The in vitro folding of rhodanese involves a competition between formation of properly folded enzyme and off-pathway inactive species. Co-solvents like glycerol or low temperature, e.g., refolding at 10 °C, successfully retard the off-pathway formation of large inactive aggregates, but the process does not yield 100% active enzyme. These data suggest that mis-folded species are formed from early folding intermediates. GroEL can capture early folding intermediates, and it loses the ability to capture and reactivate rhodanese if the enzyme is allowed first to spontaneously fold for longer times before it is presented to GroEL, a process that leads to the formation of unproductive intermediates. In addition, GroEL cannot reverse large aggregates once they are formed, but it could capture some folding intermediates and activate them, even though they are not capable of forming active enzyme if left to spontaneous refolding. The interaction between GroEL and rhodanese substantially but not completely inhibits intra-protein inactivation, which is responsible for incomplete activation during unassisted refolding. Thus, GroEL not only decreases aggregation, but it gives the highest reactivation of any method of assistance. The results are interpreted using a previously suggested model based on studies of the spontaneous folding of rhodanese (Gorovits, B. M., McGee, W. A., and Horowitz, P. M. (1998) Biochim. Biophys. Acta 1382, 120–128 and Panda, M., Gorovits, B. M., and Horowitz, P. M. (2000) J. Biol. Chem. 275, 63–70).

The in vitro folding of proteins often involves intermediates that can partition to either properly folded or mis-folded conformations (3, 4). Aggregation is one of the most important side reactions that kinetically competes with proper folding, and it often results from interactions among partially folded intermediates (5–8). Therefore, controlling aggregation can assist successful renaturation. The enzyme rhodanese (thiosulfate sulfuryltransferase, EC 2.8.1.1) is difficult to fold in vitro, and it has become an interesting model system for assisted folding. Rhodanese is monomeric, folded into two almost equal size domains, and its crystal structure is available (9). The interdomain interface is strongly hydrophobic, giving rise to strong interactions between domains. In the active enzyme, all four of the rhodanese cysteine residues are reduced (9). The successful refolding of rhodanese after denaturation requires conditions that both reduce aggregation and limit very facile sulphydryl oxidation among residues far apart in the primary sequence (10–13). Even under these conditions, some inactive, monomeric species are formed, so that 100% reactivation is never achieved. Urea-induced denaturation of rhodanese involves steps that include domain separation followed by complete denaturation of the protein (1, 14, 15). The domain-dissociated intermediate contains a high degree of secondary structure, and it displays extensive hydrophobic exposure (14, 16, 17). Consequently, it can form large aggregates that can be monitored by light scattering (1, 17). Aggregation of rhodanese can be slowed by limiting the collision among active intermediates by lowering the protein concentration (13) including detergent micelles in the refractionation solutions (18), lowering the temperature (19), or by binding partially folded intermediates to molecular chaperones such as GroEL (20–24). Also, the addition of co-solvents can reduce the formation of aggregates both by slowing the diffusion of intermediates, thereby reducing their interactions (25), as well as having an effect on preferential hydration (26). For example, the addition of glycerol is very effective at preventing the formation of large rhodanese aggregates (2). Chaperonin-assisted folding of rhodanese has a kinetic advantage over aggregation (27). In the present study we have shown that, even under conditions where the formation of large aggregates is largely prevented, the recovery of rhodanese is not complete. This indicates that rhodanese can mis-fold even from intermediates that are too small to scatter light significantly more than the native enzyme. Furthermore, the results suggest that GroEL can capture early folding intermediates of rhodanese, which are in equilibrium with both on-pathway and off-pathway forms. Formation of mis-folded species from monomeric or small associated folding intermediates removes early folding intermediates from solution, which would explain the substantial but still incomplete reactivation by GroEL/GroES/ATP, even at low temperatures. GroEL can also bind and reactivate some folding intermediates, which otherwise would not fold to give active protein. A model for the spontaneous folding of rhodanese has been proposed previously (1, 2). We use and extend this model to interpret the present studies. In the present study, we have provided additional experimental support for some of the pathways postulated in our earlier investigations.

