Structure of a stabilizing disulfide bridge mutant that closes the active-site cleft of T4 lysozyme

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(RECEIVED May 31, 1991; ACCEPTED August 20, 1991)

Abstract

The engineered disulfide bridge between residues 21 and 142 of phage T4 lysozyme spans the active-site cleft and can be used as a switch to control the activity of the enzyme (Matsumura, M. & Matthews, B.W., 1989, *Science* 243, 792–794). In the oxidized form the disulfide increases the melting temperature of the protein by 11 °C at pH 2.

The crystal structure of this mutant lysozyme has been determined in both the reduced and oxidized forms. In the reduced form, the crystal structure of the mutant is shown to be extremely similar to that of wild type. In the oxidized form, however, the formation of the disulfide bridge causes the α -carbons of Cys 21 and Cys 142, on opposite sides of the active-site cleft, to move toward each other by 2.5 Å. In association with this movement, the amino-terminal domain of the protein undergoes a rigid-body rotation of 5.1° relative to the carboxy-terminal domain. This rotation occurs about an axis passing through the junction of the amino-terminal and carboxy-terminal domains and is also close to the axis that best fits the apparent thermal motion of the amino-terminal domain seen previously in crystals of wild-type lysozyme. Even though the engineered Cys 21–Cys 142 disulfide links together the amino-terminal and carboxy-terminal domains of T4 lysozyme, it does not reduce the apparent mobility of the one domain relative to the other. The pronounced “hinge-bending” mobility of the amino-terminal domain that is suggested by the crystallographic thermal parameters of wild-type lysozyme persists in the oxidized (and reduced) mutant structures. In the immediate vicinity of the introduced disulfide bridge the mutant structure is more mobile (or disordered) than wild type, so much so that the exact conformation of Cys 21 remains obscure. As with the previously described disulfide bridge between residues 9 and 164 of T4 lysozyme (Pjura, P.E., Matsumura, M., Wozniak, J.A., & Matthews, B.W., 1990, *Biochemistry* 29, 2592–2598), the engineered cross-link substantially enhances the stability of the protein without making the folded structure more rigid.

Keywords: disulfide bridge; lysozyme; mutant structure; protein stability

To date, six different disulfide bridges have been engineered in bacteriophage T4 lysozyme (Perry & Wetzel, 1984; Wetzel et al., 1988; Matsumura & Matthews, 1989; Matsumura et al., 1989a,b; Anderson et al., 1990). Three of the disulfide mutants increase the thermostability of the protein. One of these, with a bridge between Cys 9 and Cys 164, has been crystallized and its structure determined at high resolution (Pjura et al., 1990).

Recently, crystals have been obtained of both the oxidized and reduced forms of the Cys 21–Cys 142 variant.

This disulfide bridge increases the melting temperature of the protein by 11 °C at pH 2.0. The location of the bridge is also of interest in that it spans the active-site cleft of the protein and can be used as a switch to turn lysozyme activity on and off (Matsumura & Matthews, 1989).

In this report we describe the crystal structure of the oxidized and reduced forms of the protein. In the reduced form the structure is very similar to that of wild type, but on oxidation the formation of the disulfide bond causes a rigid-body rotation of one domain relative to the other. Even though the disulfide bridge introduces a new, covalent link between the amino-terminal and the carboxy-terminal domains, there is no evidence that the structure of the protein as a whole is made more rigid.

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Results

Structure of the reduced form

The crystal structure of the reduced form of the 21C-142C-WT* mutant (see Methods) is very similar to wild-type, with only a small shift of the backbone in the region of Cys 142. A portion of the map showing the difference in electron density between the mutant and wild-type lysozyme in the region of the introduced cysteine residues is shown in Figure 1. At the position of the Thr 142 → Cys replacement there is negative density corresponding to the loss of the γ -hydroxyl and positive density where the γ -sulfur of the cysteine replaces the γ -methyl of the threonine. There is positive density extending from the sulfur position that appears to indicate the formation of an adduct with the β -mercaptoethanol (BME) present in the crystallization droplet (see below). Two negative density features in Figure 1 indicate the displacement of a solvent molecule and a presumed chloride ion that are bound to wild-type lysozyme in the vicinity of Thr 142. At the position of the Thr 21 → Cys replacement there is negative density indicating the loss of the γ -methyl group and positive density where the γ -sulfur of the cysteine replaces the γ -hydroxyl of the threonine.

