Domain Separation Precedes Global Unfolding of Rhodanese*

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Toru Shibatani‡, Gisela Kramer§, Boyd Hardesty§, and Paul M. Horowitz‡‡

From the ‡Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284-7760 and the §Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712

The enzyme rhodanese was investigated for the conformational transition associated with its urea unfolding. When rhodanese was treated with 0 or 3 mM urea, the activity was not significantly affected. 4.25 mM urea treatment led to a time-dependent loss of activity in 60 min. Rhodanese was completely inactivated within 2 min in 6 mM urea. The 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid fluorescence intensity was not significantly increased during 0, 3, and 6 mM urea equilibrations, and the fluorescence was dramatically increased with 4.25 mM urea, indicating that hydrophobic surfaces are exposed. After 0 and 3 mM urea equilibration, rhodanese was not significantly proteolyzed with trypsin. Treatment with 4.25 mM urea led to simultaneous formation of major 12-, 15.9-, 17-, and 21.2-kDa fragments, followed by progressive emergence of smaller peptides. The N termini of the 17- and 21.2-kDa bands were those of intact rhodanese. The N terminus of the 15.9-kDa band starts at the end of the interdomain tether. The 12-kDa band begins with either residue 183 or residue 187. The size and sequence information suggest that the 17- and 15.9-kDa bands correspond to the two domains. The 21.2- and 12-kDa bands appear to be generated through one-site tryptic cleavage. It is concluded that urea disrupts interaction between the two domains, increasing the accessibility of the interdomain tether that can be digested by trypsin. The released domains have increased proteolytic susceptibility and produce smaller peptides, which may represent subdomains of rhodanese.

Rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) is a mitochondrial enzyme that is widely distributed in mammalian tissues (1, 2). Rhodanese, which was isolated from bovine liver, is a single polypeptide having a molecular mass of 33 kDa containing 293 amino acids (3). The x-ray studies showed that the 33-kDa enzyme consists of two equal-sized globular domains: the N-terminal domain, comprising residues 1–142, and the C-terminal domain, comprising residues 159–293; the connecting loop comprises residues 143–158 (3). After synthesis, rhodanese is not processed beyond removal of the initiating methionine. Non-amino acid components are not required for the native active structure (3). In its active form, all four of the rhodanese sulfhydryl groups are reduced (1, 3). Therefore, all of the sequence information needed for folding and functions is present in the isolated protein. These properties have contributed to making rhodanese an attractive model for studying protein folding and for examining the interaction with chaperonins or heat shock proteins (4).

The majority of the proteins imported into mitochondria have their N-terminal signal sequences removed proteolytically. However, rhodanese retains the N-terminal signal sequence after it is imported into the mitochondrial matrix (5). Therefore, it has been proposed that the N-terminal signal sequence might play an important role in the structural integrity, folding, and function of rhodanese (4, 6, 7). When glutamic acid 17 was replaced with proline, the point-mutated rhodanese (E17P) had a specific activity similar to that of the wild type, but it has lower stability toward urea and decreased ability of refolding in vitro (4). The N-terminal sequence was further investigated, by constructing rhodanese mutants with various N-terminal deletions. Rhodanese with deletion of 9 amino acid residues was inactivated by lower concentration of urea and could not be refolded efficiently (6). Rhodanese species with deletions of 10–23 amino acids were expressed as inactive species and quickly eliminated from the cells (6), indicating that N-terminal sequence has a major role in maintaining the structural stability and function of rhodanese.

