The competition between protein aggregation and folding has been investigated using rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) as a model. During folding from a urea-denatured state, rhodanese rapidly forms associated species or intermediates, some of which are large and/or sticky. The early removal of such particles by filtration results in a decreased refolding yield. With time, a portion of the smaller aggregates can partition back first to intermediates and then to refolded protein, while a fraction of these irreversibly form unproductive higher aggregates. Dynamic light scattering measurements indicate that the average sizes of the aggregates formed during rhodanese folding increase from 225 to 325 nm over 45 min and they become increasingly heterogeneous. Glycerol addition or the application of high hydrostatic pressure improved the final refolding yields by stabilizing smaller particles. Although addition of glycerol into the refolding mixture blocks the formation of unproductive aggregates, it cannot dissociate them back to productive intermediates. The presence of 3.9 M urea keeps the aggregates small, and they can be dissociated to monomers by high hydrostatic pressure even after 1 h of incubation. These studies suggest that early associated intermediates formed during folding can be reversed to give active species.

The renaturation of a denatured protein typically involves a number of intermediate states (on-pathway or off-pathway) leading to either correctly folded or misfolded structures (1, 2). Protein aggregation is one of the main side reactions during refolding, and it often results from interactions among partially folded intermediates (3). In general, this aggregation, although ubiquitous and important for basic and applied problems, is difficult to study. The enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) is an interesting model for studying issues related to such problems in protein folding (4–8). This monomeric protein containing 293 amino acids (mass = 33 kDa) is folded into two independent, equal-size domains, and its crystal structure is available (9). The domains are tightly associated and the interdomain surface is highly hydrophobic. Rhodanese contains four cysteine residues, Cys-63, Cys-247, Cys-254, and Cys-263, which are all reduced in the active protein (9). During unfolding/refolding in the presence of denaturants, disulfides can form among these cysteines, leading to misfolded structures in addition to aggregation. The disulfide-containing species leading to misfolding have been characterized (10) with SD8-polyacrylamide gel electrophoresis, and the formation of aggregates during unfolding has been reported earlier (5, 11). It has been established that during unfolding, the presence of reductants such as β-ME and thiosulfate prevents the formation of most of the disulfides and better refolding yields are observed (5). However, refolding at low temperature (e.g. 12 °C), even in the presence of 200 mM β-ME, 50 mM thiosulfate, and at very low protein concentration (e.g. 3.6 μg/ml), led to incomplete recovery of activity (5, 11).

Folding intermediates formed during chemical or thermal denaturation often contain similar levels of secondary structure as the native proteins, but they contain a decreased number of tertiary contacts (12), partially or completely dissociated domains (13), and/or incorrectly formed disulfide bonds (14). As a result, such intermediates tend to be highly hydrophobic, and consequently they can easily form large aggregates and precipitate. It was suggested that, at intermediate concentrations of denaturant, the two domains of rhodanese dissociate (7, 13) to produce a form of the protein that is able to form dimers, trimers, and higher oligomers (6).

Formation of similar intermediates leading to incomplete refolding can be detected where aggregation is a major side-reaction. Indeed, the protein folding process can be described as a kinetic competition between correct folding and aggregation (15). Such competition has been observed in vitro (16) as well as in vivo (17, 18). Quantitative models have been proposed to explain the formation of aggregates during protein folding (15). Aggregation can be prevented by isolating intermediates from each other by complexing them with molecular chaperones (4, 19), utilizing detergents or mixed micelles (20), or refolding at low protein concentration (5). The stabilizing effects of some co-solvents have also been partially ascribed to decreased diffusion, which prevents protein molecules from interacting with each other during the period in which they are sensitive to interaction (21) along with the effects of preferential hydration (22).

Several publications have demonstrated that high hydrostatic pressures can dissociate specific protein oligomers (23–26). In addition, high hydrostatic pressure has recently been shown to dissociate nonspecific protein aggregates formed in vitro (8), and to facilitate an increase in the folding yield by allowing intermediates to complete folding.

In the present study, we have investigated the stability and relative size of aggregates formed during rhodanese folding on the efficiency of renaturation. On the basis of these studies, a minimum mechanism for rhodanese folding is proposed. The observations support the view that intermediate, dissociated species can be rescued and returned into a productive folding pathway.

