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Role of Amino Acid Residues in Left-handed Helical Conformation for the Conformational Stability of a Protein

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ABSTRACT **Our previous study of six non-Gly to Gly/Ala mutant human lysozymes in a left-handed helical region showed that only one non-Gly residue at a rigid site had unfavorable strain energy as compared with Gly at the same position (Takano et al., Proteins 2001; 44:233–243). To further examine the role of left-handed residues in the conformational stability of a protein, we constructed ten Gly to Ala mutant human lysozymes. Most Gly residues in human lysozyme are located in the left-handed helix region. The thermodynamic parameters for denaturation and crystal structures were determined by differential scanning calorimetry and X-ray analysis, respectively. The difference in denaturation Gibbs energy (ΔΔG) for the ten Gly to Ala mutants ranged from + 1.9 to −7.5 kJ/mol, indicating that the effect of the mutation depends on the environment of the residue. We confirm that Gly in a left-handed region is more favorable at rigid sites than non-Gly, but there is little difference in energetic cost between Gly and non-Gly at flexible sites. The present results indicate that dihedral angles in the backbone conformation and also the flexibility at the position should be considered for analyses of protein stability, and protein structural determination, prediction, and design. Proteins 2001;45:274–280.**

Key words: differential scanning calorimetry; X-ray crystallography; mutant protein; Gibbs energy; Ramachandran map; local constraints

INTRODUCTION

The Ramachandran plot[1,2] has been widely used as a convenient method for the analysis of the backbone conformation in protein structures. The dihedral angles (ϕ, Ψ) of amino acid residues in protein structures are usually inside three preferred regions that are right-handed helical, extended, and left-handed helical regions on the Ramachandran plot.[1,3] Most of non-Gly residues adopt angles that correspond to the right-handed helical and extended regions. Therefore, the Ramachandran plot is an important guide for the correctness of a protein structure. Indeed, the stereochemical quality of a protein model is often judged by it. If a residue is lying in a disallowed region, it is thought that the residue might have a strain energy associated with unfavorable values.

In the left-handed helical region, about 60% of the residues are populated by Gly,[4] suggesting that the non-Gly residues have unfavorable energies from the local steric interaction of the backbone atoms with the side-chain Cβ atom as compared with Gly at the same position. However, our previous study of six non-Gly to Gly/Ala mutant human lysozymes in the left-handed helical region showed that for human lysozyme the unfavorable steric interaction between the side chain Cβ and backbone occurred at only one rigid position among the six left-handed non-Gly sites.[5] This result suggests that there are both unfavorable and favorable sites for non-Gly in left-handed helical structures on the Ramachandran plot, and that many left-handed non-Gly positions in native protein structures are not unfavorable sites for non-Gly residues. Here, we examine Gly to Ala mutant proteins in the left-handed region, in order to better understand the role of the left-handed residues in protein stability; whether the strain energies of left-handed non-Gly residues depend on their flexibility, and whether left-handed Gly positions in native protein structures have more unfavorable sites for non-Gly residues than left-handed non-Gly positions in native protein structures.

The Ramachandran plot[1] of human lysozyme[6] is as shown in Figure 1. Most residues are in extended (around $\phi = -100°$ and $\Psi = 120°$), right-handed helical (around $\phi = -75°$ and $\Psi = -50°$) or left-handed helical (around $\phi = 60°$ and $\Psi = 30°$) regions. Human lysozyme has 11 Gly residues. Most of them are located in the left-handed region. In this study, we examine ten Gly to Ala mutants of human lysozyme: the stability and structures of the mutant human lysozymes were determined by calorimetry and X-ray crystal analysis, respectively. If an introduced Ala residue had an unfavorable steric interaction between the side-chain Cβ and the backbone, the Ala mutant would be expected to be less stable than the wild-type protein. The present and previous studies of left-handed residues of mutant human lysozymes[5] will be used to gain a better

