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

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ABSTRACT: The contribution of hydrogen bonds to the conformational stability of human lysozyme was investigated by the combination of calorimetric and X-ray analyses of six Tyr → Phe mutants. Unfolding ΔG and unfolding ΔH values of the Tyr → Phe mutant proteins were changed by from +0.3 to −4.0 kJ/mol and from 0 to −16 kJ/mol, respectively, compared to those of the wild-type protein. The net contribution of a hydrogen bond at a specific site to stability (ΔG_{HB}^{wild}), considering factors affected by substitutions, was evaluated on the basis of X-ray structures of the mutant proteins. In the present study, one of six mutant proteins was suitable for evaluating the strength of the hydrogen bond. ΔG_{HB}^{wild} for the intramolecular hydrogen bond at Tyr124 was evaluated to be 7.5 kJ/mol. Results of the analysis of other mutants also suggest that hydrogen bonds of the hydroxyl group of Tyr, including the hydrogen bond with a water molecule, contribute to the stabilization of the human lysozyme.

Hydrogen bonds are ubiquitous in proteins and their contribution to the conformational stability is of fundamental importance, as is the hydrophobic interaction. Although numerous studies on the contribution of a hydrogen bond to the stability have been reported, there remains a controversy, i.e., whether hydrogen bonds contribute to the stability, and if so, how much do they contribute? For example, experimental studies by Myers and Pace (*1*) have shown that hydrogen-bonded polar groups make a favorable contribution to globular proteins. In contrast, two theoretical studies have concluded that the polar groups contribute little or not at all to protein stability (*2*, *3*).

Honig and Yang (*2*) argue that the difference in unfolding Gibbs energy between a wild-type and the mutant protein does not correspond to the net contribution of a hydrogen bond. The effect of a single amino acid substitution on protein stability depends highly on the location of the mutation sites and its environment in the protein structure. Especially when a single amino acid substitution is used to disrupt a hydrogen bond, the protein may be left with an unsatisfied hydrogen donor or acceptor that is buried in the interior. To extract the binding energy of a hydrogen bond, structural changes due to an amino acid substitution must be examined in detail and the effects quantitatively considered. A useful approach to estimate the net contribution is to systematically analyze thermodynamic data obtained from calorimetry and combine this with X-ray analyses of the structures for a series of single mutant proteins.

To date, several mutational studies of the contribution of hydrogen bonding to the protein stability have been reported (*4*−*7*). However, there are fewer examples that have been investigated by both calorimetry and X-ray analysis, as compared with mutational studies for the evaluation of the hydrophobic effect (*8*−*13*). According to Janin (*14*) there is low success in the estimates of the contribution of polar groups to the conformational stability, due to the lack of high-resolution X-ray structures for the single mutants. To evaluate the contribution of a hydrogen bond to the conformational stability, we carried out the calorimetric and X-ray studies of six tyrosine to phenylalanine mutants of human lysozyme.

The positions of the six Tyr residues in human lysozyme are shown in Figure 1 (*11*, *15*). The hydroxyl groups of Tyr20, Tyr54, and Tyr124 participate in intramolecular hydrogen bonds, i.e., Oη of Tyr20 with HNη and HNϵ of Arg101, Oη of Tyr54 with Oδ of Asp67, and Oη of Tyr124 with HNϵ of Trp34. The hydroxyl groups of Tyr38, Tyr45, Tyr54, and Tyr124 form hydrogen bonds with well-ordered water molecules that have temperature factors of 13.8, 24.7, 24.1 and 34.9 Å^2, respectively (*11*). The structural characteristics of the Tyr residues in the wild-type structure are listed in Table 1.

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[‡] Coordinates for the six human lysozyme mutants have been deposited in the Brookhaven Protein Data Bank under the following PDB file names: Y124F, 1WQM; Y20F, 1WQN; Y38F, 1WQO; Y45F, 1WQP; Y54F, 1WQQ; and Y63F, 1WQR.

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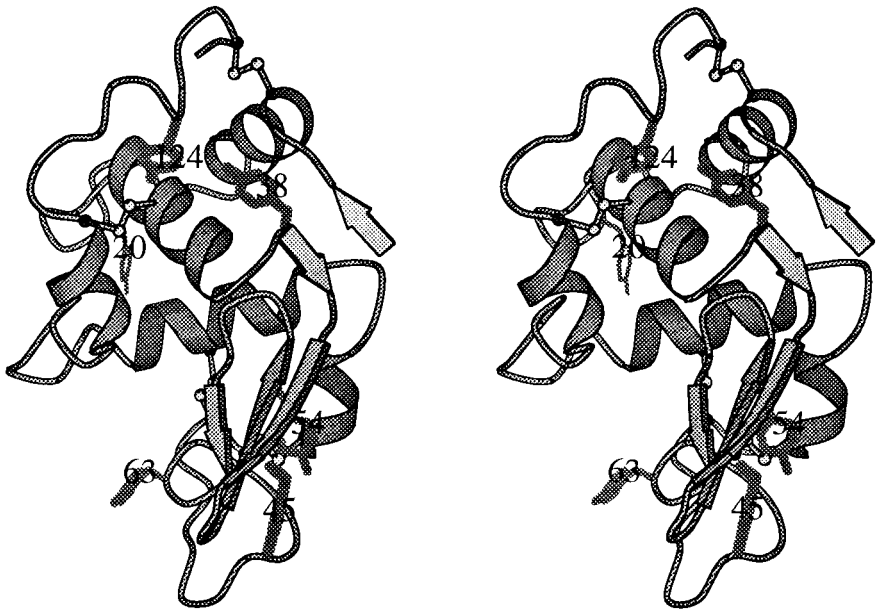