EXPERIMENTAL PROCEDURES

Materials

Urea was of electrophoresis purity (Bio-Rad), and BCA was purchased from Pierce. Other chemicals were from Sigma.

Methods

Rhodanese Purification—Recombinant bovine rhodanese was purified as described previously and stored at –70 °C as a crystalline suspension in 1.8 M ammonium sulfate containing 1 mM sodium thiosulfate (28). Rhodanese was desalted on a G-50 column before use. Rhodanese concentrations were determined using a value of \( A_{280} = 1.75 \).
Rhodanese Assay—Rhodanese activity was measured using a colorimetric method based on the absorbance at 460 nm of the complex formed between the reaction product, thiocyanate, and ferric ion (29).

GroEL and GroES Purification—GroES and GroEL were purified as described previously (30, 31). Protein concentrations were determined by the BCA method (32).

Unfolding-Folding of Rhodanese—For unfolding, 0.3 mg/ml rhodanese was denatured for at least 2 h at 25 °C in 50 mM Tris/Cl, pH 7.8, containing 8 M urea. For spontaneous folding, unfolded rhodanese was diluted to 3.6 µg/ml in 50 mM Tris/Cl, pH 7.8, containing 50 mM thiosulfate, 10 mM KCl, 10 mM MgCl₂, 0.2 M β-mercaptoethanol (folding buffer) preincubated at the desired temperature. For binding to GroEL, rhodanese was allowed to refold spontaneously in the folding buffer preincubated at a specific temperature. Aliquots were removed at different times and added to folding buffer containing 0.15 mg/ml GroEL preincubated at a desired temperature and incubated at that temperature for an additional 15 min. For reactivation from the rhodanese-GroEL complex, first 0.025 mg/ml GroES and then 2 mM ATP were added, and the incubation was continued for at least 1.5 h at 25 °C. Where indicated, additional GroEL was added, and/or additional incubation time was allowed to ensure that the chaperonin-assisted folding had come to completion. To assay the successful reactivation, 100 µl of the incubation mix were added to 1 ml of rhodanese assay mix and incubated for 10 min. The assay reaction was stopped by the addition of formaldehyde. The percent reactivation was calculated based on the activity of native enzyme. All light scattering measurements were performed as in Scheme 1. According to this model, an early intermediate, \( I \), forms rapidly when refolding is initiated for denatured rhodanese (\( U \)). This \( I \) partitions to two intermediates \( I' \) and \( I'' \). The form \( I'' \) is capable of folding to the native state (\( N \)). \( I' \) is prone to form oligomeric species (\( I_N \)) that ultimately lead to large aggregates (\( A_{\text{large}} \)). \( A_{\text{large}} \) refers to all species that are large enough to scatter light significantly more than monomeric rhodanese. All of these early folding intermediates; \( I' \), \( I'' \), and \( I'' \)' are in equilibria with each other. Since complete suppression of observable aggregates does not yield 100% active protein, it is suggested that mis-folded species can form even from the monomeric early folding intermediates. GroEL can bind early folding intermediates, and the results presented below suggest that formation of these complexes inhibits much of the intra-protein processes that would lead to inactive species. The data suggest that GroEL binds not only the early folding intermediates, but also binds some of the intermediates such as \( I_N \), which are not capable of forming active enzyme without the aid of the chaperonin. Effect of Temperature on the Unassisted Folding of Rhodanese—Fig. 1A shows the effect of temperature on the unassisted folding of rhodanese monitored by the regain of enzyme activity. At 37 °C (●), there is almost no recovery of active rhodanese, whereas the maximum recovery is ~30% at 10 °C (●) and ~10% at 25 °C (●). At the lower temperatures, the rates of activity regain are lower, and the yields are higher. This result is consistent with the idea that at lower temperatures the rates of formation of inactive species are lower. Aggregate formation is a temperature-dependent process. Fig. 1B shows via light scattering the formation of large aggregated species at different temperatures during unassisted folding. At 37 °C (●), aggregated species are formed rapidly and extensively. The light scattering reaches a maximum within 20 s (~50 × 10⁵ cps). At 25 °C (●), the final value (~22 × 10⁵ cps) is lower than that at 37 °C and requires a longer time, ~200 s, to reach equilibrium. The light scattering value at 10 °C (●) does not show any apparent time dependence, and it is only slightly higher than that of native rhodanese measured under identical conditions (6 × 10⁵ versus 3.5 × 10⁵ cps). The small final value of the light scattering at 10 °C may reflect the formation of small oligomeric species during spontaneous folding. Therefore, by lowering the temperature, the step \( I' \) [darrow] \( I'' \) [darrow] \( I_N \) [darrow] \( A_{\text{large}} \) can be limited to \( I' \) [darrow] \( I'' \) [darrow] \( I_N \). In that case, more active rhodanese would be expected to form through the partition of \( I'' \) to \( I' \), which would finally produce native enzyme. ~30% active rhodanese is formed at 10 °C compared with 10% at 25 °C (Fig. 1A). At lower temperature, more time is required to reach the equilibrium activity values. The longer time to reach the maximum activity at lower temperature is consistent with the suggestion that there is less formation of large irreversible species. Although at higher temperature, e.g. at 25 °C, the rate of reactivation of rhodanese (Fig. 1A, ▲) is faster than that at 10 °C (Fig. 1A, ●), the extent of reactivation is higher at 10 °C (Fig. 1A, ●). According to Scheme 1, the extent of reactivation is controlled by the irreversible formation of large inactive species that are formed at a higher rate at higher temperature (Fig. 1B). The poor yield of active enzyme at higher temperature is directly