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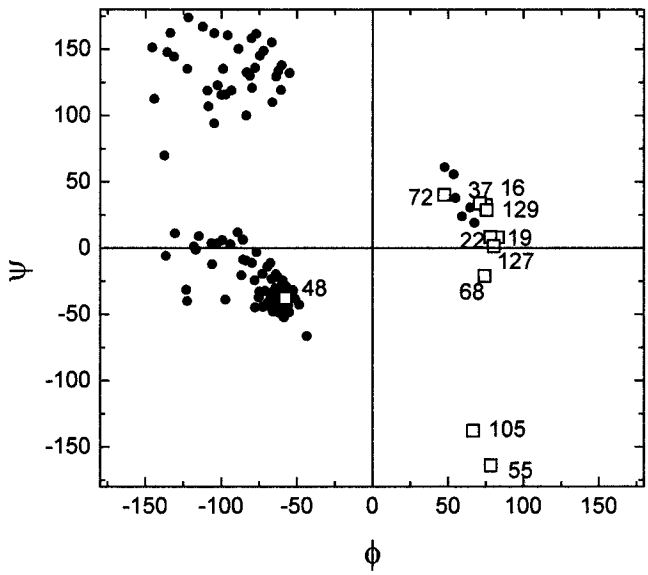

Fig. 1. Ramachandran plot[1] of human lysozyme.[6] The Gly residues modified in this study are shown as open squares.

understanding of the role of left-handed residues in protein stability.

MATERIALS AND METHODS

Mutant Proteins

Mutagenesis, expression, and purification of mutant human lysozymes examined in this study, G16A, G19A, G22A, G37A, G48A, G55A, G68A, G72A, G105A, G127A, and G129A, were performed as described.[6] For G16A, G19A, and G22A, the secretions in yeast were about 50 times lower than that of the wild-type protein. In the case of G55A, there was little secretion. All chemicals were reagent grade. Protein concentration of the mutant proteins was determined spectrophotometrically using $E^{1\%}(1\ cm) = 25.65$ at 280 nm.[7]

Differential Scanning Calorimetry (DSC)

Calorimetric measurements were carried out with a DASM4 microcalorimeter. Sample buffer was 0.05 M Gly-HCl pH 2.2 to 3.4. The data analysis of DSC was done using the Origin software (MicroCal, Inc., MA), as described previously.[6] The thermodynamic parameters for denaturation as a function of temperature were calculated using the following equations,[8]

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

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

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

assuming that ΔC_p does not depend on temperature.

X-Ray Crystal Analysis

Mutant human lysozymes, G37A, G48A, G72A, G105A, G127A, and G129A, were crystallized at pH 4.5 (2 M sodium chloride, 0.02 M sodium acetate) as described previously.[6,9] G68A was crystallized at pH 6.5 (0.2 M ammonium sulfate, 0.1 M sodium cacodylate, 15% w/v PEG 8000). The crystals of G37A, G48A, G72A, G105A, and G127A belong to the space group $P2_12_12_1$ with a crystal form identical to that of the wild-type and most mutant proteins.[6,10–15] The crystals of G68A and G129A belong to the space group $P2_12_12_1$, but the cell dimensions differ from that of the wild-type protein. The crystal form of G68A and G129A is the same as that of I56M, I56F,[16] and S82A.[17] We could not try the crystallization of G16A, G19A, G22A, and G55A because of the low expression yields.

Intensity data sets of the mutant human lysozymes were collected by the oscillation method on the Rigaku R-AXIS IV imaging plate mounted on the Rigaku RU300 for G37A, G127A, and G129A, and by synchrotron radiation at the SPring-8 on beam line 40B2 (Harima, Japan; Proposal No. 2000B0380-NL-np) for G48A, G68A, G72A, and G105A. The data were processed with the program DENZO.[18] The structures were solved by the isomorphous method using the program X-PLOR.[19] The structure was refined with the program X-PLOR[19] as described previously.[6,9]

The coordinates of mutant human lysozymes have been deposited in the Protein Data Bank under PDB file names G37A, 1IP1; G48A, 1IP2; G68A, 1IP3; G72A, 1IP4; G105A, 1IP5; G127A, 1IP6; G129A, 1IP7.

