Remarkable thermal stability of doubly intramolecularly cross-linked hen lysozyme

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In order to examine how a protein can be effectively stabilized, two intramolecular cross-links, Glu35–Trp108 and Lys1–His15, which have few unfavorable interactions in the folded state, were simultaneously introduced into hen lysozyme. Both of the intramolecularly cross-linked lysozymes, 35–108 CL and 1–15 CL, containing cross-links Glu35–Trp108 and Lys1–His15, respectively, showed increases in thermal stability of 13.9 and 5.2°C, respectively, over that of wild type, at pH 2.7. On the other hand, a doubly cross-linked lysozyme showed an increase in thermal stability of 20.8°C over that of wild type, under identical conditions. Since the sum of the differences in denaturation temperature between wild type and each of the cross-linked lysozymes was nearly equal to that between wild type and the doubly cross-linked lysozyme, we suggest that the efficient stabilization of the lysozyme molecule was the direct result of the double intramolecular cross-links.

Keywords: cross-linking/differential scanning calorimetry/lysozyme/thermal stability

Introduction

Proteins stability is related to their function: those derived from thermophiles are much more stable than proteins originating from mesophiles. Protein modification is one approach to improving a protein’s stability. However, we cannot always derive a modification strategy for increasing the stability of a protein from a mesophile from examples in thermophiles, as the stabilizing strategies are often different. For example, proteins from thermophiles rarely contain disulfide bonds, as they are decomposed at higher temperatures, above pH 6 (Aherm and Klivanov, 1984). Under the circumstances, how can we modify proteins derived from mesophiles in order to stabilize them effectively? A common approach to increasing a protein’s stability has been by means of point mutations or chemical modification (Imoto et al., 1987; Green et al., 1992; Yu et al., 1995). It has been reported previously that protein stability can be proportionally increased by the combination of several modifications (Serrano et al., 1990; Wells, 1990). However, the rule was not clearly established in the case of disulfide bonds (Wells, 1990).

Introduction of intramolecular cross-links by point mutation or chemical modification effectively increases the stability of mesophilic proteins (Johnson et al., 1978; Lin et al., 1984; Ueda et al., 1985; Pantoliano et al., 1987; Pace et al., 1988; Matsumura et al., 1989a,b). Matsumura et al. (1989b) have demonstrated that disulfide bonds, each of which singly contributes to the stability of T4 lysozyme, can achieve substantial overall improvement in stability when used in combination. However, since the denaturation temperature of mutant T4 lysozymes, in which cysteinyl residues are introduced, were all lower than that of wild type T4 lysozyme under reduced conditions, the unfavorable interactions in the folded state of these mutants would have occurred by point mutations to cysteines. Therefore, while Matsumura et al. (1989a,b) successfully improved the stability of T4 lysozyme by the incorporation of multiple intramolecular cross-links, further stabilization of T4 lysozyme by multiple intramolecular cross-linking may be possible by avoiding unfavorable interactions in the folded state.

Hen egg-white lysozyme is a small protein, which has four disulfide bonds (Imoto et al., 1972). Trials to improve its stability by point mutations or chemical modification have been conducted extensively (Imoto, 1996, 1997). So far, the greatest increase in stability has been the result of an ester bond cross-link between Glu35 and Trp108 (35–108 CL lysozyme), which stabilized the lysozyme molecule by 5.2 kcal/mol over that of wild type, at pH 2, in the presence of 1.94 M guanidine hydrochloride (Johnson et al., 1978). On the other hand, a cross-link between Lys1 and His15 introduced after treatment with N,N′-bisbromoacetyltrimethylene-diamine (1–15 CL lysozyme) also stabilized the lysozyme molecule by 2.3 kcal/mol over that of wild type, at pH 5.5, in the presence of 3 M guanidine hydrochloride (Ueda et al., 1985).

Chemical modification of the folded protein rarely affects the structure of the protein to any great extent. Indeed, few unfavorable interactions in the folded state of hen lysozyme were reported as a result of the introduction of cross-links between Glu35 and Trp108, and between Lys1 and His15 (Beddell et al., 1975; Ueda et al., 1985). Therefore, in this paper, in order to accumulate information directed towards the problem of ‘how effectively can proteins from mesophiles, such as lysozyme, be stabilized by multiple intramolecular cross-linking?’ we prepared a doubly intramolecularly cross-linked hen lysozyme by simultaneous introduction of cross-links Glu35–Trp108 and Lys1–His15, and examined its stability against thermal denaturation.

Materials and methods

Materials

Five-times recrystallized wild-type hen egg-white lysozyme was donated by QP (Tokyo).

