Unassisted Refolding of Urea Unfolded Rhodanese*

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In vitro refolding after urea unfolding of the enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) normally requires the assistance of detergents or chaperonin proteins. No efficient, unassisted, reversible unfolding/folding transition has been demonstrated to date. The detergents or the chaperonin proteins have been proposed to stabilize folding intermediates that kinetically limit folding by aggregating. Based on this hypothesis, we have investigated a number of experimental conditions and have developed a protocol for refolding, without assistants, that gives evidence of a reversible unfolding transition and leads to >80% recovery of native enzyme. In addition to low protein concentration (10 μg/ml), low temperatures are required to maximize refolding. Otherwise optimal conditions give <10% refolding at 37 °C, whereas at 10 °C the recovery approaches 80%. The unfolding/refolding phases of the transition curves are most similar in the region of the transition, and refolding yields are significantly reduced when unfolded rhodanese is diluted to low urea concentrations, rather than to concentrations near the transition region. This is consistent with the formation of "sticky" intermediates that can remain soluble close to the transition region. Apparently, nonnative structures, e.g. aggregates, can form rapidly at low denaturant concentrations, and their subsequent conversion to the native structure is slow.

Rhodanese has been difficult to reactivate after unfolding in urea or guanidine hydrochloride. Under some conditions, a small fraction of unfolded rhodanese could be reactivated to a product with high specific activity (8). However, the reactivation was inefficient, and thermodynamic reversibility was not demonstrated. Aggregation was a major competition to the regain of enzyme activity.

In general, aggregation is a significant side reaction that kinetically competes with protein refolding (9-11). Therefore, minimizing intermolecular interactions that lead to aggregation of partially folded intermediates can be an important factor in optimizing successful refolding. Complete reversibility of rhodanese unfolding was achieved by including mild detergents to minimize aggregation. It was additionally necessary, in these studies, to provide reducing conditions and to include the substrate, thiosulfate (12, 13).

In a similar vein, we have recently obtained a high recovery of activity from urea-unfolded rhodanese when proteins called chaperonins were present in the refolding buffer (14). By sequestering partially folded proteins in the form of a stable binary complex, chaperonins appear to prevent aggregation by suppressing unwanted intermolecular interactions (15-17).

In order to understand better the factors that normally limit rapid, spontaneous folding, it is important to find conditions under which unassisted folding of rhodanese can be achieved. The present study focuses on optimizing spontaneous refolding from urea, and we report aspects of the unfolding/refolding processes under conditions of optimum reversibility. Rhodanese activity was used to monitor unfolding/refolding since this parameter has proven to be a sensitive indicator of subtle changes in the structure of this enzyme (3). The availability of a protocol giving reversible folding will make it possible not only to understand the folding potentials of the native protein, but also it will provide the basis for understanding the increased folding efficiency conferred by the assistance of molecular chaperones or detergents.

EXPERIMENTAL PROCEDURES

Materials—Urea was of electrophoresis purity, purchased from Bio-Rad. All other reagents were of analytical grade. Rhodanese was prepared as described previously and stored at -70 °C as a crystalline form containing ferric ions and the reaction product, thiocyanate (19). The assay was initiated by adding microgram quantities of the enzyme, and the reaction was stopped by adding formaldehyde.

Assay—Rhodanese activity was measured by a colorimetric method based on the absorbance at 490 nm of the complex formed between ferric ions and the reaction product, thiosulfate (18). Rhodanese concentration was determined using a value of A490nm = 1.75 (19).

Unfolding—Refolding—For unfolding/refolding studies, rhodanese at 90 μg/ml was unfolded in 8 M urea for at least 30 min. After unfolding, the protein was diluted to 3.6 μg/ml and allowed to refold in the presence of 200 mM BME, 50 mM sodium thiosulfate, and 50 μM dithiothreitol.

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* The abbreviation used is: BME, β-mercaptoethanol.
4 M thiocyanate, with time. That spontaneous refolding of urea-unfolded rhodanese is directly diluted to low concentrations from the initial activity is very low (see below and Ref. 3). Progress curves observed in some refolding experiments, the enzyme activity at any time is given by the slope at that time point.

