The Crystal Structure of a Mutant Human Lysozyme C77/95A with Increased Secretion Efficiency in Yeast*

The three-dimensional structure of a mutant human lysozyme, C77/95A, in which residues Cys77 and Cys95 were replaced by alanine, was determined at 1.8-Å resolution by x-ray crystallography. The properties of this mutant protein have been well characterized with respect to its thermal stability and secretion efficiency in a yeast expression system. The overall three-dimensional structure of C77/95A was found to be essentially identical to that of the wild-type human lysozyme, although the coordinates were shifted by more than 0.6 Å and the thermal factors of the main-chain atoms were increased in the vicinity of residue 77. The reduction in thermal stability of this mutant has been previously explained by an increase in entropy of the unfolded state. In addition, a packing defect (cavity) produced by the removal of the disulfide bond was detected in the three-dimensional structure of C77/95A. This cavity can also be a reason why the stability of the protein is reduced because the free energy of the folded state could be expected to increase. The increased secretion efficiency cannot be due mainly to the three-dimensional structure, but may possibly be related to some event in the pathway of protein secretion. One of the possibilities might involve molecular flexibilities in the secondary or tertiary structure for lack of one of the disulfide bonds.

Human lysozyme (EC 3.2.1.17) belongs to a class of lysozymes of the chicken type (c-type). The enzyme is composed of 130 amino acid residues with four disulfide bonds: Cys67-Cys130, Cys90-Cys119, Cys60-Cys85, and Cys77-Cys95. The formation of a disulfide bond in a globular protein is expected to play an important role in the structural stability (1-3) and in the folding of a protein (4). The mechanism of stabilizing protein structure by the introduction of a disulfide bond has been discussed in connection with results obtained from thermodynamic measurements and structural studies (5,6). However, there are few reports concerned with structural changes in a protein molecule caused by the removal of a disulfide bond.

Our previous results, using a mutant C77/95A in which Cys77 and Cys95 were replaced by alanine residues, indicate that C77/95A has almost the same lytic activity against Micrococcus lysodeikticus as the wild-type enzyme and that the disulfide bond of Cys77-Cys95 does not affect the folding of human lysozyme (8). Further, the thermal stability of C77/95A was reduced by 14 °C (9) and secretion of the mutant was increased over eight times that of the wild-type lysozyme in the yeast secretion system (8). Moreover, other mutant human lysozymes, P110 (10, 11), C77A (12), C95A (11), and C81A (13), all of which lack the disulfide bond Cys77-Cys95, have been found to show increased secretion efficiency in yeasts and also in animal cell culture. Among many mutant proteins thus far reported, C77/95A is unique in that it shows increased secretion efficiency without any significant change in enzymatic activity.

These results suggest that some general rules might exist concerning structure on one hand and the secretion efficiency and thermal stability on the other in human lysozymes. Thus, C77/95A was considered an attractive target for studying these relationships. This report presents the three-dimensional structure of C77/95A refined to 1.8-Å resolution and analyzes the consequences of removing a disulfide bond, viz. Cys77-Cys95, on the structure, thermal stability, and secretion efficiency of human lysozyme.

MATERIALS AND METHODS

Crystalization and Data Collection—The mutant C77/95A was produced by the yeast expression system as previously described (7,8). The wild-type human lysozyme was purchased from The Green Cross Corp. (Osaka, Japan). Crystals of C77/95A were grown from a solution containing 20 mg/ml protein, 2.5 mM NaCl as a precipitant and 30 mM sodium phosphate buffer at pH 6.0 in a chamber controlled at 13.0 °C. The typical size of a rod-shaped crystal was 0.4 × 0.4 × 0.8 mm³. The crystal data were almost the same as those of the wild-type human lysozyme (Table I). Diffraction data were collected at 90 °C up to 1.76-Å resolution by an automated oscillation camera system (DIP-100) equipped with an "Imaging Plate" (14,15) on a rotating anode generator (M18X) operated at 50 kV, 90 mA with CuKα radiation. The exposure time/frame was 33.3 min. The crystals were oscillated by 2° for each frame around the a* and b* axes. For each crystal, intensity data could be collected for a total of 50 frames at 1.76-Å resolution before significant crystal damage occurred by x-ray radiation. Diffraction intensities were evaluated with the program system ELMIS (16) on a SUN-3 workstation and were processed using the program package PROTEIN (17) in a VAX-8810 system.

Structure Refinement of C77/95A Lysozyme—In a previous crystallographic study of the wild-type human lysozyme, the three-dimensional structure was refined at 1.5-Å resolution (18). But only coordinates of the protein molecule, except solvent molecules, were described in the Protein Data Bank. In order to determine the parameters of the solvent molecules, the crystal structure of the wild-type enzyme was independently refined at 1.76-Å resolution. The coordinates of the wild-type human lysozyme were subjected to rigid group refinement with the program TRAREF (19). After several cycles of the refinements, (2F, F·) and (F, F·) maps were calculated with the program PROTEIN. Cys77 and Cys95 of the model were replaced by alanine and fitted to the respective electron densities on an Evans & Sutherland PS-300 graphics system by the program FRODO (20). The solvent molecules were selected from peaks which appeared in both electron density maps and made reasonable hydrogen bonds with the protein atoms or other solvent molecules. All solvent molecules were fixed to their occupancies of 1.0 and those with high temperature factors of more than 50 Å² were omitted from the model in the final stage of the refinement. The models were refined by the program package PROTSF/PRIOLSQ (21) in a vector computer, FACOM VP4000.

