Contribution of Salt Bridges near the Surface of a Protein to the Conformational Stability[†,‡]

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ABSTRACT: Salt bridges play important roles in the conformational stability of proteins. However, the effect of a surface salt bridge on the stability remains controversial even today; some reports have shown little contribution of a surface salt bridge to stability, whereas others have shown a favorable contribution. In this study, to elucidate the net contribution of a surface salt bridge to the conformational stability of a protein, systematic mutant human lysozymes, containing one Glu to Gln (E7Q) and five Asp to Asn mutations (D18N, D49N, D67N, D102N, and D120N) at residues where a salt bridge is formed near the surface in the wild-type structure, were examined. The thermodynamic parameters for denaturation between pH 2.0 and 4.8 were determined by use of a differential scanning calorimeter, and the crystal structures were analyzed by X-ray crystallography. The denaturation Gibbs energy (ΔG) of all mutant proteins was lower than that of the wild-type protein at pH 4, whereas there was little difference between them near pH 2. This is caused by the fact that the Glu and Asp residues are ionized at pH 4 but protonated at pH 2, indicating a favorable contribution of salt bridges to the wild-type structure at pH 4. Each contribution was not equivalent, but we found that the contributions correlate with the solvent inaccessibility of the salt bridges; the salt bridge contribution was small when 100% accessible, while it was about 9 kJ/mol if 100% inaccessible. This conclusion indicates how to reconcile a number of conflicting reports about role of surface salt bridges in protein stability. Furthermore, the effect of salts on surface salt bridges was also examined. In the presence of 0.2 M KCl, the stability at pH 4 decreased, and the differences in stability between the wild-type and mutant proteins were smaller than those in the absence of salts, indicating the compensation to the contribution of salt bridges with salts. Salt bridges with more than 50% accessibility did not contribute to the stability in the presence of 0.2 M KCl.

Globular proteins frequently contain some salt bridges between oppositely charged residues in the folded conformation (*1*, *2*). It is recognized that salt bridges are important for the structure of proteins, as hydrophobic effects and hydrogen bonds are (*3*). Especially, surface salt bridges have attracted considerable attention; highly stable proteins from thermophilic and hyperthermophilic organisms have an abundance of surface salt bridges relative to mesophilic homologues, suggesting that surface salt bridges increase the stability of thermophilic proteins (*4*−*7*). However, the contribution of surface salt bridges to protein stability has been a source of controversy even today. Some experimental studies with mutant proteins have shown that surface salt bridges do not contribute to the stability (*8*−*13*). These results contradict the reports that surface salt bridges stabilize protein structures (*14*−*17*).

Mutational analysis is a useful approach for estimating the contribution of some factors to the conformational stability of a protein. However, the effects of mutations on protein stability differ from site to site, depending on their environment in the structure. Under these circumstances, information from systematic mutant proteins concerning hydrophobic effects and hydrogen bondings succeeds in providing the net contribution of these factors to protein stability (*18*−*25*). In the case of salt bridges, several studies have been reported. However, in many cases, each has focused on only a given salt bridge, resulting in only explanation of a certain surface salt bridge and causing the controversy. Furthermore, some reports have shown that a surface salt bridge of a protein contributes to the stability, but another one of the same protein does little (*26*, *27*). These results strongly suggest that data from systematic mutant proteins substituted at various sites should be accumulated and discussed on the basis of their structural features, to estimate the contribution of surface salt bridges to protein stability.

Human lysozyme has six salt bridges near the surface of the structure as listed in Table 1 (*18*). Figure 1 illustrates the structure of human lysozyme and the position of these

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[‡] The coordinates for mutant lysozymes described in this paper have been deposited in the Brookhaven Protein Data Bank under the following PDB file names: E7Q, 1EQ4; D102N, 1EQ5; and D120N, 1EQE.

