Rhodanese Can Partially Refold in Its GroEL−GroES−ADP Complex and Can Be Released to Give a Homogeneous Product†

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ABSTRACT: Molecular chaperones GroEL and GroES facilitate reactivation of denatured rhodanese which folds poorly unless the process is assisted. The present work tests the hypothesis that more extensively unfolded forms of rhodanese bind tighter than those forms that appear later in the folding pathway. The study of the interaction of different urea-induced forms of rhodanese with GroEL suggests that species preceding the domain folded form bind directly and productively to GroEL. Rhodanese partially folds while in the GroEL−GroES−ADP complex, but it does not significantly reach an active state. Partially folded rhodanese can be released from the GroEL−GroES−ADP complex by subdenaturing concentrations of urea as a homogeneous species that is committed to fold to the native conformation with little or no partitioning to the aggregated state. Dilution of denatured rhodanese to the same final concentration gives less active enzyme and significant aggregation. Urea denaturation studies show that active rhodanese released from complexes behaves identically to native enzyme, while spontaneously folded rhodanese has a different stability. These results are interpreted using a previously proposed model based on studies of unassisted rhodanese folding [Gorovits, B. M., McGee, W. A., and Horowitz, P. M. (1998) *Biochim. Biophys. Acta 1382*, 120−128. Panda, M., Gorovits, B. M., and Horowitz, P. M. (2000) *J. Biol. Chem. 275*, 63−70].

The molecular chaperones are multimeric proteins that play a major role in folding, trafficking, and assembly of proteins in the cell. The most widely characterized molecular chaperones are $GroEL_{14}$, a 14 subunit oligomer (14mer) from *Escherichia coli* and its regulatory protein $GroES_7$, a 7mer. In vivo, the GroE system is involved in the folding of ∼5−10% of proteins chains to their native three-dimensional conformations (*3*, *4*). GroEL is able to bind labile folding intermediates and prevent irreversible side reactions that compete with folding (*5*−*7*). For productive binding and release of substrate proteins under most circumstances, ATP binding and hydrolysis are necessary (*8*). In some cases, it is also dependent on the presence of GroES (*4*, *8*, *9*). Molecular chaperones do not contain any information for determining proper folding. The information for the folding of a polypeptide chain into its functional three-dimensional conformation is present solely in the amino acid sequence (*10*).

Multidomain proteins often fold with low efficiency in vitro due to unproductive interactions among domains during folding process (*11*). The enzyme rhodanese (thiosulfate transferase, EC 2.8.1.1) has become an important model for studying molecular chaperone-mediated protein folding (*5*, *9*, *12*−*14*). This monomeric enzyme is folded into two, independent, equal-size domains. The domains are tightly associated, and the interdomain interface is highly hydrophobic. The active form of the enzyme contains four cysteine residues that are all reduced (*15*). Its crystal structure is available (*15*, *16*). There are two main difficulties in reactivating rhodanese after complete unfolding. First, oxidation of one or more sulfhydryl groups leads to the formation of disulfide-linked misfolded conformations that are difficult to reduce. Second, folding intermediates of rhodanese contain solvent exposed hydrophobic surfaces, making them prone to aggregation (*12*, *17*). The yield of active enzyme from spontaneous refolding is very low predominantly because of these non-native folding reactions. Successful recovery of active rhodanese can be achieved only by the suppression of these two side reactions. Binding of unfolded protein to GroEL and subsequent addition of GroES and ATP to the stable binary complex lead to significantly reactivated rhodanese. Normally, the reactivation of rhodanese from the GroEL−rhodanese complex needs subsequent addition of GroES and ATP (*13*, *14*). This requirement of GroES and ATP can be removed by the addition of urea (*18*). Thus, when stable GroEL−rhodanese complexes are treated with 2.5 M urea, active enzyme is released.

In this investigation we show that low concentrations of urea that do not denature rhodanese can release active enzyme from the initial complexes as a homogeneous species that is committed to reach the native conformation. In this way, urea can be used as a probe to study the interaction between rhodanese and GroEL. We show that for successful binding to GroEL, rhodanese must be denatured beyond its domain folded form. The results suggest that early folding intermediates from urea denaturation are preferentially bound.

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Rhodanese can partially fold in the GroEL−GroES−ADP complex to yield a conformation that is committed to fold to its native conformation. This partially folded state inside the GroEL cavity is not active.

EXPERIMENTAL PROCEDURES

Reagents. Urea was of electrophoresis purity and was purchased from Bio-Rad. SDS, acrylamide, and bis-acrylamide were from Fischer Scientific (Pittsburgh, PA). [^{14}C]Iodoacetic acid was from Amersham. BCA[1] was purchased from Pierce (Rockford, Ill). Other chemicals were from Sigma.

Methods. (i) 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 (*19*). Rhodanese was desalted on a G-50 column before use. Rhodanese concentration was determined using a value of $A_{0.1\%,280nm} = 1.75$ (*20*).

(ii) 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 (*20*).

