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ORIGINAL RESEARCH PAPER

Refolding of partially and fully denatured lysozymes

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Abstract Lysozyme refolding with high yields sometimes results from incomplete denaturation. Dithiothreitol (DTT) is a reductant commonly used to reduce and unfold disulfide-stabilized lysozymes. Through the use of fluorescence spectroscopy to access the extent of denaturation, we found that the rate and extent of denaturation highly depended on the concentration of DTT. Further, the denaturation exhibited a two-phase transition at a high DTT concentration with DTT at >100 mM and long denaturation time (>24 h) being needed for complete denaturation. A low DTT concentration and a short denaturation time resulted in fast refolding with high activity recovery, while a high DTT concentration and a long denaturation time resulted in slow refolding with low activity recovery. Hence, the renaturation of disulfide-containing lysozyme was highly affected by the extent of denaturation.

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Introduction

Production of foreign recombinant proteins often results in the formation of inclusion bodies from which protein recovery and renaturation are critical steps. To demonstrate the effectiveness of refolding methods, denatured proteins are often obtained by purposely unfolding native ones. The yield of the refolded protein can then be easily calculated.

Denaturation of native proteins containing no disulfide bonds is a process by which hydrophobic interactions and hydrogen bonds are destabilized. An excess amount of a chaotropic agent, such as guanidine hydrochloride or urea, is added to disrupt these interactions (Terashima and Suzuki 1995). However, some proteins, such as lysozyme, are further stabilized by disulfide bonds. There are four disulfide bonds stabilizing the conformation of hen egg white lysozyme and the use of a chaotropic agent alone is incapable of denaturing it. Therefore, a reducing agent such as dithiothreitol (DTT) or mercaptoethanol is usually used together with the chaotropic agent to unfold disulfide bond-stabilized proteins. The extent and rate of unfolding/denaturation are determined by the concentration of the reducing agent, which

also affects the subsequent refolding process. Many refolding studies do not clearly report the final state of the unfolded protein. As shown in Table 1, the denaturation conditions in the literature are seldom the same, implying that partially unfolded proteins might have been used in some refolding studies, and thus the effectiveness of the refolding method is still unknown for fully denatured proteins.

We therefore attempted to investigate the effect of partial unfolding/denaturation on lysozyme refolding. The extent of unfolding needs to first be characterized. However, the presence of DTT results in very high absorbance that masks the protein ellipticity and thus hinders the determination of unfolding by circular dichroism (Panda and Horowitz 2000). So, only the intrinsic fluorescence spectrum of tryptophan has been adopted to represent the extent of protein denaturation (Touch et al. 2004). When proteins are exposed to a chaotropic reagent, tryptophans in the inner hydrophobic moiety are exposed to the bulk solution, and a red shift in the wavelength at the maximum emission intensity is often observed (Copeland 1993; Ladokhin 2000).

A direct dilution method is used to refold the lysozyme. In addition, a low concentration of a chaotropic agent is used to prevent the aggregation of partially folded proteins and to subsequently improve the refolding yield (Lanckriet and Middelberg 2004). The optimum ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) varies as the amount of residual DTT varies (Raman and Ramakrishna 1996). Hence, the GSH/GSSG ratio in the presence of DTT is critical. Our results indicated that activity recovery, even for fully reduced/denatured lysozyme, can be improved by up to 80–90% under optimal conditions.

Materials and methods

Materials

Hen egg white lysozyme (EC 3.2.1.17, MW: 14.5 kDa, 42,500 units/mg) and dried cells of *Micrococcus lysodeikticus* were purchased from Sigma. Other chemicals used throughout this study were of reagent grade.

Enzyme activity assays

Lysozyme activity was determined by the rate of lysis of *Micrococcus lysodeikticus* cells. A lysozyme solution (0.12 ml) was added to a suspension (0.63 ml) of *M. lysodeikticus* (0.4 mg/ml) in 50 mM phosphate buffer (pH 7.0). The decrease in turbidity was monitored at 450 nm (OD_{450}) at 25°C over several minutes. Lysozyme activity was calculated from the slope of the curve of OD_{450} vs. time in the linear range. One unit of lysozyme activity produced a decrease in OD_{450} of 0.003467/min (1 cm light path).