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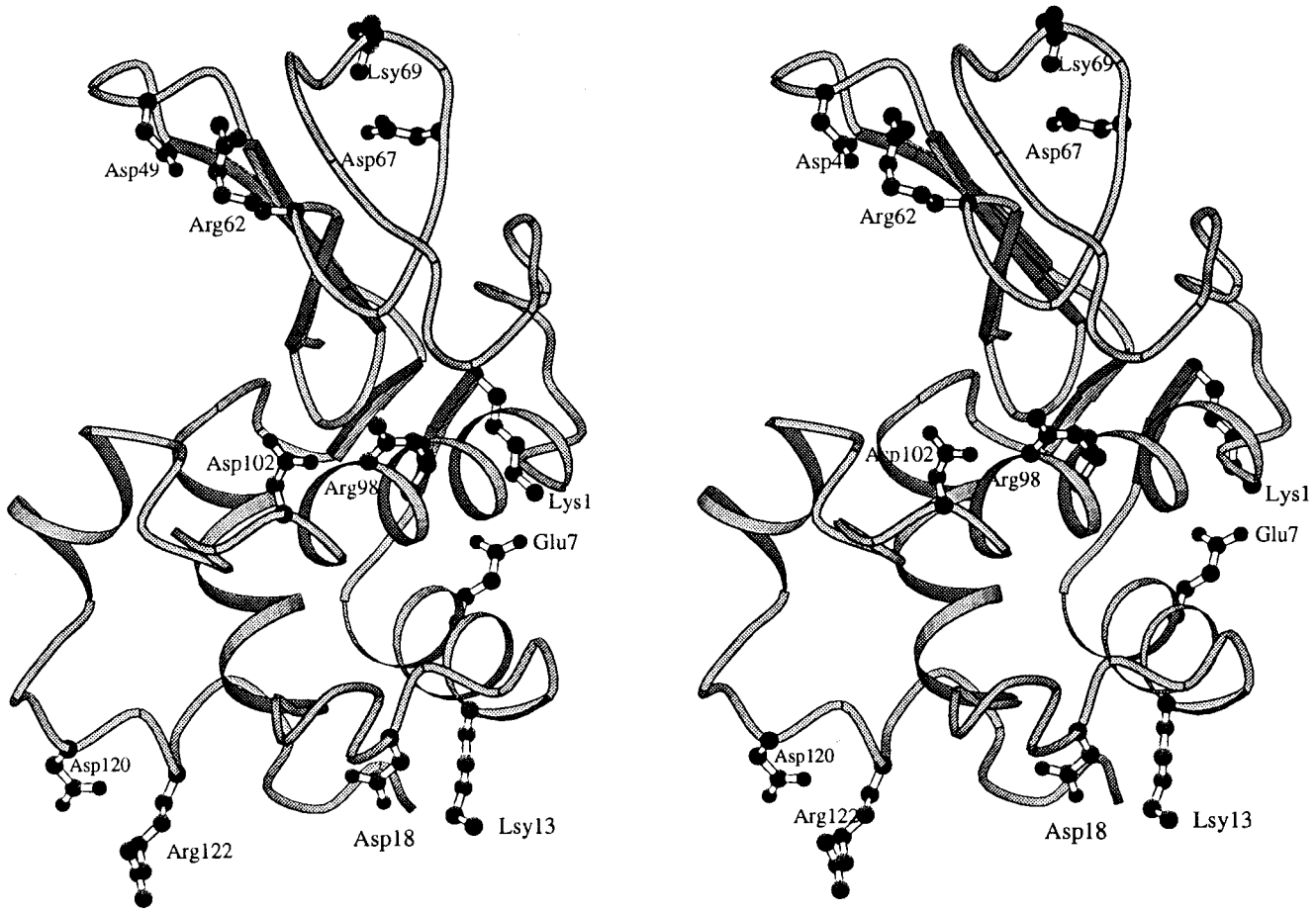

FIGURE 1: Stereo drawing of the wild-type human lysozyme structure. The locations of the six surface salt bridges are indicated. The structure was generated with the program MOLSCRIPT (*48*).

Table 1: Surface Salt Bridges[a] in the Wild-Type Structure[b]

		distance (Å)	ASA (%)[c]
Glu7−Lys1	Glu7 Oε2−Lys1 Nζ	2.94	33
Asp18−Lys13	Asp18 Oδ2−Lys13 Nζ	4.66	43
Asp49−Arg62	Asp49 Oδ2−Arg62 Nε	2.94	50
Asp67−Lys69	Asp67 Oδ2−Lys69 Nζ	3.81	12
Asp102−Arg98	Asp102 Oδ2−Arg98 Nη2	3.04	48
Asp120−Arg122	Asp120 Oδ1−Arg122 Nη2	4.59	78

[a] The presence of salt bridges is inferred when Asp or Glu side-chain carbonyl oxygen atoms are found to be within 6.0 Å distance from the nitrogen atoms in Arg, Lys, and His side chains. The salt bridge is counted only once, even if more than one nitrogen−oxygen atom pair within 6.0 Å in a given residue forming a salt bridge is found. [b] Takano et al. (*18*). [c] ASA (%) is the average ASA (%) value of oxygen and nitrogen atoms participating in the salt bridges: ASA (%) = $(ASA^{fold}/ASA^{extend}) \times 100$. ASA^{extend} is the ASA value of an extended conformation (*46*) as the reference value for the denatured state, assuming that these atoms in the denatured state should be fully exposed to solvent. The ASA values are calculated by the procedure of Connolly (*47*).

salt bridges. The ASA[1] values of oxygen and nitrogen atoms participating in the six salt bridges range from 12% to 78% in the native structure compared with the denatured state (Table 1). Although these bridges are located almost on the surface in the native state (Figure 1), they have some solvent inaccessibility because the accessible surface area does not distinguish between atoms just below the protein surface and those in the core of the protein (*28*). The criterion for a residue to be classified as being in the interior of a protein is that the residue has less than 5% accessibility (*29*−*31*).

