Positive Contribution of Hydration Structure on the Surface of Human Lysozyme to the Conformational Stability*

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Water molecules make a hydration structure with the network of hydrogen bonds, covering on the surface of proteins. To quantitatively estimate the contribution of the hydration structure to protein stability, a series of hydrophilic mutant human lysozymes (Val to Ser, Tyr, Asp, Asn, and Arg) modified at three different positions on the surface, which are located in the α-helix (Val-110), the β-sheet (Val-2), and the loop (Val-74), were constructed. Their thermodynamic parameters of denaturation and crystal structures were examined by calorimetry and by x-ray crystallography at 100 K, respectively. The introduced polar residues made hydrogen bonds with protein atoms and/or water molecules, sometimes changing the hydration structure around the mutation site. Changes in the stability of the mutant proteins can be evaluated by a unique equation that considers the conformational changes resulting from the substitutions. Using this analysis, the relationship between the changes in the stabilities and the hydration structures for mutant human lysozymes substituted on the surface could be quantitatively estimated. The analysis indicated that the hydration structure on protein surface plays an important role in determining the conformational stability of the protein.

Water is the natural medium for protein molecules and has a significant influence on the dynamics, stability, and function of the molecules (1, 2). These water molecules produce the hydration structure on the protein surface by forming hydrogen bonds with protein atoms and/or other water molecules (3, 4). Because most of the ordered water molecules are found to interact with protein atoms, it is believed that the hydration structure is almost an integral part of the protein (5). The hydration structures might affect protein stability, but the degree that the hydration structures contribute to protein stability has been unknown.

X-ray crystallography is one of several useful techniques for investigating the hydration structures of proteins. The hydration structure of proteins has been investigated by crystallographic experiments at ambient temperature (6–8). However, most of the crystallographic studies at ambient temperature have been concerned with only stable hydration structures. The mobile hydration water molecules show no appreciable peaks in the scattering density maps. A recent cryogenic method (9–12) has provided detailed and systematic analyses of entire hydration structures (3). The cryogenic crystal structures reveal the details of the hydration structures resulting from the decrease in the thermal vibrations at low temperature (3, 13).

As the first step in understanding the contribution of the hydration structure to protein stability, the relationship between the changes in stability and hydration structure resulting from the amino acid substitution on the protein surface, measured by physicochemical experiments and cryogenic x-ray analysis, respectively, should be elucidated. Mutagenesis studies have shown that the contribution of the substitutions on the surface position to the protein stability is not negligible, but on the average is somewhat smaller than in the interior (14–16). However, because an amino acid substitution affects the contribution of not only the hydration structure but also various stabilization factors to the conformational stability, the contribution of the hydration structure to protein stability cannot be simply estimated. In fact, the contributions of even the same kind of substitutions on the surface of proteins to their stabilities have been changed depending on the environment of the mutation sites (14). Considering these facts, systematic surveys are necessary to understand the role of the hydration structure. Human lysozyme (130 residues), which has been extensively examined (17), is a good model protein for the study of systematic mutants. The contributions of several stabilization factors to the stabilities of mutant human lysozymes have been evaluated by a unique equation considering the conformational changes caused by the substitutions (18–20).

In this study, three different positions (Val-2, Val-74, and Val-110) on the surface of the human lysozyme were focused on. These positions are located in different secondary structures (β-sheet, loop, and α-helix, respectively), and 72, 75, and 71% of the residues are exposed, respectively. In our previous study (19), the hydrophobic mutants (Val to Gly, Ala, Ile, Leu, Met, or Phe) substituted at these three positions have been examined. The results have shown that the local hydration structures of the mutant proteins, the stability changes of which cannot be explained by the contribution of several stabilization factors, were significantly affected by the substitutions. To quantitatively evaluate the contribution of the hydration structures, a series of hydrophilic mutants replaced with Ser, Tyr, Asp, Asn, or Arg at Val-2, Val-74, and Val-110, were constructed. The thermodynamic parameters for denaturation of the mutant proteins were determined using differential scanning calorimetry, and crystal structures were examined by x-ray crystallography at 100 K, respectively. The introduced polar residues made hydrogen bonds with protein atoms and/or other water molecules (3, 4).

