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"Loose Folding" and "Delayed Oxidation" Procedures Successfully Applied for Refolding of Fully Reduced Hen Egg White Lysozyme

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Several factors and/or procedures were examined quantitatively to improve the refolding yields of hen egg white lysozyme from its fully denatured and reduced state. Firstly, we found that refolding treatments were better conducted at lower lysozyme concentrations. The refolding yield decreased from 70% to less than 5% by increasing the lysozyme concentration from 1 to 36 μM in the refolding solution, probably due to aggregation. Secondly, in order to reduce the aggregation and improve the efficiency of refolding, we applied the "loose folding" procedure which required the incubation in the presence of about 2 M urea. The refolding of the lysozyme, studied at 17.4 μM, increased the yield to 80% yield in the presence of 2 M urea compared with a 30% yield in the absence of urea. Furthermore, we obtained a dramatic refolding yield of more than 95% in an experiment conducted at a concentration of 1.1 μM lysozyme, in the presence of 2 M urea. Finally, we examined the "delayed oxidation" procedure which meant that conformational folding preceded formation of disulfide bonds. The application of this procedure resulted in increases of 5—10% in the refolding yield. These procedures are expected to be useful in improving the refolding yield of precipitated proteins, for example, formed during recombinant DNA protein syntheses.

Keywords protein refolding; hen egg white lysozyme; aggregation; urea; disulfide bond; loose folding

Introduction

Recent advances in recombinant DNA technology have made it possible to obtain large amounts of commercially important proteins. Frequently, recombinant proteins produced in this way are unable to fold correctly within the host cell but form insoluble inactive aggregates known as inclusion bodies.[1,2] Industrially, such insoluble aggregates need to be dissolved in denaturing solvents such as guanidinium chloride (GdnHCl), urea, or detergent and the solubilized protein must be artificially refolded into its original native structure, followed by oxidation of thiol groups, if necessary.[3,4] The refolding yield of the protein from the unfolded state depends on the specific amino acid sequence of the protein itself and on the conditions of refolding. In general, protein aggregation and/or misfolding are considered to be major problems leading to low recovery of the correctly folded native protein.[5-7] It is likely that the polymorphic nature of the molten globule state is a cause of formation of these undesirable products. Theoretically, various interactions stabilizing the three-dimensional structure of the proteins, *i.e.*, hydrophobic interaction, ionic interaction, hydrogen-bonding, disulfide-bonding, must be controlled hierarchically and/or kinetically by means of systematically programmed refolding conditions to allow each protein to reach its thermodynamically favorable energy minimum.[8]

We have recently reported the possibility of replacing GdnHCl by urea plus LiCl for the unfolding of globular protein.[9] Hydrophobic interactions and ionic interactions, both of which occurred with GdnHCl were separated in the case of urea plus LiCl. Separation of two intrinsically independent interactions made it easy to select suitable refolding conditions by varying the concentrations of urea or LiCl independently.

The purpose of the present research is to establish the optimal refolding strategy for hen egg white lysozyme (lysozyme), taking account of its hydrophilic, rather than hydrophobic, character among the many globular proteins. In other words, as far as the design of refolding conditions for lysozyme is concerned, great care must be taken to construct its weak hydrophobic core correctly before proceeding to the final refolding procedure, as will be discussed later. The unfolding and refolding of lysozyme *in vitro* have been studied in detail.[10-15] The unfolded lysozyme with four intact disulfide bonds in 6 M GdnHCl solution could be refolded spontaneously by simple dilution of the denaturants.[9] However, in the case of unfolded and fully reduced lysozyme, there are some difficulties, such as enhanced liability to precipitate formation, incorrect disulfide bond formation, *etc.*, encountered during the refolding process. The recovered enzymatic activity of refolded and oxidized lysozyme, using a glutathione (GSH) redox system, has been reported to be as high as 70—80%.[10,11,15]

In the present study, we examined quantitatively various factors and/or procedures to improve the refolding yields of lysozyme. We were able to obtain an almost 100% refolding yield from fully unfolded and reduced lysozyme. The following three factors were considered to be very important in facilitating the refolding of lysozyme by preventing it from aggregating: 1) lower lysozyme concentration, 2) application of a "loose folding" procedure which means incubation for refolding in the presence of about 2 M urea, 3) application of a "delayed oxidation" procedure which means conformational refolding to take place before the formation of disulfide bonds.

Materials and Methods

Materials Hen egg white lysozyme (lysozyme) recrystallized three times was purchased from Sigma. *Micrococcus lysodeikticus* dried cell walls were also obtained from Sigma. Urea, dithiothreitol (DTT), GSH and oxidized glutathione (GSSG) were purchased from Nacalai Tesque. All other chemicals used were of analytical grade.