In this study, we constructed six mutant proteins, E7Q, D18N, D49N, D67N, D102N, and D120N, which would be expected to delete one salt bridge. The DSC measurements of the mutant human lysozymes were carried out between pH 2.0 and 4.8, and the crystal structures of E7Q, D102N, and D120N were determined. There was little difference in stability between the wild-type and mutant proteins at pH 2, but all mutant proteins were destabilized to various degrees compared with the wild-type protein around pH 4. On the basis of the structural features of surface salt bridges, the thermodynamic data could be analyzed and the net contribution of a surface salt bridge to the protein stability could be estimated. Furthermore, the effect of salts on surface salt bridges was also examined.

MATERIALS AND METHODS

Mutant Proteins. Mutagenesis, expression, and purification of mutant human lysozymes examined in this study were performed as described (*18*). All chemicals were reagent grade. Protein concentration was determined spectrophotometrically using $E^{1\%}(1\ cm) = 25.65$ at 280 nm (*32*).

Differential Scanning Calorimetry. Calorimetric measurements were carried out with a DASM4 microcalorimeter. Sample solutions were 0.05 M glycine hydrochloride buffer

[1] Abbreviations: ASA, accessible surface area; DSC, differential scanning calorimetry; T_d, denaturation temperature.

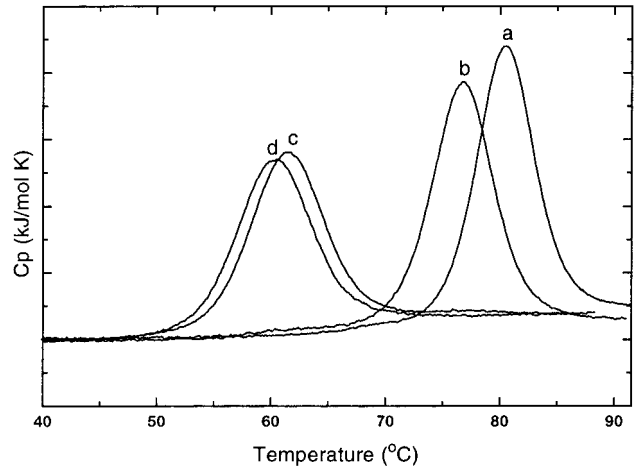

FIGURE 2: Typical excess heat capacity curves in the absence of KCl of (a) wild type at pH 4.35, (b) E7Q at pH 4.43, (c) wild type at pH 2.46, and (d) E7Q at pH 2.42. The increments in excess heat capacity are 10 kJ/(mol·K).

between pH 2.0 and 4.0, and 0.02 M sodium acetate buffer between pH 4.0 and 4.8, regardless of the presence of 0.2 M KCl. The data analysis of DSC was done using the Origin software (MicroCal, Inc., MA), as described previously (*18*). The thermodynamic parameters for denaturation as a function of temperature were calculated from the following equations:

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

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

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

under the assumption that ΔC_p does not depend on temperature (*33*).

X-ray Crystal Analysis. Mutant human lysozymes examined in this study were crystallized at pH 4.5 as described previously (*18, 24*). The crystals of E7Q, D102N, and D120N were grown to a suitable size for structural analysis. All of the crystals belong to the space group $P2_12_12_1$ with a crystal form identical to that of the wild-type protein (*18*).

The intensity data set for the mutant human lysozymes was collected by synchrotron radiation at the Photon Factory (Tsukuba, Japan; Proposal 99G099) on beam line 18B with a Weissenberg camera (*34*). The data were processed with the program DENZO (*35*). The structures were solved by the isomorphous method with the program X-PLOR (*36*). The structure was refined with the program X-PLOR (*36*) as described previously (*18, 24*).

RESULTS

DSC Measurements of the Wild-Type and Mutant Human Lysozymes. To measure the changes in conformational stability of the mutant human lysozymes in the cases of the absence and presence of KCl, we examined the heat denaturation of the wild-type and mutant proteins by DSC. The DSC measurements were carried out in the acidic pH region (pH 2.0−4.8) where the heat denaturation of human lysozyme is highly reversible even in the presence of 0.2 M KCl. Figure 2 shows excess heat capacity curves of the wild-type and mutant human lysozymes at near pH 2.4 and 4.4. The transition curves appear to have a single peak and are symmetrical. Each protein considered in this study showed an excess heat capacity curve with similar characteristics. The denaturation temperature (T_d) and the calorimetric enthalpy change (ΔH) were obtained directly from an analysis of these curves.