FIGURE 1: Location of the six tyrosine residues in human lysozyme. The structure was generated by the program MOLSCRIPT (*24*). The circles with dots represent sulfur atoms of disulfide bonds.

Table 1: Structural Features of Tyr Residues in the Wild-Type Protein (*11*)

position	secondary structure	buried side chain (%)	buried OH group (%)	*B*-factor of the Oη atom (Å^2)	hydrogen bonding partner	hydrogen bonding distance[a] (Å)	*B*-factor of the partner (Å^2)
Tyr 20	no	70	70	19	Nϵ Arg101	3.06	32
					Nη Arg101	3.24	20
Tyr 38	sheet	85	95	13	O water	2.78	14
Tyr 45	sheet	41	53	20	O water	2.67	25
Tyr 54	sheet	89	84	10	Oδ Asp67	2.60	10
					O water	2.82	24
Tyr 63	no	24	0	46			
Tyr124	no	87	72	20	Nϵ Trp34	3.05	14
					O water	2.90	35

[a] 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 atoms oxygen or nitrogen.

Most of the ΔG and ΔH values for unfolding of the six Tyr → Phe mutants examined were lower than those of the wild-type protein. X-ray structures of the mutant proteins showed that the features of structural changes by the replacements of Tyr with Phe were diverse. On the basis of these structural changes, the $\Delta\Delta G$ values of the mutant proteins will be analyzed and the contribution of a hydrogen bond to the conformational stability will be estimated.

EXPERIMENTAL PROCEDURES

Mutant Proteins. Mutagenesis, expression, and purification of the Tyr mutant human lysozymes were performed as described (*11*). The concentration of the mutant proteins was determined spectrophotometrically by using $E^{1\%}(1\text{ cm}) = 24.71$ at 280 nm with the correction by the different molar absorption coefficient between Tyr and Phe residues (*16*).

Differential Scanning Calorimetry. Calorimetric measurements and data analyses were carried out as described (*11*). A DASM4 adiabatic microcalorimeter equipped with an NEC personal computer was used. The scan rate was 1.0 K/min. The sample solutions were prepared by dissolution in 0.05 M glycine buffer between pH 2.4 and 3.3, and the sample concentrations were about 1 mg/mL. Data analysis was done with Origin software (MicroCal Inc., Northampton, MA).

X-ray Crystallography. Tyrosine mutants of human lysozyme were crystallized by gradually increasing the concentration of NaCl in the reservoir solution (final concentrations 1.6−1.9 M NaCl and 20 mM acetate, pH 4.5) using the hanging drop vapor diffusion method. The crystals of all mutants were isomorphous with our wild-type crystals (*11*).

Diffraction data to 1.59 Å resolution were collected at 10 °C using a Rigaku R-AXIS IIC image plate mounted on a Rigaku RU-300 rotating anode generator (Cu Kα, 40 kV, 200 mA) and were processed with the programs provided by Rigaku. The data below 1.8 Å resolution were excluded from the refinements, due to their weak intensities.

The wild-type structure, with water molecules and the hydroxyl oxygen atom of tyrosine deleted, was used as the starting model for the refinements of each mutant. The mutant structure was refined with the program X-PLOR (*17*). The *B*-factor restraints were performed with the parameters of Tronrud (*18*). To use the comparison between the wild-type and mutant structures, we recalculated the refinements of the wild-type structure using the same parameters.

RESULTS

Differential Scanning Calorimetry of Tyr Mutant Human Lysozymes. Figure 2 shows typical excess heat capacity

