Alteration Around the Active Site of Rhodanese during Urea-induced Denaturation and Its Implications for Folding*

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The enzyme rhodanese contains two globular domains connected by a tether region and associated by strong hydrophobic interactions. The protein has proven to be very difficult to refold without assistance to prevent oxidation and aggregation. For this study, the active site cysteine 247, near the interdomain region, was modified with the environmentally sensitive fluorescent probe, 2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS), to yield a derivative that reversibly unfolds. Structural transitions during urea unfolding/refolding were complex and multiphasic. Increasing urea concentrations increased the IAANS fluorescence intensity and polarization. Both values reached maxima at 4 M urea, where there is a concomitant large exposure of hydrophobic sites as reported by both IAANS and the noncovalent fluorescent probe, bis-ANS. The exposure of the hydrophobic sites arises from the decrease in strong interaction between the domain interfaces, which lead to their partial separation. This correlates with the loss of activity of the unlabeled enzyme. Above 4.5 M urea, there is progressive loss of rigid, hydrophobic surfaces, and both fluorescence and polarization of IAANS decrease, with accompanying loss of secondary structure. These results are consistent with a folding model in which there is an initial, rapid hydrophobic collapse of the denatured form to an intermediate with native like secondary structure, with exposed interdomain, hydrophobic surfaces. This step is followed by adjustment of the domain-domain interactions and the proper positioning of reduced cysteine 247 at the active site.

Rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) is a mitochondrial enzyme (1, 2), that catalyzes the transfer of sulfur from thiosulfate to cyanide. It is a monomeric protein having a molecular mass of 33 kDa containing 293 amino acids (3). It is folded into two independent, equal-size domains, and its crystal structure is available (3). The domains are tightly associated, and the interdomain interface is highly hydrophobic. The active form of rhodanese contains four cysteine residues (63, 247, 254, and 263) that are all reduced. The cysteine 247 is at the active site of rhodanese and it forms a persulfide linkage with the sulfur transferred from thiosulfate (3, 4).

From the crystal structure, it is evident that none of the cysteine residues are in the position to form disulfide linkages easily. The crystal structures of sulfur substituted (ES),1 sulfur depleted (E), and carboxymethylated (CMR), in which active site cysteine is modified by iodoacetic acid, are virtually identical (3, 5).

Refolding of the denatured protein is difficult because of the competition from aggregation and sulphydryl oxidation (6). The active site sulfhydryl is always involved in the formation of disulfide bond formation during refolding (7). In the carboxymethylated rhodanese (CMR), the active site cysteine is blocked by chemical modification, and it shows highly reversible refolding from the urea denatured state,2 as it prohibits the sulphydryl oxidation. Urea unfolded rhodanese has been suggested to occur in at least two stages. The first stage is associated with the loss of activity, and extensive exposure of hydrophobic surfaces (9). This transition increases the proteolytic susceptibility of the tether region, residues 143–158, which the x-ray results show are tightly bound to the surface of the protein (10). This region is not susceptible to trypsin up to 3 M urea. At 4.25 M urea, tether region becomes loose and can be cleaved by trypsin producing the two globular N- and C-terminal domains (10–13). The second stage of denaturation involves unfolding of this intermediate state.

In the present paper, we have studied the conformational change in and around the active site of the enzyme during urea induced unfolding and its implication for the folding of rhodanese. This is the first time that the vicinity of the active site has been directly probed during reversible folding. We have modified the active site cysteine with the environmentally sensitive fluorescent probe, IAANS. The results are consistent with a model, which involves initial collapse of the unfolded protein to give folded domains followed by their association to yield the native, active conformation.

EXPERIMENTAL PROCEDURES

Reagents and Protein—Urea was of electrophoresis purity, purchased from Bio-Rad. All other reagents used were of analytical grade. IAANS and bis-ANS were purchased from Molecular Probe Inc. (Eugene, OR). Recombinant bovine liver rhodanese was purified as described previously and stored at −70 °C as a crystalline suspension in 1.8 M ammonium sulfate. It was desalted on Sephadex G-50 column before use (14). Rhodanese concentration was determined by using a value of A280 nm = 1.75 (15).

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 reaction product thiocyanate (3). The assay was initiated by adding microgram quantities of rhodanese, and the reaction was stopped by the addition of formaldehyde.

