutant of His 15 is more stable than wild type because of hydrophobic interactions, in spite of the loss of hydrogen bonds. This suggests that the residual hydrophobicity at this position enhances the protein stability.

In this report, we prepared a series of mutant lysozymes

[1] To whom correspondence should be addressed. E-mail: imoto@imm1.phar.kyushu-u.ac.jp

Abbreviations: H15G, mutant lysozyme with His 15 mutated to Gly; H15A, mutant lysozyme with His 15 mutated to Ala; H15V, mutant lysozyme with His 15 mutated to Val; H15F, mutant lysozyme with His 15 mutated to Phe; GdnHCl, guanidine hydrochloride; $\Delta G(H_2O)$, Gibbs free energy of denaturation in the absence of GdnHCl; $\Delta G$tr, the free energy of transfer of an amino acid from water to ethanol or dioxane solutions. The value for Gly is set to zero.

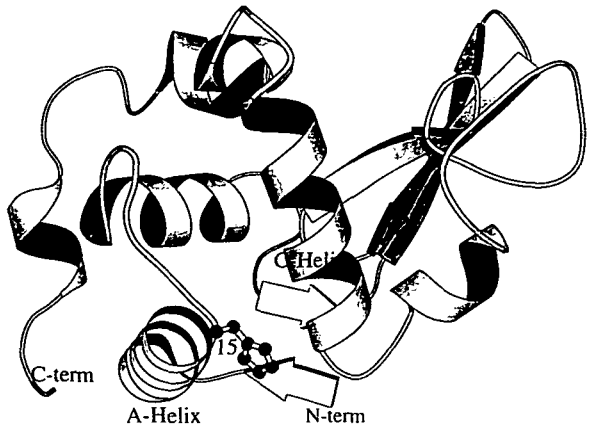


Fig. 1. **Ribbon drawing showing the location of residue 15 in hen egg white lysozyme.** The side chain is shown as a ball-and-stick model. This figure was made with the MOLSCRIPT program (*8*).

at position 15 and analyzed their stabilities with the increase in hydrophobicity of the mutated residues using X-ray crystallography.

## MATERIALS AND METHODS

*Materials*—Restriction enzymes, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were purchased from either Takara Shuzo (Kyoto) or New England Biolabs (Beverly). *Micrococcus luteus* and DNA sequencing kits (Sequenase) were purchased from Sigma and Amersham Japan (Tokyo), respectively. CM-Toyopearl 650M, a cation-exchange resin for the purification of secreted hen lysozymes, was obtained from Tosoh (Tokyo). A Wakopak 5C18 column (mesh 200) was obtained from Wako Pure Chemicals Institute (Osaka). Other chemicals were of analytical or biochemical grade.

*Mutant Lysozymes*—Mutant lysozymes (H15G, H15A, H15V, and H15F) were prepared according to the literature (*11*). The structures of the mutagenic primers used for site-directed mutagenesis to replace His 15 with Gly, Ala, Val, and Phe were 5′-ATGAAGCGTGGCGGACTTG-3′ (sense), 5′-CAAGTCCGGCACGCTTCAT-3′ (anti-sense), 5′-CAAGTCCGACACGCTTCAT-3′ (anti-sense), and 5′-CAAGTCCGAAACGCTTCAT-3′ (anti-sense), respectively. The mutations in the lysozyme gene were confirmed using a DNA sequence analyzer.

*Purification and Identification of Lysozyme Secreted by Yeast*—Each transformant of *Saccharomyces cerevisiae* AH22 was cultivated at 30°C for 125 h for expression and secretion of the respective mutant lysozyme (*11*). Purification (ion-exchange chromatography) and identification (peptide mapping, amino acid sequencing, and amino acid composition) of the lysozymes were carried out as reported previously (*12*).

