Structural and Thermodynamic Responses of Mutations at a Ca$^{2+}$ Binding Site Engineered into Human Lysozyme

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Structural determinants of Ca$^{2+}$ binding sites within proteins typically comprise several acidic residues in appropriate juxtaposition. Three residues (Ala-83, Gln-86, and Ala-92) in human lysozyme are characteristically mutated to Lys, Asp, and Asp, respectively, in natural Ca$^{2+}$ binding lysozymes and α-lactalbumins. The effects of these mutations on the stability and Ca$^{2+}$ binding properties of human lysozyme were investigated using calorimetry and were interpreted with crystal structures. The double mutant, in which Glu-86 and Ala-92 were replaced with Asp, clearly showed Ca$^{2+}$ binding affinity, whereas neither point mutant showed Ca$^{2+}$ affinity, indicating that both residues are essential. The further mutation of Ala-83→Lys did not affect the Ca$^{2+}$ binding of the double mutant. The point mutations Ala-83→Lys and Glu-86→Asp did not affect the stability, whereas the mutation Ala-92→Asp was about 1.3 kcal/mol less stable. Structural analyses showed that both Asp-86 and Lys-83 were exposed to solvent. Side chains of Asp-86 and Asp-91 were rotated in opposite directions about $\chi_1$ angle, as if to reduce the electrostatic repulsion. The charged amino acids at the Ca$^{2+}$ binding site did not significantly affect stability of the protein, possibly because of the local conformational change of the side chains.

Calcium binding proteins are known to take part in several important functions in biological systems (1). Ca$^{2+}$ binding sometimes accompanies conformational changes, which are considered to be responsible for biological functions such as signal transduction and the formation of macromolecular complexes. Ca$^{2+}$ binding sites usually consist of several acidic residues chelating to bound Ca$^{2+}$ in a pentagonal bipyramidal manner (2). The close locations of acidic residues in a Ca$^{2+}$ binding site should negatively affect protein stability due to charge repulsion between the acidic residues. How does the introduction of charged residues within a calcium binding site affect the stability and Ca$^{2+}$ binding behavior? There are some reports describing the effect of mutations within Ca$^{2+}$ binding sites on the stability of the protein (3, 4). Our approach in this paper is to introduce the minimum perturbation by amino acid replacement and determine the effect of these mutations on the stability and the Ca$^{2+}$ binding function using calorimetry. Furthermore, these effects are interpreted in terms of the structural information from x-ray crystallography. For this purpose, we chose human lysozyme because lysozymes are known to be a suitable model for studying protein function and stability (3, 5–7). More than 90 sequences of chicken type lysozymes and α-lactalbumins are in the sequence data base. Among these lysozymes, several have been found to have Ca$^{2+}$ binding ability (8–11). Three residues (Ala-83, Gln-86, and Ala-92) in human lysozyme are usually mutated to Lys, Asp, and Asp, respectively, in natural Ca$^{2+}$ binding lysozymes and α-lactalbumins as shown in Fig. 1. We have already found that only the double mutation (Gln-86→Asp and Ala-92→Asp) in human lysozyme resulted in the formation of a Ca$^{2+}$ binding site (5). The precise analyses of the stability (3, 5, 6) and the Ca$^{2+}$ affinity (7) using calorimetry have also been performed, and the high resolution structural data of the wild type (12) and Ca$^{2+}$ binding mutant (13) lysozymes from x-ray crystallography are available. Here we show the effect of the subsequent mutations (Gln-86→Asp, Ala-92→Asp, and Ala-83→Lys) on the Ca$^{2+}$ binding properties, conformational stabilities, and tertiary structures of these mutants. It was found that both aspartic acids (Asp-86 and Asp-92) are essential for Ca$^{2+}$ binding. The introduction of an aspartic acid at the Ala-92 position resulted in destabilization of lysozyme, whereas the introduction of an aspartic acid at the Gln-86 position or the introduction of positive charge the Ala-83 position did not affect the stability. The observed instability with the Ala-92→Asp mutation is proposed to result from the difference in the hydration effect.

EXPERIMENTAL PROCEDURES

Materials—Klenow fragment of DNA polymerase I and restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) and Takara Shuzo (Kyoto, Japan). T4 DNA ligase was from New England Biolabs, Inc. (Beverly, MA). Human lysozyme was purchased from Green Cross Corp. (Tokyo, Japan). α-Lactalbumin and Micrococcus lysodeikticus were from Sigma. Glycol chitin was kindly provided by Prof. T. Imoto. Other chemicals were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Enzyme reactions were carried out under the conditions recommended by the suppliers.