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1 The abbreviation used is: β-ME, 2-mercaptoethanol.
**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant bovine rhodanese (thiosulfate:cyanide sulfutfurtransferase, EC 2.8.1.1) was purified as described previously, and was stored at −70 °C as a crystalline suspension in 1.8 M ammonium sulfate containing 1 mM sodium thiosulfate (27). For the experiments, a concentrated rhodanese stock solution (typically 10–20 mg/ml) free from ammonium sulfate was prepared by gel filtration using a G-50 Sephadex column and eluting with 0.1 M sodium phosphate, pH 7.6. Rhodanese concentrations were determined using a value of 1.25 mg/ml = 1.75 (28). In the experiments for refolding and filtration of aggregates done at low protein concentrations (~3.6 μg/ml), it was not possible to accurately determine [protein] by using the standard BCA (Pierce) or Coomassie (Pierce) assays. In these cases, a standard dilution curve was made using the fluorescence emission intensity of rhodanese from 1–20 mg/ml. After the dilution state was normalized by denaturing the samples in 2% SDS (final concentration). The fluorescence intensities were measured at the emission maximum, which was at 344 nm for all samples (Fig. 6, inset). Other experimental details are in the legend for Fig. 6. The standard curve was linear to 20 μg/ml with a near zero intercept and slope of 0.050 ± 0.003 cpsi/μg (see Fig. 6, inset). Quantitation by fluorescence intensity measurements of total protein after SDS denaturation avoids errors in the measurements that might result from small differences in the misfolded species, intermediates passing through the filter, and different unfolded species in the filtrate. In the present study, it was not possible to isolate the contributions of individual species to the total recovered protein.

Urea was electrophoresis purity from Bio-Rad. All other reagents were of analytical grade. Solutions containing glycerol and β-ME were prepared by redissolving rhodanese inactivation, glycerc stock solutions were used within 10 days.

**Standard Buffer**—A standard buffer containing 200 mM β-ME, 50 mM sodium thiosulfate, and 50 mM Tris-HCl, pH 7.8, was used throughout this study (5). Buffers containing glycerol were made by adding appropriate amounts from a 8 M stock solution of glycerol to the other reagents and then making up the final volume of the solution followed by making it up to the pH. All stock solutions were filtered through 0.2-μm (Nalgene) syringe filters.

**Rhodanese Assay**—Rhodanese activity was measured by a colorimetric method (monitored at 460 nm) based on the formation of the complex between ferri cyanide and one of the reaction products, thiocyanate (28). Aliquots of 25–50 μl of the incubating enzyme were added to 1 ml of assay mixture and incubated for 5–10 min. The assay mixture was at pH 8.6 and consisted of 1:1:1 (volume) of 0.15 M sodium thiosulfate, 0.15 M KCN, and 0.12 M KHPO₄. The reaction was stopped by adding 0.5 ml of 18% formaldehyde solution. Color was developed by adding 1.5 ml of ferric nitrate solution. The presence of small amounts of β-ME, urea, or glycerol after dilution of the enzyme in the assay mixture did not interfere with the assay. The activities of the refolded rhodanese were normalized with respect to the activity of a sample of native enzyme obtained under assay conditions used in the present study.

**Unfolding and Refolding**—Unfolded rhodanese (U) was freshly prepared at 300 μg/ml by adding calculated amounts of native enzyme into 8 M urea, 50 mM Tris, 50 mM sodium thiosulfate, 1 mM β-ME, pH 7.8, and equilibrated for at least one hour (7). Refolding was achieved by diluting the unfolded protein into the standard refolding buffer, maintaining a final protein concentration of 3.6 μg/ml. All measurements were made at 25 °C. The regain of enzyme activity was used to monitor successful refolding. The refolding time was varied for the kinetics study. At the indicated times, the protein solutions were filtered by centrifugation for 15–20 s at 6,000 × g using a 0.22-μm Ultrafree-MC filter unit (Millipore). Identical results were achieved by filtering through Nalgene 0.2-μm (cellulose acetate) syringe filters using sterile plastic syringes (Becton-Dickinson). The filtrate was collected and analyzed for rhodanese activity, and protein concentration was measured by comparing fluorescence intensities of the solutions with that of native rhodanese under identical conditions.

**The Sizes of Aggregates Formed during Rhodanese Refolding**—were monitored by dynamic light scattering measurements employing a Brookhaven laser light scattering instrument (Brookhaven Instrument Corp.). Buffers contained Nalgene 0.2-μm syringe sifter and samples were prepared as described by the manufacturer to avoid contamination by dust or presence of tiny bubbles that would contribute to scattering. The laser was set at 488 nm, and scattering was monitored at a 90° angle. One-ml samples were used in cylindrical glass tubes. All the measurements were made at 25 °C. Appropriate corrections were applied for scattering by buffer. After refolding was initiated, the distributions of particle sizes were determined at the indicated times. Data were collected and analyzed using Dynamic Light Scattering software 9KDSLW (beta version 1.2, 1995) provided by Brookhaven Instruments. Sizes were calculated using the diffusion coefficient measured by the dynamic light scattering method as described by Cleland and Wang (29). Protein aggregation under filtered pressure was studied using a spectrophotometer (ISS, Inc.) as described previously (8). The pressure bomb employed was similar to the one described by Paladini and Weber (23). Native rhodanese was diluted into a solution containing 50 mM Tris-HCl, pH 7.8, 200 mM β-ME, 50 mM thiosulfate, maintaining a final urea concentration of 3.9 M and a final protein concentration of 0.3 mg/ml. After various times of incubation the samples were pressurized to a pressure of 0.001 and 2 kbar. The intensity of the scattered light was monitored at 90° using light at 400 nm.

