Contribution of Intra- and Intermolecular Hydrogen Bonds to the Conformational Stability of Human Lysozyme[†,‡]

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ABSTRACT: In globular proteins, there are intermolecular hydrogen bonds between protein and water molecules, and between water molecules, which are bound with the proteins, in addition to intramolecular hydrogen bonds. To estimate the contribution of these hydrogen bonds to the conformational stability of a protein, the thermodynamic parameters for denaturation and the crystal structures of five Thr to Val and five Thr to Ala mutant human lysozymes were determined. The denaturation Gibbs energy (ΔG) of Thr to Val and Thr to Ala mutant proteins was changed from 4.0 to −5.6 kJ/mol and from 1.6 to −6.3 kJ/mol, respectively, compared with that of the wild-type protein. The contribution of hydrogen bonds to the stability ($\Delta\Delta G_{HB}$) of the Thr and other mutant human lysozymes previously reported was extracted from the observed stability changes ($\Delta\Delta G$) with correction for changes in hydrophobicity and side chain conformational entropy between the wild-type and mutant structures. The estimation of the $\Delta\Delta G_{HB}$ values of all mutant proteins after removal of hydrogen bonds, including protein−water hydrogen bonds, indicates a favorable contribution of the intra- and intermolecular hydrogen bonds to the protein stability. The net contribution of an intramolecular hydrogen bond ($\Delta G_{HB[pp]}$), an intermolecular one between protein and ordered water molecules ($\Delta G_{HB[pw]}$), and an intermolecular one between ordered water molecules ($\Delta G_{HB[ww]}$) could be estimated to be 8.5, 5.2, and 5.0 kJ/mol, respectively, for a 3 Å long hydrogen bond. This result shows the different contributions to protein stability of intra- and intermolecular hydrogen bonds. The entropic cost due to the introduction of a water molecule (ΔG_{H_2O}) could be also estimated to be about 8 kJ/mol.

Globular proteins contain hundreds of intramolecular hydrogen bonds, an average of 1.1 intramolecular hydrogen bonds per residue, in the native states (*1*). Many studies of mutant proteins with respect to intramolecular hydrogen bonds have shown that intramolecular hydrogen bonds stabilize the protein structures (*2*−*10*). Furthermore, intermolecular hydrogen bonds between protein and water, or water and water molecules, also exist in protein structures. The solvent water molecules are bound in the interior or on the surface of proteins. There are questions about whether intermolecular hydrogen bonds contribute to the conformational stability and, if so, how much they contribute.

The effect of intermolecular hydrogen bonds on protein stability can be experimentally investigated using (i) mutant proteins to remove hydrogen bonds between protein and water molecules and (ii) mutant proteins to introduce water molecules which form hydrogen bonds with the protein atoms. Mutant S80A, Y38F, and Y45F human lysozymes that have been described (*8*, *10*) correspond to the first category. At these mutation sites, the hydrogen bonds with water molecules in the wild-type structure are removed due to mutation. The contributions of the hydrogen bonds to the stability have been estimated to be 4−7 kJ/mol with an analysis considering some factors that affect the stability due to mutation. In the second category, mutant proteins with water molecules newly introduced by substitution have been found in T4 lysozyme (T151S) (*11*), barnase (I76A) (*12*), and human lysozyme (I106A, I59A, and I59G) (*13*). The new water molecules form hydrogen bonds with the protein molecules. The stability of these mutant proteins is higher than expected, despite unfavorable energy cost due to the decrease in entropy caused by transferring the water molecule from the solvent to the interior of the protein (*14*). These reports have suggested the favorable contribution of intermolecular hydrogen bonds to the stability of a protein. However, how much they contribute remains to be estimated. Therefore, to quantitatively evaluate the role of intermolecular hydrogen bonds in the conformational stability of a

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[‡] Coordinates have been deposited in the Brookhaven Protein Data Bank as PDB file names 1CJ6 (T11A), 1CJ7 (T11V), 1CJ8 (T40A), 1CJ9 (T40V), 1CKC (T43A), 1CKD (T43V), 1CKF (T52A), 1CKG (T52V), and 1CKH (T70V).

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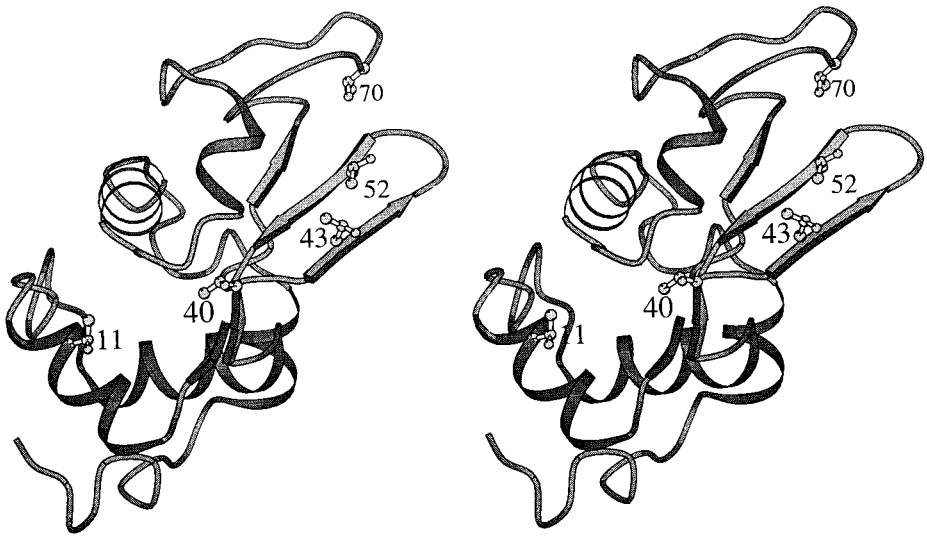

FIGURE 1: Stereodrawing of the wild-type human lysozyme structure. The locations of the five Thr residues are indicated. The structure was generated with the program MOLSCRIPT (*40*).

protein, more abundant and detailed information must be accumulated for intermolecular hydrogen bonds, and also for intramolecular ones, using systematic mutant proteins.

Mutational analysis, measurements of the changes in conformational stability ($\Delta\Delta G$)[1] due to the mutation of a hydrogen-bonded residue to one incapable of hydrogen bonding, is one of the useful methods for extracting the energy of hydrogen bonding to protein structures (*7*). The problems with this approach, however, are that mutations alter the properties of amino acid residues such as hydrophobicity which affects the stability, and mutations also affect other sites far from the substitution site, despite small structural changes (*15*). To avoid these problems, it is necessary to determine the crystal structures of mutant proteins and to assemble a large set of mutant stability−structure data. Studies on the stability and structure of more than 70 mutant human lysozymes have been reported (*8*, *10*, *13*, *15*−*20*), and the effects of changes in structural properties due to mutation on the conformational stability of human lysozyme, such as hydrophobicity, have been quantified using the stability−structure database of the mutant proteins (*20*). This data set and analysis should be useful in estimating the contribution of several types of hydrogen bonds to protein stability.