Protein denaturing

Various amounts of dithiothreitol (DTT) were added to the denaturing buffer (8 M urea, 0.2 M NaCl, 1 mM EDTA, and 0.1 M Tris/HCl; pH 8.3) to denature the lysozyme. The extent of denaturation was monitored by the intrinsic fluorescence of tryptophan. The excitation wavelength was

Table 1 Conditions for lysozyme unfolding

References	Lysozyme (mg/ml)	Denaturant	DTT (mM)	Buffer	Unfolding time (h)
Chang and Liu (2006)	5	8 M urea	10	0.1 M Tris, pH 8.6	24
Katoh and Katoh (2000)	5–35	8 M urea	10	0.1 M Tris, pH 8.5	2
Dong and Wang (2002)	30–80	8 M urea	30	0.1 M Tris, pH 8.5	1.33
Lu et al. (2005; Lu and Zhang 2006)	50	8 M urea	30	0.1 M Tris, pH 8.6	3
Buswell and Middelberg (2002)	3–25	8 M urea	32	0.05 M Tris, pH 8.0	1
Li and Liu (2004)	10	6 M Gdn/HCl	150	0.1 M Tris, pH 8.6	3
Shimizu and Fujimoto (2000)	20	6 M Gdn/HCl	150	0.1 M Tris, pH 8.6	15
Batas and Chaudhuri (1995)	10–80	8 M urea	150	0.1 M Tris, pH 8.6	2
Gu and Su (2001)	2.6–30	8 M urea	200	0.1 M Tris, pH 8.7	4–5

280 nm, and the emission spectrum from 300 to 400 nm was recorded to determine the wavelength of the maximum emission and the red-shift.

Effects of the concentration of oxidized glutathione (GSSG) on lysozyme refolding

Fully reduced/denatured lysozyme (1 mg/ml) was obtained by incubating the lysozyme in a denaturing buffer containing 40 mM DTT for more than 30 h. The fully denatured lysozyme was then reactivated by 100-fold dilution in the refolding buffer (1 mM EDTA and 0.1 M Tris/HCl; pH 8.3) containing various amounts of GSSG.

Effects of partial unfolding/denaturation on protein refolding

Partially denatured lysozyme (5 mg/ml) was obtained either by 30 h of reduction in denaturing buffers containing various amounts of DTT, or by incubation in 0.2 M DTT denaturing buffer for various lengths of time. The partially denatured lysozyme was refolded by a 50-fold dilution in the refolding buffer, and the refolding processes were monitored by the blue shift in the wavelength at the maximum emission intensity.

Results and discussion

Denaturation of lysozyme

Lysozyme was not unfolded by urea alone

Native and urea-treated lysozymes were monitored by the intrinsic fluorescence of tryptophans in the protein, as shown in Fig. 1(a). Native lysozyme had been stored in a Tris/HCl buffer (0.1 or 1 mM EDTA; pH 8.3) for 24 h, and the wavelength at maximum emission intensity (λ_{max}) was 341.2 nm. Urea-treated lysozyme, incubated in another Tris/HCl buffer plus 8 M urea and 0.2 M NaCl for 24 h, had a λ_{max} of 339.2 nm showing that 8 M urea did not disrupt the tertiary structure of the lysozyme.

