Acid pH-induced Conformational Changes in Bovine Liver Rhodanese*

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The enzyme rhodanese is greatly stabilized in the range pH 4–6, and samples at pH 5 are fully active after several days at 23 °C. This is very different from results at pH > 7, where there is significant loss of activity within 1 h. A pH-dependent conformational change occurs below pH 4 in a transition centered around pH 3.25 that leads slowly to inactive rhodanese at pH 3 (t1/2 = 22 min at pH 3). The inactive rhodanese can be reactivated by incubation under conditions required for detergent-assisted refolding of denatured rhodanese. The inactive enzyme at pH 3 has the far-red absorbance of the intrinsic fluorescence spectrum shifted to 345 nm from 335 nm, which is characteristic of native rhodanese at pH > 4. At pH 3, rhodanese shows increased exposure of organized hydrophobic surfaces as measured by 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid binding. The secondary structure is maintained over the entire pH range studied (pH 2–7). Fluorescence anisotropy measurements of the intrinsic fluorescence provide evidence suggesting that the pH transition produces a state that does not display greatly increased average flexibility at tryptophan residues. Pepsin digestibility of rhodanese follows the pH dependence of conformational changes reported by activity and physical methods. Rhodanese is resistant to proteolysis above pH 4 but becomes increasingly susceptible as the pH is lowered. The form of the enzyme at pH 3 is cleaved at discrete sites to produce a few large fragments. It appears that pepsin initially cleaves close to one end of the protein and then clips at additional sites to produce species of a size expected for the individual domains into which rhodanese is folded. Overall, it appears that in the pH range between pH 3 and 4, titration of groups on rhodanese leads to opening of the structure to produce a conformation resembling, but more rigid than, the molten globule state that is observed as an intermediate during reversible unfolding of rhodanese.

Conformational changes have been implicated in rhodanese activity. The charges on ionizable amino acids are critical to its structure and function (4). It has been suggested that in vivo control of rhodanese sulfurbtransferase activity is exerted by side chain phosphorylation at serine 124, which results in a conformational change due to changes of ionic interactions within the protein (3).

The importance of charge interactions makes it of interest to investigate the conformational potentials of rhodanese as a function of pH. Kinetic and physical measurements at pH 5 were interpreted as showing that there were conformational changes during catalysis, and these changes were slow relative to the enzyme turnover (5). However, no information about rhodanese conformation has been obtained at pH values less than 5.

Low pH may be useful in stabilizing rhodanese and providing conditions suitable for informative studies of folding. Rhodanese folding at high pH is inefficient because of competition by aggregation and the extreme sensitivity to oxidation of the rhodanese sulfhydryl groups (6). Refolding is possible at high pH, but reductants must be used, and aggregation must be prevented by inclusion of detergents (6) or proteins called chaperonins (heat shock proteins) (7–9).

The sensitivity of rhodanese to oxidation stems from the fact that it contains 4 reduced cysteine residues. Oxidation, or other reaction at cysteine 247, is associated with loss of activity. This sulfhydryl reactivity is responsible for the instability of rhodanese at high pH. Sulfhydryls are generally less oxidizable at low pH, and these conditions would be expected to stabilize rhodanese. Rhodanese can form folding intermediates that have characteristics of molten globules (10). The molten globule states of proteins are described as compact with a high degree of secondary structure but with loosened tertiary interactions (11). Low pH structural transitions in several proteins give states that are like molten globules (12–14). It has been suggested that rhodanese intermediates are in the molten globule state when they are complexed with chaperonins during assisted refolding (7–9).

In the present work, we have examined the structural behavior of the enzyme rhodanese at acid pH. We find that rhodanese is greatly stabilized at acid pH, and it can adopt conformational state(s) that appear to be molten globule(s).

MATERIALS AND METHODS

All reagents used were analytical grade. 1,1′-Bis(4-anilino)naphthalene-5,5′-disulfonic acid, dipotassium salt (bis-ANS)† was obtained from Molecular Probes (Junction City, OR). Unless otherwise indicated, the buffer used throughout was 10 mM sodium citrate adjusted to the desired pH. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described.

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†The abbreviations used are: bis-ANS, 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid, dipotassium salt; 4PDS, 4,4′-dipyridyl disulfide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
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Rhodanese was prepared and stored as described previously (16). Rhodanese was incubated at 0.2 mg/ml in 10 mM citrate buffer, pH 3. Rhodanese was incubated at 20 µg/ml in 10 mM citrate buffer, pH 3. After the indicated times of incubation, the enzyme was assayed in the standard assay.