Initial refinement of the reduced Cys 21–Cys 142 structure commenced with alanine residues at these two positions. Based on maps with coefficients $(2F_o - F_c)$ and phases from the partially refined structure, the Cys 21 and Cys 142 side chains were added using the model-building program FRODO (Jones, 1982). It became clear, however, that the electron density at Cys 142 could not be completely accounted for by cysteine alone. Rather, this cysteine appeared to have formed an adduct with BME that had been added to the crystallizing system to ensure a reducing environment. Such an adduct was therefore included as part of the refined model. The final R-factor was 15.4% at 1.8 Å resolution. Additional

Table 1. Data collection and refinement statistics^a

Protein	21C-142C-WT*	
	Reduced	Oxidized
Data collection		
Cell dimensions		
a, b, c (Å)	61.2, 61.2, 96.9	62.3, 62.3, 95.2
$\Delta a, \Delta b, \Delta c$ (Å)	0.0, 0.0, 0.1	1.1, 1.1, -1.6
Unique reflections	12,722	15,778
Resolution (Å)	1.8	1.7
R_{merge} (%)	7.5	7.1
Refinement		
Crystallographic residual (%)	15.4	17.6
Δ_{bond} (Å)	0.016	0.019
Δ_{angle} (°)	2.3	2.9

^a The cell dimensions of wild-type lysozyme are $a = b = 61.2$ Å, $c = 96.8$ Å (Weaver & Matthews, 1987). $\Delta a, \Delta b$, and Δc are the changes in cell dimensions of the mutant crystals relative to wild type.

statistics are given in Table 1. The quality of the map for the reduced structure is very good throughout the entire model. A section of the $(2F_o - F_c)$ map following refinement is shown in Figure 2.

A least-squares superposition between the backbone atoms of the mutant and wild-type molecules indicated a root-mean-square (rms) deviation of 0.14 Å. The low value of the rms deviation between the backbone atoms of the mutant and the wild-type molecules indicates that the introduced cysteines cause little perturbation from the overall backbone configuration of the wild-type molecule. α -carbon tracings of the wild-type and mutant backbones are shown superimposed in Figure 3 and Kinemage 1.

In the mutant structure, the side chain of Cys 21 has a χ_1 torsion angle of 45°. This value places the sulfur atom in the position occupied by the hydroxyl oxygen of

Fig. 1. Map showing the difference in electron density between reduced 21C-142C-WT* and wild-type lysozyme. Amplitudes $(F_{\text{mut}} - F_{\text{WT}})$ where F_{mut} and F_{WT} are the observed structure amplitudes for the mutant and wild-type structures. Phases from the refined structure of wild-type lysozyme. Resolution was 1.8 Å; contoured at 3σ where σ is the root-mean-square density through the unit cell.

Fig. 2. Map showing the refined density of reduced 21C-142C-WT* lysozyme. Coefficients $(2F_{\text{mut}} - F_c)$ where F_{mut} and F_c are, respectively, the observed and calculated amplitudes for the mutant structure. Phases were from the refined mutant structure. Resolution was 1.8 Å; contoured at 1σ . The superimposed protein structure corresponds to the refined model of the reduced form, including the apparent adduct of Cys 142 with BME.

Fig. 3. Superposition of the refined backbone structure of reduced 21C-142C-WT* (solid bonds) on that of wild-type lysozyme (open bonds).

Thr 21 in the wild-type molecule. The sulfur atom accepts a hydrogen bond from the main-chain amide of Glu 22 similar to that which is observed in wild type. Cys 142, which forms the apparent adduct with BME, has a χ_1 torsion angle of -55° . The resulting sulfur-sulfur distance between the S^γ atoms of Cys 21 and Cys 142 is 4.7 Å. The formation of the BME adduct causes a 0.3-Å shift of the backbone between Gln 141 and Pro 143 away from S^γ of Cys 21. This movement alleviates a close contact between the sulfur atom of the BME and S^γ of Cys 21, resulting in an interatomic distance of 3.5 Å, which corresponds to the van der Waals separation expected between two sulfur atoms.

Structure of the oxidized form

Unlike the reduced form, the oxidized form of 21C-142C-WT* exhibits large-scale shifts of the backbone atoms. Because of these coordinate shifts, and also the associated changes in unit cell dimensions (Table 1), the initial difference map for this variant was extremely noisy (not shown). The formation of the disulfide bond causes the N-terminal and C-terminal domains to rotate toward

each other, decreasing the C^α - C^α distance between residues 21 and 142 by 2.5 Å relative to the reduced molecule. As a result, the distance between the α -carbons of Cys 21 and Cys 142 is 5.9 Å, a value that is well within distances observed for naturally occurring disulfide bonds.