*Unfolding Equilibrium*—Unfolding equilibrium of lysozymes by GdnHCl was measured at pH 5.5 and 35°C by fluorescence at 360 nm (excited at 280 nm). The protein concentration was $0.9 \times 10^{-7}$ M. The buffer used was 0.1 M sodium acetate adjusted to the pH 5.5 with HCl. It has been found experimentally that the free energy of unfolding of proteins in the presence of GdnHCl is linearly related to the concentration of denatured protein (*13*). Details of the analysis were as described (*14*), and the average $m$ value of the wild type and mutant lysozymes was employed.

*X-Ray Analysis*—Crystallization was carried out using a hanging drops vapor diffusion technique at pH 4.7. Intensity data collection for the wild type and the mutant lysozymes were carried out with an automated oscillation camera system, R-AXIS IIC (RIGAKU), equipped with an Imaging Plate detector, on a Cu rotating anode generator operated at 40 keV and 120 mA at room temperature. Refinement of the structure was carried out using both programs TURBO-FRODO and X-PLOR (*15*) installed on SGI Indigo$^2$. Coordinates for all structures, wild-type, H15G, H15A, H15V, and H15F, have been deposited in the Protein Data Bank (Brookhaven National Laboratory) with codes 1UIG, 1UIE, 1UIC, 1UIF, and 1UID, respectively.

## RESULTS

*Stabilities of the Wild Type and the Mutant Lysozymes Determined with GdnHCl Denaturation*—We designed four mutants where His 15 is mutated to Gly, Ala, Val, and Phe, respectively. The mutations were confirmed by DNA sequencing and amino acid sequencing of the peptides obtained by digestion with lysylendopeptidase (data not shown). Table I shows the results of the stability measurements of the wild type and mutant lysozymes (H15G, H15A, H15V, and H15F). The value of $\Delta G$tr, an indicator of the hydrophobicity of amino acid residues obtained by Nozaki and Tanford (*16*), is also shown in Table I. All mutant lysozymes were less stable than the wild-type. Plots of $\Delta G(H_2O)$ in wild type and mutant lysozymes against $\Delta G$tr are shown in Fig. 2. A linear relationship between $\Delta G$tr and $\Delta G(H_2O)$ was not found.

*X-Ray Structures of the Wild Type and the Mutant Lysozymes*—The crystallographic data of the wild type and the mutant lysozymes are shown in Table II. The resolution of each sample was 1.95 Å (1.85 Å for H15V), $R$-merge was less than 8%, and data completeness was above 88%. The refinement of the wild type was carried out using coordinate 1HEL (*5*), and those of the mutant lysozymes were carried out using the wild type structure obtained here. As a result of the refinement, $R$-factors fell to about 17%. From the wild type structure, it is verified that the nitrogen atoms (N$\delta$1 and N$\varepsilon$2) of His 15 form hydrogen bonds with the carbonyl-oxygen atom of Ala 11 (2.7 Å) and the O$\gamma$ atom of Thr 89 (2.8 Å), respectively. There were no differences in the global structure between the wild type and the mutant lysozymes (from RMS deviation plots in Fig. 3), and the mutants also had lysozyme activity comparable to the wild type enzyme (above 83%) (*17*). Figure 4 shows the structures around position 15 in each mutant lysozyme. The local structures around position 15 in H15A and H15G were very similar to that of the wild type. Those in H15V and H15F were slightly altered due to the difference in size of substituted residues. In H15V, structural changes were observed in the main chain around position 15 and the side chain of Val 92. These changes may be considered to be caused by C$\gamma$2 atom of Val 15. On the other hand, in H15F, the structure around position 15 was expanded by the substitution of a bulky Phe residue. Table III shows all atoms within 3.4 Å from the substituted side chain atoms in H15V and H15F. In these mutants, there were several atoms with shorter distances than the standard ones. Val 92

TABLE I. **GdnHCl denaturation of wild type and mutant lysozymes at pH 5.5 and 35°C.**

| Lysozyme | $C_{1/2}$[a] (M) | $\Delta G(H_2O)$[b] (kcal/mol) | $\Delta Gtr$[c] (kcal/mol) |
|---|---|---|---|
| Wild-type | 3.62 | 10.1 | 0.5 |
| H15G | 3.34 | 9.3 | 0 |
| H15A | 3.56 | 9.9 | 0.5 |
| H15V | 3.15 | 8.8 | 1.5 |
| H15F | 3.48 | 9.7 | 2.3 |

[a]Deviations are within 0.03. [b]Deviations are within 0.12. [c]Values are from Nozaki and Tanford (*16*).