RESULTS

DSC Measurements of the Mutant Human Lysozymes

To measure the changes in the conformational stability of the mutant human lysozymes, we examined the heat denaturation of the mutant proteins by DSC. The DSC measurements were carried out in the acidic pH region (pH 2.2 to 3.4) where denaturation of the human lysozyme is highly reversible. Table I shows the denaturation temperature (T_d), and the calorimetric enthalpy (ΔH_{cal}) for each measurement for the mutant proteins. For G16A, G19A, and G22A, only one measurement at pH 2.7 was done because of their low expression yields. Using these data and Equations 1 to 3, the thermodynamic parameters for the denaturation of the mutant and wild-type proteins at the same temperature, 64.9°C, which is the denaturation temperature of the wild-type protein at pH 2.7,[6] were calculated as shown in Table II. G16A, G19A, and G22A had greatly decreased stability (more than 5 kJ/mol), but the stability changes in the other mutant proteins were smaller (less than 3 kJ/mol). This indicates that the effects of mutation are dependent on the environment of the site. Table III shows the $\Delta\Delta G$ values of Gly to Ala mutations in human lysozyme. For sites 21, 38, 50, 58, 78, and 118, the $\Delta\Delta G$ value of Gly to Ala mutation is the difference in stability between the Gly and Ala mutants at each left-handed non-Gly site.[5]

Crystal Structures of the Mutant Human Lysozymes

To investigate the structural changes due to mutations, we determined the crystal structures of the mutant human

TABLE I. Thermodynamic Parameters for Denaturation of Mutant Human Lysozymes at Different pH Values

	pH	T_d (°C)	ΔH_{cal} (kJ/mol)		pH	T_d (°C)	ΔH_{cal} (kJ/mol)
G16A	2.70	60.3	403	G72A	2.38	58.2	418
					2.64	62.6	444
G19A	2.70	59.4	427		3.16	72.2	502
G22A	2.70	57.7	322	G105A	2.30	56.1	380
					2.65	61.3	408
G37A	2.38	58.1	406		3.20	71.8	469
	2.60	62.4	435				
	3.18	72.4	498	G127A	2.20	54.6	394
					2.60	61.6	435
G48A	2.40	61.1	397		3.16	71.1	494
	2.68	66.0	427				
	3.20	75.1	490	G129A	2.67	64.9	417
					2.79	67.1	427
G68A	2.60	62.8	431		2.80	67.2	431
	2.72	64.9	456				
	3.00	69.2	477				

TABLE II. Thermodynamic Parameters for Denaturation of Mutant Human Lysozymes at 64.9°C, pH 2.7[†]

	T_d (°C)	ΔT_d (°C)	ΔC_p[a] (kJ/mol K)	ΔH (kJ/mol)	$\Delta\Delta H$[b] (kJ/mol)	$\Delta\Delta G$[c] (kJ/mol)
Wild-type[d]	64.9		6.6	477		
G16A[e]	60.3	−4.6	(6.6)	433	−44	−5.8
G19A[e]	59.4	−5.5	(6.6)	463	−14	−7.4
G22A[e]	57.7	−7.2	(6.6)	370	−107	−7.5
G37A	64.0	−0.9	6.4	450	−27	−1.2
G48A	66.4	+1.5	6.7	421	−56	+1.9
G68A	64.5	−0.4	6.9	450	−27	−0.5
G72A	63.8	−1.1	6.0	458	−19	−1.5
G105A	62.8	−2.1	5.7	429	−48	−2.6
G127A	63.2	−1.7	6.1	456	−21	−2.3
G129A	65.4	+0.5	5.4	417	−60	+0.6

[a]ΔC_p was obtained from the slope of ΔH versus T_d, except for G16A, G19A and G22A.
[b]$\Delta\Delta H = \Delta H$ (mutant) − ΔH (wild).
[c]$\Delta\Delta G = \Delta G$ (mutant) − ΔG (wild).
[d]Data taken from ref. 6.
[e]The ΔH and $\Delta\Delta G$ values at 64.9°C of these mutant proteins were calculated using the ΔC_p value of the wild-type protein, 6.6 kJ/mol K.

lysozymes by X-ray analysis. The data collection and refinement statistics of the mutant proteins are summarized in Table IV. The overall structures of the mutant proteins were similar to that of the wild-type protein. The superimposed structures of the wild-type and mutant proteins in the vicinity of each mutation site are illustrated in Figure 2.

