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Structure of a Glutathionylated Human Lysozyme: a Folding Intermediate Mimic in the Formation of a Disulfide Bond

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Abstract

The three-dimensional structure of a mutant human lysozyme, C77A-a, in which the residue Cys77 is replaced by alanine, has been refined to an R value of 0.125 using 8230 reflections in the resolution range 10.0–1.8 Å. It has been shown that C77A-a, in which the counterpart of Cys77 (Cys95) is modified with glutathione, has been shown to mimic an intermediate in the formation of the disulfide bond Cys77–Cys95 during the folding of human lysozyme [Hayano, Inaka, Otsu, Taniyama, Miki, Matsushima & Kikuchi (1993). *FEBS Lett.* **328**, 203–208]. An earlier structure demonstrates that its overall structure is essentially identical to that of the wild-type protein and served as the starting model. The refined model includes atoms for all protein residues (1–130), 20 glutathione atoms and 113 water atoms. Further refinement shows more clearly the details of the protein, the bound glutathione molecule and solvent structure. However, the main-chain folding and the atomic thermal factors of the loop region from Thr70 to Leu79 were highly affected by the binding of the glutathione molecule, as compared with those of the wild-type protein. The bound glutathione shifted the main-chain atoms from Val74 to Ala77 by more than 6.0 Å, and the temperature factors of the atoms in the loop region were quite high (more than 40 Å^2), indicating that the backbone conformation of this region is highly flexible and that the loop region is not folded in the specific conformation observed in the wild-type protein. These results strongly suggest that the loop structure in human lysozyme is folded later than the other regions of the protein *in vivo*, as observed in *in vitro* folding. Since the bound glutathione is efficiently and irreversibly dissociated by protein disulfide isomerase, the glutathione molecule may act as a protecting group to prevent the formation of an incorrect disulfide bond in the protein folding process *in vivo*.

Introduction

Understanding the mechanism of protein folding is important to expand our knowledge of the structure–function relationships of proteins. Investigations of folding intermediates have been carried out using far- and near-UV, circular dichroism (Kuwajima, Hiraoka, Ikeuchi & Sugai, 1985; Ikeuchi, Kuwajima, Mitani & Sugai, 1986), and NMR techniques (Radford, Dobson & Evans, 1992) to understand how a globular protein folds *in vitro*. These results suggest that the folding of a globular protein involves partially structured intermediates and multiple pathways *in vitro*. On the other hand, little is known about the mechanism of protein folding and the structures of folding intermediates *in vivo*.

We have focused our studies on the isolation and the analysis of mutant human lysozymes, which mimic folding intermediates. Our strategy is to inhibit the formation of native disulfide bonds by the replacement of a cysteine residue by alanine (Taniyama, Yamamoto, Kuroki, & Kikuchi, 1990; Taniyama, Seko & Kikuchi, 1990) or by the introduction of a non-native disulfide bond (Taniyama, Kuroki, Omura, Seko & Kikuchi, 1991; Kanaya & Kikuchi, 1992). Human lysozyme is a model protein with four disulfide bonds: Cys6—Cys128, Cys30—Cys116, Cys65—Cys81 and Cys77—Cys95. It has been suggested previously that the formation of the Cys77—Cys95 bond occurs at the final step in the

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folding of human lysozyme *in vivo* (Kikuchi, Taniyama, Kanaya, Takao & Shimonishi, 1990). In addition, the mutant human lysozyme C77A, in which Cys77 is replaced by alanine, was secreted from yeast in two distinct forms, C77A-a and C77A-b, with different specific activities (Taniyama, Seko *et al.*, 1990). The former has a Cys95 modified with a glutathione and the latter has a free thiol at Cys95. The mixed disulfide with glutathione at Cys95 was suggested to be formed not after folding, but during folding (Taniyama, Seko *et al.*, 1990). A preliminary structural analysis of C77A-a showed that a glutathione molecule is rigidly bound to Cys95 by a disulfide bond and several hydrogen-bonding interactions (Hayano *et al.*, 1993). The disulfide bond between Cys95 and the glutathione molecule is not accessible to solvent, but the C77A-a form was converted specifically to the C77A-b form, which has the essentially identical three-dimensional structure compared with that of the wild-type enzyme (Kuroki *et al.*, 1992), by protein disulfide isomerase (PDI) (Hayano *et al.*, 1993). From these results, the C77A-a protein was predicted to possibly mimic the intermediate just prior to the formation of the Cys77—Cys95 bond (Hayano *et al.*, 1993). A mutant human lysozyme, AC83/91, also yielded two proteins, termed the a and b forms (Taniyama *et al.*, 1991). The AC83/91-a protein has Cys83 and Cys91 both modified with glutathione, and the AC83/91-b protein has the non-native disulfide bond Cys83—Cys91. This result also suggests that the a form is an intermediate of the b form *in vivo*, as in the case of bovine pancreatic trypsin inhibitor (BPTI) *in vitro* (Staley & Kim, 1990).

