High Hydrostatic Pressure Can Reverse Aggregation of Protein Folding Intermediates and Facilitate Acquisition of Native Structure

Boris M. Gorovits and Paul M. Horowitz*

Department of Biochemistry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284

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ABSTRACT: The present work demonstrates that high hydrostatic pressure can increase protein folding by reducing nonspecific aggregation. Protein aggregation is one of the main side reactions that competes with protein folding, and it typically results from interactions among partially folded intermediates. It is known that oligomeric proteins can be dissociated by the application of high hydrostatic pressure. Since protein aggregates can be described as nonspecific protein oligomers, it can be predicted that they can be completely or partially dissociated by pressure. The enzyme rhodanese is prone to slow aggregation in 3.9 M urea, and it is widely used as a model for the folding of a protein which readily aggregates. In the present study, it was demonstrated that this aggregation process could be completely reversed under high hydrostatic pressure. Release of the pressure led to renewed protein aggregation. In addition, it was demonstrated that refolding of urea-denatured rhodanese at 2 kbar pressure led to an increased yield of the native enzyme. The final recovery was increased up to ∼25% in contrast to ∼5% recovery observed under ambient pressure. The recovery can be further increased in the presence of 4 M glycerol, where 56% of the protein was recovered by treatment with high pressure. These observations suggest that some protein aggregation can be limited without the use of chemical additives, and they show that the pressures needed to maintain solubility are considerably less than those typically required for dissociation of specific oligomers and unfolding of polypeptide chains.

Partial denaturation of proteins caused by chemical denaturants or temperature often results in formation of folding intermediates that contain almost the same level of the secondary structure as the native protein, but decreased number of the tertiary contacts (*1*), unpaired domains (2), or incorrectly formed disulfides bonds (*3*). As a result, such intermediates tend to be highly hydrophobic and consequently can easily precipitate, forming large aggregates. Formation of similar intermediates can be detected during protein refolding where aggregation is a major side reaction.

Formation of aggregates can be observed during in vitro (*4*) and in vivo folding (*5*). *In vivo*, interaction of such intermediates with molecular chaperones can protect folding proteins from aggregation (*6*). Sequestering of the protein intermediate into the central cavity of the molecular chaperone GroEL was proposed to be the first step during GroE-assisted protein folding (*6*). A number of folding protocols in vitro are based on the principle of isolating folding intermediates, thereby preventing their interaction. For example, interaction of folding intermediates with liposomes in the form of large unilamellar vesicles or detergent micelles (*7*) can prevent protein aggregation and improve the final refolding yields (*8*).

The ability of hydrostatic pressure to dissociate oligomeric proteins was theoretically predicted and experimentally confirmed (*9*). One explanation of this effect is based on the assumption of the existence of small "free volumes" at the intersubunit boundaries. The structural constraints imposed by the interacting structures determine imperfection of the contact area between subunits in the oligomeric protein. The small molecules of the solvent can be packed much better on the surface of the intersubunit interfaces after their dissociation. This phenomenon was shown with several protein models (*10*). The prediction can be made that protein aggregates, produced under certain conditions and containing protein intermediates bound to each other through mainly hydrophobic interactions, can be dissociated under high hydrostatic pressure. This assumes that such interaction can produce free volumes similar to ones found on the interfaces of completely folded oligomeric proteins. Since nonspecific aggregates pack less precisely, they may be expected to contain large total free volumes which will result in requiring lower hydrostatic pressures for dissociation. Other sources of the pressure sensitivity are discussed below.

The enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) has become an important model for studying issues related to the problems of protein folding (*11*, *12*). This monomeric protein is folded into two independent, equal-size domains, and a crystal structure is available (*13*). The domains are tightly associated, and the interdomain interface is highly hydrophobic. Rhodanese undergoes an unfolding transition when subjected to increasing concentrations of urea (*2*, *14*). At intermediate urea concentrations, rhodanese forms a molten globule-like

* Author to whom correspondence should be addressed at Department of Biochemistry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284. Telephone: (210) 567-3737. Fax: (210) 567-6595.