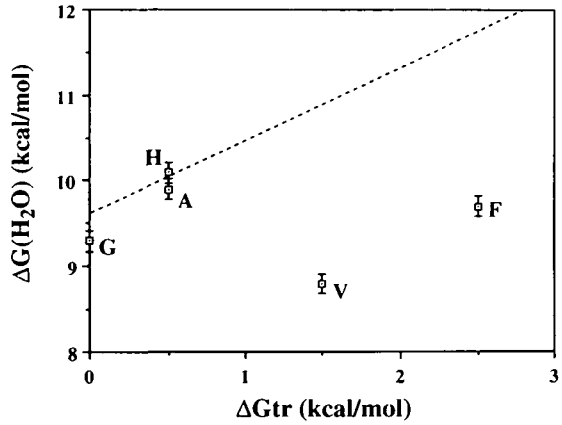


Fig. 2. **Plots of $\Delta G(H_2O)$ in wild type and mutant lysozymes against $\Delta Gtr$.** The dotted line shows the line of slope 0.81. See "DISCUSSION" for the dotted line.

TABLE II. **Crystallographic data collection and refinement statistics.**

| Lysozyme | Wild-type | H15G | H15A | H15V | H15F |
|---|---|---|---|---|---|
| Data collection | | | | | |
| Cell dimensions (Å) | | | | | |
| $a=b=$ | 79.18 | 79.44 | 79.56 | 79.07 | 78.52 |
| $c=$ | 37.96 | 37.76 | 37.84 | 38.00 | 38.44 |
| Resolution (Å) | 1.95 | 1.95 | 1.95 | 1.85 | 1.95 |
| Unique reflections ($F \geq 1\sigma[F]$) | 8,543 | 8,629 | 8,690 | 9,431 | 8,089 |
| Completeness (%) | 92.1 | 93.2 | 93.8 | 88.4 | 89.4 |
| $R$-merge[a] (%) | 4.96 | 5.76 | 5.16 | 5.03 | 7.73 |
| Refinement | | | | | |
| $R$-factor[b] (%) | 16.6 | 17.0 | 16.9 | 17.4 | 16.7 |
| $\Delta$bond length (Å) | 0.009 | 0.010 | 0.009 | 0.009 | 0.009 |
| $\Delta$bond angle (°) | 1.445 | 1.467 | 1.501 | 1.531 | 1.496 |

[a]$R$-merge $= (\Sigma(I - \langle I \rangle)/\Sigma I)$. [b]$R$-factor $= (\Sigma(F_o - F_c)/\Sigma F_o)$.

moved to the exterior in H15G and to the interior in H15V (Fig. 4, A and C). Interestingly, in H15G and H15A (Fig. 4, A and B), two water molecules were observed in the cavity where the His side chain had been located in the wild type. On the other hand, there was no water molecule around residue 15 in H15V and H15F (Fig. 4, C and D). The solvent-accessible surface area of residue 15 in all mutant lysozymes (H15G: 25 Å$^2$, H15A: 25 Å$^2$, H15V: 22 Å$^2$, H15F: 36 Å$^2$) was almost the same as that of the wild type (30 Å$^2$).