**Scheme 1.** \( U \), denatured; \( N \), native state.

The abbreviations used are: cps, counts/s; \( I \), \( I' \), \( I'' \), different early folding intermediates; \( I_N \), oligomeric intermediates; \( A_{\text{large}} \), large aggregates.
Temperature. But once the complex has been formed, spontaneous refolding, temperature plays a vital role in formation added and incubated at 25 °C for an additional 1.5 h. During incubated at that temperature for 15 min. GroES and ATP are rhodanese is added to folding buffer containing GroEL that has three temperatures indicates a recovery of rhodanese activity A large. These data indicate that formation of large aggregates (A), (B), and at 37 °C (C). Therefore, within the GroEL-rhodanese complex, intra protein continued, a maximum of 80% active enzyme is recovered.

As seen in Fig. 1, lower temperatures retard the formation of large aggregates, which can either form active rhodanese through formation of I’’, which is very fast, as there is a large drop in reactivation of rhodanese by GroEL is very slow at 10 °C. Even after 3800 s of spontaneous folding, capturable and reactivatable intermediates are present in solution. The final yield of active rhodanese at 10 °C in assisted folding is much higher than that obtained in the unassisted process (Fig. 2B). This result suggests that at the lower temperature there are some intermediates present in the solution that during unassisted refolding do not form the active protein. But GroEL can capture them and allow them to successfully fold to active enzyme upon the addition of GroES and ATP. So GroEL can also bind and reactivate some species that will not spontaneously form active enzyme. This is demonstrated by the observation in Fig. 2B that the plateau values for spontaneous and GroEL folding are not coincident, i.e. 20% more active rhodanese is formed at 10 °C with the chaperonin system. Although large aggregated species are not formed at 10 °C, we still do not achieve 100% reactivation either by assisted or by unassisted folding. At 10 °C, the maximum reactivation of active rhodanese from GroEL/GroES/ATP is ~80%. This suggests that monomeric or small folding intermediates can also form misfolded species, which removes early folding intermediates and leads to incomplete recovery of the active protein (Scheme 1).

