Stabilization of phage T4 lysozyme by engineered disulfide bonds

(thermostability/lysozyme/protein structure)

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ABSTRACT Four different disulfide bridges (linking positions 9–164, 21–142, 90–122, and 127–154) were introduced into a cysteine-free phage T4 lysozyme at sites suggested by theoretical calculations and computer modeling. The new cysteines spontaneously formed disulfide bonds on exposure to air in vitro. In all cases the oxidized (crosslinked) lysozyme was more stable than the corresponding reduced (noncrosslinked) enzyme toward thermal denaturation. Relative to wild-type lysozyme, the melting temperatures of the 9–164 and 21–142 disulfide mutants were increased by 6.4°C and 11.0°C, whereas the other two mutants were either less stable or equally stable. Measurement of the equilibrium constants for the reduction of the engineered disulfide bonds by dithiothreitol indicates that the less thermostable mutants tend to have a less favorable crosslink in the native structure. The two disulfide bridges that are most effective in increasing the stability of T4 lysozyme have, in common, a large loop size and a location that includes a flexible part of the molecule. The results suggest that stabilization due to the effect of the crosslink on the entropy of the unfolded polypeptide is offset by the strain energy associated with formation of the disulfide bond in the folded protein. The design of disulfide bridges is discussed in terms of protein flexibility.

The design of proteins with enhanced stability is one of the major goals of protein engineering. Among the physical forces that maintain the tertiary structure of proteins, disulfide bonds can make a substantial contribution (1). This knowledge has, therefore, stimulated several attempts to introduce new disulfide bonds to improve protein stability. To date, disulfide bonds have been engineered into dihydrofolate reductase (2), T4 lysozyme (3, 4), subtilisin (5, 6), and a repressor (7). The addition of new disulfides has not, however, always increased stability. One aspect of this problem is that knowledge of the mechanism by which crosslinks, such as disulfide bonds, stabilize or destabilize proteins is limited.

In this study, we have designed and constructed four different disulfide mutants of phage T4 lysozyme. The results illustrate the principles by which the engineered disulfide bonds stabilize the enzyme and suggest a strategy for the selection of potential disulfides that are most likely to increase the stability of a protein.

MATERIALS AND METHODS

Design of Disulfide Bridges. Possible sites for the introduction of disulfide bridges were sought by the following three steps. (i) The coordinates of pairs of cysteine residues in disulfide bridges (total of 295 pairs) in known protein X-ray structures were collected (8). Next, all potential pairs of residues to be engineered in T4 lysozyme (9) were scanned to find pairs that satisfied the following three criteria: The residues must be separated by at least 20 residues along the polypeptide chain, both residues must have γ atoms (This criterion excluded alanine and glycine, which may, in retrospect, have been unnecessarily restrictive.), and these γ atoms must be closer together than 6 Å. Each of the 295 known Cys–Cys pairs was then fitted to each of the selected pairs using least-squares superposition of corresponding coordinates (12 atoms for each pair). The lowest root-mean-square deviation after superposition was used to rank the selected pairs (see geometry in Table 1).

(ii) The second step involved energy minimization to estimate the strain associated with formation of the disulfide bond. Energy minimization, with defined parameters (10), was confined to a small “molten zone” that included one residue on each side of the pair of residues being replaced, allowing only the six residues to move. The strain energy was calculated as the difference between the energy of the protein with and without disulfide bond formation (see strain energy in Table 1). The energy of the crosslinked form was found to always be higher than that with uncrosslinked form by at least 4 kcal/mol (1 cal = 4.184 J).

(iii) The third step involved an analysis of stabilizing interactions, such as hydrophobic interactions, hydrogen bonds, and van der Waals' contacts that existed in the wild-type (WT) structure. Ideally, the introduction of cysteine pairs should minimize the loss of preexisting interactions that stabilize the WT structure. Final candidates were examined by simulating the disulfide bridge with the use of computer graphics (11).

Table 1 and Fig. 1 summarize the data and locations for the four sites that were selected. It has previously been shown that a single mutation at position 3 (Ile-3 → Cys) in T4 lysozyme forms a disulfide bond with WT Cys-97 and stabilizes the enzyme (3, 12, 13). Our procedure correctly predicted that the 3–97 site is one of the best candidates, and the relevant parameters are also included in Table 1. Although the 21–142 site has a very unfavorable calculated strain energy, it was anticipated that the presumed “hinge-bending” motion at the active-site cleft in T4 lysozyme (9) would facilitate the formation of this link.