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The atomic coordinates and structure factors (code 1GF8 (V2S), 1GF9 (V2Y), 1GFA (V2D), 1GF9 (V2N), 1GF9 (V2R), 1GFH (V74Y), 1GFJ (V74D), 1GFK (V74N), 1GFR (V74R), 1GFT (V110Y), 1GFU (V110D), 1GFV (V110N), and 1INU (V110R)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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scanning calorimetry (DSC), and the crystal structures were determined by cryogenic x-ray analysis at 100 K. Various changes in the hydration structure resulting from the interaction between these polar residues and water molecules around the mutation sites were observed. The role of surface hydrophilic residues and hydration structures in the conformational stability of a protein will be discussed along with the changes in the stability and structure caused by the substitution.

EXPERIMENTAL PROCEDURES

**Mutant Proteins**—The mutagenesis, expression, and purification of the mutant human lysozymes were performed as described previously (21). Only one mutant, V110S, could not be obtained because there was no occurrence during the transformation of the yeast. The protein concentration was spectrophotometrically determined using $E^{1%}_{280} = 25.69$ at 280 nm for the mutant human lysozymes (22), except for the Tyr-substituted mutants. The concentration of the Tyr mutant proteins was spectrophotometrically determined using $E^{1%}_{280} = 26.59$ at 280 nm with a correction for the increase in the molar absorption coefficient of Tyr (23).

**DSC**—Calorimetric measurements and data analyses were carried out as described previously (21). For the measurements, a DASM4 adiabatic microcalorimeter equipped with an NEC personal computer was used. The sample buffer for measurements was 0.05 M Gly-HCl. Each protein was measured from three to five times at different pH points between pH 2.6 and 3.5. At each condition, one measurement was done. The data analysis was done using Origin software (MicroCal, Northhampton, MA). The thermodynamic parameters for denaturation of the wild-type protein at pH 2.7 were calculated using Equations 1–3. WT, wild type.

### Table I

| Residues | Position 2 | Position 74 | Position 110 |
|----------|------------|-------------|--------------|
| pH       | $T_d$      | $\Delta H$  | $\Delta H$  |
|          | °C         | kJ mol⁻¹     | kJ mol⁻¹     |
| Ser      | 2.65       | 59.4        | 431          |
|          | 2.83       | 62.9        | 452          |
|          | 3.09       | 67.1        | 481          |
|          | 3.24       | 69.4        | 498          |
|          | 3.45       | 73.0        | 519          |
| Tyr      | 2.83       | 65.9        | 452          |
|          | 2.91       | 67.4        | 465          |
|          | 3.20       | 72.3        | 498          |
|          | 3.21       | 72.0        | 502          |
|          | 3.24       | 73.0        | 507          |
| Asp      | 2.66       | 59.6        | 431          |
|          | 2.96       | 64.9        | 469          |
|          | 3.01       | 65.7        | 473          |
|          | 3.14       | 68.0        | 485          |
|          | 3.30       | 70.2        | 502          |
|          | 3.01       | 66.3        | 490          |
|          | 3.22       | 69.3        | 506          |
| Arg      | 2.60       | 62.1        | 431          |
|          | 2.88       | 66.4        | 452          |
|          | 2.99       | 68.6        | 469          |
|          | 3.15       | 70.7        | 477          |
|          | 3.22       | 74.1        | 502          |
| Val (WT) | 64.9 ± 0.5 | 6.6 ± 0.5   | 477 ± 4      |
| Ser      | 60.5 ± 0.2 | 5.5 ± 0.1   | 465 ± 2      |
| Tyr      | 63.7 ± 0.2 | 7.6 ± 0.4   | 444 ± 3      |
| Asp      | 60.4 ± 0.2 | 6.6 ± 0.2   | 466 ± 2      |
| Asn      | 60.8 ± 0.2 | 7.1 ± 0.3   | 476 ± 3      |
| Arg      | 63.6 ± 0.4 | 5.9 ± 0.3   | 445 ± 3      |