To determine the extent to which the individual domains of oxidized 21C-142C-WT* retained the conformations seen in wild-type lysozyme, the refined model of the oxidized mutant was superimposed onto the wild-type structure using two different transformations. One superposition (Fig. 4) was based on residues belonging to the amino terminal domain (residues 13-69). The other superposition (Fig. 5) was determined by including only those residues belonging to the carboxy-terminal domain (residues 70-162). Atoms within these groups of residues were superimposed by least squares onto the corresponding wild-type atoms. Residues 1-12 were omitted from the calculations as this part of the structure is not clearly associated with either domain. Figures 6 and 7 show the corresponding superpositions of the α -carbons of the two molecules.

Figures 4 and 6 and Kinemage 2 show the results of the superposition based on the amino-terminal domains.

Fig. 4. Shift plot showing the discrepancy between the backbone atoms of oxidized 21C-142C-WT* and wild-type lysozymes. The superposition of the two structures is based on residues 13-69 (indicated by the horizontal bar). The value plotted for each residue is the average discrepancy of the four backbone atoms N, CA, C, and O.

Fig. 5. Discrepancy between the α -carbons of oxidized 21C-142C-WT* and wild-type lysozymes when their optimal superposition is based on residues 70-162 (indicated by the horizontal bar).

Clearly the overall structure of the N-terminal domain is conserved in the mutant structure. The atoms included in the superposition have an average rms discrepancy of 0.25 Å. In this orientation, the residues that belong to the amino-terminal domain are seen to correspond well, whereas those residues that belong to the carboxy-terminal domain are seen to shift up to 2.1 Å from the wild-type positions (Fig. 4). Note, however, that the backbone

configuration close to the introduced cysteine residue, Cys 21, is not well conserved. The largest deviation within the superimposed N-terminal domain (0.8 Å) is centered on residue 21 (Fig. 4). This deviation, which extends over about five residues, suggests that local readjustment of the backbone has occurred in association with the formation of the disulfide bond.

The results of the superposition based on the carboxy-

Fig. 6. Superposition of the backbone of oxidized 21C-142C-WT* (solid bonds) on that of wild-type lysozyme (open bonds), with the molecules aligned as in Figure 4.

Fig. 7. Superposition of 21C-142C-WT* (solid bonds) and wild-type lysozyme (open bonds), corresponding to Figure 5.

terminal domains are shown in Figures 5 and 7 and Kinemage 3. As with the amino-terminal domains, there is good agreement within the domain being superimposed, but larger deviations from the wild-type structure outside this region. The backbone atoms included in the superposition of the carboxy-terminal domains have an rms deviation of 0.25 Å. Conversely, those residues belonging to the amino-terminal domain are now seen to shift up to 2.1 Å from their wild-type counterparts. As in the superposition of the amino-terminal domains, the backbone in the vicinity of the introduced cysteine is seen to be somewhat distorted (0.6 Å). The distortion, in this case centered on Cys 142, again extends over about five residues.

In addition to the examination of backbone positions, a survey of side-chain positions was carried out for the two domains. Other than the interdomain motion, no large movements of side chains were observed except on

the surface of the molecule in residues with high thermal factors. The coordinates of such residues are subject to greater experimental uncertainty.

The thermal factors of the backbone atoms of the oxidized 21C-142C-WT* mutant and wild-type lysozyme are compared in Figure 8. Although the thermal factors for the two lysozymes are similar for much of the respective structures, in the vicinity of the introduced cysteine sites they are very different. A large increase in the B values is observed in the vicinity of the introduced disulfide bond, especially for residue 21. Cys 21 has a main-chain B value of 56 Å² in the oxidized mutant structure as opposed to 32 Å² for Thr 21 in wild-type lysozyme. In the reduced structure of 21C-142C-WT* lysozyme the backbone thermal factors are very similar to those of wild-type lysozyme not only for the backbone atoms in general but also at the two cysteine sites (Fig. 9).

Although the effects of the disulfide formation are read-

Fig. 8. Comparison of the backbone thermal factors of oxidized 21C-142C-WT* (thick line) with wild-type lysozyme (thin line). For each residue the plotted value is the average of the thermal parameters for the N, C, C α , and O atoms.