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RESULTS

Structure Refinement and Final Model of C77/95A—The refined model of the wild-type lysozyme included 107 water molecules and the crystallographic R factor was 0.167. The diffraction data of C77/95A were 96.1% complete to 1.76-A resolution. The refined model finally consisted of the protein and 101 water molecules. An (Fo-Fc) map calculated after the final cycle of refinement was very clean with no peaks exceeding 0.3e^2. The refinement parameters and the weighting parameters used in the refinements are listed in Tables II and III, respectively. The coordinate error was estimated to be about 0.15 A, based on the statistics of Luzzati (17).

Comparison of the Three-dimensional Structures of the Wild-type and Mutant Human Lysozymes—The location of the mutation sites in the wild-type human lysozyme is shown in Fig. 1. Before calculating relative coordinate shifts of corresponding atoms in C77/95A and the wild-type lysozymes, a transformation matrix was applied to the C77/95A coordinates, in order to eliminate the effects of rigid body rotation and translation of the C77/95A molecule. Fig. 2, a and c, show the shifts in atomic coordinates of main-chain atoms, N, Cα, C, and O, of the C77/95A from those of the wild-type enzyme. The root-mean-square deviation was 0.10 A between the two, when those of the residues from Thr20 to Val99 were not included in the statistics. It was 0.25 A for all atoms including the side-chain atoms and corresponding molecules around the protein. Significant shifts for main-chain atoms were found around both of the mutation sites, where the Cα atoms of the residues 77 and 95 moved by 0.55 and 0.44 A, respectively. That of Gly82 also moved by 0.47 A.

The overall averaged B-factor of the main-chain atoms was 14.7 A^2. Fig. 2b shows the B-factors of main-chain atoms in each residue and the differences between those of the wild-type lysozyme and C77/95A, where the crystallographic B values of individual atoms were averaged to reduce local fluctuations. The maximum difference was 13.0 A^2 at Ala76. The B-factors from Ala72 to His86 were significantly increased in C77/95A, but the absolute values were less than 25.0 A^2.

The electron densities for main- and side-chain atoms in this region were very clearly distinguishable in C77/95A. The B-factors of the main-chain atoms around the other mutation site of the residue 95 were almost the same as those of the wild-type human lysozyme. There were no significant changes in the B-factors in other regions of C77/95A. As with the positions of the protein atoms, those of the corresponding solvent molecules bound to the protein molecule were also essentially identical in the structures of C77/95A and the wild-type human lysozyme.

DISCUSSION

Structure of Mutant Human Lysozyme C77/95A—Residue 77, which is one of the mutation sites, is located in a large loop which consists of residues Cys46 to Cys81 (Fig. 1). This loop does not contain any typical secondary structure such as an α-helix or a β-strand. The residues in the loop can be divided into two regions based on the structural environment: regions Cys46 to Pro51 and Gly72 to Cys81. The main- and side-chain atoms in the former have several interactions with other regions of the protein molecule and three internal solvent molecules. These nonbonded interactions are conserved in the structure of C77/95A as in the case of the wild-type enzyme. On the other hand, the main-chain is exposed to solvent in the latter region and the coordinate shifts could be expected to be large as a result of the removal of the disulfide bond because the main-chain has few interactions with either other parts of the protein molecule or neighboring protein molecules in the crystal. Their B-factors were also affected by the mutation as were the positions of the atoms in the region. Average B-factors of main-chain atoms were 12.5 and 14.2 A^2 in the structures of the wild-type lysozyme and C77/95A, respectively. They were 15.4 and 25.3 A^2 in the region of Gly72 to His86 in the wild-type lysozyme and C77/95A, respectively. The B-factors in the region of Gly72 to His86 definitely increased in the structure of C77/95A (Fig. 2b). The significant atomic shifts and increased B-factors of main-chain atoms suggest an increase in the flexibility of the main-chain from Gly72 to His86 in C77/95A.

While residue Cys77 is located in the loop, residue Cys96 at the other mutation site is in a middle of a long α-helix,
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consisting of residues Ala90 to Val128, in the center of the lysozyme molecule (Fig. 1). Both termini of this α-helix interact with other regions of the protein by various hydrogen bonds. In the middle of this helix, the main- and side-chain atoms of Val95 to Lys97 interact with other regions of the protein only by hydrophobic interactions. Significant atomic shifts of the main-chain atoms could be detected by about 0.4 Å only around position 95 (Fig. 2a) and the B-factors of the Cα atom of residue 95 was 9.6 and 11.0 Å² in the wild-type lysozyme and C77/95A, respectively (Fig. 2b). Therefore, this α-helix is slightly distorted at position 95 by the disulfide bond Cys97-Cys106 in the structure of the wild-type lysozyme.

