Interactive Intermediates Are Formed during the Urea Unfolding of Rhodanese*

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Structural transitions have been studied on the pathway for urea denaturation of rhodanese. Unlike guanidinium hydrochloride, urea gives no visible precipitation. Increasing urea concentrations cause a transition in which the enzyme activity is completely lost by 4.5 M urea, and there is a shift of the intrinsic fluorescence maximum from 335 nm for the native enzyme to 350 nm. There is a maximum exposure of organized hydrophobic surfaces at 4.5 M urea as reported by the fluorescence of 1,1'-bi(4-anilino)naphthalene-5,5'-disulfonic acid. Above 4.5 M urea, this probe reports the progressive loss of organized hydrophobic surfaces. The polarization of the intrinsic fluorescence falls with increasing urea concentrations in a complex transition showing that rhodanese flexibility increases in at least two phases. Rhodanese becomes increasingly susceptible to digestion by subtilisin between 3.5 and 4.5 M urea, giving rise to large fragments. At urea concentrations >5 M, rhodanese is completely digested. There is a small increase in the rate of sulfhydryl accessibility between 3.5 and 4.5 M urea, but there is a large increase in the sulfhydryl accessibility above 4.5 M urea. Dimethyl suberimidate cross-linking shows the presence of associated species in 5-5 M urea, but there are few cross-linkable species at lower or higher urea concentrations. These results are consistent with a model in which urea unfolding of rhodanese is associated with the initial production of a species having organized regions of structure with exposed hydrophobic surfaces separated by flexible elements.

Refolding of the denatured enzyme rhodanese (thiosulfate sulfurtransferase; EC 2.8.1.1) is difficult because of competition from aggregation and because of sulfhydryl oxidation (1). When denaturation was attempted using guanidinium HCl, almost all of the rhodanese precipitated from solution (2). Successful refolding of rhodanese can be achieved under conditions that include the use of assistants such as detergents (3, 4), or proteins called chaperonins (6) to limit aggregation, together with reducing agents and the substrate thiosulfate. Folding with detergents revealed intermediates that had the properties of molten globules, but it was not clear whether the detection of the intermediates required the presence of detergents (7). This led to the general question: which aspects of the denaturation are properties of the protein, and which parts depend on the interaction of the protein with the particular assistant used in renaturation? Thus, the specific question arose as to whether one could detect intermediate states of rhodanese in the absence of detergents, lipids, or chaperonins? An opportunity appeared recently to approach this question when it was demonstrated that reversible folding was possible for rhodanese without assistants (8). Rhodanese was shown previously that urea unfolding was shown to follow a reversible path, and in this process, there was no precipitation, indicating that aggregated states, at least those of the size formed in guanidinium HCl, were not present in urea.

In the present paper, we have studied the urea unfolding of rhodanese in the absence of detergents, chaperonins, or liposomes under conditions that have been shown previously to produce an unfolding transition that approximated the reversible pathway (8). Under these conditions, it is demonstrated here that the major transition leading to enzyme inactivation is not associated with total unfolding of the polypeptide chain; instead, it produces a state with a considerable degree of structure and a maximum exposure of organized hydrophobic surfaces. Apparently, it is this type of structure that must be protected by interactions with accessory substances for the successful high yield reactivation of rhodanese after denaturation.

EXPERIMENTAL PROCEDURES

Reagents and Proteins—All the reagents used were of analytical grade. Bovine liver rhodanese (9) and recombinant rhodanese (10) were prepared as described previously and stored at −70 °C as a crystalline suspension in 1.8 M ammonium sulfate. Rhodanese concentration was determined using a value of $A_{280 	ext{nm}}$ = 1.78 (11) and a molecular mass of 33 kDa (12).

Rhodanese Assay—Rhodanese activity was measured by a colorimetric method based on the absorbance at 480 nm of the complex formed between ferric ion and the reaction product thiocyanate (12).

Rhodanese Denaturation—Rhodanese was typically denatured in 50 mM Tris, pH 7.6, at a protein concentration of 10 μg/mL. The buffer contained 200 mM 2-mercaptoethanol and 50 mM thiosulfate, which were shown previously to be necessary to prevent oxidative damage (13). Samples were denatured at each urea concentration for at least 3 h before measurements were made. Typically, rhodanese assays used 10 μL of enzyme solution for 10 min, and time courses of product formation were used to detect nonlinearities in the progress curves.