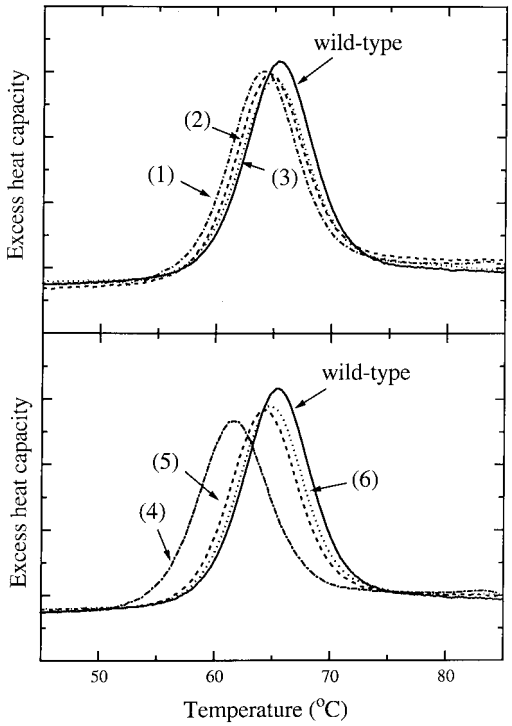

FIGURE 2: Typical excess heat capacity curves of the wild-type and mutant human lysozymes. (1) Y124F at pH 2.70; (2) Y38F at pH 2.71; (3) Y63F at pH 2.73; (4) Y54F at pH 2.69; (5) Y20F at pH 2.74; (6) Y45F at pH 2.71. The increments of excess heat capacity were 10 kJ/(mol K).

curves obtained from differential scanning calorimetric recordings of the six mutant human lysozymes at pH 2.7. Each protein considered in this study gave an excess heat capacity curve with characteristics similar to those of the wild-type protein (*11*), although their peak temperatures differed at the same pH. An acidic pH region was chosen for the measurement because of the higher reversibility of the thermal denaturation of the mutant proteins as well as the wild-type protein. The denaturation temperature (T_d), the denaturation enthalpy changes, calorimetric (ΔH_{cal}) and van't Hoff (ΔH_{vH}), and the denaturation heat capacity change (ΔC_p) were obtained directly from analyses of the heat capacity curves (Table 2). The denaturation temperatures decreased linearly with decreasing pH, in the pH range from 2.4 to 3.3, for all the mutant human lysozymes. The temperature dependence of the calorimetric enthalpies of the mutant proteins examined at different pHs varied showing that the ΔC_p values for the mutant proteins were affected to different magnitudes by the same kind of substitution. Each slope almost coincided with the average of the ΔC_p values obtained from the excess heat capacity curves, within experimental errors (Tables 2 and 3). The thermodynamic parameters of denaturation as a function of temperature can be calculated from 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 values are assumed to be independent of temperature (*19*).

Table 2: Thermodynamic Parameters for Denaturation of the Mutant Human Lysozymes (Tyr→Phe) at Different pHs

protein	pH	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	ratio $\Delta H_{cal}/\Delta H_{vH}$	ΔC_p[a] (kJ/mol K)
Y20F	3.18	72.0	510	540	0.94	2.5
	3.06	69.8	498	531	0.94	5.2
	2.89	66.8	481	515	0.93	7.1
	2.74	64.3	456	494	0.92	5.3
	2.58	61.1	444	477	0.93	7.0
avg					0.93	6.5 ± 1.4
Y38F	3.25	72.4	515	527	0.98	4.7
	3.10	70.3	506	519	0.98	4.0
	2.92	67.2	490	506	0.97	6.1
	2.71	64.6	473	490	0.97	4.3
					0.97	4.9 ± 1.1
Y45F	3.13	72.5	506	531	0.95	6.8
	2.89	68.7	485	510	0.95	2.7
	2.71	64.9	464	490	0.95	4.8
	2.67	64.6	469	490	0.96	5.6
	2.48	61.4	456	481	0.95	3.7
avg					0.95	4.5 ± 1.7
Y54F	3.13	69.1	490	519	0.94	4.7
	2.99	67.0	477	502	0.95	5.6
	2.82	64.0	452	477	0.95	8.0
	2.69	61.7	439	469	0.94	3.7
avg					0.94	5.5 ± 2.3
Y63F	3.17	72.7	515	540	0.95	2.8
	3.00	69.5	490	510	0.96	2.5
	2.73	65.0	464	485	0.96	4.9
	2.58	61.9	444	469	0.95	6.4
avg					0.95	5.3 ± 0.8
Y124F	3.06	69.7	506	523	0.97	7.6
	2.70	64.0	473	494	0.96	6.4
	2.68	63.4	469	485	0.97	4.5
	2.53	60.8	452	469	0.96	6.2
avg					0.96	6.0 ± 1.2

[a] ΔC_p was obtained from each calorimetric curve.

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

protein	T_d (°C)	ΔT_d (°C)	ΔC_p[a] [kJ/(mol K)]	ΔH_{cal} (kJ/mol)	$\Delta\Delta G$ (kJ/mol)	$\Delta H_{cal}/\Delta H_{vH}$
wild type	64.9 ± 0.5		6.6 ± 0.5	477 ± 4	(0)	0.95
Y20F	63.4 ± 0.1	−1.5	6.5 ± 0.5	466 ± 4	−2.1 ± 0.1	0.93
Y38F	64.3 ± 0.2	−0.6	5.4 ± 0.1	475 ± 2	−0.8 ± 0.2	0.97
Y45F	65.1 ± 0.3	+0.2	4.6 ± 0.4	469 ± 4	+0.3 ± 0.4	0.95
Y54F	61.9 ± 0.2	−3.0	7.0 ± 0.4	461 ± 2	−4.0 ± 0.2	0.94
Y63F	64.2 ± 0.2	−0.7	6.4 ± 0.3	463 ± 2	−1.0 ± 0.2	0.95
Y124F	63.8 ± 0.2	−1.1	6.1 ± 0.1	477 ± 1	−1.5 ± 0.2	0.96

[a] ΔC_p was obtained from the slope of ΔH against T_d.

