Interactions between the GroE Chaperonins and Rhodanese

MULTIPLE INTERMEDIATES AND RELEASE AND REBINDING

(Received for publication, May 11, 1995, and in revised form, July 12, 1995)

Kirk E. Smith‡ and Mark T. Fisher‡

From the Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160

Efficient renaturation of urea-denatured rhodanese using the chaperonin GroE system requires GroEL, GroES, and ATP. At high concentrations this renaturation also requires the substrate thiosulfate to have been present during GroEL-rhodanese complex formation. When thiosulfate is present the GroEL-rhodanese complex can be concentrated to greater than 1 mg/ml rhodanese with little effect on the efficiency of renaturation. However, if complex is formed in the absence of thiosulfate, renaturation of rhodanese in the presence of thiosulfate shows a critical concentration of approximately 0.4 mg/ml, above which renaturation yields drop dramatically. This critical concentration appears to be related to an aggregation event in the refolding of rhodanese. The nucleotide free or ADP-bound form of GroEL also binds to rhodanese that has been either already renatured or never denatured. The bound rhodanese has no activity but can be released from GroEL with ATP recovering 90% of control activity. The data presented herein support a release and rebinding mechanism for the GroE-assisted refolding of rhodanese. It also suggests GroEL binds several protein folding intermediates along the entire refolding pathway.

In vivo, molecular chaperones such as hsp70/hsp40 and the chaperonins prevent the accumulation of inappropriate protein aggregates and enhance the acquisition of native protein structure. For instance in Escherichia coli, chaperonins are known to bind to a host of partially folded proteins (1, 2) and act in concert with the upstream (hsp70/40) molecular chaperones (3, 4) to prevent misfolding and aggregation. While the mechanism through which this is accomplished has been the subject of intense research, it is still unclear exactly how chaperones assist protein folding.

The most thoroughly studied chaperonin system is the GroE proteins (GroEL and GroES) isolated from E. coli. Results from in vitro protein folding experiments indicate that the GroE chaperonins inhibit protein misfolding and aggregation (5–7). Two models have been proposed to explain this observation. The first model suggests that the GroE system sequesters the folding protein inside a cavity of the GroEL chaperonin and allows the protein to fold in a shielded environment through repeated ATP-dependent release and rebinding reactions (7, 10, 11). The second model suggests that the protein is released from GroEL and folds free in solution but will rebind to the chaperonin if it is not committed to fold to the native state. In this latter model, protein aggregation is prevented by decreasing the concentration of free, aggregation-prone, folding intermediates.

Although early evidence suggested that the protein folding intermediates could bind and become sequestered inside the central cavity of GroEL (12, 13), later studies have indicated that the protein folding intermediate is easily accessible at the GroEL surface (14). Furthermore, structural studies indicate that the central cavity is not continuous through the GroEL, and its volume may not be large enough to accommodate an expanded protein folding intermediate (15, 16).

These structural constraints do not preclude the possibility that the protein folding intermediates undergo some folding while bound to GroEL. Gray and Fersht (17) have suggested that some refolding of barnase can occur while it is in contact with GroEL. They found that an increase in the GroEL concentration alone slows the spontaneous folding rate of barnase to a constant non-zero level rather than producing a continuous decline as expected for a simple bimolecular reaction. In addition, GroEL has been found to induce formation of α-helices in small unstructured polypeptides (18).

It has become clear that secondary structural elements are dictated by non-local as well as local interactions (19, 20). Rapid induction of defined secondary structural elements can arise from constraining conformational search space (21) and by increasing the concentration of the potential hydrophobic interactive surfaces (22). Therefore, it is conceivable that the folding pathways for unstructured polypeptides can be influenced by interaction with the hydrophobic peptide binding surface of the chaperonins (8, 9).

The complete release model also has experimental support. Numerous investigators have examined the lifetime of the protein folding intermediate-GroE complex and have observed a rapid release of non-native intermediates following ATP addition (23–25). In this model, the folding protein may still undergo a number of rebinding and release cycles but in contrast with the former hypothesis, released intermediates do not fold in a sequestered environment.