(iii) Preparation of ^{14}C-Labeled Rhodanese. ^{14}C-Labeled rhodanese was prepared by carboxymethylation of the active site cysteine residue (Cys 214) using ^{14}C-labeled iodoacetic acid. The method followed was the same as previously described (*21*, *22*).

(iv) Unfolding-Refolding of Rhodanese. For unfolding, 9 μM of rhodanese was denatured for at least 2 h at 25 °C in 50 mM TrisCl, pH 7.8, and containing 8 M urea. For spontaneous refolding, unfolded rhodanese was diluted to 0.11 μM and allowed to refold in 50 mM TrisCl, pH 7.8, containing 50 mM thiosulfate, 10 mM KCl, 10 mM $MgCl_2$, 0.2 M β mercaptoethanol (refolding buffer) for 18 h. The results were independent of the presence of a reductant during denaturation. For binding to GroEL, denatured rhodanese was diluted to 0.11 μM and allowed to refold in the refolding buffer containing 2.5 μM/protomer GroEL and 2.5 μM/protomer GroES and, last, either 2 mM ADP or ATP. The incubation was continued for at least 1.5 h at 25 °C.

For equilibrium denaturation of either native rhodanese, spontaneously refolded rhodanese or the GroEL−GroES−rhodanese−ADP complex, samples were diluted to give 0.11 μM of rhodanese in refolding buffer to which the indicated urea concentrations were added, and they were allowed to incubate either for 3 h or for 18 h. Both the profiles were essentially identical for all the sample described above.

To assay the successful reactivation, 100 μL of the incubating enzyme were added to 1 mL of assay mix and incubated for 10 min prior to stopping the reaction. For GroEL−GroES mediated rhodanese refolding, 15 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid was used in the assay mix to stop the further refolding during assay. To measure the activity during equilibrium unfolding and refolding, the same concentration of urea was added to the assay buffer and the profile was identical to that with no urea in the assay buffer. The percent reactivation was calculated based on the activity of native enzyme that had been subjected to the refolding conditions.

(v) GroEL and GroES Purification. $GroES_7$ and $GroEL_{14}$ were purified as described previously (*23*, *24*). Protein concentration was determined by the BCA method (*25*).

(vi) Preparation of [^{14}C]GroEL. ^{14}C-Labeled $GroEL_{14}$ was prepared by reductive methylation using sodium cyanoborohydride and [^{14}C]formaldehyde in 0.1 M sodium phosphate, pH 8 (*26*).

(vii) Preparation of Monomeric GroEL with Urea. Monomeric GroEL was prepared by incubating GroEL in refolding buffer which was made 2.5 M in urea for 1 h at 25° C (*27*, *28*).

(viii) Gel Electrophoresis of GroEL−GroES−Rhodanese−ADP Complex. Nondenaturing gel electrophoresis was used as described previously (*26*). Tris borate buffer (2 M), pH 8.5, was used to get clearer bands. Gel was dried under vacuum onto Whatman 3 MM paper. Radiolabel was detected by using a storage phosphor screen and a PhosphorImager from Molecular Dynamics.

(ix) Measurement of Percent of Rhodanese Productively Bound to GroEL. Rhodanese (9 μM) was incubated at different urea concentrations in 50 mM TrisCl, pH 7.8, for 16 h at 25 °C. Incubated samples were diluted to 0.11 μM in refolding buffer containing 2.5 μM/protomer GroEL and 2.5 μM/protomer GroES. ATP (2 mM) was added, and the samples were incubated at 25 °C for 90 min. Aliquots (100 μL) of each sample were assayed for activity by incubating the assay mixtures for 10 min at 25 °C. For a control, 0.11 μM of native rhodanese was incubated for 3 h at 25 °C in refolding buffer to which was added different concentrations of urea. Aliquots (100 μL) of each sample were assayed for 10 min at 25 °C. The same control experiment was performed using 9 μM native rhodanese denatured in 50 mM TrisCl, pH 7.8, containing different concentrations of urea for 14 h at 25 °C. These samples were then diluted to 0.11 μM in refolding buffer containing corresponding concentration of urea and incubated further at 25 °C for 2 h. The profile was identical to the former one (data not shown), which indicated that 3 h of incubation gave equilibrium values. All activity values were normalized using 100% activity for native rhodanese subjected to refolding conditions. To calculate the percent of rhodanese productively bound to GroEL, normalized activity values of the control set were subtracted from the corresponding normalized activity values of rhodanese in the presence of GroEL−GroES−ATP.

(x) Fluorescence Spectroscopic Studies and Light Scattering. All fluorescence measurements were performed using a Fluorolog-3 (Jobin Yvon-Spex) spectrofluorimeter. For tryptophan fluorescence measurement, the excitation wavelength was set at 295 nm and emission spectra were taken from 315 to 500 nm. The excitation and emission slit widths were each 1 nm. For light scattering experiments, both excitation and emission wavelengths were set at 340 nm with 0.5 nm slit widths. All fluorescence values were corrected using appropriate buffer blanks.

