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Design and creation of a Ca^{2+} binding site in human lysozyme to enhance structural stability

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ABSTRACT **A Ca^{2+} binding site like an EF-hand motif was designed and created in human lysozyme by replacing both Gln-86 and Ala-92 with aspartic acids by site-directed mutagenesis. The mutant human lysozyme (D86/92-lysozyme) was expressed and secreted by yeast. One Ca^{2+} was found to bind one molecule of the purified protein with the binding constant 5.0×10^6 M^{-1}. The enzymatic activity of holo-D86/92-lysozyme against glycol chitin at 40°C was 2-fold higher than that of the native lysozyme. Maximal activity of the holo-D86/92-lysozyme was observed at 80°C, where its relative activity normalized to the value at 40°C was 6-fold and 17-fold higher than those of the native and apoenzymes, respectively. The activities of the native lysozyme and apo-D86/92-lysozyme were maximum at 65°C–70°C. Moreover, D86/92-lysozyme was more stable against protease digestion than the native lysozyme. These results indicate that the creation of the calcium binding site like an EF-hand motif in the human lysozyme enhances its structural stability.**

There are several known engineering approaches to improve the structural stability of a protein. Two strategies are available to stabilize a protein molecule on the basis of the free energy difference between the native and unfolded states. One is to increase the free energy of the unfolded state by the entropy loss of the unfolded state, introducing additional disulfide bonds (1), replacing a residue by a proline, or replacing a glycine by a more bulky residue (2). The other is to decrease the free energy of the native state by introducing intramolecular interactions in the protein molecule, such as hydrogen bonds (2) and hydrophobic (3) and electrostatic interactions (4, 5).

In some proteins, metal ions are known to act as stabilizers through their electrostatic interactions. One example is α-lactalbumin, the tertiary structure of which is stabilized by Ca^{2+} (6). Some Ca^{2+} binding proteins often have a common structure called an EF-hand motif at the Ca^{2+} binding site (7). This motif structure is composed of a loop between two orthogonal helices. A conformational change of this EF-hand structure results from Ca^{2+} binding. Altered interactions between the EF-hand and the target proteins, resulting from such conformational changes, play important roles in biological regulatory systems (8, 9). Recent x-ray crystallographic studies revealed that the Ca^{2+} binding site of α-lactalbumin has some characteristic features of the EF-hand motif (10). Chicken-type (c-type) lysozymes are some of the enzymes in which structure and function have been most investigated (11) and are thought to have the same ancestral protein as α-lactalbumin (12). In spite of the differences in their biological functions, the amino acid sequences and the tertiary structures of c-type lysozymes are very similar to those of α-lactalbumin (13, 14). Although c-type lysozymes have mostly lost the Ca^{2+} binding ability, a loop structure similar to the EF-hand motif is conserved in their structures (10). These facts suggest that c-type lysozymes should possibly bind Ca^{2+} just as α-lactalbumin by idealizing amino acids in the loop region. Moreover, the binding of Ca^{2+} to the idealized region might be expected to increase the structural stability of the lysozyme molecule.

In the present study, a Ca^{2+} binding site was designed in human lysozyme and both Gln-86 and Ala-92 in the loop structure were replaced by aspartic acids by site-directed mutagenesis. This engineered lysozyme was named D86/92-lysozyme. The D86/92-lysozyme gene was expressed in the yeast secretion system (15), by which various mutant lysozymes have already been generated (16, 17). The results demonstrate that the design was successful in enhancing the structural stability of human lysozyme with Ca^{2+} binding to the protein.

MATERIALS

Klenow fragment of DNA polymerase I and restriction enzymes were purchased from Boehringer Mannheim and Takara Shuzo (Kyoto, Japan). T4 DNA ligase was from New England Biolabs. Human lysozyme, α-lactalbumin, Pronase, and *Micrococcus lysodeikticus* were from Sigma. Glycol chitin and $(NAG)_3$ (the β-1,4-linked trimer of *N*-acetyl-D-glucosamine) were from Seikagaku Kogyo (Tokyo). The fluorescent reagent Fura-2 and other chemicals were from Wako Pure Chemical (Osaka, Japan). Enzyme reactions were carried out under the conditions recommended by the suppliers.

