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Influence of Mutations of the N-Cap Residue, Gly4, on Stability and Structure of Hen Lysozyme

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Hen lysozyme, with three α-helices (A, B, and C), is a c-type lysozyme. In these lysozymes, Ser24 and Asp88 located at the N-cap position in the B- and C-helix, respectively, are mostly conserved, but residue 4 at the N-cap position in A-helix is variable. To investigate the effect of mutation at position 4 on the stability of hen lysozyme, we prepared five mutant lysozymes and examined their stabilities and structures. Gly4Pro lysozyme (G4P), in which Gly4 was replaced by Pro, was less stable by 8.8 kJ/mol than the wild-type lysozyme, possibly because the side chain at position 7 is shifted away from the A-helix. The other mutant lysozymes were of almost equal stability to the wild-type lysozyme, although the hydrogen bonds of the amide groups at positions N1-N3 in the A-helix were absent or altered. These results indicated that various mutations at the N-cap position in the A-helix would be allowed as long as the negative charge of Glu7 at the N-terminus stabilized the A-helix.

Key word: helix, lysozyme, site-directed mutagenesis, stability, X-ray crystallography.

Proteins are unstable in nature, and this instability may be important for their physiological functions, it is desirable to improve their stabilities when we use proteins as drugs or catalyst and so on. Such improvement of stability is one of the purposes of protein engineering, and comparative biology is an important method of obtaining information on the stability of proteins. For example, the amino acid residue at position 62 is proline in the thermophilic RNaseH but histidine in the mesophilic enzyme (*1*). The amino acid residues at positions 109, 121, 175, 208, 261, 270, 290, and 378 positions in the thermophilic oligo-1,6-glucosidase is proline, whereas the corresponding residues in the mesophilic enzyme are other amino acids (*2*). These results indicate that the prolyl residues stabilized these thermophilic proteins. And it was confirmed that the mutation of some amino acid residues to proline stabilized the mesophilic RNaseH and oligo-1,6-glucosidase (*1, 2*).

An N-cap residue is defined as the first residue in a helix whose α-carbon is within the cylinder defined by the helix (*3*). A survey of 215 α-helices in the structures of 45 different globular proteins indicated that the most common residues at the N-cap position are Ser, Asn, Gly, Asp, and Thr, in order of decreasing frequency. Why the selection of these residues proved advantageous in the evolution of proteins is not clear, but both protein folding and protein stabilizing roles have been proposed for the N-cap residues (*3, 4*).

Hen lysozyme is a c-type lysozyme, for many of which the primary structures have been determined. Differences in the primary structures of c-type lysozymes may correlate with the differences in the activity (*5*), stability (*6*), and other properties. C-type lysozymes have three α-helices (A, B, and C), the N-cap position of which are occupied by a conserved Ser24 in the B-helix and a conserved Asp88 in the C-helix, but various amino acids at position 4 in the A-helix (*5-15*) (Table I and Fig. 1). The most common residues at position 4 are Glu, Gly, Ser, and Asp. In this paper, we focused on why various amino acids can occupy position 4 in c-type lysozymes. Employing a system we had previously constructed for the expression and secretion of mature hen lysozyme by yeast (*7*), we prepared five mutant lysozymes: as their most common mutants, Gly4Glu (G4E), Gly4Ser (G4S), and Gly4Asp (G4D) as common mutants; Gly4Ala (G4A) as a control; and Gly4Pro (G4P) as an common mutant. We compared their stabilities and their structures as revealed X-ray crystallography using the molecular replacement method.

MATERIALS AND METHODS

Materials—Restriction enzymes, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were purchased from Takara Shuzo (Kyoto) or New England Biolabs (Beverly). DNA sequencing kits (Sequenase) were purchased from Amersham Japan (Tokyo). CM-Toyopearl 650M, a cation-exchange resin for the purification of secreted hen lysozymes, was obtained from Tosoh (Tokyo). All other chemicals were of analytical grade for biochemical use.