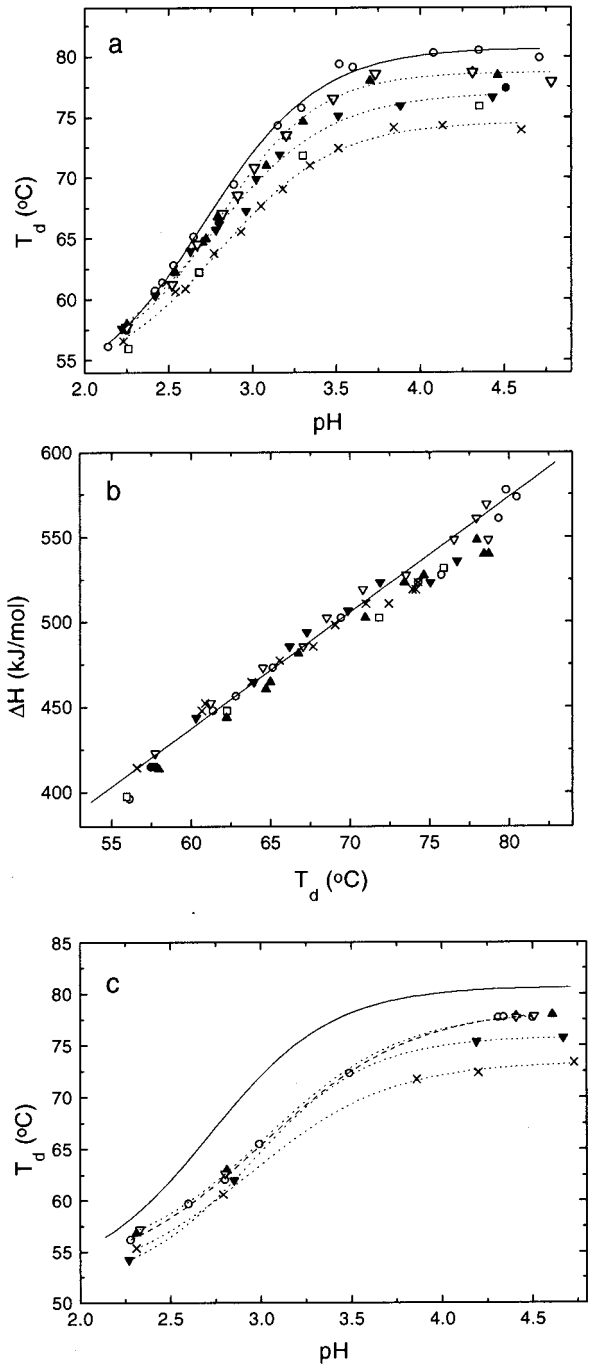

FIGURE 3: (a) pH dependence of the denaturation temperature (T_d) and (b) T_d dependence of the calorimetric enthalpy changes (ΔH) in the absence of KCl. (c) pH dependence of the denaturation temperature (T_d) in the presence of 0.2 M KCl. (○) Wild type; (▼) E7Q; (□) D18N; (●) D49N; (×) D67N; (▲) D102N; (▽) D120N. The solid and dashed lines in panel c are the pH dependence of T_d of the wild-type protein in the absence and presence of KCl, respectively. The dotted lines in panels a and c are the pH dependence of T_d of E7Q, D67N and D102N. The lines in panels a and c are obtained by fitting the data to the equation $f(x) = (A_1 - A_2)/[1 + \exp\{(x - x_0)/dx\}] + A_2$. The line in panel b is obtained by least-squares fitting of the data of the wild-type human lysozyme.

(i) Stability in the Absence of KCl. Figure 3a shows the pH dependence of T_d for the wild-type and mutant human

Table 2: Thermodynamic Parameters for Denaturation of the Wild-Type and Mutant Human Lysozymes in the Absence of KCl at pH 2.2 and 4.0

	T_d (°C)	ΔT_d (°C)	ΔG^a (kJ/mol)	$\Delta\Delta G^b$ (kJ/mol)
		(A) pH 2.2		
wild type	57.5		−10.1	
E7Q	57.8	+0.3	−9.7	+0.4
D18N	56.0	−1.5	−12.1	−2.2
D49N	57.5	0	−10.1	0
D67N	56.8	−0.7	−11.1	−1.0
D102N	58.1	+0.6	−9.3	+0.7
D120N	57.8	+0.3	−9.7	+0.4
		(B) pH 4.0		
wild type	80.1		22.7	
E7Q	76.2	−3.9	16.7	−6.0
D18N	75.5[c]	−4.6	15.6	−7.1
D49N	77.4[d]	−2.7	18.5	−4.2
D67N	74.0	−6.1	13.3	−9.4
D102N	78.3	−1.8	19.9	−2.8
D120N	78.3	−1.8	19.9	−2.8

[a] At 65 °C. The ΔG values were obtained from eqs 1−3. ΔH (65 °C) = 477 kJ/mol. ΔC_p = 6.7 kJ/(mol K) (*18*). [b] $\Delta\Delta G = \Delta G$ (mutant) − ΔG (wild). [c] T_d at pH 4.3. [d] T_d at pH 4.5.