METHODS

Strain and Media. *Saccharomyces cerevisiae* AH22R$^-$ (*mata, leu2, his4, can1, pho80*) (18) was used for the host strain and cultivated in modified Burkholder medium (19) supplemented with 8% sucrose.

DNA. Oligonucleotides were synthesized by the phosphoramidite method (20) with a model 380A DNA synthesizer (Applied Biosystems) and purified by HPLC on a TSK gel ODS-120T (Toyo-Soda, Tokyo).

Oligonucleotide-Directed Mutagenesis. Plasmid pGEL125, which contains the cloned yeast glyceraldehyde-3-phosphate dehydrogenase promoter and DNA encoding the chemically synthesized chicken lysozyme signal sequence and human lysozyme sequence, was used as a starting material for mutagenesis (16). To replace both Gln-86 and Ala-92 by aspartic acid, oligonucleotide-directed mutagenesis developed by Zoller and Smith (21) was carried out using 5′-

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Abbreviations: D86/92-lysozyme, mutant human lysozyme in which Gln-86 and Ala-92 are replaced by aspartic acids; $(NAG)_3$, β-1,4-linked trimer of *N*-acetyl-D-glucosamine; c-type lysozyme, chicken-type lysozyme.

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GCTTTGCTTGACGACAACATTGCTGATGATGTTGCC-3′ (36-mer) as a primer.

Sequencing and Plasmid Construction. To confirm the mutation, a Takara M13 sequencing kit (Takara Shuzo) was used for sequencing by the dideoxynucleotide method (22). The wild-type human lysozyme gene in pGEL125 was replaced by the *Xho* I fragment of the mutated gene to obtain pERI8811.

Purification of the D86/92-Lysozyme. After a 72-hr cultivation of *S. cerevisiae* AH22R$^-$/pERI8811 at 30°C, 5 liters of the culture supernatant was subjected to purification as described (16).

Determination of Amino Acid Composition. Protein samples (0.2 mg) were hydrolyzed in 6 M HCl under vacuum at 110°C for 20 hr, and the amino acid composition was analyzed with a Hitachi 835 amino acid analyzer (Hitachi, Japan). Free SH group was determined with Ellman's reagent (23) according to the procedure of Honda *et al.* (24).

Measurement of CD Spectra. CD spectra of the lysozymes were measured with a J-600 spectropolarimeter (Japan Spectroscopic, Tokyo) in a range between 200 and 250 nm at room temperature. The proteins were dissolved in 5 mM Tris·HCl buffer (pH 8.0) and the concentration was adjusted to 0.5 mg/ml.

Enzymatic Analyses of the Lysozymes. The lytic activities of the native and the holo-D86/92-lysozymes were determined by lysis of *M. lysodeikticus* cells (0.2 mg/ml) according to the procedure of Kikuchi *et al.* (16). Activities of the lysozymes using glycol chitin as a substrate were measured in 0.1 M acetate buffer (pH 5.5) at 40°C as described (25).

Preparation of Apo- and Holoproteins. To prepare the holo-D86/92-lysozymes and holo-α-lactalbumin, a lyophilized sample was dissolved in 10 mM calcium chloride solution and incubated at 40°C for 1 hr. The free calcium ions were removed from the protein solution by gel filtration with a Sephadex G-25 (medium) (Pharmacia) column (1 × 20 cm) equilibrated with 0.01 M Hepes buffer at pH 7.1. The preparations of the apo-D86/92-lysozyme and apo-α-lactalbumin were performed according to the method previously reported (26).

Calcium Analysis. The number of Ca^{2+} binding to the D86/92-lysozyme and α-lactalbumin was determined with a Hitachi 170-10 atomic absorption photometer (Hitachi, Japan). The binding constants of Ca^{2+} to the D86/92-lysozyme and bovine α-lactalbumin molecules were determined through the titration of Fura-2 by adding small amounts of 0.4 mM calcium chloride in 0.01 M Hepes buffer at pH 7.1 according to the method of Nitta *et al.* (26).

Determination of Dissociation Constants of $(NAG)_3$. Dissociation constants of the substrate analog $(NAG)_3$ to the native, apo-, and holo-D86/92-lysozymes were determined by UV difference spectroscopy in 0.1 M acetate buffer (pH 5.5) at 40°C (27) using a Hitachi U-3200 spectrophotometer.