Preparation of cross-linked lysozymes

1–15 CL lysozyme (Ueda et al., 1985) and 35–108 CL lysozyme (Imoto and Rupley, 1973) were prepared as described previously. For preparation of doubly CL lysozyme, where cross-links exist between both Lys1 and His15 and between Glu35 and Trp108, 50 mg 1–15 CL lysozyme was dissolved in 3 ml 1 mM acetate buffer at pH 5.5. Ten microliters of KI-I2 solution (0.48 M KI containing 0.02 M I2) was
added to the protein solution five times at room temperature over 2 h. After the addition of KI–I₂, the reaction mixture was applied directly to an ion-exchange chromatography column (Bio-Rex 70, Bio-Rad, Hercules, CA) equilibrated with 0.02 M borate buffer at pH 10. The column (2 × 70 cm) was eluted with a gradient of 1000 ml 0.02 M borate buffer at pH 10 and 1000 ml of the same buffer containing 0.1 M NaCl. The protein elution was monitored by absorbance at 280 nm. The desired protein fraction (120 ml) was collected and diluted to 300 ml with H₂O. In order to concentrate the protein solution, the solution was adjusted to pH 4 by adding acetic acid and applied to the ion-exchange chromatography column (CM-Toyopearl 650 M, 1.5 × 20 cm; Tosoh, Tokyo) equilibrated with 0.1 M acetate buffer at pH 4. The desired protein was eluted with the same buffer containing 1 M NaCl, dialyzed against distilled water at 4°C and lyophilized. The yield of doubly CL lysozyme based on 1–15 CL lysozyme was 10%.

Characterization of cross-linked lysozyme

Ultraviolet absorption spectrum measurements of doubly CL lysozyme were taken using a Perkin Elmer Lambda 10 spectrophotometer. For lytic activities, 50 µl lysozyme solution (50–100 µg/ml) was added to 3 ml 0.25 mg/ml Micrococcus luteus solution (Sigma, St. Louis, MO) and the decrease in turbidity at 450 nm was monitored.

Differential scanning calorimetry (DSC)

Before the DSC measurements were taken, a second chromatography step was performed on the 35–108 CL and doubly CL lysozymes using ion-exchange HPLC (CM-Toyopearl 650 S, 4.6 × 300 mm; Tosoh, Tokyo) to remove any impurities that had formed during the storage of these cross-linked lysozymes. The column was eluted with 200 ml 0.02 M borate buffer at pH 10 and 200 ml of the same buffer containing 0.3 M NaCl at a flow rate of 1.0 ml/min. The protein elution was monitored by absorbance at 280 nm. The desired fraction was collected, followed by dialysis against 50 mM Gly–HCl buffer, pH 2.0–3.0 at 4°C, exhaustively. Other samples for DSC measurements were dissolved in 50 mM Gly–HCl buffer at pH 2.0–3.0 and were dialyzed against the respective buffers at 4°C, exhaustively. Differential scanning calorimetry measurements were carried out with a VP-DSC calorimeter (Microcal, Northampton, MA). The scan rate was 0.5 or 1.0 K/min. Protein concentrations (25–45 µM) were checked using a amino acid analyzer after acid hydrolysis. Data analyses were done using the Origin Software (Microcal).

Results and discussion

Since the cross-linking between Glu35 and Trp108 in lysozyme is via an ester bond, the cross-link may be unstable, especially in alkaline conditions. The preparation of 1–15 CL lysozyme has to be conducted under alkaline conditions (Ueda et al., 1985). Therefore, we introduced the cross-link between Glu35 and Trp108 into 1–15 CL lysozyme, which had been prepared following the method described previously (Ueda et al., 1985) (see Materials and methods). According to the report (Imoto et al., 1973), the last peak eluted on the ion-exchange column was the desired protein, and this protein fraction was collected and dialyzed. The obtained lysozyme had no activity against Micrococcus luteus. From its UV spectrum, the ratio of absorbance at 250 nm to that at 280 nm was 1.96, which is consistent with the formation of an ester bond between Glu35 and Trp108 (Imoto and Rupley, 1973). Moreover, amino acid analysis of the collected protein confirmed the formation of a cross-link between Lys1 and His15 in the presence of the alkyl reagent (data not shown). From these results, we concluded that the collected protein did indeed contain two cross-links, Lys1–His15 and Glu35–Trp108 (doubly CL lysozyme). Since the cross-link between Glu35 and Trp108 in 35–108 CL lysozyme may be unstable due to the ester bond, a second chromatography step was performed on doubly CL lysozyme and 35–108 CL lysozyme immediately before the DSC measurements to remove any impurities. In Figure 1, a typical chromatogram of doubly CL lysozyme shows two distinct peaks. The derivative in the main peak was collected and dialyzed against 50 mM Gly–HCl buffer, pH 2.0–3.0 at 4°C, exhaustively. The derivative in the minor fraction may be an oxindolealanine 108 lysozyme derivative, judging from its ultraviolet spectrum, which is a product of 35–108 CL lysozyme hydrolysis (Imoto et al., 1973).