Purification and Identification of Mutant Lysozymes Secreted by Yeast—Mutant lysozymes (G4A, G4S, G4D,

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Abbreviations: GdnHCl, guanidine hydrochloride; G4A, a mutant lysozyme where Gly4 is mutated to Ala; G4S, a mutant lysozyme where Gly4 is mutated to Ser; G4D, a mutant lysozyme where Gly4 is mutated to Asp; G4E, a mutant lysozyme where Gly4 is mutated to Glu; G4P, a mutant lysozyme where Gly4 is mutated to Pro.

TABLE I. **Primary structures of hen, duck-1, duck-III, chachalaca, human, mouse P, cat milk, pig-I lysozymes around the A-helix (5-15), and the N-caps of other α-helices.**

	1 4[a] 10 20	24[a]	88[a]	References
Hen	KVFGRCELAA AMKRHGLDNY	S	D	*22*
Duck-1 (pekin)	- - YS- - - - - - - - - - L- - - - -	S	D	*23*
Duck-III (kaki)	- - YE- - - - - - - - - - L- - - - -	S	D	*24*
Chachalaca	- I YK- - - - - - - - - - Y- - - - -	S	D	*25*
Human	- - - E- - - - - R TL- - L- M- G-	S	D	*26*
Mouse P	- - YN- - - - - R I L- - N- M- G-	K	D	*27*
Cat milk[b]	- I - PK- - - - R KL- AE- MN- F	S	D	
Pig-I	- - YD- - - F- R I L- KS- M- G-	S	D	*28*
	A-helix			

[a]Positions 4, 24, and 88 are the N-cap in the A-helix (5-15), B-helix (25-35), and C-helix (89-101), respectively. [b]Unpublished results.

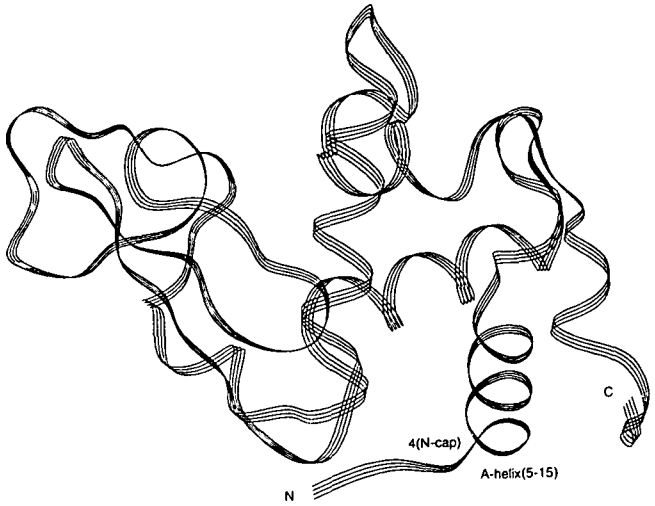

Fig. 1. **Ribbon drawing of the backbone conformation of hen lysozyme.** The residue at position 4 is located at the N-cap of the A-helix (5-15).

G4E, and G4P) were prepared as described before (*7*). Transformants of the yeast *Saccharomyces cerevisiae* AH22 were cultivated at 30°C for 125 h for expression and secretion of the lysozymes. Purification (ion-exchange chromatography) and identification (peptide mapping, amino acid sequencing, and amino acid composition) of the lysozymes were carried out as before (*8*). Each mutation was confirmed by DNA sequencing and peptide analysis (*8*).

Stabilities of Mutant Lysozymes Determined with GdnHCl Denaturation—Unfolding equilibria of mutant lysozymes by guanidine hydrochloride (GdnHCl) were measured at pH 5.5 and 35°C by fluorescence at 360 nm (excited at 280 nm). The protein concentration was 9.3×10^{-7} M, and the buffer used was 0.1 M sodium acetate adjusted to pH 5.5 with HCl. Details of the analysis were as described (*8*). Namely, by assuming a two-state transition for unfolding, the equilibrium constant between the folded (N) and the unfolded (D) state, $K_D=D/N$, and the free energy of unfolding, $\Delta G=-RT\ln K_D$, at a given concentration of GdnHCl were calculated from each unfolding curve, and a least-squares analysis was used to fit the data to the equation

$$\Delta G=\Delta G(H_2O)-m[\mathrm{GdnHCl}] \quad (1)$$

where $\Delta G(H_2O)$ is the value of ΔG in the absence of GdnHCl and m is a measure of the dependence of ΔG on GdnHCl concentration. The midpoint of GdnHCl denaturation is $C_{0.5}=\Delta G(H_2O)/m$, since $\Delta G=0$ at $C_{0.5}$. The values of $\Delta\Delta G(H_2O)$ were calculated from Eq. 1 using the average m value of the wild-type and mutant lysozymes.