The backbone structures around the mutation site in the mutant structures were apparently unchanged. However, there were subtle differences in the dihedral angles of the mutated residues. Figure 3 shows the dihedral angles of the mutated residues in the wild-type structure and each mutant structure. In the left-handed helical region (residues 37, 68, 72, 127, and 129), each Ala residue has both a smaller ϕ value and a larger Ψ value than those of the corresponding Gly residues. Similar observations have been reported for mutant proteins of human lysozyme,[5,20] hen egg white lysozyme,[21] RNase H,[22] and T4 lysozyme.[23] This is because a non-Gly residue in the left-handed region would have a closer contact between its Cβ and the carbonyl oxygen in the preceding residue (n-1) when the ϕ and Ψ values move to larger and smaller values, respectively.

TABLE III. $\Delta\Delta G$ Value of Gly to Ala Mutations in Human Lysozyme

Mutation[a]	$\Delta\Delta G$ (kJ/mol)	Mutation[b]	$\Delta\Delta G$ (kJ/mol)
G16A	−5.8	G21A	+0.7
G19A	−7.4	G38A	−0.7
G22A	−7.5	G50A	+0.7
G37A	−1.2	G58A	−4.0
G48A	+1.9	G78A	−0.1
G68A	−0.5	G118A	+0.6
G72A	−1.5		
G105A	−2.6		
G127A	−2.3		
G129A	+0.6		

[a]This work (Table II).
[b]Difference between Ala and Gly mutant proteins at the left-handed non-Gly sites in human lysozyme.[5]

TABLE IV. X-Ray Data Collection and Refinement Statistics of Mutant Human Lysozymes

	G37A	G48A	G68A	G72A	G105A	G127A	G129A
Data collection							
Cell (Å)							
a	56.39	56.33	42.25	56.47	56.40	56.27	41.87
b	61.67	60.96	64.32	61.62	61.44	61.34	63.65
c	32.37	32.91	111.12	32.50	32.62	32.74	111.32
Resolution	1.8	1.8	1.8	1.8	1.8	1.8	1.8
No. of measured reflections	35,510	56,165	133,246	55,173	53,713	37,478	81,918
No. of independent reflections	10,945	10,905	28,656	10,477	10,321	10,384	23,164
Completeness (%)	99.4	98.6	99.0	95.0	93.1	96.5	95.4
R_{merge} (%)[a]	3.9	3.2	4.1	2.9	5.5	5.0	7.0
Refinement							
No. of atoms	1,317	1,292	2,509	1,292	1,303	1,284	2,472
Resolution (Å)	8.0–1.8	8.0–1.8	8.0–1.8	8.0–1.8	8.0–1.8	8.0–1.8	8.0–1.9
No. of used reflections	9,801	10,671	28,090	10,279	10,175	10,185	22,948
Completeness (%)	90.6	98.3	98.7	94.6	93.7	93.8	96.4
R-factor[b]	0.158	0.179	0.176	0.171	0.174	0.184	0.193

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

DISCUSSION

Effect of Gly to Ala Mutations on Protein Stability

The thermodynamic analysis showed that the stability changes ($\Delta\Delta G$) due to the Gly to Ala mutations ranged from +1.9 to −7.5 kJ/mol (Table II). However, for seven mutant proteins, G37A, G48A, G68A, G72A, G105A, G127A, and G129A, the changes in stability ($\Delta\Delta G$) were small, less than 3 kJ/mol (Table II). This result suggests that these positions have little local steric hindrance between the Cβ and backbone atoms. Here, since the crystal structures of the seven mutant proteins were determined, we can estimate the stability change ($\Delta\Delta G$) from the empirical structure-based calculation using the wild-type and mutant structures.[16,24] In this case, these mutations did not change the number of hydrogen bonds and water molecules, so that the changes in stability due to mutation ($\Delta\Delta G_{est}$) could be expressed by Equation 4,[16,24]

$$\Delta\Delta G_{est} = \Delta\Delta G_{HP} + \Delta\Delta G_{ent} \quad (4)$$

where $\Delta\Delta G_{HP}$ and $\Delta\Delta G_{ent}$, represent the changes in ΔG due to a hydrophobic effect, and the side chain or backbone conformational entropy of a mutation residue, respectively. Each $\Delta\Delta G$ can be expressed with each parameter in terms of the conformational change as shown in the following equations,[16,24]