Fully unfolded lysozyme could only be obtained after DTT was added to the urea-treated sample (0.01 mg protein/ml). As shown in Fig. 1(b), the

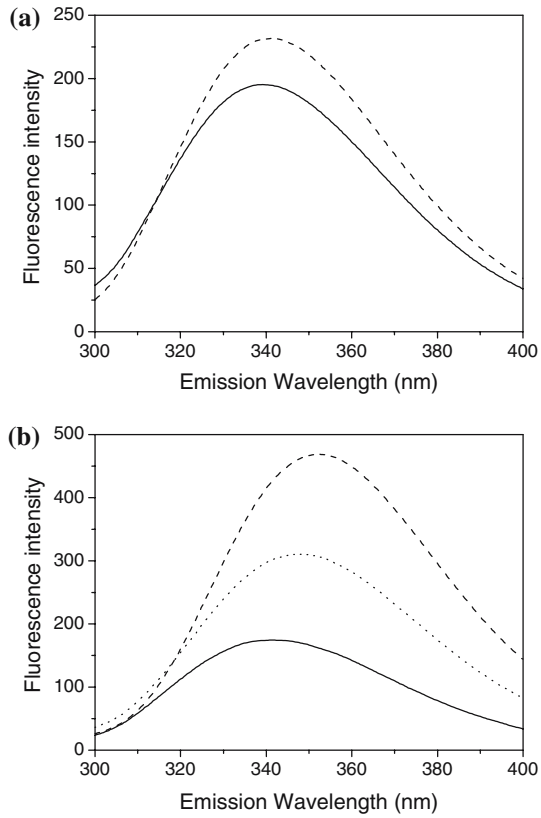

Fig. 1 Fluorescence spectrum of 0.01 mg lysozyme/ml. (**a**) Native lysozyme (- - -) freshly prepared in 0.1 M (pH 8.3) Tris buffer containing 1 mM EDTA, λ_{max} = 341.2 nm. Urea-treated lysozyme (—) denatured in denaturing buffer for 1 h, λ_{max} = 339.2 nm. (**b**) Lysozyme incubated for 1 min (—) with λ_{max} = 342.4 nm, 4 h (- - -) with λ_{max} = 348 nm, 24 h (···) with λ_{max} = 352 nm in denaturing buffer containing 1 mM DTT. The denaturing buffer contained 8 M urea, 0.2 M NaCl, and 1 mM EDTA in 0.1 M (pH 8.3) Tris buffer

λ_{max} shifted to a longer wavelength as the incubation time increased. The λ_{max} of fully denatured lysozyme was 352 nm. The fully unfolded lysozyme not only had a longer λ_{max} wavelength, but also had a higher emission intensity than the native and urea-treated lysozymes. The results confirmed that the disulfide bonds are critical for stabilizing lysozymes (Touch et al. 2004).

Effect of the concentration of DTT on lysozyme unfolding

By monitoring the unfolding process through the fluorescence spectrum of a 0.01 mg/ml lysozyme

solution, the unfolding rate strongly depended on the concentration of DTT. In Fig. 2(a), when lysozyme was denatured in a buffer containing 8 M urea and 2.8 μM DTT, the peak of the fluorescence spectrum, λ_{max}, shifted only slightly even after 30 h of incubation. As the concentration of DTT increased to 0.2 mM, lysozyme rapidly unfolded with a λ_{max} of 348 nm and then slowly underwent a second phase of unfolding to λ_{max} of 352 nm. At DTT concentrations higher than 10 mM, lysozyme rapidly unfolded to the final state of λ_{max} = 352 nm (Fig. 2).

The unfolding rate was also dependent on the lysozyme concentration. With lysozyme at 5 mg/ml, two-phase unfolding was observed at DTT above 10 mM (Fig. 2b). The rates of the first phase of unfolding were similar when lysozyme (5 mg/ml) was incubated in 30, 100, and 200 mM DTT. The concentration of DTT affected only the rate of the second phase of unfolding.