**a** The error is ±0.1 °C.

**b** The error is ±13 kJ mol⁻¹.

**c** The data for this mutant have been measured (28).

**d** This mutant protein was not obtained (see “Experimental Procedures”).

### Table II

| Residue | Position 2 | Position 74 | Position 110 |
|---------|------------|-------------|--------------|
| pH      | $T_d$      | $\Delta H$  | $\Delta H$  |
|         | °C         | kJ mol⁻¹     | kJ mol⁻¹     |
| Val (WT) | 64.9 ± 0.5 | 6.6 ± 0.5   | 477 ± 4      |
| Ser      | 60.5 ± 0.2 | 5.5 ± 0.1   | 465 ± 2      |
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**a** $\Delta C_p$ was obtained from the slope of $\Delta H$ vs. $T_d$.

**b** $\Delta G = \Delta G$(mutant) − $\Delta G$(wild). The minus value indicates the destabilization of mutant protein.

**c** Takano et al. (28).
X-ray Structural Analysis—The mutant human lysozymes were crystallized as described elsewhere (21, 25). All crystals belong to space group P2₁,2₁,2₁ with a crystal form identical to that of the wild type and of most mutant proteins. The intensity data were collected at 100 K by the oscillation method on a Rigaku RAXIS IV imaging plate mounted on a Rigaku RU300 for the other mutants. Their structures were solved by the isomorphous method using the program X-PLOR (27) as described previously (21, 25). The data set for V74S has been collected, and its structure has been solved at 100 K (28).

Detection of solvent molecules was done using the program FLAPPER (21), as described previously (19). The criteria for selecting solvent molecules were to have hydrogen bonding geometry contacts of 2.4–3.5 Å with protein atoms or with the existing solvent, excluding contacts to carbon atoms within 3.2 Å, to have temperature factors of less than 0.2 and 0.3 Å², respectively. Many of the detected water molecules interacted with the protein main and side chains relative to the wild-type structure. In most cases, however, the large enthalpy changes were offset by the entropy changes. Therefore, under the existing circumstances, no correlation between the changes in enthalpy and structural changes upon mutation was found. For instance, the ∆H value of V110D was the smallest among the mutants in this study, 386 kJ/mol, whereas that of the wild-type protein was 477 kJ/mol (Table II). This unfavorable enthalpy term might be compensated by the favorable entropy term because this mutant protein was stabilized by 0.7 kJ/mol as a result of the substitution. These quite large changes in entropy and enthalpy might be caused by the change in the structural features, but the structural change in this mutant was quite small.

Structure of Mutant Human Lysozymes—The data collection and refinement statistics for the hydrophilic mutant human lysozymes are summarized in Table III. As described above, all of the intensity data were collected at 100 K. Although the wild-type structure at 100 K seemed to be the same fold as the wild-type structure at 283 K, the B-factors for the protein atoms and the water molecules at 100 K were significantly smaller than those at 283 K (19), as previously reported (29). All structures of the mutant proteins were similar to the wild-type structure; the root mean square differences in the main chains and side chains relative to the wild-type structure were less than 0.2 and 0.3 Å, respectively.