Strain and Media—Saccharomyces cerevisiae AH22R (α, leu2, his4, can1, phe80) (14) was used for the host strain and cultivated in modified Burkholder medium (15) supplemented with 5% sucrose.

DNA—Oligonucleotides were synthesized using a model 380A DNA Synthesizer (Applied Biosystems, Foster City, CA) and purified by high pressure liquid chromatography on a TSK gel ODS-120T (Toyobo, Tokyo, Japan).

Oligonucleotide-directed Mutagenesis—Plasmid pERI8811, which contains the cloned yeast glyceroldehyde-3-phosphate dehydrogenase promoter and DNA encoding the chemically synthesized chicken lysozyme signal sequence and mutant human lysozyme sequence (Q86D/A92D), was used as a starting material for mutagenesis (5). To obtain the mutant human lysozymes (As3K86D/A92D, Q86D, and A92D), Ala-83, Asp-86, or Asp-92 in mutant human lysozyme (Q86D/A92D) were replaced by lysine, glutamine, and alanine, respectively. Oligonucleotide directed mutagenesis developed by Zoller and Smith (16) was carried out using 5′-GAGGCGAGACGGCATCGAATGTTGTC-3′ (27-mer) and 5′-GCAATGTGGCTCGAAGCAAGC-3′ (23-mer) for A92D mutant, and 5′-GTAAGCAATTGTCAGAAGA-3′ (21-mer) for Q86D mutant.
**RESULTS**

**Enzymatic Activities and Ca\(^{2+}\) Affinities of the Mutant Lysozymes**—The lytic activities of the mutant lysozymes measured at pH 6.2 and 25 °C are summarized in Table II. In the presence of Ca\(^{2+}\), the lytic activity of all mutant lysozymes is lower than that of the wild type. The lytic activity of the mutant lysozymes having Ca\(^{2+}\) binding affinity (Q86D/A92D and A83K/Q86D/A92D) is approximately 70% of that of the wild type. In the absence of Ca\(^{2+}\) (in the presence of 5 mM EDTA), the lytic activity of all mutant lysozymes is almost the same as that of the wild type. Because the net charge of the protein affects the lytic activity, the enzymatic activity against glycol lysis of *M. lysodeikticus* cells (0.2 mg/ml) according to the procedure of Kikuchi et al. (18). Activities of the lysozymes using glycin chitin as a substrate were measured in 0.1 M acetate buffer (pH 5.5) at 40 °C as described previously (19).

**Measurement of Thermal Stability of the Mutant Human Lysozyme**—Thermal stability of the wild type and mutant human lysozymes were determined by differential scanning calorimetry using a DASM4 microcalorimeter (20, 21) as described previously (3). The scan rate was 1.0 K/min, and the lysozyme concentrations used were 1.5 mg/ml. Sample solutions were prepared by dissolving the lysozymes in 0.05 M sodium acetate buffer (pH 4.5) with and without 10 mM CaCl\(_2\). The pH of the sample solution was confirmed before and after each measurement. Calorimetric (\(\Delta H_{\text{cal}}\)) and van’t Hoff enthalpies (\(\Delta H_{\text{vH}}\)) were calculated using the computer program ORIGIN (MicroCal Inc.).

**Affinities of the Mutant Lysozymes**—The affinity of the Ca\(^{2+}\) to mutant lysozymes was determined by using a MicroCal OMEGA titration calorimeter (23). 2 mg/ml of the protein solution was prepared in 0.05 M sodium acetate buffer (pH 5.5), and the lysozyme concentrations used were 1.5 mg/ml. Sample solutions were prepared by dissolving the lysozymes in 0.05 M sodium acetate buffer (pH 5.5) with and without 10 mM CaCl\(_2\). The pH of the sample solution was confirmed before and after each measurement. Calorimetric (\(\Delta H_{\text{cal}}\)) and van’t Hoff enthalpies (\(\Delta H_{\text{vH}}\)) were calculated using the computer program ORIGIN (MicroCal Inc.).