Constitution and Expression of Mutant Lysozymes. WT T4 lysozyme contains two cysteine residues at positions 54 and 97. To avoid possible thiol/disulfide interchange reaction with the newly engineered disulfides, Cys-54 and Cys-97 were replaced with threonine and alanine, respectively (4). The cysteine-free T4 lysozyme (designated either 54'T-97A or WT*) was used as a template to create the four mutants by site-directed mutagenesis (14): Ile-9 → Cys and Leu-164 → Cys (9C–164C-WT*); Thr-21 → Cys and Thr-142 → Cys (21C–142C-WT*); Ser-90 → Cys and Gln-122 → Cys (90C–122C-WT*); Asp-127 → Cys and Arg-154 → Cys (127C–

Abbreviations: WT, wild-type; Tm, melting temperature.
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for completely to also were other disulfide bond, however, in itself reduced form. This indicated by oxidized and reduced forms, respectively. The disulfide bond obtained by mutating Ile-3 → Cys and Cys-54 → Thr was also constructed Glu-11 and Asp-20 are the active-site residues. (Inset) Sizes of the loops formed by the respective disulfide bridges.

154C-WT*). The 3–97 disulfide mutant (3C–54T) was constructed by mutating Ile-3 → Cys (and Cys-54 → Thr) in WT lysozyme. The resulting five mutants were expressed in *Escherichia coli* and purified as described (15).

Nonreducing SDS/PAGE (5, 6) and reversed-phase HPLC (3, 4) have been used to assay the formation of disulfide bonds. However, neither SDS/PAGE nor reversed-phase HPLC was always effective in distinguishing between the oxidized (crosslinked) and reduced (noncrosslinked) form of the mutant lysozymes. This problem was overcome by using an ion-exchange HPLC (see below). Immediately after purification some mutants were found to be a mixture of the oxidized and reduced form. On standing under slightly alkaline conditions, however, these mutants converted completely to the oxidized form. Disulfide formation was also indicated by the fact that no free cysteine thiols could be detected with Ellman’s reagent (data not shown).

**RESULTS**

**Enzyme Activity.** Table 2 shows activities of the mutant and WT lysozymes. Because WT* enzyme has essentially the same activity as WT lysozyme, any changes in activity of the mutants can be attributed to the introduction of new cysteines. 9C–164C-WT* is as active as WT both in the oxidized and reduced forms, indicating that neither the replacement with cysteines nor the formation of the disulfide bond at this site perturbs activity. As described (4), formation of the 21–142 disulfide bond, however, leads to the complete abolition of activity, presumably because this bridge across the active-site cleft prevents substrate from entering the active site of the enzyme (see Fig. 1). To a much smaller degree, the other two mutants, 90C–122C-WT* and 127C–154C-WT*, were also less active in the oxidized form compared with the reduced form. This tendency is most obvious in 90C–122C-WT*, where the introduction of the two new cysteines in itself does not change the activity at all. Although both these disulfide bonds are far from the active site (see Fig. 1), it is possible that a structural perturbation, due to the disulfide formation, might alter the mode of interaction with the peptidoglycan of *E. coli* cell wall (17). In this context, it is interesting to note that reduced activity has been seen when other mutations are introduced in the same region of the enzyme surface [80% reduction for Pro-86 → Arg (18), 50% for Gly-156 → Asp (19), and 96% for Glu-128 → Lys (17)]. In principle, however, it appears possible to introduce disulfide bonds without sacrificing enzyme activity, unless the mutations perturb substrate binding or catalysis (see, for examples, 3C–54T and 9C–164C-WT*).

**Stability of Mutant Lysozymes.** T4 lysozyme can be reversibly unfolded (denatured) by heating under controlled conditions, with the compact denatured form (T4d) being able to refold to the native form (T4n). As is seen below, the WT* lysozyme is more stable than the WT lysozyme, and the disulfide bonds provide additional stabilization to the protein. The disulfide bonds of the mutant proteins can be reduced by dithiothreitol, resulting in an increase in activity. The activity of WT* enzyme after incubation with 10 mM dithiothreitol for 4–16 hr as described in the legend for Fig. 3. Measurements for the reduced enzymes were done in the buffer containing 5 mM dithiothreitol.

**Table 2. Relative activities of T4 lysozyme variants**

<table>
<thead>
<tr>
<th>Variant</th>
<th>Oxidized activity</th>
<th>Reduced activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>WT* (i.e., 54T–97A)</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>3C–54T (i.e., 3C–97C)</td>
<td>96†</td>
<td>90†</td>
</tr>
<tr>
<td>9C–164C-WT*</td>
<td>106</td>
<td>99</td>
</tr>
<tr>
<td>21C–142C-WT*</td>
<td>0‡</td>
<td>68‡</td>
</tr>
<tr>
<td>90C–122C-WT*</td>
<td>58</td>
<td>102</td>
</tr>
<tr>
<td>127C–154C-WT*</td>
<td>52</td>
<td>79</td>
</tr>
</tbody>
</table>

Activities were measured at 23°C by the turbidity assay (16). The reduced enzymes were prepared by incubation with 10 mM dithiothreitol for 4–16 hr as described in the legend for Fig. 3. Measurements for the reduced enzymes were done in the buffer containing 5 mM dithiothreitol.