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

$$\Delta\Delta G_{ent} = -T\Delta S_{conf} \quad (6)$$

where ΔASA_{NP} and ΔASA_{P} represent the differences in ASA (accessible surface area) of the non-polar (C/S) and polar (O/N) atoms, respectively, of all residues in a given protein upon denaturation, and $\Delta\Delta$ASA means the difference in ΔASA between the wild-type and mutant proteins[16,25]; and ΔS_{conf} is the difference in the backbone entropy between Gly and non-Gly in the denatured state.[26] Using Equations 4 to 6, the $\Delta\Delta G_{est}$ values at 65°C were calculated for five mutant human lysozymes, G37A, G48A, G72A, G105A, and G127A, whose crystal form was the same as that of the wild-type structure. Figure 4 shows the correlation between $\Delta\Delta G_{est}$ and the experimental values ($\Delta\Delta G_{exp}$) of the five Gly to Ala mutants and the ten non-Gly to Gly/Ala mutants in the left-handed region.[5] The estimated stability roughly agrees with the experimental stability, except for Q58G,[5] indicating that the stability changes of these Gly to Ala mutant proteins do not include the effect of local steric strain.

In contrast, the G16A, G19A, and G22A mutations decreased the stability more than 5 kJ/mol (Table II). The destabilization due to the Gly to Ala mutations is larger than that at position 58 (Table III) where the Ala residue has unfavorable strain energy as compared with the Gly residue.[5] This suggests that at these left-handed positions, residues 16, 19, and 22 have some local steric constraints.

Relationship Between Stability and Local Structure

As described above, the present results suggest that there are both unfavorable and favorable sites for non-Gly residues in the left-handed helical conformations. What is different between the unfavorable and favorable sites for non-Gly? Our previous study using non-Gly to Gly/Ala mutant human lysozymes in the left-handed helical region showed that only the non-Gly residue at a rigid site had unfavorable strain energy as compared with Gly at the same position.[5] Consequently, we examined the relation between stability and flexibility of the mutation sites. Figure 5 shows that the relationship between the stability changes for the Gly to Ala mutations and B-factors at the mutation sites in the wild-type human lysozyme[6] as a measure of flexibility. For the positions with low B-factors, residues 16, 19, 22, and 58, the Gly to Ala mutations result in large decreases in stability. In contrast, there is little difference in stability due to the Gly to Ala mutations in