To minimize the error of estimation from the experimental results, the thermodynamic parameters of the denaturation of the six mutant proteins are compared at the denaturation temperature (64.9 °C) of the wild-type protein at pH 2.7, as shown in Table 3. The ΔH values of all the mutant proteins were directly obtained near 65 °C at different pHs. T_d and ΔG of the mutant proteins were lower than that of the wild-type protein except for Y45F. ΔH values of the mutant proteins were smaller than that of the wild-type protein except for Y124F. The changes in ΔG ranged from +0.3 to −4.0 kJ/mol, and the changes in ΔH ranged from 0 to −16.0 kJ/mol, compared with the wild-type protein. The changes in ΔH were bigger than those in ΔG, and the ΔH values of each mutant protein were substantially different

Table 4: X-ray Data Collection and Refinement Statistics for Tyr → Phe Mutants

	Y20F	Y38F	Y45F	Y54F	Y63F	Y124F
(A) Data Collection						
crystal system	orthorhombic					
space group	$P2_12_12_1$					
cell dimension (Å)						
a	56.72	56.65	56.78	56.77	56.71	56.72
b	61.05	61.02	60.97	61.07	60.96	61.12
c	33.80	33.74	33.81	33.88	33.83	33.74
resolution (Å)	1.59	1.59	1.59	1.59	1.59	1.59
no. of measured reflections	34844	48488	43579	34462	48753	43246
no. of independent reflections	13928	15639	14557	13271	14363	13520
completeness of data (%)	84.7	94.8	88.1	80.1	86.8	81.5
R_{merge}[a] (%) [intensity]	6.06	6.53	5.28	3.82	6.34	6.49
(B) Refinement						
no. of protein atoms	1028	1028	1028	1028	1028	1028
no. of solvent atoms	192	224	175	173	168	204
resolution range (Å)	8−1.8	8−1.8	8−1.8	8−1.8	8−1.8	8−1.8
no. of reflections used	10055	10803	10366	9369	10150	9795
completeness (%) [$F_{obs} \geq 3\sigma$]	89.6	96.5	92.3	82.6	90.5	87.3
R factor[b]	0.165	0.170	0.174	0.164	0.165	0.164
rms of deviations						
bonds (Å)	0.008	0.009	0.007	0.008	0.009	0.009
angles (deg)	1.50	1.54	1.42	1.52	1.54	1.52
average of *B*-factors						
backbone (Å^2)	14.0	13.9	14.4	14.6	13.3	14.2
side chain (Å^2)	19.5	17.5	19.6	19.6	18.4	19.3

[a] $R_{merge} = 100\sum|I_{h,i} - \langle I_h\rangle|/\sum I_{h,i}$, where $I_{h,i}$ are individual values, and $\langle I_h\rangle$ is the mean value of the intensity of reflection *h*. [b] $R_{factor} = \sum||F_o| - |F_c||/\sum|F_o|$.

from each other despite the same kinds of substitutions. The results indicate that the destabilization of the mutant proteins differs, depending on the structural feature of the mutation sites.

Changes in denaturation Gibbs energy ($\Delta\Delta G$) by the substitution of Tyr with Phe have been examined in several proteins, such as five RNase T1 mutants (4.8 ∼ −8.5 kJ/mol) (*5*), three barnase mutants (0.4 ∼ −4.8 kJ/mol) (*4*), and seven staphylococcal nuclease mutants (0 ∼ −10.0 kJ/mol) (*6*). They show that the values of $\Delta\Delta G$ range from 4.8 to −10.0 kJ/mol. For some of them, the values seem to correlate with the strength of the hydrogen bonds found in the respective wild-type proteins, but there are significant exceptions and the reasons could not be explained. In general, mutants with deletions of intramolecular hydrogen bonds observed in the wild-type crystal structure tend to have more negative $\Delta\Delta G$ values.

X-ray Structures of the Mutant Human Lysozymes. Data collection and refinement statistics for the six mutant human lysozymes are summarized in Table 4. The overall X-ray structures of the examined mutant proteins were essentially identical to the wild-type structure. In particular, the main-chain atoms of all mutants shift less than 0.2 Å, except for the region including the mutation site in Y124F or the loop region around Ala73 in several mutants. The crystal structures of the mutant proteins in the vicinity of the amino acid substitutions are shown in Figure 3. The replacements of Tyr residues with Phe mainly cause structural changes at the mutation site or affect hydrogen-bond partners. The features of the changes differ from each other.