## DISCUSSION

RMS deviation (Fig. 3) between the wild type and the

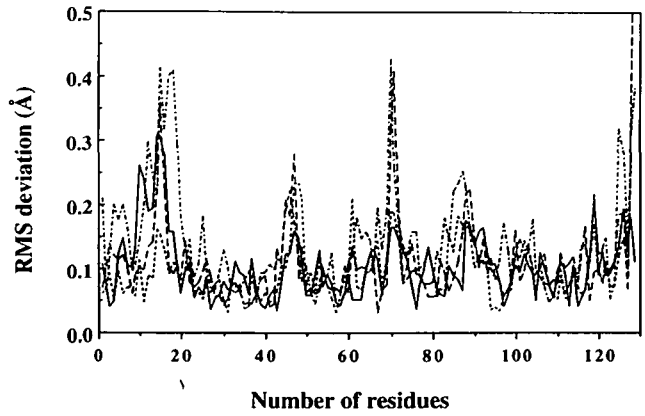


Fig. 3. **Plots of the RMS deviation of main chain for wild type *versus* H15G (—), H15A (·····), H15V (----), and H15F (–·–).**

TABLE III. **Contact distances for substituted side chains at residue 15.**

| Closest protein atoms | Side-chain atom and distance (Å) | | | |
|---|---|---|---|---|
| | H15V | | H15F | |
| Met 12 O | Cγ2 | 3.06 | – | – |
| Ala 11 O | – | – | Cδ1 | 2.75 |
| Arg 14 Nη2 | – | – | Cζ | 3.27 |
| Thr 89 Oγ | – | – | Cε2 | 3.32 |
| | – | – | Cζ | 3.20 |

mutant lysozymes indicated that their overall structures were the same, and that differences in stability between them may depend on limited structural changes around the mutated region. A Leu residue is conserved at position 15 in most chicken-type lysozymes, but other amino acid residues such as Ser, His, Met, Asn, Tyr, Phe, and Glu (*10, 18-20*) are also present. Shih and Kirsch measured the stability of a mutant lysozyme where His 15 in hen lysozyme is replaced with Leu (*10*). Although they did not solve the structure of this mutant, they stated that hydrophobic interactions enhanced the protein stability by referring turkey lysozyme structure (*10*). This fact indicates that hydrophobicity contributes to stability at this position. Matsumura *et al.* (*1*) and Yutani *et al.* (*2*) have demonstrated a linear correlation between an increase in hydrophobicity at the mutated position and an increase in stability. However, we could not find a linear correlation. As the accessibility at position 15 is almost equal to that at Ile 3 in T4 lysozyme (about 20%), we hypothesize that the hydrophobic effect at position 15 in hen egg white lysozyme should be similar to that at Ile 3 in T4 lysozyme. When we drew in Fig. 2 the straight line of slope 0.81 that was obtained for the correlation at Ile 3 in T4 lysozyme, H15V and H15F were less stable by about 2 kcal/mol than the corresponding points on the line.

Pakula and Sauer have suggested that a hydrophobic residue located on the protein surface causes a reverse hydrophobic effect (*21*). We should consider a reverse hydrophobic effect here, since His 15 in hen lysozyme is located on the protein surface. As mentioned above, solvent accessibility of residue 3 in T4 lysozyme was reported to be around 20%, almost the same as that of His 15 in hen lysozyme. Because a reverse hydrophobic effect was not considered for residue 3 in T4 lysozyme, a residue partially