X-Ray Crystallography of Mutant Lysozymes—Mutant lysozymes were crystallized from 50 mM sodium acetate at pH 4.7 and 6.5 containing 0.9-1.2 M NaCl, the same conditions as those for crystallization of native lysozyme (*9*). Mutant lysozymes were crystallized by vapor diffusion using the hanging-drop method. In shape, the crystals were isomorphous with that of the wild-type lysozyme: they belonged to the tetragonal system, space group $P4_32_12$. The intensity data out to below 2.0 Å were collected at room temperature on a RIGAKU R-AXIS II C area detector using a RIGAKU RU-300 rotating anode source operating at 40 kV and 120 mA. The intensity data were reduced using the RIGAKU PROCESS software package. Because the mutant lysozyme crystals were isomorphous with that of the wild-type lysozyme ($P4_32_12$, cell dimensions $a=b=79.21$, and $c=37.97$ Å), the coordinates of the wild-type lysozyme were used as the starting model for the refinement. These structures of mutant lysozymes were refined using X-PLOR 3.1 (*10*). These structures were fitted to Sim-weighted difference-electron density maps using the program package TURBO-FRODO 5.1 (*11*) on SGI indigo^2. The first refinements dropped the R-values of mutant lysozymes below 23.0%. Then water molecules were added to the coordinates, and the next refinements were carried out. The final refinements of the structures including water molecules gave R-values below 18.0%.

RESULTS

The Stabilities of Mutant Lysozymes Determined with GdnHCl Denaturation—The unfolding transition of the wild-type lysozyme and G4A, G4S, G4D, G4E, and G4P induced by GdnHCl was analyzed by observing changes in the fluorescence (emission at 360 nm, excited at 280 nm) as a function of denaturant concentration at pH 5.5 and 35°C. The denaturation of these mutant lysozymes may be explained as an equilibrium between N and D, because the denaturation data gave an approximate fit with the two-state model. $\Delta G(H_2O)$ is the value of ΔG in the absence of GdnHCl, and m is a measure of the dependence of ΔG on GdnHCl concentration. The midpoint of GdnHCl denaturation is referred to $C_{0.5}$. $\Delta G(H_2O)$ was calculated using $C_{0.5}$ and the average m value of the wild-type and the mutant lysozymes. The values of $\Delta\Delta G(H_2O)$ are calculated by Eq. 2.

$$\Delta\Delta G(H_2O) = \Delta G(H_2O)_{MUTANT} - \Delta G(H_2O)_{WILD} \quad (2)$$

The average values of $C_{0.5}$, m, $\Delta G(H_2O)$, and $\Delta\Delta G(H_2O)$ from two or three experiments are shown in Table II. In comparison with the wild-type lysozyme at pH 5.5, G4D was more stable by 0.6 kJ/mol, G4S had the same stability, G4A and G4E lysozymes were less stable by 1.1 kJ/mol, and G4P lysozyme was less stable by 8.8 kJ/mol. Thus, the mutant lysozymes other than G4P were of similar stability to the wild-type lysozyme. On the other hand, in G4D and G4E, where Gly is mutated to negatively charged residues, G4D was more stable by 1.7 kJ/mol than G4E.

TABLE II. **Thermodynamic parameters characterizing the GdnHCl denaturation of mutant lysozymes at pH 5.5 and 35°C.**

Lysozyme	$C_{0.5}$ (M)	m (kJ/mol/M)	$\Delta G(H_2O)$[a] (kJ/mol)	$\Delta\Delta G(H_2O)$[b] (kJ/mol)
G4G (wild)	3.62±0.01	12.3	43.1	0
G4A	3.53±0.01	11.1	42.0	−1.1
G4S	3.62±0.01	12.3	43.1	0
G4D	3.67±0.02	11.9	43.7	0.6
G4E	3.53±0.01	11.4	42.0	−1.1
G4P	2.88±0.03	13.0	34.3	−8.8

[a]Evaluated by using the mean value of m=11.9. [b]Based on the wild-type lysozyme.