Based on these observations, the C77A-a protein is possibly the first *in vivo* folding intermediate isolated thus far, and is an attractive target to provide insight into the structure of a folding intermediate and the protein-folding process *in vivo*. To investigate the details of protein structure modified by the glutathione, we carried out the further refinement of the C77A-a protein. Here, we report the highly refined structure of the C77A-a protein and the structural changes induced by the dissociation of the glutathione by PDI. These observations demonstrate a mechanism of protein folding that is characterized by structural changes associated with disulfide-bond formation at the last step of the folding process *in vivo*.

Experimental

The mutant lysozyme C77A-a was prepared as previously reported (Taniyama, Seko *et al.*, 1990). The protein was crystallized using a modification of the published method and a seeding technique (Inaka, Taniyama, Kikuchi, Morikawa & Matsushima, 1991; Inaka, Kuroki, Kikuchi & Matsushima, 1991). Crystals of C77A-a were grown from a solution containing 20 mg ml^{-1} protein, 2.5 *M* NaCl as a percipitant, and 30 m*M* sodium phosphate buffer at pH 6.0 in a temperature-controlled chamber at 293 K. A preliminary structural analysis of the mutant has been already reported (Hayano *et al.*, 1993). Diffraction data were collected at 282 K up to 1.76 Å resolution by an automated oscillation camera system (DIP-100) equipped with an imaging plate on a rotating-anode generator (M18X) operated at 50 kV, 90 mA with Cu $K\alpha$ radiation. The structure of C77A-a was determined by a molecular-replacement method, using the atomic coordinates of the wild-type human lysozyme as a starting model, with the programs *PROTEIN* (Steigemann, 1974) and *TRAREF* (Huber & Schneider, 1985). Further refinement of the mutant was carried out using the stereochemically restrained least-squares refinement method, as implemented in the program package *PROTIN/PROLSQ* (Hendrickson, 1985) at 1.80 Å resolution. The model of the glutathione molecule was constructed from the crystal structure of γ-L-glutamyl-L-cysteinyl-L-glycine at 120 K (Gorbitz, 1987). The program *CONEXN* (Pähler & Hendrickson, 1990) has been used to incorporate the stereochemical restraints information for the glutathione group into the dictionary of ideal groups used by the program *PROTIN*, which prepares the input files for the refinement program *PROLSQ*. After several cycles of the refinements, $2F_o - F_c$ and $F_o - F_c$ maps were calculated with the program *PROTEIN*. The model modification was carried out on an Evans and Sutherland PS390 graphics system using the program *FRODO* (Jones, 1978). The solvent molecules were selected from peaks which appeared in both electron-density maps and made reasonable hydrogen bonds with the protein atoms and other solvent molecules. Since an experiment using fast atom bombardment mass spectrometry showed that Cys95 is linked to one glutathione molecule in C77A-a (Taniyama, Seko *et al.*, 1990), the occupancy of glutathione atoms was fixed at 1.0. Other solvent molecules were also fixed to their occupancies of 1.0, and those with temperature factors (B factors) of more than 50 Å^2 were omitted from the model in the final stage of the refinements.