In this study, Thr to Val and Ala mutant proteins of human lysozyme were highlighted. These substitutions introduce little steric hindrance. Both side chains of Val and Ala are incapable of forming hydrogen bonds, but are different from each other on the scale of hydrophobicity. The human lysozyme consists of five Thr residues: Thr11, -40, -43, -52, and -70. Thr11 is in an α-helix, and Thr40, -43, and -52 are in β-strands in the wild-type structure. The OH groups of Thr11, -40, -52, and -70 are almost buried and form hydrogen bonds with protein or water molecules in the wild-type structure. The structural characteristics of the Thr residues in the wild-type human lysozyme are listed in Table 1. The positions of the five Thr residues in the human lysozyme are shown in Figure 1. The thermodynamic parameters for denaturation of the mutant proteins were determined using differential scanning calorimetry (DSC), and the crystal structures were determined by X-ray analysis. These and previous results with mutant human lysozymes with respect to hydrogen bonds (*8*, *10*, *19*, *20*) were used to gain a better understanding of the contribution of intra- and intermolecular hydrogen bonds to protein stability.

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

	secondary structure	buried % of the side chain	buried % of the OH group	hydrogen bonding partner[b]	hydrogen bonding distance[c] (Å)
Thr11	helix	80	83	Glu7 O	2.92
Thr40	strand	99	100	Lys1 N	2.81
				water O	2.80
Thr43	strand	52	6		
Thr52	strand	91	97	water O	2.83
Thr70		100	100	Ser61 Oγ	2.77
				Asp67 Oδ	2.80
				water O	2.92

[a] From Takano et al. (*16*). [b] As hydrogen bonding partners of Thr residues, water molecules on the protein surface with only one hydrogen bond are excluded. [c] 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 and nitrogen, respectively.

MATERIALS AND METHODS

Mutant Proteins. Mutagenesis, expression, and purification of the Thr mutant human lysozymes were performed as previously described (*16*). The concentration of the mutant proteins was spectrophotometrically determined using an $E^{1\%}$(1 cm) of 25.65 at 280 nm (*21*).

Differential Scanning Calorimetry (DSC). Calorimetric measurements and data analyses were carried out as previously described (*16*). For measurements, the DASM4 adiabatic microcalorimeter equipped with an NEC personal computer was used. The sample buffer for measurements

[1] Abbreviations: ASA, solvent-accessible surface area; ASP, atomic solvation parameter; ΔC_p, heat capacity change; $\Delta\Delta G$, difference in ΔG between the wild-type and mutant proteins; ΔG, Gibbs energy change; ΔH_{cal}, calorimetric enthalpy change; ΔH_{vH}, van't Hoff enthalpy change; DSC, differential scanning calorimetry; HB, hydrogen bond; HP, hydrophobicity; rms, root-mean-square; T_d, denaturation temperature.

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

protein	pH	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	protein	pH	T_d (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)
T11A	3.22	73.4	499	502	T11V	3.19	73.0	490	506
	3.02	71.4	485	498		2.81	68.7	477	490
	2.82	68.5	481	485		2.71	66.2	460	469
	2.74	66.8	469	481		2.51	62.9	435	448
	2.58	63.7	439	464					
T40A	3.10	68.6	490	506	T40V	3.10	68.7	481	514
	3.00	66.5	477	490		3.00	66.3	469	498
	2.85	63.3	448	477		2.85	63.2	460	473
	2.70	59.4	418	427		2.70	59.2	389	418
	2.50	56.6	408	431		2.50	57.0	404	435
T43A	3.08	69.7	477	477	T43V	3.20	75.4	473	506
	2.90	67.1	464	469		3.02	73.0	452	494
	2.74	64.5	452	456		2.71	68.4	439	477
	2.54	61.1	435	439		2.51	65.0	418	469
T52A	3.10	68.9	456	494	T52V	3.10	68.7	464	477
	3.00	66.9	452	477		3.00	66.6	448	473
	2.85	64.4	439	469		2.85	64.2	439	460
	2.70	61.9	431	456		2.70	62.1	427	448
	2.50	58.8	413	435		2.50	58.8	413	435
T70A	3.10	67.1	460	473	T70V	3.10	70.3	498	515
	3.00	64.7	448	460		3.00	67.6	481	506
	2.85	62.2	431	444		2.85	65.3	469	490
	2.70	60.0	423	435		2.70	62.6	448	464
	2.50	57.3	412	418		2.50	59.5	427	444

was 0.05 M Gly-HCl (pH 2.5−3.3). Data analysis was performed 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 values are assumed to be independent of temperature (*22*).

X-ray Structural Analysis. Mutant human lysozymes were crystallized as previously described (*8*, *16*). All crystals belong to space group $P2_12_12_1$, but the crystal cell dimensions of T52V differed from those of the wild type and most mutant proteins.

All intensity data except for those for T70A were collected by the oscillation method on the Rigaku R-AXIS IIC imaging plate mounted on the Rigaku RU300 rotating anode X-ray generator. The data were processed with the software provided by Rigaku. For T70A, the two data sets were collected by the oscillation method on the Rigaku R-AXIS IV imaging plate mounted on the Rigaku RU300 rotating anode X-ray generator. The data were processed with the software provided by Rigaku. Another data set for T70A was collected by synchrotron radiation at the Photon Factory on beam line 6B with a Weissenberg camera (*23*). The data were processed with DENZO (*24*).

The structures except for T52V were refined with the program X-PLOR (*25*) as previously described (*8*, *16*). For T52V, the structure was determined by the molecular replacement technique using the program AMoRe (*26*) and refined with the program X-PLOR (*25*) as previously described (*20*).

Calculation of ASA Values. The accessible surface area (ASA) values of the proteins were calculated by the procedure of Connolly (*27*) with a probe radius of 1.4 Å as previously described (*17*, *20*). The ASA values of the denatured state were calculated using an actual polypeptide with an extended conformation (*28*).

RESULTS

Stability of Mutant Human Lysozymes

DSC measurements of Thr to Val/Ala mutant human lysozymes were carried out at acidic pHs (2.5−3.3) where the denaturation of human lysozyme is reversible. Table 2 shows the denaturation temperatures (T_d), the calorimetric enthalpies (ΔH_{cal}), and the van't Hoff enthalpies (ΔH_{vH}) of each measurement for the mutant proteins. The T_d values are sensitive to changes in pH and increase linearly with increasing pH for all the proteins that were studied. 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. The heat capacity changes (ΔC_p) of denaturation were obtained from the slopes of the plot of ΔH_{cal} versus T_d.

The denaturation Gibbs energy (ΔG) of Thr to Val and Thr to Ala mutant proteins was changed from 4.0 to −5.6 kJ/mol and from 1.6 to −6.3 kJ/mol, at pH 2.7, respectively, compared to that of the wild-type protein. Even for the same kind of substitution, Thr to Val or Thr to Ala, the effects of the mutations on the stability were different, depending on each mutation site. Moreover, for positions 11, 40, and 52, the substitutions with Val and Ala brought similar stability changes to each other, but the stabilities of Thr to Val and Thr to Ala mutants at positions 43 and 70 were quite different from each other.