**RESULTS**

**Unfolding/Refolding of Rhodanese in Urea at 24 °C**—The reversibility of the unfolding process was studied by monitoring enzyme activity. Activity has proven to be the most sensitive monitor of the overall integrity of the rhodanese molecule (3). For example, in detergent-assisted unfolding/refolding, the activity follows a two-state transition, and activity is the first property lost and the last property regained when the kinetics of urea-induced unfolding/folding are monitored.

There was very little reactivation when urea-unfolded rhodanese was diluted into buffer at 24 °C at a final protein concentration of 50 µg/ml, as reported previously (3). These conditions, in the presence of the detergent lauryl maltoside (5 mg/ml), give 90% reactivation. Previous studies of the detergent-assisted refolding indicated that aggregation was largely responsible for low yields of spontaneous refolding. Accordingly, we attempted to refold rhodanese at lower protein concentrations. In Fig. 1, the effects of urea on enzyme at 3.6 µg/ml are shown. Fig. 1A shows the partial reversibility of the unfolding of rhodanese at 24 °C. Some activity is regained under these conditions, but it is never more than 35%; even after 24 h. No activity was regained if the refolding was attempted at 50 µg/ml. The raised base line for the unfolding transition in Fig. 1A (▲) reflects the fact that rhodanese is directly diluted to low concentrations from the native state, and the small recovery at urea concentrations >4 M represents activity regain after dilution into the assay mix and incubation for 10 min. Initial slopes of progress curves for enzyme diluted from high urea concentrations show the initial activity is very low (see below and Ref. 20). The unfolding profile shows that the activity is almost completely lost by 3.75 M urea. To allow comparison, Fig. 1B shows the same transitions as above, except the experimental data have been normalized so that both transitions have approximately the same span. This figure shows that the curves are nearly coincident in the transition region, with a transition midpoint at 3.25 M urea.

**Temperature Dependence of the Refolding of Rhodanese**—Initial refolding experiments performed at 24 °C, as above, gave a maximum recovery of approximately 35%. Fig. 2 shows that spontaneous refolding of urea-unfolded rhodanese is strongly temperature-dependent. At 42 °C, very little (3%) reactivation was observed after incubation for 60 min. However, at temperatures below 42 °C the maximum yield of spontaneously reactivated rhodanese rose steadily, such that a maximum of the recoverable activity (>65%) was obtained at or below 12 °C.

**Dependence of the Refolding of Rhodanese at 24 °C on the Concentration of Protein and Urea**—The extent of reactivation of rhodanese shows an interdependence of the concentrations of protein and urea (Fig. 3). The concentrations of urea employed, 0–3 M, had little effect on native rhodanese (see Fig. 1). At a very low rhodanese concentration (0.5 µg/ml), the recovery was inversely dependent on the concentration of urea in the refolding buffer. For example, in 3 M urea, the recovery was 3%, but the percentage increased almost linearly when the concentration of urea was reduced. A maximum recovery of about 30% was obtained at the lowest concentration of urea (0.5 M).

A different dependence was seen at intermediate concentrations of protein (between 1–3.6 µg/ml). Here, the percentage of recovery was not very dependent on the urea concentration and ranged between 30 and 40% (Fig. 3).

At the highest protein concentrations (>10 µg/ml), the
nonequilibrium behavior in the unfolding/refolding transitions for rhodanese at 10 °C; kinetics were investigated. Fig. 5A shows the refolding kinetics at 10 °C. The reactivatable activity that had yet to be regained was calculated using the following expression.

\[
\% \text{ inactive} = 100 \times (\% \text{ recovery}_0 - \% \text{ recovery}_t) / (\% \text{ recovery}_0)
\]

where (\% recovery), is the percentage recovery of enzymatic activity at selected times, t, or 3 h (\infty) after dilution of the unfolded enzyme in the refolding buffer. There was no further recovery of activity after 3 h of incubation in the refolding buffer at 24 °C. As shown, the activity was regained in a single, first-order process with a half-time of approximately 30 min.