Results and discussion

The completeness of the diffraction data of the C77A-a crystal was 83.9% at 1.80 Å resolution. The crystal data and the data-processing statistics are shown in Table 1. Even at the initial stage of the structural refinement of the C77A-a protein, the electron density of the bound glutathione molecule was easily identified in the $2F_o - F_c$ and $F_o - F_c$ maps (Fig. 1*c*). However, electron densities for the region from Val74 to Ala77 could not be found in the $F_o - F_c$ maps, even at the final stage of the refinement, which will be discussed later. The refined model finally consisted of the protein, the glutathione, and 113 water molecules, and the crystallographic R value was 0.125 for 8230 independent reflections

Table 1. *Crystal data and X-ray data-processing statistics*

	Wild-type	C77A-a
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell constants (Å)		
a	56.50	57.19
b	60.89	60.96
c	33.83	33.22
Resolution (Å)	∞–1.76	∞–1.76
No. of measured reflections	43577	30257
No. of independent reflections	11487	9501
Averaged R_{symm} (%)*	2.80	3.79
R_{merge} (%)†	4.98	6.67

* R_{symm} provides the agreement between symmetry-related intensities in the same film.

† $R_{merge} = \sum |I - \langle I \rangle| / \sum |\langle I \rangle| \times 100\%$.

between 10.0 and 1.8 Å. An $F_o - F_c$ map calculated after the final cycle of the refinement was very clean with no peaks exceeding 0.3 eÅ^{-3}. The refinement parameters are listed in Tables 2 and 3. The coordinate error was estimated to be about 0.15 Å, based on the statistics of Luzzati (Luzzati, 1952). The coordinates and individual *B* factors of the present molecules have been deposited in the Protein Data Bank* (Bernstein *et al.*, 1977).

* Atomic coordinates have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1HNL). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: AS0675).

The crystal structure of glutathione has been already determined and refined at 120 K by the X-ray diffraction method (Gorbitz, 1987), and the atomic model was used as an initial model for the bound glutathione in the C77A-a protein. Little is known about the tertiary structure of a glutathione molecule interacting with a protein. Figs. 1(*a*) and 1(*b*) show the three-dimensional structures of the glutathione molecules observed in a single crystal and in C77A-a, respectively. The backbone conformations as well as the side-chain conformations were quite different between the two glutathione molecules. It is not surprising that the conformation of the glutathione is affected by binding to the protein, since the glutathione molecule, which is a tripeptide, γ-L-glutamyl-L-cysteinyl-L-glycine, may have high conformational flexibility. The bound glutathione molecule formed a typical disulfide bond between its cysteine residue and Cys95 of the protein, which has the same conformation as the disulfide bond Cys77—Cys95 observed in the wild-type human lysozyme. There were six hydrogen-bonding interactions; three were direct interactions with the protein molecules and others were water-mediated interactions. Moreover, there were many van der Waals contacts between the glutathione and the protein. The averaged *B* factor of the glutathione atoms was 26.4 Å^2, which is comparable to that of the side-chain atoms in the protein molecule (19.0 Å^2). Thus, the glutathione is bound to the protein rigidly. The locations