Fluorescence Measurements—Fluorescence measurements were made on an SLM 5000C fluorometer (SLM Instruments, Urbana, IL). Intrinsic fluorescence emission spectra were measured at various urea concentrations in solutions containing 50 mM Tris-HCl, pH 7.8, at a protein concentration of 50 μg/mL at 23 °C, unless indicated otherwise. Excitation was at 280 nm (band pass, 5 nm) and the emission was either monitored at 335 nm or recorded as a spectrum from 300 to 450 nm.

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to 400 nm (band pass, 7.5 nm). The temperature was controlled by circulating water through the cell holder. Solutions were equilibrated for 20 min before addition of the enzyme. For solutions containing 10 μM bis-ANS, 1,1'-bitol-4-anilino)naphthalene-5,5'-dissulfonic acid, the excitation wavelength was 395 nm, and the emission was either monitored at 482 nm or recorded as a spectrum from 480 to 600 nm. Individual samples were used for the bis-ANS measurements. As a control for the ability of urea to disrupt interactions between bis-ANS and hydrophobic surfaces, the complex of 10 μM bis-ANS with 0.9% 7-cyclodextrin was prepared and titrated with urea under the same conditions as used for the protein. Measurements of the polarization of the intrinsic fluorescence were made with excitation at 300 nm and emission at 345 nm. For each sample the determined value was the average of 10 measurements. The readings were stable for 24 h. Polarization readings were corrected for any instrumental artifacts as described by the manufacturer.

**Results**

**Urea-induced Inactivation of Rhodanese Is Correlated with a Transition in Its Intrinsic Fluorescence**—Fig. 1 shows the enzymatic activity (open circles) and the wavelength of the intrinsic fluorescence maximum (closed circles) for rhodanese as functions of increasing urea concentration. The activity falls in a transition that occurs between approximately 3 and 4.5 M urea with 50% inactivation at about 3.7 M urea. The intrinsic fluorescence shifts from approximately 335 nm, characteristic of the native protein, to about 351 nm that is characteristic of the denatured protein. The transition in fluorescence correlates with that observed in the activity. True activity beyond 4.5 M urea appears to give about 10-12% of the initial activity, because there is some reactivation during the assay. This reactivation has been previously demonstrated (1). There was no appearance of visible turbidity associated with the activity loss as has been reported for denaturation with guanidinium HCl.

**Increased Hydrophobic Exposure Is Associated with Urea-induced Inactivation**—The wavelength maximum for the fluorescent probe bis-ANS was used to monitor the exposure of hydrophobic surfaces (15). Fig. 2 shows that, as the urea concentration is increased from 0 to 4.5 M urea, the fluorescence of bis-ANS shifts to shorter wavelengths in a transition that follows the loss in activity. The wavelength maximum reaches its lowest value, 500 nm, at the point where the activity has fallen to a minimum. As the concentration of urea is increased further, the fluorescence wavelength maximum shifts back toward the red, and it reaches 540 nm at 8 M urea. Thus, the maximum hydrophobic exposure in rhodanese occurs at 4.5 M urea, a concentration giving minimum activity. As the urea concentration is increased beyond 4.5 M, there is a continual decrease in hydrophobic exposure. This apparently indicates that rhodanese is not fully unfolded at 4.5 M urea, and the transition that is being monitored by both the intrinsic fluorescence and the enzyme activity does not represent a transition between the native and the fully unfolded protein.

Fig. 3 shows that the intensity for the bis-ANS fluorescence changes in a complex transition (Fig. 3, closed triangles). As
Rhodanese Intermediates Can Be Detected without Using Assistants

The decrease in bis-ANS fluorescence with increasing urea concentration above 4.5 M could be due either to a shift of the binding equilibrium due to increased bis-ANS solubility and/or to a disruption of organized hydrophobic surfaces on rhodanese as the protein denatured. A control with γ-cyclodextrin was used to address the issue. The γ-cyclodextrin enhanced the fluorescence of bis-ANS, presumably as a result of binding to the hydrophobic cavity in the center of the cyclodextrin ring. Increasing urea concentrations, over the range used in the titration of the protein, did not cause any significant fall of the bis-ANS fluorescence (data not shown), so there was no significant shift of the binding equilibrium between bis-ANS and the γ-cyclodextrin. Thus, it is likely that the decrease of bis-ANS fluorescence at the higher urea concentrations in the protein titration is due to a loss of the rhodanese structure, and it is not primarily due to interference with interactions between rhodanese and bis-ANS. This distinction is necessary, because, as a protein is unfolded by chemical denaturants, it is expected that hydrophobic residues become more exposed to the solvent. However, the feature that is important for the maximum binding of bis-ANS is not simply having individual exposed residues, but having those residues be part of an organized hydrophobic surface. Additionally, since the protein unfolds because denaturants can disrupt hydrophobic interactions, it is not expected that hydrophobic probes would bind as tightly, and the γ-cyclodextrin control was a potential way of sorting out these differences. The bis-ANS intensity, as noted above, reflects the same features observed in the bis-ANS wavelength maximum shift shown in Fig. 2. There is a gentle rise in intensity to 3 M urea and then there is a steep rise that follows the loss of activity. The maximum of the fluorescence intensity occurs in the region of the maximum loss of activity. The steeply falling intensity beyond 4.5 M urea indicates the loss of organized hydrophobic surfaces. The maximum in the bis-ANS intensity due to competing effects is in the same region as the maximum shift in the wavelength maximum. The activity profile is shown for comparison (open circles), and the activities are lower at the higher urea concentrations in this figure compared with Fig. 1, because these experiments are done at higher protein concentration, which requires a shorter incubation in the assay and, therefore, less opportunity for recovery in the assay.