†Similar activities have been reported (3).
‡From ref. 4.
Stability of the Disulfide Bonds. Stability of a disulfide bond can be assessed by determining the equilibrium constant ($K_{eq}$) for the reduction of the enzyme ([Enz]) by dithiothreitol ([DTT]) (1, 5). That is, $K_{eq}$ for the reaction

$$[\text{Enz}]_{\text{red}} + [\text{DTT}]_{\text{ox}} = [\text{Enz}]_{\text{ox}} + [\text{DTT}]_{\text{red}}$$

[1]

can be calculated as

$$K_{eq} = \frac{[\text{Enz}]_{\text{red}}[\text{DTT}]_{\text{ox}}}{[\text{Enz}]_{\text{ox}}[\text{DTT}]_{\text{red}}}$$

[2]

Cation-exchange HPLC was found to give a quantitative separation of the reduced (red) and oxidized (ox) enzymes (data not shown); the mutant lysozymes were treated with mixtures of oxidized and reduced dithiothreitol, and the fraction of the enzyme in the oxidized and reduced forms was determined.

The results are summarized in Fig. 3. The concentrations of reduced dithiothreitol in the redox buffer needed to produce equal amounts of reduced and oxidized enzymes were $0.023$ mM, $0.30$ mM, $0.56$ mM, and $1.9$ mM for 127C–154C-WT*, 90C–122C-WT*, 21C–142C-WT*, and 9C–164C-WT*, respectively. This indicates that the order of the stability of disulfide bonds is as follows: 9–164 > 21–142 > 90–122 > 127–154. We have assumed that the loss of reduced dithiothreitol and formation of oxidized dithiothreitol due to air oxidation is negligible during the reaction under nitrogen-purged conditions (5). Taking into account that each of the

![Fig. 2. Schematic diagram showing the $T_m$ values of the T4 disulfide mutants relative to WT lysozyme. $T_m$ of the thermal denaturation was measured at pH 2.0 as described (20). $\Delta T_m$ is the change in $T_m$ of the oxidized (S–S) and the reduced (SH HS) forms of the mutant relative to WT ($T_m$ = 41.9°C (21)). $\Delta T_m$ values for the five oxidized lysozymes (left to right) were −2.4, −0.3, 4.8, 11.0, and 6.4°C, respectively, whereas $\Delta T_m$ values for the reduced enzymes were −5.4, −5.8, −1.9, −2.7, and −6.5°C, respectively. The rectangle shown for each mutant is defined by the $T_m$ values of the oxidized and reduced enzymes relative to WT. The oxidized enzymes were prepared by air exposure under slightly alkaline conditions (pH 8.0). The reduced enzymes were prepared as follows. Purified protein (=1 mg/ml) was treated with 6 M guanidine hydrochloride/20 mM dithiothreitol/1 mM EDTA/50 mM Tris-HCl, pH 8.3. After incubation for 4 hr at 23°C, the mixture was extensively dialyzed under a nitrogen purge at 4°C against 0.2 M KCl/1 mM EDTA/0.1 mM dithiothreitol, pH 2.0.](http://example.com/fig2.png)

![Fig. 3. Stability of engineered disulfide bonds in T4 lysozyme to reduction by dithiothreitol (DTT).](http://example.com/fig3.png)
DISCUSSION

Thermodynamic Cycle. The stabilization of the disulfide-crosslinked protein can be analyzed in terms of the following thermodynamic cycle:

\[
\begin{align*}
\Delta G_1 & \quad \text{U} & \quad \text{N} \quad \Delta G_4 \\
\Delta G_2 & \quad \text{U} & \quad \text{N} \quad \Delta G_4
\end{align*}
\]

where \( \text{N} \) is native oxidized enzyme, \( \text{U} \) is unfolded oxidized enzyme, and \( \text{U} \) is unfolded reduced enzyme. If the free energy of conformational stability for unfolding in the oxidized (crosslinked) enzyme is \( \Delta G_1 \), in the reduced (noncrosslinked) enzyme is \( \Delta G_4 \), and the free energy of disulfide formation in the native enzyme is \( \Delta G_3 \) and in the unfolded form is \( \Delta G_2 \), then

\[
\Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4. \tag{3}
\]

The difference in the free energy of stabilization between the oxidized and reduced enzyme \( \Delta [\Delta G_{\text{ox-red}}] \) should be equivalent to the free energy difference between \( \Delta G_1 \) and \( \Delta G_4 \); hence,

\[
\Delta [\Delta G_{\text{ox-red}}] = \Delta G_1 - \Delta G_4 \tag{4}
\]

\[
= \Delta G_3 - \Delta G_2. \tag{5}
\]

\( \Delta [\Delta G_{\text{ox-red}}] \) can be estimated from the \( T_m \) values of the oxidized and reduced proteins (Fig. 2). \( \Delta G_3 \), the free energy of disulfide formation in the native enzyme can also be estimated relative to dithiothreitol from the \( K_m \) values in dithiothreitol redox buffer. These values are given in Table 3.