The ΔH values of the mutant human lysozymes that were examined were changed from −58 to −14 kJ/mol. The range of the changes in ΔH was larger than that in ΔG, indicating

Table 3: Thermodynamic Parameters for Denaturation of Mutant Human Lysozymes (Thr → Ala and Thr → Val) 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	0
T11A	66.1 ± 0.6	+1.2	5.7 ± 1.0	453 ± 7	+1.6
T11V	65.9 ± 0.3	+1.0	5.4 ± 0.9	450 ± 6	+1.3
T40A	60.2 ± 0.6	−4.7	7.2 ± 0.5	462 ± 5	−6.3
T40V	60.7 ± 0.3	−4.2	6.7 ± 0.9	459 ± 8	−5.6
T43A	63.8 ± 0.2	−1.1	4.9 ± 0.1	453 ± 0	−1.5
T43V	68.1 ± 0.3	+3.2	4.9 ± 0.7	419 ± 5	+4.0
T52A	62.0 ± 0.2	−2.9	3.5 ± 0.5	445 ± 5	−3.8
T52V	62.1 ± 0.3	−2.8	5.0 ± 0.3	442 ± 3	−3.6
T70A	60.2 ± 0.5	−4.7	5.0 ± 0.3	448 ± 2	−6.2
T70V	62.8 ± 0.4	−2.1	6.6 ± 0.2	463 ± 2	−2.9

[a] ΔC_p was obtained from the slope of ΔH_{cal} vs T_d. [b] From Takano et al. (*16*).

that the large enthalpy changes were offset by the entropy changes in most cases. The determination of ΔH values for mutant proteins with DSC must be important for understanding the mechanism of protein stabilization. However, in this paper, the changes in ΔH values due to mutation were not correlated with changes in hydrogen bonds.

Structures of Mutant Human Lysozymes

Table 4 lists X-ray data collection and refinement statistics for Thr to Val/Ala mutant human lysozymes. The crystal form of T52V was different from those of the wild type and the other Thr mutants. There were two T52V molecules in an asymmetric unit. In the case of T70A, even though the data collection for T70A was performed three times using different crystals, the electron densities around residue 70 were obscure in any case so that the structure around the mutation site could not be determined. The overall X-ray structures of the mutant proteins that were determined were essentially identical to that of the wild-type structure. The crystal structures in the vicinity of the mutation sites except for T70A are illustrated in Figures 2−6. For each mutant protein, the structural changes were observed as described below.

Thr11 → Val and Thr11 → Ala. Thr11 is located in an α-helix (residues 5−14), and the side chain forms a hydrogen bond with the carbonyl oxygen of Glu7, which is in the same α-helix. The substitutions of Thr11 with Val and Ala caused little structural change, except for the removal of the hydrogen bond between Thr11 and Glu7 (Figure 2). The rms deviations for main chain atoms between the wild-type and mutant structures, T11V and T11A, were 0.08 and 0.09 Å, respectively.

Thr40 → Val and Thr40 → Ala. The substitutions of Thr40 caused structural changes larger than those of Thr11, especially around residue 40. The rms deviations for main chain atoms between the wild-type and mutant structures, T40V and T40A, were 0.12 and 0.15 Å, respectively, and those within 6 Å from Cα at residue 40 were 0.21 and 0.29 Å, respectively.

The side chain of Thr40 in the wild-type structure forms two hydrogen bonds. One is an intramolecular hydrogen bond with Lys1, and the other is an intermolecular hydrogen bond with a water molecule which has two more intermolecular hydrogen bonds with protein atoms. In the T40A structure, two hydrogen bonds in which Thr40 participated were removed (Figure 3a), but the replacement of Thr by Val at position 40 removed one more hydrogen bond which had been formed between the water molecule and Leu85, as the result of the movement of the water molecule, in addition to the two hydrogen bonds in which Thr40 participated (Figure 3b).

Thr43 → Val and Thr43 → Ala. Thr43 is located on the protein surface and in a β-strand (residues 42−46). The side chain of Thr43 forms no hydrogen bond in the wild-type lysozyme. The mutation of Thr43 to Ala affected the β-strand. The rms deviation for main chain atoms between the wild-type and mutant T43A structures was 0.10 Å, but that in the β-strand was 0.52 Å (Figure 4a). In contrast, the structural changes of the β-strand and the overall structure in T43V were not so great. The side chain conformation of Val43, however, was quite different from the corresponding one of the parent Thr residue (Figure 4b). The γ-carbon atoms of Val43 interact with other hydrophobic residues, such as Leu85.

Thr52 → Val and Thr52 → Ala. The side chain of Thr52 forms one intermolecular hydrogen bond with a water molecule in the wild-type protein. The water molecule forms one more intermolecular hydrogen bond with a protein atom. In the mutant structure, T52A, the former hydrogen bond was deleted, but the water molecule was located in the same position with the latter hydrogen bond (Figure 5a). The T52V crystal form (type III) was different from those of the wild type and most other mutant proteins (type I), and those of I56M, I56F, and S82A (type II) (*10*, *20*). There were two T52V molecules (T52V1 and T52V2) in an asymmetric unit.

Table 4: X-ray Data Collection and Refinement Statistics for Thr to Val/Ala Mutant Human Lysozymes

	data collection								refinement					
	cell dimensions (Å)			resoluion	measured	independent	completeness	R_{merge}[a]		solvent	resolution		completeness	
	a	*b*	*c*	(Å)	reflections	reflections	(%)	(%)	atoms	atoms	(Å)	reflections	(%)	R_{factor}[b]
T11A	56.72	61.11	33.86	1.8	27 429	10 057	87.8	6.8	1299	272	8−1.8	9683	86.0	0.150
T11V	56.74	60.98	33.95	1.8	30 207	10 154	88.6	4.9	1307	278	8−1.8	9873	87.7	0.150
T40A	56.78	61.24	33.67	1.8	26 157	10 645	93.1	5.4	1254	227	8−1.8	10 218	91.0	0.161
T40V	56.86	61.15	33.73	1.8	34 021	11 049	96.5	5.8	1292	263	8−1.8	10 632	94.5	0.155
T43A	56.67	61.06	33.80	1.8	33 379	10 679	93.5	4.2	1290	263	8−1.8	10 392	92.6	0.151
T43V	56.64	61.04	33.91	1.8	32 373	11 166	97.5	4.4	1283	254	8−1.8	10 884	96.8	0.151
T52A	56.51	61.25	33.00	1.8	22 975	9611	84.1	7.5	1296	269	8−1.8	8896	81.1	0.170
T52V	65.26	106.67	39.54	2.0	31 949	16 216	83.6	10.0	2361	303	8−2.2	11 543	81.5	0.166
T70V	56.50	60.63	32.56	1.8	24 020	9394	86.6	7.1	1302	273	8−2.0	6838	87.1	0.183