To determine the thermodynamic parameters for the denaturation of the wild type and modified lysozymes, DSC measurements were performed at acidic pH (pH 2.0–3.0) where the denaturation of wild type lysozyme is reversible. Typical DSC patterns for wild-type and doubly CL lysozyme at pH 2.85 are shown in Figure 2. Doubly CL lysozyme showed a single transition on denaturation, as indicated by a similar peak shape to that of wild type. Thus, we considered doubly CL lysozyme to be homogeneous under the measuring conditions. In order to examine whether or not the stability of doubly CL lysozyme and 35–108 CL lysozyme, both of which contain an ester bond between Glu35 and Trp108, can be treated with the equilibrium two-state model, ΔH_cal/ΔH_VH values for the denaturation in the DSC profiles of the first heating of wild type and modified lysozymes were obtained at pH 2.85. The value of ΔH_cal/ΔH_VH in the DSC profiles of doubly CL lysozyme and 35–108 CL lysozyme were 0.8, whereas those of the wild type and 1–15 CL lysozymes were almost unity. Privalov (1979) has suggested that the denaturation of a protein can be treated with the equilibrium two-state model when the value of ΔH_cal/ΔH_VH in the DSC profile of a protein approaches unity. In the second heating of doubly CL lysozyme, there appeared to be a small transition around a similar position to that of 1–15 CL lysozyme, indicating...
that the thermal denaturation of doubly CL lysozyme was irreversible to some extent on DSC measurement.

Imoto et al. (1973) reported that an ester bond between Glu35 and Trp108 in hen lysozyme was hydrolyzed by heating at pH 2. We set the measuring conditions of DSC at an acidic pH where the thermal denaturation of the wild type was reversible. After heating until the denaturation temperature of doubly CL lysozyme, we found a small amount of ester bond cleaved protein and no aggregation in doubly CL lysozyme solution after ion-exchange HPLC. Therefore, we judged that \( \Delta H_{\text{calc}}/\Delta H_{\text{calc}} (0.8) \) in the DSC profiles of doubly CL lysozyme and 35–108 CL lysozyme depended on a partial irreversibility due to the cleavage of the ester bond. Moreover, the denaturation temperature of doubly CL lysozyme at a scan rate of 0.5 K/min was lower by 1.0°C than at a scan rate of 1.0 K/min; the denaturation temperature of the wild type lysozyme was almost identical under both measuring conditions. The stability of the resulting lysozyme produced by the cleavage of the ester bond, oxindolealanine 108 lysozyme, was less than that of 35–108 CL lysozyme, due to the lack of an intermolecular cross-link. Therefore, the production of oxindolealanine 108 during the first heating may increase with a decrease in scan rate and this may cause the difference in the denaturation temperature between above conditions. From these results, we concluded that the thermal denaturation of the doubly CL and 35–108 CL lysozymes, which both contain an ester bond between Glu35 and Trp108 in the lysozyme molecule, proceeded irreversibly to some extent, we could not evaluate the thermodynamic parameters of these cross-linked lysozymes for reversible thermal denaturation. Therefore, to understand better the contribution of each cross-link on the stabilization of the lysozyme molecule, each free energy change of denaturation of the modified lysozyme \( [\Delta G(T)]= \) calculated according to the equations below.

\[
\Delta H(T) = \Delta H(T_m) - \Delta C_p (T_m - T) \quad (1)
\]

\[
\Delta S(T) = \Delta H(T_m)/T_m - \Delta C_p \ln (T_m/T) \quad (2)
\]

\[
\Delta G(T) = \Delta H(T) - T \Delta S(T) \quad (3)
\]

where \( T_m, T \) and \( \Delta C_p \) are the denaturation temperature of the wild type lysozyme (in kelvin), the denaturation temperature of the modified lysozyme (in kelvin) and difference in heat capacity between the folded and unfolded state of wild type lysozyme, respectively. The values of \( \Delta H(T_m) \) and \( \Delta C_p \) for wild type lysozyme were 114 ± 1 kcal/mol (at 65.6°C) and 1.6 ± 0.2 kcal/(mol·K), respectively, at pH 2.7 and were obtained by a series of denaturation experiments of wild type lysozyme at pH 2.0–3.0. Each free energy change of denaturation at the denaturation temperature of modified lysozymes is also shown in Table I.