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protein atoms and each other via hydrogen bonds. The hydration water molecules formed aggregates of various shapes and dimensions, and some of the aggregates even covered the hydrophobic residues by forming oligomeric network arrangements (3, 30). The total number of water molecules observed in the crystal structures (Table III) and the conservation of hydration water molecules between the wild-type and mutant human lysozymes (see “Experimental Procedures”) were different to a certain extent among the mutant crystals. This may reflect the original crystal quality and the difference of the quenching of hydration water molecules by a flash cooling. These differences were mostly observed in the regions apart from protein surface. Nakasako (3) has reported using trypsin crystals that most hydration sites in the room temperature structure are occupied by water molecules at 100 K and the hydration sites in the crystal contact area are poorly conserved in three different crystal forms. Nakasako suggests that the molecular packing in the crystallization process artificially produced the hydration sites in the contact area. Around the residues 2, 74, and 110 in human lysozyme crystals where they are not directly involved in crystal contacts, the number of water molecules was not significantly different unless the residue was substituted. There were 24, 22, and 32 water molecules within 10 Å from Ca atom of the residues 2, 74, and 110, respectively, in the wild-type structure, and 24.0 ± 1.1, 22.2 ± 1.5, and 32.5 ± 1.5 water molecules on the average within 10 Å from Ca atom of the residues 2, 74, and 110, respectively, in unrelated mutant structures, V74X, V110X, and V2X, respectively, where X means G, A, I, L, M, F, S, Y, D, N, and R (19).

On the other hand, the number of water molecules around the residues 2, 74, and 110 are 20–26 in V2X, 19–25 in V74X, and 30–34 in V110X, respectively. Analyzing the conservation of hydration sites shows that 73, 73, and 77% of the total water molecules around the residues 2, 74, and 110 (within 10 Å from Ca atom), respectively, in the wild-type human lysozyme structure, were conserved in unrelated mutant structures, V74X, V110X, and V2X, respectively, on the average, and totally the number of water molecules is almost same. When these residues were substituted, however, 58, 60, and 63% of the water molecules around each mutation site in the wild-type crystal were occupied by water molecules in the V2X, V74X, and V110X crystals, respectively, on the average. These results show that discussions about the hydration water molecules around the residues 2, 74, and 110 in human lysozyme crystals are possible and useful.

The structures of the Val-2, Val-74, and Val-110 mutants in the vicinity of the mutation sites are illustrated in Figs. 1, 2, and 3, respectively. (i) Val-2 Mutants—In the wild-type structure (Fig. 1a), Oδ1 of Asn-39 forms a hydrogen bond with the N of Lys-1 via a water molecule, and Nε of Asn-39 forms a hydrogen bond with a water molecule, which is the end of the hydration structure covering the side chain of Val-2. In the mutant structures, all the introduced polar side chains formed the hydrogen bond(s) with the protein atoms or water molecules. The side chains of Tyr-2 in V2Y, Asp-2 in V2D, Asn-2 in V2N, and Arg-2 in V2R formed hydrogen bonds with water molecules, resulting in changes in the hydration structure covering residue 2 (Fig. 1, (a)).
The side chains of Ser-2 in V2S and Asp-2 in V2D also formed a hydrogen bond with that of Asn-39, destroying the hydrogen bonds between O\textsubscript{81} of Asn-39 and N of Lys-1 via the water molecule (Fig. 1, b and d).

(ii) Val-74 Mutants—All of the polar residues introduced at position 74 formed hydrogen bond(s) with water molecule(s), not with the protein atoms (Fig. 2). However, these polar residues did not substantially change the hydration structure around residue 74. The hydration structure and hydrogen bond networks changed slightly.

(iii) Val-110 Mutants—The substituted polar residues at position 110 hardly affected the hydration structure around there (Fig. 3). The side chains of Asp-110 in V110D and Asn-110 in V110N formed hydrogen bonds (Fig. 3, c and d). In the case of V110Y and V110R, however, the side chains of the introduced polar residues, Tyr-110 and Arg-110, respectively, did not form any hydrogen bonds in their crystal structures (Fig. 3, b and e), suggesting that these residues interact with mobile water molecules, which could not be detected in the crystal structure analysis.