Unfolding and refolding of rhodanese may be achieved through intermediate structures, but the natures of any intermediates are not clear. Proteases have been utilized to proteolyze rhodanese in order to map the exposed sites of rhodanese and to understand some aspects of its three-dimensional conformation (4, 7–10). When rhodanese was oxidized, proteases, including subtilisin, chymotrypsin, V8, and trypsin, produced bands equivalent to the size of a rhodanese domain, indicating that rhodanese exhibits conformational change with oxidation and the interdomain tether of rhodanese is exposed and become susceptible to protease digestion (9). Furthermore, under acidic conditions, digestion with pepsin revealed loosening of the structure with the production of species that could have been subdomains of rhodanese (10). In other experiments, native rhodanese was digested with immobilized trypsin and produced a significant amount of 31-kDa species. The N terminus of the 31-kDa band indicated that rhodanese was cleaved at residue 45 (7). However, the absence of this N-terminal region did not increase proteolytic susceptibility of the rhodanese tether region. The sequence of rhodanese would indicate that the interdomain tether of rhodanese should be susceptible to proteolytic attack, but attempts to proteolyze this region in native rhodanese have been unsuccessful, presumably because of conformational constraint imposed (9, 11, 12). This is confirmed by the x-ray results, which show that the sequence 143–158 is tightly bound to the surface of the protein.

We investigated the structural integrity of rhodanese by loosening the tertiary structure with a various concentration of urea and assessment using tricine-based SDS-PAGE, which resolves low molecular weight peptides (13). The conformational change of rhodanese was examined with respect to ac-
tivity, fluorescence, and tryptic digestion with time course.

With the methods presented here, we could produce an unfolding condition in which tryptic digestion released the two globular domains of rhodanese, which were subsequently digested into smaller peptides. This process was associated with inactivation as well as significant increase of the hydrophobic exposure of rhodanese. These data were further supported by the N-terminal analysis of the digested peptides. These results demonstrate that rhodanese unfolds initially by disrupting the interactions between its two globular domains, which then open further to reveal possible subdomains.

EXPERIMENTAL PROCEDURES

Materials

Urea was from Bio-Rad. SDS, acrylamide, and bis-acrylamide were from Fischer Scientific (Pittsburgh, PA). Silver nitrate and sodium thiosulfate was from Aldrich. The gel apparatus was purchased from Idea Scientific (Minneapolis, MN). 1,1'-Bi(4-anilino)naphthalene-5,5'-disulfonic acid (bis-ANS) was from Molecular Probes, Inc. (Eugene, OR). Trypsin (sequence grade-modified) was from Promega Corp. (Madison, WI). Immobilon-P transfer membrane is a product of Millipore Corp. (Bedford, MA). Amido Black was from ICN Pharmaceuticals, Inc (Cleveland, OH). Other chemicals were from Sigma.

Methods

Rhodanese Purification—Recombinant bovine rhodanese was purified as described previously and stored at 70 °C as a crystalline suspension in 1.8 M ammonium sulfate containing 1 mM sodium thiosulfate (14). Rhodanese was desalted on a G-50 column before use (15). Rhodanese concentrations were determined using a value of A280 nm = 1.75.

Rhodanese Assay—Rhodanese activity was measured by a colorimetric method based on the absorbance at 460 nm of the complex formed between ferric ion and the reaction product, thiocyanate (16). Rhodanese activity was measured by a colorimetric method based on the absorbance at 460 nm of the complex formed between ferric ion and the reaction product, thiocyanate (16).

Fluorescence Measurement—bis-ANS fluorescence measurements were performed using an SLM 48000S fluorometer (SLM Instruments, Urbana, IL). The excitation wavelength was 399 nm, and the emission was recorded as a spectrum from 450 to 550 nm. The excitation and emission slit widths were 1 nm. The collection of recorded spectra showed that the maximum intensity of the bis-ANS fluorescence was observed at ~500 nm (data not shown). Therefore, the data were plotted as the fluorescence intensities obtained with the excitation 399 nm and the emission at 500 nm. The scanned samples consisted of 10 µg/ml of rhodanese, 10 µM bis-ANS, 200 µM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.6, and 50 mM sodium thiosulfate with 0, 3, 4.25, or 6 M urea. The scanning was carried out at several times between 0 and 185 min as indicated under "Results." Rhodanese was added to the unfolding solution at t = 0.