Measurement of Thermal Stability. The thermal stabilities of the native, apo-, and holo-D86/92-lysozymes were examined by measuring the enzymatic activities in the temperature range 30°C–100°C using glycol chitin as a substrate in 0.1 M acetate buffer at pH 5.5.

Measurement of Stability Against Protease Digestion. Ten micrograms of native or holo-D86/92-lysozyme was digested with 10 μg of Pronase in 0.01 M Hepes buffer (pH 7.1) containing 0.1 M KCl and 10 mM $CaCl_2$ at 40°C. The time course of the remaining activity of the lysozyme was measured, and the half-life was calculated from the decrease in its activity.

RESULTS

Design of Ca^{2+} Binding Site in Human Lysozyme. To construct a Ca^{2+} binding site in human lysozyme, the Ca^{2+} binding site of α-lactalbumin was chosen as a model structure. Based on the x-ray crystallographic results for α-lactalbumin, the calcium ligands were confirmed to be the backbone carbonyl groups of Lys-79 and Asp-84 and the carboxyl groups of Asp-82, Asp-87, and Asp-88 (7). The partial sequences from residues 80–90 of α-lactalbumins and c-type lysozymes from several species were aligned by the program IDEAS (28) and are shown in Fig. 1*A*. The residue numbers shown are those of human lysozyme. As already stated, α-lactalbumin binds Ca^{2+} in nearly the same manner as Ca^{2+} binding proteins having a typical EF-hand motif. All c-type lysozymes except equine and pigeon lysozyme lack two or three aspartic acids at position residues 86, 91, and 92. In human lysozyme, the carboxyl group of Asp-91 is present, but the other two carboxyl groups have been replaced by Gln-86 and Ala-92. The equine and pigeon lysozymes, in which all three carboxyl groups are present, have recently been reported to have a Ca^{2+} binding potential (26, 29). Leu-84, Leu-85, and Ile-89 in human lysozyme are well-conserved hydrophobic residues among c-type lysozymes and α-lactalbumin. Ile-89, in particular, is a conserved hydrophobic residue in all the sequences in Fig. 1. Replacing these hydrophobic residues may cause a structural deformation around the loop. Considering the above, the replacement of both Gln-86 and Ala-92 by two aspartic acids was thought to be the most effective way to bind Ca^{2+}. Using molecular

A) C-Type Lysozymes and α-Lactalbumins

	82											93
D86/92-Lysozyme	Ser	Ala	Leu	Leu	Asp	Asp	Asn	Ile	Ala	Asp	Asp	Val
Human Lysozyme	Ser	Ala	Leu	Leu	Gln	Asp	Asn	Ile	Ala	Asp	Ala	Val
Hen Lysozyme	Ser	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala	Ser	Val
Equine Lysozyme	Ser	Lys	Leu	Leu	Asp	Glu	Asn	Ile	Asp	Asp	Asp	Ile
Pegion Lysozyme	Ser	Lys	Leu	Arg	Asp	Asp	Asn	Ile	Ala	Asp	Asp	Ile
Human α-Lactalbumin	Asp	Lys	Phe	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp	Ile
Bovine α-Lactalbumin	Asp	Lys	Phe	Leu	Asp	Asp	Asp	Leu	Thr	Asp	Asp	Ile
		*			**		*			**	**	

B) Other Lysozymes and EF-hand

	51											62
T4 Lysozyme	Gly	Arg	Asn	Cys	Asn	Gly	Val	Ile	Thr	Lys	Asp	Glu
	106											117
Goose Lysozyme	Thr	Trp	Asn	Gly	Glu	Val	His	Ile	Thr	Gln	Gly	Thr
	51											62
Carp Parvalbumin	Asp	Gln	Asp	Lys	Ser	Gly	Phe	Ile	Glu	Glu	Asp	Glu
(EF-hand)	**		**		**		*		**			**

FIG. 1. Sequence alignments of loop regions in various proteins. Aligned by the program IDEAS (28). *, Position of backbone carbonyl ligand; **, position of the side-chain carboxyl ligand. The centers of the loops (boxed) are structurally similar.

graphics, both Gln-86 and Ala-92 of human lysozyme were replaced by aspartic acids and their dihedral angles were adjusted. This resulted in a spherical space of ≈4.5 Å diameter among these carboxyl groups, where a Ca^{2+} might possibly be positioned. The modeled structure is shown in Fig. 2.