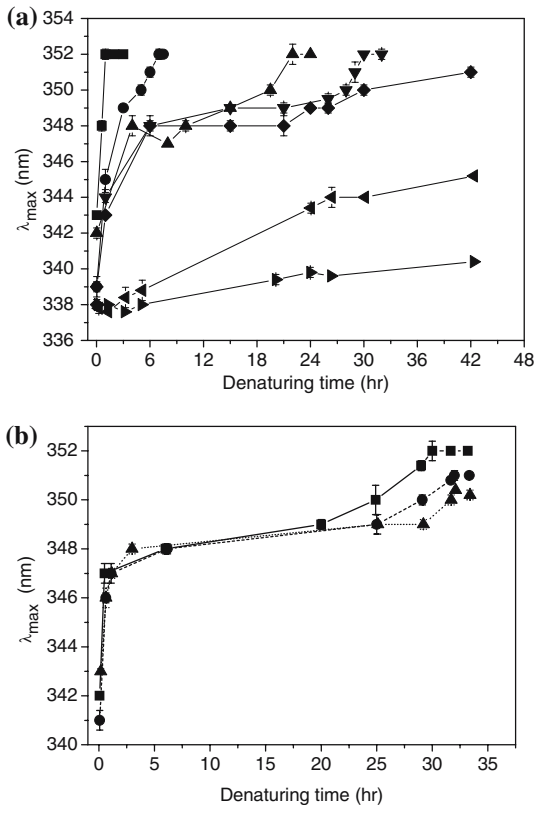

Fig. 2 Extent of denaturation of lysozyme. (**a**) Lysozyme at 0.01 mg/ml was denatured in denaturing buffer containing 10 (■), 4 (•), 1 (▲), 0.4 (▼), 0.2 (◆), 0.014 (◀), and 0.0028 (▶) mM DTT. (**b**) Lysozyme at 5 mg/ml was denatured in 8 M urea denaturing buffer containing 0.2 (■), 0.1 (•), and 0.031 (▲) M DTT. The denaturation was monitored by the λ_{max} of intrinsic fluorescence of tryptophan residues of lysozyme (n = 3)

Optimization of lysozyme refolding by direct dilution

Effect of the concentration of GSSG on lysozyme refolding

The GSH/GSSG ratio is critical for refolding disulfide bond-containing proteins. As the residual DTT from the denatured lysozyme solution could function as a reducing agent, like GSH, only oxidized glutathione (GSSG) was needed for lysozyme refolding. The oxidoreductive factor (α), defined as the molar ratio of GSSG to DTT, was used to evaluate its effect on lysozyme refolding. A small amount of residual chaotropic agent was beneficial to protein refolding. A low concentration of guanidinium hydrochloride or urea prevented aggregate formation during protein refolding (Gu and Su 2001). Our results indicated that the addition of 1.5 M urea to the refolding buffer significantly decreased the amount of lysozyme aggregates (data not shown). So, the effect of the GSSG concentration on the refolding yield is shown in Fig. 3 with the addition of 1.5 M urea to the refolding buffer.

The fully reduced/denatured lysozyme was more difficult to refold than partially denatured lysozyme in most other research because a higher GSSG concentration was needed to overcome the influence of the residual DTT and to re-form the reduced disulfide bonds. When the concentration of GSSG was lower than the residual concentration of DTT ($\alpha < 1$), there was almost no recovery of lysozyme activity, even after being refolded for 3 h. When increasing the value of α, the refolding rate and activity recovery also increased. Our results are similar to those reported by Raman and Ramakrishna (1996) but differ from results obtained by Hevehan and Clark (1996). Our results showed that low-activity recovery was obtained when α was less than 1 and the recovery increased with increasing α. However, Hevehan and Clark (1996) reported that the refolding yield

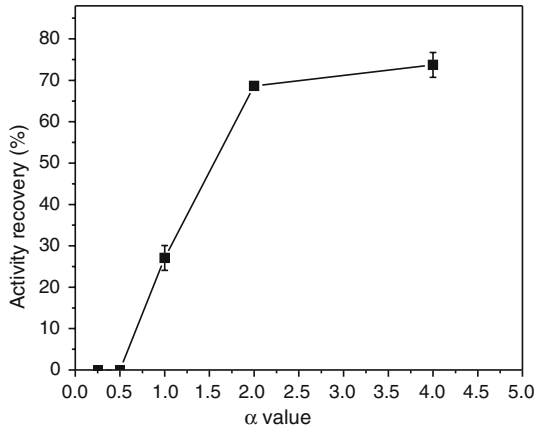

Fig. 3 Effect of the oxidized glutathione (GSSG) concentration added to the refolding buffer on activity recovery. Fully reduced/denatured lysozyme was refolded by a 50-fold dilution (at 0.1 mg/ml) in 0.1 M (pH 8.3) Tris buffer containing 1.5 M urea and 1 mM EDTA, with different α values (α = molar ratio of GSSG to DTT) in 0.1 M (pH 8.3) Tris buffer for 3 h. The specific activity of native lysozyme (100% activity) was 42,500 units/mg). The mass recovery was determined by the protein absorbance at 280 nm, and all values were between 95% and 97% (n = 3)