To investigate rhodanese refolding under high hydrostatic pressure, samples were pressurized up to 2 kbar at 5–10 min after dilution of the denatured protein. These samples were incubated for 70 min, depreserved, and the activities were analyzed as described above. The activity of the native protein kept at atmospheric pressure was taken as 100%.

Rhodanese was covalently labeled by succinimidyl 1-pyrenenbutyrate as described previously (30). Briefly, protein at a final concentration of 85 μg/ml was dissolved in triethanolamine hydrochloride (50 mM, pH 7.8). Succinimidyl 1-pyrenenbutyrate was diluted from a stock solution in distilled triethanolamine to a final concentration of 15 mg/ml. The sample was incubated for 2 h at room temperature and then dialyzed against Tris-HCl (50 mM, pH 7.8) containing 0.1 mM β-ME to exchange the buffer and remove unreacted labeling reagent.

Pyrene-labeled rhodanese was used to monitor protein aggregation under pressure. Rhodanese covalently labeled with pyrene was diluted into the standard buffer containing 3.9 M urea. In some cases, unlabeled rhodanese was added to maintain the final protein concentration at 0.3 mg/ml. At the indicated times, samples were pressurized as described above. The polarization of the pyrene fluorescence was measured using an ISS fluorometer (ISS, Inc.). Samples were excited at 345 nm, and the emission was detected at 400 nm. Kinetic experiments were done as discussed in Fig. 1 legend. The data were fitted to either mono- or bi-exponential first-order equations: 

\[
\frac{\Delta A}{A} = A_0 \exp (-k_1 t) + A_1 \exp (-k_2 t) + A_2 \exp (-k_3 t) + A_3 \exp (-k_4 t) + A_4,
\]

where \(A_0, A_1, A_2, A_3, A_4\) and \(k_1, k_2, k_3, k_4\) are the amplitudes and the pseudo-first order rate constants, respectively. The independent variable \(t\) was the percentage of activity recovered at time \(t\). The pseudo-first order rate constants \(k_1\) and \(k_2\) and the amplitudes \(A_0, A_1, A_2\) and \(A_3\) were obtained from iterative non-linear least squares regression of the data using the Origin software program (MicroCal).

**RESULTS**

**Aggregates Formed during Rhodanese Refolding Exist in Equilibrium with Rhodanese Folding Intermediates**—Rhodanese at 300 μg/ml was unfolded in 8 M urea (see “Experimen al Procedures”) and then diluted to give a final urea concentration of 96 mM and 4 M glycerol to allow rhodanese to refold (Fig. 1). The refolding of rhodanese is considerably slower in the presence of glycerol (7) than without it, thus permitting observation of the kinetics of the process by the methods used here. The plateau of these kinetic traces from the single exponential fits correspond to the maximum yields (\(A_0\) in “Experimental Procedures”). For example, under these conditions, about 35% of the activity could be recovered with a \(t_{1/2}\) of 10 min at [rhodanese] = 3.8 μg/ml (Fig. 1, open circles). The curves in Fig. 1 show that the final yield decreased as the rhodanese concentration increased as reported earlier (7). The observed rates from single-exponential fits were \(k_{1(0)-500} = 0.067 ± 0.007, 0.066 ± 0.005, 0.17 ± 0.01, 0.28 ± 0.13, \) and \(0.27 ± 0.04 \text{ min}^{-1}\) for 3.6, 10, 15, 30, and 50 μg/ml rhodanese, respectively. The calculated yields were 35, 30, 28, 18, and 14% at 3.6, 10, 15, 30, and 50 μg/ml rhodanese, respectively. 4 M glycerol was present in the standard buffer (Fig. 1). The concentration dependence of the observed rates and the maximum yields derived from the data in Fig. 1 are shown in Fig. 2 (top and bottom panels, respectively). Such non-linear dependences of both the rate and maximum regain of activity are consistent with previous results (5). These observations highlight the complex mechanism involving competitive pathways for folding and
aggregation (see “Discussion”). Samples at different protein concentrations were derived from the same unfolded rhodanese solution in 8 M urea to avoid experimental errors due to any difference in unfolded states of rhodanese. In this method, the urea concentrations in the final refolding mixtures depended on the dilution ratio. This variation in urea from 0 to 1.33 M in the final refolding mixtures did not cause significant changes in the enzyme activity of control samples whether glycerol was present or not. Experiments were repeated using a protocol in which the final mixtures were supplemented with urea so that the different rhodanese concentrations were refolded at the same final urea concentration of 1.33 M (Fig. 2, bottom panel). These results show that the results do not depend on differential carryover of urea.