Sulphydryl titrations used 4,4′-dipyridyl disulfide (4PDS) as follows (19): 250-µl samples of rhodanese were mixed with 250 µl of 8 mM 4PDS and 500 µl of 4% SDS. After incubation for 15 min the absorbances were read at 324 nm. A molecular extinction coefficient of 19,800 M⁻¹ cm⁻¹ was used for 4-thiopyridone produced by cleavage of the 4PDS and a molecular weight of 35,000 for rhodanese.

Circular dichroism (CD) measurements were recorded with a Jasco model J-500 spectropolarimeter, and the data were treated as reported previously (20).

Fluorescence spectra were recorded with an SLM-Aminco 500C spectrophotofluorometer. Anisotropy measurements were used to get estimates of the average rotational relaxation time, τ, for trypthophan residues as a function of pH relative to the τ at pH 5, ρₜ₅, as a standard. This function, although approximate, is expected to be sensitive to any large changes in tryptophan flexibility. The ratio of ρₜ₅ to that at a given pH, ρₜ₅/pₜ₅, were derived as follows. Anisotropy measurements were made with excitation at 280 nm while monitoring fluorescence emission at the wavelength maximum of each sample. Anisotropies, r, can be interpreted in terms of ρ as given by the Perrin-Weber equation (21).

\[ r/\tau = 1 + 3\tau/\rho \]

rₜ is the limiting anisotropy in the absence of molecular rotation and was taken to be 0.17 for 280-nm excitation from measurements of N-acetyltryptophanamide in glycerol at 5 °C. The value is consistent with those reported previously (22, 23). τ is the lifetime of the excited state, which is proportional to the quantum yield. The latter quantity was estimated in the present study from the areas under the measured fluorescence spectra (24). The validity of this approach was verified by comparisons between fluorescence spectra and directly measured average lifetimes for sulfur-free and sulfur-containing rhodanese (25).

The ratio of the rotational relaxation time at pH 5 to that at any other pH, from the above equation, is given by the following equation.

\[ \rho/\rho_{5.8} = r/\tau_{5.8}/[1/(r_{5.8} - 1)/r_{5.8} - 1/r_{5.8}] \]

ρ is inversely proportional to the rate of rotational motion, so slower motion is associated with smaller ratios.

RESULTS

Rhodanese Is Stable between pH 7 and pH 4 and Is Inactivated at Lower pH Values—Fig. 1 shows that rhodanese was progressively inactivated by preincubation at pH levels lower than 4 in a transition centered at about pH 3.25. The enzyme was totally inactivated at pH 3 and below. These data indicate that the recovery of enzyme activity was rapid on a time scale of the assay, down to pH 4. There was then reduced recovery of enzyme activity as the pH was reduced further, and the enzyme appeared inactive if the preincubation was at pH < 3 (however, see below). Even after 2 days at room temperature (23 °C), samples preincubated at pH 5 were still fully active. If this incubation was performed at pH values > 7, the enzyme would have been inactivated within 2.5 h (26).

Time Course of Inactivation at pH 3—Fig. 2 shows the time course of inactivation for a sample of rhodanese incubated at pH 3. The t₁/₂ for this first order process is 22 min. These data show that the response was not simply due to the titration of a side chain which would normally be expected to be more rapid. Rather, it is consistent with a slower, rate-limiting process, such as a conformational change.

Rhodanese Sulphydryl Titors After Incubation at pH 3—Even after extensive incubation at pH 3, the 4PDS assay described under “Materials and Methods” gave measured A₂₈₄ = 0.45 for a rhodanese concentration of 0.2 mg/ml. This corresponds to 3.75 SH groups/rhodanese molecule, which is consistent with the 4 sulphydryl groups expected from the known sequence (27, 28). Thus, even as the activity decreased, the enzyme continued to show 4 SH groups. Therefore, all sulphydryl groups appeared to be reduced in the inactive enzyme so that oxidation to a disulfide is not an explanation for the inactivation.

Low pH Produces a State of Rhodanese That Is Inactive but Can Recover to a Small Degree in the Enzyme Assay at pH 8.6—Fig. 3 shows a progress curve representing the product...

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Fig. 3. Time course of product formation in a rhodanese assay at pH 8.6 after preincubating the enzyme at pH 3. Rhodanese was preincubated for 2 h at 50 μg/ml in 10 mM citrate buffer, pH 3. The enzyme was diluted to a final concentration of 0.5 μg/ml into 15 ml of standard assay solution. At the indicated times, 1-ml aliquots were removed and tested for the content of the reaction product, thiocyanate. The ordinate gives assay absorbances that are proportional to the thiocyanate formed.

