Contribution of Hydrogen Bonds to the Conformational Stability of Human Lysozyme: Calorimetry and X-ray Analysis of Six Ser → Ala Mutants[†,‡]

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ABSTRACT: To further examine the contribution of hydrogen bonds to the conformational stability of the human lysozyme, six Ser to Ala mutants were constructed. The thermodynamic parameters for denaturation of these six Ser mutant proteins were investigated by differential scanning calorimetry (DSC), and the crystal structures were determined by X-ray analysis. The denaturation Gibbs energy (ΔG) of the Ser mutant proteins was changed from 2.0 to −5.7 kJ/mol, compared to that of the wild-type protein. With an analysis in which some factors that affected the stability due to mutation were considered, the contribution of hydrogen bonds to the stability ($\Delta\Delta G_{HB}$) was extracted on the basis of the structures of the mutant proteins. The results showed that hydrogen bonds between protein atoms and between a protein atom and a water bound with the protein molecule favorably contribute to the protein stability. The net contribution of one intramolecular hydrogen bond to protein stability (ΔG_{HB}) was 8.9 ± 2.6 kJ/mol on average. However, the contribution to the protein stability of hydrogen bonds between a protein atom and a bound water molecule was smaller than that for a bond between protein atoms.

Globular proteins form 1.1 hydrogen bonds per residue when they fold (*1*), suggesting that hydrogen bonds are one of the important factors stabilizing the folded conformations of proteins (*2*−*6*). The net contribution of hydrogen bonds to the conformational stability of proteins, however, remains controversial (*3*).

A useful approach for estimating the contribution of hydrogen bonds to stability is analysis of the thermodynamic data of a single mutant protein in which a hydrogen-bonded residue is substituted with one incapable of hydrogen bonding, e.g., Tyr to Phe. However, it has been shown that the loss in stability per one deleted hydrogen bond varies from case to case (*7*−*14*). This suggests that factors other than hydrogen bonds are affected by substitution and contribute to the overall stability of the mutant proteins. Therefore, the net contributions of a hydrogen bond at a specific site to stability may be estimated, by correcting for the effect of extra factors. To do so requires the structures of the single mutants (*15*). For mutant experiments related to hydrogen bonds, unfortunately, there are only a few examples that have been investigated by structural analysis (*10*, *14*, *16*), although numerous studies have been reported for mutant proteins.

In this study, the calorimetric and X-ray structural analyses of six Ser to Ala mutant human lysozymes are reported. Earlier, a similar study of six Tyr to Phe mutants was reported (*14*). Human lysozyme is an $\alpha + \beta$ protein, and consists of two domains. The active site is located in the cleft positioned between the two domains. The positions of the six Ser residues in the human lysozyme are shown in Figure 1. The hydroxyl groups of Ser24, Ser36, Ser51, and Ser61 participate in the intramolecular hydrogen bonds, i.e., Oγ of Ser24 with Nδ of Asn27, Oγ of Ser36 with O of Ile56, Oγ of Ser51 with Oδ of Asp49 and Nδ of Asn60, and Oγ of Ser61 with Oγ of Thr70 and N of Thr52. The hydroxyl group of Ser80 is hydrogen bonded with a water molecule which has another hydrogen bond with a protein atom. The hydroxyl groups of Ser24 and Ser82 also form hydrogen bonds with water molecules, which surround the surface of the protein and do not form other hydrogen bonds with the protein atoms. The structural characteristics of the Ser residues in the wild-type structure are listed in Table 1. On the basis of the mutant structures, the thermodynamic data will be analyzed and the net contribution of hydrogen bonding at a specific site to the protein stability will be estimated.

EXPERIMENTAL PROCEDURES

Mutant Proteins. Mutagenesis, expression, and purification of Ser mutant human lysozymes were performed as described previously (*17*). The concentration of the mutant proteins was spectrophotometrically determined using an $E^{1\%}$(1 cm) of 25.65 at 280 nm (*18*).

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[‡] Coordinates have been deposited in the Brookhaven Protein Data Bank under PDB file names 1B5U (S24A), 1B5Y (S36A), 1B5V (S51A), 1B5W (S61A), 1B5X (S80A), and 1B5Z (S82A).