RESULTS

Rhodanese Should Be Extensively Unfolded Prior to Binding to GroEL. Rhodanese unfolds through intermediates that represent a separation of its two domains in a process

[1] Abbreviations: CMR, carboxymethylated derivative of rhodanese; BCA, bicinchonic acid; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; EcDHFR, *Eschericia coli* dihydrofolate reductase.

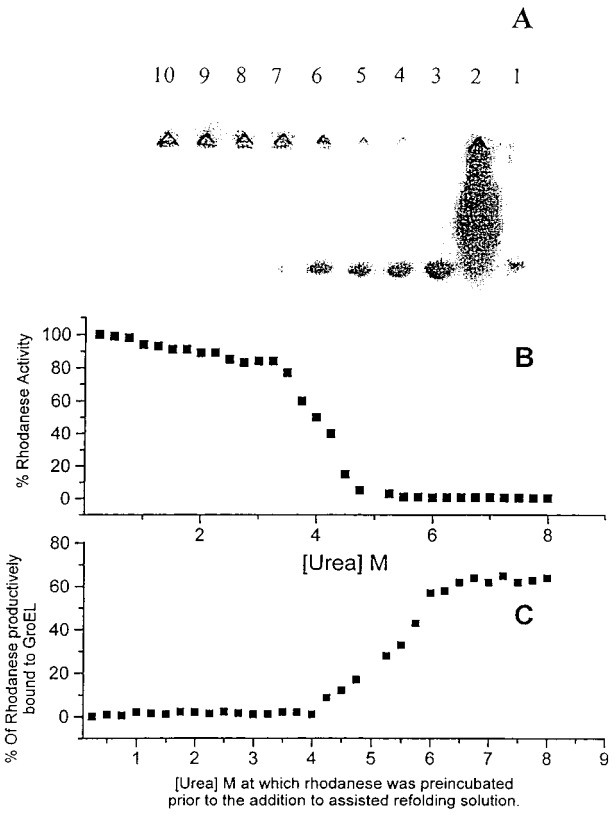


FIGURE 1: Effect of urea on the binding of rhodanese to GroEL−GroES complex and its release as active protein. (A) Phosphoimage analysis of the binding to GroEL−GroES of ^{14}C carboxymethylated rhodanese ([^{14}C]CMR) treated with different urea concentrations. Lane 1 corresponds to native [^{14}C]CMR, lane 2 is [^{14}C]GroEL, lanes 3−10 correspond to GroEL−GroES−[^{14}C]CMR−ADP complexes, where [^{14}C]CMR samples were preincubated at 0.5, 4, 4.5, 4.75, 5, 6, 7, and 8 M urea, respectively. (B) Activity detected urea denaturation of rhodanese. (C) Rhodanese productively bound to GroEL−GroES as a function of urea concentration. Rhodanese was incubated at different urea concentrations before dilution into refolding buffer. Reactivation was measured after addition of ATP and incubation for 90 min. All activity values of B and C were normalized using 100% activity for the same concentration of the native rhodanese subjected to refolding conditions. Calculation for the % of rhodanese productively bound to GroEL and experimental details are described in the Experimental Procedures.

that precedes complete unfolding (*29*). The first transition, which occurs at 3−4 M urea, is associated with domain separation, followed by a second transition leading to complete denaturation (*1*, *30*, *31*). Figure 1A shows a native gel of the complex formed by mixing ^{14}C-labeled rhodanese, CMR (^{14}C), denatured at different urea concentrations, and GroEL−GroES−ADP (Materials and Methods). Only rhodanese is observable on this gel. The upper bands represent rhodanese in the complex, while the lower bands show uncomplexed rhodanese. This method permitted the measurement of actual complex formation without having to rely on activity measurements. Binding of rhodanese to GroEL is observed starting with samples that had been preincubated at urea concentrations of >4 M, and only a small fraction of complex is found at lower urea concentrations. Above 5 M urea, rhodanese contains no detectable secondary structure (*1*, *21*, *31*). These data in Figure 1A clearly indicate that rhodanese must be presented to GroEL in a form that is unfolded beyond the domain folded state in order to form a complex with GroEL.

Reactivation-Based Method for Assessing Interactions of Rhodanese with GroEL. A second method was used to assess the binding of rhodanese by measuring the ability to refold rhodanese that had been pretreated with different concentrations of urea. Figure 1B is a control showing the activity-detected denaturation of native rhodanese treated at different urea concentrations. The activity remains constant up to ~3.5 M urea and then declines with no detectable activity beyond 6 M. Figure 1C shows the results of an experiment in which native rhodanese was preincubated at different urea concentrations and then diluted into refolding buffer containing GroEL, GroES, and ATP (see Materials and Methods). Figure 1C is derived from the differences in activity between the GroEL−GroES treated samples and the control urea denaturation. The data are presented as the percent of rhodanese that is productively bound to GroEL as a function of the urea concentration used for preincubation. Zero on the ordinate indicates the same activities for rhodanese that was released from the complex and native rhodanese and indicates no productive binding of rhodanese. Higher values indicate that rhodanese has been bound and productively refolded (described in detail in the Experimental Procedures). The activity values of the complex were very similar to that for native rhodanese up to 4 M urea. Beyond this concentration, the difference in the activity values and hence the binding of rhodanese to GroEL−ES increased and a reached maximum when the rhodanese had been pretreated at ~6 M urea, where rhodanese lacks detectable secondary structure (*1*, *20*, *30*).