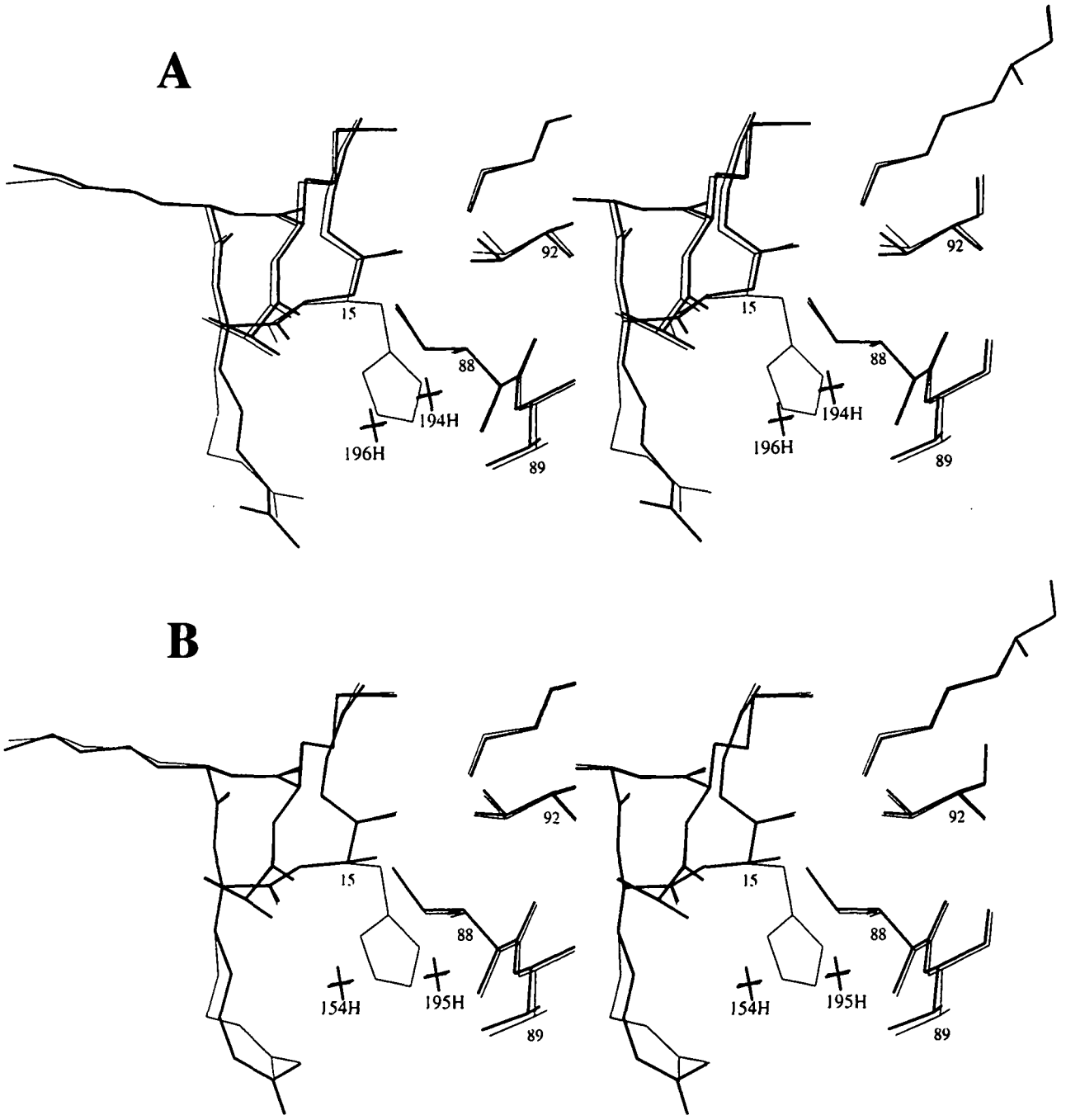


Fig. 4, A and B

exposed to solvent in a protein may not always cause a reverse hydrophobic effect. Moreover, we found from X-ray structures that the solvent-accessible surface areas of residue 15 in mutant lysozymes employed here were almost the same as each other. Therefore, even if a reverse hydrophobic effect occurs at residue 15 in hen lysozyme, it would appear to be similar in all mutants employed here.