Fig. 9. Comparison of the backbone thermal factors of reduced 21C-142C-WT* lysozyme (thick line) with wild-type (thin line).

ily apparent from the large-scale shifts of the domains, the electron density for the residues in the region of the disulfide bond is poorly defined, particularly on the amino-terminal side (residues 21-23) (Fig. 10). Not only is the electron density ill defined, but also a stereochemically reasonable model could not be found that would account for the density in this region. The torsion angles for the bonds constituting the disulfide bridge are as follows: Cys 21, $\chi_1 = -8^\circ$, $\chi_2 = 148^\circ$, $\chi_3 = 99^\circ$, $\chi'_2 = -69^\circ$, $\chi'_1 = -63^\circ$, Cys 142 (see Richardson, 1981, for the definition

of these angles). The geometry best resembles the right-handed hook described by Richardson (1981). The value of χ_1 , however, corresponds to an extremely high-energy conformation because the β -carbon of Cys 21 is in an eclipsed position relative to the main-chain nitrogen of Glu 22. This clearly indicates the inadequacy of the model in this vicinity. In an attempt to resolve the unsatisfactory aspects of the model in this area, residues 19-23 were omitted, additional refinement was carried out, and a new map was calculated. The new map, however,

Fig. 10. Map showing the electron density, after refinement, of oxidized 21C-142C-WT* lysozyme. Coefficients and conventions as in Figure 2 except that the map is contoured at 0.8σ .

suggested that these residues had already been optimally placed, and no further improvement could be made. The thermal factors for the atoms C^α , C^β , and S^γ of Cys 21 were, respectively, $43.1 \text{ \AA}^2$, $32.9 \text{ \AA}^2$, and $78.7 \text{ \AA}^2$. For Cys 142 the B-values for C^α , C^β , and S^γ were $27.2 \text{ \AA}^2$, $57.5 \text{ \AA}^2$, and $57.5 \text{ \AA}^2$. These high values, especially for S^γ of Cys 21, suggest that there is either genuine disorder or errors in the model that cannot be resolved. Away from this region, the electron density is well defined and can be modeled with good stereochemistry and reasonable temperature factors. This indicates that the presumed disorder is localized to the region surrounding the introduced disulfide bond.

Discussion

The engineered Cys 21–Cys 142 disulfide bridge in T4 lysozyme spans the active-site cleft of the molecule. Residue 21 lies at the tip of a relatively mobile loop of irregular secondary structure in the amino-terminal domain, and residue 142 is found at the carboxyl end of a short stretch of α -helix belonging to the carboxy-terminal domain. In the wild-type structure the α -carbons of Thr 21 and Thr 142 are separated by a distance of $8.1 \text{ \AA}$. In the oxidized mutant structure the separation is decreased to $5.9 \text{ \AA}$. The wild-type C^α – C^α separation is outside the range of $4.6 \text{ \AA}$ to $7.4 \text{ \AA}$ observed for C^α – C^α distances between cysteine residues forming disulfide bonds in known protein structures (Thornton, 1981). Notwithstanding the fact that residues 21 and 142 might have been too far apart to be bridged by a disulfide link, there were two factors that encouraged us to proceed with the attempt. First, there was evidence from the refined crystal structure of wild-type lysozyme (Weaver & Matthews, 1987) that the amino-terminal domain undergoes hinge-bending motion, even within the constraints of contacts within the crystal lattice. Such hinge-bending motion might allow residues 21 and 142 to move closer together without necessarily introducing significant strain in the lysozyme structure.

The second motivation for introduction of the 21–142 S–S bridge was to determine its effect on activity. Indeed, it was subsequently found that the 21–142 disulfide provided a catalytic switch (Matsumura & Matthews, 1989).

In the oxidized form, the disulfide occludes the active-site cleft and totally eliminates catalytic activity. On addition of a suitable reducing agent, however, the bridge opens, and catalytic activity is largely restored.

The determination of the structure of the “reduced” form shows that the species in the crystals is actually the mixed-disulfide of Cys 21 with BME. We attribute the presence of this mixed-disulfide in part to the relatively high concentration of BME (60 mM) that was necessary to obtain X-ray quality crystals (see Materials and methods). Dr. T.E. Creighton (pers. comm.) has also pointed out that the mixed-disulfide is the intermediate in the formation of a protein disulfide bond, and the stability of the mixed-disulfide in this case indicates that the next step, formation of the protein disulfide, is slow in the folded protein.