The activity was measured as the initial slope of progress curves of the formation of product versus time as shown in Fig. 5B. This initial slope was taken as representative of the activity of the enzyme in the buffer just before dilution into the assay mix. Curves representing activity regain from urea concentrations higher than the transition were nonlinear, i.e., they showed an induction period preceding the linear steady state (Fig. 5B). This behavior has been noted previously for detergent-assisted refolding (20), and it indicates that reactivation is occurring during the assay. Linear steady state behavior from t = 0 was noted for samples preincubated at urea concentrations lower than the transition region (data not shown; Ref. 20).

Kinetics of Unfolding at 10 °C—Fig. 6 shows that the rate of unfolding depends on the concentration of urea. In the vicinity of the unfolding transition, unfolding can take many

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**Fig. 3.** Effects of protein and urea concentration during rhodanese refolding at 24 °C. Activity measurements were carried out 60 min after diluting unfolded rhodanese to the indicated urea and protein concentrations. Each curve represents a separate urea concentration in the refolding buffer. ●, 0.5 M urea; ○, 1 M urea; ▲, 2 M urea; △, 3 M urea. The final concentrations of the protein ranged from 0.3 to 50 μg/ml.

**Fig. 4.** Unfolding and refolding of urea-unfolded rhodanese at 10 °C. The experimental conditions were similar to those described under “Experimental Procedures” and in Fig. 1, but both unfolding and refolding were at 10 °C. ▲, unfolding experiments; ●, refolding experiments. Samples were incubated for 20 h after folding or unfolding prior to assay.

recovery ranged from 14 to 38%. The higher concentrations of urea gave higher recoveries of activity, the reverse of the behavior at the lowest protein concentrations. For example, at 25 μg/ml rhodanese, the recovery ranged from 1 to 18%, and maximum recovery was obtained when the concentration of urea was the highest. Finally, for a protein concentration of 50 μg/ml, the recoveries that were obtained were very low, being less than 8% at all urea concentrations in the refolding buffer, but the recoveries still increased with increasing urea concentrations.

Unfolding/Refolding of Rhodanese in Urea at 10 °C—Fig. 4 shows the reversibility of urea-induced unfolding of rhodanese at 10 °C. The process, even at such a low, near optimal temperature, does not appear to be fully reversible. The maximum recovery of activity upon refolding was approximately 80%. The maximum recovery (80%) was higher than shown in Fig. 2 (60%). However, the recoveries described in Fig. 2 were obtained after 1 h of incubation in the refolding buffer, compared with 24 h in Fig. 4. Thus, the time of incubation may be a critical parameter. The results shown in Fig. 4 (10 °C) are also distinct from those at 24 °C (Fig. 1), especially in the transition region. At 24 °C, the unfolding/refolding transitions are essentially superimposable (see Fig. 1B), but at 10 °C, curves have become separated. In comparing the two temperatures, it is seen that the unfolding transition accounts for most of the difference, and this transition appears to be shifted from 3.2 M (24 °C) to almost 4 M (10 °C). This behavior reflects slow kinetics in the transition region at the lower temperature (see below).

Kinetics of Refolding at 10 °C—To understand the apparent
DISCUSSION

A thermodynamically reversible unfolding process for rhodanese, unfolded in urea or guanidinium chloride, has only previously been observed in the presence of BME, thiosulfate, and a detergent such as lauryl maltoside (3, 4). The requirements for BME and thiosulfate are due to the sensitivity of rhodanese's sulfhydryl groups (3). Oxidation of these groups changes the conformation of rhodanese, resulting in exposure of hydrophobic surfaces (21, 22). These studies showed that reversibility of rhodanese folding can be achieved when aggregation is minimized and oxidative inactivation is prevented.

Previous studies detected some regain of rhodanese activity after dilution of denaturants (8), but they could not distinguish between inactivation/activation, which may follow different paths, and reversibility, in which the same path is followed for folding and unfolding. Thus, it was not possible to construct transition profiles showing the extent to which unfolding was "thermodynamically" reversible for rhodanese unfolded without accessory substances.