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structure that has a high level of hydrophobic exposure and a considerable amount of secondary structure (*2*). These folding intermediates are prone to aggregation that is effectively irreversible under normal conditions. We have investigated the ability of high hydrostatic pressure to dissociate aggregates formed under conditions of partial denaturation of rhodanese and the ability of such pressure to improve final refolding yield of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. Urea was electrophoresis purity, purchased from Bio-Rad. All other reagents were of analytical grade. Bovine rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) was purified as described earlier and stored at −70 °C as a crystalline suspension in 1.8 M ammonium sulfate containing 1 mM sodium thiosulfate (*15*). Rhodanese concentration was determined using a value of $A^{0.1\%}_{280\ nm} = 1.75$ (*16*).

Standard Buffer. Standard buffer (200 mM βME, 50 mM sodium thiosulfate, and 50 mM Tris-HCl, pH 7.8.) was used throughout the study.

Rhodanese Assay. Rhodanese activity was measured by a colorimetric method based on the absorbance at 460 nm of the complex formed between ferric ions and the reaction product, thiocyanate (*16*). The assay was initiated by adding microgram quantities of the enzyme, and the reaction was stopped by adding formaldehyde.

Protein Aggregation under Pressure. Native rhodanese was diluted into a solution containing 50 mM Tris-HCl, pH 7.8, 200 mM βME, 50 mM thiosulfate, maintaining the final urea concentration at 3.9 M. The final protein concentration was 0.3 mg/mL. The sample was pressurized after various times of incubation. The pressure was varied between 0.001 kbar (atmospheric pressure) and 2 kbar. The pressure bomb similar to the one previously described by Paladini and Weber (*9*) was employed. A fluorometer (ISS, Inc.) was employed to monitor the intensity of light scattering at 400 nm and the tryptophan intrinsic fluorescence (sample was excited at 280 nm and fluorescence was detected at 350 nm).

Rhodanese Unfolding−Refolding. For the unfolding studies, rhodanese was unfolded in 8 M urea for at least 60 min, a time sufficient to ensure denaturation (*14*). After unfolding, the protein was diluted to 3.6 μg/mL and allowed to refold for the indicated time in the presence of 200 mM βME, 50 mM sodium thiosulfate, and 50 mM Tris-HCl, pH 7.8. The regain of enzyme activity was used to monitor successful refolding. Refolding under these conditions is rapid, and no further recovery is observed after 5 min (*12*, *14*). Aliquots containing 25−50 μL of the incubating enzyme were added to 1 mL of assay mix and incubated for 5−10 min before determining the amount of product formed. The percent reactivation was calculated based on the activity of native enzyme that had been subjected to the refolding conditions. The refolding time was varied for the kinetics study.

To investigate rhodanese refolding under high hydrostatic pressure, samples were pressurized up to 2 kbar 5 min after dilution. Standard buffer was used. Glycerol (4 M final concentration) was added as indicated. Samples were incubated for 70 min, and activity was analyzed as described above. Activity of the native protein kept at the ambient conditions was taken as 100%.