**Crystallographic data, x-ray processing statistics, and refinement statistics of the mutant human lysozymes**

| Crystal data and x-ray processing statistics | Q86D | A92D | A83K/Q86D/A92D |
|---------------------------------------------|------|------|-----------------|
| Space group                                  | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\) |
| Cell constants (\(\AA\))                     | a    | b    | c               |
| Resolution (\(\AA\))                         | 1.80 | 1.80 | 1.80 |
| Number of measured reflections               | 32762| 32762| 32762 |
| Number of independent reflections            | 10159| 10159| 10159 |
| Remap                                        | 6.88 | 6.88 | 6.88 |
| Final refinement parameters                  |      |      |                 |
| Resolution used                              | 2.00–1.80 | 2.00–1.90 | 2.00–1.80 |
| Number of used reflections                   | 39946| 39946| 39946 |
| R-factor                                     | 0.161| 0.164| 0.172 |

**Fig. 1.** Amino acid sequence of the mutants corresponding to the Ca\(^{2+}\) binding site from natural lysozyme and \(\alpha\)-lactalbumin.
of Ca$^{2+}$, only the $T_d$ of the A83K/Q86D/A92D mutant lysozyme was increased ($T_d = 89.3 \, ^\circ C$), indicating that this mutant has strong Ca$^{2+}$ binding ability. $T_d$ values of the Q86D mutant lysozyme (80.3 $\, ^\circ C$) were not affected by the presence of Ca$^{2+}$, which is the same as that of the wild type lysozyme.

The $\Delta H_{cal}$ values of the A83K/Q86D/A92D, Q86D, and A92D lysozymes in the absence of Ca$^{2+}$ at their denaturation temperature were determined to be 123.1, 135.3, and 134.1 kcal/mol, respectively (Table IV). These values are comparable with the values of the wild type and apo-Q86D/A92D lysozymes at the same temperature. In addition, the $\Delta H_{cal}$ values of the mutants in the presence of 10 mM CaCl$_2$ at their $T_d$ were found to be 145.1, 134.0, and 131.5 kcal/mol, respectively. The ratios of $\Delta H_{cal}/\Delta H_{cal}^{T_d}$ for every mutant was about 0.95, which is also similar to that of the wild type and Q86D/A92D lysozymes (3), indicating two state denaturation (22). The heat capacity changes ($\Delta C_p$) in the denaturation of the mutants were also similar to that of the wild type and Q86D/A92D lysozyme reported previously (3, 5).

**Three-dimensional Structures of the Mutant Human Lysozymes**—Both the Q86D and A92D mutants were crystallized in space group P2$_1$2$_1$2$_1$, the same as that of the wild type, apo-, and holo-Q86D/A92D lysozyme reported previously (13). Three-dimensional structures of the Q86D and A92D mutants were determined at 1.8 Å and 1.9 Å resolution by x-ray crystallography. The structures were refined to R-factor of 16.1 and 19.2%, respectively. The overall structures of Q86D and A92D were quite similar to that of the wild type and apo- and holo-Q86D/A92D lysozyme. The root mean square deviations between the wild type and the Q86D and A92D mutants for the main chain Ca atoms were 0.278 and 0.309 Å, respectively.

The A83K/Q86D/A92D mutant lysozyme was crystallized in space group P2$_1$. The tertiary structure of A83K/Q86D/A92D was determined by the molecular replacement method with the program X-sight (MSI) using the structure of holo-Q86D/A92D lysozyme (13) as the initial search model. Four molecules (chains A–D) were found in the asymmetric unit. The structure of A83K/Q86D/A92D lysozyme was refined to an R-value of 17.2%. Each molecule in the asymmetric unit was similar to that of the wild type (root mean square deviation, $\leq 0.3$ Å) and holo-Q86D/A92D mutant (root mean square deviation, $\leq 0.4$ Å) lysozymes.

The structures in the vicinity of the residues at positions 86, 91, and 92 in Q86D, A92D, and A83K/Q86D/A92D mutant lysozymes are shown in Fig. 2. No Ca$^{2+}$ bound in this region was observed in the Q86D and A92D mutants. In the structure of the Q86D mutant lysozyme, the conformation of the side chain of Asp-86 is rotated around the $\chi$1 angle toward solvent, which is similar to that seen in the structure of apo-Q86D/A92D lysozyme. The water molecules occupied the same position relative to the bound Ca$^{2+}$. The water configuration is quite similar to that of the wild type (Fig. 2a). In the structure of A92D mutant lysozyme, there is no significant conformational change observed in the side chain of this region in comparison with that of the wild type. However, the network of water molecules in the structure of the A92D mutant is quite different from that of the wild type and is rather similar to the structure of apo-Q86D/A92D lysozyme (Fig. 2b). The water molecule seen in the structure of A92D mutant lysozyme is considered to be a sodium ion, because at least five interactions within a 3-Å distance are observed. The changes in the structure of the Q86D and A92D lysozymes are consistent with the results from isothermal titration calorimetry, which show that no Ca$^{2+}$ binds to the Q86D and A92D mutant lysozymes. In the structure of the A83K/Q86D/A92D mutant lysozyme, one Ca$^{2+}$ ion was found at the same position as that of the Q86D/A92D mutant lysozyme. Three side chain oxygens from Asp-86, Asp-91, and Asp-92 chelate to the bound Ca$^{2+}$ as seen in the structure of the Q86D/A92D mutant lysozyme (Fig. 2c). With the other two water molecules, as well as two main chain