The size distributions of the aggregates that formed during the rhodanese folding were determined by light scattering measurements performed at various times after initiating refolding, as described under “Experimental Procedures.” The average diameters of particles at the maxima of the curves and the average widths at half-height of the distributions provide a reasonable description of the multimers relevant to the aims of this study (29, 31). Within the first minute after initiating folding, the lowest curve in Fig. 3 shows that particles with average diameters of \( \sim 225 \text{ nm} \) can be detected (the diameter of native rhodanese is \( \sim 5.6 \text{ nm} \)). The total intensity of the scattered light quickly increased within the time of manual mixing (less than 1 min), indicating that the particles observed were formed quickly. The average size of the particles increased from \( \sim 225 \text{ nm} \) to \( \sim 330 \text{ nm} \) over 44 min (highest curve, Fig. 3). The heterogeneity of the ensemble also increased during this interval as evidenced by the increase of width at half-height of the distributions from \( \sim 94 \text{ nm} \) at 1 min to \( \sim 200 \text{ nm} \) at 44 min.

The fates of intermediates or particles formed during refolding were tested by a double dilution method in which refolding was initiated at a high concentration for various periods before being diluted further. Thus, refolding was started (diluting the unfolded protein into 4 M glycerol-containing buffer) at 50 \( \mu \text{g/ml} \) rhodanese. After different times (Scheme 1), aliquots of the refolding rhodanese were further diluted to a final concentration of 3.6 \( \mu \text{g/ml} \) and then immediately assayed. Fig. 4 shows that, within 30 s after the refolding was started, the protein regained about 35% of the control activity, and dilution did not dissociate the aggregates that had already formed at that instant. The recoverability of activity continued to fall
exponentially. The data (Fig. 4, solid circles, solid line) were fit to a biphasic rate expression (see “Experimental Procedures”), and two rate constants were evaluated for the resolved components (Fig. 4, dashed and dotted lines). The faster observed rate ($k_{obs} = 0.68 \pm 0.07 \text{ min}^{-1}$) corresponds to a half-life of 1.02 min, whereas the slower rate ($k_{obs} = 0.035 \text{ min}^{-1}$) corresponds to a half-life of about 19.8 min. The important observation from this analysis is that the fast phase reaches a plateau corresponding to a biphasic rate expression (see “Experimental Procedures”), after which there is a slow, further loss of activity (Fig. 4, dashed line). We have no information regarding the slow process, but it could be due to additional loss of recoverability by the formation of misfolded structures from any of the postulated intermediates. This suggests the existence of off-pathway intermediates that contribute to the loss of recoverable activity due to the time-dependent formation of aggregated species. If the species responsible for the loss were reversible aggregates formed simply because of high enzyme concentration, then they would have dissociated upon further dilution resulting in recovered activities of about 35% at these final concentrations. In that case, a plot of percentage of activity recovered versus time would have been a horizontal straight line at ~35%, instead of the observed exponential decrease in Fig. 4.

Some of the off-pathway particles formed on dilution of denatured rhodanese could be filtered through a 0.22-μm filter, a process requiring between 15 and 20 s. The results are shown in Fig. 5. When the solutions were filtered early in the refolding process (closed squares, filtration at ~3 min), the renaturation of the protein in the filtrate initially was at the expected value compared with an unfiltered sample (open squares), and the activity increased with time to give a final yield that was decreased to ~58% of what would have been observed in the absence of filtration. Thus, a portion of the protein that would have renatured in solution was removed on the filter (compare the closed square and the open square at 45 min). However, a portion of the protein that did pass through the filter was not active initially, but it continued to fold as shown (solid squares). Thus, there were two forms of initially inactive protein that could fold in a time-dependent manner, one was retained on the filter and the second could pass the filter. An analogous result was observed for a solution filtered 9 min after initiating renaturation (closed diamonds). Here, there was a smaller total increase in recovered activity from ~55% immediately after filtration to a maximum recovery of ~75%, a value still below the unfiltered control. When the solution was filtered after 29 min of refolding (open diamonds), the final concentration of the active enzyme was very close to that in the unfiltered solution. It was thus apparent from the reduced final yields that filtration could remove species that were able to contribute to the yield of refolding. The time dependent increase in activity in the filtrates showed that some inactive species could pass through the filter and become active with time. The results in Fig. 6 show that the amount of total protein in the filtrate rapidly increased over the first 5 min and then more slowly, consistent with the idea that reduced recovery of activity after filtration (Fig. 5) correlates with the removal of protein rather than with inactivation of species formed during incubation. It may be noted that, in control experiments, only 2–5% of native or urea denatured rhodanese samples were lost on the 0.2-μm filters (data not shown), whereas these filters retained very large fractions of protein when refolding rhodanese solutions were filtered.