Table 3: Thermodynamic Parameters for Denaturation of the Wild-Type and Mutant Human Lysozymes in the Presence of 0.2 M KCl at pH 2.3 and 4.5

	T_d (°C)	ΔT_d (°C)	ΔG^a (kJ/mol)	$\Delta\Delta G^b$ (kJ/mol)
		(A) pH 2.3		
wild type	56.2		−10.4	
E7Q	54.6	−1.6	−12.2	−1.8
D67N	55.2	−1.0	−11.5	−1.1
D102N	56.7	+0.5	−9.8	+0.6
D120N	56.9	+0.7	−9.6	+0.8
		(B) pH 4.5		
wild type	77.7		17.0	
E7Q	75.5	−2.2	11.6	−5.4
D67N	72.5	−5.2	9.9	−7.1
D102N	77.9	+0.2	17.3	+0.3
D120N	77.8	+0.1	17.3	+0.2

[a] At 65 °C. The ΔG values were obtained from eqs 1−3. ΔH (65 °C) = 423 kJ/mol. ΔC_p = 6.8 kJ/(mol K). [b] $\Delta\Delta G = \Delta G$ (mutant) − ΔG (wild).

Table 4: X-ray Data Collection and Refinement Statistics of Mutant Human Lysozymes

	E7Q	D102N	D120N
	(A) Data Collection		
cell (Å)			
a	56.23	56.43	56.44
b	61.33	61.81	62.02
c	32.77	32.38	32.50
resolution (Å)	1.8	1.8	1.8
no. of measured reflections	45018	49195	36612
no. of independent reflections	10682	10400	9654
completeness (%)	97.3	94.9	87.4
R_{merge}[a] (%)	3.8	3.7	2.5
	(B) Refinement		
no. of atoms	1287	1271	1264
no. of solvent atoms	258	242	235
resolution (Å)	8.0−1.8	8.0−1.8	8.0−1.8
no. of reflections used	10 390	10 051	9401
completeness (%)	95.7	92.6	86.1
R factor[b]	0.179	0.188	0.173

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

lysozymes. The stabilities decreased as the pH decreased below pH 3.5, but the T_d values did not change above pH 4. It has been reported that there is little pH dependence of the stability of lysozyme from pH 4 to neutral pH (*37*). The results also indicate that all mutant proteins are less stable than the wild-type protein around pH 4, but the changes in stability between them near pH 2 are smaller than those at pH 4. The T_d dependence of the calorimetric enthalpy changes (ΔH) is shown in Figure 3b. The enthalpy changes and heat capacity changes in the Glu to Gln and Asp to Asn mutant proteins were equivalent to those of the wild-type protein; ΔH (65 °C) = 477 kJ/mol and ΔC_p = 6.7 kJ/(mol K) (*18*). The amino acid residues Glu and Gln or Asp and Asn are isosteric and have similar hydrophobicity (*38*), resulting in small changes in ΔH. For other mutant human lysozymes, the changes in ΔH ranged from −37 to +3 kJ/mol for five Ile to Ala mutants (*20*), from −23 to +10 kJ/mol for six Ser to Ala mutants (*22*), and from −58 to −14 kJ/mol for 10 Thr to Val/Ala mutants (*23*). In the case of other proteins, the DSC analysis of RNase A has shown that the $\Delta\Delta H$ value of N67D RNase A is −5 kJ/mol, while that of N67isoD RNase A is −28 kJ/mol (*39*).

The T_d and ΔG (65 °C) values at pH 2.2 and 4.0 in the absence of KCl for the wild-type and mutant human lysozymes are summarized in Table 2.

(ii) Stability in the Presence of KCl. The DSC measurements in the presence of 0.2 M KCl for the wild type, E7Q, D67N, D102N, and D120N were performed. The pH dependence of T_d is shown in Figure 3c. Around pH 4, the stability of all proteins in the presence of KCl decreased more than those in the absence of KCl, and the changes in stability between the wild-type and mutant proteins in the presence of KCl were smaller than those in the absence of KCl. These results suggest an effect of salts on the stability through surface salt bridges. The T_d and ΔG (65 °C) values at pH 2.3 and 4.5 in the presence of KCl for the wild-type and mutant human lysozymes are summarized in Table 3.