Amino Acid Composition of the D86/92-Lysozyme. The amino acid composition of the D86/92-lysozyme was confirmed (data not shown). In the D86/92-lysozyme, the number of glutamine and alanine residues was reduced by one, and the number of aspartic acid residues was increased by two. The numbers of other residues were the same as those of the native lysozyme. These results indicate that the D86/92-lysozyme has the same amino acid composition as designed. The number of free SH groups was determined to be <0.01 mol per D86/92-lysozyme molecule by using Ellman's reagent. The value was almost the same as that of native human lysozyme. It indicates that the D86/92-lysozyme has no free SH group.

CD Spectra. CD spectra of the native, apo-, and holo-D86/92-lysozyme were measured in 5 mM Tris·HCl buffer at pH 8.0. The data were expressed in terms of mean residue ellipticity [θ] in Fig. 3. The spectra of the apo- and holo-D86/92-lysozyme in the region from 210 to 250 nm were almost the same as that of the native lysozyme. This indicates that the replacement of both Gln-86 and Ala-92 by aspartic acid does not significantly influence the secondary structure of the D86/92-lysozyme whether Ca^{2+} binds or not. This is also confirmed by the preliminary results of NMR measurements.

The Number of Calcium Ions and the Dissociation Constant. The number of Ca^{2+}, which binds to the native human lysozyme, the D86/92-lysozyme, and α-lactalbumin, was determined by atomic absorption spectra as summarized in Table 1. No Ca^{2+} bound the native lysozyme, whereas one Ca^{2+} bound one molecule of D86/92-lysozyme and one molecule of bovine α-lactalbumin. The binding constant of the D86/92-lysozyme was $5.0 \times 10^6\ M^{-1}$, which is comparable to the binding constant of α-lactalbumin ($4.0 \times 10^7\ M^{-1}$) as shown in Table 1. These results indicate that one Ca^{2+} tightly binds one molecule of the D86/92-lysozyme as strongly as α-lactalbumin.

Enzymatic Activity and Dissociation Constant of the $(NAG)_3$–Lysozyme Complex. The lytic activities of the native and holo-D86/92-lysozymes measured in 0.1 M phosphate buffer (pH 6.2) are shown in Table 2. Because of the difficulty in excluding Ca^{2+} that originated from bacterial cells, the lytic activity of the apo-D86/92-lysozyme was not measured. The lytic activity of the holo-D86/92-lysozyme was 95% that of the native lysozyme.

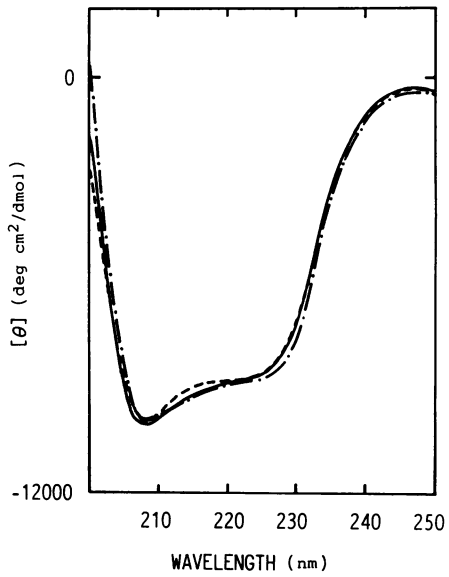

FIG. 3. CD spectra of the native (—), apo- (----), and holo- (-·-) D86/92-lysozymes measured in 5 mM Tris·HCl buffer (pH 8.0) at room temperature.

The enzymatic activities of the native, apo-, and holo-D86/92-lysozymes against glycol chitin are also shown in Table 2. The enzymatic activities of the apo- and holo-D86/92-lysozyme were higher than that of native human lysozyme under these conditions. The dissociation constants of the substrate analog $(NAG)_3$ to the lysozymes are also summarized in Table 2. The dissociation constants of the apo- and holo-D86/92-lysozymes were $1.0 \times 10^{-4}\ M^{-1}$ and $0.8 \times 10^{-4}\ M^{-1}$, respectively. These values are almost the same as that of the native lysozyme.