Fig. 4. Time-dependent reactivation of rhodanese after preincubation at acid pH. Rhodanese was preincubated at the indicated pH levels as in Fig. 1 for 2 h. After preincubation, each sample was diluted to a final concentration of 0.5 μg/ml into a reactivation mixture consisting of 0.5 mg/ml lauryl maltoside, 200 mM β-mercaptoethanol, 50 mM sodium thiosulfate, and 200 mM sodium phosphate, pH 7.4. At a given time after dilution into refolding buffer, the enzyme was assayed for activity in the standard assay at 0.5 μg/ml. The data correspond to times of incubation after low pH treatment as follows: 0 h (filled circles), 0.5 h (filled triangles), 1 h (filled squares), 2 h (open circles), and 4 h (open squares).

Fig. 5. Intrinsic fluorescence of rhodanese at acid pH. A, intrinsic fluorescence spectra of rhodanese at 100 μg/ml in 10 mM citrate buffer at the indicated pH values were taken using an excitation wavelength of 280 nm. The spectra were measured at the following pH values. For the group with maxima near 330 nm the spectra from the lowest are: pH 7.6, 5.0, 4.0, and 3.5 (highest spectrum). For the spectra with maxima at wavelengths longer than 340 nm the spectra were from spectra that were collected for samples like those in A. The left ordinate refers to the fluorescence intensities (filled squares, native rhodanese). The right ordinate refers to the wavelength of the maximum fluorescence (upright triangles, native rhodanese). Inverted triangles correspond to wavelength maxima for rhodanese samples that were in 8 M urea at each pH. Diamonds correspond to wavelength maxima for samples of N-acetyl tryptophanamide at 12 μM in the same buffers used for the native rhodanese samples.
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Data from both studies show that a transition began in the region of pH 3.5 and extended to pH 3. This transition was accompanied by a long wavelength shift and a decrease in the maximum fluorescence intensity. As the pH was lowered from pH 7 to pH 3.5, the main effect was a relatively modest increase in the maximum intensity. Thus, the initial region of activity loss, seen in Fig. 1 between pH 4 and pH 3.5 was not accompanied by any major shift in the fluorescence wavelength maximum, although there might have been a slight increase in the fluorescence intensity. The activity loss in this region was at most 20% to approximately pH 3.5. As indicated in Fig. 5B, between pH 3.5 and 3 there was a significant decrease in the maximum intensity and a significant red shift bringing the maximum of this spectrum to 345 nm. It is interesting that the nonlinearity in progress curves was not really apparent until the pH was close to 3. During reversible unfolding of rhodanese at high pH, the fluorescence maximum at 345 nm is associated with folding intermediates that have characteristics of what have been called molten globule states (15). Fig. 5B shows that the protein never displayed a maximum that reached or exceeded 350 nm, which would be expected for a protein whose tryptophans were fully accessible to the solvent, as by unfolding. Thus, the activity of the enzyme was zero for a state that had a fluorescence wavelength maximum of 345 nm. Comparisons can be made using controls for fully exposed tryptophan residues from spectra of N-acetyl tryptophanamide (diamonds, the lowest curve) and for rhodanese unfolded in 8 M urea (inverted triangles, second lowest curve). These data are consistent with a considerably longer wavelength shift to 352–355 nm for fully exposed tryptophans, which is more than 5 nm longer than is observed for the state that was reached by rhodanese at pH 3.

To the extent that the classification scheme of Burstein (29) can be relied on in the present instance, the wavelength maximum of rhodanese fluorescence at pH 3 would correspond to the average tryptophan residue being partially exposed to the solvent. This conclusion is similar to that reached in investigations of folding intermediates that were observed in detergent-assisted refolding of rhodanese (10, 15).

Hydrophobic Surfaces Are Exposed at low pH—Fig. 6A shows fluorescence spectra of the hydrophobic probe, bis-ANS, in the presence of rhodanese as a function of pH. These data indicate that, as the pH was lowered, the hydrophobic binding sites for bis-ANS increased. Previous studies provided evidence that increased exposure of hydrophobic surfaces is characteristic of the molten globule state (11). As the pH was lowered in the present case, there was a considerable increase in bis ANS fluorescence intensity and a red shift that is characteristic of increased exposure of hydrophobic surfaces (30).

Fig. 6B collects together fluorescence parameters for bis-ANS binding to rhodanese. The wavelength maximum (squares, left ordinate) and the fluorescence intensity at 490 nm (triangles, right ordinate) are shown as functions of pH. The major change occurred between pH 3.5 and 3, although there was a small change that began at pH 4. These data show that the wavelength maximum and the fluorescent intensity changed together. Model studies of solvent effects show that these two shifts occur in parallel when the environment of the probe changes. If the pH change was simply changing the proximity or access of the probe to quenching group(s), one would expect a greater change in intensity than in wavelength. Therefore, the results observed here with rhodanese are consistent with the conclusion that organized hydrophobic surfaces are being exposed as the protein passes through the transition which is centered at about pH 3.25.