**DISCUSSION**

**Estimation of the Stabilities for the Mutant Proteins Substituted at Three Different Exposed Positions**—The network of hydrogen bonding formed the hydration structure. It might be apparent that changes in the hydration structure contribute to protein stability. To understand the effect on protein stability caused by changes in the hydration structure, it is necessary to estimate the contributions of the changes in various factors resulting from the substitutions to the protein stability and subtract these contributions from the experimental results. These stabilization factors have been studied using mutant proteins in which the substitutions would affect each stabilization factor of the proteins (31–40). However, the same types of substitutions have given different results, depending on the differences in the environments surrounding the substitution residues and structural changes because of the mutation (21, 41–46). Recently, it has been proposed that the changes in stability of each mutant human lysozyme are represented by a unique equation, considering the conformational changes caused by the mutations (18, 19). In these studies, by a least-squares fit of the experimental Gibbs energy changes upon denaturation (\(\Delta G\text{\textsubscript{exp}}\)) of 54 mutant human lysozymes to the equation, the contribution of the major stabilization factors, such as the hydrophobic effect, hydrogen bonding in the interior of the protein, water molecules introduced in the interior of the protein, and propensity of the secondary structure, to protein stability has been estimated. The difference in the Gibbs energy changes upon denaturation between the wild-type and mutant proteins (\(\Delta G\)) is expressed by Equation 4 (18, 19).

\[
\Delta G = \Delta G_{\text{HP}} + \Delta G_{\text{conf}} + \Delta G_{\text{HB}} + \Delta G_{\text{H2O}} + \Delta G_{\text{pro}} - \alpha \Delta \Delta \text{ASA}_{\text{NP}} \\
+ \beta \Delta \Delta \text{ASA}_{\theta} - T \Delta \Delta S_{\text{conf}} + \gamma \nu_{\text{HB}}^2 + \delta \Delta N_{\text{H2O}} + \varepsilon_{\text{pro}} \Delta \text{pro}_{\text{adj}} + \varepsilon_{\theta} \Delta \text{pro}_{\theta}
\]  

(Eq. 4)

\(\Delta G_{\text{HP}}, \Delta G_{\text{conf}}, \Delta G_{\text{HB}}, \Delta G_{\text{H2O}}, \text{and } \Delta G_{\text{pro}}\) represent the changes in \(\Delta G\) resulting from the hydrophobic effect, the conformational entropy of the side chain at the mutation site, the formation and removal of hydrogen bonding in the interior of the protein, the introduction of water molecules in the interior of the protein, and the propensity of the secondary structure of the protein, respectively.
The substituted residue, respectively; ΔASA_{NP} and ΔASA_{P} represent the differences in the ASA (accessible surface area) of the non-polar (C/S) and polar atoms (N/O) of all residues in a protein upon denaturation, respectively; ΔΔASA means the difference in ΔASA between the wild-type and each mutant protein; S_{conf} is the conformational entropy defined by Doig and Sternberg (47); r_{HB} is the length of the hydrogen bonds; ΔN_{H2O} is changes in the number of water molecules introduced by the substitutions; and pro[α] and pro[β] are the α-helix and the β-sheet propensities, respectively, of the residue defined by Chou and Fasman (48) (revised by Koehl and Levitt (Ref. 49)). The parameters in Equation 4 are α = 0.178 kJ mol⁻¹ Å⁻², β = -0.013 kJ mol⁻¹ Å⁻², γ = 15.53 kJ Å⁻¹ mol⁻¹, δ = -7.79 kJ mol⁻¹, ε_{α} = 5.07 kJ mol⁻¹, ε_{β} = 2.32 kJ mol⁻¹ (18, 19).