Thermal Stability and the Stability Against Protease Digestion. The temperature dependencies of enzymatic activity of

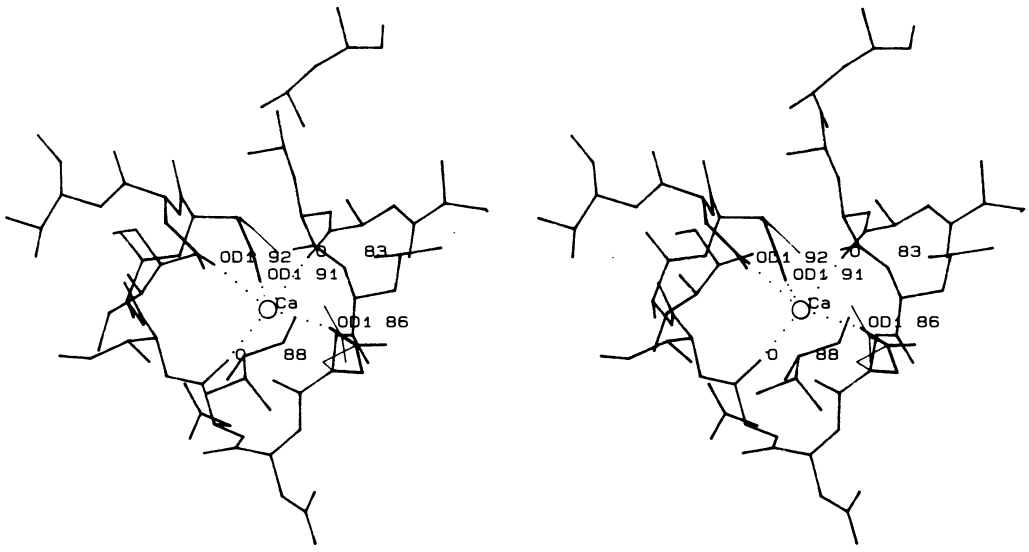

FIG. 2. Stereoview of the modeled structure of the Ca^{2+} binding site in D86/92-lysozyme. The structure was obtained after replacing both Gln-86 and Ala-92 by aspartic acids and adjusting the dihedral angles of Asp-86, Asp-91, and Asp-92 by using the program INSIGHT on PS350 molecular graphics. The thin line on the side chain reveals the original conformation of x-ray structure. The circle reveals the position of Ca^{2+}. See text for details.

Table 1. Binding number of Ca^{2+} and its binding constant to native lysozyme, D86/92-lysozyme, and α-lactalbumin

Protein	Concentration, μM		Ca^{2+} per protein molecule	Binding constant, M^{-1}
	Calcium	Protein		
Human lysozyme (native)	1.0	24.4	0.02	ND
D86/92-lysozyme (mutant)	40.0	41.9	0.94	5.0×10^6
Bovine α-lactalbumin	75.0	83.0	0.90	4.0×10^7

ND, not detected by the present method.

the native, apo-, and holo-D86/92-lysozymes are shown in Fig. 4. The activities of all three lysozymes are normalized so that they have the same activities at 40°C. The maximal activity of the holo-D86/92-lysozyme was observed at 80°C, where its normalized activity was 6-fold and 17-fold higher than those of the native and the apoenzymes, respectively. The maximal activities of the apo-D86/92-lysozyme and the native lysozyme were observed at ≈65°C and ≈70°C, respectively. Holo-D86/92-lysozyme was more stable than the native lysozyme, and the apo-D86/92-lysozyme was less stable than the native lysozyme.

The half-lives of the remaining activity against the Pronase digestion of the native and holo-D86/92-lysozymes were 58 min and 70 min, respectively, assuming that the digestion proceeded as a pseudo-first-order reaction (30). The holo-D86/92-lysozyme was found to be more stable against the protease digestion than the native lysozyme.