Simple exposure of individual hydrophobic residues is not expected to provide sufficiently extensive regions for interaction with bis-ANS molecules.

Pepsin Digestibility Follows the Low pH Transition and Reveals Intermediates in the Process—Fig. 7 shows SDS-PAGE results that demonstrate increased proteolytic susceptibility of rhodanese as the pH is lowered. Pepsin is specific for aromatic, hydrophobic, and acidic residues. Fig. 7A (upper panel) shows that rhodanese was resistant down to at least pH 3.5 (lane 3), and it became increasingly digestible as the pH was decreased below pH 3. Digestion under these conditions produced of molecular weight products with only a hint of intermediate species. The low molecular weight products are not limit products, but can be digested further (Fig. 7B; lane 1 shows no detectable protein bands). For Fig. 7A, digestion was for a constant time interval of 10 min, and the amount of pepsin was chosen so that the same number of pepsin units were added at each pH (see "Materials and Methods").

Fig. 7B shows that species were produced with intermediate susceptibility to pepsin. Pepsin concentrations increase from right to left with zero pepsin in lane 7. The lane with 5% pepsin (w/w) (lane 1) shows that rhodanese could be completely digested. The parent band (33 kDa) disappeared to give large fragments which could be further digested to give fragments, which either did not remain on the gel or were not visualized. At intermediate pepsin concentrations (e.g. lanes 4 or 5), discrete intermediates appeared, first giving two bands just below the parent, and then, at higher pepsin concentration, producing a series of species below those, that increased.
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Fig. 7. SDS-gel electrophoresis showing the pH dependence of the pepsin digestibility of rhodanese. A, pH dependence of pepsin digestibility of rhodanese. Digestion was at 25 °C for 10 min using 200 μl of rhodanese at 0.1 mg/ml. The same number of pepsin units was added to each sample as described under "Materials and Methods." Digestion was stopped by addition of 0.1 M Tris, pH 8.9, to each sample. 7.5 pg of rhodanese were electrophoresed in each lane. 12.5 ng of rhodanese were digested with various concentrations of pepsin for the fixed time of 1 h. The percent pepsin (w/w) that was used was as follows, from the right lane: pH 5.0 (lane 1, 128.5 ng of pepsin), 4.0 (lane 2, 73.0 ng), 3.5 (lane 3, 54.0 ng), 3.0 (lane 4, 50.0 ng), 2.5 (lane 5, 46.0 ng), 2.0 (lane 6, 29.1 ng). B, pepsin concentration dependence of the digestion of rhodanese at pH 3.0. Rhodanese (0.2 mg/ml) was digested with various concentrations of pepsin for the fixed time of 1 h. The percent pepsin (w/w) that was used was as follows, from the right lane: pH 5.0 (lane 1, 128.5 ng of pepsin), 4.0 (lane 2, 73.0 ng), 3.5 (lane 3, 54.0 ng), 3.0 (lane 4, 50.0 ng), 2.5 (lane 5, 46.0 ng), 2.0 (lane 6, 29.1 ng). The positions of molecular weight standards that were used are marked on the left by arrows. Their identity and their molecular mass (kDa) are (from the top): phosphorylase B, 97.4; bovine serum albumin, 66.2; ovalbumin, 42.7; carbonic anhydrase, 29.1; soybean trypsin inhibitor, 21.5; and lysozyme, 14.4. Other details are as in panel A or "Materials and Methods."

as the pepsin concentration was increased further. Susceptible bonds become present at low pH, in a clear transition around pH 3.25. These data indicate a structural transition that gives rise to an open structure with protease sensitive regions separating resistant regions, thus giving rise to the observed large fragments.

There Is No Significant Loss of Secondary Structure in the Low pH Structural Transition—Fig. 8 shows the pH dependence of the molar ellipticity measured at 220 nm (filled circles). For comparison, results are presented for samples of rhodanese that were denatured in 8 M urea (filled squares) to show the response for extensively unfolded protein. There was a slight increase in the magnitude of the ellipticity at 220 nm as the pH was reduced to pH 3. Certainly, there was no loss of regular secondary structure. Below pH 3 there was a slight increase in ellipticity, but in no case did the ellipticity approach that expected for an extensively unfolded protein.