The ΔG values of a series of the mutant proteins substituted at three different exposed positions can be estimated using the parameters of Equation 4 and the structural information for mutant proteins obtained by x-ray analysis. First, the contribution of the hydration structure and hydrogen bonds formed by the introduced polar residue to protein stability were not included in the estimated ΔΔG values, because the contribution of the hydrogen bond on the surface of the protein has been unknown and it is apparently different from that in the interior. ΔΔG_{H2O} and ΔΔG_{H2O} in Equation 4 represent the contributions of the hydrogen bond and introduced water molecule, respectively, in the interior of the protein. In this case, ΔΔG_{H2O} and ΔΔG_{H2O} were assumed to be zero. The ΔS_{conf} values were corrected corresponding to the degree of exposure of the substituted residue calculated from the crystal structure of each mutant. Fig. 4a shows a correlation between the ΔG (ΔΔG_{exp}) measured and ΔG (ΔΔG_{est}) estimated from Equation 4 using the above parameters for the mutant human lysozymes. The crosses are 54 mutant human lysozymes used for the determination of each coefficient in Equation 1 (18, 20, 21, 25, 44–46, 50, 51). The largest deviation between ΔΔG_{exp} and ΔΔG_{est} among these 54 mutant proteins was less than 5 kJ/mol (S.D. = 2.7 kJ/mol). The solid and open circles represent the hydrophilic mutants (Val to Ser, Tyr, Asp, Asn, and Arg) examined in the present study and the hydrophobic mutants (Val to Gly, Ala, Ile, Leu, Met, and Phe) (19), respectively, substituted at Val-2 (green), Val-74 (red), and Val-110 (blue) (S.D. = 3.5 kJ/mol). The estimated value agreed with the experimental value, but with a few exceptions.

The Contribution of Hydration Structure to Protein Stability—There were some mutant proteins for which the deviations between ΔΔG_{exp} and ΔΔG_{est} in Fig. 4a were greater than 5 kJ/mol. They are V2G, V2F, and V74G (hydrophobic mutants), and V2R and V74S (hydrophilic mutants). In the case of the hydrophobic mutants, the substitutions in V2G and V2F, whose ΔΔG_{exp} values are lower by 6.4 and 7.1 kJ/mol than the ΔΔG_{est}, respectively, destroy the hydration structure observed in the wild-type structure (19). On the other hand, the substitution in V74G, whose ΔΔG_{exp} value is higher by 7.9 kJ/mol than the ΔΔG_{est}, strengthens the hydration structure (19). For V2R and V74S (Figs. 1f and 2b, respectively), the hydration structures observed in the wild-type structure seemed to change because of these substitutions. However, the hydration
Contribution of Hydration Structure to Protein Stability

Fig. 4. a, the relation between the experimental and estimated $\Delta G$ values for the Val-2 (green circles), Val-74 (red circles), and Val-110 (blue circles) mutants. The solid and open circles denote the hydrophilic mutants (the present study) and hydrophobic mutants (19), respectively. The crosses are 54 mutant human lysozymes used for the determination of each coefficient in Equation 4. b, the results of the fitting using 32 surface mutants to obtain the parameters of Equation 7. The dotted line represents $y=x$. The dashed lines represent the error bars of $\pm 5 \text{kJ/mol}$.  

![Diagram](http://example.com/diagram.png)

$\Delta G = \Delta G_{\text{HS}} + \Delta G_{\text{HB}} + \Delta G_{\text{inter}} + \Delta G_{\text{vapor}} + \Delta G_{\text{H}_2\text{O}}$ (Eq. 5)

As mentioned above, $\Delta G_{\text{HS}}$ and $\Delta G_{\text{H}_2\text{O}}$ in Equation 4 represent the contributions of hydrogen bond and introduced water molecule, respectively, in the interior of the protein. Therefore, $\Delta G_{\text{HS}}$ and $\Delta G_{\text{H}_2\text{O}}$ were assumed to be zero in this case. Using Equation 4, the $\Delta G_{\text{HS}}$ could be calculated as follows.

$\Delta G_{\text{HS}} = \Delta G - (\Delta G_{\text{HB}} + \Delta G_{\text{inter}} + \Delta G_{\text{vapor}}) = \Delta G - (0.178 \Delta \Delta S_{\text{exp}} - 0.013 \Delta \Delta S_{\text{Asp}} - 3.15 \Delta \Delta S_{\text{Asn}} + 5.07 \Delta \rho_{\text{pro}} + 2.32 \Delta \rho_{\text{H}_{2}\text{O}})$ (Eq. 6)

Because several hydration water molecules interacting with protein atoms and each other via hydrogen bonds formed the hydration structure, the contribution of the hydration structure to protein stability ($\Delta G_{\text{HS}}$) might include the contribution of the hydrogen bond on the surface of the protein, which is apparently different from that in the interior. In addition, $\Delta G_{\text{HS}}$ might include an entropic effect of the water molecule hydrated on the surface of the protein, which is also different from that in the interior. Here, the contribution of the hydrogen bonding on the surface and the water molecule introduced on the surface to protein stability were represented as $\Delta G_{\text{HB}}^{\dagger}$ and $\Delta G_{\text{H}_2\text{O}}^{\dagger}$, respectively. The change in $\Delta G$ because of the changes in the hydration structure between the wild-type and mutant proteins can then be expressed as follows.