There is no binding of rhodanese to GroEL, when it is treated below 4 M urea. Highest reactivation was found when rhodanese was denatured at 6 M urea and above. This observation indicates again that rhodanese could only bind extensively to GroEL when it was unfolded beyond the domain folded form before presentation to GroEL.

Active Rhodanese Undergoes Partial Refolding within the GroEL−GroES−ADP Complex and Can Be Released by Subdenaturing Concentrations of Urea. GroEL−GroES−ADP−rhodanese complexes formed after denaturing rhodanese with 8 M urea were very stable, and they showed insignificant activity (<4%) even after 24 h of incubation [Figure 2B (●), and Table 1]. This is in contrast to previous reports that active enzyme could be recovered from GroEL−GroES−ADP−rhodanese complexes (*38*). Table 1 shows the results of reactivation experiments performed under the conditions used previously and in the present studies. The previous conditions give recoveries of rhodanese from the ADP-containing complexes of 4% or less (Table 1, buffer conditions MA and MB). When these results are normalized to the activity recovered with ATP, the calculated recovery is 81% (Table 1, 37° C, buffer MA), which is close to the percent recovery reported earlier (*38*). However, neither ATP nor ADP returned significant absolute activity, and for the purposes of the present study we consider the ADP-containing complexes to be essentially inactive (compare ATP vs ADP with either RB or RBA in Table 1). The most important buffer components for successful refolding of rhodanese from GroEL−GroES−ATP are the combination of sodium thiosulfate and β-ME. Table 1 shows that in the presence of those two components, buffer MA shows small but significant refolding of rhodanese in the presence of ATP. Active rhodanese could be released from the complexes using

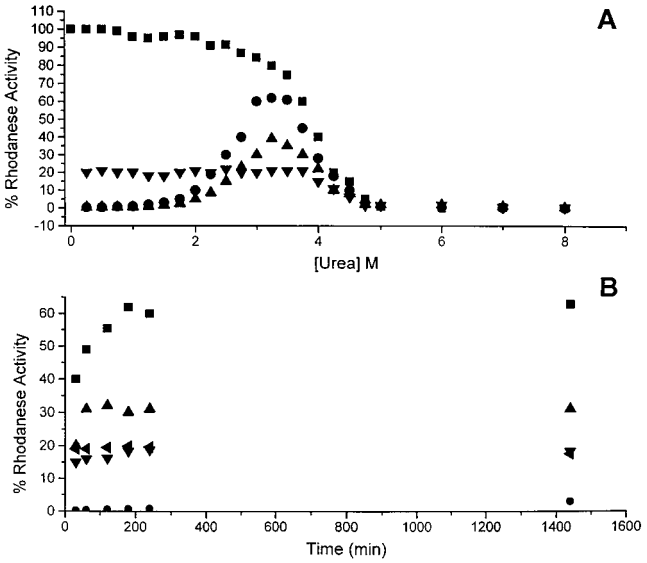


FIGURE 2: Rhodanese can be released as active protein from GroEL−GroES−ADP complexes using subdenaturing concentrations of urea. (A) (■) Native rhodanese (0.11 μM) was incubated in refolding buffer to which was added different concentrations of urea, for 3 h at 25 °C. (●) Denatured rhodanese (described in Experimental Procedures) was diluted into refolding buffer containing 0.15 mg/mL GroEL, 0.025 mg/mL GroES, and 2 mm ADP and incubated at 25 °C for 15 min. The complex was then incubated at different urea concentrations for 3 h at 25 °C. (▲) Identical to samples with solid circle (●), but prepared in the absence of GroES. (▼) Spontaneously refolded rhodanese (described in Experimental Procedures) was incubated at different urea concentrations for 3 h at 25 °C. (B) Kinetics of the formation of active rhodanese from (■) GroEL−GroES−ATP, where the reaction was initiated by the addition of 2 mM ATP, (▲) GroEL−GroES−ADP in the presence of 2.5 M urea, (●) GroEL−GroES−ADP, (▼) GroEL (monomeric)−GroES−ADP, and (triangle left solid) spontaneous refolding in refolding buffer. Experimental details are described in Experimental Procedures.