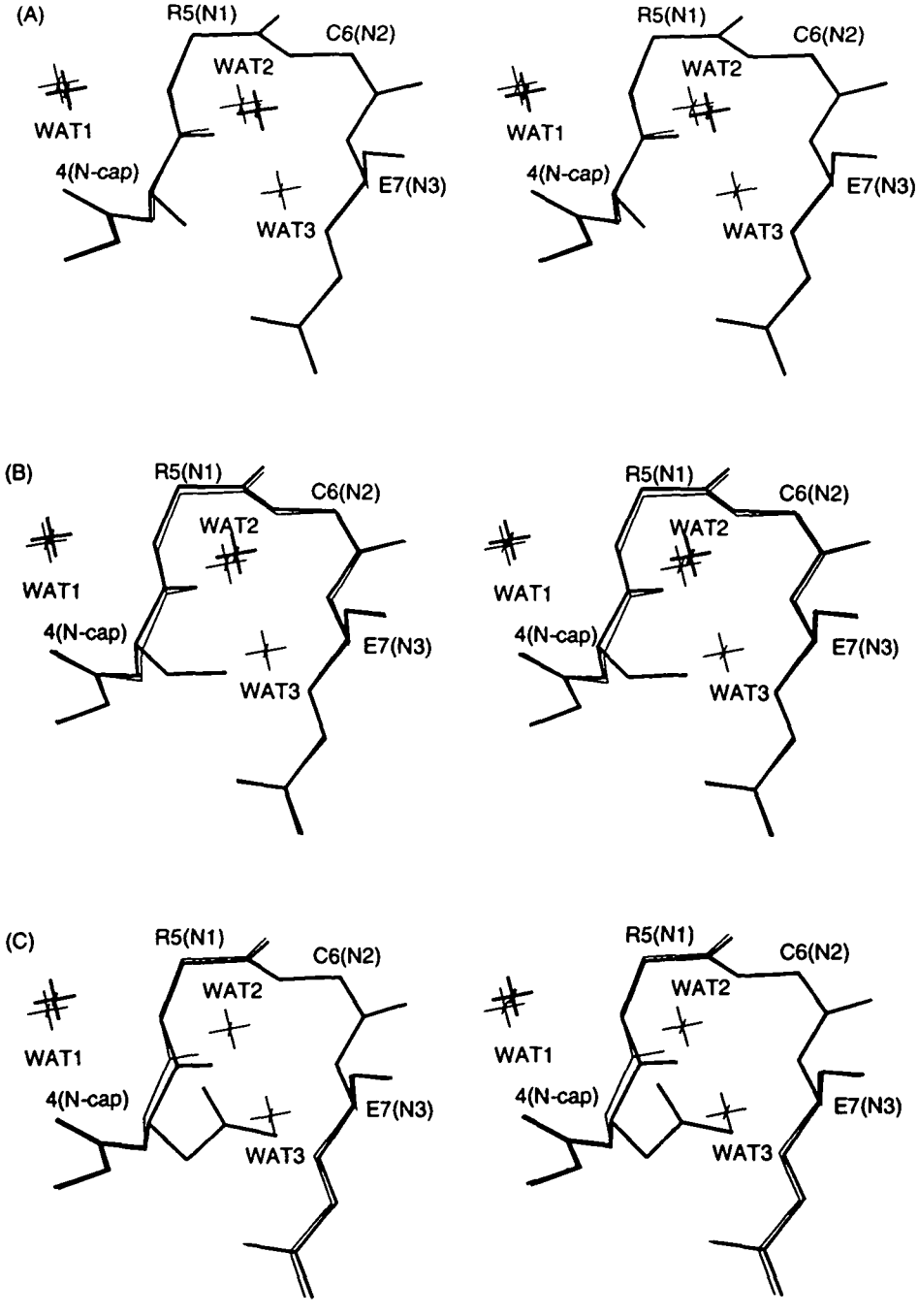

Fig. 2 (continued on next page)

Crystal Structures of Mutant Lysozymes—The crystallographic data collection and refinement statistics for the crystal structures of the wild-type and mutant lysozymes are summarized in Table III. The refined structures of these mutant lysozymes were in good agreement with that of the wild-type lysozyme. Superimposing each mutant lysozyme on the wild-type lysozyme, we calculated the root mean square (r.m.s.) deviation in coordinates of main chains to be 0.07 Å for G4A, 0.10 Å for G4S, 0.11 Å for G4D, 0.11 Å for G4E, and 0.11 Å for G4P.