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

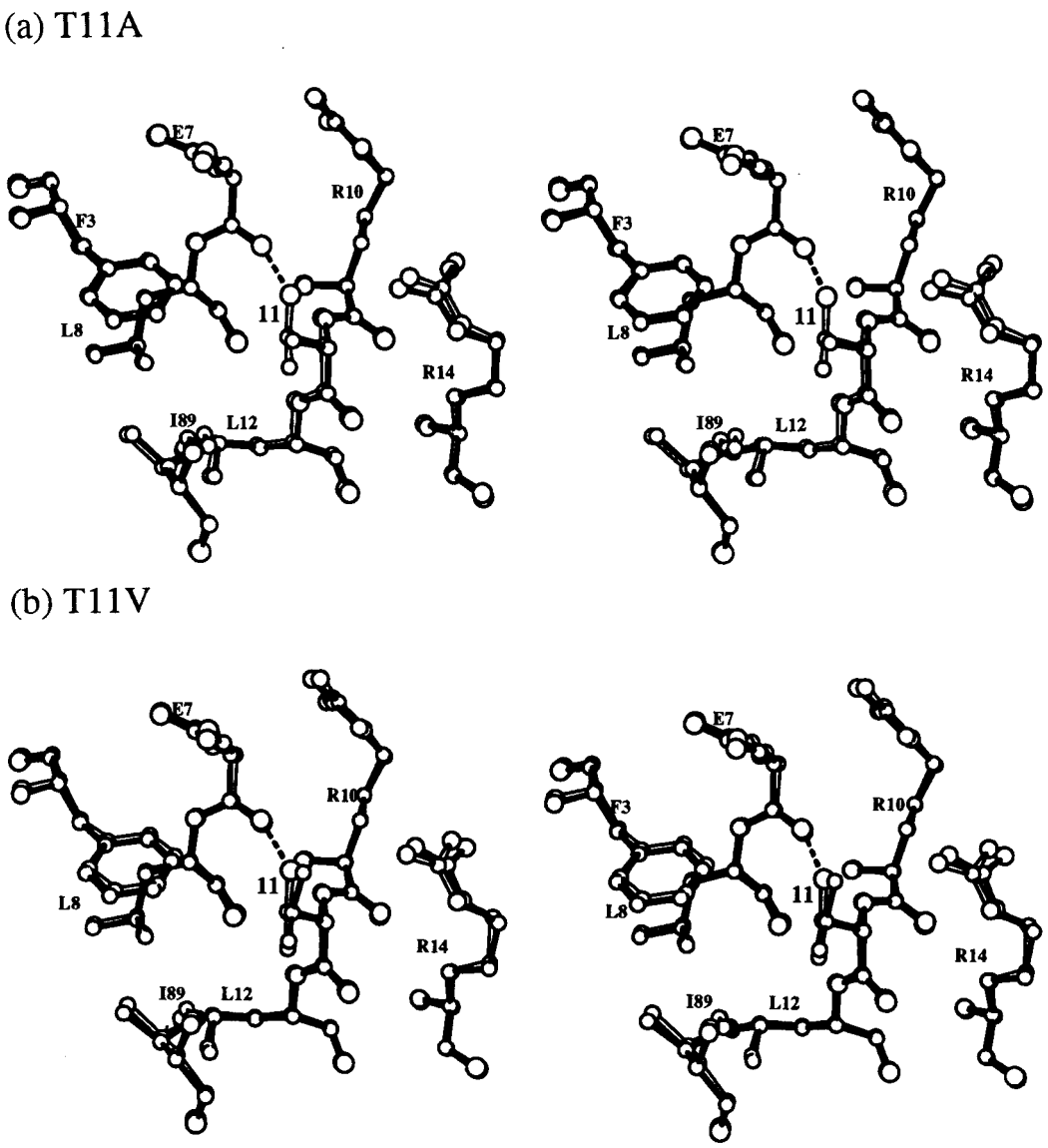

FIGURE 2: ORTEP (*41*) views showing the structure in the vicinity of the mutation sites. Panels a and b depict T11A and T11V, respectively. The wild-type (white bonds) and mutant structures (black bonds) are superimposed. Solvent water molecules are drawn as white circles (wild-type) and crossed circles (mutants). The broken lines represent hydrogen bonds.

In both structures, T52V1 and T52V2, the hydrogen bonds between residue 52 and the water molecule were removed. The water molecule was observed in the T52V2 structure (Figure 5c), but it disappeared in T52V1 (Figure 5b), because the position of 52 in the T52V1 structure was within the crystal contact region in the type III crystal form.

Thr70 → Val and Thr70 → Ala. In the wild-type protein, the side chain of Thr70 participates in a hydrogen bond network that includes residues Thr52, Tyr54, Ser61, and Asp67. Thr70 forms two intra- and one intermolecular hydrogen bonds. The water molecule hydrogen bonding with Thr70 has two other hydrogen bonds with protein atoms. The substitution of Thr70 with Val caused the structural changes around the site, especially in the loop region, residues 68−78 (Figure 6). The rms deviation for main chain atoms between the wild-type and T70V structures was 0.35 Å, and that in the loop region was 0.88 Å.

Three sets of data with different crystals, including one for which synchrotron radiation was used, were collected for crystals of T70A with a suitable size for X-ray analysis. However, the electron densities around residue 70, especially in the loop region, residues 68−73, were very poor in any case. This means the flexibility of the loop region was high. It, therefore, seems that the hydrogen bond network in which Thr70 participated plays an important role in fixing the loop structure.

DISCUSSION

Estimate of the Contribution of Hydrogen Bonds to the Stability of a Protein

In mutational analyses involving hydrogen bonds in protein structures, a hydrogen bonding residue is usually replaced with a residue incapable of hydrogen bonding. It seems that the contribution of a hydrogen bond to protein stability is directly obtained by measuring the difference in stability between the wild-type and mutant proteins, $\Delta\Delta G$. However, the $\Delta\Delta G$ value reflects a total change in stability upon mutation, so the $\Delta\Delta G$ value also includes some components other than that of the hydrogen bond. Myers and Pace (*7*) have assumed that $\Delta\Delta G$ can be represented by the additive