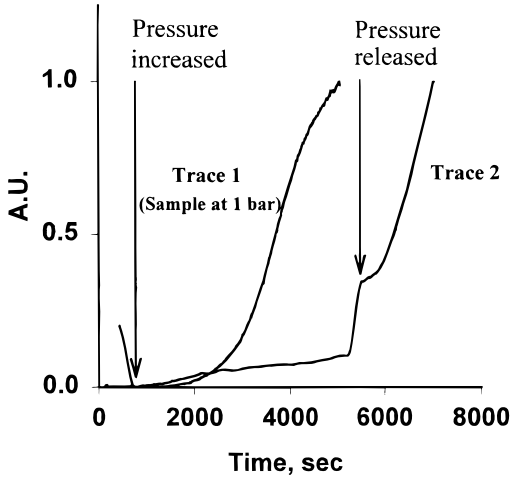


FIGURE 1: Rhodanese aggregation is inhibited under high hydrostatic pressure. Native rhodanese was diluted into a solution containing 50 mM Tris-HCl, pH 7.8, 200 mM βME, and 50 mM thiosulfate, maintaining a final urea concentration 3.9 M. The final protein concentration was 0.3 mg/mL. Light scattering was monitored on an ISS, Inc. fluorometer, and the measurements were started at 2 and 7 min for the sample examined at 1 bar (trace 1) and 2 kbar (trace 2), respectively, after the initial dilution of the enzyme. Pressure was increased at 10 min and released at 120 min (shown by arrows).

RESULTS AND DISCUSSION

Chemical unfolding of rhodanese by urea results in a complex transformation of the protein structure. Two transitions can be observed (*2*, *14*). At 3.9 M urea, the protein is partially unfolded to form a molten globule-like state (*2*, *14*), and in the standard refolding buffer, rhodanese slowly forms aggregates. This process is protein concentration-dependent, and it displays a considerable lag time (Figure 1, trace 1). The duration of the lag time also depends on the protein concentration, and it approaches a plateau at the protein concentrations equal or higher than 0.3 mg/mL. The existence of such a lag period is very useful, since the high-pressure system employed in this study requires 5−7 min for assembly. A typical example of the time-dependence of the scattered light intensity for a rhodanese solution at ambient pressure is shown in Figure 1, trace 1. At the protein concentration of 0.3 mg/mL there is ∼2500 s lag time before a rapid increase in the intensity of the scattered light. The existence of the lag period indicates that during this time there exists a slow equilibrium among rhodanese folding intermediates. As a result, an intermediate that is highly prone to aggregation is formed. It is possible that smaller aggregates, undetectable by the method employed in this study, are formed during the lag time.

The native structure of rhodanese contains two domains tightly bound to each other by noncovalent, mainly hydrophobic, interactions. It has been suggested (*17*) that partial denaturation of the enzyme results in the dissociation of these domains, and there follows the formation of nonproductive dimers, trimers, and higher-order oligomers. Such aggregation involves hydrophobic cross interactions between domains of different protein molecules and is less specific than the native intramolecular domain interactions. Therefore, it might be expected that large interdomain free volumes can be formed. To investigate this possibility, rhodanese was diluted into 3.9 M urea and pressurized up to 2 kbar within

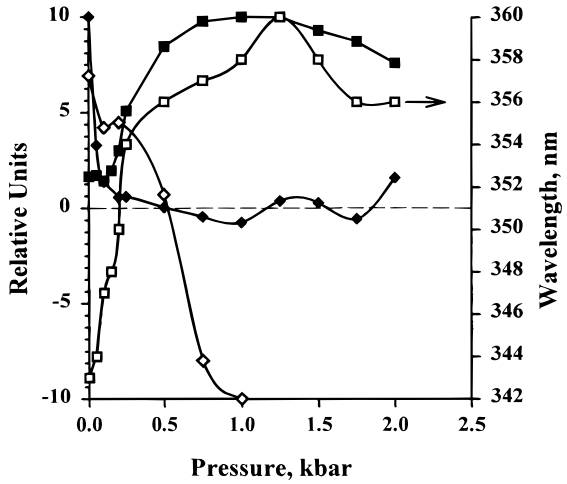


FIGURE 2: To determine minimal pressure that is able to prevent rhodanese aggregation, a sample was prepared as in Figure 1. After the precipitation started, pressure was gradually increased with an increment that varied from 0.05 to 0.25 kbar, maintaining constant pressure at the indicated values for 5−20 min during which measurements were made of fluorescence intensities (solid squares), wavelength maxima (open squares), and the rates of the protein aggregation (open diamonds). In a separate experiment, a protein sample, prepared as in Figure 1, was pressurized up to 2 kbar and after 1 h incubation, pressure was gradually decreased monitoring the rate of the protein aggregation (solid diamonds) following the protocol described above. Rates of protein aggregation and fluorescence intensity at 350 nm shown are normalized.