TABLE III. **Crystallographic data collection and refinement statistics of lysozymes.**

Lysozyme	G4G[a] (wild)	G4A[a]	G4S[a]	G4D[b]	G4E[b]	G4P[a]
Data collections						
Cell dimensions						
a, b(Å)	79.21	79.10	79.05	78.84	78.72	79.24
c(Å)	37.97	38.12	38.06	38.21	38.43	37.83
Resolution (Å)	1.75	1.87	1.86	1.82	1.82	1.86
Unique reflections $[F>1\sigma(F)]$	11,948	9,877	8,948	10,035	10,120	9,486
Completeness (%)	93.2	93.2	83.7	88.8	89.0	89.2
R-merge (%)[c]	3.69	4.76	8.30	7.97	5.23	5.93
Refinement						
R-factor (%)[d]	16.8	17.1	17.4	17.3	17.4	17.3
Δ bond length (Å)	0.009	0.009	0.010	0.010	0.009	0.010
Δ bond angle (°)	1.448	1.475	1.513	1.500	1.486	1.534
Number of water molecules	98	68	71	64	64	62

[a]Crystallized at pH 4.7. [b]Crystallized at pH 6.5. [c]R-merge $=\Sigma|F_i - F|/\Sigma F_i$, where F_i are repeated measurements of equivalent structure amplitudes and F is the average value of F_i. [d]R-factor $=\Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$.

(1) G4G wild-type lysozyme: The N-terminal neighbor of the A-helix in the wild-type lysozyme (thin line) is shown in Fig. 2A. The amide groups at positions N1, N2, and N3 in the A-helix of the wild-type lysozyme formed hydrogen bonds with three water molecules, WAT1, WAT2, and WAT3, respectively (Table IV).

(2) G4A: The N-terminal neighbor of the A-helix in G4A (thick line) is shown in Fig. 2A. The amide groups at positions N1 and N2 in the A-helix in G4A formed hydrogen bonds with two water molecules, WAT1 and WAT2, respectively. The amide groups at position N3 did not form a hydrogen bond (Table IV).

(3) G4S: The N-terminal neighbor of the A-helix in G4S (thick line) is shown in Fig. 2B. The amide groups at positions N1, N2, and N3 in the A-helix formed hydrogen bonds with two water molecules, WAT1 and WAT2, and the side chain of Ser4, respectively (Table IV). Therefore, the two hydrogen bonds between the backbone and the side chains at the N-cap and N3 positions (Ser4 and Glu7) were found to form a capping box (*12*).

(4) G4D: The N-terminal neighbor of the A-helix in G4D (thick line) is shown in Fig. 2C. The amide group at position N1 in the A-helix formed hydrogen bond with one water molecule (WAT1) or the side chain of Asp4. The amide groups at position N2 did not form a hydrogen bond (Table IV). The amide group at position N3 in the A-helix formed a hydrogen bond with the side chain of Asp4. View from the N-terminus of the A-helix in G4D (thick line) and G4E (thin