**Nature of the Unproductive Larger Aggregates—** An experiment was performed to investigate the nature of larger aggregates that formed after the off-pathway intermediates incubated sufficiently. Formation of aggregates large enough to scatter light significantly was monitored using a SPF-500C (SLM, Aminco) spectrophuorometer with excitation and emission wavelengths fixed at 400 nm. The results are shown in Fig. 7. The kinetic trace (curve A) corresponds to aggregation formed at [rhodanese] = 10 μg/ml (t_{1/2} ~350 s) in standard buffer without glycerol. Curve B shows that the presence of 4 M glycerol at t = 0 virtually eliminates the aggregation monitored by this method. The other kinetic traces (C–F) represent the results of experiments done as follows; rhodanese was refolded in standard buffer without glycerol at 20 μg/ml and then, after
various times, aliquots were diluted to 10 μg/ml by adding an equal volume of buffer containing 8 M glycerol so that the final reaction condition was: 50 mM Tris-HCl, 50 mM thiosulfate, 200 mM b-ME, pH 5.7, and 4.0 M glycerol (the condition for trace B). Individual experiments were done for such glycerol additions after initially refolding the protein in non-glycerol-containing buffer for 15 s (trace C), 1 min (trace D), 5 min (trace E), and 15 min (trace F). The jumps seen at the beginning are artifacts due to a combination of closing and opening of shutters, and mixing. The relevant observation from these experiments is that the amounts of large aggregates that form in the solution are not reversed to productive smaller ones even by the addition of glycerol. However, the formation of such non-productive aggregates can be prevented in the very early stages by the presence of glycerol.

Rhodanese Aggregation Can Be Controlled by Pressure—
Rhodanese slowly aggregates under mild denaturing conditions near the transition zone (3.9 M urea in standard refolding buffer) (8). This process, monitored by the intensity of the scattered light, has a rather long induction period of ~1000 s (Fig. 8, trace B). Light scattering measurements of these solutions in the presence of 3.9 M urea indicated the accumulation of particles with a diameters of ~40 nm after incubation for 1800 s. Prolonged incubation under these conditions resulted in formation of slightly larger particles with diameters of 50–90 nm (data not shown). Thus, although the protein concentration

![Figure 4](image1.png)

**FIG. 4.** Dilution of refolding enzyme at different stages of refolding. Unfolded rhodanese was diluted in to 4 M glycerol-containing buffer at 50 μg/ml and then the refolding mixture was diluted to 3.6 μg/ml of protein at different times (Scheme 1) with the same 4 M glycerol-containing buffer. The assays were done immediately after the final dilution. The solid line through the data is from fit to a biphasic exponential as described under “Experimental Procedures.” The dotted line and the dashed line represent the resolved fast and slow phases that were generated using the parameters obtained from the biphasic fit of the data.

![Figure 5](image2.png)

**FIG. 5.** Large protein aggregates can be retained on the 0.22-μm filter. Rhodanese was unfolded as in Fig. 1. Refolding was initiated by diluting unfolded enzyme to the standard buffer (see “Experimental Procedures”) in the presence of 4 M glycerol. The increase in activity with time for an unfiltered control is shown by open squares (■). Solutions of refolding rhodanese were filtered after the 3rd (●), 9th (△), and 29th (○) minute of the refolding process. At the indicated times, aliquots were withdrawn, and the amounts of active enzyme were measured. Activities were normalized to the highest activity observed, which was taken as 100%.

![Figure 6](image3.png)

**FIG. 6.** Percentage of total protein retained in the filtrate after filtering the refolding solutions. Unfolding and refolding were done as described in Fig. 5, except that the final protein concentration was 10 μg/ml. At different times after refolding was initiated, 1-ml aliquots were withdrawn and filtered through 0.2-μm syringe filter (see “Experimental Procedures”), made 2% (by weight) SDS (sodium dodecyl sulfate), heated over boiling water for 5 min, cooled, and fluorescence intensity scanned (excitation = 280 nm, emission = 300–450 nm). The fluorescence maxima of these solutions were at 344 nm. The percentage of total protein present in these solutions was calculated from the scans of a native control where known amounts of protein were treated with SDS, heated, cooled, and scanned in a similar manner under identical conditions as the samples. The plot indicates a systematic non-linear increase of protein content with refolding time at filtration. The inset shows the concentration dependence of the fluorescence intensities of control solutions of SDS denatured native rhodanese.
was considerably higher here (300 μg/ml) than in the light scattering experiments shown in Fig. 3 (3.6 μg/ml), the presence of urea led to a reduced particle size, but it did not eliminate association.