10 min. The intensity of the scattered light increased very slowly (Figure 1, trace 2). Protein aggregation quickly started after the pressure was released. The small decrease in the scattered intensity immediately after pressurizing the sample and its increase after depressurizing are artifacts related to the optical properties of the system used.

It is important to note that the lag time (the time between the pressure release and the time when noticeable increase in the scattered light intensity was observed) is considerably shorter after the pressure is released compared to the lag time at atmospheric pressure (less than 300 s vs approximately 2500 s, respectively). This demonstrates that while under pressure the protein underwent conformational changes, and the critical concentration of the intermediate that is prone to the precipitation was achieved. No changes in the protein conformation during incubation at high pressure (2 kbar) could be detected by measuring the tryptophan intrinsic fluorescence of the enzyme at 350 nm (data not shown). No significant change in the intensity was detected during incubation at high pressure. The apparent intensity of the intrinsic fluorescence did decrease when the pressure was gradually decreased from 2 kbar to 1 bar (Figure 2, solid squares). At the same time, a transition of the wavelength maximum of the tryptophan fluorescence was observed (Figure 2, open squares). Pressures above 0.5 kbar produced a considerable red shift in the wavelength maximum of the rhodanese tryptophan spectrum which reached 360 nm at 1.25 kbar, suggesting that under such conditions, tryptophan moieties of the protein exist in close contact with the solvent. Interestingly, such a red shift has been observed for chymotrypsin and lysozyme under conditions of their pressure-induced denaturation (>5 kbar) (*9*, *18*).

The minimum pressure that can prevent protein aggregation was sought (Figure 2, solid diamonds). Here, protein was diluted into 3.9 M urea and pressurized up to 2 kbar. After 1 h incubation, the pressure was gradually decreased, and the time-dependence of the light scattering intensity was monitored at each pressure. The slopes of the corresponding time courses are plotted as a function of pressure (Figure 2, solid diamonds). The scattered light intensity starts to slowly grow at pressures less than 0.5 kbar, and it rapidly increases at pressure less than 0.2 kbar. The same pressure is able to stop protein aggregation, as is shown in Figure 2, open diamonds. Here, precipitation was started at 1 bar, and pressure was gradually increased while monitoring kinetics of the increase in light scattering intensity. Aggregation was completely reversed at pressure higher than 0.5 kbar.

A very steep transition was observed in an experiment designed to investigate the equilibrium between low molecular weight forms of rhodanese and protein aggregates at 3.9 M urea (data not shown). In this experiment, a rhodanese solution containing 3.9 M urea was pressurized up to 2 kbar. Pressure was then gradually reduced, and the sample was incubated to allow the system to equilibrate at each individual pressure. No significant scattering was observed at pressures that prevent rhodanese from aggregation. When pressure was reduced to less than 0.2 kbar, the intensity of the scattered light rapidly increased, reaching a value equal to an unpressurized sample (data not shown). These results reflect the high degree of cooperativity for the aggregation process. In addition, during aggregation, rhodanese forms a number of different oligomeric forms (*17*) which results in a high degree of heterogeneity of the precipitating species. As a result, methods that have been previously described for the calculation of the equlibrium constant and the reaction volume changes for discrete protein oligomers cannot be applyed here.

These results demonstrate that protein aggregation can be reversed by high hydrostatic pressure. Indeed, when rhodanese was pressurized at 2 kbar for 80 min and pressure was released, the intensity of the scattered light started to increase. After approximately 30 min incubation at 1 bar, pressure was adjusted up to 2 kbar again which resulted in the complete dissociation of protein aggregates and a decrease of the scattered light intensity to the initial value (Figure 3). This sequence was repeated at least three times without any considerable change in the baseline value. This is the first demonstration that protein aggregation can be reversed by pressure.