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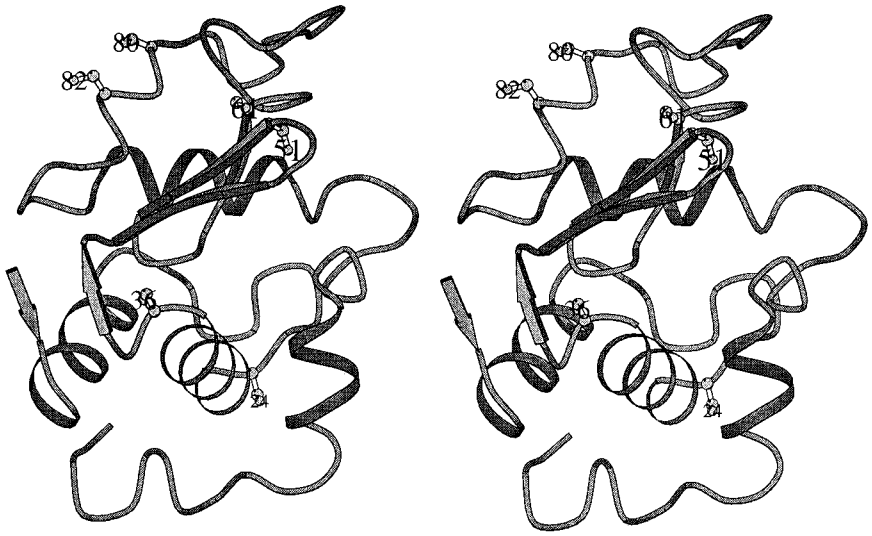

FIGURE 1: Stereodrawing of the wild-type human lysozyme structure. The locations of the six Ser residues are denoted. The structure was generated with MOLSCRIPT (35).

Differential Scanning Calorimetry (DSC). Calorimetric measurements and data analyses were carried out as described previously (*17*). For measurement, the DASM4 adiabatic microcalorimeter equipped with an NEC personal computer was used. Data analysis was carried out using the Origin software (MicroCal Inc., Northampton, MA).

The thermodynamic parameters for denaturation as a function of temperature were calculated using the following equations.

$$\Delta H(T) = \Delta H(T_d) - \Delta C_p(T_d - T) \quad (1)$$

$$\Delta S(T) = \Delta H(T_d)/T_d - \Delta C_p \ln(T_d/T) \quad (2)$$

$$\Delta G(T) = \Delta H(T) - T\Delta S(T) \quad (3)$$

where the ΔC_p[1] values are assumed to be independent of temperature (*19*).

X-ray Structural Analysis. Mutant human lysozymes were crystallized as previously described (*14*, *17*). All crystals belong to space group $P2_12_12_1$, but the crystal cell dimensions of S82A differed from those of the wild type and most mutant proteins, including the other Ser mutants. The crystal form of S82A is the same as the crystal forms of I56M and I56F mutants (*20*).

In general, the crystallization of the Ser to Ala mutants was difficult and the obtained crystals were smaller than those of the mutants previously reported (*14*, *17*, *20*−*24*). For S24A, S36A, S51A, and S61A, the data set was collected using synchrotron radiation at the Photon Factory (Tsukuba) on beam line 6B, 6A, or 18B with a Weissenberg camera (*25*). The data were processed with DENZO (*26*). For S80A, the data set was collected at 100 K using synchrotron radiation at the SPring-8 (Harima) on beam line 41XU (Proposal 1998A0182-NL-np). For S82A, the data set was collected by the oscillation method on a Rigaku R-AXIS IIC imaging plate mounted on a Rigaku RU300 rotating anode X-ray generator. The data were processed with software provided by Rigaku.

The structures of the mutant proteins were determined by the isomorphous method. The structures were refined with X-PLOR (*27*) as previously described (*14*, *17*).

Calculation of the ASA Value. The accessible surface area (ASA) values of the proteins were calculated by the procedure of Connolly (*28*) with a probe radius of 1.4 Å (*17*, *20*, *23*). The ASA values of the denatured state were calculated using an actual polypeptide with an extended conformation (*29*).

Table 1: Structural Features of Ser Residues in Wild-Type Human Lysozyme[a]

position	secondary structure	buried % of the side chain	buried % of the OH group	hydrogen bonding partner	hydrogen bonding distance[b] (Å)
Ser24		46	82	Nδ of Asn27	2.85
				(O water)[c]	2.85
Ser36		100	100	O of Ile56	2.72
Ser51	sheet	98	96	Oδ of Asp49	2.79
				Nδ of Asn60	2.84
Ser61	sheet	100	100	Oγ of Thr70	2.77
				N of Thr52	2.98
Ser80		28	32	O water	2.78
Ser82		11	0	(O water)[c]	2.52

[a] From Takano et al. (*17*). [b] The length of a hydrogen bond between a solvent molecule and a protein atom, or between protein atoms, represents the distance between the solvent oxygen and the protein atom oxygen or nitrogen, or between the protein atom oxygen or nitrogen, respectively. [c] A water molecule is on protein surface and forms no other hydrogen bonds with protein atoms.