Results from identical experiments at 37 °C are shown in Fig. 2C. There is almost no active rhodanese formed during unassisted folding, which may be due to fast formation of irreversible aggregates. The loss of early folding intermediates is very fast, as there is a large drop in reactivation of rhodanese from ~75 to ~7% within 20 s. Fig. 1B shows that at 37 °C, light scattering reaches a maximum during this time. The loss in reactivation of rhodanese as spontaneous folding proceeds at 37 °C is not due to inactivity of GroEL at 37 °C because the t = 0 value corresponds to GroEL that has been preincubated at this temperature and yields ~75% reactivation (t = 0 point in Fig. 2C), a value similar to those observed at 25 °C (Fig. 2A) and 10 °C (Fig. 2B). Native rhodanese is stable at 37 °C under these buffer conditions, so inactivity of the protein is due to processes involving the intermediates and not to inactivation of properly folded rhodanese. The high light scattering value and formation of inactive rhodanese during both assisted and unassisted folding suggest that at 37 °C, the reaction I’ → I’’ → I₀ → Aₕₐₙ₉₉ is very rapid.
mediates. Beyond cies and maintains the equilibrium among early folding inter-glycerol retards the formation of irreversible aggregated spe-
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GroEL is not due to slower diffusion of rhodanese onto GroEL. Before the addition of GroES and ATP, the reduced efficiency of
GroEL and rhodanese were incubated together for 15 min
Rhodanese was denatured
during assisted and unassisted folding.

Fig. 3. Effect of 4 M glycerol on reactivation of rhodanese during assisted and unassisted folding. Rhodanese was denatured as described under “Methods.” ●, denatured rhodanese was added to folding buffer containing 4 M glycerol, preincubated at 25 °C. Spontaneous folding was done at 25 °C. Aliquots were taken after the noted time intervals and assayed. ■, spontaneously folded rhodanese was made as above. Aliquots were taken after the indicated times and added to folding buffer containing 4 M glycerol and GroEL. The complex was incubated at 25 °C for 15 min. GroES and ATP were added and incubated at 25 °C for 1.5 h. The activity of native rhodanese in folding buffer containing 4 M glycerol was taken as 100%.

In all experiments, additional incubation of the GroEL samples gave no increase in reactivation. Similarly, introduction of additional GroEL to all samples at 3600 s gave no increased reactivation. Thus, the plateau values shown are taken as the equilibrium values.

Effect of Glycerol on the Folding Pathway of Rhodanese—It has been shown that glycerol inhibits the formation of large aggregates (Fig. 1B, ▼), which form irreversibly from smaller ones during rhodanese folding (2). If this is the case, then glycerol should show similar effects on the reactivation of rhodanese as that observed at 10 °C. Fig. 3 shows the effect of 4 M glycerol on the unassisted folding of rhodanese (●) at 25 °C. The rate of formation and the final yield of active enzyme is similar to that found at 10 °C (Fig. 2B), ~30%, which is considerably higher than value obtained at 25 °C (Fig. 2A, ~10%).