In the case of Y20F, the side chain atoms of Arg 101, which forms a hydrogen bond with the hydroxyl group of Tyr20 in the wild-type protein, move by ca. 0.5 Å toward Phe20 (Figure 3a). Their temperature factors increase substantially compared with those of the wild-type protein: averaged values of temperature factors for the side-chain atoms of Arg101 are 41 Å^2 for Y20F and 24 Å^2 for the wild-type protein.

On the other hand, the backbone atoms in the vicinity of Phe124 in the Y124F mutant move slightly away from Trp34, which is the hydrogen-bond partner of the hydroxyl group of Tyr124 in the wild-type protein (Figure 3f). The solvent water near Phe124 moves by ca. 2.3 Å and makes a new hydrogen bond with the Nϵ of Trp34. The break of the hydrogen bond between the hydroxyl group of Tyr124 and the Nϵ of Trp34 does not affect the position of Trp34.

In the wild-type protein, the hydroxyl group of Tyr54 participates in a hydrogen-bond network that includes the carboxyl oxygens of Asp67, the hydroxyl group of Ser61, the hydroxyl groups and the amide groups of the main chain of Thr52 and Thr70, and three well-ordered water molecules with temperature factors of 13, 21, and 24 Å^2, respectively. The replacement of Tyr by Phe at position 54 reduces the two kinds of hydrogen bonds in the network but causes little change in the position of atoms that make up the network (Figure 3d).

The hydroxyl group of Tyr38 makes a hydrogen bond with an internal water with a temperature factor of 14 Å^2, which is as small as the value observed for the average of the main-chain atoms. The water also makes hydrogen bonds to two other waters. When Tyr38 is replaced by Phe, the very well-ordered water moves slightly away from Phe38, keeping the two hydrogen bonds with the waters, and the temperature factor increases by ca. 20 Å^2 (Figure 3b).

The water molecule that makes a hydrogen bond with the hydroxyl group of Tyr45 in the wild-type protein is located in the same position in the crystal structure of Y45F, although the temperature factor of the water increases by ca. 13 Å^2 over the 25 Å^2 observed in the wild-type protein (Figure 3c). The break of the hydrogen bond between the water and