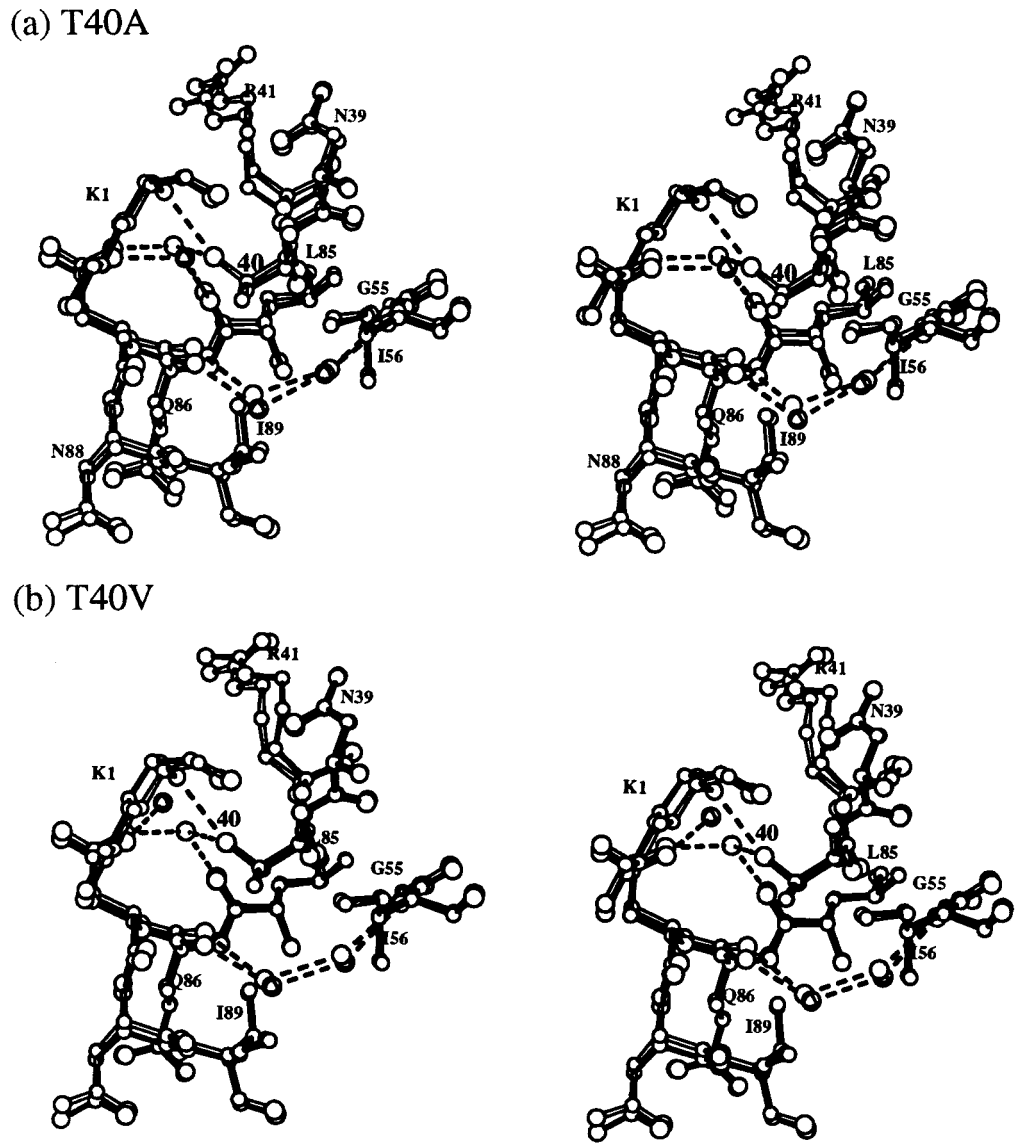

FIGURE 3: ORTEP (*41*) views showing the structure in the vicinity of the mutation sites. Panels a and b depict T40A and T40V, respectively. All conventions are as described in the legend of Figure 2.

contribution of each factor, which mainly affects protein stability.

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

Here, $\Delta\Delta G_{HB}$, $\Delta\Delta G_{HP}$, and $\Delta\Delta G_{conf}$ are contributions due to changes in hydrogen bonds, in the hydrophobic effect, and in the conformational entropy of the residue being mutated, respectively. The $\Delta\Delta G_{HP}$ and $\Delta\Delta G_{conf}$ values are based on the *n*-octanol hydrophobicity scale of Fauchere and Pliska (*29*) and the mean $T\Delta S_{conf}$ values given by Doig and Sternberg (*30*), respectively. This estimation, however, does not include the effect of structural changes due to mutation. It has been reported that the changes in accessible surface area (ASA) of the overall structure upon denaturation correlate with the stability changes due to mutation (*13, 15, 17*). Funahashi et al. (*20*) evaluated the parameters of the hydrophobic effect, which are proportional to the ASA.

$$\Delta\Delta G_{HP} = 0.178\Delta\Delta ASA_{nonpolar} - 0.013\Delta\Delta ASA_{polar} \quad (5)$$

Here, $\Delta\Delta ASA_{nonpolar}$ represents the change in ΔASA of the nonpolar atoms (C and S atoms) upon denaturation between the wild-type and mutant proteins, and $\Delta\Delta ASA_{polar}$ represents the change in ΔASA values of the polar atoms (N and O atoms). The proportional coefficients, 0.178 and −0.013, in eq 5 are empirical parameters. Takano et al. (*10*) proposed that the contribution of the hydrogen bonds to protein stability can be obtained as follows:

$$\begin{aligned}\Delta\Delta G_{HB} &= \Delta\Delta G - (\Delta\Delta G_{HP} + \Delta\Delta G_{conf}) \\ &= \Delta\Delta G - (0.178\Delta\Delta ASA_{nonpolar} - \\ &\qquad 0.013\Delta\Delta ASA_{polar} - T\Delta\Delta S_{conf}) \quad (6)\end{aligned}$$

Here, the $\Delta\Delta G$ values of the mutant human lysozymes experimentally determined were those at 64.9 °C, because the most reliable thermodynamic parameters could be obtained near 65 °C where many experimental data with high accuracy for the mutant proteins were obtained (Table 2). Then, the $\Delta\Delta G_{HB}$ values obtained from eq 6 are those at 64.9 °C, but the temperature dependence of the contribution of hydrogen bonds to protein stability is not great (*31*). In

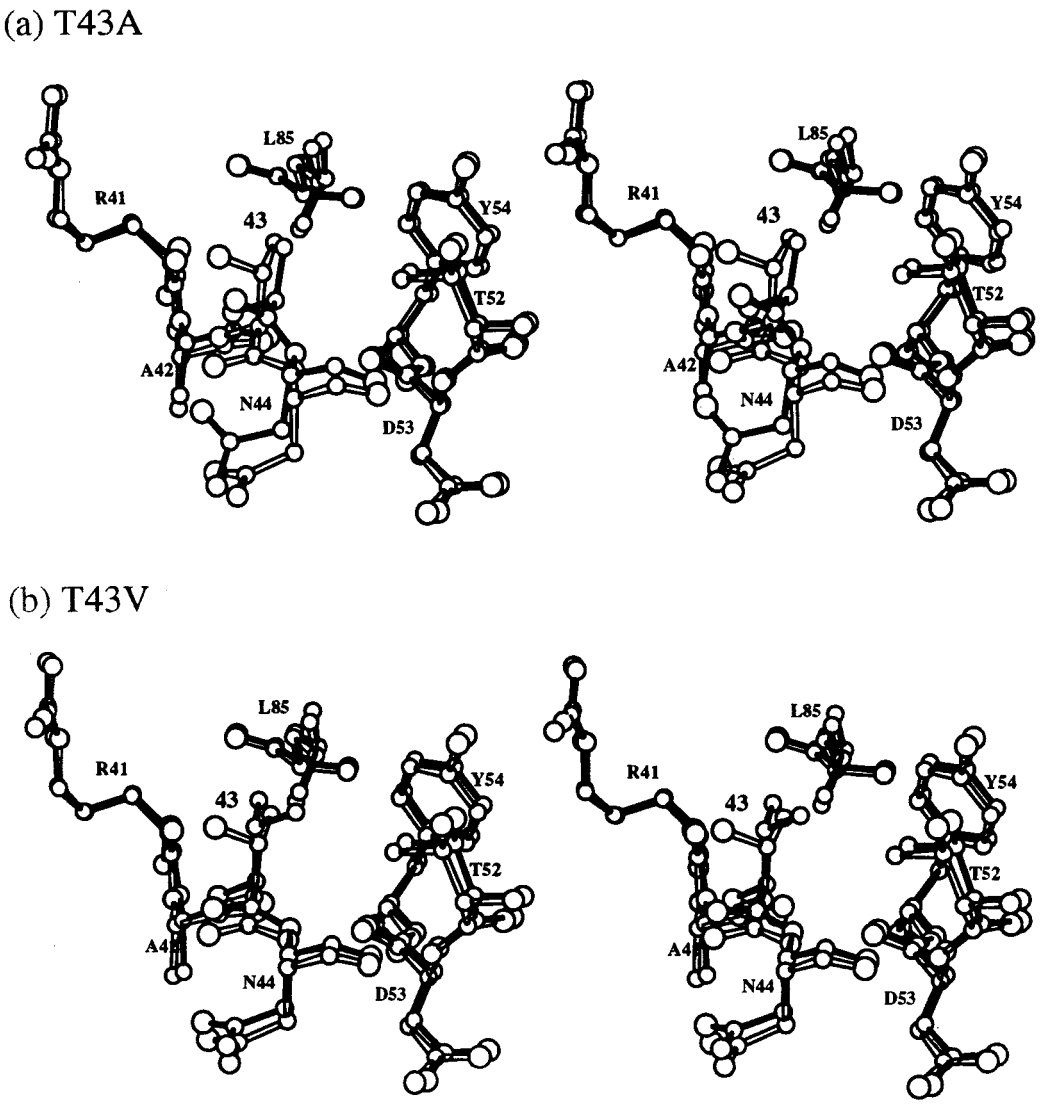

FIGURE 4: ORTEP (*41*) views showing the structure in the vicinity of the mutation sites. Panels a and b depict T43A and T43V, respectively. All conventions are as described in the legend of Figure 2.

the cases of Thr to Val and Thr to Ala mutations, the values of $T\Delta\Delta S_{conf}$ at 65 °C are −3.1 and −5.1 kJ/mol, respectively (*30*).