Lysozyme Assay *Micrococcus lysodeikticus* suspension (0.25 mg/ml, initial turbidity at 450 nm, about 1.0 absorbance unit) was prepared in

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50 mM sodium phosphate buffer at pH 6.2.[16] To 3.0 ml of cell suspension, a protein solution of between 10 and 100 μl was added and mixed well in a 1.0 cm path length cuvette. The reaction was carried out at 25 °C. Care was taken to keep the ionic strength carried in the assay solution below 0.02 M.[17] The decrease in turbidity was measured at 450 nm in a Hitachi UV-228 double beam spectrophotometer for 3 min. Daily standardizations using native lysozyme were carried out. The concentrations of protein was determined spectrophotometrically: $A_{280}^{1\%}=23.7$ for reduced lysozyme, $A_{280}^{1\%}=26.3$ for native lysozyme.[10]

Preparation of Fully Reduced Lysozyme The fully reduced lysozyme was prepared using a slight modification of the procedure of Perraudin *et al.*[15] Lysozyme solution (10 mg/ml) in 0.1 M Tris–HCl/10 M urea/65 mM DTT/1.0 mM EDTA, pH 8.5, was incubated for 2 h at 40 °C under a nitrogen atmosphere. The solution was then acidified to pH 3 with glacial acetic acid. The mixtures were applied to a column (1.4 × 50 cm) of Sephadex G-25 (coarse grade) which was equilibrated and eluted with 0.1 M acetic acid. The fractions containing reduced lysozyme were lyophilized and stored under nitrogen at −20 °C. The assay, using Ellman's reagent,[18] gave 7.5—8.5 free thiol groups per lysozyme molecule, as expected from complete reduction.

Refolding Procedure Refolding was performed according to the method of Saxena and Wetlaufer[10] with slight modifications. Generally, the lyophilized reduced lysozyme was dissolved in 0.1 M acetic acid or in 0.1 M sodium acetate buffer containing 8 M urea at pH 5.0. The solution was then rapidly diluted 10-fold with renaturation buffer which consisted of 0.1 M Tris–HCl/1.0 mM EDTA/3 mM GSH/0.3 mM GSSG at pH 8.0. The diluted solution was incubated at 38 °C for different periods and the enzymatic activity assayed. The refolding yields of lysozyme were given as percentages relative to the native lysozyme of an equimolar concentration. In the case of experiments to investigate the effect of residual urea concentrations on the refolding, the desired amount of urea was added to renaturation buffer. In order to examine the effect of a "delayed oxidation" procedure, reduced lysozyme was first diluted with buffer in the absence of GSH and GSSG, then GSH and GSSG added 1 min later. In a few experiments, instead of using lyophilized, reduced lysozyme, the freshly reduced lysozyme solution 100 μM each for every experiment was directly diluted 100-fold with renaturation buffer which consisted to 0.1 M Tris–HCl/1.0 mM EDTA/1.0 mM GSH/0.5 mM GSSG, pH 8.0. In this system, the refolding yield was similar to that obtained using a lyophilized, reduced sample. Other experimental modifications are presented under Results and Discussion.

Results and Discussion

Protein Concentration Dependency We examined the effect of lysozyme concentrations on the refolding of the fully denatured and reduced lysozyme. Denatured and reduced lysozyme, at various concentrations in 0.1 M acetic acid, was rapidly diluted 10-fold with renaturation buffer which consisted of 0.1 M Tris–HCl/1 mM EDTA/3 mM GSH/0.3 mM GSSG, at pH 8.0, at 38 °C with vigorous stirring. Aliquots of the solution were subjected to the activity assay after various periods of incubation at 38 °C. Refolding yield was given as the percentage of recovered activity relative to the native lysozyme solution of the corresponding concentration. Figure 1 shows the time course of refolding yields on lysozyme concentration ranging between 1.1 and 36.0 μM. The refolding yields were strongly dependent on the concentration of lysozyme in the renaturation buffer. At a lysozyme concentration of 1.1 μM, the refolding yield increased rapidly, reaching about 70% in 90 min. In contrast, the final refolding yield obtained with a 17.4 μM lysozyme solution was below 30%. Furthermore, at the lysozyme concentration of 36 μM, very little activity was recovered even after long incubation periods. Thus, we have demonstrated that high refolding yields are achieved at lower lysozyme concentrations. As a matter of fact, in the case of low refolding yields with protein concentrations greater than 11.8 μM, a white suspension was observed in the renaturation buffer immediately after dilution. Evidently, the loss of activity is strongly linked to such aggregate formation, caused by intermolecular interactions. These results have been quantitatively well documented in a recent paper which studied the competition between renaturation and aggregation during the refolding of lysozyme.[14] Thus, one could expect that suppression of aggregation, by lowering the enzyme concentration, may result in an increased yield of active protein in the refolding process.