nondenaturing concentrations of urea. Figure 2A shows the activity of native rhodanese (■), spontaneously refolded rhodanese (▼), and rhodanese−GroEL complexes [+GroES (●), −GroES (▲)] each of which were treated at different urea concentrations. To make the initial complex with GroEL, rhodanese was first denatured in 8 M urea and then diluted in refolding buffer containing GroEL, GroES, and ADP which was added last. The final concentration of urea was 0.1 M. The preformed complexes were then treated with different urea concentrations, and the activities were compared with the effect on rhodanese that had been spontaneously folded and containing no GroEL−GroES. There was no detectable reactivation of rhodanese from the complexes up to ~2 M urea. The recovered activity increased with further increases in urea concentration and reached a maximum around 3.5−3.75 M. Beyond this concentration, there was a decrease in activity and the transition profile was virtually identical to that of native and spontaneously refolded protein. Maximum activity found with spontaneously refolded rhodanese was around 20%, whereas maximum activity found with the rhodanese released from the complex was 60% (●). In the absence of GroES, a maximum of 40% activity (▲) could be recovered. These data show that there is extra reactivation if GroES is present in the complex, and they are consistent with a picture in which binding of rhodanese to GroEL and sequestration of the protein inside the cavity formed within the GroEL−GroES

Table 1: Percentage of Rhodanese Activity Recovered from GroEL−GroES under Different Conditions[a]

temp (°C)	buffer[c]	2 mM ADP	0.2 mM ADP	5 mM ATP
	RB	3.5	4	100
25	RBA	3.3	4.2	100
	MA[b]		3.5	4
	MB[b]		3	3.6
	RB, no β-ME			10
	RB, no thiosulfate			22
	RB, no β-ME, no thiosulfate			1
	MA with β-ME			10
	MA with thiosulfate			6
	MA with β-ME and thiosulfate			20
	RB	4	2.6	100
37	RBA	2	3.2	100
	MA[b]		3.4	4.2
	MB[b]		2.1	3

[a] Urea (0.11 μM) denatured rhodanese was added to different buffers containing 2.5 μM/protomer GroEL and 2.5 μM/protomer GroES and incubated either at 25 or 37 °C for 15 min. ADP or ATP was added and incubated at 25° C for 90 min and at 37 °C for 60 min. The activity of rhodanese released by ATP from buffer RB was taken as 100%. [b] Conditions used as in ref *38*. [c] Buffers used, RB: 50 mM TrisCl, 50 mM sodium thiosulfate, 10 mM KCl, 10 mM $MgCl_2$, pH 7.8, 0.2 M β-mercaptoethanol. RBA: 50 mM TrisCl, 50 mM sodium thiosulfate, 80 mM KCl, 5 mM $MgCl_2$, 20 mM NaCl, pH 7.8, 0.2 M β-mercaptoethanol. MA: 20 mM MOPS−KOH, 80 mM KCl, 5 mM magnesium-acetate, 20 mM NaCl, pH 7.2. MB: 20 mM MOPS−NaOH, 5 mM KCl, 12 mM magnesium-acetate, pH 7.2

complex plays a positive role on the reactivation of rhodanese. From Figure 2B, it is evident that maximum recoveries of active rhodanese from the complexes were achieved by 40 min. Therefore, all the activity values reported in Figure 2A, which were measured after 3 h incubation at 25 °C, were the equilibrium values.

Figure 2B shows the time course of the effect of urea on the reactivation of rhodanese from GroEL−GroES−ADP complex, refolding of rhodanese from GroEL−GroES-ATP complex, and refolding in the presence of monomeric GroEL. Maximum activity achieved from GroEL (14 mer)−GroES−ATP complex was 60%, and it was ~40% when rhodanese was released from GroEL−GroES−ADP complex at 2.5 M urea, whereas both rhodanese that had been spontaneously refolded or refolded in the presence of monomeric GroEL were 20%. Thus, monomeric GroEL, prepared as described in the Experimental Procedures, had no effect on the reactivation of rhodanese. Recently it had been demonstrated that rhodanese binding requires at least two subunits of GroEL (*31*). The data presented here suggests that rhodanese underwent at least partial refolding within the GroEL cavity, and it could be released in a refoldable conformation at urea concentrations that do not perturb the native rhodanese structure. The GroEL−GroES complexes formed here showed little or no rhodanese activity, so whatever folding occurs in the complexes does not give rise significantly to active enzyme. The activity must arise in a step following the urea-induced release from the complex.

Binding to GroEL−GroES−ADP Complexes Helps Rhodanese Form Homogeneous, Nativelike Species. The next question addressed was whether there was any difference among native rhodanese, spontaneously refolded rhodanese, and rhodanese released by 2.5 M urea from GroEL−GroES−