The human lysozyme molecule consists of two domains. Since the disulfide bond Cys77-Cys95 joins these domains and is formed at the final stage of folding (11), it was expected that the overall structure of C77/95A would be affected by the removal of this disulfide bond. As described above, some small but significant differences were found between the structures of the wild-type lysozyme and C77/95A. It is therefore possible to conclude that the catalytic active site residues of human lysozyme (23), were 0.08 and 0.04 Å, respectively, and those of Trp44, Asp102, and Ala106, which have interactions with the substrate molecules in the active site cleft, were also less than 0.1 Å (Fig. 2a). Therefore, we should not expect significant structural changes in the active site and the substrate-binding region of C77/95A. These results are in good agreement with the observations that C77/95A has almost the same relative activity against M. lysodeikticus as the wild-type enzyme (8).

Relation between Structure of C77/95A and Increased Secretion—It is surprising that structural changes caused by removal of the disulfide bond Cys77-Cys106 were not so large in C77/95A and were localized only around the mutation sites. Our previous results using the yeast secretion system indicate that mutants lacking one of the disulfide bonds, Cys97-Cys128, Cys93-Cys116, or Cys95-Cys116, do not secrete efficiently (8), but all mutants of human lysozyme, C77/95A (8), P110 (10, 11), C77A (12), C95A (11), and C81A (13), in which the disulfide bond of Cys77-Cys95 is cleaved, do secrete with increased efficiency. The three-dimensional structures of P110 and C77A have been determined and refined at 1.8-Å resolution. Two proteins (A, B, C, and D) were purified from the mutant, P110 (Val110→Pro) (10). X-ray structure analysis revealed that the Cys77-Cys95 bond in B (designated P110-B) was cleaved. The B-factors of the main-chain atoms from Gly77 to His95 also increased significantly in P110-B and C77A. Moreover, it has been suggested that protease digestion of proteins proceeds via their unfolded states (24), and stability against protease digestion is closely related to the unfolding rate constant (25). It has been suggested that the positions of the amino acid residues attacked by protease correspond well with the degrees of positional fluctuation (26). Since the mutants, C77/95A, C77A, C95A, and C81A were less stable against trypsin digestion in solution than the wild-type lysozyme (data not shown), it is conceivable that the unfolding rate constant of the mutant is increased. However, secretion efficiency cannot

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readily be attributed to the three-dimensional structures of C77/95A in the folded state, since the overall structures were not so changed. Nevertheless, the possibility cannot be ignored because removal of the disulfide bond increased the flexibility of the molecule, as shown in the crystal structures and also in solution. Such flexibility, caused by some changes in the degree of freedom of the tertiary structure of C77/95A, could increase secretion efficiency by affecting some step in the folding pathway in vivo or the transport of the protein through the membrane. Based on the present evidence, it is apparent that no conclusive answer can be given for secretion efficiency of C77/95A at this time.

Relation between Structural Changes and Protein Stability—The thermal stability of a protein depends on the difference in free energy between its folded and unfolded states and the decrease in the free energy of the unfolded state can be one of the reasons for destabilizing the protein (27). The disruption of a disulfide cross-link decreases the free energy of its unfolded state because of the increase in entropy (6). The removal of the disulfide bond Cys^77-Cys^95 reduced the thermal stability of the molecule because the unfolding temperature of C77/95A decreased by about 14.0 °C, compared with that of the wild-type lysozyme. The reduction of the thermal stability could be well explained by an increase in entropy of the unfolded state (9). In addition to the results from these thermal experiments, some changes were found in
the folded structure of C77/95A. In the structure of the wild-type lysozyme, S' atoms of Cys77 and Cys95 are surrounded by many hydrophobic residues, Phe57, Ile59, Trp60, Leu79, Leu84, Val96, and Trp169, forming a large hydrophobic core. Notably, the S' atom of Cys77 has van der Waal's contacts with the C3 atom of Trp64 and the C4 atom of Lys88, while the S' atom of Cys95 has contacts with the C3 atom of Ile99 and the C4 atom of Leu104 at a distance of about 4.0 Å (Fig. 3). Thus, the S' atoms of Cys77 and Cys95 and side-chain atoms of residues in this hydrophobic core were closely packed together without any extra space. However, a small evacuated space was created by the removal of the two S' atoms of residues 77 and 95. The side chain atoms of residues Ile99, Trp104, and others have van der Waal's contacts with S' atoms in the wild-type lysozyme. The relation between hydrophobic interactions and stability of proteins has been examined in other biochemical (28) and structural studies (29, 30).

In summary, structural analysis of C77/95A shows that removal of the disulfide bond Cys77-Cys95 in human lysozyme does not affect the overall structure of the molecule, but affects the flexibility of the local loop structure around one of the mutation sites at position 77. However, changes in the flexibility of the tertiary structure of the molecule might affect some process in the folding of the protein and its translocation across the cell membrane. Moreover, the cavity created by this mutation affects the stability of the protein. Thus, data obtained in this study support the concept that introduction of a disulfide bond tends to stabilize the structure of a protein molecule.

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