Rhodanese Modification (RHAANS)—25-Fold molar excess of IAANS in N,N-dimethylformamide was added to 2 mg/ml rhodanese in 0.2 M sodium phosphate, pH 7.6. A 20-fold molar excess of KCN over rho-

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1 The abbreviations used are: ES, the sulfur substituted form of rhodanese; IAANS, 2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid; RHAANS, rhodanese modified by IAANS; E, rhodanese without transferred sulfur; CMR, carboxymethylated derivative of rhodanese; bis-ANS, 4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid.

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rhodanese was added to this solution and incubated at room temperature in the dark. The buffer was flushed with nitrogen to remove oxygen, as sulphhydryl oxidation involving the active site cysteine was a major reaction (5), which could compete with the active site cysteine modification. The reaction was monitored by following the loss of activity of the protein. When the enzyme lost ~50% activity, the reaction mixture was chromatographed on Sephadex G-50 to remove excess IAANS and KCN and eluted with 0.2 M sodium phosphate, pH 7.6. The incorporation ratio was in the range of 0.6–0.7. The position of RHAANS both by fluorescence and Coomassie Brilliant Blue stain in SDS-polyacrylamide gel electrophoresis was identical to rhodanese.

Denaturation of Rhodanese and RHAANS—Both rhodanese and RHAANS were denatured in 0.2 M sodium phosphate, pH 7.6, containing different concentrations of urea, at a protein concentration of 1 μM. For equilibrium denaturation, protein samples were incubated at room temperature, for 18 h. All the reaction mixtures except those used for CD measurements contained 1 mM dithiothreitol. The fluorescence of the bound probe changes in a complex transition. As the urea concentration increases, there is little change in the intensity up to 2 M urea. Then, there is a steep rise in intensity up to 4.5 M urea, beyond which there is a steep fall. This rise in fluorescence intensity correlates with the loss of activity of rhodanese from unlabeled enzyme (9). The increase in fluorescence intensity is due to an increase in accessible hydrophobicity around the active site, where the environmentally sensitive probe is covalently bound. Since this method modifies cysteine 247, which is at the interdomain interface, the result reflects that the two-domain structure of rhodanese is stabilized by strong hydrophobic interface that can be exposed by perturbation with urea. A similar complex transition is observed when bis-ANS binding to rhodanese is monitored at different urea concentrations (9). This transition is completely reversible (Fig. 1). Thus, this profile suggests that there are two transitions during urea induced unfolding of rhodanese. The first transition involves the exposure of hydrophobic surfaces around the active site cysteine (247), so that the environmentally sensitive probe attached to it is in more hydrophobic environment and hence shows fluorescence enhancement. Since the structural perturbations occur around the active site, it also accounts the loss in activity associated with this step. The second transition is the global melting of the protein, which destroys all the hydrophobic structures, and there is a decrease in fluorescence with the increase of urea concentration beyond 4.5 M urea. The complete reversibility indicates that the involvement of active site cysteine in the sulphhydryl oxidation has a major role in nonreversibility of refolding that is normally observed with rhodanese.