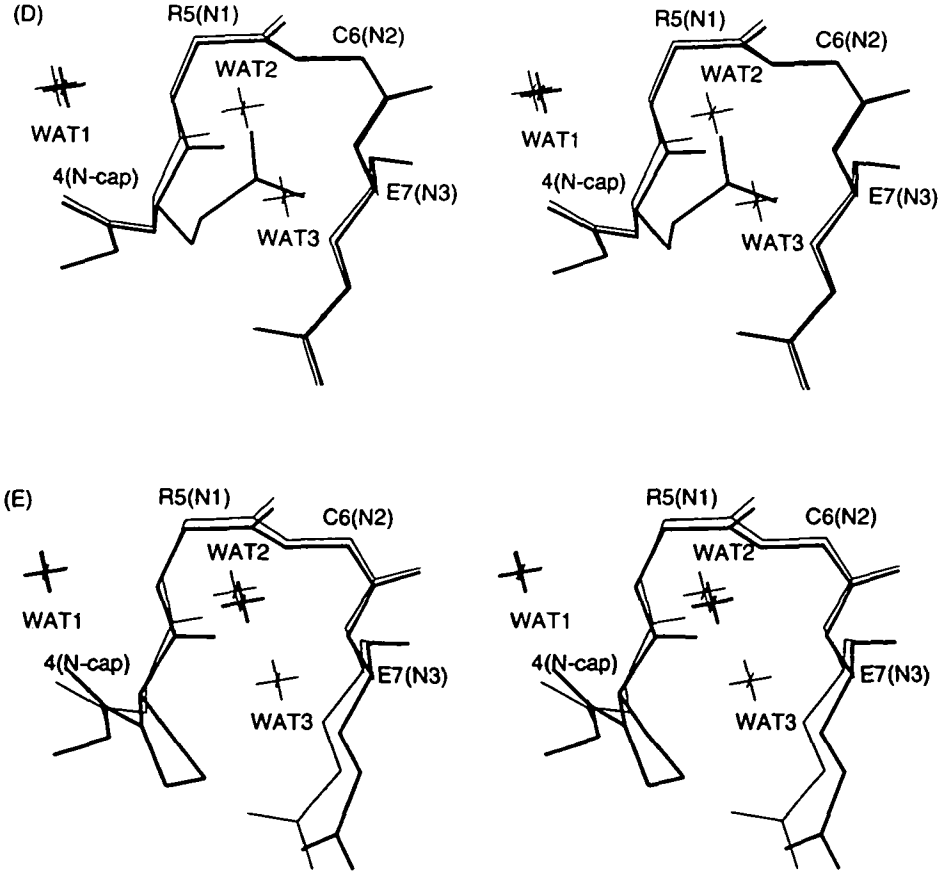

Fig. 2. **Superposition of the structures of each lysozyme (thick line) on that of the wild-type lysozyme (thin line).** The amide groups at positions N1, N2, and N3 form hydrogen bonds with WAT1, WAT2, and WAT3, respectively. (A) G4A, (B) G4S, (C) G4D, (D) G4E, and (E) G4P.

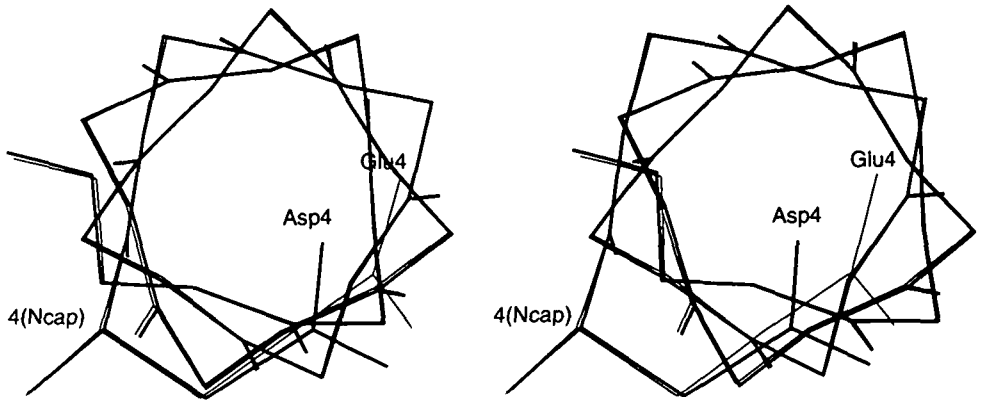

Fig. 3. **Crystal structure of the region near the N-terminus of the A-helix (5-15) in G4D (thick line) and G4E (thin line).**