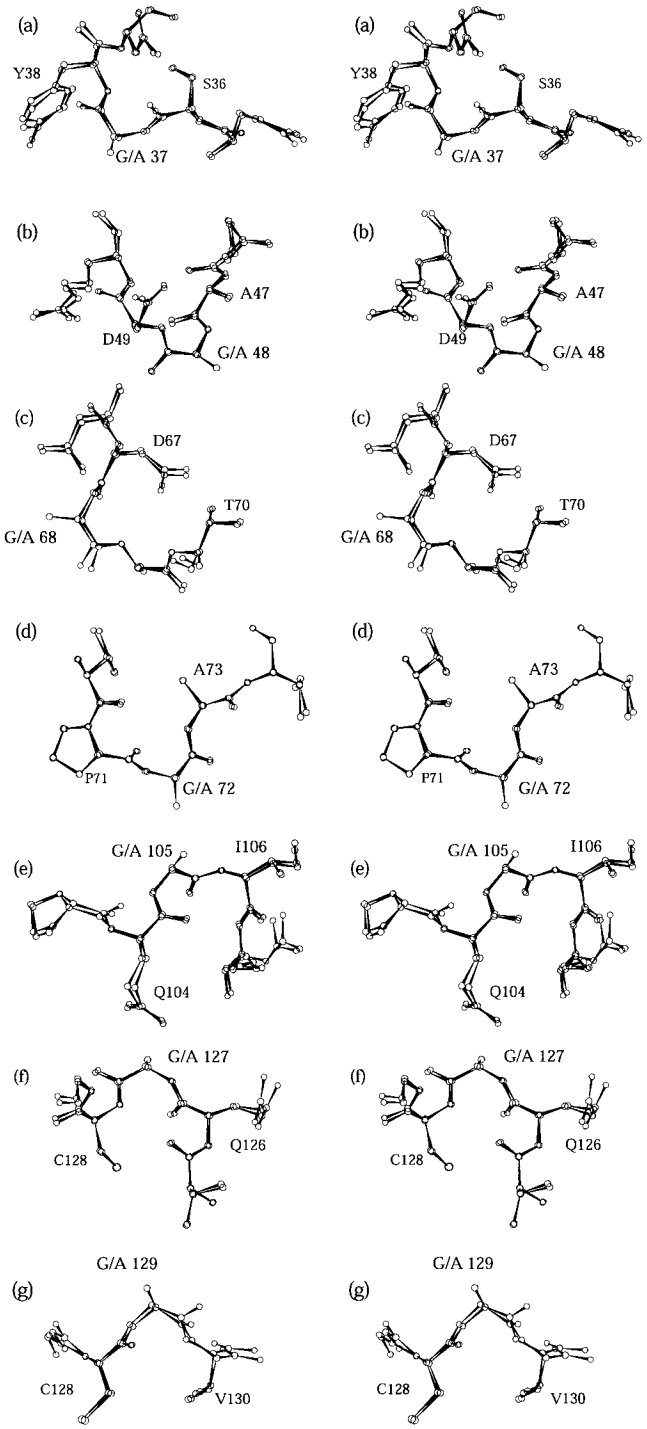

Fig. 2. Stereodrawings of the structures in the vicinity of the mutation sites for (**a**) G37A, (**b**) G48A, (**c**) G68A, (**d**) G72A, (**e**) G105A, (**f**) G127A, and (**g**) G129A of human lysozymes. The wild-type and mutant structures are superimposed.

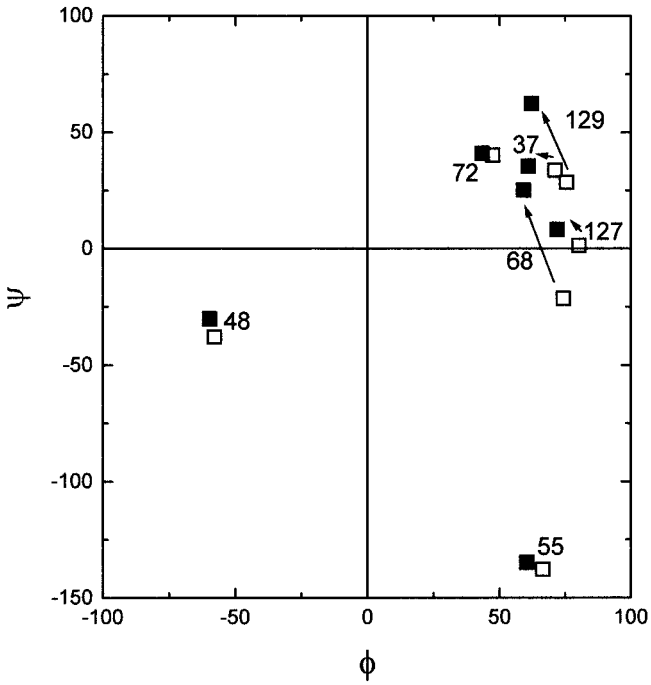

Fig. 3. Ramachandran plot[1] of the Gly residues in the wild-type human lysozyme structure (open squares), and each Ala residue in the mutant structure (closed squares).