RESULTS

Differential Scanning Calorimetry (DSC) of Ser Mutant Human Lysozymes

To determine the thermodynamic parameters of the denaturation of Ser to Ala mutant human lysozymes, DSC measurements were taken at acidic pHs between 2.4 and 3.2 where the denaturation of the human lysozyme is reversible. Table 2 shows the denaturation temperature (T_d), the calorimetric enthalpy (ΔH_{cal}), the van't Hoff enthalpy (ΔH_{vH}),

[1] Abbreviations: ASA, solvent accessible surface area; ΔC_p, heat capacity change; ΔΔASA, changes in surface area exposed upon denaturation; $\Delta\Delta G$, changes in stability; ΔH_{cal}, calorimetric enthalpy change; ΔH_{vH}, van't Hoff enthalpy change; DSC, differential scanning calorimetry; HB, hydrogen bond; HP, hydrophobic effect; T_d, denaturation temperature.

Table 2: Thermodynamic Parameters for Denaturation of Mutant Human Lysozymes (Ser → Ala) at Different pHs

	pH	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{cal}/\Delta H_{vH}$	ΔC_p (kJ mol^{-1} K^{-1})
S24A	3.09	70.3	494	515	0.96	5.9
	2.82	65.7	469	490	0.96	6.5
	2.66	62.5	444	464	0.96	7.0
	2.46	58.8	423	439	0.96	6.1
S36A	3.09	68.7	477	498	0.96	5.1
	2.84	64.0	448	473	0.95	5.2
	2.67	60.8	435	456	0.95	5.5
	2.47	57.1	412	431	0.96	5.5
S51A	3.11	70.8	502	527	0.95	5.7
	2.86	66.7	481	506	0.95	6.5
	2.69	64.2	464	494	0.94	6.0
	2.51	61.0	444	473	0.94	6.2
S61A	3.10	68.3	473	494	0.96	5.5
	2.84	63.6	448	464	0.97	4.8
	2.71	60.7	427	444	0.96	5.6
	2.51	57.0	414	431	0.96	4.6
S80A	3.11	73.6	540	565	0.96	5.8
	2.85	69.1	510	540	0.94	5.6
	2.69	66.2	498	527	0.94	5.6
	2.50	62.7	473	502	0.94	5.9
S82A	3.12	73.5	523	556	0.94	5.9
	2.85	68.9	502	536	0.94	5.0
	2.70	66.0	485	515	0.94	5.3
	2.51	62.5	464	490	0.95	5.9

and the heat capacity change (ΔC_p) of each measurement for the mutant proteins. The thermodynamic parameters of denaturation at a constant temperature (64.9 °C) and pH 2.7 were calculated using these data as shown in Table 3. S24A, S36A, S51A, and S61A were destabilized, but S80A and S82A were stabilized by substitution, compared with the wild-type protein. For the stabilized mutants, the substituted residues in the wild-type structure are mostly exposed to solvent and exhibit no intramolecular hydrogen bonds (Table 1).

The changes in enthalpy, $\Delta\Delta H$, upon mutation were also substantially different from each other, depending on the structural feature of the mutation sites. In most cases, however, the large enthalpy changes were offset by the entropy changes. So, under the existing circumstances, it was difficult to correlate the changes in enthalpy with structural changes upon mutation.

X-ray Structural Analysis of Ser Mutant Human Lysozymes

The data collection and refinement statistics for six mutant human lysozymes are summarized in Table 4. The structures in the vicinity of the mutation sites are illustrated in Figure 2.

S24A. In the wild-type structure, the hydroxyl group of Ser24 participates in hydrogen bonding with the amino group of Asn27 and a water molecule with no other hydrogen bonds. In the S24A mutant protein, these hydrogen bonds disappeared and the water molecule moved by 1.0 Å and made a new hydrogen bond with another solvent molecule. Finally, the replacement of Ser with Ala at position 24 removed the intramolecular hydrogen bond (Figure 2a).

S36A. The hydroxyl group of Ser36 buried in the interior of a protein forms a hydrogen bond with the main chain oxygen of Ile56. The substitution of Ser36 with Ala deleted the hydrogen bond (Figure 2b). The S36A mutant protein had a structure in the vicinity of Ala36 very similar to the wild-type one, except for the deletion of the hydroxyl group and hydrogen bond. The structure of S36A, which has a mutation site in the interior of the α-domain, was more identical to the wild-type structure than those of S51A and S61A substituted in the interior of the β-domain. The rms deviation for the Cα atoms between the wild type and S36A was ca. 0.1 Å. On the other hand, the rms deviation between the wild type and S51A or S61A was ca. 0.2 Å.

Table 3: Thermodynamic Parameters for Denaturation of Mutant Human Lysozymes (Ser → Ala) at the Denaturation Temperature (64.9 °C) of the Wild-Type Protein at pH 2.7

	T_d (°C)	ΔT_d (°C)	ΔC_p[a] (kJ mol^{-1} K^{-1})	ΔH_{cal} (kJ/mol)	$\Delta\Delta G$ (kJ/mol)
wild-type[b]	64.9 ± 0.5		6.6 ± 0.5	477 ± 4	
S24A	63.3 ± 0.2	−1.6	6.3 ± 0.3	461 ± 2	−2.2
S36A	61.4 ± 0.1	−3.5	5.5 ± 0.3	455 ± 3	−4.7
S51A	64.2 ± 0.2	−0.7	6.0 ± 0.3	468 ± 2	−1.0
S61A	60.6 ± 0.2	−4.3	5.3 ± 0.5	454 ± 4	−5.7
S80A	66.3 ± 0.1	1.4	6.0 ± 0.4	487 ± 3	2.0
S82A	66.0 ± 0.2	1.1	5.3 ± 0.3	479 ± 2	1.6

[a] ΔC_p was obtained from the slope of ΔH_{cal} vs T_d. [b] From Takano et al. (*17*).