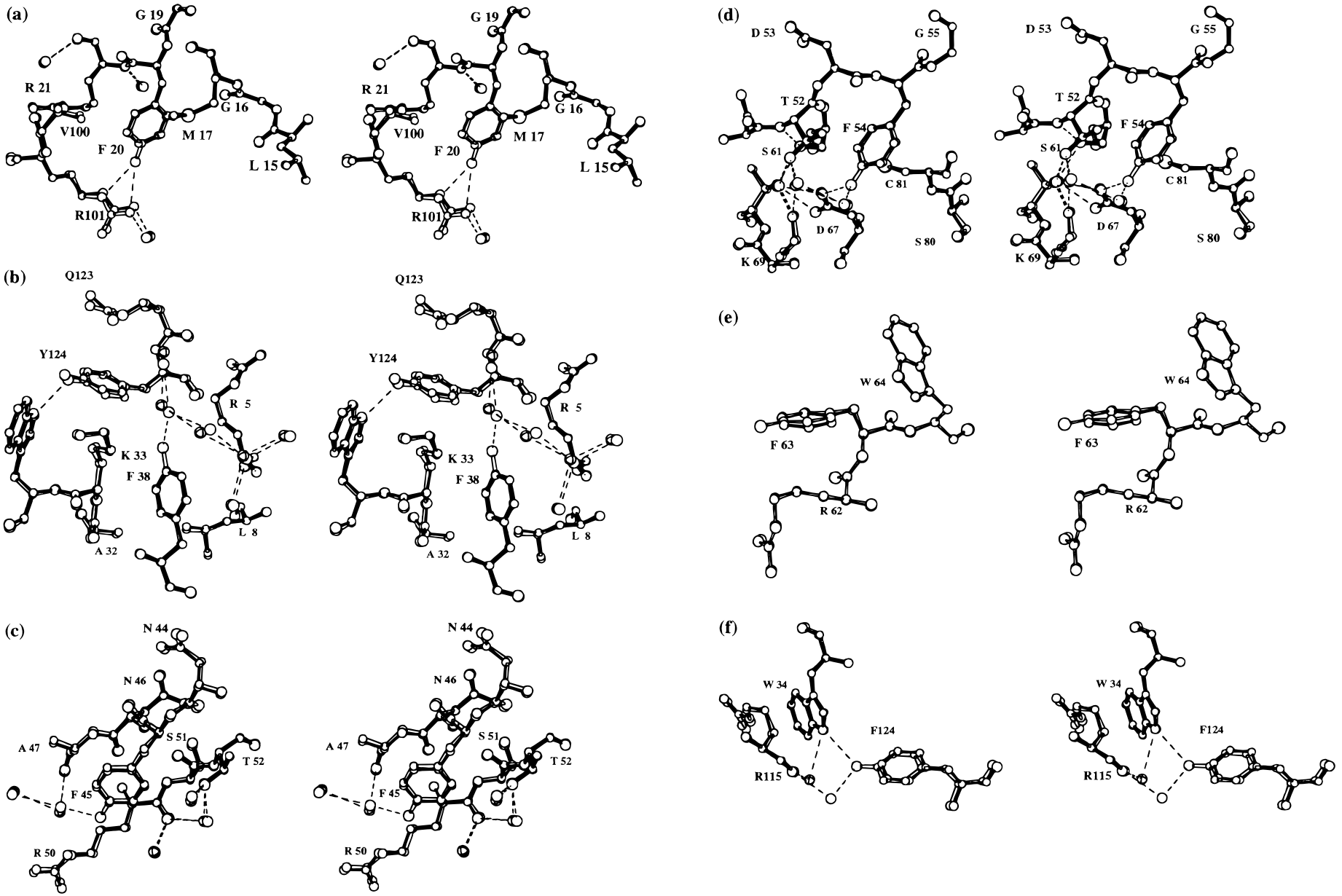

FIGURE 3: ORTEP (*25*) views showing the structure in the vicinity of the mutation sites. The wild-type (open bonds) and mutant structures (filled bonds) are superimposed. Panels a−f represent Y20F, Y38F, Y45F, Y54F, Y63F, and Y124F, respectively. Solvent water molecules are drawn as open circles (wild type) and crossed circles (mutants). The broken lines indicate hydrogen bonds.

Table 5: Estimation of $\Delta\Delta G_{site}$, $\Delta\Delta G_{other}$, and Corrected $\Delta\Delta G$ (kJ/mol)

mutant	$\Delta\Delta G_{site}$ (kJ/mol)		$\Delta\Delta ASA_{HP}^{other}$ [c] (kJ/mol)	$\Delta\Delta G_{other}$ [d] (kJ/mol)	$\Delta\Delta G$ (kJ/mol)	
	$\Delta\Delta G_{tr}$ [a]	$\Delta\Delta G_{conf}$ [b]			measured	corrected[e]
Y20F	3.3	2.4	11.34	1.4	−2.1	−9.2
Y38F	4.5	2.4	16.67	2.0	−0.8	−9.7
Y45F	2.5	2.4	−1.99	−0.2	0.3	−4.4
Y54F	4.0	2.4	−16.57	−2.0	−4.0	−8.4
Y63F	0	1.1[f]	−7.79	−0.9	−1.0	−1.2
Y124F	3.4	2.4	1.30	0.2	−1.5	−7.5

[a] (Fraction of OH group exposed upon denaturation at a mutation site) × $\Delta\Delta G_{tr(Y\rightarrow F)}$ (*20*). [b] $\Delta\Delta G_{conf} = -T\Delta S_{conf}$ (*21*). [c] $\Delta\Delta ASA_{HP}^{other} = \Delta\Delta ASA_{HP}^{all} - \Delta\Delta ASA_{HP}^{site}$. [d] $\Delta\Delta G_{other} = (0.12)\Delta\Delta ASA_{HP}^{other}$ (*12, 13*). [e] Measured $\Delta\Delta G$ values have been corrected by $\Delta\Delta G_{site}$ and $\Delta\Delta G_{other}$. [f] See discussion in text.

the hydroxyl group of Tyr45 does not affect the other hydrogen bonds the waters participate in.

The replacement of Tyr by Phe at position 63 causes an ordering of the phenyl ring as shown by the fact that the average temperature factor of the six-membered ring in Y63F (22 Å^2) is smaller than in the wild-type protein (33 Å^2) (Figure 3e). Indeed, the alternate conformation of the side chain of Tyr63 is unambiguously observed in the frozen crystal (100 K) of the wild-type protein (M. Nakasako, K.T., & K. Y., unpublished results).

DISCUSSION

How To Estimate Gibbs Energy of Hydrogen Bonding. The removal of a hydroxyl group by the substitution of Tyr to Phe of human lysozyme changed the conformational stabilities of the mutant proteins by +0.3 to −4.0 kJ/mol compared with the wild-type protein. The difference in unfolding Gibbs energy between the wild-type and the mutant proteins ($\Delta\Delta G_{exp}$) is described by eq 4. Unfolding Gibbs energy of the wild-type protein experimentally determined (ΔG_{exp}^{wild}) is divided into the contribution of a hydrogen bond at a specific site to stability (ΔG_{HB}^{wild}), the contribution at the mutation site excluding the contribution of the hydrogen bond (ΔG_{site}^{wild}), and other contributions to stability (ΔG_{other}^{wild}). The mutant protein (ΔG_{exp}^{mutant}) is divided into the contribution at the mutation site (ΔG_{site}^{mutant}) and the other contribution ($\Delta G_{other}^{mutant}$).

$$\Delta\Delta G_{exp} = \Delta G_{exp}^{mutant} - \Delta G_{exp}^{wild} \quad (4)$$

$$= (\Delta G_{site}^{mutant} + \Delta G_{other}^{mutant}) - (\Delta G_{HB}^{wild} + \Delta G_{site}^{wild} + \Delta G_{other}^{wild}) \quad (5)$$

$$= \Delta\Delta G_{site} + \Delta\Delta G_{other} - \Delta G_{HB}^{wild} \quad (6)$$