Rhodanese aggregation was successfully reversed when the solution was pressurized to 2 kbar (Fig. 8, trace 2). In this experiment, aggregation was allowed to proceed for different times (1800, 2400, and 4200 s). After each interval, when the pressure was adjusted to 2 kbar, the intensity of the scattered light was reduced to the same values through three cycles of pressurization (Fig. 8, trace 2). This demonstrates that no matter how long the aggregation was allowed to proceed, hydrostatic pressure was able to reverse the aggregation. These results complement previous results, where only the reversibility of large aggregates by pressure was demonstrated (8) but its effect on incubated aggregates was not investigated.

To have a better understanding of the state of the protein under high hydrostatic pressure, fluorescently labeled rhodanese was used as a reporter. Rhodanese was labeled by succinimidyl 1-pyrenebutyrate (see “Experimental Procedures”), and it was mixed with unlabeled protein under conditions inducing aggregation. Polarization of the pyrene fluorescence was used to monitor the size of the protein aggregates (Fig. 9). The long fluorescence lifetime (150-ns average; Ref. 30) of succinimidyl 1-pyrenebutyrate permits monitoring of the formation of relatively large moieties (30).

Incubation of the protein at 3.9 M urea and atmospheric pressure resulted in a substantial time dependent increase in the polarization of pyrene fluorescence (Fig. 9, trace 1) with a hint of an induction period. Protein aggregation under these conditions forms relatively large particles that include the labeled rhodanese, a process that is reflected in the increase of the pyrene fluorescence polarization. When the solution was pressurized to 2 kbar after incubation for about 950 s, the polarization decreased to the initial value indicating dissociation of the aggregates into monomers (Fig. 9, trace 2). Releasing the pressure resulted in an increase in polarization (Fig. 9, trace 2). The value of the polarization that was observed under high pressure (0.033 ± 0.005) is very close to that observed for the sample of pyrene-labeled rhodanese in the absence of urea (0.030 ± 0.001). In a control experiment, the polarization of pyrene-labeled native rhodanese was determined as a function of pressure in the absence of urea. No change in the polarization was detected upon increasing pressure from 1 bar up to 2 kbar. These polarization measurements are complementary to those by light scattering, since the polarization is more sensitive to the formation of small species, while the light scattering is always dominated by the larger species. For example, calculations using the observed polarizations and the known properties of rhodanese suggest that the labeled enzyme exists as a monomer in solution. Therefore, protein aggregates formed in the presence of 3.9 M urea appear to be completely dissociated when the protein solution is pressurized to 2 kbar.

High Hydrostatic Pressure at Early Stages of Folding Can Improve Refolding Yields—The ability of high hydrostatic pressure to dissociate protein aggregates under mild denaturing conditions suggests that pressure can be used to influence the equilibria among protein aggregates formed during rhodanese folding. When a solution of rhodanese was pressurized within 5–6 min after diluting from the denaturing conditions and refolding was allowed to occur under high pressure, the final
yield of the native enzyme was increased (Table I, standard buffer, containing 4 M glycerol). This can be explained by the ability of high pressure to reverse formation of large aggregates. Conformational changes in the large aggregates after long incubation are indicated, since, after sufficient time, high pressure is no longer effective in renaturing the enzyme. Thus, if rhodanese is incubated for 65 min before pressurization, the final yield of activity is 32%, which is comparable to that observed for enzyme that was refolded at 1 bar. On the other hand, if pressure was applied only 5 min after the start of renaturation, the final activity reached 56.6%, a value that was almost twice that observed for the sample refolded at 1 bar. In the absence of glycerol, the yields of refolding were about 24% and 5% at 2 kbar and 1 bar, respectively. These relative percentage yields at 2 kbar and at atmospheric pressure are consistent with earlier results in 4 M glycerol-containing refolding buffer (8), although in those studies the effect of pressure at different refolding times and on the regain of activity was not studied. These results (Table I) demonstrate that even though applying pressure dissociated the aggregates (Fig. 8), the activity could be regained to a maximum (of about 56%) only when the refolding mixture was pressurized at early (5–15 min) but not at very late stages (65 min) of folding. Thus, changes within the aggregates must occur as a function of time.

**DISCUSSION**

Native rhodanese contains two, independently folded, equal-sized domains that are tightly associated at an interface containing a substantial number of hydrophobic residues. As rhodanese unfolds, it forms at least one stable intermediate that displays extensive hydrophobic exposure (6). During formation of these states near the unfolding transition zone between 3 and 4.5 M urea with $U_{1/2} \approx 3.7$ M, only minor changes can be detected in its secondary (13) and tertiary (7) structures. The $U_{1/2} = 3.7$ M represents the concentration of urea (50 mM Tris, pH 7.6, 50 mM sodium thiosulfate, and 200 mM β-ME) at which 50% of initial rhodanese activity is observed (6). In these intermediates, it has been suggested that the domains remain largely intact but dissociated (7). As a result, the protein is able to form intermolecular interactions, which lead to aggregation. In addition to aggregation, other problems such as disulfide formation and misfolding contribute to its less than complete reactivation (see Introduction).