Effect of Urea Concentration in the Refolding Solution ("Loose Folding" Effect) In order to improve the refolding yields, especially at higher lysozyme concentrations, experiments were conducted using a urea-containing renaturation buffer. The effect of the urea concentration in the renaturation buffer is shown in Fig. 2 for an experiment conducted at a lysozyme concentration of 17.7 μM. In the absence of urea, the refolding yield at a lysozyme concentration of 17.7 μM is usually less than 30% due to irreversible aggregation during renaturation. As seen in

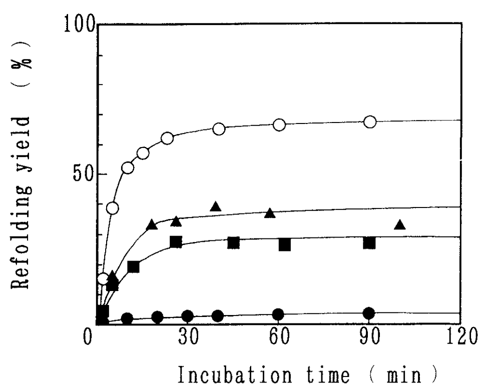

Fig. 1. Effect of Lysozyme Concentration on the Refolding Yield

Denatured and reduced lysozyme dissolved at various concentrations in 0.1 M acetic acid were rapidly diluted 10-fold with renaturation buffer which consisted of 0.1 M Tris–HCl/1 mM EDTA/3 mM GSH/0.3 mM GSSG at pH 8.0 and at 38 °C with vigorous stirring. The lysozyme concentrations in the renaturation buffer were: 1.1 μM (○), 11.8 μM (▲), 17.4 μM (■), 36.0 μM (●). After various periods of the refolding, their recovered activities were assayed. The refolding yields were given as the percentage of recovered activity relative to the native lysozyme solution of an equimolar concentration.

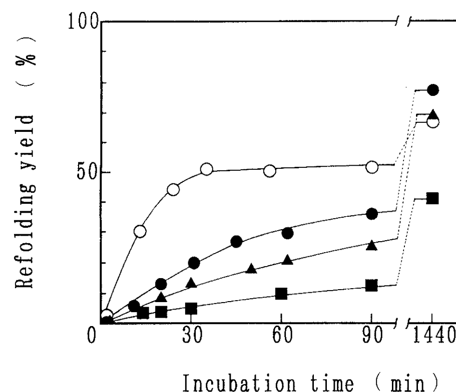

Fig. 2. Effect of Urea Concentration on the Refolding at a Lysozyme Concentration of 17.7 μM

Denatured and reduced lysozyme solutions in 0.1 M sodium acetate buffer containing 8 M urea, pH 5.0, were rapidly diluted 10-fold with renaturation buffer which consisted of 0.1 M Tris–HCl/1 mM EDTA/3 mM GSH/0.3 mM GSSG at pH 8.0, to give the desired final concentration of urea. The final urea concentrations in the renaturation buffer were: 0.8 M (○), 2.0 M (●), 2.6 M (▲), 4.4 M (■). The lysozyme concentration in all the renaturation buffers was 17.7 μM. Activity was assayed after various periods of refolding at 38 °C. The refolding yields were given as the percentage of recovered activity relative to the native lysozyme solution of 17.7 μM.

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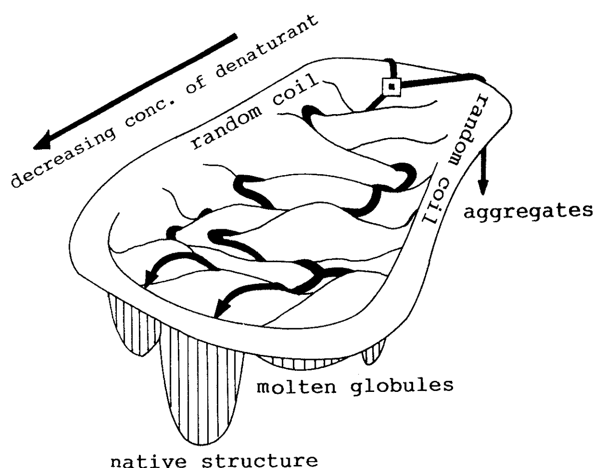