The free energy change of denaturation of doubly CL lysozyme was calculated to be approximately 8.0 kcal/mol. The free energy change of denaturation of a protein in aqueous solution is at most 5–15 kcal/mol at physiological temperature. Therefore, the present stabilizing energy (8.0 kcal/mol) resulted from these double cross-links may be comparable with the whole free energy change of denaturation of a protein. Moreover, the sum of the difference in the denaturation temperatures between the wild type lysozyme and each cross-linked lysozyme was nearly equal to that between the wild type lysozyme and doubly cross-linked lysozyme. This indicates that the efficient stabilization of a protein molecule may be attained by the introduction of multiple intramolecular cross-links.

In this paper, we succeeded in the preparation of a modified lysozyme effectively stabilized against thermal denaturation by the introduction of two intramolecular cross-links. We found that the thermal denaturations of doubly CL lysozyme and 35–108 CL lysozyme proceeded irreversibly to some extent, and as a result, the denaturation temperatures of doubly CL lysozyme and 35–108 CL lysozyme obtained by DSC measurements were unfortunately underestimated. However, we consider that the irreversibility of the denaturation of doubly CL lysozyme and 35–108 CL lysozyme only affect thermal denaturation. However, since the thermal denaturations of doubly CL lysozyme and 35–108 CL lysozyme, which both contain an ester bond between Glu35 and Trp108 in the lysozyme molecule, proceeded irreversibly to some extent, we could not evaluate the thermodynamic parameters of these cross-linked lysozymes for reversible thermal denaturation. Therefore, to understand better the contribution of each cross-link on the stabilization of the lysozyme molecule, each free energy change of denaturation of the modified lysozyme \( [\Delta G(T)]= \) calculated according to the equations below.

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\Delta G(T) = \Delta H(T) - T \Delta S(T) \quad (3)
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where \( T_m, T \) and \( \Delta C_p \) are the denaturation temperature of the wild type lysozyme (in kelvin), the denaturation temperature of the modified lysozyme (in kelvin) and difference in heat capacity between the folded and unfolded state of wild type lysozyme, respectively. The values of \( \Delta H(T_m) \) and \( \Delta C_p \) for wild type lysozyme were 114 ± 1 kcal/mol (at 65.6°C) and 1.6 ± 0.2 kcal/(mol·K), respectively, at pH 2.7 and were obtained by a series of denaturation experiments of wild type lysozyme at pH 2.0–3.0. Each free energy change of denaturation at the denaturation temperature of modified lysozymes is also shown in Table I.

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In this paper, we succeeded in the preparation of a modified lysozyme effectively stabilized against thermal denaturation by the introduction of two intramolecular cross-links. We found that the thermal denaturations of doubly CL lysozyme and 35–108 CL lysozyme proceeded irreversibly to some extent, and as a result, the denaturation temperatures of doubly CL lysozyme and 35–108 CL lysozyme obtained by DSC measurements were unfortunately underestimated. However, we consider that the irreversibility of the denaturation of doubly CL lysozyme and 35–108 CL lysozyme only affect

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**Table I. Thermodynamic parameters for denaturation of wild type and mutant lysozymes at pH 2.7**

<table>
<thead>
<tr>
<th>Lysozyme</th>
<th>( T_m ) (°C)</th>
<th>( \Delta G ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>65.6 ± 30.4</td>
<td>–0</td>
</tr>
<tr>
<td>1–15 CL</td>
<td>70.8 ± 0.6</td>
<td>–1.7</td>
</tr>
<tr>
<td>35–108 CL</td>
<td>79.5 ± 0.4</td>
<td>–5.1</td>
</tr>
<tr>
<td>Doubly CL</td>
<td>86.4 ± 0.5</td>
<td>–8.0</td>
</tr>
</tbody>
</table>

\( \Delta G(T) \) is the difference in free energy change between the wild type and modified lysozymes at the denaturation temperature of the modified lysozyme.

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\( \Delta G(T) \) is the difference in free energy change between the wild type and modified lysozymes at the denaturation temperature of the modified lysozyme.

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**Fig. 2.** DSC profiles of the wild type and doubly CL lysozymes. Protein samples were –30 μM in 50 mM Gly–HCl buffer at pH 2.85. The scan rate was 1.0 K/min.
their thermal denaturations in a small way, because their thermal transitions were apparently single for the denaturation because their peak shapes were similar to that of the wild type lysozyme. Therefore, the present finding that the sum of the difference in the increasing denaturation temperature between the wild type lysozyme and each cross-linked lysozyme was nearly equal to that between the wild type lysozyme and doubly cross-linked lysozyme may be significant to show that the efficient stabilization of a protein molecule can be attainable by such modifications. This information is of help when designing de novo proteins, and the finding will contribute to protein engineering.

References

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