Crystal Structures of Mutant Human Lysozymes. Data collection and refinement statistics of the mutant proteins E7Q, D102N, and D120N are summarized in Table 4. The overall structures of the examined mutant proteins were essentially identical to that of the wild-type protein. The structures in the vicinity of each mutation site are illustrated in Figure 4. The major structural changes in the mutant proteins were as follows.

(i) E7Q. The mutant protein, E7Q, contains Gln at position Glu7. In the wild-type structure, the side-chain atoms of Glu7, Oϵ2 and Oϵ1, interact with Nζ of Lys1 by the 2.94 Å salt bridge with a hydrogen bond and with N of Glu4 by the 3.28 Å hydrogen bond, respectively. The result from the substitution of Glu7 with Gln is that Oϵ2 and Oϵ1 of Glu7 were replaced by Nϵ2 and Oϵ1 of Gln7, respectively. In the E7Q structure, the distance between Nϵ2 of Gln7 and Nζ of Lys1 is 3.35 Å, and Oϵ1 of Gln7 forms a hydrogen bond with N of Glu4 by 3.03 Å. One salt bridge with a hydrogen bond was totally removed by the substitution (Figure 4a).

(ii) D102N. Oδ2 of Asp102 forms a salt bridge with a hydrogen bond with Nη2 of Arg98 (3.04 Å) (Table 1). In the D102N structure, Oδ1 of Asn102 hydrogen-bonds with Nη2 of Arg98 (3.33 Å). Finally, the substitution of Asp with Asn at position 102 removed one salt bridge with a hydrogen bond but formed one hydrogen bond (Figure 4b).

(iii) D120N. Oδ1 of Asp120 forms a salt bridge with Nη2 of Arg122 (4.59 Å) in the wild-type protein (Table 1). The replacement of Asp by Asn at position 120 reduced the salt