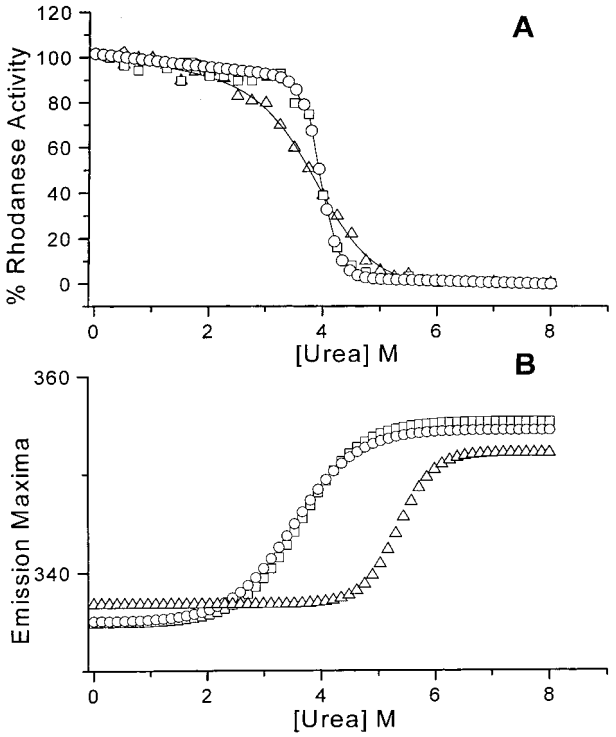


FIGURE 3: Equilibrium denaturation curves for native rhodanese released from the GroEL−GroES−ADP complex by 2.5 M urea and spontaneously refolded rhodanese. (□) Native rhodanese (0.11 μM) was incubated at different urea concentrations for 3 h at 25 °C. (○) Denatured rhodanese (described in Experimental Procedures) was diluted into refolding buffer containing GroEL, GroES, and ADP and incubated at 25 °C for 15 min. Urea (2.5 M) was added and incubated at 25 °C for 90 min. Released rhodanese was incubated at different urea concentrations for 3 h at 25 °C. (△) Spontaneously refolded rhodanese (described in Experimental Procedures) was incubated at different urea concentrations for 3 h at 25 °C. In panel A, activities of all the samples are plotted as a function of urea concentration. In panel B, tryptophan emission maxima of all the samples were plotted against urea concentration. Samples were excited at 295 nm with 1 nm slit width, and emission was recorded from 310 to 450 with 1 nm band slit. All fluorescence values were corrected using reagent blanks.

ADP complexes. We used 2.5 M urea to release rhodanese from GroEL−GroES−ADP complex, as at this urea concentration, native rhodanese retains its active conformation (*21*, *31*). Figure 3 shows the equilibrium denaturation profile for native, spontaneously refolded and urea-released rhodanese. When activity was measured as a function of urea concentration, the profiles for native and urea-released rhodanese were the same, with a sharp transition extending from ~3.5 to 4.5 M urea. For spontaneously refolded rhodanese, the transition was less sharp. Figure 3A showed the activity as a function of urea concentration for all three species. These data suggest that the active protein released from the complex behaved identically to native rhodanese, but spontaneously refolded rhodanese had somewhat different stability toward urea-induced inactivation, and the somewhat broader transition could indicate heterogeneity. When the conformation was followed using the emission maximum of tryptophan as a function of urea (Figure 3B), spontaneously refolded rhodanese showed more apparent stability toward urea denaturation than either native or released rhodanese, each of which showed almost identical transitions. Rhodanese contains eight tryptophan residues that are distributed throughout the two domains. Changes in the emission maximum reflect the global structure of the protein. The emission maximum measures all the released protein, and not just the active protein as in Figure 1A. The apparently enhanced stability of spontaneously refolded rhodanese may be due to formation of disulfide linked and aggregated product, which need higher urea concentrations for complete denaturation (see below). These results indicate that binding in GroEL complexes helps protect rhodanese from side reactions that lead to formation of non-native and aggregated species. It is likely that some folding occurs on GroEL, and there is not simply the release of intermediates that partition into species as is the case with spontaneously folded rhodanese. For example, there is no formation of large aggregates from the species released from GroEL (see below).

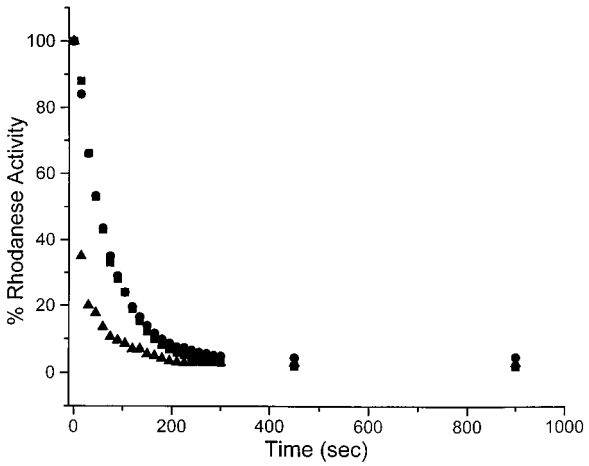


FIGURE 4: Kinetics of the denaturation of native rhodanese, rhodanese released from the GroEL−GroES−ADP complex at 2.5 M urea and spontaneously refolded rhodanese. (■) Native rhodanese (0.3 mg/mL) was incubated at 2.5 M urea in 50 mM TrisCl, pH 7.8 for 90 min at 25 °C. Preincubated rhodanese was diluted to 0.11 μM in refolding buffer that was made 6 M in urea, and 100 μL aliquots were assayed as a function of time. (●) Denatured rhodanese (described in Experimental Procedures) was diluted into refolding buffer containing GroEL, GroES, and ADP and incubated at 25 °C for 15 min. Urea (2.5 M) was added and incubated at 25 °C for 90 min. Released rhodanese at 0.11 μM was incubated at 6 M urea in refolding buffer at 25 °C, and 100 μL aliquots were assayed as a function of time. (▲) Spontaneously refolded rhodanese (described in Experimental Procedures) was incubated at 2.5 M urea concentrations for 90 min at 25 °C. It was diluted to 0.11 μM in refolding buffer that was made 6 M in urea, and 100 μL aliquots were assayed as a function of time at 25 °C.