Reversibility was shown when aggregation was minimized by the use of the detergent lauryl maltoside, supporting the view that hydrophobic interactions play an important role in refolding. In the absence of detergents, we have recently observed that rhodanese efficiently reactivates when the chaperonin proteins, cpn60 and cpn10 from Escherichia coli, are present during refolding (14). The combination of thiosulfate and BME was also required to prevent oxidative inactivation in this case. The replacement of detergent with the chaperonins suggested that these proteins were responsible for the suppression of unwanted hydrophobic interactions, in agreement with their proposed role in the in vivo folding process (29-26). It was also demonstrated that cpn60 contains hydrophobic surfaces (14). These may stabilize folding intermediates through hydrophobic interactions and, therefore, prevent "incorrect" interactions that can lead to aggregated states.

In the present study, we have demonstrated that significant recovery of activity of urea-unfolded rhodanese can be obtained in the absence of either detergents or chaperonins, when intermolecular interactions that lead to aggregation are suppressed (3). Successful, nonassisted refolding involved lowering the concentration of the protein, lowering the temperature, and optimizing the concentration of urea during refolding. The requirement for urea implies that the best yield of refolding would be achieved by an intermediate dilution of the perturbant before its final removal.

In this nonassisted process, no significant recovery was obtained when unfolded rhodanese was diluted into buffer at protein concentrations exceeding 10 μg/ml. This is in contrast with the detergent-assisted refolding of rhodanese where complete recovery was observed at a protein concentration of 50 μg/ml (3). These results are consistent with previous reports showing a concentration dependence of the ability to reactivate a fraction of unfolded rhodanese, as well as of the stability of native enzyme (8, 27). Both reactivation and stability display optima at low protein concentrations. These optima were interpreted as resulting from a balance of two effects; high protein concentrations encourage aggregation, whereas low protein concentrations increase susceptibility both to surface adsorption and to limiting amounts of adventitious reactants, such as reactive oxygen species (27). Similarly, in the present study, we find that even under reducing conditions, a strong protein concentration dependence is observed for the reversible unfolding of rhodanese. The results indicate that there is an optimum concentration of the enzyme at which maximum recoveries can be obtained.

Nonassisted refolding is strongly temperature-dependent. Previous results on the temperature dependence of aggregation of rhodanese at high protein concentration suggested that hydrophobic interactions were involved in the competition between the refolding and aggregation (3, 28). This was supported by the observation that the aggregation rate is reduced at lower temperatures (3), where the hydrophobic effect is weakened. Our results indicate that almost complete recovery of activity can be achieved when a combination of low protein concentration, low temperature, and conditions that help to prevent oxidation are used for refolding rhodanese.

The present results are in accord with a model that was proposed to account for the role of detergents and chaperonins in rhodanese folding.

\[
N \rightleftharpoons I_1 \quad \text{fast} \quad I_1 \rightarrow I_2 \quad \text{slow} \quad I_2 \rightarrow D
\]

The major feature of this model is that aggregation kinetically competes with reactivation. Native rhodanese (N) passes through metastable intermediate(s) (I, I) in the course of unfolding/refolding. Activity is lost at an early stage, accompanied by relatively small structural changes, i.e. \( N \rightarrow I \). Higher urea concentrations are required to fully unfold the enzyme, i.e. \( I \rightarrow D \). Further, the observations in Fig. 5A indicate that \( I \rightarrow N \) is rate limiting, which is consistent with previous suggestions (4).

A major side reaction that competes with the refolding process is aggregation. Noncovalent aggregation has been previously reported as a side reaction in the reconstitution of unfolded oligomeric enzymes, e.g. lact dehydrogenase (29, 30). For rhodanese, aggregation can become effectively irreversible. Aggregation is protein concentration-dependent and has been suggested to involve intermediates that are still compact and have extensive, exposed hydrophobic surfaces represented here, collectively, as \( I \). In the model, above, the step leading from \( I \) to the aggregate (denoted as involving \( n \)
molecules of I₂, i.e. nL₂) is concentration-dependent, and the high order of this reaction can make it appear to be cooperative (31). Aggregation could also involve the unfolded species, D. However, partially folded species that rapidly form on dilution of unfolded rhodanese (4, 20) are prime candidates, since they possess organized hydrophobic surfaces. As with most aggregation reactions, the dissolution of large aggregates is expected to be slow and, therefore, appear to be irreversible. In contrast, the rate of aggregation can be controlled to a great extent by decreasing the concentration of rhodanese, as well as the temperature of the refolding reaction.