S51A. In the wild-type structure, the hydroxyl group of Ser51 participates in two hydrogen bonds. The structure around position 51 of S51A was slightly different from that of the wild-type lysozyme. The deletion of the hydroxyl group resulted in shifts of the residues which participate in the hydrogen bonds with the hydroxyl group of Ser51 in the wild-type structure. The side chain of Asn60 moved by ca. 0.9 Å, and the temperature factors for the side chain atoms increased by ca. 10 Å^2, compared with those observed in the wild-type structure. The side chain of Asn49 shifted by ca. 0.6 Å, but the temperature factors did not change. The changes (>0.25 Å) for the main chain atoms were observed in the regions of positions 46−50 and 71−74. The two intramolecular hydrogen bonds were totally removed by the replacement of Ser51 by Ala (Figure 2c).

S61A. The deletion of the hydroxyl group of Ser61 resulted in the movement (>0.25 Å) of the regions consisting of residues 60, 61, and 69−74. However, the pattern of the hydrogen bond network surrounding residue 61 did not change, and two hydrogen bonds of Ser61 with Thr52 and Ser61 with Thr70 were deleted (Figure 2d).

S80A. The hydroxyl group of Ser80 in the wild-type structure makes one hydrogen bond with a water molecule which has a low-temperature factor (10.6 Å^2) and another hydrogen bond with O of Asn66. The replacement of Ser80 by Ala deleted one protein···water hydrogen bond. The water molecule exists at the same position and made a hydrogen bond with Asn66 in the structure of S80A (Figure 2e).

S82A. The crystal form of the S82A mutant was different from the others. There were two S82A molecules in the asymmetric unit. The rms deviations for the Cα atoms between the two molecules and between the wild type and each S82A molecule were 0.34, 0.38, and 0.31 Å, respectively. The changes were mainly on residues 43−49, 67−73, and 117−120, which were far from position 82. The values of the total molecular accessible surface area for two S82A molecules were about 7100 Å^2, and smaller than those

Table 4: X-ray Data Collection and Refinement Statistics for Ser to Ala Mutants

	S24A	S36A	S51A	S61A	S80A	S82A
(A) Data Collection						
crystal system	orthorhombic					
space group	$P2_12_12_1$					
cell dimensions (Å)						
a	56.96	56.98	56.69	56.57	56.36	64.67
b	60.98	61.19	60.79	60.71	62.62	110.30
c	33.78	33.95	33.68	33.52	32.55	43.64
resolution (Å)	1.80	2.20	2.17	2.17	2.00	1.81
no. of measured reflections	47257	19862	17842	29351	23122	68449
no. of independent reflections	10868	6155	5960	6404	7913	23175
completeness (%)	95.2	95.7	92.2	97.7	95.7	79.4
R_{merge} (%)[a]	3.6	9.1	7.0	6.0	7.1	5.1
data collection	PF (BL18B)	PF (BL6B)	PF (BL6A)	PF (BL6A)	SPring-8 (BL41XU)	RaxisIIC
(B) Refinement						
no. of protein atoms	1028	1028	1028	1028	1028	2056
no. of solvent atoms	234	169	128	147	241	257
resolution range (Å)	8.00−1.80	8.00−2.20	8.00−2.17	8.00−2.17	8.00−2.00	8.00−2.20
no. of reflections used	10355	5866	5731	6217	7207	14157
completeness (%)	92.0	92.9	89.7	96.9	89.2	88.0
R-factor[b]	0.156	0.148	0.161	0.160	0.152	0.190
rmsd						
bonds (Å)	0.008	0.009	0.009	0.009	0.008	0.009
angles (deg)	1.50	1.53	1.60	1.55	1.53	1.48
average of *B*-factors						
main chain (Å^2)	9.7	8.3	7.0	8.2	8.9	15.3
side chain (Å^2)	16.7	14.4	11.5	12.9	12.2	18.8

[a] $R_{merge} = 100 \times \Sigma|I - \langle I\rangle|/\Sigma\langle I\rangle$. [b] $R\text{-factor} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$.

(7300 Å^2) of the wild-type and mutant structures in the other usual crystal form. The hydroxyl group of Ser82 in the wild-type protein hydrogen bonds with a water molecule on the protein surface, but the hydroxyl group of Ser82 in crystal structures of other mutant human lysozymes does not always hydrogen bond with surface water (*14*, *17*, *20*−*23*). In both structures of S82A, the hydrogen bond and water molecule disappeared (Figure 2f).