Equation 6 can be rearranged to

$$\Delta G_{HB}^{wild} = \Delta\Delta G_{site} + \Delta\Delta G_{other} - \Delta\Delta G_{exp} \quad (7)$$

For the correction at a mutation site ($\Delta\Delta G_{site}$), the increase in hydrophobicity due to deletion of the hyroxyl group of Tyr residue (*20*) and conformational entropy between Tyr and Phe residues (*21*) should be considered, as described by Myers and Pace (*1*). $\Delta\Delta G_{other}$ can be evaluated from the structural changes due to the mutation, if X-ray structures of the wild-type and mutant proteins are available. It has been reported that Ile to Val and Val to Ala substitutions in the human lysozyme affect not only the mutation sites but also other parts far from the sites, although the structural changes were not great (*11*−*13*). The subtle structural changes caused by rearrangements of the overall structure can change the accessible surface area of all hyrophobic residues exposed upon denaturation ($\Delta\Delta ASA_{HP}$) and are equivalent to changes in stability ($\Delta\Delta G_{HP}$) (0.12 kJ mol^{-1} Å^{-2}) (*12, 13*). Thus, we can estimate the contribution of hydrogen bonds to protein stability. The estimation of $\Delta\Delta G$ due to changes in ASA ($\Delta\Delta ASA_{HP}^{other} = \Delta\Delta ASA_{HP}^{all} - \Delta\Delta ASA_{HP}^{site}$) of all hydrophobic groups excluding the mutation site exposed upon denaturation ($\Delta\Delta G_{HP}$), changes in hydrophobicity due to the deletion of the hyroxyl group of a Tyr residue ($\Delta\Delta G_{tr}$), and conformational entropy change between Tyr and Phe residues ($\Delta\Delta G_{conf}$) are listed in Table 5, on the basis of the difference between the wild-type and mutant structures. $\Delta\Delta G_{HP}$ assigned to $\Delta\Delta G_{other}$, and $\Delta\Delta G_{tr}$ and $\Delta\Delta G_{conf}$ to $\Delta\Delta G_{site}$. The stability changes depending on what happens to the remaining member of the hydrogen-bonding pair in the mutant proteins could be included in the term of $\Delta\Delta G_{other}$. In the present paper, this effect will be discussed on the basis of the comparison with the structural changes in the high-resolution X-ray structures of the six Tyr → Phe mutant human lysozymes. When $\Delta\Delta G_{other}$ contains only $\Delta\Delta G_{HP}$ in this estimation, the corrected $\Delta\Delta G$ ($= \Delta\Delta G_{exp} - \Delta\Delta G_{site} - \Delta\Delta G_{other}$) in Table 5 consists of the lost ΔG_{HB}^{wild}. The remaining factors are discussed below.

Relationship between Corrected $\Delta\Delta G$ Values and Structural Changes at Each Mutation Site. Table 1 shows structural characteristics of six Tyr residues of the human lysozyme, and Gibbs energy changes of each component contributing stability affected by the substitution are shown in Table 5. For calculation of the corrected $\Delta\Delta G$ value, $\Delta\Delta G_{other}$ can be partly estimated from $\Delta\Delta ASA_{HP}$, but some of them cannot be quantitatively estimated in the present condition. Then, it is important to describe in detail structural changes due to the mutation, for example, about the remaining group of a hydrogen-bonding pair. In this session, what the corrected $\Delta\Delta G$ value of each mutant reflects is discussed separately below.

(1) Y124F. The accessible surface area (ASA) of the hydroxyl group of Tyr124 in the wild-type human lysozyme is 72%. The hydroxyl group makes a hydrogen bond with NϵH of Trp34 and with a water molecule, whose *B*-factor is larger than those of the other water molecules ordered by Tyr (Table 1). In the case of Y124F, the water molecule moves by 2.3 Å and makes a hydrogen bond with NϵH of

Trp34. The remaining members (NϵH of Trp34 and a water molecule) of the hydrogen-bonding pair in the mutant protein make a hydrogen bond to each other as a new pair. $\Delta\Delta ASA_{HP}$ of the mutant protein was small (Table 5), suggesting that the effect of the substitution was localized at the substitution site. Considered these facts, this mutant protein might be a good example for estimating the net effect that a hydrogen bond has on protein stabilization. The difference in the hydrophobicity and the conformational entropy between Tyr and Phe residues might be stabilized by 5.8 kJ/mol and the increase in $\Delta\Delta$ASA of all hydrophobic groups excluding the mutation site is 0.2 kJ/mol (Table 5). As the measured $\Delta\Delta G$ value of the mutant protein is -1.5 kJ/mol, the corrected $\Delta\Delta$G value is -7.5 kJ/mol. The correction value should correspond to ΔG_{HB}^{wild} of one hydrogen bond lost at position 124. This represents the net effect of a hydrogen bond on conformational stability.