### Table II

| Lysozyme          | Lytic activity | Activity against glycol chitin |
|-------------------|----------------|--------------------------------|
|                   | 1 mM CaCl$_2$ | 5 mM EDTA                      | 10 mM CaCl$_2$ |
| Wild type         | 100           | 100                            | 100            |
| Q86D              | 89            | 101                            | 88             |
| A92D              | 79            | 100                            | 75             |
| Q86D/A92D         | 67            | 92                             | 140            |
| A83K/Q86D/A92D    | 67            | 92                             | 120            |

**Table III**

| Lysozymes                  | Number of bound Ca$^{2+}$ | $K_a$ (mM) | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ (kcal/mol K) |
|---------------------------|---------------------------|------------|-----------------------|-----------------------|-------------------------|
| Wild type                 | not detected              |            |                       |                       |                         |
| Q86D                      | not detected              |            |                       |                       |                         |
| A92D                      | not detected              |            |                       |                       |                         |
| Q86D/A92D                 | 1.0                       | $1.9 \times 10^4$ | $-7.3$                | 1.6                   | 8.9                     |
| A83K/Q86D/A92D            | 1.0                       | $3.9 \times 10^4$ | $-7.8$                | 2.2                   | 10.0                    |

**Table IV**

| Lysozyme | [Ca$^{2+}$] | $T_d$ ($^\circ C$) | $\Delta H_{cal}$ (kcal/mol) | $\Delta H_{vH}$ (kcal/mol) | Ratio | $\Delta C_p$ (kcal/mol K) |
|----------|-------------|-------------------|-----------------------------|-----------------------------|-------|---------------------------|
| Wild type | 0.0         | 80.3              | 138.4                       | 146.5                       | 0.95  | 1.71                      |
| Q86D     | 0.0         | 80.3              | 135.3                       | 139.5                       | 0.97  | 1.11                      |
| A92D     | 0.0         | 77.0              | 134.1                       | 141.4                       | 0.95  | 1.30                      |
| Q86D/A92D| 0.0         | 76.5              | 130.6                       | 136.9                       | 0.95  | 1.12                      |
| A83K/Q86D/A92D | 0.0         | 77.0              | 132.1                       | 137.7                       | 0.96  | 1.39                      |
| Wild type | 10.0        | 80.0              | 136.0                       | 144.2                       | 0.94  | 1.02                      |
| Q86D     | 10.0        | 80.3              | 134.0                       | 143.0                       | 0.94  | 1.75                      |
| A92D     | 10.0        | 77.2              | 131.5                       | 142.0                       | 0.93  | 1.15                      |
| Q86D/A92D| 10.0        | 89.2              | 142.2                       | 155.6                       | 0.90  | 0.80                      |
| A83K/Q86D/A92D | 10.0        | 89.3              | 145.1                       | 156.0                       | 0.91  | 1.25                      |

* Previously reported (3).
FIG. 2. Tertiary structure of the loop region corresponding to the Ca$^{2+}$ binding site. a, structural comparison between the wild type and Q86D mutant lysozymes. b, structural comparison between A92D mutant and apo-Q86D/A92D lysozyme. c, structural comparison between Holo A83K/Q86D/A92D and holo-Q86D/A92D mutant lysozymes.

Fig. 3. Side chain conformational responses to the subsequent mutagenesis and the binding of Ca$^{2+}$.
ca2 + Asp-92 and the positively charged Lys-83 are conserved in residues. Three chelating aspartic acids, Asp-86, Asp-91, and sites appear to provide a counter ion charge to the chelating binding sites, such as a Ca2 +

no stabilization was observed in the presence of Ca2 +

of the three mutations, Gln-86 already exists in wild type human lysozyme (Fig. 1), the effect mutants are the same as those of the holo-Q86D/A92D and the indications on the stability is summarized, in which the mutants Q86D/A92D and A83K/Q86D/A92D). This indi-

the presence of 10 mM Ca2 +

mutants are the same as those of the wild type lysozyme (3).