Kinetic Comparison of Stability of Rhodanese Released from GroEL−GroES−ADP Complex by Urea, with Native and Spontaneously Refolded Rhodanese. Because of the possible differences in sensitivity of activity to urea as seen above for spontaneously folded rhodanese, the denaturation kinetics were studied for active released rhodanese, native, and spontaneously refolded rhodanese. Figure 4 shows the loss of activity as a function of the time of incubation in 6 M urea. Released rhodanese showed almost identical kinetics to the native enzyme, but spontaneously refolded rhodanese denatured more rapidly. The rate constants were 0.015 ± 0.00038, 0.01489 ± 0.0005, and 0.064 ± 0.005/min for native, released and spontaneously refolded rhodanese, respectively. Therefore, the data are consistent with the view that the active site of released rhodanese is conformationally similar to the native enzyme, whereas active, spontaneously refolded rhodanese, as prepared in these experiments, appears

Table 2: Light Scattering of Rhodanese Samples under Different Conditions[a]

sample	τ_{340}
native rhodanese	17 039
spontaneously refolded rhodanese	320 660
GroEL−GroES−rhodanese−ADP (released)	17 512
GroEL−rhodanese−ADP (released)	18 079

[a] Light scattering values of native, spontaneously refolded rhodanese at 2.5 M urea and rhodanese released from GroEL−GroES−ADP and GroEL−ADP complex at 2.5 M. urea. The buffer used was 50 mm TrisCl, pH 7.8, containing 50 mM thiosulfate, 0.2 M βME, 10 mM KCl, and 10 mM $MgCl_2$. All light scattering values were corrected using blanks containing all the components described above except rhodanese.

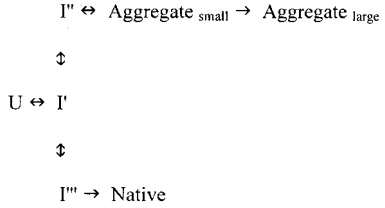
as species with lower kinetic stabilities toward urea. The same experiment monitored by tryptophan fluorescence yielded similar rate constants, 0.0099 ± 0.0001/min for native, 0.0098 ± 0.0001/min for released and 0.011 ± 0.0001/min for spontaneously refolded rhodanese (data not shown), which shows that the global structural stabilities are very similar.

Rhodanese, Bound to GroEL Can Be Released as Homogeneous Species. Table 2 shows the light scattering values of native rhodanese, spontaneously refolded rhodanese, and rhodanese released from the GroEL−GroES−ADP complex by urea. The spontaneously refolded rhodanese showed very high light scattering indicating the presence of large aggregated species. But the light scattering of urea-released rhodanese was very similar to that of native rhodanese. Therefore, there were no large aggregates in the product released from the complex. Although it is possible that small associated species such as dimers might be formed, these have never been reported. These data suggest that rhodanese is released from the complex as a refoldable, homogeneous species that does not aggregate.

DISCUSSION

Urea unfolded rhodanese reactivates poorly due to formation of aggregates and disulfide-linked misfolded species (*12*, *33*). When these problems are minimized, it has been suggested that rhodanese refolds through a series of intermediates (*1*, *2*). Efficient refolding is observed in the presence of molecular chaperones such as GroEL (*5*, *34*). Our results indicate that rhodanese must be presented to GroEL as a form that is unfolded beyond its domain folded form for successful binding and subsequent reactivation. Rhodanese retains most of its secondary structure and activity up to 4 M urea. Proteolytic susceptibility and exposure of organized hydrophobic surfaces also increase in this urea concentration range (*30*). During urea induced denaturation of rhodanese, domain separation and exposure of hydrophobic sites precedes global unfolding of the enzyme. Domain separation starts at ∼3 M urea (*29*, *30*). Bis-ANS binding, which is a measure of exposed hydrophobic sites, is maximum at 4 M urea. Complete denaturation of rhodanese is observed above 5 M urea. No appreciable binding of rhodanese to GroEL is observed when the enzyme is preincubated below 4 M urea. Highest reactivation is found when the enzyme is denatured above 6 M urea where it does not contain any measurable secondary structure (*21*, *31*). Similar observations have been reported with EcDHFR, which has been reported to bind GroEL as an unfolded form (*35*). Since there is almost no binding to GroEL of rhodanese denatured at 4 M urea (Figure 1, panels A and B), it is clearly indicated that that only rhodanese that has been extensively unfolded before being presented to GroEL can bind to GroEL. The domain-separated form does not appear to bind appreciably under the conditions used here. It has been suggested that rhodanese forms a molten globule like intermediate within the GroEL cavity (*9*). The limited proteolysis data has suggested that domains of rhodanese are formed inside the GroEL complex (*36*). From these observations and our data, it can be suggested that the domain folded, molten globule like conformations of rhodanese are formed from the initially bound forms. This would indicate that rhodanese progresses from the initially bound state(s) to later intermediates while bound to the complex, but they do not reach the native state in the complexes we studied. From previous study with protenase K cleavage, it has been found that ∼40% of the rhodanese bound to GroEL is capped by GroES, thus forming a cis complex in the presence of ADP (*37*−*39*). We find that rhodanese GroEL complexes without GroES (a model for rhodanese in trans complexes) yield 40% active enzyme on treatment with urea. Thus, in the present case, 24% of the regained activity in the presence of GroES can be ascribed to the cis complexes (40% regain × 60% trans complex). The total recovery in the mixed cis/trans complexes is 60%. Thus, the excess 36% reactivation (60−24%) can be ascribed to the 40% cis complex that is formed, which indicates a 90% reactivation by urea of rhodanese from the cis complex.