The absence of irreversible, nonproductive aggregates makes more I’ available, which can form active rhodanese through I’” (Scheme 1). So one may expect higher reactivation of rhodanese by the chaperonin system. The loss of reactivation of active rhodanese from GroEL/GroES/ATP (■) shows very slow time dependence, as spontaneous folding proceeds in the presence of 4 M glycerol. GroEL itself is less efficient in releasing active rhodanese in 4 M glycerol (see the t = 0 point in Fig. 3). Since GroEL and rhodanese were incubated together for 15 min before the addition of GroES and ATP, the reduced efficiency of GroEL is not due to slower diffusion of rhodanese onto GroEL. The maximum reactivation of rhodanese obtained is in the range of 40%, when denatured rhodanese was added to folding solution containing GroEL and 4 M glycerol. In the absence of glycerol, the maximum reactivation is ~70%. The higher yield of active rhodanese during unassisted folding and the slow loss of early folding intermediates that can be captured by GroEL may indicate that in the presence of glycerol, there is partitioning of I between I’” and I’” as we observed at 25 °C, but glycerol retards the formation of irreversible aggregated species and maintains the equilibrium among early folding intermediates. Beyond ~800 s of spontaneous refolding, no early folding intermediates are present that can be captured by GroEL, as there is no further reactivation of rhodanese by GroEL/GroES/ATP. Since glycerol appears to inhibit the for-
mation of large aggregates, the lack of capturable intermediates beyond 800 s may be due to formation of mis-folded species from monomeric intermediates such as I’, I”, and I’” (Scheme 1). It appears then that the presence of intermediates like I’ ultimately determines the higher reactivation during folding either through the formation of I’” or by being captured by GroEL. At lower temperatures or in the presence of a high concentration of glycerol formation of active rhodanese is the predominant pathway during unassisted folding, as there is no appreciable formation of large, irreversible aggregates. However, the suppression of aggregation still does not yield 100% active rhodanese as we get only a maximum yield of 30% at 10 °C and in the presence of 4 M glycerol during unassisted folding. These data suggest that I’, I”, and I’” form mis-folded species, which lead to incomplete recovery of active rhodanese during spontaneous folding. The incomplete recovery of active hen lysozyme has been reported (33). It has been shown that spontaneous folding in buffer produced 38% active protein, whereas in 3.44 M glycerol, the activity regained was 42%. Therefore, competition among off-pathway aggregation, formation of mis-folded species from folding intermediates, and interconversion of species such as I” to I’ dictate the final yield of the active rhodanese.

Formation of Large Aggregated Species during Unassisted Folding Dictates the Final Yield of Active Rhodanese—According to Scheme 1, irreversible formation of large, unproductive aggregated species during folding is a major contributor to the inefficient reactivation of rhodanese during both assisted and unassisted folding. If GroEL could reverse the large aggregated species, one should expect lower light scattering in the presence of GroEL. Fig. 4 shows the light scattering of the species formed by spontaneous folding that was allowed to proceed for the noted times at different temperatures and then followed by the addition of GroEL. The shapes of the profiles for the light-scattering values of spontaneously folded rhodanese in Figs. 1B and 4 are similar. At 10 °C, there is no time-dependent increase in the scattering (●), and the value is close to that of native rhodanese under identical conditions. This result is consistent with the idea that at low temperature, intermediates are formed that are not large enough to scatter light. The addition of GroEL does not have any effect on the scattering values. At 37 °C, there is very fast formation of large aggregates, which is irreversible under the conditions used here. Aggregation favors removal of folding intermediates through the process I’ → I’ →
If GroEL could capture these large aggregates through the formation of monomeric intermediates, the scattering value in the presence of GroEL should be lower. But at 37 °C, the rapidly enhanced light scattering remains unchanged in the presence of GroEL (compare Fig. 1B with Fig. 4, ▲). The profile at 25 °C (■) shows time-dependent increase in the scattering values as spontaneous folding proceeds. It reaches a maximum value by 400 s. By this time, the release of active rhodanese by GroEL/GroES/ATP reaches its minimum level (Fig. 2A). This time dependence of the light scattering may reflect the fact that in the initial stages of spontaneous folding (less than 400 s) early folding intermediates are present that could be captured by GroEL. But at the later stage large aggregates predominate (Scheme 1). Thus, constant light-scattering values in the presence of GroEL (Fig. 4) indicate that GroEL cannot reverse large aggregates once they have formed.

From our data, we suggest that GroEL can bind folding intermediates like I′, I″, and I‴. Even in the absence of the aggregate-forming pathway, mis-folding can occur. These processes remove productive folding intermediates from solution, which results in incomplete recovery of active rhodanese either during unassisted folding or from GroEL/GroES/ATP even at lower temperatures. GroEL can recover some species, which themselves would not form active protein if left to the spontaneous process. Binding to GroEL significantly inhibits intra-protein inactivation of rhodanese.

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The Aggregation State of Rhodanese during Folding Influences the Ability of GroEL to Assist Reactivation
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*J. Biol. Chem.* 2001, 276:28739-28743.
doi: 10.1074/jbc.M102500200 originally published online June 7, 2001

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