Proteolysis by Trypsin—Rhodanese (final concentration, 0.2 mg/ml) was unfolded for 3 h at 23 °C in buffers containing 200 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.6, and 50 mM sodium thiosulfate with either 0, 3, 4.25, or 6 M urea. The unfolded rhodanese was proteolyzed with 1% trypsin (w/w) at 23 °C. The incubation times are described in the legends of Figs. 3 and 4. The reduction of rhodanese activity during urea unfolding. Rhodanese was treated with 0 (closed circles), 3 (open circles), 4.25 (closed squares), and 6 (open squares) M urea unfolding solution containing 200 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.6, and 50 mM sodium thiosulfate. The activity was monitored at 23 °C for 0–120 min. The rhodanese concentration was 0.2 mg/ml. Rhodanese activity is indicated as the absorbance at 460 nm.

RESULTS

Accelerated Inactivation of Rhodanese with Increased Concentration of Urea—Rhodanese was treated with 0, 3, 4.25, and 6 M urea. Rhodanese activity was measured during 2 h of equilibration with urea. As shown in Fig. 1, activity was not significantly perturbed when rhodanese was treated with 0 (closed circles) or 3 (open circles) M urea. With time, the rhodanese activities were reduced to approximately 90% of the original activity because of rhodanese oxidation. Rhodanese was completely inactivated with both 4.25 and 6 M urea equilibration. With 6 M urea (Fig. 1, open squares), the activity was completely lost in 2 min, whereas rhodanese was slowly inactivated (t1/2 ~ 20 min) by treatment with 4.25 M urea (closed squares).

Hydrophobic Surface Exposure Assessed by bis-ANS Fluorescence—Rhodanese was treated with 0, 3, 4.25, and 6 M urea, and the exposure of the hydrophobic surfaces was assessed with the fluorescence probe, bis-ANS. The bis-ANS fluorescence increases when it binds to hydrophobic sites. The bis-ANS fluorescence intensity was measured at times between 0 and 185 min as indicated in Fig. 2. Unfolding with 0 (Fig. 2, closed circles) and 3 (open circles) M urea did not significantly increase the fluorescence intensity, indicating that hydrophobic surfaces of rhodanese were not significantly exposed with those urea concentrations. The bis-ANS fluorescence dramatically increased with time when rhodanese was treated with 4.25 M urea (Fig. 2, closed squares). This increase in fluorescence intensity indicated that rhodanese was partially unfolded and the hydrophobic surface was increased. In contrast, the bis-ANS fluorescence was not increased when the enzyme was unfolded with 6 M urea (Fig. 2, open squares). This indicated that rhodanese was completely unfolded and contained very few organized hydrophobic surfaces.