The complete release and rebinding mechanism has been criticized on the grounds that the released protein would still have the tendency to aggregate. Since inappropriate aggregation is a concentration-dependent process, the complete release and rebinding mechanism predicts that at high concentrations, the released folding intermediates would aggregate before re-binding to the chaperonin. To determine if a critical aggregation concentration can be observed in a chaperonin-dependent folding reaction, the chaperonin-dependent refolding of rhodanese was monitored as a function of concentration.

Rhodanese was used as a substrate for the following reasons: 1) it requires the entire chaperonin system throughout the refolding process to fold efficiently (28), 2) the GroEL-rhodanese complex is stable over time (26), and 3) GroEL-protein substrate complexes can be easily concentrated (27).
Results presented herein indicate that concentration-dependent aggregation can be observed for the chaperonin-assisted folding of rhodanese. Under certain solution conditions, rhodanese exhibits a concentration-dependent decline in recovered activity and an increase in aggregation.

EXPERIMENTAL PROCEDURES

Materials—Rhodanese was purchased from Sigma. Ultrapure urea was purchased from ICN. Centricon ultrafiltration units were from Amicon. Coomassie Blue protein assay reagent was from Pierce. All other chemicals and water were the best quality available.

Chaperonin proteins GroEL and GroES were purified as described previously (27). Purity of the chaperonin proteins was followed using second derivative spectral analysis (29). Protein that had no detectable contribution from tryptophan (as determined by the 290–294 nm peak-trough value) was considered pure (27). Unless otherwise stated, the reconstitutions were carried out in the presence of the complete GroE system containing equimolar amounts of GroEL and GroES dimers (1 mol of GroE = 14 mol of GroEL monomer + 7 mol of GroES monomer).

Denaturation of Rhodanese—Rhodanese was solubilized in Buffer A (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 10 mM DTT, 0.5 mM dithiothreitol) and its concentration determined spectroscopically using the extinction coefficient of 1.75 for a 0.1% solution (30). Rhodanese was denatured by dilution to concentrations between 0.5 and 10 mg/ml in 8 M urea, 10 mM DTT, 50 mM sodium thiosulfate for 2 h on ice. Denaturation was carried out in the presence or absence of 50 mM sodium thiosulfate. Rhodanese was allowed to incubate in the urea for 2–4 h. No detectable differences in the denaturation or percent recovery was noted within this time frame.

GroE-Rhodanese Complex Formation—Denatured rhodanese was rapidly diluted 1/100 into Buffer A containing either no additional protein or GroE at a 1:1 or 2:1 molar ratio with rhodanese. In some experiments the buffer also contained 50 mM sodium thiosulfate as explained in results. The buffers were pre-equilibrated to 37°C, and the complex was incubated at 37°C for 15 min before additional manipulations.

Concentration of the GroE-Rhodanese Complex—The GroE-rhodanese complexes formed as described above were concentrated using ultrafiltration units with a molecular weight cutoff of 30,000. The membranes of the ultrafiltration units were washed with deionized water prior to use, and the protein solutions were concentrated at room temperature in a bench-top centrifuge (1000 g) for 15–45 min. The degree of concentration was determined by comparing protein concentration before and after centrifugation as determined using a colorimetric protein assay. The extent of concentration was also determined using 280 nm absorbance of the samples before and after concentration.

Second derivative spectral analysis was also used to follow the rhodanese in the presence of large excesses of GroE proteins. Second derivative spectra of proteins can be used to measure the ratio of phenylalanine, tyrosine, and tryptophan in a protein. Since the GroE proteins contain no tryptophan (33), we used the presence of a tryptophan spectralsignatureat291–294nm as an indication of the presence of rhodanese. In addition, we were able to use the ratio of the peak trough differences at 283–287 nm and 291–294 nm as a relative measure of the GroE to rhodanese ratio (34). This ratio was checked before and after concentration to assure that rhodanese and GroE concentrations were uniform. No significant change in the ratio of peak trough values were ever detected during the concentration. The presence of ATP, sodium thiosulfate, or DTT did not interfere with the second derivative peak-trough measurements.