DISCUSSION

Estimation of Gibbs Energy Changes of Hydrogen Bonding ($\Delta\Delta G_{HB}$)

The stability of mutant proteins with intramolecular hydrogen bonds deleted by substitution, S24A, S36A, S51A, and S61A, decreased when compared with that of the wild-type protein. The differences in stability between the wild-type and mutant proteins, $\Delta\Delta G$, might show the contribution of a hydrogen bond to protein stability. However, the loss of stability per one deleted hydrogen bond was different for each mutant. This indicates that factors other than the hydrogen bonds affect the stability of the mutant proteins. If a mutation site is in the interior of a molecule, changes in hydrophobicity are introduced due to substitution. In this study, the contribution due to other factors was offset as follows.

Myers and Pace (*6*) have assumed that $\Delta\Delta G$ can be represented by the additive contribution of each factor, which mainly affects protein stability.

$$\Delta\Delta G = \Delta\Delta G_{HP} + \Delta\Delta G_{conf} + \Delta\Delta G_{HB} \quad (4)$$

where $\Delta\Delta G_{HP}$, $\Delta\Delta G_{conf}$, and $\Delta\Delta G_{HB}$ represent the contributions of changes in the hydrophobic effect, side chain conformational entropy, and hydrogen bonds, respectively. Equation 4 can be rearranged into eq 5.

$$\Delta\Delta G_{HB} = \Delta\Delta G - (\Delta\Delta G_{HP} + \Delta\Delta G_{conf}) \quad (5)$$

In the estimation of Myers and Pace (*6*), $\Delta\Delta G_{HP}$ is the contribution of the hydrophobic effect depending on the accessibility of a mutation residue on the basis of the *n*-octanol hydrophobicity scale of Fauchere and Pliska (*31*), ΔG_{tr}. $\Delta\Delta G_{conf}$ is based on the mean $T\Delta S_{conf}$ values given by Doig and Sternberg (*30*). Therefore, the contribution of a hydrogen bond by Myers and Pace (*6*), $\Delta\Delta G_{HB}$, is as follows.

$$\Delta\Delta G_{HB} = \Delta\Delta G - (\Delta\Delta G_{tr} + \Delta\Delta G_{conf}) \quad (6)$$

Each $\Delta\Delta G_{tr}$ value is calculated using only the wild-type structure. However, other contributions to stability, $\Delta\Delta G_{other}$, such as the effects of structural changes due to mutation, are not contained in eq 6. Takano et al. (*17*, *21*−*23*) have shown that the stability changes of a series of hydrophobic mutant human lysozymes correlate with changes in the accessible surface area of all hydrophobic residues exposed upon denaturation ($\Delta\Delta ASA_{HP}$) because the substitutions affect not only the mutation site but also other parts of the protein far from the site, although the structural changes are not large. Yamagata et al. (*14*) have considered this effect and have introduced a new term to give

$$\begin{aligned}\Delta\Delta G_{HB} &= \Delta\Delta G - (\Delta\Delta G_{tr} + \Delta\Delta G_{other} + \Delta\Delta G_{conf}) \\ &= \Delta\Delta G - [\Delta\Delta G_{tr} + 0.12 \times \Delta\Delta ASA_{HP(other)} + \\ &\qquad \Delta\Delta G_{conf}] \quad (7)\end{aligned}$$

where $\Delta\Delta ASA_{HP(other)}$ is calculated using the surface area of all hydrophobic residues excluding the mutation residue of the wild-type and mutant structures and the coefficient (0.12 kJ mol^{-1} Å^{-2}) is the value given by Takano et al. (*22*, *23*).