did not increase with an increasing amount of GSSG. On the contrary, the results reported by Raman and Ramakrishna (1996) were similar to ours. Our data indicated that an α value equal to 2 was sufficient for the oxidative refolding of lysozyme (data not shown).

Effect of incomplete denaturation on lysozyme refolding

Many instances of superior refolding might have resulted from incomplete unfolding, which resulted from either a low amount of reducing agent or a short unfolding time. We examined the possible effects of incomplete denaturation on subsequent refolding by denaturing lysozyme at different DTT concentrations and for different denaturation times. The refolding of lysozyme was carried out under the obtained optimal conditions: urea at 1.5 M and an α value of 2. The rate and yield of refolding were monitored by fluorescence spectroscopy and an activity assay. Lysozyme at 5 mg/ml was denatured in denaturing buffer containing 1.5, 7, 35, or 200 mM DTT for 30 h. The unfolded protein was then refolded

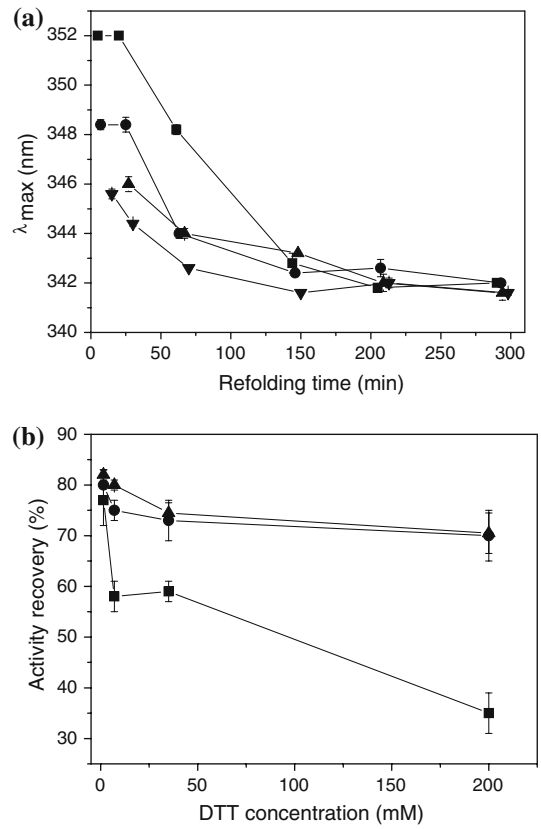

Fig. 4 Effect of partial denaturation on lysozyme refolding. Lysozyme (5 mg/ml) was denatured in denaturing buffer containing different DTT concentrations for 30 h and were refolded by a 50-fold dilution of refolding buffer (α = 2) containing 1.5 M urea. (**a**) Refolding process of lysozymes with different extents of denaturation caused by different DTT concentrations: 200 (■), 35 (•), 7 (▲), and 1.5 (▼) mM DTT, monitored by the blue-shift of λ_{max} in the fluorescence spectrum (with excitation at 280 nm). (**b**) Activity recovery of lysozymes with different extents of denaturation over time: 100 (■), 220 (•), and 320 (▲) min. The specific activity of native lysozyme (100% activity) was 42,500 units/mg). The mass recovery was determined by protein absorbance at 280 nm, and all values were between 95% and 97% (n = 3)

by a 50-fold dilution. As shown in Fig. 4(a), at the beginning, the lysozyme was completely reduced/denatured by 200 mM DTT and had a λ_{max} of 352 nm in the fluorescent spectrum, and the lysozyme, partially denatured by 1.5, 7, or 35 mM DTT, had a λ_{max} of 346–348 nm. As shown in Fig. 4(b), the fully unfolded lysozyme slowly refolded toward the native state, but the partially unfolded lysozyme had faster refolding rates.