Ideally, aggregation could be avoided completely by using extremely low protein concentrations and temperatures. However, as the protein concentration is decreased, detrimental processes other than aggregation are seen, and the overall efficiency of reactivation decreases. These processes may include surface effects as well as oxidative reactions, driven by limiting quantities of reactants. For temperature, although recoveries may continue to increase as the temperature is lowered, slow kinetics make the refolding process less practical.

Rhodanese folding appears to conform to the "thermodynamic hypothesis" in that the native structure is thermodynamically most stable. However, practical folding to the native structure requires that the folding pathway be controlled so as to avoid kinetically trapping metastable conformers. Thus, biological protein-folding mechanisms may be designed to provide kinetic control.

REFERENCES
1. Westley, J. (1973) Adv. Enzymol. Relat. Areas Mol. Biol. 39, 327-368
2. Ogata, K., Dae, X., and Volini, M. (1989) J. Biol. Chem. 264, 2718-2725
3. Tandon, S., and Horowitz, P. M. (1989) J. Biol. Chem. 264, 9869-9866
4. Tandon, S., and Horowitz, P. M. (1990) J. Biol. Chem. 265, 5967-5970
5. Ploegman, J. H., Drent, G. H., Kalk, K. H., Hol, W. G. J., Heinrikson, R. L., Keim, P., Weng, L., and Russell, J. (1978) Nature 273, 1245-1249
6. Hol, W. G. J., Lijk, L. J., and Kalk, K. H. (1983) Fundam. Appl. Toxicol. 3, 370-376
7. Miller, D. M., Delgado, R., Chirgwin, J. M., Hardies, S. C., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 4686-4691
8. Horowitz, P. M., and Simon, D. (1986) J. Biol. Chem. 261, 13887-13891
9. Mitre, A., and King, J. (1989) Bio/Technology 7, 690-696
10. Jaenicke, R. (1987) Prog. Biophys. Mol. Biol. 49, 117-237
11. Fischer, G., and Schmid, F. X. (1990) Biochemistry 29, 2205-2212
12. Tandon, S., and Horowitz, P. (1986) J. Biol. Chem. 261, 15615-15618
13. Tandon, S., and Horowitz, P. M. (1987) J. Biol. Chem. 262, 4486-4491
14. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 13044-13049
15. Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keeffe, D. P., and Lorimer, G. H. (1990) Biochemistry 29, 5665-5671
16. Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1988) Nature 342, 884-889
17. Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 337, 44-47
18. Horowitz, P. (1978) Anal. Biochem. 86, 751-753
19. Sörbo, B. H. (1953) Acta Chem. Scand. 7, 1129-1136
20. Horowitz, P. M., and Criscimagna, N. L. (1990) J. Biol. Chem. 265, 2576-2583
21. Horowitz, P. M., and Bowman, S. (1987) J. Biol. Chem. 262, 8728-8733
22. Horowitz, P. M., and Criscimagna, N. L. (1988) J. Biol. Chem. 263, 10278-10283
23. Østergaard, J., Horwich, A. L., Neupert, W., and Hartl, F. U. (1999) Nature 341, 125-130
24. Rothman, J. E. (1989) Cell 59, 591-601
25. Ellis, R. J. (1953) Semin. Cell Biol. 1, 25-38
26. Georgopoulos, C., and Ang, D. (1990) Semin. Cell Biol. 1, 25-38
27. Aird, B. A., and Horowitz, P. M. (1988) Biochim. Biophys. Acta 956, 30-38
28. Horowitz, P., and Bowman, S. (1987) J. Biol. Chem. 262, 5587-5591
29. Zettlmeissl, G., Rudolph, R., and Jaenicke, R. (1979) Biochemistry 18, 5567-5571
30. Rudolph, R., Zettlmeissl, G., and Jaenicke, R. (1979) Biochemistry 18, 5572-5575
31. Horowitz, P. M., and Bowman, S. (1987) J. Biol. Chem. 262, 8728-8733