Refolding and Analysis of Rhodanese—Rhodanese release and refolding was initiated by the addition of 5 mM ATP to the GroE-rhodanese complex (Fig. 3). All samples were supplemented to 50 mM sodium thiosulfate at the same time as ATP addition in order to stabilize the refolded rhodanese. At specified times after initiating refolding, aliquots were analyzed either by enzyme activity assay or gel filtration HPLC.

Recovery of enzymatic activity was used as a measure of recovery of "native" rhodanese structure. Rhodanese activity was measured using a discontinuous assay as described by Westley (30). For determining the amount of interaction between rhodanese and GroEL, proteins were separated by gel filtration HPLC on a TSK-3000SW gel filtration column equilibrated with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.5 mM EDTA. The HPLC used was a Waters 600S controller 626 pump with...
column used, the free GroEL coelutes with groEL-rhodanese and groEL-groES complexes. However, rhodanese in the GroEL-rhodanese complex can be distinguished from GroEL and GroES by the tryptophan contribution to its second derivative spectra. Second derivative spectral analysis was performed on each peak eluting from the column (Fig. 3, inset). For complexes formed in the presence of sodium thiosulfate there is no significant difference between the chromograms monitored at 280 nm before and after 15-min incubation with 5 mM ATP (Fig. 3A). This is probably due to the GroE proteins being present at 21-fold molar excess over rhodanese (14 GroEL and 7 GroES per complex). Under these conditions the released rhodanese is difficult to detect. However a difference can be seen in the second derivative spectral analysis of the GroE-rhodanese complex. For this peak, a significant decrease was seen in the tryptophan contribution indicated by the 290–294 nm peak-trough value after incubation with ATP (inset), indicative of a decrease in the amount of tryptophan containing rhodanese bound to the GroEL complex. When the GroE-rhodanese complex was formed in the absence of sodium thiosulfate and then analyzed as described above before and after incubation with 5 mM ATP for 15 min, a similar change in the second derivative of the GroE/complex peak was observed. In addition, a peak eluting at the void volume of the column was also detected after incubation with ATP. The elution time and spectrum of this void volume peak mirrored that observed for aggregated rhodanese (urea denatured and diluted into Buffer A alone). The appearance of this peak in samples where the complex was formed without thiosulfate indicates that under these conditions GroE can no longer prevent inappropriate aggregation at high rhodanese concentrations. At final concentrations between 0.5 and 2.0 mg/ml rhodanese, this void volume peak was not observed even though activity recovery is low. Since aggregation is a concentration-dependent event, concentrations between 0.5 and 2.0 mg/ml rhodanese may not be high enough to form an aggregate that is excluded from the TSK-3000SW column. Second derivative analysis of the base-line spectra in these chromatograms shows a very weak "protein signature" in the 280–300 nm region which suggests that ill-defined aggregates may spread out over the elution profile (data not shown).

Interaction of GroEL with Native Rhodanese—While characterizing the GroE-assisted rhodanese refolding reaction at 37 °C, it was observed that activity versus refolding time was not a simple profile. There is both an activation and inactivation reaction occurring at the same time in these samples. In addition, when the GroE-rhodanese complex was formed in the presence of thiosulfate, concentrated, and then diluted back to specified rhodanese protein concentration before incubation in 5 mM ATP, 50 mM sodium thiosulfate. Rhodanese activity was measured after incubation at 37 °C for 30 min. These data represents three experiments. Data are represented as enzyme activity determined after 30-min incubation at 37 °C.