Several models for failure of protein folding invoke partitioning of folding intermediates between productive folding and formation of aggregates (3, 29, 32–34). The rhodanese refolding pathway has been partially described earlier (7), and it is
revised and extended on the basis of our present results in Scheme 2 (states denoted with * define the partial pathway described in Ref. 7). In this model, \( U \) is an unfolded state, \( I, I' \), and \( I'' \) are intermediates, \( N \) designates the native state, and \( A_{G_1} \) and \( A_{G_2} \) are off-pathway aggregated intermediate species leading to the formation of large aggregates. After initiating folding by dilution of the denatured enzyme, rhodanese is suggested to undergo fast contamination to form state \( I(11) \), which rapidly partitions to the forms \( I \) and \( I'' \). Form \( I'' \) is able to fold to the active enzyme, while form \( I \) requires a slow conformational transition to the state \( I \) and then \( I'' \) in order to form active protein. State \( I \) is also prone to aggregation that becomes irreversible as the aggregates become large. The concentration dependence and high order of the aggregation steps result in lowering the yield of refolding at high protein concentrations (5, 7). This aggregate-forming pathway is reflected in the concentration dependence of the refolding rates (Fig. 1 and top panel of Fig. 2) that were previously reported both in the absence (5) and presence of glycerol (7). As suggested previously (7), these steps lead to the counterintuitive observations seen here. Thus, the slow recovery path \((I > I > I'' > N)\) is reduced at high protein concentrations, because then \( I'' \rightarrow A_{G_1} \), leaving the faster folding steps \( I > I'' > N \) to dominate the mechanism. This explains the observation that, as the protein concentration increases, the refolding yield decreases but the folding rate increases. This mechanism is somewhat similar to that discussed for refolding of carbonic anhydrase in the presence of 1 m guanidinium chloride (33). In addition to aggregation, each of the intermediates can undergo misfolding (5, 10) as denoted by dashed arrows in Scheme 2. Therefore, even though glycerol could eliminate irreversible aggregation \( A_{G_1} \rightarrow A_{G_1} \), only 35% of the initial activity could be recovered. Incomplete recovery of activity on refolding has been reported for hen egg lysozyme and explained in a similar manner, and it was shown that the hen egg lysozyme, on refolding from 8 m urea, regained 38% of its activity in buffer and 42% in 3.44 m glycerol (35).

The filtration experiments show that some aggregates can be retained on the 0.22 \( \mu m \) filters. As seen from Figs. 5 and 6, the concentration of total protein including the active enzyme in the filtrate continued to increase and there was continued folding in the filtrate. This suggests that the solution contained smaller productive intermediates, which could pass through the filter. In addition, the reduced levels of recovery after filtration at early times show that not all of the protein retained on the filters is composed of unproductive large aggregates, but rather it contains species that can produce active enzyme in solution.

Dissociation of \( A_{G_1} \) and conformational transitions of \( I \) to the state \( I \) and then \( I '' \) in order to form active protein can also account for the slow kinetics of rhodanese refolding observed in the filtrate. The equilibrium between \( A_{G_1} \), which is very sticky, and \( A_{G_2} \), which is relatively less sticky, can be slowed down in 4 m glycerol. Therefore, the irreversible formation of \( A_{G_1} \) can be retarded. However, when glycerol was added after the refolding had proceeded to some extent, the further formation of large aggregates stopped, but the ones that had already formed did not dissociate (traces C–F in Fig. 7).

High hydrostatic pressure is generally found to dissociate protein oligomers and aggregates, and the present results are consistent with the idea that high hydrostatic pressure can affect both specific and nonspecific protein aggregates (24, 25). When the rhodanese solution was pressurized shortly after the renaturation was started, the final refolding yield was increased (Table I). This effect can be explained by the ability of pressure to shift the equilibrium between the small and sticky \( A_{G_1} \) species toward formation of monomeric folding intermediate \( I'' \) via \( I'(2) \). As a result, the amount of refolded native-like enzyme can be substantially increased.

In conclusion, high concentrations of glycerol prevent the irreversible formation of large unproductive aggregates, and high hydrostatic pressure dissociates the small sticky aggregates that are off the pathway of rhodanese folding. In addition, results from this investigation provide a mechanism for the previously reported phenomenon (5) showing that rhodanese refolding is considerably more efficient when the denatured enzyme is diluted to urea concentrations close to the unfolding transition (weakly native conditions), compared with dilutions to very low urea concentrations. Thus, non-denaturing concentrations of urea help to solubilize intermediates that can contribute to folding and prevent formation of large irreversible aggregates. These results demonstrate that aggregation can go through stages, and small aggregates can be rescued to give active protein, if intervention is applied early enough in the aggregation process. It is hoped that this information will generate additional interest both in understanding the folding mechanisms of proteins that are susceptible to aggregation, and in devising conditions to control aggregation in the recovery of active proteins.