DISCUSSION

In the present study, the designed D86/92-lysozyme was found to bind one Ca^{2+} per protein molecule as shown in Table 1. The binding constant of D86/92-lysozyme was comparable to those of bovine α-lactalbumin (see Table 1) and one of the EF-hands, parvalbumin ($K_a = 2 \times 10^6$) (31). If the Ca^{2+} were only to bind to the surface of the D86/92-lysozyme molecule, the binding constant would be expected to be as low as those of phospholipase A_2 (2.5×10^3) (31) and concanavalin A (3.3×10^3) (31). Therefore, the high affinity observed for Ca^{2+} suggests that D86/92-lysozyme binds Ca^{2+} in the same manner as α-lactalbumin.

It is known that there are four groups of lysozymes: c-type lysozymes, goose-type lysozymes, T4 phage lysozymes, and bacterial lysozymes. On the basis of the structural and functional similarities, it was reported that three of the groups, c-type, goose-type, and T4 lysozymes, probably had a common evolutionary precursor, even though their amino acid sequences have no detectable similarity (32–34). The loop region, similar to an EF-hand motif, that was discovered in c-type lysozymes and α-lactalbumin (10) is expected to be observed in T4 lysozyme and goose lysozyme. Fig. 1*A* shows the amino acid sequences of this loop region in c-type lysozymes and α-lactalbumins aligned by the program IDEAS (28). The sequences of the structurally corresponding region of T4 and goose-type lysozymes and the sequence of a typical EF-hand motif were also aligned by the program IDEAS, and they are summarized in Fig. 1*B*. There is no detectable similarity between c-type lysozymes (Fig. 1*A*) and T4 and goose lysozymes (Fig. 1*B*) as previously mentioned (32, 33, 35). But in T4 lysozyme, although only a few aspartic acids and glutamic acids are found in this region, the sequence is rather homologous to the typical EF-hand motif taken from carp parvalbumin. The sequence of goose lysozyme also has a weak homology to that of the EF-hand motif. The region indicated in Fig. 1 forms a similar loop structure in the

Table 2. Enzymatic activities and dissociation constants of $(NAG)_3$ in the presence of native lysozyme and D86/92-lysozymes

Lysozyme	Enzymatic activity, %		Dissociation constant, $\times 10^{-4}$ M
	Lysis	Glycol chitin	
Native	100	100	1.0
D86/92-lysozyme (apo)	ND	110	1.1
D86/92-lysozyme (holo)	95	150	0.8

All enzymatic values are normalized to a value of 100 for the activity of native lysozyme. Lysis was determined by lysis of bacterial cell walls from *M. lysodeikticus* in 50 mM phosphate buffer (pH 6.2) at 25°C. ND, not detected. Activity against glycol chitin was determined in 0.1 M acetate buffer (pH 5.5) at 40°C. The dissociation constant was determined by UV difference spectroscopy in 0.1 M acetate buffer (pH 5.5) at 40°C.

α-lactalbumin (10, 14), c-type lysozymes (36, 37), T4 lysozyme (38), and goose lysozyme (35). The box in Fig. 1 encloses the residues that are most structurally similar when superimposed. In the typical EF-hand motif and T4 lysozyme, the loop is between two α-helices. In the c-type lysozyme, the former helix is replaced by a 3_{10}-helix. In goose lysozyme, the former helix is absent. From the investigations described above, T4 lysozyme has a more idealized site like an EF-hand motif than human lysozyme, suggesting that it is also possible to stabilize T4 lysozyme by creating the Ca^{2+} binding site. If a Ca^{2+} binding site can be introduced in T4 lysozyme, it becomes more acceptable that the three kinds of lysozymes are evolved from a common precursor as mentioned previously (32, 33, 35). The similarities between the loop structures in T4 lysozyme and the EF-hand motif also suggest that the lysozymes may have some evolutionary relationship with the proteins having the EF-hand motif as a calcium binding site.