Polarization of the Intrinsic Fluorescence of Rhodanese Reveals a Complex Transition during the Loosening of Protein Structure—Polarization of the intrinsic fluorescence was measured for rhodanese during the unfolding transition (open squares, Fig. 3). The polarization fell in a complex transition during the urea perturbation. The transition appears not to be a smooth two-state transition, and it looks as if there are at least two transitions. Part of the polarization loss occurs in the region of the activity loss, between approximately 3.75 and 4.5 M urea. In fact, the activity may fall at somewhat lower urea concentrations than the polarization change. There is a more gradual change in the polarization as the urea concentration is raised further, and the lowest polarization is observed at approximately 7 M urea.

Proteolysis by Subtilisin Reflects Urea-induced Transitions—Fig. 4 shows that rhodanese is quite resistant to digestion by subtilisin when equilibrated between 0 and 3 M urea. The protein becomes increasingly susceptible to digestion in a transition between 3 and 5 M urea. The protein is very susceptible to proteolysis at 5 and 6 M urea. Between 3.5 and 4.5 M urea, digestion produces discrete high molecular weight fragments as opposed to the behavior at and above 5 M urea where digestion produces only small fragments. Subtilisin was chosen because of its broad specificity, so that proteolytic patterns reflect exposure of the structure rather than the specificity of the protease. These data indicate that rhodanese behaves, at concentrations of urea that give minimum activity and maximum hydrophobic exposure, as if there are elements of organized structure separated by proteolytically susceptible regions.

Rhodanese Sulfhydryl Accessibility Reflects Urea-induced Structural Changes—The four sulfhydryl groups of rhodanese are all reduced in the native state, and these sulfhydryl groups are very resistant to reaction with DTNB when the protein is fully folded (18). Fig. 5 shows rate constants for the reaction of DTNB with rhodanese as a function of the urea concentration. The sulfhydryl groups of rhodanese react slowly with

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Urea concentration dependence of bis-ANS fluorescence intensity and the polarization of the intrinsic protein fluorescence shown together with the enzyme activity. Rhodanese was prepared in individual samples at 40 μg/ml, and separate samples were used for polarization and bis-ANS fluorescence. Intrinsinc polarization was measured with excitation at 300 nm, and emission was monitored at 345 nm. Readings were stable for 24 h. All other conditions were the same as noted in the legend to Fig. 2.

0 2.5 3 3.5 3.75 4 4.25 4.5 5 6

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** The lability of rhodanese to proteolysis by subtilisin as a function of the urea concentration. Rhodanese at 200 μg/ml was equilibrated at various urea concentrations and then was digested with 1% subtilisin for 30 min before being stopped by phenylmethysulfonyl fluoride. The lanes on the SDS gel shown here correspond to increasing urea concentrations. From the left they are 0, 2.5, 3, 3.5, 3.75, 4, 4.25, 4.5, 5, and 6 M urea, respectively.
DTNB up to 3.5 M urea. Beyond this urea concentration, a transition begins that leads to increasingly rapid reaction. At 4 M urea, where the activity is zero, and the first phase of the polarization drop is completed, there is still no large change in the rate of reaction of the sulphydryl groups. Sulphydryl accessibility does not increase substantially until the region of 5 M urea, where rhodanese becomes completely susceptible to protease and where bis-ANS binding has substantially decreased. It is interesting that, at 5 M urea, the bis-ANS intensity is almost at its minimum, whereas the shift in the wavelength maximum of the bis-ANS that is bound has not quite shifted to its maximum extent, which may indicate that some hydrophobic surfaces are still present, although they are only weakly able to bind bis-ANS. If this is the case, then there are still organized hydrophobic surfaces while the sulphydryl titer has increased substantially. Thus, there are several identifiable stages in the denaturation profile of rhodanese.