Table 5 lists $\Delta\Delta G_{HB}$ values estimated using eq 6 and the number of hydrogen bonds changed by substitution for the Thr to Val/Ala mutant human lysozymes and the other mutant human lysozymes previously reported, Tyr to Phe (*8*), Ser to Ala (*10*), and Ile to Thr (*19*) mutant proteins. The data in Table 5 indicate that the mutant proteins which removed hydrogen bonds have negative $\Delta\Delta G_{HB}$ quantities. On the contrary, I56T which formed one intermolecular hydrogen bond by the substitution has a positive $\Delta\Delta G_{HB}$ quantity. These values show the favorable contribution of hydrogen bonds, including protein−water hydrogen bonds, to the conformational stability of the protein. Moreover, the mutant proteins which removed more than two hydrogen bonds, T40A, T40V, T70V, Y20F, S51A, and S61A, mostly exhibited larger negative values of $\Delta\Delta G_{HB}$ than those which removed one hydrogen bond, T11A, T11V, Y38F, Y45F, Y54F, Y124F, S24A, S36A, S80A, and I56T. The $\Delta\Delta G_{HB}$ values of T43A, T43V, and Y63F which did not affect the number of hydrogen bonds by substitution were nearly zero. These results indicate that the estimated $\Delta\Delta G_{HB}$ values are reasonable. Table 5 also shows the contribution of one hydrogen bond to the stability of a protein, ΔG_{HB}, demonstrating the average contribution of one intra- or intermolecular hydrogen bond to protein stability to be 7.9 ± 2.6 kJ/mol.

Contribution of Hydrogen Bonds to the Stability of Each Thr Mutant Human Lysozyme

The contribution of one hydrogen bond of each Thr residue in the human lysozyme to the stability was examined as follows. The T52V and T70A mutant proteins were excluded, because the crystal form of T52V was different from that of the wild-type protein, and the structure around Ala70 in T70A was not determined.

Thr40 (T40A and T40V) and Thr70 (T70V). The side chain of Thr40 forms two hydrogen bonds in the wild-type structure: one intra- and one intermolecular hydrogen bond. The $\Delta\Delta G_{HB}$ value of T40A was −14.7 kJ/mol, but that of T40V was −20.8 kJ/mol (Table 5). T40A removed just two hydrogen bonds. On the other hand, the substitution of Thr40 to Val removed one more intermolecular hydrogen bond, a total of three hydrogen bonds. For one hydrogen bond, the

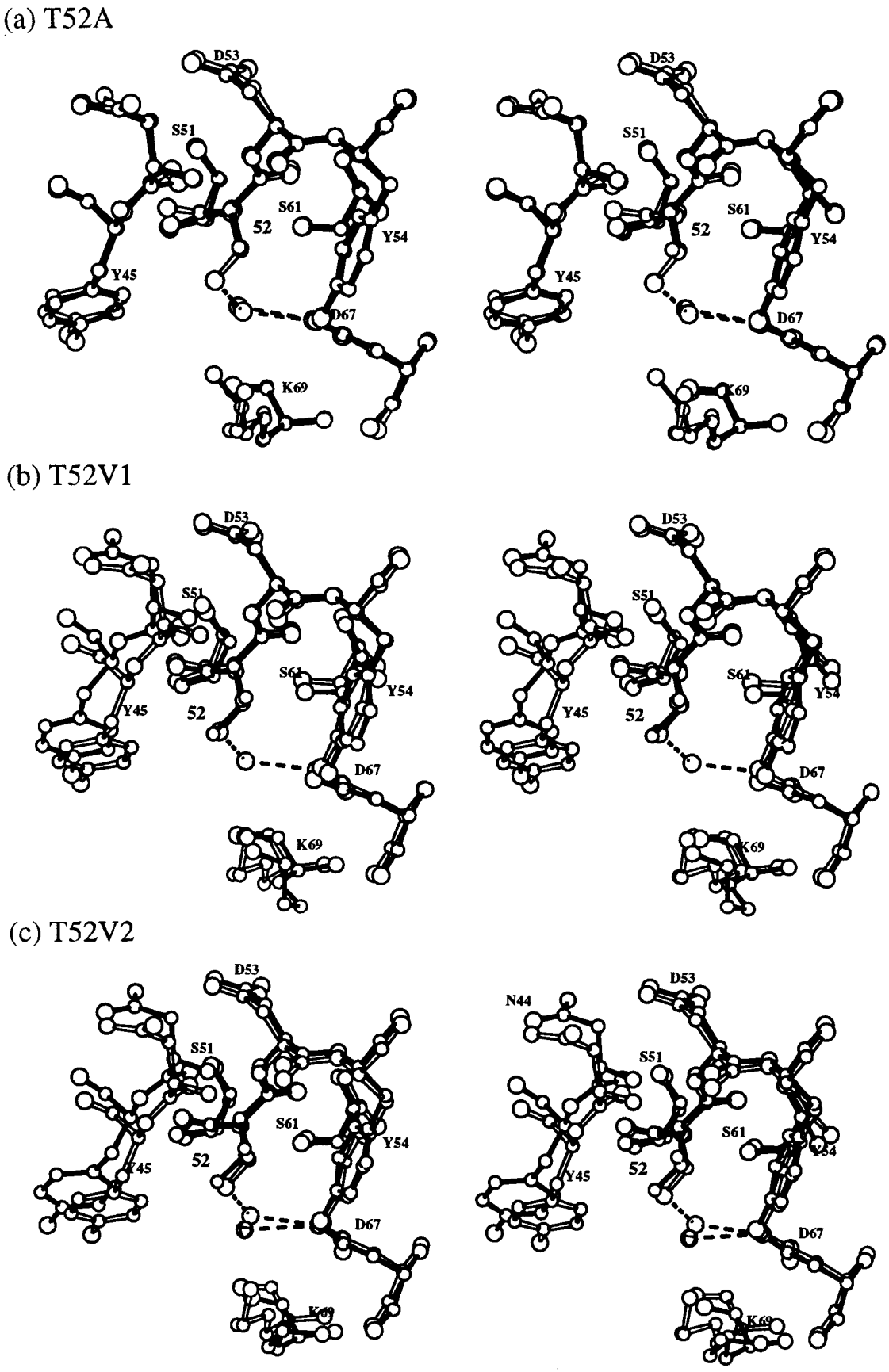

FIGURE 5: ORTEP (*41*) views showing the structure in the vicinity of the mutation sites. Panels a−c depict T52A, T52V1, and T52V2, respectively. All conventions are as described in the legend of Figure 2.

ΔG_{HB} value of T40V was 6.9 kJ/mol, comparable with that of T40A (7.4 kJ/mol).