Scheme 1



Although the present work does not establish the properties of folding intermediates, the data can be interpreted in terms of a model that was proposed previously for the spontaneous folding of rhodanese, which is summarized in Scheme 1 (*1*, *2*).

On the basis of this scheme, oligomeric GroEL (14-mer) efficiently captures rhodanese presented as an early folding intermediate (e.g., I′) to give a very stable complex, and the bound rhodanese is inactive (Figure 2B). This complex is also stable toward urea up to 2 M urea. Beyond this range, there is release of rhodanese from the complex, as evident from the increase in activity found in the solution. This activity is not due to dissociation of the complex, followed by spontaneous refolding of rhodanese, as the activity of the released rhodanese is always higher than that of spontaneously refolded rhodanese (e.g., 60% compared to 20% from spontaneous refolding). There is also a significant effect of the addition of GroES, a regulatory protein for GroEL. In its absence, the maximum activity found with released rhodanese is 40%. These data suggest that binding to GroEL and sequestration within the cavity in the presence of GroES helps rhodanese to undergo partial refolding. Since no significant activity (<4%) is found either from GroEL−

rhodanese−ADP or from the GroEL−GroES−rhodanese-ADP complex, complete refolding of rhodanese to the native state does not take place inside the cavity of these complexes, which would show rhodanese activity. This observation agrees with the data shown by Hartl et al. (*40*). The report that ADP can release rhodanese activity from rhodanese−GroEL−GroES complexes referred to very different conditions from those used here (*37*) and, at best, corresponded to a very low level of activity compared with appropriate controls (Table 1). Further, the results suggest that urea releases rhodanese from GroEL−GroES−ADP complex as a homogeneous species in a refoldable state, which readily folds to its native conformation, whereas spontaneously refolded rhodanese contains both heterogeneous active and inactive species. The orientation of the active site in released rhodanese is identical to that in the native rhodanese, whereas it is more perturbed in spontaneously refolded rhodanese (Figure 4), though the global stability of the three species are similar.

According to our results and the model in Scheme 1, rhodanese would be preferentially released from its complexes with GroEL as I‴, which is capable of forming native rhodanese. If at 2.5 M urea, rhodanese were to be released as I′, there would be partitioning to I″ and I‴, and I″ would lead to the formation of aggregates. Since there is no significant aggregation, rhodanese must be released as I‴, which is not capable of readily aggregating. Our results suggest that GroEL interacts readily with very early folding intermediates of rhodanese, e.g., I′ (dominant band of complex between CMR and GroEL is observed at urea concentration higher than 5 M) and less well with the intermediates occurring further along the folding pathway, either I″ or I‴ (relatively small amounts of complex are formed between GroEL and CMR below 5 M urea). Further, it is suggested that GroEL releases rhodanese as I‴, which readily folds to native state. EcDHFR also shows similar behavior in that a late folding intermediate of EcDHFR gets released from the GroEL cavity (*35*). Though GroEL may bind some of the late folding intermediates of rhodanese, it could not release active protein, as there is no substantial activity found after preincubation below 5 M urea. No aggregated product is found in released rhodanese, indicating complete inhibition of the formation of intermediate, I″, whose formation would lead to aggregation.

Therefore, for the initial binding, subsequent sequestration, and productive folding, rhodanese unfolded beyond the domain folded state must be presented to GroEL−GroES. The interaction of this form of rhodanese with GroEL−GroES suppresses irreversible side reactions, which would lead to the formation of non-native conformations and aggregated species. The ability of urea to release active rhodanese from complexes with GroEL suggests that the fundamental interactions leading to reactivation of bound proteins do not depend on the binding or hydrolysis of ATP.

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