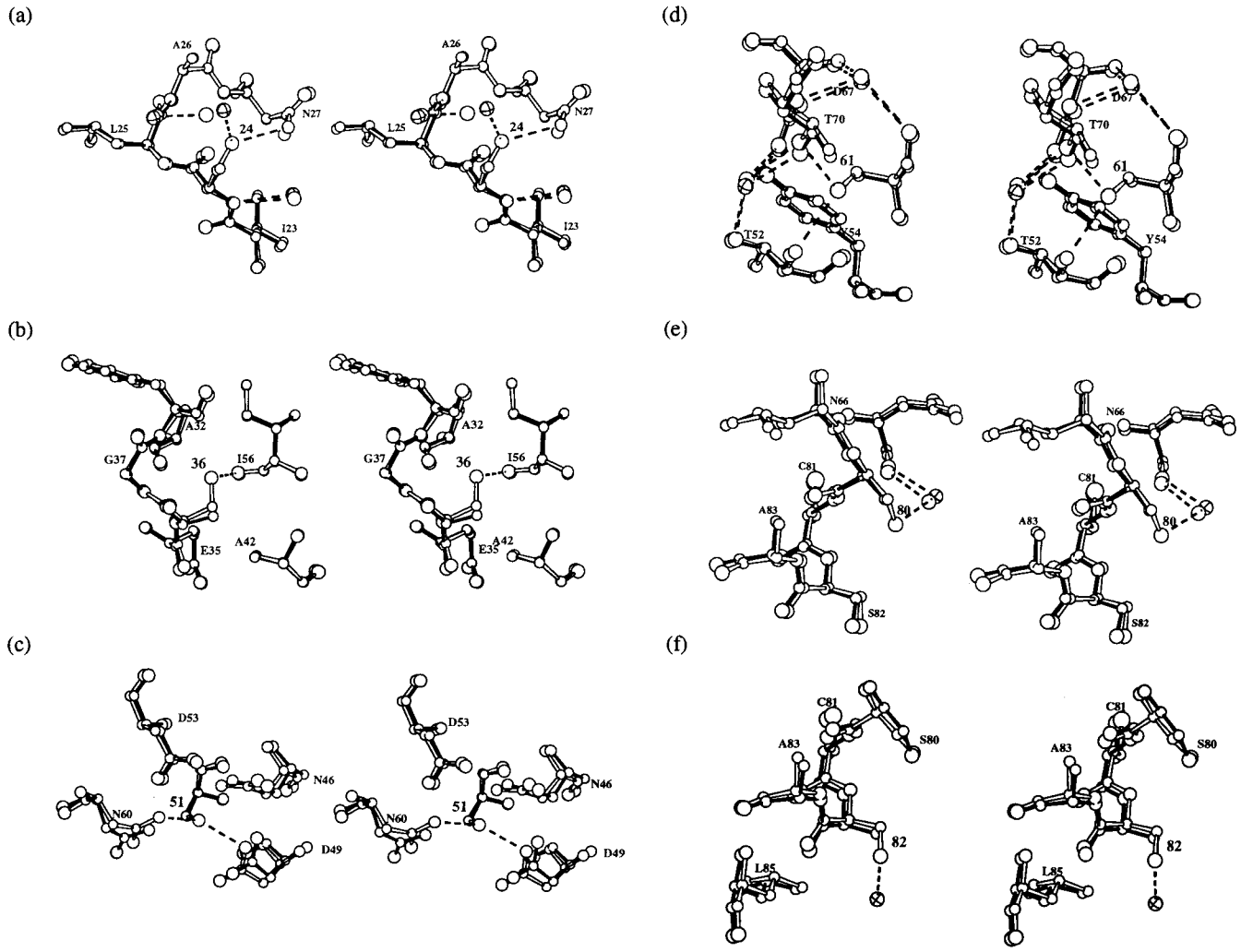

FIGURE 2: Stereodrawing of structures in the vicinity of mutation sites. The structures of the wild type (white bonds) and mutants (black bonds) are superimposed. Panels a−f represent S24A, S36A, S51A, S61A, S80A, and S82A, respectively. Solvent water molecules are drawn as crossed circles (wild type) and white circles (mutants). Broken lines represent hydrogen bonds. The structures were generated with ORTEP (*36*).

In eq 7, the effects of all hydrophobic residues are considered but those of the hydrophilic residues are not. Recently, Funahashi et al. (*20*) have evaluated the hydrophobic effect of the overall structure on protein stability using the large mutant stability and structure database of human lysozymes as follows.

$$\Delta\Delta G_{HP} = 0.178 \times \Delta\Delta ASA_{nonpolar} - 0.013 \times \Delta\Delta ASA_{polar} \quad (8)$$

where $\Delta\Delta ASA_{nonpolar}$ and $\Delta\Delta ASA_{polar}$ represent the difference in ΔASA of nonpolar (carbon) and polar (nitrogen and oxygen) atoms upon denaturation between the overall wild-type and mutant proteins, respectively (*20*). The values of $\Delta\Delta ASA_{nonpolar}$ and $\Delta\Delta ASA_{polar}$ are calculated using both the wild-type and mutant crystal structures. The coefficients in eq 8, 0.178 for $\Delta\Delta ASA_{nonpolar}$ and −0.013 for $\Delta\Delta ASA_{polar}$, are comparable with the atomic solvation parameters (ASP) (*32*−*34*) based on the hydrophobicity of residue burial of Fauchere and Pliska (*31*). Therefore, in this study, the contribution of the hydrogen bond, $\Delta\Delta G_{HB}$, can be more accurately estimated as follows.

$$\Delta\Delta G_{HB} = \Delta\Delta G - (\Delta\Delta G_{HP} + \Delta\Delta G_{conf}) = \Delta\Delta G - (0.178 \times \Delta\Delta ASA_{nonpolar} - 0.013 \times \Delta\Delta ASA_{polar} + \Delta\Delta G_{conf}) \quad (9)$$