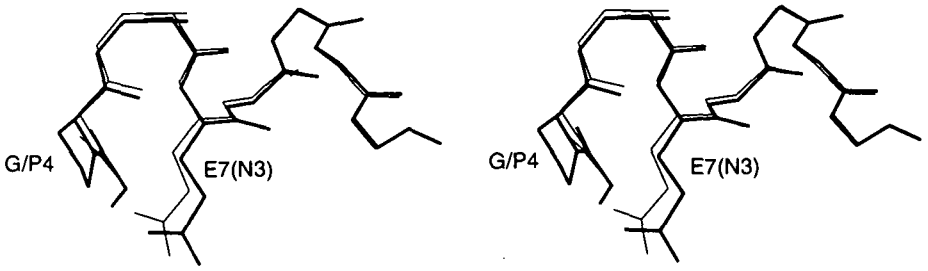

Fig. 4. **Crystal structure of the region near the N-terminus of the A-helix (5-15) in G4P (thick line) and the wild-type lysozyme (thin line).**

TABLE IV. **Hydrogen bonding of the amide groups at positions N1, N2, and N3 in the A-helix of lysozymes.**

H-donor	H-acceptor	Distance (Å)					
		G4G (wild)	G4A	G4S	G4D	G4E	G4P
Arg5N (N1)	WAT1	3.00	2.78	2.85	2.88	2.81	2.95
	Asp4Oγ1				2.82		
Cys6N (N2)	WAT2	2.93	2.85	2.85			2.87
	Glu4Oε1					2.91	
Glu7N (N3)	WAT3	3.15					
	Ser4Oγ			3.27			
	Asp4Oγ2				3.21		
	Glu4Oε2					3.00	

line) are shown in Fig. 3. The side chain of Asp4 in G4D was located closer to the center of the A-helix than that of Glu4 in G4E.

(5) G4E: The N-terminal neighbor of the A-helix in G4E (thick line) is shown in Fig. 2D. The amide group at position N1 in the A-helix formed a hydrogen bond with one water molecule (WAT1). The amide groups at positions N2 and N3 in the A-helix in G4E lysozyme formed hydrogen bonds with the side chain of Glu7 (Table IV).

(6) G4P: The N-terminal neighbor of the A-helix in G4P (thick line) is shown in Fig. 2E. The amide groups at positions N1 and N2 in the A-helix formed hydrogen bonds with two water molecules, WAT1 and WAT2, respectively. The amide groups at position N3 did not form a hydrogen bond (Table IV). Because of the mutation of Pro4, the orientation of the side chain of Glu7 was shifted away from the A-helix (Fig. 4).

DISCUSSION

Based on crystallographic data, Richardson and Richardson reported that such amino acid residues as Asn, Ser, Asp, and Gly frequently occupy the N-cap position in α-helix (*3*). Some scientists have investigated the stabilities of mutant proteins where such amino acid residues were introduced at the N-cap position. From studies on barnase (*13*), T4 lysozyme (*14*), growth hormone (*15*), and histidine-containing protein (*16*) mutant proteins with Asp or Glu at the N-cap position were more stable than Ala, possibly as the result of a charge-helix macrodipole interaction. On the other hand, mutants of barnase (*13*), T4 lysozyme (*14*), and histidine-containing protein (*16*), with Ser or Thr at the N-cap position were more stable than those with Ala, because of hydrogen bond formation between the side chain at the N-cap position and the amide group at N3. In the present paper, we investigated the structures and stabilities of mutant lysozymes where the amino acid residue at position 4 was mutated to Ala, Ser, Asp, Glu, and Pro, because there are various amino acids at this position in naturally occuring c-type lysozymes. The stabilities of mutant lysozymes other than G4P were similar to that of the wild-type lysozyme.

Earlier experimental results (*14-17*) indicate that the electrostatic interaction between the charged side chain at the N-cap position and the helix macrodipole stabilizes proteins. This is consistent with the present finding that G4D was slightly more stable than the wild-type lysozyme, and G4E was less stable than the wild-type lysozyme. Since the side chain of Asp4 in G4D was located closer to the center of the A-helix than that of Glu4 in G4E (Fig. 3), the helix-charge macrodipole interaction in G4D was stronger than that in G4E. The structural difference between G4D and G4E may thus be involved in the difference between them in stability.