**RESULTS**

Reversible Unfolding of Modified Rhodanese (RHAANS)—Fig. 1 shows the unfolding and refolding of RHAANS in the presence of 1 mM dithiothreitol. The fluorescence of the active site-labeled enzyme is used to monitor the local structure of rhodanese. The data were recorded 18 h after preparing the samples. The fluorescence of the bound probe changes in a complex transition. As the urea concentration increases, there is little change in the intensity up to 2 M urea. Then, there is a steep rise in intensity up to 4.5 M urea, beyond which there is a steep fall. This rise in fluorescence intensity correlates with the loss of activity of rhodanese from unlabeled enzyme (9). The increase in fluorescence intensity is due to an increase in accessible hydrophobicity around the active site, where the environmentally sensitive probe is covalently bound. Since this method modifies cysteine 247, which is at the interdomain interface, the result reflects that the two-domain structure of rhodanese is stabilized by strong hydrophobic interface that can be exposed by perturbation with urea. A similar complex transition is observed when bis-ANS binding to rhodanese is monitored at different urea concentrations (9). This transition is completely reversible (Fig. 1). Thus, this profile suggests that there are two transitions during urea induced unfolding of rhodanese. The first transition involves the exposure of hydrophobic surfaces around the active site cysteine (247), so that the environmentally sensitive probe attached to it is in more hydrophobic environment and hence shows fluorescence enhancement. Since the structural perturbations occur around the active site, it also accounts the loss in activity associated with this step. The second transition is the global melting of the protein, which destroys all the hydrophobic structures, and there is a decrease in fluorescence with the increase of urea concentration beyond 4.5 M urea. The complete reversibility indicates that the involvement of active site cysteine in the sulphhydryl oxidation has a major role in nonreversibility of refolding that is normally observed with rhodanese.
ing of RHAANS in Urea—Fig. 2 shows the polarization of the bound probe in RHAANS during unfolding and refolding transitions, at different urea concentrations. The profile shows a complex transition like that monitored by fluorescence intensity (Fig. 1). There is only a modest change in polarization of bound IAANS up to ~2 M urea. Beyond this, there is a sharp rise in polarization up to ~3.5 urea. At higher concentrations of urea, polarization values fall sharply and become limiting above ~6.5 M urea. This transition is completely reversible (Fig. 2). The increase in polarization values indicates that the fluorophore attached to active site cysteine (247) is in more rigid environment at around 3.5 M urea. There are two possibilities, which can account for the enhanced hydrophobic (increase in fluorescence of the covalently bound probe) and rigid environment (increase in polarization of the bound probe) in 3.5 M urea. One is at 3.5 M urea, rhodanese may form a sticky intermediate that can associate, giving rise to aggregated product. The other possibility is that 3.5 M urea may loosen up the interdomain interactions, exposing the hydrophobic interface to enter into the hydrophobic interdomain interface. The interdomain space may impose restriction on the mobility of the bound probe. The visible light scattering experiments do not show any associated species up to 5 M urea (data not shown), which rules out the first possibility. Recent studies have been shown that the interdomain interaction can be perturbed at 4.25 M urea, making the hinge region susceptible to tryptic digestion (9, 10).

Structural Transition as Monitored by Ellipticity Change—Both rhodanese and RHAANS respond similarly when structural changes are followed by monitoring ellipticity at 222 nm as a function of urea concentrations. Fig. 3 shows that there is no change in ellipticity of rhodanese or RHAANS up to 4.5 M urea. This result clearly indicates that there is no global change in the structure of the protein. Beyond this urea concentration, there is steep fall in ellipticity, which accounts for...
complete loss of secondary structure between 4.5 and 7 M urea.

Secondary Structure of Rhodanese and RHAANS in 0, 4.5, and 8 M Urea—Fig. 4, A and B, show the CD spectra of rhodanese and RHAANS in 0, 4.5 and 8 M urea, respectively. There are no differences in the spectra of either rhodanese or RHAANS in 0 and 4.5 M urea, which indicates that there is no global change in the structure up to 4.5 M urea. In 8 M urea, there is substantial decrease in CD signal, which accounts for the global melting of the protein, producing random coil structure.

Accessibility of the Bound IAANS Toward Collisional Quencher—Fig. 5 shows the Stern-Volmer plot of quenching by acrylamide of IAANS bound to rhodanese and RHAANS in 0, 4.5, and 8 M urea. In 8 M urea, all the fluorescence is available for solvent quenching, as it produces a straight line with virtually zero slope. The values of $K_{SV}$ in 0 and 4.5 M urea are 6.9 $\pm$ 0.18 and 5.79 $\pm$ 0.04, respectively. The $K_{SV}$ values are very similar. The slight additional protection of bound fluorophore in 4.5 M urea (slightly lower $K_{SV}$ value) may be due to shielding of the active site cysteine within the interdomain interface, which correlates with the higher fluorescence and polarization of IAANS bound to that cysteine residue, as seen above.