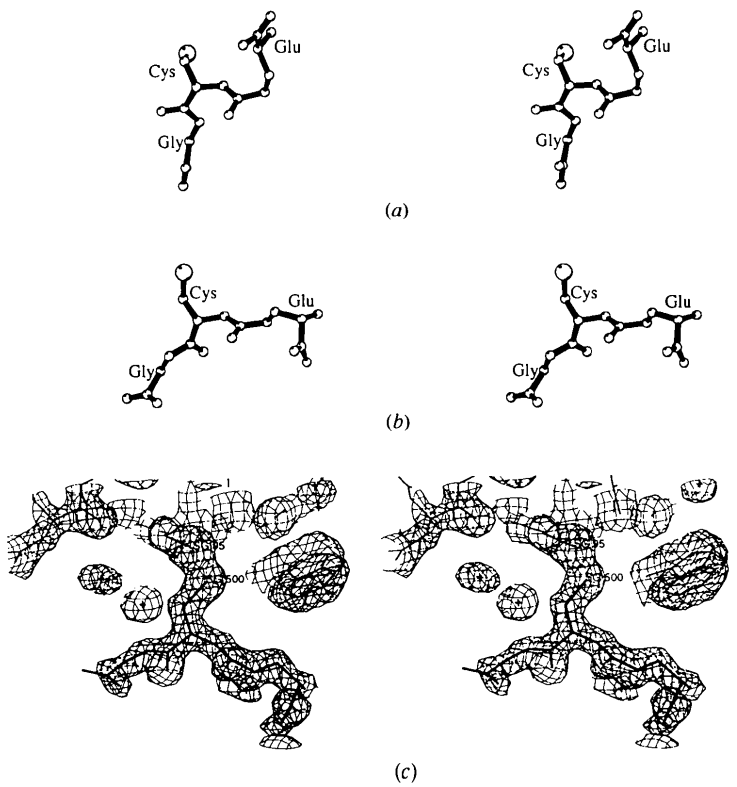


Fig. 1. Stereo drawings of the atomic structure of glutathione molecules. (*a*) Observed in the crystal of γ-L-glutamyl-L-cysteinyl-L-glycine at 120 K (Gorbitz, 1987) and (*b*) in the C77A-a crystal. (*c*) The final $2F_o - F_c$ electron density around the bound glutathione molecule.

Table 2. *Final refinement parameters*

No. of atoms	1161
No. of solvent atoms	113
No. of glutathione atoms	20
No. of parameters	4565
Resolution range (Å)	10.0–1.80
No. of reflections used	8254
R factor (final)*	0.125

* R factor $= \sum ||F_o| - |F_c|| / \sum |F_o|$.

Table 3. *Refinement statistics of the* 1.8 Å *structure of the C77A-a lysozyme*

Restraints	Root-mean square deviation*	Target σ†	No. of parameters
Distance (Å)			
Bond	0.017	0.020	1070
Angle	0.040	0.040	1447
Planar	0.050	0.050	376
Planar groups (Å)	0.014	0.020	950
Chiral groups (Å^3)	0.159	0.150	148
Non-bonded contacts (Å)			
Single torsion	0.167	0.3	359
Multiple torsion	0.182	0.3	259
Possible hydrogen bond	0.221	0.3	96
Torsion angles (°)			
Peptide plane (ω in °)	2.6	3.0	143
Staggered (±60° or 180°)	17.6	15.0	185
Orthonormal (±90°)	20.2	20.0	14
Isotropic *B* factors (Å^2)			
Main-chain bond	1.425	1.5	549
Main-chain angle	2.313	2.0	691
Side-chain bond	2.283	2.0	521
Side-chain angle	3.552	2.5	757

* R.m.s. deviation from ideality.
† The weight for each restraint was $1/\sigma^2$.

of the mutation site and the bound glutathione molecule are shown in Fig. 2. A comparison of the three-dimensional structures of the wild-type and the C77A-a lysozymes shows that several effects on the protein structure occurred due to the modification of Cys95 with the glutathione molecule. The overall structures of the C77A-a and the wild-type lysozymes were essentially identical to each other, since the root-mean-square deviation for all of the main-chain atoms between these proteins was 1.0 Å. However, the deviation was reduced to only 0.17 Å when the main-chain atoms of the residues from Thr70 to Leu79 were excluded in the statistics. Figs. 3(*a*) and 3(*c*) show the shifts in the atomic coordinates of the main-chain atoms of the C77A-a mutant from those of the wild-type protein. Significant shifts were found around the mutation site, where the C_α atoms of the residues from Val74 to Ala77 move more than 6.0 Å. The carbonyl O atom of Arg62 also moves by 0.96 Å. Thus, the bound glutathione affects the local structure of the loop region from Thr70 to Leu79. These stuctural changes in the loop region are not observed in the other mutant human lysozymes, C77/95A (Cys77 and Cys95 are replaced by alanine) (Inaka, Taniyama *et al.*, 1991) and C77A-b (Kuroki *et al.*, 1992), which lack the disulfide bonds of Cys77—Cys95. Structural analyses of C77/95A and C77A-b indicate that not only the backbone structures, but also the side-chain conformations are identical to those of the wild-type protein. These results demonstrate that the structural changes observed in the C77A-a mutant are not caused by the disruption of the disulfide bond Cys77—Cys95, but by the binding of the glutathione molecule to Cys95.