(2) Y45F. The hydroxyl group of Tyr45 is 47% exposed on the surface and interacts strongly with a water molecule. The water molecule also interacts with the carbonyl of Ala47 in the wild-type protein and is located in the same position in the crystal structure of Y45F, although the *B*-factor is increased. In this case, one hydrogen bond between the hydroxyl group and a water molecule is lost. Judged from the small ΔG_{HP}^{wild} value of ΔASA_{HP}^{other}, the structural changes of Y45F due to the mutation might be localized in the substitution site. Therefore, the force of the lost hydrogen bond could be estimated to be -4.4 kJ/mol after the correction, although increase in the mobility of the partner water molecule was not considered.

(3) Y38F. The hydroxyl group of Tyr38 in the wild-type protein is 95% buried and it binds to the perfectly ordered internal water with a lower *B*-factor (13.8 Å^2, Table 1). The water also forms hydrogen bonds with two other waters. The removal of the hydroxyl group resulted in the movement of the ordered water molecule and in an increase of the *B*-factor, but two hydrogen bonds between waters still remain. The corrected value of -9.7 kJ/mol indicates that the lack of the hydrogen bond of Tyr38 with the internal water molecule and the movement of the hydrogen-bond network of waters in the interior of the protein destabilizes the protein. These results from Y45F and Y38F consist of our finding that a hydrogen bond between the polar group and a water molecule in a cavity created in the interior of the protein contributes favorably to the stability (*13, 23*).

(4) Y20F. The hydroxyl group of Tyr20 in the wild-type human lysozyme is 30% buried. The hydroxyl group forms two intramolecular hydrogen bonds with NϵH and NηH of Arg101 (Figure 3a): one has a normal hydrogen-bond distance and the other is a very weak hydrogen bond. Due to the substitution of Tyr to Phe, the loss of two hydrogen bonds causes the increase in the *B*-factor of the side chain of Arg101 and the distance between Cζ at position 20 and Nϵ of Arg101 was shorter by 0.25 Å. This suggests that the movement of Arg101 in the wild-type protein was restricted by the hydrogen bond with the hydroxyl group of Tyr20. The corrected $\Delta\Delta G$ value is -9.2 kJ/mol. The value of 9.2 kJ/mol is slightly larger than ΔG_{HB}^{wild}estimated from Y124F and might be small as compared with ΔG_{HB}^{wild} corresponding to the summation of a normal and a weak hydrogen bond. This may suggest that the destabilization by the deletion of the two hydrogen bonds is compensated by entropic effect due to increased movement of Arg101.

(5) Y54F. The hydroxyl group of Tyr54 is 84% buried in the wild-type protein and makes hydrogen bonds with the carboxyl oxygen of Asp67 and a water molecule in a hydrogen-bond network. The removal of the hydroxyl group at Tyr54 in the hydrogen-bond network reduces the two kinds of hydrogen bonds but causes little change in the position of the atoms that join to the network. In this case, the conformational stability was the lowest and the measured $\Delta\Delta G$ value of the mutant protein was -4.0 kJ/mol. The corrected $\Delta\Delta G$ value is -8.4 kJ/mol. The negative corrected value is smaller than the summation (11.9 kJ/mol) of the estimated ΔG_{HB}^{wild} values of Tyr at position 45 corresponding to the lost energy due to the deletion of a hydrogen bond with water and that of position 124 corresponding to an intramolecular hydrogen-bond energy. This may show that the contribution per hydrogen bond to stability in the presence of a network is not equivalent to that in the isolated state. The remaining members of the hydrogen bond in the mutant protein still make hydrogen bonds with the same partners as those in the wild-type protein.

(6) Y63F. In the wild-type protein, the hydroxyl group of Tyr63 is 100% solvent-exposed, and it does not appear that the residue has a strong interaction with a water molecule from the X-ray structure (Table 1). Because the side chain of Tyr63 might have an alternate conformation, the side-chain entropy might be higher. This partly compensated the difference in side-chain entropy between Tyr and Phe as shown in Table 5, when we recalculated the side-chain entropy according to the method of Doing and Sternberg (*21*). The experimental value was -1.0 kJ/mol and the correction value was also nearly negligible, indicating that our correction methods are reasonable.

CONCLUSION

The net contribution of the intramolecular hydrogen bond to the conformational stability of a protein could be estimated to be 7.5 kJ/mol, using the most suitable model mutant human lysozyme for the evaluation. Myers and Pace (*1, 23*) have summarized results that show that hydrogen bonds stabilize proteins and that the average net stabilization is 1−2 kcal/mol (4.2−8.4 kJ/mol) per intermolecular hydrogen bond. Our values agree with their conclusions. In addition to finding similar values, we could give a more detailed account of the reasons for the difference in the stability between each Tyr mutant and the wild-type protein. If we can obtain thermodynamic parameters for a series of mutant proteins, combined with X-ray structures of the mutant proteins, the contribution of a hydrogen bond in various situations to stability would be better understood.

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