However, studies on barnase (*13*) and T4 lysozyme (*14, 17*) demonstrated that mutant proteins with Asp or Glu at

the N-cap position were more stable by 1.7 to 6.4 kJ/mol than the corresponding mutant proteins with Ala. If the charge-helix macrodipole interaction between the charged side chain at position 4 and the A-helix had contributed to the stabilization in G4D and G4E, these mutant lysozymes should have been more stable. Therefore, a destabilizing factor may operate around the N-terminus of the A-helix. The mutant hen lysozyme where Glu7 was mutated to Gln was less stable by 4.9 kJ/mol than the wild-type lysozyme (*18*), and the mutant with Asn27 mutated to Asp was more stable by 1.5 kJ/mol than the wild-type (*6*). Since residues 7 and 27 are located at position N3 in the A-helix and B-helix, respectively, charge-helix macrodipole interactions between the charged side chain at position N3 and the α-helix should contribute to stabilization. The negative charge at Glu7 mainly stabilizes the A-helix at the N-terminus. Moreover, the short distance between the side chains at the N-cap and N3 positions may give rise to electrostatic repulsion between these side chains, resulting in the lower extent of stabilization than was expected in G4D and G4E.

While G4A and G4S were structurally different from the wild-type lysozyme, their stabilities were similar. In G4A, the hydrogen bond between the amide group at N3 (residue 7) in the A-helix and WAT3 water molecule was absent. In G4S, the WAT3 water molecule was replaced by the hydroxyl group of Ser4. The hydroxyl group of Ser4 in G4S formed an end-cap consisting of a hydrogen bond between the amide group at position N3 and the side chain at the N-cap position. Therefore, the reason for the similar stabilities of G4A and G4S to the wild-type lysozyme may depend on the fact that the N-terminal neighborhood (N1, N2, and N3) in the A-helix of hen lysozyme was exposed to solvent molecules. Thus, G4A, G4S, G4D, and G4E were of similar stability to the wild-type lysozyme, indicating that some mutations of the amino acid residue at position 4 in c-type lysozymes were possible without affecting the stability of the global structure.

On the other hand, Nicholson *et al.* have suggested that when a protein is stabilized by replacing a certain amino acid residue with proline, the dihedral angles of the residue replaced and of the introduced prolyl residue should be considered (*19*). The dihedral angles of the amino acids at positions 3 and 4 in the wild-type lysozyme are in the allowed ranges. However, G4P was less stable by 8.8 kJ/mol than the wild-type lysozyme, and G4P was found to lack the hydrogen bond between the amide group at position 4 and the side chain at position 7. Thus one reason for the reduced stability of G4P may be the loss of the hydrogen bond. However, as Shirley *et al.* demonstrated that most intramolecular hydrogen bonds contributed 5.4 ± 2.5 kJ/mol to the stability of structures such as globular proteins (*20*), the reduction in stability of G4P is larger than would be expected from the loss of one hydrogen bond. The mutant lysozyme where Glu7 was mutated to Gln was less stable by 4.9 kJ/mol than the wild-type lysozyme due to the loss of a charge-helix macrodipole interaction (*18*). From X-ray crystallography, the conformation of side chains of Glu7 did not change in mutant lysozymes other than G4P, in which this side chain was shifted away from the N-terminus of the A-helix. Consequently, the other reason for the reduced stability of G4P may be the decrease in the charge-helix macrodipole interaction between the charged side chain at position 7 and the A-helix. In cat milk lysozyme, the amino acid residue at position 4 is Pro. This lysozyme has a calcium binding site (86-92). Because the lysozymes which have calcium-binding sites are stable (*21*), a Pro residue at the N-cap position in the A-helix is thought to be permissible in this case.

Finally, we refer to the reason why various amino acids are present at position 4 in c-type lysozymes. From the results above, several mutations at position 4 did not greatly affect the stabilization of the A-helix in c-type lysozymes. Also, the negative charge at Glu7 stabilized the lysozyme molecule (*18*). The estimated stabilization free energy was 4.9 kJ/mol. This value was larger than the level of fluctuation of the stability of mutant lysozyme where Gly4 was mutated to Ala, Ser, Asp, or Glu. The conformation of Glu7 was affected little in these mutant lysozymes. Thus, we concluded that various mutations at the N-cap position in A-helix were allowable because the negative charge of Glu7 at the N-terminus was the dominant factor stabilizing the A-helix.

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