Thiosulfate affects the folding of rhodanese at high concentrations. Rhodanese (3 mg/ml) was denatured in 8 M urea, 10 mM DTT, in the presence (●) or absence (○) of 50 mM sodium thiosulfate on ice. GroE-rhodanese complex was formed by diluting denatured rhodanese 1/100 into Buffer A containing an equimolar amount of GroEL and GroES (0.9 µM) with or without sodium thiosulfate. The complex was concentrated by ultrafiltration and then diluted back to specified rhodanese protein concentration before incubation in 5 mM ATP, 50 mM sodium thiosulfate. Rhodanese activity was measured after incubation at 37 °C for 30 min. These data represents three experiments. Data are represented as enzyme activity determined after 30-min incubation at 37 °C.
Complex formed in the presence (A) or absence (B) of sodium thiosulfate as described in the legend to Fig. 2 was concentrated to 3 mg/ml rhodanese, diluted to 2.5 mg/ml, and then either analyzed directly or incubated with 5 mM ATP, 50 mM sodium thiosulfate at 37 °C for 15 min before analysis. Samples were analyzed by passing them over a 1-ml Sephadex G25-300 spin column to remove excess salts and ATP and then injecting them onto a TSK-3000SW column equilibrated in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 mM EDTA. Inset, second derivative spectra of the GroEL/complex peak before and after incubation in ATP.
GroE (Fig. 5A). In addition, the second derivative analysis of the GroEL/complex peak shows an increase in the 290–294 nm peak-trough value (Fig. 5B). These changes suggest that the rhodanese in these mixtures is binding to the GroEL over time and eluting from the gel filtration column as a GroEL-rhodanese complex. After a 15-min incubation with 5 mM ATP, there is a small increase in the area of the rhodanese peak in the 280 nm chromatogram and a complete loss of the 290–294 nm peak-trough in the second derivative spectra of the GroEL/complex peak, suggesting that the rhodanese has been released from the GroEL. The second derivative spectra in Fig. 5B have been normalized to emphasize the changes in the 290–294 nm region. There are also small changes in the absolute values at 283 and 287 nm resulting from an increase in the total amount of protein in the GroEL/complex peak as rhodanese and/or GroES bind to GroEL.

**DISCUSSION**

The question of how chaperonins function to assist in the folding of nascent polypeptide is one of great complexity. Chaperonins are known to assist in the in vitro refolding of a variety of proteins of both procaryotic and eucaryotic origin. The requirements for efficient refolding vary from protein substrate to protein substrate such that some protein folding intermediates can be released from GroEL by the addition of the co-chaperonin GroES or ATP/ADP alone, whereas others require the presence of both GroES and ATP together.

To test whether non-native folding intermediates were dissociating from GroEL, Wiessman et al. (25) used mutant non-functional forms of GroEL to trap folding intermediates that were released from the wild-type GroEL complexes. Results indicated that released folding intermediates of rhodanese and ornithine decarboxylase bind to and become trapped on the mutant GroEL. This mutant form cannot release the folding
intermediates in the presence of ATP and inhibits the folding when the refolding was initiated from wild type GroEL-protein folding intermediate complexes. This suggests that the wild-type GroEL releases non-native folding intermediates that rebind to mutant GroEL and become trapped. These observations could not rule out the possibility that the protein folding intermediates shuttle between GroEL dimers through a direct transfer mechanism. However, this latter mechanism is without experimental support and is simply speculative.

Our initial finding that rhodanese can be refolded from a GroEL complex at concentrations as high as 2.5 mg/ml rhodanese is intriguing. If a release and rebinding mechanism is operative, the formation of a GroEL-rhodanese complex in the presence of thiosulfate may result in an intermediate that has either an extremely high or non-existent critical aggregation concentration. It is interesting to note that the total protein concentration in the refolding milieu (including rhodanese, GroEL, and GroES) is in excess of 30 mg/ml, and yet rhodanese recovery is near 100% of the control activity. However, when refolding is attempted with a slightly different form of protein folding intermediate that binds to GroEL in the absence of thiosulfate, there is a concentration above which the recovery of activity decreases significantly. This suggests that rhodanese may bind to GroEL in a minimum of two conformers whose bound structures depend on the absence or presence of thiosulfate in the complex. Upon release, these different intermediates appear to exhibit drastically different critical aggregation concentrations.