Fig. 3(*b*) shows the *B* factors of the main-chain atoms in each residue and the differences between those of the wild-type and the C77A-a lysozymes, where those of the individual atoms were averaged to reduce local fluctuations. The average *B* factor for the main-chain atoms of the C77A-a lysozyme (12.7 Å^2) was comparable to that of the wild-type lysozyme (10.7 Å^2) when the *B* factors of the residues from Thr70 to Leu79 were not included in the statistics. It can be concluded that the mutation at position 77 and the modification of Cys95 with the glutathione molecule do not affect the overall thermal factors of the protein molecule. However, large differences in the *B* values were observed at the loop region, specifically from Ala73 to Ala77, in C77A-a, where these atomic *B* factors were more than 50 Å^2. Even at the final stage of the structural refinement of C77A-a, the electron densities for Val74, Asn75, Ala76 and Ala77 could not be found clearly in the $2F_o - F_c$ maps. These results show that the loop region is highly flexible and is not folded in the specific conformation that is observed in the wild-type protein. On the other hand, the other regions are folded in the same conformation as that of the wild-type protein.

As described in the *Introduction*, the C77A-a mutant drew our attention because the specific attachment of glutathione at Cys95 may mimic an intermediate just prior to the formation of the native disulfide bond Cys77—Cys95 in the folding process of human lysozyme.

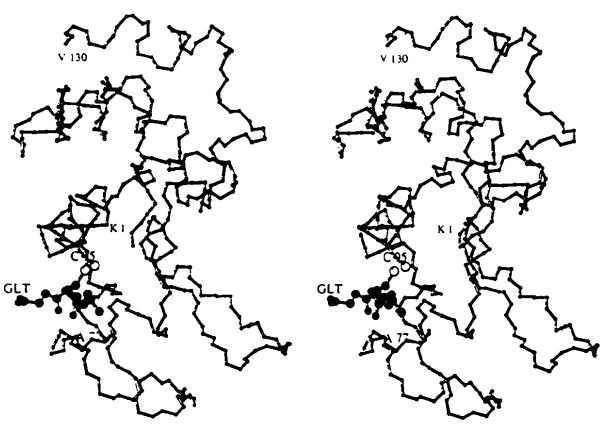
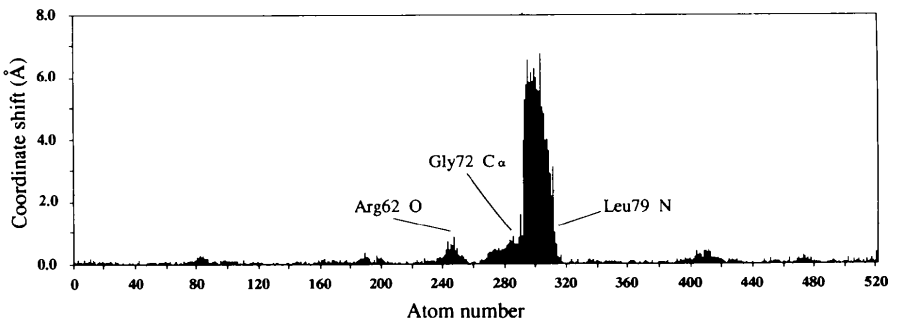
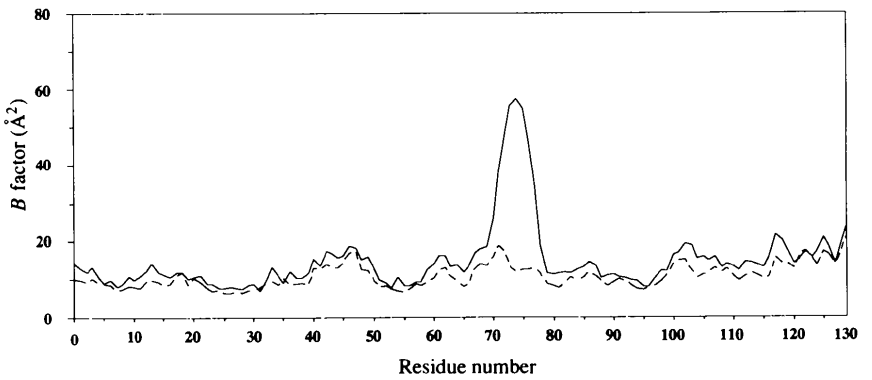


Fig. 2. Stereo drawing of the backbone of the refined structure of the C77A-a mutant. The C_α atoms of Lys1, Ala77, Cys95 and Val130 are labeled. The atoms of the bound glutathione molecule are indicated by black spheres and the glutathione is labeled as GLT.