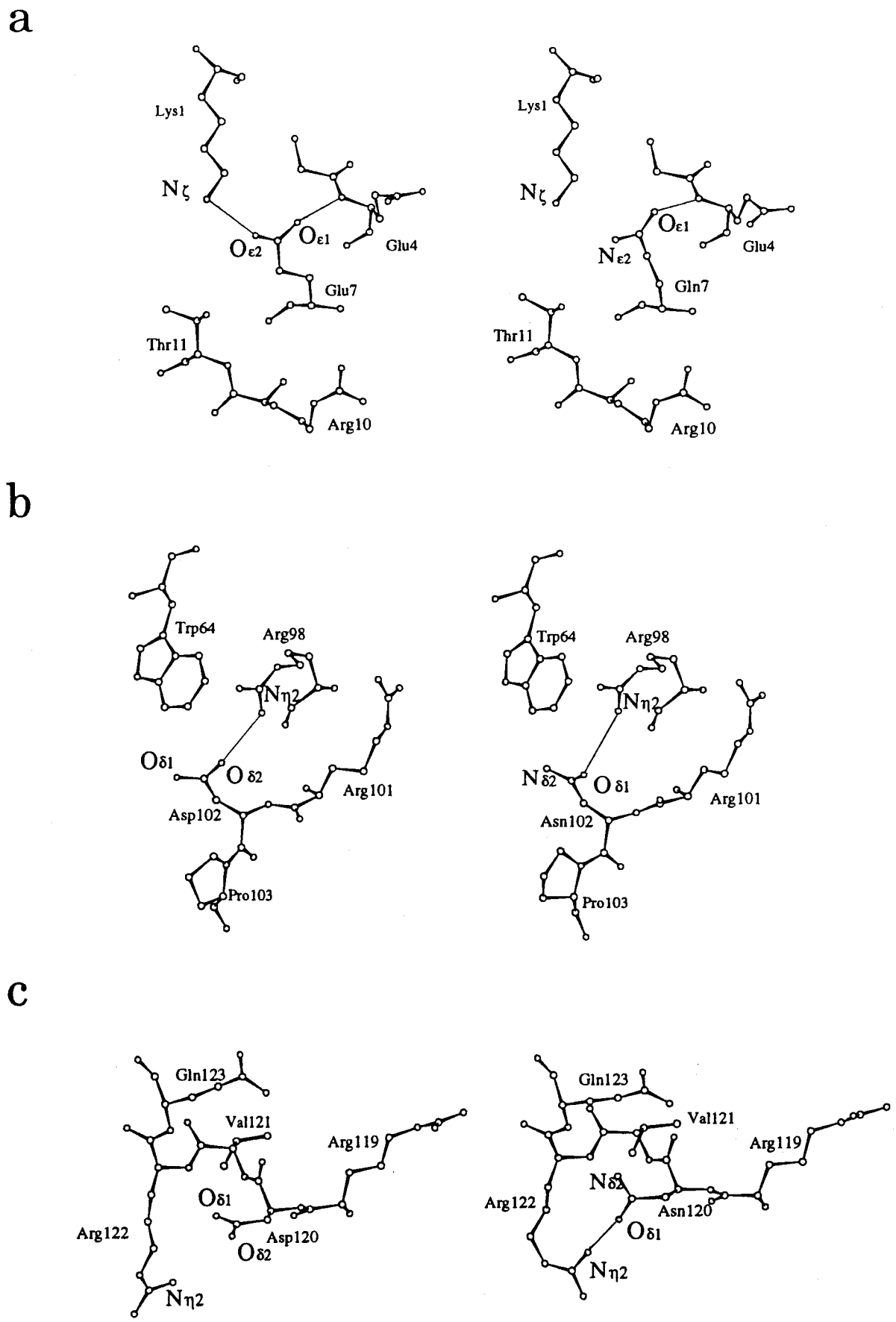

FIGURE 4: Structures in the vicinity of mutation sites: (a) E7Q, (b) D102N, and (c) D120N. The wild-type and mutant structures are drawn at the left and right sides, respectively. The thin lines represent hydrogen bonds.

Table 5: ΔG_{SB} Values of Mutant Human Lysozymes

	ΔG_{SB}[a] (kJ/mol) with no KCl	ΔG_{SB}[b] (kJ/mol) with 0.2 M KCl
E7Q	6.4	3.6
D18N	5.1	
D49N	4.2	
D67N	8.4	6.0
D102N	3.5	0.3
D120N	3.2	0.6

[a] $\Delta G_{SB} = \Delta\Delta G_{SB}/\Delta N_{SB} = (\Delta\Delta G_{pH4.0} - \Delta\Delta G_{pH2.2})/(-1)$. [b] $\Delta G_{SB} = \Delta\Delta G_{SB}/\Delta N_{SB} = (\Delta\Delta G_{pH4.5} - \Delta\Delta G_{pH2.3})/(-1)$.

bridge but formed one hydrogen bond between Oδ1 of Asn120 and Nη2 of Arg122 (2.52 Å) (Figure 4c).

DISCUSSION

Contribution of a Salt Bridge to the Conformational Stability of Human Lysozyme (ΔG_{SB}). The pH dependence of the stability (T_d) in the absence of KCl for human lysozymes and the ΔG values at pH 4.0 and 2.2 are shown in Figure 3a and Table 2, respectively. The ΔG values of the mutant proteins decreased around pH 4, compared with that of the wild-type protein, whereas there was little difference between them around pH 2. This suggests that the Glu and Asp residues are ionized at pH 4 but protonated at pH 2, resulting in a favorable effect of the surface salt bridges in the wild-type structure at pH 4.

The $\Delta\Delta G$ values at pH 4 might include not only the effect of salt bridges ($\Delta\Delta G_{SB}$) but also other effects due to mutation ($\Delta\Delta G_{others}$). The other effects can be estimated from the difference in ΔG between the wild-type and mutant proteins ($\Delta\Delta G$) at pH 2.2, where salt bridges are not formed. In the present case, it is thought that effects other than salt bridges due to mutation ($\Delta\Delta G_{others}$) are mainly the changes in the side-chain conformational entropy of substituted residues ($\Delta\Delta G_{conf}$), the hydrophobic effects of the overall structure ($\Delta\Delta G_{HP}$), and the surface hydrogen bonds of the mutation residues ($\Delta\Delta G_{sHB}$). On the basis of the method by Funahashi et al. (*25*), the ($\Delta\Delta G_{conf} + \Delta\Delta G_{HP}$) values of E7Q, D102N, and D120N could be calculated from the mutant structures as -0.6, $+1.1$, and $+1.1$ kJ/mol, respectively. These values were corresponding to the $\Delta\Delta G$ values at pH 2.2 (Table 2). Here, the structural analysis of E7Q, D102N, and D120N showed that the changes in the surface hydrogen bonds of the mutation residues would be -1, 0, and $+1$, respectively. These results suggest that a hydrogen bond on the protein surface appears to make a small contribution to protein stability. The contribution of a hydrogen bond in the interior of a protein to the conformational stability has been estimated to be about 9 kJ/mol (*23*).