Thr70 has three hydrogen bonds: two intramolecular bonds and one intermolecular hydrogen bond. In the T70V

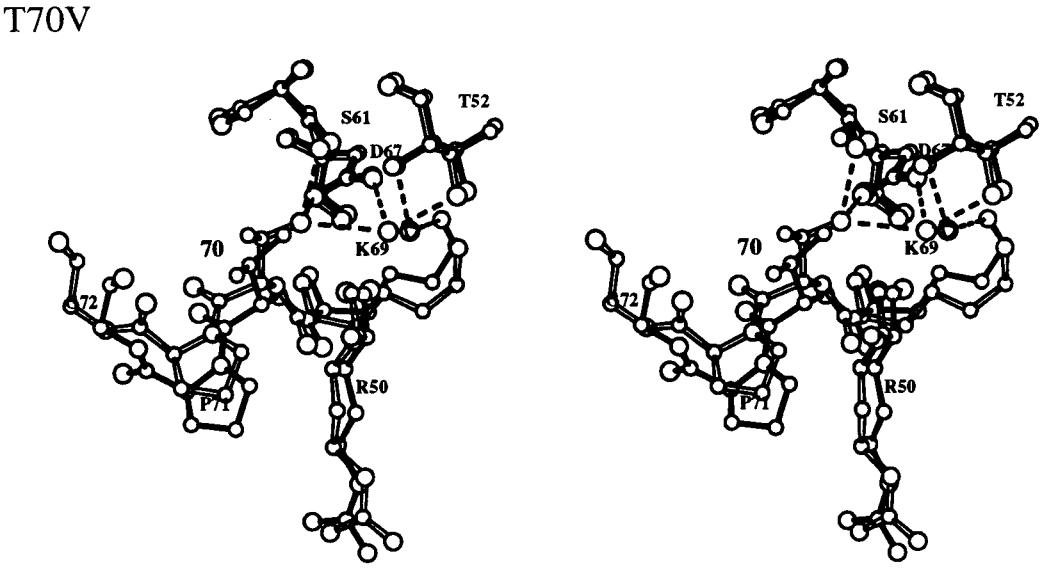

FIGURE 6: ORTEP (*41*) views showing the structure in the vicinity of the mutation sites for T70V. All conventions are as described in the legend of Figure 2.

Table 5: Changes in the Number of Hydrogen Bonds Due to Substitution and $\Delta\Delta G_{HB}$ Values of Mutant Human Lysozymes

	protein−protein[a]	protein−water[b]	$\Delta\Delta G_{HB}$[c] (kJ/mol)	ΔG_{HB}[d] (kJ/mol)
T11A	−1		−1.9	1.9
T11V	−1		−8.3	8.3
T40A	−1	−1	−14.7	7.4
T40V	−1	−2	−20.8	6.9
T43A			0.2	−
T43V			−0.9	−
T52A		−1	−6.1	6.1
T70V	−2	−1	−22.6	7.5
Y20F[e]	−2		−12.5	6.3
Y38F[e]		−1	−4.4	4.4
Y45F[e]		−1	−6.9	6.9
Y54F[e]	−1		−9.7	9.7
Y63F[e]			−1.8	−
Y124F[e]	−1		−10.5	10.5
S24A[f]	−1		−9.9	9.9
S36A[f]	−1		−13.9	13.9
S51A[f]	−2		−16.4	8.2
S61A[f]	−2		−24.8	12.4
S80A[f]		−1	−6.7	6.7
I56T[g]		+1	5.3	5.3
				7.9 ± 2.6[h]

[a] Changes in the number of hydrogen bonds between protein atoms due to substitution. [b] Changes in the number of hydrogen bonds between the protein atom and water molecule due to substitution. [c] $\Delta\Delta G_{HB} = \Delta\Delta G$(measured) − ($\Delta\Delta G_{conf} + \Delta\Delta G_{HP}$) (eq 6). [d] $\Delta G_{HB} = \Delta\Delta G_{HB}$/(change in no. of hydrogen bonds). [e] From Yamagata et al. (*8*). [f] From Takano et al. (*10*). [g] From Funahashi et al. (*19*). [h] Average per hydrogen bond.

structure, these hydrogen bonds disappeared. The reduction in the $\Delta\Delta G_{HB}$ value for T70V was 22.6 kJ/mol; i.e., the contribution per one hydrogen bond, ΔG_{HB}, was 7.5 kJ/mol, which was similar to those of T40A and T40V.

Thr52 (T52A). T52A removed one intermolecular hydrogen bond between Thr52 and a water molecule due to substitution. The contribution of the hydrogen bond (ΔG_{HB}) was 6.1 kJ/mol, slightly lower than the average value, 7.9 kJ/mol. For the mutant proteins, Y38F, Y45F, S80A, and I56T (*8*, *19*), which removed or formed one protein−water hydrogen bond, the ΔG_{HB} values are 4.4−6.9 kJ/mol, suggesting that the contribution of the protein−water hydrogen bond to protein stability is smaller than that of the intramolecular one.

Thr11 (T11A and T11V). T11A and T11V deleted one intramolecular hydrogen bond between Thr11 and Glu7. The ΔG_{HB} value of T11V was 8.3 kJ/mol, but that of T11A was only 1.9 kJ/mol. This may be caused by the effect of secondary structural propensity on the stability of T11A. It has been known that the secondary structural propensity affects the stability of mutant proteins substituted on the α-helix (*15*, *32*−*36*). The helix propensities of Thr/Val and Ala are quite different. The stabilization effect caused by α-helix propensity of the mutation of Val to Ala on the α-helix was about 4 kJ/mol (*15*, *33*). The ΔG_{HB} value of T11A might then be greater if the effect of secondary structural propensity was introduced in the estimation of the ΔG_{HB} values.

Contribution of an Intramolecular Hydrogen Bond and an Intermolecular Hydrogen Bond between Protein and Water Molecules to the Stability of a Protein

The results presented here and previous results (*8*, *10*) suggest that the contribution of intramolecular hydrogen bonds to protein stability is different from that of intermolecular hydrogen bonds with water molecules. To estimate the contribution of protein−water hydrogen bonds to protein stability, $\Delta\Delta G_{HB}$ was divided into the contributions of intra- and intermolecular hydrogen bonds as follows:

$$\Delta\Delta G_{HB} = \Delta\Delta G_{HB[pp]} + \Delta\Delta G_{HB[pw]} \quad (7)$$

where $\Delta\Delta G_{HB[pp]}$ and $\Delta\Delta G_{HB[pw]}$ are the contribution of intramolecular hydrogen bonds and of protein−water hydrogen bonds, respectively. Because the magnitude of the electrostatic interaction between two atoms is proportional to a reciprocal of the distance between them, $\Delta\Delta G_{HB[pp]}$ and $\Delta\Delta G_{HB[pw]}$ are normalized by the length of a hydrogen bond, r_{HB}.

$$\Delta\Delta G_{HB}(\gamma_{[pp]},\gamma_{[pw]}) = \gamma_{[pp]}\sum(r_{HB[pp]})^{-1} + \gamma_{[pw]}\sum(r_{HB[pw]})^{-1} \quad (8)$$