REFERENCES
1. Baldwin, R. L. (1995) Fed. Des. 1, R1–R8
2. Hagihara, Y., and Goto, Y. (1998) in Molecular Chaperones in the Life Cycle of Proteins (Pinc, A. L., and Goto, Y., eds) pp. 1–33, Marcel Dekker, New York
3. Jaenicke, R., and Seckler, R. (1997) in Advances in Protein Chemistry (Wetzel, R., ed) pp. 1–59, Academic Press, New York
4. Mendoza, J. A., Lorimer, G. H., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 10973–10976
5. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 13587–13591
6. Horowitz, P. M., and Butler, M. (1993) J. Biol. Chem. 268, 2500–2504
7. Gorovitz, B. M., McGee, W. A., and Horowitz, P. M. (1998) Biochim. Biophys. Acta 1382, 120–128
8. Gorovitz, B. M., and Horowitz, P. M. (1998) Biochemistry 37, 6132–6135
9. Ploegman, J. H., Drent, G., Kalk, K. H., Hol, W. G., Heinrikson, R. L., Reim, P., Weng, L., and Russell, J. (1978) Nature 272, 124–129
10. Horowitz, P. M., and Hua, S. (1995) Biochim. Biophys. Acta 1249, 161–167
11. Tandon, S., and Horowitz, P. M. (1989) J. Biol. Chem. 264, 8959–8966
12. Ploegman, J. H., Drent, G., Kalk, K. H., Hol, W. G., Heinrikson, R. L., Reim, P., Weng, L., and Russell, J. (1978) Nature 272, 124–129
13. Horowitz, P. M., and Criscimagna, N. L. (1990) J. Biol. Chem. 265, 2576–2583
14. Erhank, J. J., and Creighton, T. E. (1991) Nature 350, 518–520
15. Kieflhuber, T., Rudolph, R., Kohler, H. H., and Buchner, J. (1991) Biol. Technology 9, 825–829
16. Zettlmeisel, G., Rudolph, R., and Jaenicke, R. (1979) Biochemistry 18, 5567–5571
17. King, J., Haase, C., and Yu, M.-H. (1987) in Protein Engineering (Oxender, D. L., and Fox, C. F., eds) pp. 109–121, Alan R. Liss, Inc., New York
18. Klein, J., and Dhirajati, P. (1985) Appl. Environ. Microbiol. 61, 1220–1225
19. Fink, A. L., Goto, Y. (eds) (1998) in Molecular Chaperones in the Life Cycle of Proteins, Marcel Dekker, Inc., New York
20. Tandon, S., and Horowitz, P. M. (1987) J. Biol. Chem. 262, 4486–4491
21. Creighton, B. A., and Matthews, C. R. (1990) Biochemistry 29, 2149–2154
22. Timasheff, S. N. (1995) in Methods in Molecular Biology (Shirley, B. A., ed) pp. 253–269, Humana Press, Totowa, NJ
23. Paladini, A. A., Jr., and Weber, G. (1981) Biochemistry 20, 2587–2593
24. Weber, G. (1996) in Protein Interactions, Chapman and Hall Inc., New York
25. Markley, J. L. E. (1996) High-Pressure Effects in Molecular Biophysics and Enzymology, Oxford University Press, New York
26. Prehoda, K. E., Moober, E. S., and Markley, J. L. (1998) Biochemistry 37, 5785–5790
27. Miller, D. M., Delgado, R., Chirgwin, J. M., Hardies, S. C., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 4686–4691
28. Serbe, H. B. (1953) Acta Chem. Scand. 7, 1129–1136
29. Cleland, J. L., and Wang, D. L. (1990) Biochemistry 29, 11072–11078
30. Gorovitz, B. M., Ybarra, J., Seale, J. W., and Horowitz, P. M. (1997) J. Biol. Chem. 272, 26999–27007
31. Murphy, R. M., Yarmush, M. L., and Colton, C. K. (1991) Biopolymers 31, 1289–1295
32. Goldberg, M. E., Rudolph, R., and Jaenicke, R. (1991) Biochemistry 30, 2790–2797
33. Xie, Y., and Wettlaufer, D. B. (1996) Protein Sci. 5, 517–523
34. Jaenicke, R. (1998) Bioch. 379, 237–243
35. Klionsan, A. M. (1997) Trends Biotechnol. 13, 97–101