$\Delta G_{\text{HS}} = \Delta G_{\text{HB}}^{\dagger} + \Delta G_{\text{H}_2\text{O}}^{\dagger} = \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{HB}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \delta^\dagger \Delta N_{\text{H}_2\text{O}}$ (Eq. 7)

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$\Delta G_{\text{HS}} = \Delta G_{\text{HB}}^{\dagger} + \Delta G_{\text{H}_2\text{O}}^{\dagger} = \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{HB}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \delta^\dagger \Delta N_{\text{H}_2\text{O}}$ (Eq. 7)

$\Delta G_{\text{HS}}$ might include an entropic effect of the water molecule hydrated on the surface of the protein, which is also different from that in the interior. Here, the contribution of the hydrogen bonding on the surface and the water molecule introduced on the surface to protein stability were represented as $\Delta G_{\text{HB}}^{\dagger}$ and $\Delta G_{\text{H}_2\text{O}}^{\dagger}$, respectively. The change in $\Delta G$ because of the changes in the hydration structure between the wild-type and mutant proteins can then be expressed as follows.

$\Delta G_{\text{HS}} = \Delta G_{\text{HB}}^{\dagger} + \Delta G_{\text{H}_2\text{O}}^{\dagger} = \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{HB}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \delta^\dagger \Delta N_{\text{H}_2\text{O}}$ (Eq. 7)

$\Delta G_{\text{HS}}$ might include an entropic effect of the water molecule hydrated on the surface of the protein, which is also different from that in the interior. Here, the contribution of the hydrogen bonding on the surface and the water molecule introduced on the surface to protein stability were represented as $\Delta G_{\text{HB}}^{\dagger}$ and $\Delta G_{\text{H}_2\text{O}}^{\dagger}$, respectively. The change in $\Delta G$ because of the changes in the hydration structure between the wild-type and mutant proteins can then be expressed as follows.

$\Delta G_{\text{HS}} = \Delta G_{\text{HB}}^{\dagger} + \Delta G_{\text{H}_2\text{O}}^{\dagger} = \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{HB}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \delta^\dagger \Delta N_{\text{H}_2\text{O}}$ (Eq. 7)

$\Delta G_{\text{HS}}$ might include an entropic effect of the water molecule hydrated on the surface of the protein, which is also different from that in the interior. Here, the contribution of the hydrogen bonding on the surface and the water molecule introduced on the surface to protein stability were represented as $\Delta G_{\text{HB}}^{\dagger}$ and $\Delta G_{\text{H}_2\text{O}}^{\dagger}$, respectively. The change in $\Delta G$ because of the changes in the hydration structure between the wild-type and mutant proteins can then be expressed as follows.

$\Delta G_{\text{HS}} = \Delta G_{\text{HB}}^{\dagger} + \Delta G_{\text{H}_2\text{O}}^{\dagger} = \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{HB}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \gamma_{\text{H}_{2}\text{O}} [\Delta G_{\text{H}_2\text{O}}^{\dagger}] + \delta^\dagger \Delta N_{\text{H}_2\text{O}}$ (Eq. 7)
shown that the dynamic behavior of protein molecules can be quantitatively estimated using the contributions of the hydration structure to protein stability could be quantitatively estimated. These values are quite reasonable and would be useful for further understanding the structural dynamics and the principles of protein folding. This is the first report indicating that the contribution of the hydration structure to protein stability could be quantitatively estimated.