The exact role of thiosulfate in these experiments is impossible to determine from these data, but it is fairly clear that thiosulfate influences the folding pathway of rhodanese. It is tempting to speculate that thiosulfate donates a sulfur that binds directly to rhodanese folding intermediates and shifts the intermediate population toward more native form, thereby decreasing the conformational space that rhodanese must search in order to fold correctly. Upon release from GroEL, this sulfur-bound form may obtain native structure more rapidly and therefore be less prone to aggregation. Goldberg and Guillou (21) report that guanidine-denatured hen egg white lysozyme rapidly collapses into a compact intermediate upon dilution of the denaturant. The amount of secondary structure detectable in this intermediate differs greatly, depending on whether or not the unfolded protein has intact disulfide bridges. If the unfolded protein is reduced, this intermediate has much less secondary structure than when refolding is initiated from the non-reduced enzyme. The suggestion is that the disulfide bonds constrain the 3rd structure of the protein folding intermediate and facilitate folding to the native structure by limiting the conformational search space. We suggest that thiosulfate has a similar effect on rhodanese. The fact that the crystal structure of rhodanese has been determined with a hetero-atom of sulfur bound and that thiosulfate is one of the substrates for the enzymatic reaction lend support to this speculation. However, this hypothesis remains to be tested.

In the refolding experiments, the observed decline in rhodanese activity seen at long time periods could be due to thermal inactivation, aggregation, or covalent damage to the rhodanese conformation. However, if this were the case, GroEL would be expected to protect the rhodanese from conformational damage, at least in the case of thermal inactivation (26). In the experiments presented here, increasing the concentration of GroEL leads to a more rapid decrease in activity, suggesting that, in fact, the GroEL is sequestering the rhodanese in an inactive form. We have also demonstrated the binding of native rhodanese to GroEL under conditions which maintain rhodanese activity in the absence of GroEL. This bound form is released and reactivates after ATP addition. Although it is impossible to determine the structure of the bound rhodanese from these data, the form that binds must be in equilibrium with the native structure. Support for the existence of this equilibrium comes from the observation that there is GroEL concentration dependence in the rate of inactivation and that the released form regains native activity. We have yet to determine the lifetime of the complex at 37 °C.

Although these results appear to conflict with previous reports where no interactions between rhodanese and either wild-type or mutant GroEL were observed (25), the solution conditions used here were significantly different. The GroEL-native rhodanese interaction appears to be favored under our conditions of high protein concentrations, low ATP, and physiological temperatures. Martin et al. (32) report that addition of GroEL alone increases the rate of thermal inactivation of dihydrofolate reductase over that seen for dihydrofolate reductase alone or dihydrofolate reductase in the presence of GroEL, GroES, and MgATP. This is comparable with what we see with rhodanese at 37 °C. Our results may also explain the decreased yield of active rhodanese seen by Mendoza et al. (26) as the GroE-rhodanese ratio was increased from 2:1 to 8:1.

The data presented here provides support for the complete release and rebinding model. This does not rule out the possibility that some folding may occur while the protein folding intermediate is bound directly to or sequestered by GroEL. For example, glutamine synthetase has a limited time of interaction with GroEL (28) and yet is released in a different conformation (i.e., not aggregation prone) as compared with the pre-GroEL-influenced intermediate (27). The proportion of folding that is induced by direct interaction between folding intermediates and GroEL is probably dependent on the nature of the folding substrate. However, given the limited evidence for folding on GroEL (17, 18) more data supporting protein folding in a sequestered environment is needed.

We also present evidence of an interaction between GroEL and a native or near-native form of rhodanese which suggests that GroEL binds to multiple intermediates in the path from unfolded rhodanese to native rhodanese. These individual intermediates may be free in solution at some time and have varying critical aggregation concentrations and folding properties. In this model the nucleotide free or ADP-bound form of GroEL functions as a buffer to prevent the accumulation of high concentrations of aggregation-prone folding intermediates in solution.