Partially unfolded lysozyme was also obtained by varying the unfolding time. Lysozyme at 5 mg/ml was incubated in a buffer containing 200 mM DTT for different times. The refolding of lysozyme was also carried out at a urea concentration of 1.5 M and an α value of 2. As shown in Fig. 5(a), the λ_{max} in the fluorescent spectrum of the fully unfolded lysozyme (those being denatured for 30 h) returned slowly to the native state with a λ_{max} of 341 nm. The lysozyme in the intermediate state (those being denatured for 8 h) refolded much faster, and slightly denatured lysozyme (those being denatured for 1 h) rapidly returned to the native state. The activity assay showed similar behaviors. As shown in Fig. 5(b), 80% activity recovery could easily be achieved if the lysozyme had been exposed to the unfolding buffer for only 1 h. But it took 4 h for the fully unfolded sample to recover 60% of its activity.

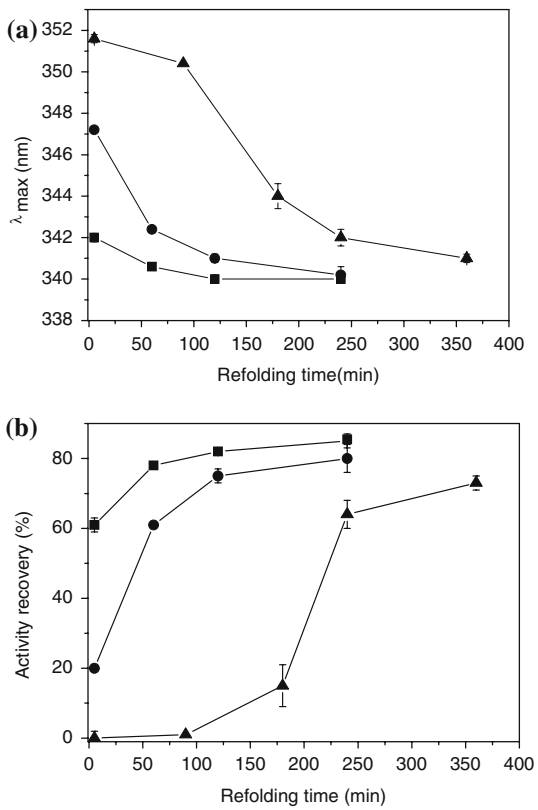

Fig. 5 Effect of partial denaturation on lysozyme refolding. Lysozyme (5 mg/ml) was denatured in 8 M urea and 200 mM DTT denaturing buffer for different lengths of time: 1 (■), 8 (•), and 30 (▲) h and then refolded by a 50-fold direct dilution in refolding buffer (α = 2) containing 1.5 M urea. (**a**) Refolding process of lysozymes with different extents of denaturation caused by varying denaturing times and monitored by the blue-shift of λ_{max} in the fluorescence spectrum (with excitation at 280 nm). (**b**) Activity recovery of lysozymes with different extents of denaturation over time. The specific activity of native lysozyme (100% activity) was 42,500 units/mg. The mass recovery was determined by the protein absorbance at 280 nm, and all values were between 95% and 97% (n = 3)

Conclusions

Many refolding methods have been proposed to efficiently renature proteins to their native state. However, the state of the unfolded protein has not been carefully examined. Partially unfolded proteins can easily be refolded with high activity recovery. But the refolding of fully unfolded proteins is much more difficult. Our studies showed that it requires a high concentration of a reducing agent and a long incubation time to fully denature lysozyme. The amount of the reducing agent needed to fully unfold lysozyme is also dependent on the protein concentration. Further, lysozyme in a partially unfolded intermediate state might be obtained before undergoing refolding. The effectiveness of refolding methods might be misjudged if the unfolded state has not been clearly characterized.

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