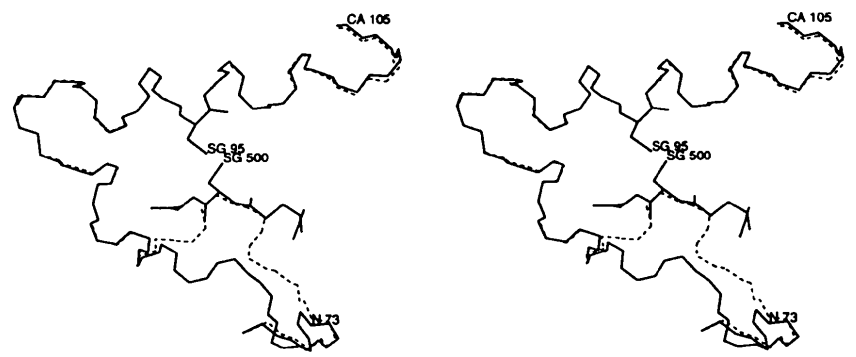
In light of the glutathionylation seen in mutant proteins, such as C77A-a and AC83/91-a (Taniyama *et al.*, 1991), and the oxidative environment maintained by glutathione disulfide in the endoplasmic reticulum (ER) (Hwang, Sinskey & Lodish, 1992), glutathionylation is considered to be the important event occurring during the synthesis and folding of nascent polypeptides. If this is the case, the structural analysis of the C77A-a mutant demonstrates the first three-dimensional structure of an intermediate in the folding process. The folding process of a protein *in vitro* has been reported through studies of disulfide formation in oxidative refolding (Creighton, 1978), protection from hydrogen exchange (Schmid & Baldwin, 1979), effects of amino-acid substitutions (Matouschek, Kellis, Serrano, Bycroft & Fersht, 1990), partially folded states (Baum, Dobson, Evans & Hanley, 1989), and polypeptide analogues of folding intermediates (Staley & Kim, 1990). Recently, it has been reported that the folding of hen egg-white lysozyme *in vitro* involves partially structured intermediates and multiple pathways (Radford, Dobson & Evans, 1992), and that the α-helical domain folds faster that the β-sheet domain. The loop region from Thr70 to Leu79 of the C77A-a mutant, which showed high flexibility and was not folded, belongs to the β-sheet domain in human lysozyme (Fig. 4). Further, the disulfide bond Cys77—



(*a*)

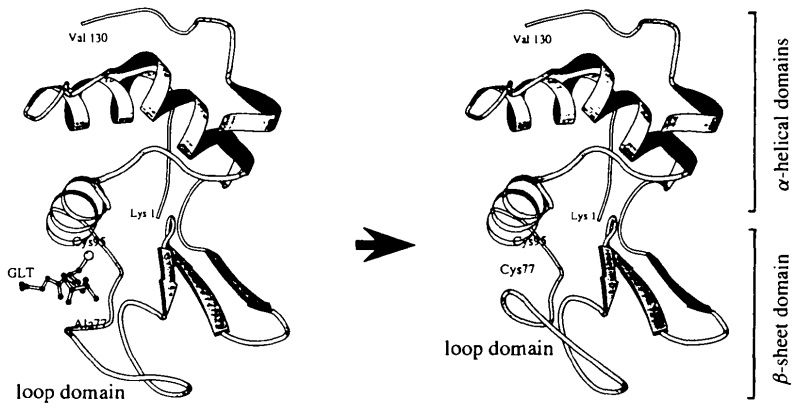


(*b*)



(*c*)

Fig. 3. Structural changes between the wild-type and the C77A-a lysozymes. (*a*) 'Shift plot' shows the differences in the coordinates of the main-chain atoms (N, C_α, C and O) common to the refined structures of the C77A-a and the wild-type human lysozymes. (*b*) The solid line shows the thermal factors averaged for the main-chain atoms of C77A-a, and the broken line shows those of the wild-type human lysozyme. (*c*) Stereo drawings of the backbones of the C77A-a (thick line) and wild-type (broken line) human lysozymes from Thr70 to Gly105. The N atom at 73, the C_α atom at 105 and Sγ atoms at 95 and the glutathione molecule are labeled.