The determination of the structures of the oxidized and reduced forms of 21C-142C lysozyme vindicate the assumptions that went into the design of this variant. As anticipated, in the oxidized form of the protein there is a hinge-bending rotation (see below and Kinemage 2) that brings residues 21 and 142 about $2.5 \text{ \AA}$ closer together than in the wild type (and in the reduced) structures, and permits the formation of the S–S bridge. The amino-terminal and carboxy-terminal domains move essentially as rigid units. The backbone conformations and positions of side chains within the domains in the mutant enzyme retain essentially the wild-type structure, except for some local shifts near the disulfide bond and some possible rearrangement of mobile surface side chains. Because the hinge-bending of the two domains is seen in the crystal structure of the oxidized variant, but not the reduced form (Kinemage 1), it is clearly associated with the formation of the S–S bridge and is not due to the Thr 21 $\rightarrow$ Cys and Thr 142 $\rightarrow$ Cys replacements per se.

The change in the backbone structure of the Cys 21–Cys 142 disulfide bridge mutant relative to wild-type lysozyme can also be analyzed by using a difference-distance matrix (Nishikawa et al., 1972). Such a plot (Fig. 11) shows the change in the distance between every pair of α -carbon atoms in the mutant structure relative to wild type. Regions of structure that are structurally unchanged in the mutant structure (i.e., the C_i^α – C_j^α distances remain constant) appear as featureless regions in

the difference-distance plot. Such regions in Figure 11 include residues 1–20, 24–80, 80–140, and 144–162. It can also be seen from Figure 11 that the larger region including residues 1–80 is essentially invariant except for movement in the vicinity of residue 21, i.e., one of the cysteine sites. Similarly, the domain including residues 80–162 (the C-terminal domain) is also essentially unchanged except for movement in the vicinity of Cys 142.

Previously the crystallographic thermal displacement parameters seen in the structure of wild-type lysozyme

were analyzed in terms of rigid body motion either about a point or about an axis. The best axis to account for the apparent displacement of the N-terminal domain passed approximately through the α -carbons of residues 5 and 71 (Fig. 12; Kinemage 3) (Weaver & Matthews, 1987).

In the case of the oxidized 21C–142C–WT* lysozyme there is a rigid-body movement of one domain relative to the other. This can be analyzed by first superimposing the C-terminal domain of 21C–142C–WT* lysozyme on that of wild-type lysozyme (Fig. 7). Then, in this co-

Fig. 11. Difference-distance plot showing the difference between C^α - C^α distances in wild-type lysozyme and in the oxidized Cys 21-Cys 142 mutant. The function plotted is $\Delta r_{ij} = (r_{ij})_{WT} - (r_{ij})_{mut}$ where r_{ij} is the separation in ångströms between the i th and j th α -carbon in the wild-type or mutant structure. Solid contours are plotted at values of 0.5 Å, 1.0 Å, 1.5 Å, and 2.0 Å; negative contours plotted at -0.5 Å.

Fig. 12. Backbone of wild-type lysozyme showing the locations of two different axes of motion. The axis labeled thermal analysis best fits the crystallographic thermal parameters of the amino terminal domain within the crystal structure of wild-type lysozyme (Weaver & Matthews, 1987). The axis labeled SS defines interdomain motion associated with the formation of the 21C-142C-WT* disulfide. This motion consists of a rotation of 5.1° about the SS axis, together with a translation of $0.26 \text{ \AA}$ along the axis.

ordinate system, one can determine the additional transformation to bring the N-terminal domains of the two lysozymes into register. To help visualize the result, this transformation can be expressed as the combination of a rotation about a certain axis plus a translation along the same axis. Such an analysis reveals that rigid-body motion of the N-terminal domain associated with formation of the 21C-142C disulfide bond consists of a rotation of 5.1° and an axial translation of $0.26 \text{ \AA}$. The location of the axis (Fig. 12; Kinemage 2) is remarkably close to that determined from analysis of the thermal parameters. It should be stressed, however, that the apparent agreement between these two axes of motion should not be pressed too closely, in part because the axis determined from analysis of the thermal parameters was not well defined, and, in addition, this axis considers only the apparent motion of the N-terminal domain within the wild-type crystals and ignores any motion of the C-terminal part of the molecule.