Fig. 3. A Schematic Potential Energy Surface Indicating the Polymorphic Nature of the Protein Folding Process

Fig. 2, however, refolding in the presence of 2 M urea gave a final yield of approximately 80% after an overnight incubation. The suspension, which could be observed in the absence of urea under such long-term incubation, was never observed. On the other hand, higher concentrations of urea (4.4 M) had an adverse effect on the refolding yield, although formation of a suspension was not observed. Furthermore, the apparent rate of refolding in the presence of urea was slower than that in the absence of urea. Consequently, it took almost 24 h to obtain the maximum refolding yield. One explanation for the above results is illustrated in Fig. 3. Unfolded, fully reduced lysozyme in a random coil state could be induced to refold by dilution of the denaturants. The "loose folding" state could be obtained at relatively low urea concentrations, such as 2 M, or it might be related to the so-called "molten globule"[19]-like structures. In this stage of refolding, the system exhibits a polymorphic nature consisting of many possible three-dimensional conformations of the loosely folded lysozyme, each searching for its correct structure by crossing decreased activation energy barriers produced by the presence of 2 M urea. An other important point is that, throughout these long reaction times, intermolecular interactions leading to the formation of aggregates are prevented from operating effectively by the presence of 2 M urea. At high urea concentrations, such as 4.4 M, however, no substantial conformation could form owing to the extremely weakened intramolecular interactions necessary to form the three-dimensional structure of lysozyme. Of course, intermolecular interactions are weak. Thus, it seems important to maintain a "loose folding" state while in 2 M urea solution before refolding takes place in order to achieve good refolding yields.

Furthermore, we examined the effect of urea at a lower lysozyme concentration of 1.1 μM. Recovery of activity at this lysozyme concentration was always about 70%, without the loose folding procedure using 2 M urea, as seen in Fig. 1. The refolding yield under conditions similar to those reported earlier by Saxena and Wetlaufer was 70—80%,[10] while others have reported it to be 30—40% in the recent literature.[14] An experiment to improve the refolding yields of lysozyme at a concentration of 1.1 μM was performed using renaturation buffers containing different amount of urea, as seen in Fig. 4. Refolding yields at a concentration

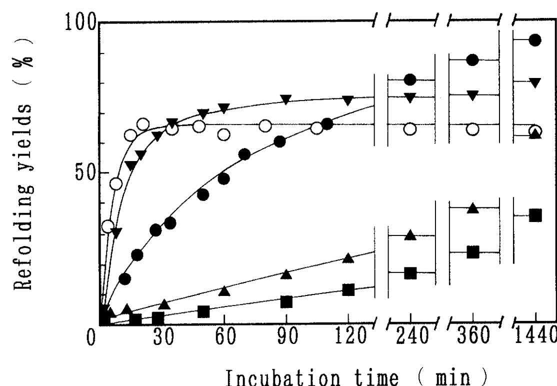

Fig. 4. Effect of Urea Concentration on the Refolding, Tested at a Low Lysozyme Concentration of 1.1 μM

The freshly reduced lysozyme solution, prepared in 0.1 M Tris–HCl/1 mM EDTA/8 M urea/10 mM DTT at pH 8.5, was directly diluted 100-fold with renaturation buffer which consisted of 0.1 M Tris–HCl/1.0 mM EDTA/1.0 mM GSH/0.5 mM GSSG at pH 8.0 and at 38 °C, to give the desired final concentration of urea. The urea concentrations in the renaturation buffer were: 0 M (○), 1 M (▼), 2 M (●), 4 M (▲), 4.8 M (■). The lysozyme concentration in all the renaturation buffers was 1.1 μM.

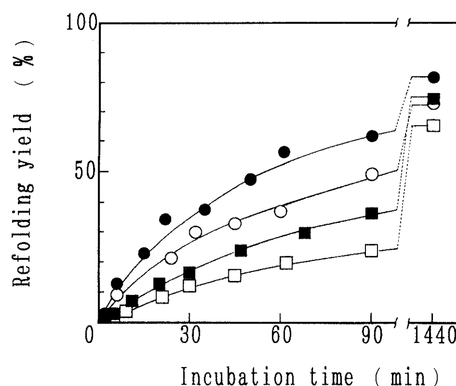

Fig. 5. Effect of the "Delayed Oxidation" Procedure on the Refolding

The results using the "delayed oxidation" procedure in the presence of 1.8 M urea (●) and 2.6 M urea (■) were shown compared with control experiments without this delay in the presence of 1.8 M (○) and 2.6 M urea (□). Denatured and reduced lysozyme at 177 μM was diluted 10 fold with 0.1 M Tris–HCl at pH 8.0 which contained different concentrations of urea to given a final urea concentration of 1.8 or 2.6 M, at room temperature. Then, after 1 min, reoxidation of this lysozyme solution was initiated by the addition of GSH and GSSG to give an initial concentration of 3 and 0.3 mM, respectively. The mixture was incubated for various periods at 38 °C and the activity determined. The control experiments, without a delay, were conducted using renaturation buffer containing 1.8 or 2.6 M urea in the presence of 3 mM GSH and 0.3 mM GSSG.