Dimethyl Suberimidate Cross-linking Reveals Urea-induced Protein Association—The strong exposure of hydrophobic surfaces at 4 M urea introduces the possibility that association of interactive intermediates was occurring even though these associated species were not large enough to cause significant scattering of visible light as was observed in the guanidinium HCl unfolding. Fig. 6 shows the results of DMS cross-linking of rhodanese that had been equilibrated at various urea concentrations. With increasing urea concentration, those concentrations giving evidence of incompletely denatured rhodanese give rise to a small amount of associated species. For example, there are no associated species observed from 1 to 3 M urea. At 4 and 5 M urea, there is clear formation of cross-linked species that correspond in molecular weight to dimers and trimers. At 6 M urea, there is a diminished amount of cross-linking, and at 7 M urea, there is virtually no cross-linking observed.

DISCUSSION

The structural transition leading to inactivation is not associated with complete unfolding of rhodanese. Urea-induced unfolding apparently occurs in at least two stages, and the first stage is associated with loss of activity and a sufficient opening of the rhodanese structure to give what appears to be exposed tryptophan residues. However, the structure that is so produced still has (a) restricted mobility of those same tryptophan residues, (b) large regions of exposed hydrophobic surface, and (c) enough retained structure so that the exposure of proteolytic cleavage sites leads to the formation of discrete large fragments, rather than complete proteolysis. In addition, even though the sulphydryl groups in rhodanese are all reduced in the native structure, there is still significant restriction to the access of the reagent DTNB up to 4.5 M urea. This description is similar to the properties described for the molten globule states that many proteins, including rhodanese, adopt in the course of reversible unfolding (7, 19, 20).

The concentration of urea that leads to complete loss of activity (4–4.5 M) gives a protein that is apparently not denatured to the random coil, but instead it forms sticky species that can associate, but they do not form large enough aggregates to give visible light scattering as would be seen in guanidinium HCl denaturation.

The results demonstrated here are consistent with Model I.

![Diagram of Model I](image)

In this model, native enzyme, when perturbed by increasing urea concentrations, initially forms intermediates that, while compact, contain exposed hydrophobic surfaces. As the urea concentration is increased, there are further increases in the opening of the structure. This structural opening may be related to a partial separation of the two domains into which the single polypeptide chain is folded. As the urea concentration is increased further, structures that perhaps can be best envisioned as beads on a string form to give the kind of structure that can give rise to the pattern observed in the subtilisin digestion. Finally, in the region of 6 M urea, the protein completely unfolds, finally, to give a structure that would be more like the random coil normally pictured for a denatured protein. Circular dichroism measurements are consistent with a random coil conformation of rhodanese at 6 M.
urea (7, 21). This model would be in keeping with the multiple intermediates that have been observed in the binding of rhodanese to the chaperonin cpn60 and by the results that indicate that the interaction with cpn60 involves more than one part of the rhodanese structure (22). These results would also be in keeping with findings from liposome disruption studies that suggest that more than one portion of the rhodanese structure is important for interactions with liposomes (24). The formation of these types of potentially interactive intermediates may be the reason that efficient refolding requires assistants such as detergents or liposomes or the chaperonin protein cpn60.

There are some common elements that are present after any perturbation of the rhodanese structure. For example, perturbation by both urea and guanidinium HCl have the common characteristic that intermediates are formed with sticky surfaces that can be diverted to associated species. Even in the absence of significant light scattering, there can be significant association that can short-circuit refolding. This last is important to emphasize, because many recent folding studies using rhodanese and similar proteins focus on the ability of chaperonins and assistants to influence light scattering after dilution of the protein from denaturants (6, 15, 23). Thus, it is possible to suggest a common model that can be used to understand irreversible denaturation, unassisted folding, chaperonin-assisted folding, detergent-assisted refolding, and liposome-assisted refolding that we observed previously with rhodanese. In this common model, as shown above, rhodanese can adopt partially folded intermediate states with strongly exposed interactive surfaces that can lead to small associated species which can give rise to large aggregates. This aggregation would give states that are kinetically prevented from folding quickly, and the folding could become so slow that it appears irreversible. By stabilizing these intermediates, they can be protected from interactions, and folding would be favored. Thus, in keeping with numerous previous studies on protein folding, it is likely that the control of folding intermediates can control the fates of proteins.

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