Proteolysis of Rhodanese by Trypsin—Rhodanese was equilibrated in buffers with 0, 3, 4.25, and 6 M urea for 3 h at 23 °C.
Rhodanese was then digested with trypsin for 30 min, and the digested rhodanese was subjected to tricine-based SDS-PAGE, as described under "Experimental Procedures." As shown in Fig. 3, rhodanese was not susceptible to digestion when it was equilibrated with 0 or 3 M urea, indicating that rhodanese structure was still compact and thereby resistant to proteolytic attack by trypsin. When the enzyme was treated with 4.25 M urea, rhodanese became susceptible to trypsin and was digested to produce distinct fragments, which were highly reproducible. Trypsin did not efficiently proteolyze rhodanese unfolded in 6 M urea because trypsin is susceptible to denaturation by 6 M urea. Subtilisin, a nonspecific protease, is more resistant to urea denaturation and digested 6 M urea-unfolded rhodanese to only small fragments, and it also produced a discrete high molecular weight fragment with 4.25 M urea (8). Digestion of 4.25 M urea-unfolded rhodanese by trypsin was investigated at 0, 1, 2, 5, 10, 20, 30, and 60 min to determine whether particular fragments appear in the early phase of trypsic digestion or whether all digested fragments seen in Fig. 3 appear simultaneously. Fig. 4 shows the time course of trypsic digestion of 4.25 M urea-equilibrated rhodanese. The kinetic experiments were performed with two different forms of the enzyme, the ES form and the E form. As shown in Fig. 4, no significant difference was observed between the digestion patterns of the two forms. Four major bands appeared after 1 min incubation with trypsin, and then other fragments started appearing. The four bands are approximately 21, 17, 15.9, and 12 kDa. Because these four major bands appeared in the early phase, identification of these tryptic cleavage sites could indicate how the rhodanese structure was partially unfolded. After rhodanese was digested with trypsin, samples were subjected to tricine-based SDS-PAGE, and the digested fragments on the gel were transferred to Immobilon-P membrane. The fragments were subjected to N-terminal protein sequencing. The N terminus of the 17-kDa band was the same as that of intact rhodanese. The sequence information as well as the size of the band indicated that the 17-kDa band represents the N-terminal domain of rhodanese. The N-terminal sequence of the 15.9-kDa band showed that cleavage occurred before Ser-159, the first amino acid of the C-terminal domain, providing strong evidence, together with the size of the fragment, that this band is the C-terminal domain of rhodanese. These results suggested that the 4.25 M urea-equilibrated rhodanese loosened the interaction of the N- and C-terminal domains, and the tether region of rhodanese was easily accessible to trypsin digestion. The N-terminal sequence of the 21.2-kDa band corresponded to the N terminus of native rhodanese, suggesting that this species contains the N-terminal domain, as well as part of the C-terminal domain. In addition, the 12-kDa band sequence showed that it contained two fragments that were close in size and comigrated within the resolution of the gel system. One fragment started with Ala-183, and the second fragment started with Tyr-187, both of which are located in the C-termi-

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nal domain. These data indicated that the 21.2- and 12-kDa bands might also be directly cleaved from the full-length rhodanese. Overall, 4.25 M-activated rhodanese might be susceptible to tryptic digestion at two major locations, the tether region and the 183–187 region in the C-terminal domain.

**DISCUSSION**

In order to obtain information on the effect of the domains structure of rhodanese and its unfolding, we investigated its structure by using urea to create the partially unfolded enzyme. Because rhodanese consists of two equal-sized domains connected by a linker region that is located on the surface of the molecule, rhodanese has been a very important model for studying structure and refolding, as well as function. Folding intermediates of rhodanese have been investigated to explain aspects of unfolding, stability, and binding of the enzyme to the molecular chaperone (8, 20, 21). However, little is known about the details of these structures. Therefore, it was very important to obtain the structure information on intermediate conformations involved in folding. Our studies have increased our understanding of intermediate unfolded states of rhodanese.

When rhodanese was partially unfolded with 4.25 M urea, hydrophobic regions on rhodanese were exposed. This conformation has been described as “beads on a string” (8), in which rhodanese unfolds to give compact regions separated by less structured regions. The model was proposed based on results in which the urea-unfolded rhodanese was partially digested by a nonspecific protease, subtilisin, to produce many distinct bands (8). However, there has been little direct experimental evidence to support the hypothesis that rhodanese was digested according to its domains. The sequence connecting the domains of rhodanese is located on the surface of the molecule. Therefore, it was assumed that it should be easily proteolyzed to release the two domains. However, many methods have failed to demonstrate that the tether region is accessible to the protease digestion (4, 7–10). We investigated the intermediate state of rhodanese by using the time course as well as SDS-PAGE to examine the course of the digestion more carefully. Another point of these experiments is that we utilized a specific protease, trypsin, to digest the partially unfolded enzyme. Previous studies have performed using nonspecific subtilisin (8). The peptides from this digestion could not be identified because of their variable N termini. The present studies used the more specific protease, trypsin, which cleaves peptide bonds only at the C terminus of arginine and lysine residues. Rhodanese has 36 trypsin cleavage sites (20 arginine residues and 16 lysine residues). From the N-terminal sequencing, the major digested fragments, the 17- and 15.9-kDa bands represent the N- and C-terminal domains, respectively. The results indicated that rhodanese was unfolded to intermediate states in which the interactions between the two domains were loosened, and the connecting loop was accessible to protease attack. Two other digested fragments, the 21.2- and 12-kDa bands also appeared at 1 min of digestion. The 12-kDa band has an N terminus of Ala-183 or Tyr-187. The sequencing data, as well as the size information, indicated that those bands were also generated from full-length rhodanese. These data suggested that rhodanese might be unfolded to have two major proteolytic access sites: the tether region and the 183–187 region in the C-terminal domain. We have investigated whether the ES form and the E form have different digestion patterns. Tryptic digestion demonstrated that the digestion patterns of both 4.25 M urea-unfolded forms were the same over the time course studied. This study is consistent with data from x-ray crystallography showing that ES and E have very limited structural differences, which are mostly confined to the active site (19).