In the wild type structure, two nitrogen atoms (N$\delta$1 and N$\varepsilon$2) of the imidazole ring of His 15 form hydrogen bonds with the carbonyl-oxygen atom of Ala 11 and the O$\gamma$ atom of Thr 89, respectively. In the mutants, these hydrogen bonds are lost due to the replacement of the His residue. Interestingly, however, X-ray structural analysis revealed that two water molecules were fixed to form hydrogen bonds with neighboring residues in H15G and H15A. Alber *et al.* (*22*) have demonstrated from analysis of the stabilities and structures of mutants of T4 lysozyme that the preservation of hydrogen bonds is important for protein stability, even if these bonds involve water molecules. Therefore, the stabilization by the hydrogen bonds involving two water molecules in H15G and H15A may be equivalent to the stabilization by the hydrogen bonds involving His 15 in the wild type. The destabilization in H15G and H15A, on the other hand, may be explained

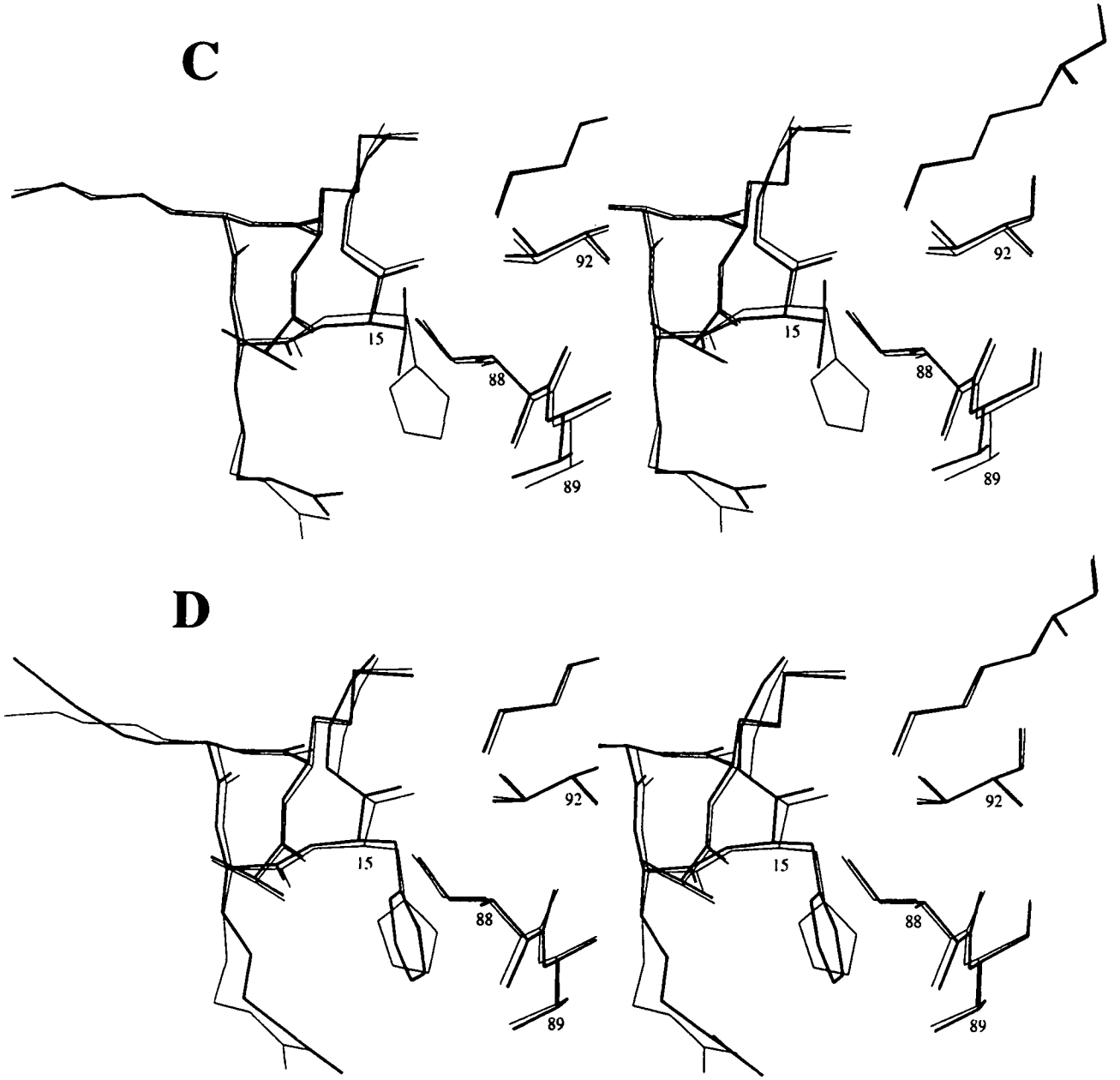


Fig. 4. **Stereo view showing the superposition of the structure of wild type (thin line) and mutant lysozymes (thick line).** A: H15G, B: H15A, C: H15V, D: H15F. Residues around the mutation are shown.

solely by the decrease in hydrophobicity at position 15. X-ray structural analysis showed that H15V and H15F did not have the water molecules as in H15A and H15G, and therefore they lost the hydrogen bonds of the wild type. We consider that the instability of H15V and H15F can be attributed to the loss of these hydrogen bonds.

In general, the distance between a carbon atom and an oxygen atom and between a carbon atom and a nitrogen atom is about 3.22 and 3.25 Å, respectively (*23*). Karpusas *et al.* (*4*), who designed cavity-filling mutants in T4 lysozyme, noted that several unfavorable interactions produced by mutation caused instability. In H15V, the shorter distance (3.06 Å in Table III) between the C$\gamma$2 atom of Val 15 and the carbonyl-oxygen atom of Met 12 may cause instability of lysozyme molecule due to unfavorable van der Waals' contact. In H15F, the shorter distance (2.75 Å in Table III) between the C$\delta$1 atom of Phe 15 and the carbonyl-oxygen atom of Ala 11 may cause instability of lysozyme molecule due to unfavorable van der Waals' contact. On the other hand, the distances between the C$\zeta$ atom of Phe 15 and the O$\gamma$ atom of Thr 89, the C$\varepsilon$2 atom of Phe 15 and the O$\gamma$ atom of Thr 89, and the C$\zeta$ atom of Phe 15 and the N$\eta$2 atom of Arg 14 were 3.20, 3.32, and 3.27 Å, respectively (Table III). Because there is little difference between the latter three distances and the allowed distances, we concluded that these contacts had little effect on the stability of the lysozyme molecules. Thus, we concluded that the instabilities of H15V and H15F