For the Ser and Tyr mutant human lysozymes, the $\Delta\Delta G$ values from each estimation are listed in Table 5. As the crystal form of S82A was different from that of the wild type, its $\Delta\Delta G_{HB}$ values were not estimated. The values of $\Delta\Delta G_{HB}$ from three estimations for each mutant protein were different but similar, except for the cases of S51A and S61A. The changes in $\Delta\Delta G_{HB}$ values for S51A and S61A were larger than those of the equivalent values estimated by Myers and Pace (*6*) and by Yamagata et al. (*14*). In the wild-type structure, the positions of 51 and 61 are in the β-domain and participate in the hydrogen bond network formed by the side chains of some hydrophilic residues. The substitutions of Ser51 and Ser61 caused some structural changes in the β-domain. For the estimations, Myers and Pace (*6*) do not consider the structural changes due to substitution and Yamagata et al. (*14*) do only the structural changes of hydrophobic residues.

Table 5: Changes in the Number of Hydrogen Bonds Due to Substitution and $\Delta\Delta G$ Values (kJ/mol) for Mutant Human Lysozymes

	change in no. of protein···protein HBs[a]	change in no. of protein···water HBs[b]	$\Delta\Delta G$ (measured)	$\Delta\Delta G_{conf}$[c]	$\Delta\Delta G_{tr}$[d]	$\Delta\Delta G_{other}$[e]	$\Delta\Delta G_{HP}$[f]	$\Delta\Delta G_{HB}$[g]	$\Delta\Delta G_{HB}$[h]	$\Delta\Delta G_{HB}$[i]	ΔG_{HB}[j]
S24A	−1	−1[k] (+1)[l]	−2.2	5.2	1.6	1.1	2.5	−9.0	−10.1	−9.9	9.9[n]
S36A	−1		−4.7	5.2	2.0	1.5	4.0	−11.9	−13.4	−13.9	13.9
S51A	−2		−1.0	5.2	1.9	0.3	10.1	−8.1	−8.4	−16.4	8.2
S61A	−2		−5.7	5.2	2.0	−2.3	13.9	−12.9	−10.6	−24.8	12.4
S80A		−1	2.0	5.2	0.6	−1.2	3.5	−3.8	−2.6	−6.7	6.7
Y20F[m]	−2		−2.1	2.4	3.3	1.4	8.0	−7.8	−9.2	−12.5	6.3
Y38F[m]		−1	−0.8	2.4	4.5	2.0	1.1	−7.7	−9.7	−4.4	4.4
F45F[m]		−1	0.3	2.4	2.5	−0.2	4.8	−4.6	−4.4	−6.9	6.9
Y54F[m]	−1	−1[k]	−4.0	2.4	4.0	−2.0	3.3	−10.4	−8.4	−9.7	9.7[n]
Y63F[m]			−1.0	1.1[m]	0	−0.9	−0.3	−2.1	−1.2	−1.8	−
Y124F[m]	−1		−1.5	2.4	3.4	0.2	6.6	−7.3	−7.5	−10.5	10.5

[a] Change in the number of hydrogen bonds between protein atoms due to substitution. [b] Change in the number of hydrogen bonds between a protein atom and a water molecule due to substitution. [c] From Doig and Sternberg (*30*). [d] (Fraction of OH groups exposed upon denaturation at a mutation site) × $\Delta\Delta G_{tr(S\rightarrow A,orY\rightarrow F)}$ (*31*). [e] $\Delta\Delta G_{other} = 0.12 \times \Delta\Delta ASA_{HP(other)}$ (*22, 23*). [f] $\Delta\Delta G_{HP} = 0.178 \times \Delta\Delta ASA_{nonpolar} - 0.013 \times \Delta\Delta ASA_{polar}$ (*20*). [g] $\Delta\Delta G_{HB} = \Delta\Delta G$(measured) − ($\Delta\Delta G_{conf} + \Delta\Delta G_{tr}$) (eq 6) (*6*). [h] $\Delta\Delta G_{HB} = \Delta\Delta G$(measured) − ($\Delta\Delta G_{other} + \Delta\Delta G_{conf} + \Delta\Delta G_{tr}$) (eq 7) (*14*). [i] $\Delta\Delta G_{HB} = \Delta\Delta G$(measured) − ($\Delta\Delta G_{conf} + \Delta\Delta G_{HP}$) (eq 9). [j] $\Delta G_{HB} = \Delta\Delta G_{HB}$/(change in the no. of HBs). [k] Hydrogen bond with a water molecule on the protein surface forming no other hydrogen bonds with protein atoms. [l] Hydrogen bond between water molecules on the protein surface. [m] From Yamagata et al. (*14*). [n] Hydrogen bonds related with water molecule on the protein surface were not considered.