Protein aggregation is one of the most important obstacles to protein folding. Isolation of the folding intermediates results in the increased yield of native protein. *In vivo*, molecular chaperones can exert this function (*6*), since they that can bind to the non-native protein conformers preventing their interaction with other folding intermediates.

Chemically unfolded rhodanese refolds with a relatively low yield that depends on the temperature and final concentration of the protein in the refolding mixture (*19*). Such spontaneous folding can be improved by introducing detergents (*8*) or cosolvents such as glycerol (*20*, *14*). The effect of detergents has been explained by their ability to form micelles that can include folding intermediates and therefore isolate them from each other preventing protein from aggregation. Given the idea of the ability of the high hydrostatic pressure to reverse formation of protein aggregates, rhodanese refolding was performed under 2 kbar.

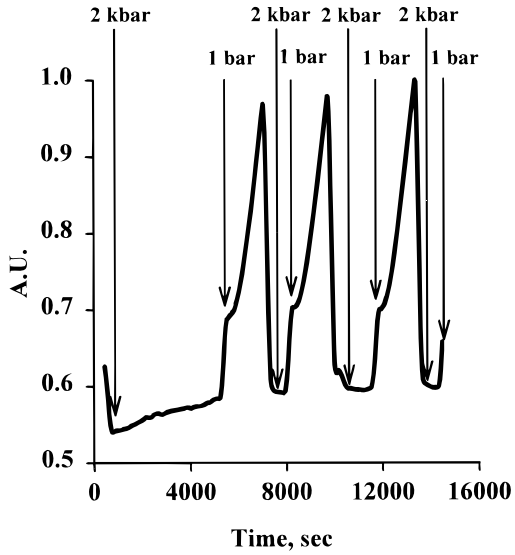
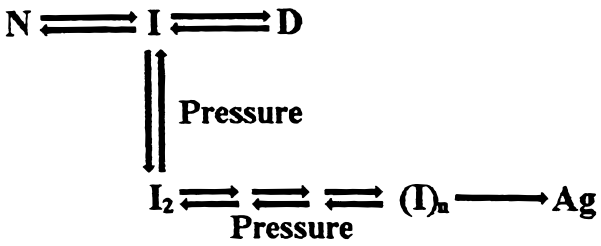


FIGURE 3: The reversibility of the rhodanese aggregation under pressure. A protein sample was prepared as in Figure 1. Pressure was increased up to 2 kbar at 10, 120, 165, or 225 min Pressure was released at 90, 135, 195, or 240 min (shown by arrows).

Scheme 1



Protein was initially unfolded at 6 M urea. After dilution into the refolding buffer, pressure was adjusted up to 2 kbar after 6 min of incubation. Refolding was allowed to continued for 60 min. The result indicated that the final yield was considerably increased compared to the sample refolded under 1 atm pressure (24.4 and 5.2%, respectively). It has been reported that the final yield of the native enzyme can be increased in the presence of glycerol (*14*). When rhodanese was refolded under 2 kbar hydrostatic pressure as described above and in the presence of 4 M glycerol, the final yield of the native enzyme was found to be 56% compared to 30.6% observed at ambient pressure. Maximizing the final yield of active enzyme in all these studies was hindered by technical difficulties related to the complexity of the pressure apparatus which requires assembly of the pressure cell after initiating refolding. Thus, solutions of refolding rhodanese could only be pressurized after a delay of up to 10 min. During this delay, a portion of the enzyme can form irreversible aggregates, thereby reducing the final concentration of the native protein. However, relatively high value of the reaction volume change can be suggested based on the low pressure that is able to prevent rhodanese aggregation under the described conditions (<0.5 kbar).