There Is No Large Change in Average Tryptophan Flexibility during the Acid-induced Structural Transition in Rhodanese—Fig. 9 shows the approximate, average tryptophan rotational relaxation as assessed from the anisotropy of the intrinsic fluorescence as a function of pH as described under "Materials and Methods." Smaller numbers in Fig. 9 correspond to lower relative tryptophan mobilities. Data from several experiments are superimposed in this figure. The lower curve (filled circles) indicates that the average tryptophan mobility did not change until the pH was brought below 4, and then the mobility increased slightly as the pH was lowered further. For comparison, the upper curve presents data for rhodanese in 8 M urea (filled squares), which show that the tryptophan mobility was high and constant to pH 3 for these fully denatured samples. Thus, the state of rhodanese that is adopted at pH 3 does not appear to have extremely high tryptophan flexibility.

DISCUSSION

The results in this paper show that rhodanese is stable at acid pH values down to at least pH 4. This is quite different from its behavior at pH levels greater than pH 7, where inactivated species are not reactivatable (26). Between pH 4 and at least pH 2, a conformational process produces an inactive state of rhodanese that can be reactivated. The
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inactive state at pH 3 can be partially reactivated during the assay (pH 8.6), and it can be reactivated fully by incubation in buffers containing additives that have previously been shown to be required for detergent-assisted refolding (15).

The inactive state of rhodanese produced at pH 3 has characteristics that have been described for the so-called molten globule state formed during protein unfolding/folding. This state is characterized by loosened tertiary interactions, low enzyme activity, high accessibility of water to what were formerly internal residues in the protein, exposure of hydrophobic surfaces, and substantial retention of secondary structure. These characteristics of rhodanese at pH < 3 are similar to some of those properties observed for intermediate(s) in the detergent-assisted refolding (25). This pH behavior is similar to observations with several proteins including β-lactamase, and it is characteristic of the class of proteins (Class II in the scheme in Ref 14) that enter the molten globule state at low pH but do not denature further.

It is interesting to consider the nature of potential ionizable group(s) associated with the processes occurring around pH 3. In the simplest case, the data suggest that the ionizable group(s) would have a pKₐ in the region of pH 3-3.5. The N-terminal sequence of rhodanese has been suggested to be involved in its folding, stabilization, and organelle targeting (1, 7, 27). Models for rhodanese unfolding invoke initial changes in the N-terminal residues 1-25 (1). This N-terminal sequence may also be important in binding to the chaperonin proteins that facilitate the folding of rhodanese (32-34). Rhodanese has a single glutamic acid in the N-terminal sequence (Glu³), and this residue is appropriately positioned with respect to basic residues so that an amphiphilic α helix involving residues 11-22, which is seen in the α-rx-ray structure, can be stabilized by ionic interactions between Glu³ and positively charged residues that are appropriately positioned in the N-terminal sequence. Thus, protonation of Glu³ could influence the stability of this helix as has been suggested for these types of helices (35).

The x-ray structure of rhodanese shows additional features relevant to the present effects. Extensive hydrophobic interactions provide most of the energy for association of the two domains. The mutual orientation of these domains appears to be fixed, in part, by salt bridges, Glu⁷₁-Arg⁴⁸₈ and Arg⁴¹⁰-Asp²₉⁷, within the contact area (36). Thus, the conformational effects of pH may be influenced by titration of groups that are responsible for interdomain orientation.

The proteolytic patterns observed are consistent with a loosening of connections between elements of structure that remain resistant to proteolysis, rather than a general global loosening or unfolding. The structure of rhodanese lends itself to partial unfolding, since it is composed of independent domains and an N-terminal sequence that is fixed to the protein surface by noncovalent interactions. Results of pepsin digestion suggest that major fragments are produced that correspond to cleavage at one end of the protein followed by formation of fragments corresponding in size to single domains.

The limited number of pepsin cleavage points correlates well with the lack of extensive flexibility indicated by the tryptophan anisotropy, as well as with the incompletely open tertiary structure as assessed by intrinsic fluorescence. The deepening of the ellipticity at pH 3 may indicate that there is some structural reorganization that leads to a slight increase in regular secondary structure as the protein undergoes an overall change in tertiary structure at pH 3.

The conformational states proposed for rhodanese at acid pH have some characteristics similar to those states proposed in the chaperonin or detergent assisted refolding of this enzyme (1, 2, 15). That is, rhodanese folds to states that are more open than the native protein but which are still compact. Although this structure is open, some rigidity of the backbone might be expected at low pH from the stiffening due to electrostatic repulsions within a globular protein having an overall positive surface charge with no negatively charged side chains.

REFERENCES