The average thermal factors (B-factor) of the main chain for the wild type, Q86D, and A92D lysozymes are similar to those of the wild type including the carbonyl groups from Lys-83 and Asn-88, a total of seven oxy-

D

lar to that of apo- and holo-Q86D/A92D as reported previously

D

1.2

1.3 kcal/mol less stable than the wild type, which is similar to the apo-Q86D/A92D and apo-A83K/Q86D/A92D lysozymes, indicating that the mutation Ala-83 to Lys did not affect the stability but the mutation Ala-92 to Asp resulted in 1.3 kcal/mol destabilization.

To interpret the stability in terms of the structural information, high resolution structural data were obtained from x-ray crystallography. Although these aspartic side chains are located within 5 Å distance to chelate Ca2 + (holo-Q86D/A92D and holo-A83K/Q86D/A92D lysozymes in Figs. 2c and 3), the introduction of aspartic acid at position 86 resulted in a shift of the side chain conformation toward the outside of the side as if to reduce the charge repulsion to Asp-91 (Q86D lysozyme in Fig. 3). The charged groups of Asp-86, Asp-92, and Lys-83 are found to be different as summarized in Fig. 3. If both aspartic

charges because of the solvent shielding. It has been reported that an ion pair located on the surface of a protein does not always affect the stability of the protein (30). On the other hand, a destabilization was observed for the mutation Ala-92 → Asp. One explanation for this destabilization is the difference in hydration between the Ala and Asp side chains upon unfolding. The tertiary structure of the mutant A92D indicated that the side chain of Asp-92 is almost buried and the side chain conformation was quite similar to those of the apo-and holo-Q86D/A92D lysozyme (Fig. 3). According to Oohatake and Ooi (31, 32), the difference in hydration free energy between alanine and aspartate is calculated to be about 2.5 kcal/mol at 80 °C. Because Asp-92 in the mutant and Ala-92 in the wild type structures are almost buried with the accessible surface areas of these amino acids calculated to be less than 3 Å2, the difference in hydration is considered to be the major part of the instability (1–2 kcal/mol in Table V) observed in the mutants having Asp-92. The smaller than expected effect on stability may be caused by the weak binding of Na+ ion to Asp-92 in the native state as seen in the crystal structures of the A92D and apo-Q86D/A92D lysozyme.

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**DISCUSSION**

Several acidic residues accompanying some basic residues are characterized as associated to make a Ca2 + binding site in Ca2 + binding proteins. Acidic residues are found to directly chelate to Ca2 + in a pentagonal bipyramidal manner (2, 13), and some basic residues located in the vicinity of Ca2 + binding sites appear to provide a counter ion charge to the chelating residues. Three chelating aspartic acids, Asp-86, Asp-91, and Asp-92 and the positively charged Lys-83 are conserved in Ca2 + binding lysozymes and α-lactalbumins. Because Asp-91 already exists in wild type human lysozyme (Fig. 1), the effect of the three mutations, Gln-86 → Asp, Ala-92 → Asp, and Ala-83 → Lys on the stability and affinity of Ca2 + was investigated using calorimetry. In Table V, the effect of the mutations on the stability is summarized, in which ΔΔG at 80 °C was calculated according to Becktel and Shellman (27) by assuming that ΔS and ΔC, values of Ca2 + bound and unbound mutants are the same as those of the holo-Q86D/A92D and the wild type lysozymes, respectively, as reported previously (3). In the presence of 10 mM CaCl2, the mutant A83K/Q86D/A92D showed about 3.6 kcal/mol stabilization, which is similar to that of Q86D/A92D lysozyme. Other mutants, Q86D and A92D lysozymes, did not show any stabilization in the presence of Ca2 +. According to Shellman (28, 29), the existence of ligand binding sites, such as a Ca2 + binding site, should improve the stability of the protein in the presence of Ca2 +. Therefore, the addition of 10 mM CaCl2, which is about 100 times in excess of the protein concentration, should result in stabilization of the protein. In both Q86D and A92D mutant lysozymes, however, no stabilization was observed in the presence of Ca2 + (unlike the mutants Q86D/A92D and A83K/Q86D/A92D). This indi-
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