Although the hinge-bending motion results in overall coordinate shifts of up to $2 \text{ \AA}$ (Fig. 5) this motion is achieved by conformational changes in the interdomain region that are much less ($\leq 0.3 \text{ \AA}$). In the case of the mutant Met 6 $\rightarrow$ Ile (Faber & Matthews, 1990), which exhibited much larger hinge-bending motion than is the case here, the interdomain movement resulted primarily from adjustments in the three backbone segments 12-14, 58-60, and 79-80 (highlighted in Kinemage 2). The first of these is in the strand that connects the first α -helix to the amino-terminal domain and the other two are at the beginning and end of the long interdomain helix. Comparison of the oxidized and reduced 21C-142C structures (Figs. 6, 7) suggests that small adjustments (~ 0.1 - $0.2 \text{ \AA}$) in the same three regions permit the observed hinge-bending movement, but these adjustments are sufficiently

small as to be almost at the level of the uncertainty in the coordinates.

Because the electron density in the vicinity of Cys 21, especially, is so poorly defined in the oxidized structure (Fig. 10), it is not possible to describe reliably the detailed geometry of the disulfide bridge. Indeed, it is not obvious that a single conformation occurs in the crystal structure. Several atypical and strained geometries have been reported for nonnative disulfide bridges (Katz & Kosciakoff, 1986, 1990; Ecker et al., 1989). In the present instance, however, the most likely explanation for the tenuous density in the vicinity of Cys 21, as well as the lack of a stereochemically reasonable model, is the presence of disorder. It is not possible to distinguish between static disorder, in which two or more distinct but different conformations occur in the crystal, or dynamic disorder, in which there are large-scale fluctuations about a single low-energy-minimum conformation. The conformation of the disulfide bridge corresponds to that of a right-handed spiral, and it has been noted that the right-handed spirals observed in nature show a much greater diversity than the left-handed spirals (Richardson, 1981; Thornton, 1981).

Because of the ambiguity of the 21C-142C-WT* structure in the vicinity of Cys 21 it is not possible to determine whether the closure of the active-site cleft changes the solvent structure in this region.

It might be that the weak density in the vicinity of Cys 21 could be due to a mixed population of oxidized and reduced molecules in the crystal. This does not seem very probable. Because of the large shifts of the domains that are observed on disulfide formation, it seems unlikely the two species would cocrystallize. Even if the two species were able to coexist in the same crystal lattice, one might expect to see degradation of density throughout

the molecule. This is not obviously the case. Neither is it observed in the crystal structure of the reduced mutant. Therefore the most likely explanation remains that the disulfide bond is disordered and displays a mixture of conformations.

Thermal stability

Of the engineered disulfide bridges described to date in T4 lysozyme, that between Cys 21 and Cys 142 is the most effective in increasing the thermostability of the protein. In the oxidized form, the bridge increases the melting temperature of the protein by 11 °C relative to wild type.

Why is this bridge so effective? One contributing factor is the size of the loop created by the S-S bridge, but loop size alone is not a dominant factor. According to theory, the contribution to protein stability of a disulfide bridge through reduction in entropy of the unfolded polypeptide is expected to be proportional to the logarithm of the number of residues in the loop (Pace et al., 1988) and therefore increases only slowly as the size of the loop is increased.

As has been discussed elsewhere, the contribution to protein stability from a given disulfide bridge depends not only on the reduction of entropy in the unfolded state but also on enthalpic effects that could occur in both folded and unfolded forms (Creighton, 1988; Pace et al., 1988; Matsumura et al., 1989a). To be maximally effective in increasing thermostability a disulfide bridge should not introduce strain into the folded protein structure. It has been suggested that one way to avoid strain is to engineer S-S bridges into flexible parts of a protein (Matsumura et al., 1989a). If the primary benefit of a disulfide bridge comes from its effect on the unfolded protein, then this benefit can be realized irrespective of the location of the bridge within the folded protein. According to this line of reasoning, a disulfide bridge does not have to make the folded protein structure more rigid in order to be effective in increasing stability.

This is consistent with the previously described crystal structure of the Cys 9–Cys 164 mutant of T4 lysozyme (Pjura et al., 1990). Cys 164, introduced at the extreme carboxy-terminus of T4 lysozyme, formed a disulfide bridge to Cys 9 and increased the melting temperature of the protein by 6.4 °C (Matsumura et al., 1989a), but the S-S bridge did not noticeably reduce the apparent disorder in the vicinity of residue 164.