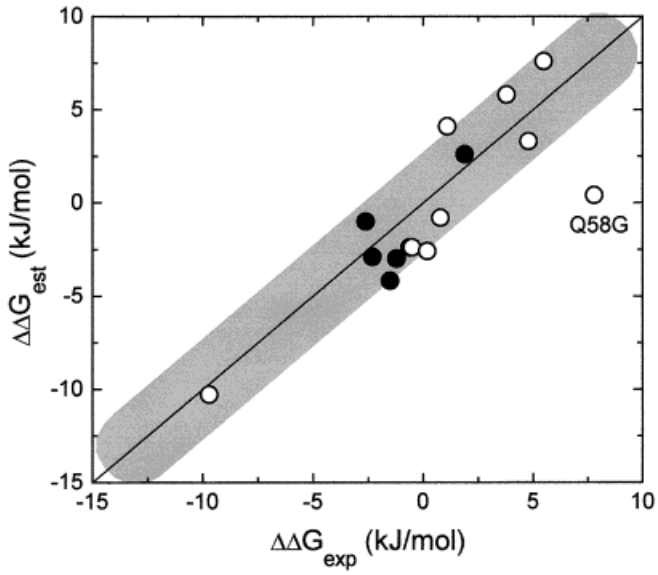

Fig. 4. The relation between the experimental ΔΔG value ($\Delta\Delta G_{exp}$) and estimated ΔΔG value ($\Delta\Delta G_{est}$) for the Gly to Ala mutants (closed circles) and non-Gly to Gly/Ala mutants (open circles)[5] of human lysozymes. The gray region represents the standard deviation of the data (SD = 1.94).

the residues which have higher B-factors. This observation has also been reported recently for mutant hen egg white lysozymes.[21] It can be confirmed from our systematic mutational analysis for left-handed residues in human lysozyme that Gly is more favorable at rigid sites in the left-handed region than non-Gly, but there is little difference in energetic cost between Gly and non-Gly at flexible sites. The Ramachandran plot[1,2] has been widely used as a convenient method for the analysis of the backbone conformation in protein structures. Our results indicate that we should consider not only dihedral angles in backbone conformation but also the flexibility for protein structural determination, prediction, and design.

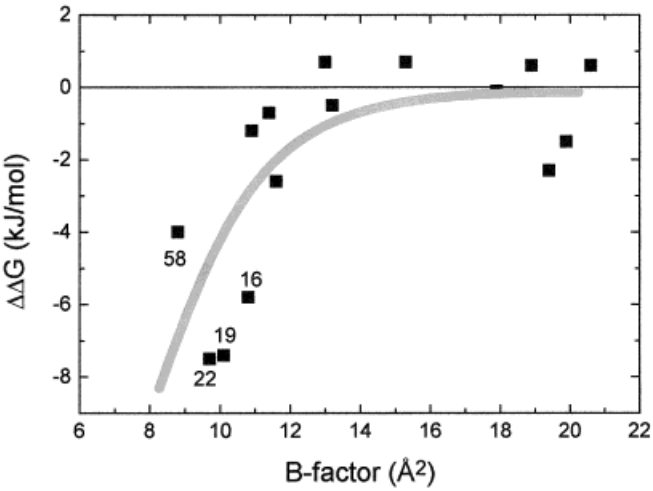

Fig. 5. The relation between the stability changes for the Gly to Ala mutations and the average backbone B-factors of the mutation sites in the wild-type human lysozyme.[6]

Role of Left-Handed Residues in the Structure of a Protein

Our previous study using non-Gly to Gly/Ala mutant human lysozymes in the left-handed helical region has shown that human lysozyme has unfavorable local steric interaction at only one site among the six left-handed non-Gly sites.[5] However, in the left-handed helical region of protein structures, about 60% of the residues are populated by Gly.[4] These results suggest that non-Gly substitutions would be more unfavorable at left-handed Gly positions than at left-handed non-Gly positions in the native structures. The present study shows that human lysozyme has unfavorable local steric interaction at three sites (residues 16, 19, and 22) among the eight left-handed Gly sites. The number of unfavorable sites for non-Gly at the left-handed Gly positions (three cases out of eight) is higher than that at the left-handed non-Gly positions (one case out of six). This indicates that left-handed Gly positions in native protein structures are more unfavorable for non-Gly than left-handed non-Gly positions in native protein structures.

CONCLUSIONS

It has been postulated that non-Gly residues in the left-handed helical region would have unfavorable energies from local steric interactions of the backbone atoms with the side chain Cβ atom. In the present (Gly to Ala) and previous (non-Gly to Gly/Ala) studies,[5] we systematically examined mutant proteins at residues in the left-handed region in human lysozyme. It can be concluded that (1) the left-handed positions do not always have unfavorable strain energies for non-Gly residues, (2) only rigid left-handed sites are unfavorable for non-Gly residues, and (3) in the native structures of proteins, substitutions for left-handed Gly positions are more unfavorable than for left-handed non-Gly positions.

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