Contribution of Hydrogen Bonds to the Stability of Human Lysozyme

The mutant proteins removing intramolecular hydrogen bonds by substitution, S24A, S36A, S51A, S61A, Y20F, Y54F, and Y124F, exhibited a significant change in $\Delta\Delta G_{HB}$, but the estimated $\Delta\Delta G_{HB}$ of the Y63F mutant protein, of which the substitution residue had no hydrogen bonds in the wild-type structure, was slight (Table 5). These results indicate that the estimated $\Delta\Delta G_{HB}$ values are reasonable and the intramolecular hydrogen bonds favorably contribute to the stability of the human lysozyme. Moreover, mutant proteins, Y38F, Y45F, and S80A, deleting one hydrogen bond with a water molecule tightly bound in the protein molecule also showed the loss of $\Delta\Delta G_{HB}$, indicating that the intermolecular hydrogen bonds between the protein and bound water molecules also stabilize the protein structures. The contribution of hydrogen bonds of each Ser residue to the stability of the human lysozyme is discussed separately below, including the results of the Tyr mutants (*14*).

S36A. S36A removed one intramolecular hydrogen bond between Ser36 and Ile56 due to substitution. The contribution of the hydrogen bond (ΔG_{HB}) was 13.9 kJ/mol, slightly higher than that in Y124F (10.5 kJ/mol), which also deleted one intra-hydrogen bond by mutation. This may be caused by the difference in the length of a hydrogen bond; in the wild-type structure, the hydrogen bond of Ser36 (2.72 Å) is shorter than that of Tyr124 (3.05 Å) (*14*).

S61A. The side chain of Ser61 forms two intramolecular hydrogen bonds; one has a normal hydrogen bond length (2.98 Å), and the other is a strong hydrogen bond (2.77 Å). The $\Delta\Delta G_{HB}$ value of S61A was 24.8 kJ/mol, corresponding to the total of those of S36A and Y124F (24.4 kJ/mol). In contrast, the ΔG_{HB} value of Y20F was only 6.3 kJ/mol. This may be because the substitution deleted weak hydrogen bonds (3.24 and 3.06 Å) (*14*).

S51A. Ser51 also has two strong hydrogen bonds in the wild-type structure. However, the loss of the $\Delta\Delta G_{HB}$ value for S51A was only 16.4 kJ/mol; i.e., the contribution per one hydrogen bond, ΔG_{HB}, was 8.2 kJ/mol, which was slightly smaller than those of the S36A, S51A, and Y124F deleted intra-hydrogen bonds. The other effects not considered in the present estimation might partly offset the destabilization due to substitution. For example, a constrained conformation is released by structural change due to substitution.

S24A. S24A deleted one intramolecular hydrogen bond with Asn27 and one intermolecular hydrogen bond with a surface water, and newly formed one water···water hydrogen bond on the protein surface (Figure 2a). The $\Delta\Delta G_{HB}$ value of S24A was −9.9 kJ/mol. This value was comparable with that of Y124F which deleted one protein···protein hydrogen bond. This suggests that for S24A, the protein···water hydrogen bond deleted on the protein surface has a similar contribution to the protein stability with the water···water hydrogen bond formed on the protein surface. In the case of Y54F, which deleted one intra-hydrogen bond and one inter-hydrogen bond with a surface water, the $\Delta\Delta G_{HB}$ value was −9.7 kJ/mol, comparable with those of S24A and Y124F. Therefore, it seems that hydrogen bonds related to water molecules on the protein surface contribute very little to protein stability.

S80A. The $\Delta\Delta G_{HB}$ values of S80A, Y38F, and Y45F removing one hydrogen bond with bound water molecules were −6.7, −4.4, and −6.9 kJ/mol, respectively, the average being 6.0 ± 1.4 kJ/mol. This indicates a hydrogen bond with a bound water favorably contributes to protein stability. This contribution is slightly smaller than that of an intramolecular hydrogen bond; the average ΔG_{HB} value of S24A, S36A, S51A, S61A, Y20F, Y54F, and Y124F removing intra-hydrogen bonds by substitution was 8.9 ± 2.6 kJ/mol. Funahashi et al. (*20*) have also estimated the ΔG_{HB} value to be 5.1 kJ/mol from the analysis of the mutant human lysozymes which were forming hydrogen bonds by mutation between a protein atom and water molecule, and between water molecules in the interior of the protein. These results suggest the different contributions to protein stability between hydrogen bonds with a water molecule and inter-hydrogen bonds with residues of a protein.

CONCLUSION

In this study, the more consistent net contribution of hydrogen bonds to the conformational stability of a protein could be estimated considering changes in the overall hydrophobic effect due to substitution, the side chain conformational entropy, and the experimental denaturation Gibbs energy between the wild-type and mutant proteins using Ser to Ala and Tyr to Phe mutants of human lysozyme. The net contribution of an intramolecular hydrogen bond to protein stability was about 9 kJ/mol. On the other hand, the contribution of hydrogen bonds between a protein atom and a bound water molecule was smaller than that between protein atoms.

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