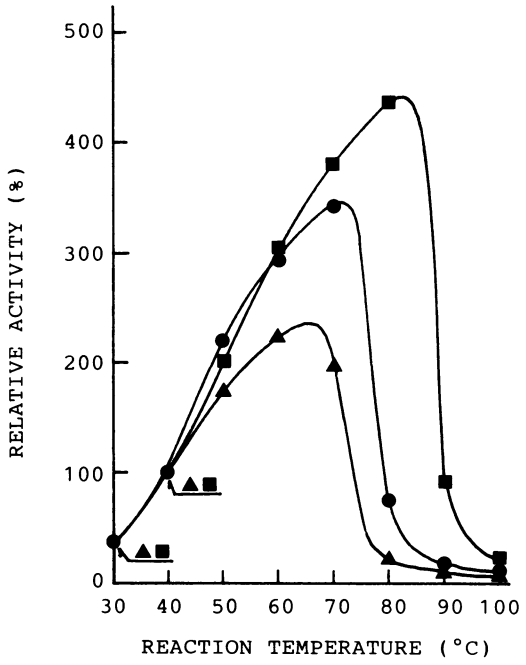

FIG. 4. Temperature dependence of the enzymatic activities of the native, apo-, and holo-D86/92-lysozymes using glycol chitin as a substrate in 0.1 M acetate buffer at pH 5.5. The activities were normalized so that all three lysozymes had the same activities at 40°C and they were plotted against temperature for the native lysozyme (●), the apo-D86/92-lysozyme (▲), and the holo-D86/92-lysozyme (■).

As shown in Fig. 4, the holo-D86/92-lysozyme is active even at a temperature >10°C higher than the native lysozyme. On the contrary, the apo-D86/92-lysozyme is ≈5°C less stable than the native lysozyme. These phenomena are understandable by the free energy decrease and increase, respectively. In the holo-D86/92-lysozyme, the strong electrostatic interaction between Ca^{2+} and its ligand groups of the protein decreases the free energy of native state. On the other hand, without Ca^{2+}, the electrostatic repulsion between negatively charged carboxyl groups destabilizes the protein structure. Recently, it was reported that the thermostability of bacterial subtilisin was increased by enhancing the specific Ca^{2+} binding affinity (4). In that case, enhancing a metal binding affinity was considered to decrease the free energy of the protein structure. In this study, the introduction of a Ca^{2+} binding site into a lysozyme molecule that originally had no Ca^{2+} binding ability gave a more drastic increase of the thermal stability.

The holo-D86/92-lysozyme is more stable against protease digestion than the native lysozyme. It has been considered that the digestion of small globular proteins with proteases proceeds via their unfolded states (39). The stability against the protease digestion has been found to be closely related to the transition rate constant from the native state to the unfolded state (23). A recent study of protease digestion of thermolysin (40) also revealed that the amino acid positions attacked by proteases correspond well to the large degrees of the positional fluctuation. Since the Ca^{2+} binding to the D86/92-lysozyme stabilizes the protein against protease digestion, it is reasonable to suppose that this is because this binding stabilizes its structure and makes the associated loop more rigid.

Since Gln-86 and Ala-92 in the native lysozyme are located on the opposite side of the protein to the active site cleft, the enzymatic activity would be expected to remain after the mutations. The lytic activity of the holo-D86/92-lysozymes was 95% that of the native lysozyme. The enzymatic activities against glycol chitin of the apo- and holo-D86/92-lysozymes were somewhat higher than that of the native lysozyme. The dissociation constants of $(NAG)_3$ for the binding of the native, apo-, and holo-D86/92-lysozymes were comparable to that of the native lysozyme, as indicated in Table 1. At present there is no precise structural information about the active site of the mutated lysozymes, so it is too early to discuss these differences in the enzymatic activities. However, it should be pointed out that from these experiments the structures of the active site of apo- and holo-D86/92-lysozymes are likely to be very similar to that of the native lysozyme.

Generally, chemical reaction rates increase with the increase in temperature. In the present study, the enzymatic activity of the holo-D86/92-lysozymes against glycol chitin increases as temperature increases up to 80°C, as shown in Fig. 4. At 80°C, the native and the apo-mutant lysozyme are inactive due to possible unfolding of the protein structures. Moreover, the maximal value of absolute activity of the holo-D86/92-lysozyme at 80°C is 1.7-fold higher than that of the native lysozyme at 70°C. These findings are important and useful for remodeling the protein in the actual application. Without any precise tuning around the active site, stabilization of a protein structure by tailoring a local region far from its active site possibly gives rise to its higher activity at high temperature, where the native protein is inactive.

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