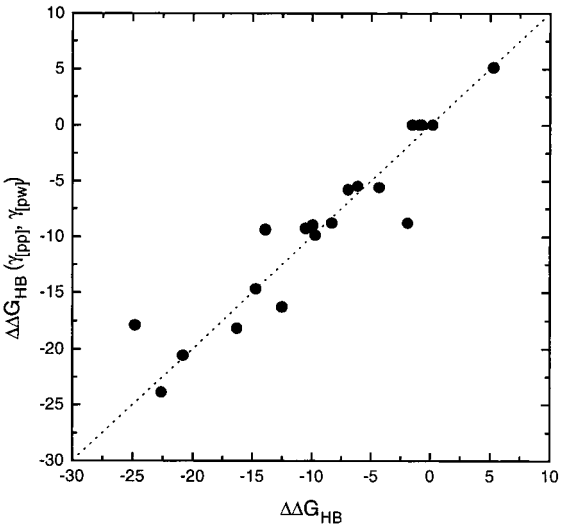

FIGURE 7: Correlation between $\Delta\Delta G_{HB}$ and $\Delta\Delta G_{HB}(\gamma_{[pp]},\gamma_{[pw]})$. The mutant proteins that were used and the $\Delta\Delta G_{HB}$ values are listed in Table 5. $\Delta\Delta G_{HB}(\gamma_{[pp]},\gamma_{[pw]})$ is represented by eq 8 (see the discussion in the text). The coefficient, *R*, is 0.94, and the standard deviation, SD, is 2.8 kJ/mol (20 points). The dotted line represents the equation $y = x$.

where $\gamma_{[pp]}$ and $\gamma_{[pw]}$ are the proportionality constants. The constants, $\gamma_{[pp]}$ and $\gamma_{[pw]}$, were estimated using the least-squares fit of the $\Delta\Delta G_{HB}$ values in Table 5 and $\Delta\Delta G_{HB}(\gamma_{[pp]},\gamma_{[pw]})$ in eq 8. Figure 7 shows the correlation between the $\Delta\Delta G_{HB}$ values listed in Table 5 and $\Delta\Delta G_{HB}(\gamma_{[pp]},\gamma_{[pw]})$ in eq 8. The correlation coefficient, *R*, was 0.94, and the standard deviation, SD, was 2.8 kJ/mol (20 points). The estimation led to a $\gamma_{[pp]}$ of 25.63 kJ Å mol^{-1} and a $\gamma_{[pw]}$ of 15.60 kJ Å mol^{-1}. These values show that the contributions of 3 Å intra- and intermolecular hydrogen bonds to protein stability are 8.5 and 5.2 kJ/mol, respectively, indicating the different contributions of intra- and intermolecular hydrogen bonds.

Contribution of an Intermolecular Hydrogen Bond between Water Molecules and the Entropic Effect Due to the Introduction of a Water Molecule to the Stability of a Protein

Globular proteins frequently have some water molecules in their cavities (*37*), and mutant proteins forming cavities occasionally contain some water molecules in their cavities (*12*, *13*, *16*, *20*, *38*). These water molecules form hydrogen bonds with protein or other bound water molecules. Here, we estimated the contribution of an intermolecular hydrogen bond between the water molecules and the entropic effect due to the introduction of a water molecule to the stability of a protein, using some mutant human lysozymes, I59A, I59G, I59S, I59T, I59V, and I106A, which include some water molecules introduced by substitution (*13*, *16*, *20*). The stability changes ($\Delta\Delta G$) of these proteins could be represented as follows:

$$\Delta\Delta G = \Delta\Delta G_{HP} + \Delta\Delta G_{conf} + \Delta\Delta G_{HB[pw]} + \Delta\Delta G_{HB[ww]} + \Delta\Delta G_{H_2O} \quad (9)$$

where $\Delta\Delta G_{HB[ww]}$ is the contribution of the water−water hydrogen bonds and $\Delta\Delta G_{H_2O}$ is the entropic effect due to the introduction of water molecules. Equation 9 is rearranged as eq 10:

$$\begin{aligned}\Delta\Delta G_{HB[ww]} + \Delta\Delta G_{H_2O} &= \Delta\Delta G - (\Delta\Delta G_{HP} + \Delta\Delta G_{conf} + \Delta\Delta G_{HB[pw]}) \\ &= \Delta\Delta G - [0.178\Delta\Delta ASA_{nonpolar} - 0.013\Delta\Delta ASA_{polar} - T\Delta\Delta S_{conf} + 15.60\sum(r_{HB[pw]})^{-1}] \quad (10)\end{aligned}$$

$\Delta\Delta G_{HB[ww]}$ and $\Delta\Delta G_{H_2O}$ could be also described by eq 11.

$$\Delta\Delta G_{HB[ww]}(\gamma_{[ww]}) + \Delta\Delta G_{H_2O}(\delta) = \gamma_{[ww]}\sum(r_{HB[ww]})^{-1} + \delta\Delta N_{H_2O} \quad (11)$$

where ΔN_{H_2O} is the change in the number of water molecules due to mutation and $\gamma_{[ww]}$ and δ are the proportionality constants. The constants, $\gamma_{[ww]}$ and δ, were estimated using the least-squares fit of the $(\Delta\Delta G_{HB[ww]} + \Delta\Delta G_{H_2O})$ values in eq 10 and $\Delta\Delta G_{HB[ww]}(\gamma_{[ww]}) + \Delta\Delta G_{H_2O}(\delta)$ in eq 11. A set of values ($\gamma_{[ww]}$ = 14.91 kJ Å mol^{-1} and δ = −7.54 kJ/mol) was found to give results in good agreement with the experimental data (R = 0.86, SD = 1.5 kJ/mol, six points). The $\gamma_{[ww]}$ value corresponds to the destabilization by 5.0 kJ/mol if a 3.0 Å long hydrogen bond is removed. This contribution was comparable with that of a water−protein hydrogen bond (5.2 kJ/mol). The δ value means that the introduction of one water molecule in the interior of a protein entropically destabilizes the protein structure by 7.5 kJ/mol. This value was identical with that estimated by Funahashi et al. (*20*), 7.8 kJ/mol. Dunitz (*14*) has also estimated an unfavorable free energy cost, maximally 10 kJ/mol at 65 °C, of transferring a water molecule from the solvent to the interior of a protein. On the other hand, a statistical study (*39*) of cavity and buried water in protein structures has shown that a buried water usually forms three or four hydrogen bonds. Because an intermolecular hydrogen bond makes a favorable contribution of 5.2 kJ/mol to the protein stability as shown above, two hydrogen bonds with a water (10.4 kJ/mol) are able to overcome the entropic cost (7.5 kJ/mol) of transferring a water molecule from the solvent to the interior of a protein. That is, a bound water molecule in a protein structure contributes favorably to the stability (*13*).

CONCLUSION

Myers and Pace (*7*) have summarized the results of mutant proteins with respect to hydrogen bonds and concluded that the net contribution of one intramolecular hydrogen bond to protein stability is 1−2 kcal/mol (4.2−8.4 kJ/mol). In this study, the contribution of three kinds of hydrogen bonds to the conformational stability of a protein could be estimated using systematic mutant proteins: 8.5 kJ/mol for a 3.0 Å long intramolecular hydrogen bond, 5.2 kJ/mol for a 3.0 Å long intermolecular hydrogen bond between the protein and water molecules, and 5.0 kJ/mol for a 3.0 Å long intermolecular hydrogen bond between water molecules. The results show the different contributions of intra- and intermolecular hydrogen bonds. This is the first report which experimentally evaluated them.

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