C77A-a
Glutathionylated folding intermediate

Wild-type human lysozyme

Fig. 4. Schematic drawing of structural difference between the C77A-a and the wild-type human lysozymes. The diagram was produced usng the program *MOLSCRIPT* (Kraulis, 1991).

Cys95 links the α-helical and β-sheet domains. It has already been suggested that the disulfide bond Cys77—Cys95 is formed at the final step in the folding of human lysozyme *in vivo* (Kikuchi *et al.*, 1990). Thus, the structural differences between the C77A-a and the wild-type human lysozymes provide some information about the structural changes that occur between an intermediate at the last step of the folding process and the completely folded protein. Although the β-sheet domain in the C77A-a mutant is not folded to the extent observed in the structure of the wild-type protein, the α-helical domain is completely folded. Structural studies of the C77A-a protein clearly demonstrated that the β-sheet domain folds later than the α-helical domain *in vivo*, as was observed *in vitro*. This result is also supported by the mutational studies of human lysozyme (Kanaya, Ishihara, Tsunasawa, Nokihara & Kikuchi, 1993). Since the β-sheet structure is constructed completely in the C77A-a mutant, it is concluded that the loop region from Thr70 to Leu79 (the loop domain) folds at the last step of the folding process *in vivo*. Therefore, if there were a cysteine residue at position 77 in C77A-a, PDI would dissociate the glutathione molecule from C77A-a to form the disulfide bond Cys77—Cys95, as seen in the wild-type protein (Fig. 4). Since the bound glutathione is dissociated by PDI irreversibly, the glutathione molecule bound to Cys95 in C77A-a may act as a protective group to prevent the formation of an incorrect disulfide bond during the protein folding.

Other interesting structural changes were observed at Tyr63, as compared with the wild-type protein. The side-chain conformation of Tyr63 also changed, due to the binding of the glutathione molecule (Fig. 5). The side-chain conformation as well as the atomic coordinates of the main-chain atoms of Tyr63 shifted by more than 0.8 Å. Since it is well known that Tyr63 interacts with the substrate molecules in the active-site cleft (Malcolm *et al.*, 1989), these results are in good agreement with the observation that the enzymatic activity of the C77A-a mutant against *Micrococcus lysodeikticus* is reduced to 26%, as compared with that of the wild-type human lysozyme (Taniyama, Seko *et al.*, 1990). The lower enzymatic activity of the C77A-a mutant is also considered to be evidence that the folding of C77A-a is not complete.

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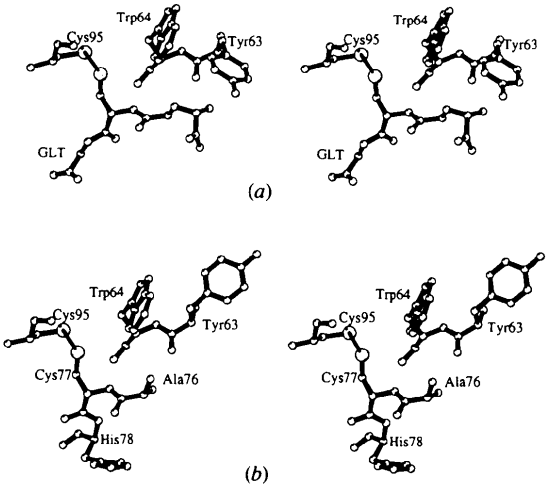

Fig. 5. Changes in the side-chain conformations of residue 63. (*a*) The atomic structure around the glutathione molecule in the C77A-a mutant. Tyr63, Trp64 and Cys95 are labeled. The glutathione molecule is labeled as GLT. S atoms of the disulfide bond Cys77–Cys95 are shown as shadowed circles. (*b*) The atomic structure around the disulfide bond Cys77–Cys95 in the wild-type human lysozyme. Residues 63, 64, 76, 77, 78 and 95 are labeled.

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