A similar result is observed in the present case. The introduction of the Cys 21–Cys 142 disulfide bridge causes a global movement of the N-terminal domain relative to the C-terminal one but, at least as judged by the crystallographic thermal factors, does not reduce mobility within the crystal. As can be seen in Figure 8 and Kinemage 3, the disulfide-bridged Cys 21–Cys 142 structure has thermal factors that are no less than those of wild-type lysozyme. Furthermore, there is actually greater motion or disorder in the vicinity of the Cys 21–Cys 142

disulfide bridge than is observed at Thr 21 and Thr 142 in wild-type lysozyme (Fig. 8; Kinemage 3). In the reduced structure, however, the mobilities of the two cysteines are essentially the same as the residues they replace. Therefore it is the formation of the disulfide bridge that causes enhanced mobility at residues 21 and 142, not the introduction of the two cysteines, *per se*.

As noted above, the thermal factors of wild-type lysozyme are consistent with the amino-terminal domain undergoing hinge-bending motion about an axis that passes through the equator of the molecule (Fig. 12). Perhaps surprisingly, the thermal factors in the crystal structure of oxidized Cys 21–Cys 142 lysozyme show no evidence that the presence of the disulfide inhibits this hinge-bending motion. This is shown most clearly by the similarity in the overall distribution of thermal factors in the mutant and wild-type structures seen in Figure 8. Residues proximal to the hinge (e.g., 70) have low mobility, whereas the most distal residues (e.g., 36) have the largest thermal parameters in both structures.

In the wild-type crystal structure it was assumed that the C-terminal domain has relatively low thermal factors because most of the crystal contacts occur in this part of the molecule. The N-terminal domain, having fewer crystal contacts, was more free to move and could display something of the hinge-bending motion that was presumed to occur in solution (Matthews & Remington, 1974; Weaver & Matthews, 1987). It appears in the disulfide-linked mutant structure that there is still an intrinsic tendency for the N-terminal domain to display the same hinge-bending motion. It appears that the retention of this flexibility induces local disorder in the vicinity of Cys 142, and, more obviously, in the region of Cys 21.

Subsequent to the design of the 21–142 disulfide bridge mutant, it has been shown that the mutant lysozyme with methionine 6 replaced by isoleucine (M6I) crystallizes in a novel form in which the hinge-bending angle between the amino-terminal and carboxyl-terminal domains varies by up to 32° (Kinemages 4, 5; Faber & Matthews, 1990). In the crystal structures of M6I the active-site cleft is more open than wild-type lysozyme. As noted above, the 21–142 disulfide closes the active-site cleft and so increases the overall range of hinge-bending angles that have been observed for different lysozyme variants (Kinemage 4). We have argued that the hinge-bending motion in M6I lysozyme is a low energy deformation (Faber & Matthews, 1990). It would not be surprising if low energy hinge-bending motion occurs not only for M6I but is a general attribute of T4 phage lysozyme.

Materials and methods

Protein purification and crystallization

As described previously (Matsumura et al., 1989a), the disulfide bonds introduced into T4 lysozyme have been

engineered in an otherwise cysteine-free protein. To this end a cysteine-free pseudo-wild-type lysozyme was first constructed by replacing Cys 54 with Thr and Cys 97 with Ala. This double mutant, C54T/C97A, is abbreviated as WT*. The identification of the engineered protein with a disulfide bridge between residues 21 and 142 is T21C/T142C/WT*, which is here further shortened to 21C-142C-WT*. Purification of this mutant protein has been described (Matsumura & Matthews, 1989).

Crystals were obtained under reducing and nonreducing conditions using the hanging-drop vapor diffusion method. A mixture of sodium and potassium phosphate was used as the precipitant for both the reduced and oxidized crystals. It has been known for a long time that T4 lysozyme crystals are very difficult to grow without the presence of BME (Matthews et al., 1973; also see below). In the present case, attempts to crystallize 21C-142C-WT* lysozyme in the presence of 1.5 mM reduced BME (Matthews et al., 1973) led to very intergrown crystals. It was presumed that this might be due to a mixture of reduced and oxidized protein molecules. Therefore a range of conditions was explored with an increasing concentration of BME in the reservoir buffers. Crystallization droplets containing 5 μ L of protein (21.5 mg/mL in 0.1 M NaKPO₄, pH 6.6, 0.55 M NaCl, 0.02% NaN₃) were mixed with an equal volume of well buffer (1.9 M Na/KPO₄, pH 7.1, 0.24 M NaCl, 30–140 mM BME) and suspended over a 1-mL volume of the same buffer. Two crystals, each 0.5 mm along the diagonal, were obtained at 60 mM BME. These grew in the space group P3₂21 with cell dimensions $a = b = 61.2$ Å, $c = 96.9$ Å and were isomorphous with wild-type crystals.