The data presented here can be accommodated by the model shown in Scheme 1, where N is native protein, I is an intermediate state, D is the denatured state, and Ag is an aggregate. Pressure can successfully reverse formation of small aggregates $(I)_n$, but it is not able to reverse large aggregates (Ag). For example, a fraction of the protein that rapidly aggregates after dilution of rhodanese from 8 M urea cannot be easily dissociated, and therefore, refolding of this portion of the protein cannot be facilitated by high pressure. During the refolding process, rhodanese undergoes conformational transitions among a wide range of folding intermediates. Some unfolded protein intermediates formed at the early stages of refolding can possibly form highly packed aggregates that cannot be dissociated by using high hydrostatic pressure.

In conclusion, a few word should be said about the possible nature of the observed phenomena. Three main causes for the pressure dissociation of protein aggregates in the solution can be suggested on the basis of studies with specific protein oligomers (*10*): 1) filling of the existing free volumes between individual protein moieties; 2) hydration of the dissociated salt linkages at the intersubunit boundaries (electrostriction effect); and 3) hydration of nonpolar groups as a result of subunit dissociation. Due to the hydrophobic nature of the protein folding intermediates, the latter one can be very important in the energetics of the pressure-induced dissociation of nonspecific protein aggregates. The result of hydration of apolar groups can depend on their density and the protein concentration (*10*). However, the relative importance of those causes for the dissociation of nonspecific protein aggregates by high pressure is unclear at present.

REFERENCES

1. Ptitsyn, O. B. (1992) *Protein Folding* (Creighton, T. E., Ed.) pp 243−300, W. H. Freeman and Co., New York.
2. Horowitz, P. M., and Criscimagna, N. L. (1990) *J. Biol. Chem. 265*, 2576.
3. Ewbank, J. J., and Creighton, T. E. (1991) *Nature 350*, 518.
4. Kiefhaber, T., Rudolf, R., Kohler, H. H., and Buchner, J. (1991) *BioTechnology 9*, 825.
5. Rudolph, R. (1996) *FASEB J. 10*, 49.
6. Ellis, R. J., and van derVies, S. M. (1991) *Annu. Rev. Biochem. 60*, 321.
7. Tandon S., and Horowitz, P. M. (1986) *J. Biol. Chem. 261*, 15615.
8. Zardeneta, G., and Horowitz, P. M. (1992) *Eur. J. Biochem. 210*, 831.
9. Paladini, A. A., and Weber, G. (1981) *Biochemistry 20*, 2587.
10. Weber, G. (1992) *Protein Interactions*, pp 235−270, Chapman and Hall, Inc., New York.
11. Horowitz, P. M. (1993) *Biocatalyst Design for Stability and Specificity*, (Hummel, M. E., and Georgiou, G., Eds.) ACS Symposium Series No. 516, pp. 167-172,
12. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem. 266*, 13044.
13. Ploegman, J. H., Drent, G., Kalk, K. H., Hol, W. G. J., Heinrikson, R. L., Keim, P., Weng, L., and Russell, J. (1978) *Nature 273*, 124.
14. Gorovits, B. M., McGee, W. A., and Horowitz, P. M. (1998) *Biochim. Biophys. Acta 1382*, 120.
15. Miller, D. M., Delgado, R., Chirgwin, J. M., Hardies, S. C., and Horowitz, P. M. (1991) *J. Biol. Chem. 266*, 4686.
16. Sorbo, B. H. (1953) *Acta Chem. Scand. 7*, 1129.
17. Horowitz, P. M., and Butler, M. (1993) *J. Biol. Chem. 268*, 2500.
18. Li, T. M., Hook, J. W., III, Drickamer, H. G., and Weber, G. (1976) *Biochemistry 15*, 5571.
19. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem. 266*, 13587.
20. Shi, L., Palleros, D. R., and Fink, A. K. (1994) *Biochemistry 33*, 7536.

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