Acknowledgment—We thank Dr. G. Helmkamp for his valuable suggestions.

REFERENCES
1. Viitanen, P. V., Gatenby, A. A., and Lorimer, G. H. (1992) Protein Sci. 1, 363–369
2. Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N., and Furtak, K. (1983) Cell 74, 905–917
3. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F.-U. (1992) Nature 356, 683–689
4. Hendrick, J. P., Langer, T., Davis, T. A., Hartl, F.-U., and Wiedmann, M. (1993) Proc. Natl. Acad. Sci. U.S.A. 100, 10216–10229
5. Goloubinoff, P. A., Gatenby, Christelier, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 342, 884–888
6. Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, J., Schmid, F. X., and Kieferhaber, T. (1991) Biochemistry 30, 1586–1591
7. Martin, J., Langer, T., Beteva, R., Schramel, A., Horwich, A. L., and Hartl, F.-U. (1997) Nature 385, 36–41
8. Fenton, W. A., Kashi, Y., Furtak, K., and Horwich, A. (1994) Nature 371, 614–619
9. Linz, Z., Schwarz, F. P., and Eisenstein, E. (1995) J. Biol. Chem. 270, 1011–1014
10. Agard, D. A. (1993) Science 260, 1902–1904
11. Hartl, F.-U. (1994) Nature 371, 557–559
12. Langer, T., Pfeifer, G., Martin, J., Baumelung, W., and Hartl, F.-U. (1992) EMBO J. 11, 4757–4765
13. Braig, K., Simon, M., Furuya, F., Haefnld, J. F., and Horwich, A. L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3978–3982
14. Ishii, N., Taguchi, H., Sasebo, H., and Yoshida, M. (1994) J. Mol. Biol. 236,
Chaperonins and Rhodanese Release and Rebinding

15. Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, S. G., Ranson, N. A., Clarke, A. R., and Saibil, H. R. (1994) Nature 371, 261–264
16. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) Nature 371, 578–586
17. Gray, T. E., and Fersht, A. R. (1993) J. Mol. Biol. 232, 1197–1207
18. Landry, T. J., and Fersht, A. R. (1993) Biochemistry 30, 7359–7362
19. Dill, K. A., Fiebig, K. M., and Chan, M. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1942–1946
20. Cohen, B. I., Presnell, S. R., and Cohen, F. (1993) Protein Sci. 2, 2134–2145
21. Goldberg, M. E., and Guillou, Y. (1994) Protein Sci. 3, 883–887
22. Dado, G. P., and Gellman, S. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1942–1946
23. Todd, M. J., Viitanen, P. V., and Lorimer, G. H. (1994) Science 265, 659–666
24. Todd, M. J., Viitanen, P. V., and Lorimer, G. H. (1994) Science 265, 659–666
25. Wiessman, J. S., Kashi, Y., Fenton, W. A., and Horwich, A. (1994) Cell 78, 693–702
26. Mendoza, J. A., Lorimer, G. H., and Horowitz, P. M. (1992) J. Biol. Chem. 266, 16973–16976
27. Fisher, M. T. (1993) J. Biol. Chem. 268, 13777–13779
28. Fisher, M. T., and Yuan, X. (1994) J. Biol. Chem. 269, 29598–29601
29. Fisher, M. T. (1994) J. Biol. Chem. 269, 13620–13636
30. Westley, J. (1981) Methods Enzymol. 77, 285–291
31. Tandon, S., and Horowitz, P. M. (1989) J. Biol. Chem. 264, 9859–9866
32. Martin, J., Horwich, A. L., and Hartl, F. U. (1992) Science 258, 995–998
33. Hemmingsen, S. M., Woolford, C., van-ver-Vies, M. S., Tilby, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988) Nature 333, 330–334
34. Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., and Irace, G. (1984) Biochemistry 23, 1871–1875