Several models would formally be possible for the structure of rhodanese in 4.25 M urea: 1) rhodanese structure is loosened globally; 2) the tether sequence is released from the rhodanese surface and the rhodanese domains are still associated; or 3) rhodanese domains separate, and the tether becomes accessible as suggested by the beads on a string model. To address this question, rhodanese was digested by trypsin, and the digested products were monitored over time by the method of the SDS-PAGE. As demonstrated in Fig. 4, rhodanese was digested into four major bands at 1 min, which were subsequently digested to smaller peptides. From this result, reaching the intermediate state through global unfolding is not possible because rhodanese would otherwise be degraded by trypsin at any cleavage site to produce variety of small peptides. The existence of intact associated domains with only a loosened tether is not likely, because then the extensive hydrophobic exposure would not be achieved as observed in Fig. 2. In addition, all rhodanese molecules would have been cleaved first only at the tether region. The most consistent model is beads on a string: that is, domain separation. This model fits the observation that treatment with 4.25 M urea resulted in increased hydrophobic exposure, as well as largely maintaining protease resistant domains.

The data also suggest that the released domains of rhodanese become more susceptible to trypsin digestion. A possible explanation is that the released domains may change to a less stable conformation. Thus, the N-terminal domains accumulated with time of trypsin digestion, whereas the C-terminal domains accumulated initially, but they began to disappear after 30 min digestion. This observation was true when digestion was performed with subtilisin instead of trypsin. In these studies, the 15.9-kDa C-terminal domain was further cleaved by trypsin at Ala-183 and Tyr-187. This is consistent with the C-terminal domain being more fragile than the N-terminal domain.

A recent report indicated that the N-terminal signal sequence of rhodanese is a key for stability of the enzyme (4, 6). It has been demonstrated that the deletion in the N-terminal signal sequence (Δ1–23) caused inactivation as well as instability of the rhodanese molecule (6). It has been demonstrated that a site-directed mutant that replaced glutamic acids with Pro-17 has lower stability to perturbation by urea and has higher hydrophobic exposure in the unperturbed condition in comparison to the wild type (4). From these previous data, the N-terminal signal sequence appeared to contribute to stability of rhodanese. However, the data presented here show little indication that the N-terminal sequence play a significant role in opening the two domains of rhodanese or that the N-terminal sequence was the major proteolytic target of trypsin. As previously shown in proteolysis with immobilized trypsin, the approximate 31-kDa band has been detected among the digested products (7), but the band was a minor species in these studies, so it is unlikely that the removal of the N-terminal region contributes to destabilize the interactions between the two globular domains of rhodanese. Therefore, the N-terminal sequence might be important for domain stability of the rhodanese structure but not be required to loosen the domain structure.

Overall, we have demonstrated that the interaction of the rhodanese domains can be loosened, using 4.25 M urea, which leads to an increase in the hydrophobic surface exposure as well as the proteolytic susceptibility in the rhodanese tether region. The results also further support the idea that the intermediate state of rhodanese can be viewed as beads on a string, i.e., a domain-separated structure connected by a proteolytically susceptible sequence.
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