A large component of \( \Delta G_2 \) is thought to derive from the decrease in configurational entropy of the unfolded protein associated with the introduction of the disulfide bridge. This entropic term \( (\Delta T S_2) \) can be estimated theoretically (22, 24) and is included in Table 3. It has been shown that the introduction of crosslinks into denatured proteins need not introduce strain (25). When this enthalpic effect \( \Delta H_3 \) is neglected, Eq. 5 reduces to

\[
\Delta [\Delta G_{\text{ox-red}}] = \Delta G_3 - \Delta T S_2. \tag{6}
\]

A direct verification of Eq. 6 is not possible because the measurements of \( \Delta [\Delta G_{\text{ox-red}}] \) and \( \Delta G_3 \) reported here were made under different conditions. In addition, we only have an estimate for the entropic part of \( \Delta G_2 \).

Nevertheless, when the lysozymes are ranked according to the stabilization associated with formation of the disulfide bond \( [\Delta [\Delta G_{\text{ox-red}}]] \) (Table 3), the proteins having greater stabilization also have a larger entropic term, \( \Delta T S_2 \). In addition, these proteins also have smaller values of \( \Delta G_3 \). Thus, the results are consistent with the expectations that large loop sizes are beneficial in contributing to the stabil-

<table>
<thead>
<tr>
<th>Variant</th>
<th>( \Delta T S_2 ) (kcal/mol)</th>
<th>( \Delta G_3 ) (kcal/mol)</th>
<th>( \Delta G_{\text{ox-red}} ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21C-142C-WT*</td>
<td>5.2</td>
<td>-2.1 ± 0.4</td>
<td>2.8</td>
</tr>
<tr>
<td>9C-164C-WT*</td>
<td>5.4</td>
<td>-1.2 ± 0.2</td>
<td>2.7</td>
</tr>
<tr>
<td>3C-54T</td>
<td>4.9</td>
<td>ND</td>
<td>1.3</td>
</tr>
<tr>
<td>21C-142C-WT*</td>
<td>3.9</td>
<td>-2.4 ± 0.2</td>
<td>1.4</td>
</tr>
<tr>
<td>127C-154C-WT*</td>
<td>3.8</td>
<td>-4.2 ± 0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

energies (Table 1) have smaller measured redox potentials (Table 3). Because of the strict stereochemical requirements (26), it is often difficult to find optimal sites for introducing disulfide bonds into proteins. The two most successful disulfides introduced into T4 lysozyme—namely, 9–164 and 21–142—both involve flexible parts of the molecule. Residue 164 is at the carboxyl terminus, which is known to be the most mobile part of the structure (9). Residues 21 and 142 are located on opposite sides of the active-site cleft (Fig. 1), where there is significant hinge-bending motion in T4 lysozyme (9). Such mobility presumably allows the protein to form the disulfide bond with the introduction of minimal strain, as shown by the low value of $\Delta G_f$ (Table 3). In contrast, the two introduced disulfides that do not increase stability (90–122 and 127–154) are both located within the carboxyl-terminal domain. This region consists of close-packed $\alpha$-helices (Fig. 1) and, as judged by crystallographic thermal parameters (9) is the most rigid part of the molecule. Thornton (26) has noted that secondary structure ($\alpha$-helix and $\beta$-sheet) often prevents formation of disulfides. In the present context, residues 90, 122, 127, and 154 are all located within $\alpha$-helices.

In summary, our results suggest that the stabilities of these engineered disulfide-bond lysozymes are determined, in large part, by a combination of the three factors mentioned above. What the two most effective disulfide bonds in T4 lysozyme have in common is a large loop size, which connects the amino- and carboxyl-terminal lobes of the molecule, and a location within a region of the molecule that is flexible. By flexible we mean a site at which the side chains and backbone can freely adjust to allow the disulfide bridge to assume optimal or near-optimal geometry. The choice of flexible sites should help not only to ensure that the protein can adjust to accommodate the stringent requirements for optimal disulfide geometry, but also to minimize the perturbation due to replacements with cysteine residues. Flexibility at a given site could be indicated by high crystallographic thermal factors, by evidence for large-scale mobility, by location close to the ends of the polypeptide chain, or by some combination of these factors.

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