were due to the loss of hydrogen bonds and unfavorable van der Waals' contacts.

Previous reports (*1, 2, 4*) have noted that the stability resulting from the mutation to a Phe residue often deviated from the linear correlation between stability and hydrophobicity due to the bulkiness of the residue, whereas the stability resulting from the mutation to a Val residue fitted the correlation. In this sense, the present findings differed from the previous data. Val 92 is located near position 15 in lysozyme. Although there was little structural change at Val 92 in H15A, a slight structural change occurred at Val 92 in H15V. This indicates that the position of the C$\beta$ atom of residue 15 is crowded, as are other part of the protein, and the $\beta$-branch in this position causes steric hindrance in hen lysozyme. This idea is consistent with the finding that there is no $\beta$-branched amino acid (Val, Ile, and Thr) at residue 15 in chicken-type lysozymes. From the above results, we concluded that His 15 stabilizes lysozyme structure by the formation of hydrogen bonds and by allowing the best packing.

This report has investigated the effect of hydrophobicity at a pocket of the protein surface using a series of mutant lysozymes and X-ray crystallography. Although no simple correlation between hydrophobicity and stability was found at this position, interesting results were obtained. Namely, in H15G and H15A, two fixed water molecules were observed at the location of the His side-chain in the wild type, and these formed hydrogen bonds. These hydrogen bonds were suggested to compensate for the reduction in stability resulting from the breakage of the wild-type hydrogen bonds. As reported previously (*1, 2*), the mutation of an amino acid residue to a more hydrophobic one provides a convenient method of stabilizing a protein. However, we found that this rule was not applicable to some residues, such as those which had formed hydrogen bonds. Therefore, we should pay closer attention to the environment of the target residue.

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