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**TABLE IV**

| Protein | ΔG_{exp} | ΔG_{int} | ΔG_{H_{ref}} | ΔG_{H_{ref}} | ΔG_{H_{ref}} | ΔG_{H_{ref}} |
|---------|-----------|----------|--------------|--------------|--------------|--------------|
|         | pp        | pw       | ww           |              |              |              |
| V2G     | -9.8      | -6.0     | -1.3         | 0            | 0.1          | -15          |
| V2A     | -6.3      | -10.5    | -6.8         | -0.4         | -1.7         | 0            |
| V2I     | 4.6       | 3.0      | 1.1          | 0.5          | 0.1          | 0            |
| V2L     | -0.2      | -0.8     | 0.3          | -0.3         | -0.8         | 0            |
| V2M     | -1.3      | -4.3     | -3.8         | 0.9          | -0.6         | 0            |
| V2F     | -3.6      | 0.0      | 4.4          | 0.2          | 1.1          | 0            |
| V2S     | -5.9      | -8.5     | -10.7        | 0.9          | 2.4          | 1.3          |
| V2Y     | -1.5      | -3.7     | -3.4         | 0.6          | 0.5          | 0            |
| V2D     | -6.0      | -5.0     | -7.8         | 0.3          | 2.1          | 0            |
| V2N     | -5.6      | -9.8     | -11.3        | 0.8          | 2.1          | 0            |
| V2R     | -1.6      | -2.2     | 2.8          | 1.1          | 1.3          | 0            |
| V74G    | -0.9      | -5.1     | -8.4         | -0.4         | 0            | 4.8          |
| V74A    | -1.5      | -4.9     | -4.3         | -0.4         | -0.1         | -3.0         |
| V74I    | 1.9       | 5.0      | 4.0          | 0.5          | 0            | -2.6         |
| V74L    | 0.8       | 1.7      | 2.2          | 0.2          | -0.1         | -4.2         |
| V74M    | 2.7       | -0.1     | 0.8          | 0.8          | 0            | -2.9         |
| V74F    | -1.2      | 0.6      | -2.6         | 0.1          | -0.1         | -0.2         |
| V74S    | 1.6       | 0.0      | 3.7          | 0.4          | -0.1         | -4.2         |
| V74Y    | -1.0      | -1.7     | -3.0         | 0.6          | -0.1         | -2.6         |
| V74D    | -1.8      | -4.0     | -4.9         | 0.3          | 0            | -3.1         |
| V74N    | -1.4      | -5.4     | -4.7         | 0.5          | -0.1         | -1.5         |
| V74R    | -0.3      | -2.4     | -2.9         | 0.2          | -0.1         | -4.1         |
| V110G   | 2.0       | -1.4     | -2.5         | -0.2         | -2.3         | -0.1         |
| V110A   | 2.2       | 3.7      | -2.0         | 0.2          | 2.9          | -0.1         |
| V110I   | 3.6       | 3.1      | 1.8          | 0.2          | 0.7          | 2.4          |
| V110L   | 0.3       | 2.8      | -0.3         | -0.3         | 2.3          | -0.2         |
| V110M   | 2.2       | 5.3      | 2.6          | 0.3          | 2.0          | -0.2         |
| V110F   | -0.2      | -1.8     | -2.9         | -0.1         | 0.4          | 0            |
| V110Y   | -0.6      | -5.5     | -4.1         | -0.2         | 0.4          | 0            |
| V110D   | 0.7       | 0.2      | 3.4          | 0.2          | 0.2          | 0.1          |
| V110N   | 0.3       | 3.5      | 2.3          | 0.1          | 0.5          | -0.1         |
| V110R   | 3.7       | 5.4      | 3.6          | -0.5         | -1.4         | -0.2         |

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The contribution of the hydration structure to protein stability could be quantitatively estimated. These values are quite reasonable and would be useful for further understanding the structural dynamics and the principles of protein folding. This is the first report indicating that the contribution of the hydration structure to protein stability could be quantitatively estimated.
Contribution of Hydration Structure to Protein Stability

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