The engineered disulfide bond in 21-142 lysozyme forms spontaneously when exposed to air under normal storage conditions (Matsumura & Matthews, 1989). In the present case, protein from an 8-month-old preparation was used. One crystal of the oxidized form was obtained using crystallization conditions similar to those for the reduced form except that 40 mM 2-hydroxyethyl disulfide (HEDS) was substituted for BME. HEDS is the oxidized form of BME, and a HEDS molecule has been identified bridging between two molecules of T4 lysozyme in the wild-type crystal structure (Bell et al., 1991). The HEDS molecule packs into a hydrophobic pocket and seems to facilitate crystallization. Five microliters of protein (21.3 mg/mL in 0.1 M Na/KPO₄, pH 6.6, 0.55 M NaCl, 0.02% M NaCl, 0.02% NaN₃) was mixed with 5 μ L of well buffer (2.1 M Na/KPO₄, pH 7.2, 0.24 M NaCl, 40 mM HEDS) and suspended over 1 mL of well buffer. One crystal 0.9 mm along the diagonal was obtained. The crystal grew in the space group P3₂21 and had cell dimensions of $a = b = 62.3$ Å, $c = 95.2$ Å. Crystals obtained under both reducing and nonreducing conditions were mounted in siliconized glass capillaries using well buffer to maintain vapor pressure and used for data collection.

Data collection and refinement

Reduced form

Twenty film packs were used to record the diffraction data from two crystals of the reduced form of the 21C-142C-WT* lysozyme mutant. Data were collected on a rotating anode X-ray generator (Cu-K α radiation at 40 kV $\times$ 150 mA) using the oscillation method as described by Schmid et al. (1981). Ten exposures, each with a 2° oscillation range, were collected at 3 h per exposure from each crystal. These crystals diffracted to a resolution of 1.8 Å.

Films were digitized using an Optronics System P-1000 photo scanner, and the data evaluated using the method of Rossmann (1979), as adapted by Schmid et al. (1981). Data processing statistics are summarized in Table 1.

The structure was refined using the TNT restrained least-squares refinement routines (Tronrud et al., 1987), together with model building on an Evans and Sutherland PS330 graphics display terminal using the molecular modeling program FRODO (Jones, 1982). The most recent version of the wild-type T4 lysozyme coordinates, T4165RF (Bell et al., 1991), was used as the starting model. Those residues that differed in the mutant, namely Cys 21, Thr 54, Ala 97, and Cys 142, were initially modeled as alanines. The overall refinement strategy used for the reduced form followed that described in detail by Dao-pin et al. (1991) for other mutant lysozymes.

Oxidized form

Twenty film packs were collected from one crystal measuring 0.6 $\times$ 0.6 $\times$ 0.5 mm of oxidized 21C-142C-WT*. A translation of the crystal was carried out halfway through data collection. The films were collected, processed, and analyzed as described above. The oxidized crystal form diffracted to a resolution of 1.7 Å. Data collection statistics are summarized in Table 1.

The wild-type lysozyme coordinates, T4165RF, were again used as a starting model, with residues 21, 54, 97, and 142 replaced by alanines. An initial round of rigid-body refinement was carried out, followed by a second round, where possible interdomain motion resulting from the formation of the disulfide bond was allowed. This was accomplished by dividing the molecule into two rigid units consisting, respectively, of residues 1–60 and 61–162. The initial R-factor before any refinement was 42.7% at 4 Å resolution. After allowing the molecule to move as a whole as a rigid body the R-factor dropped to 28.1%. The further rigid-body refinement, where the domains were treated independently, resulted in an R-factor of 20.9% at 4 Å resolution. Positional and temperature factor refinement was then carried out, and residues Cys 21, Thr 54, and Cys 142 were built into the electron density. Further positional and temperature factor refinement was performed following the scheme described by Dao-pin et al. (1991) until the R-factor con-

verged to 17.6% at 1.7 Å resolution and the stereochemistry was acceptable. Refinement statistics are summarized in Table 1. The refined coordinates will be deposited in the Brookhaven Data Bank.

Acknowledgments

We thank Joan Wozniak for expert technical assistance and Drs. H. Nicholson and L.H. Weaver for advice with data collection and computational procedures. This work was supported in part by a grant from the National Institutes of Health (GM21967) and the Lucille P. Markey Charitable Trust.

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