The net contribution of a salt bridge to protein stability (ΔG_{SB}) can be described by the following equations:

$$\Delta G_{SB} = \Delta\Delta G_{SB}/\Delta N_{SB} = (\Delta\Delta G_{pH4.0} - \Delta\Delta G_{pH2.2})/\Delta N_{SB} \quad (4)$$

where ΔN_{SB} is the changes in the number of salt bridges between the wild-type and mutant proteins. In this study, ΔN_{SB} is -1. The ΔG_{SB} values estimated are summarized in Table 5, indicating that a surface salt bridge favorably contributes to protein stability. Recently, it has been reported

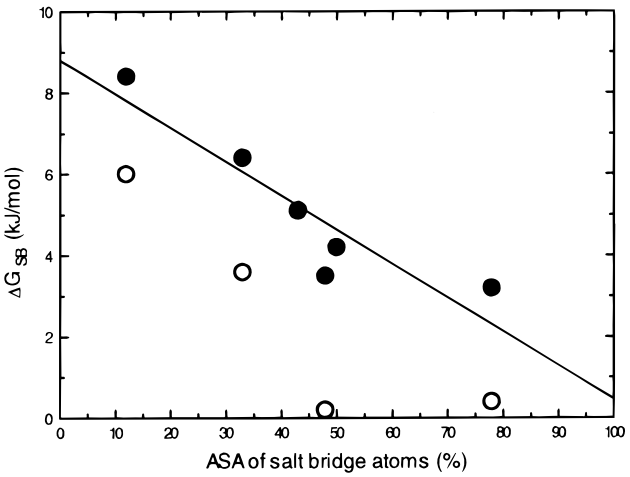

FIGURE 5: Correlation between the ASA (%) values of the salt bridge atoms and the ΔG_{SB} values. The closed and open circles represent the absence and presence of 0.2 M KCl, respectively. The solid line is obtained by least-squares fitting of six data in the absence of KCl (●).

that rational mutations of charged surface residues increase the protein stability (*40*−*42*). In Table 5, however, there are some differences in ΔG_{SB} values, representing the different contribution of a surface salt bridge to protein stability, depending on the location.

Correlation between ΔG_{SB} and ASA of Surface Salt Bridges. The ΔG_{SB} values estimated ranged from 3.2 to 8.4 kJ/mol (Table 5). This indicates that surface salt bridges variously contribute to the protein stability, depending on their structural characteristics. In the present case, there was no clear correlation between the ΔG_{SB} values and the distance of the salt bridges examined, suggesting that other effects strongly affected the ΔG_{SB} values. Here, we found that the ΔG_{SB} values correlate well with the inaccessibility of salt bridges as shown in Figure 5. The residues forming a surface salt bridge inevitably have a certain amount of surface area inaccessible to solvents, compared with their denatured state, even if located on the protein surface. This provides a different contribution of surface salt bridges, because electrostatic interactions are affected by the dielectric constant, and the dielectric constant is greatly different between the inside and the outside of a protein (*43*). Figure 5 indicates that, when fully exposed, although it is impossible, the salt bridge contribution to protein stability is little. In contrast, a salt bridge with 100% inaccessibility contributes about 9 kJ/mol, which corresponds to the contribution of a salt bridge in the interior of proteins (*44*, *45*). Kumar and Nussinov (*2*) have also reported that a buried salt bridge stabilizes a protein because it may be able to undergo favorable interactions with other charged protein atoms.

Effect of KCl on Salt Bridge. In the presence of salts, Coulombic interactions might be expected to be screened and would thus have only minimal effects on stability. For the wild-type human lysozyme, the stability in the presence of 0.2 M KCl near pH 4 was lower than that in the absence of KCl, whereas near pH 2 there was little difference between them. The mutant proteins, E7Q, D67N, D102N, and D120N, also showed the same effect of KCl as the wild-type protein did. Moreover, the decrease in stability near pH 4 of the mutant proteins was smaller than that of the wild-type protein. These results indicate that the KCl salt compensates

the contribution of salt bridges to protein stability.

The ΔG_{SB} values in the presence of 0.2 M KCl were calculated from the $\Delta\Delta G$ values at pH 2.3 and 4.5, as shown in Table 5. In the case of D102N and D120N, in which the salt bridge atoms have great accessibility, the ΔG_{SB} values in the presence of KCl were almost zero. Figure 5 also shows the correlation between the ΔG_{SB} values in the presence of KCl and the ASA of salt bridge atoms. The result indicates that salt bridges with more than 50% accessibility are no longer contributing to the stability in the presence of 0.2 M KCl. A theoretical study has shown that interactions between buried or partially buried charges are not effectively screened by mobile ions (*17*).

CONCLUSIONS

Some experimental results have shown that surface salt bridges have little contribution to protein stability (*8*−*13*). Others, however, have shown favorable contributions of surface salt bridges (*14*−*17*). In this study, we demonstrated the obvious dependence of an advantageous salt bridge contribution upon the solvent inaccessibility of salt bridge atoms: little contribution when 100% accessible and about 9 kJ/mol contribution when 100% inaccessible, which is equivalent to the contribution of a salt bridge in the interior of a protein (*44*, *45*). This finding would reconcile a number of conflicting reports and is also advantageous to an understanding of the stabilization mechanism of thermophilic proteins. Here, it should also be noted that the atoms of surface salt bridge residues have some surface area inaccessible to solvents in the native state, compared with their denatured state.

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