of 2 M urea gradually increased with time, reaching more than 95% after overnight incubation. Also in this case, 4.8 M urea did not improve the yield. In this experiment, we have demonstrated the importance of holding the protein in its "loose folding" state under mild urea concentrations.

A similar technique has been shown to be effective in improving the refolding yields of various recombinant proteins from inclusion bodies by use of chaotropic agents at non-denaturing concentrations.[4,20] We report here quantitative results on the "loose folding" effect for the refolding of lysozyme, allowing a maximum yield of more than 95% to be achieved together with a detailed discussion of the phenomenon.

"Delayed Oxidation" Effect We examined the "delayed oxidation" effect as another important procedure for increasing the refolding yield, especially in the case of refolding at higher lysozyme concentrations. Denatured and reduced lysozyme, at a concentration of 177 μM, was diluted

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10 fold with 0.1 M Tris–HCl buffer at pH 8.0 containing urea to give a final urea concentrations of 1.8 or 2.6 M at room temperature. After 1 min, reoxidation of this lysozyme was initiated by the addition of a mixed solution of GSH and GSSG to give an initial concentration of 3 and 0.3 mM, respectively in the refolding buffer. Then, the mixture was incubated for different periods at 38 °C. During the 1 min period, the refolding proceeds in the absence of GSH and GSSG. The formation of disulfide bonds is completely suppressed, because the protein solutions were degassed before use and the experiment was conducted under a nitrogen atmosphere. The control experiments without the 1 min "delay" were performed using the same renaturation buffer to examine the effect of "delayed oxidation". As shown in Fig. 5, the effect of this "delayed oxidation" procedure on refolding resulted in 5—10% higher refolding yields than those conducted without using this procedure. This result suggests that, within 1 min, the "loose folded" conformation, similar to that of the native material, might be formed in the 2 M urea, and the specific formation of disulfide bonds might be facilitated. On the other hand, a concurrent oxidation, *i.e.*, without the delayed oxidation to the refolding, might result in the formation of nonspecific, "scrambled" disulfide bond formation.

Conclusions

In our present study, we have described a strategy for refolding the unfolded and fully reduced lysozyme correctly and in high yields. Firstly, refolding procedures have to be conducted at low protein concentrations. In our results, the refolding yield decreased from 70% to less than 5% at lysozyme concentrations of 1 and 36 μM, respectively, probably owing to aggregation.

Next, in order to reduce the aggregation and improve the efficiency of refolding, we used a renaturation buffer containing urea at relatively low concentrations. As a result, the refolding yield of lysozyme studied at 17.4 μM increased in the presence of 2 M urea to 80% from an initial value of 30% obtained in the absence of urea. Furthermore, we have obtained a dramatic yield of more than 95% in an experiment conducted at a concentration of 1.1 μM lysozyme in the presence of 2 M urea. We believe that this is the highest yield so far reported for the refolding of reduced lysozyme. In "loose folding" media, lysozyme molecules can not aggregate but fold loosely to reach their own unimolecular energy minimum of the correct three-dimensional structure.

Finally, we examined the "delayed oxidation" procedure, giving refolding yields of reduced lysozyme 5—10% higher than the control experiments. This result means that, by a short-period of incubation in the presence of urea prior to reoxidation, the formation of "incorrect" disulfide bonds can be considerably suppressed by the action of the GSH–GSSG redox system.

Our strategies described above will be useful for improving the refolding yield for proteins like lysozyme. Of course, there may be many other factors influencing the refolding yield. For example, ionic strength, pH, temperature, stirring speed, *etc.*, should be carefully controlled and these experiments are now under way. However, the various factors influencing the refolding process might be different in terms of their effects on each protein. Therefore, care must be taken when applying the present results to other proteins. Our ultimate objective is to formulate quantitatively the correlations among the three main factors, *i.e.*, the refolding yields, the various interactions that stabilize proteins, and the design of the refolding environment.

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