Environment of bis-ANS Bound to Rhodanese and RHAANS—Table I shows the fluorescence intensities, emission maxima, and polarizations of bis-ANS bound to rhodanese and RHAANS, in 0, 4.5, and 8 M urea. Protein is used in large excess over bis-ANS (50:1) to minimize the effect of free bis-ANS in solution. As the urea concentration increases from 0 to 4.5 M urea, relative fluorescence of the bound bis-ANS increases from 800 to 1600 (in arbitrary unit) with rhodanese, and 900 to 1900 (in arbitrary unit) with RHAANS. The enhancement in fluorescence is accompanied by the increase in polarization values from 0.21 to 0.33 (in case of rhodanese) and from 0.22 to 0.39 (in case of RHAANS). There is also a concomitant blue shift in the bis-ANS emission maxima from 483 to 478 for rhodanese and from 486 to 478 for RHAANS. As the urea concentration is increased to 8 M urea, there is drop in fluorescence and polarization, and the emission maxima shift toward the red. These data support the idea that 4.5 M urea opens the hydrophobic interdomain interface, and the binding of a hydrophobic probe like bis-ANS to this site decreases its mobility.

**DISCUSSION**

Urea denatured rhodanese does not undergo unassisted refolding, because of the competition among refolding, aggregation, and formation of disulfide linkages (16, 17), unless assistants such as detergents (18, 19), liposomes (20), chaperonins (21), or large excesses of reductants (22) are present. Mutational studies show that cysteine 247 is the only cysteine residue responsible for the activity of the enzyme (23, 24). This cysteine is located near the cleft formed by the juxtaposition of the two domains. During oxidation, this cysteine 247 is most reactive, and always involved in the formation of intramolecular disulfides (23). Cysteine 247 can be modified exclusively,
and x-ray crystallographic data reveal that its chemical modification, e.g. by iodoacetic acid to form CMR, does not significantly alter the protein structure. This derivative has a virtually identical structure compared with sulfur free (E) and sulfur substituted (ES) forms of the enzyme both in solution and in the crystal (5). Importantly, in CMR, cysteine 247 loses its ability to form disulfide linkages with other cysteine residues during refolding. As expected, it shows excellent reversibility during refolding from urea, in the absence of any assistance (8). These observations, along with the position of the highly reactive active site cysteine at the cleft formed by two globular domains, make rhodanese a good model system to study the conformational change around the active site during folding of the protein.

Multiphasic transitions are observed for “hinge-bending” type proteins such as T4 lysozyme (25) and phosphoglycerate kinase (8). Rhodanese, whose two globular domains are linked by a connecting region consisting of a single strand of polypeptide chain also shows at least a two-step unfolding transition. The first step of urea induced unfolding involves local change in the structure as the secondary structures are similar in 0 and 4.5 m urea (Fig. 4). Major changes involved in this stage are around tether region and interdomain interface affecting the environment of the active site. In the absence of perturbation, the tether region is tightly bound to the surface of the two domains and completely inaccessible to proteolytic cleavage in the native protein. At 4.25 m urea, this interaction becomes weak, and the tether becomes susceptible to trypsin, yielding two globular domains (10). The hydrophobic interaction between two domains also decreases, resulting in opening up of interdomain interface. This in turn exposes the available hydrophobic sites, as evident from higher bis-ANS binding (Table I). The exposed hydrophobic sites are close to the active site cysteine, as there is increase in fluorescence of bound IAANS in 4.5 m urea (Fig. 1). IAANS is an environmentally sensitive probe and it is covalently linked to the active site cysteine residue, so fluorescence enhancement is solely due to increase in hydrophobicity of its environment. The loss of strong interaction between two domains, perturbed by urea, may help the reactive cysteine residue to enter into the interdomain interface. High polarization values and protection against collisional quenching (Figs. 2 and 4) at 4.5 m urea, support the idea that the probe (and the active site cysteine) can slip into the interdomain space, where it is more protected and less mobile. This accounts for the loss of activity of unlabeled rhodanese associated with the first step. Based on the observations reported here, a model is supported in which urea perturbation weakens the strong interdomain interaction in the native enzyme, and there is a partial separation between them. The active site cysteine residue can enter into this space, which is hydrophobic and rigid. This step is associated with inactivation of the unlabeled enzyme. As urea concentrations are increased further, global melting of the protein would occur. This suggestion is consistent with a model, according to which the first step in rhodanese folding involves the rapid formation of native-like domains, with exposed hydrophobic patches, separated by a flexible tether region. This would be followed by a second step that includes slow adjustment of the domain interactions, which would foster proper alignment of cysteine 247 at the active site.

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