Isolation and Characterization of Rhodanese Intermediates during Thermal Inactivation and Their Implications for the Mechanism of Protein Aggregation†

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ABSTRACT: The initial steps of heat-induced inactivation and aggregation of the enzyme rhodanese have been studied and found to involve the early formation of modified but catalytically active conformations. These intermediates readily form active dimers or small oligomers, as evident from there being only a small increase in light scattering and an increase in fluorescence energy homotransfer from rhodanese labeled with fluorescein. These species are probably not the domain-unfolded form, as they show activity and increased protection of hydrophobic surfaces. Cross-linking with glutaraldehyde and fractionation by gel filtration show the predominant formation of dimer during heat incubation. Comparison between the rates of aggregate formation at 50 °C after preincubation at 25 or 40 °C gives evidence of product−precursor relationships, and it shows that these dimeric or small oligomeric species are the basis of the irreversible aggregation. The thermally induced species is recognized by and binds to the chaperonin GroEL. The unfoldase activity of GroEL subsequently unfolds rhodanese to produce an inactive conformation and forms a stable, reactivable complex. The release of 80% active rhodanese upon addition of GroES and ATP indicates that the thermal incubation induces an alteration in conformation, rather than any covalent modification, which would lead to formation of irreversibly inactive species. Once oligomeric species are formed from the intermediates, GroEL cannot recognize them. Based on these observations, a model is proposed for rhodanese aggregation that can explain the paradoxical effect in which rhodanese aggregation is reduced at higher protein concentration.

The widely accepted mechanism for thermal inactivation of enzymes involves the reversible partial unfolding of the molecule, which is followed by chemical or conformational modification. During the later stages, the enzyme may form aggregates, incorrectly fold, or be chemically altered (*1*). In general, the association of exposed hydrophobic surfaces during unfolding gives rise to aggregated species. Similar association processes can be observed during refolding of denaturant-induced unfolded proteins (*2*−*4*). It has been suggested that, in vivo, chaperonins can interact with partially folded, newly synthesized polypeptide, thereby preventing self-association due to exposed hydrophobic surfaces (*5*), and in this way protect thermally perturbed proteins.

The enzyme rhodanese has become a valuable model for studying protein folding (*6*−*11*), as well as the functional role of protein dynamics and domain interactions (*12, 13*). It is well-known that unfolded rhodanese reactivates poorly due to formation of "sticky" intermediates that can form aggregates and disulfide-linked misfolded species (*6*). Refolding conditions that minimize the formation of aggregates and disulfide-linked species increase the recovery of active enzyme. These conditions include the following: refolding at lower temperature (*6, 14*), or in the presence of mild detergents (*15*); binding to liposomes or to GroEL (*7, 8*); or in the presence of a very large excess of reducing agents (*6, 13*). The highest recovery is observed using GroEL/GroES/ATP, although the yields are less than 100% (*14*). It has been reported that rhodanese is very sensitive to thermal inactivation, and it irreversibly forms precipitate even at low protein concentration (*16*). Reversibility after thermal inactivation is observed when rhodanese is covalently coupled to an insoluble support, thus preventing aggregation of the protein (*16*). The thermal protection effect by GroEL on the inactivation of rhodanese has been reported. During thermal inactivation, the enzyme forms irreversible aggregates, but the aggregation is prevented if GroEL is present in the solution (*17*). A similar effect is found with mitochondrial aspartate aminotransferase (*18*). At higher protein concentrations, rhodanese appears to protect itself from heat inactivation (*19*). It has been proposed that rhodanese may form small oligomeric species that protect the enzyme from heat inactivation.

In this work, we have studied the heat-modified, active conformation of rhodanese. We have shown that during the early stage of heat inactivation, rhodanese forms an intermediate that readily gives rise to active associated species. GroEL can capture this early intermediate, but not the associated species. Addition of GroES and ATP successfully releases the active protein. These associated species do not scatter light to an appreciable extent, which implies that these

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are either dimers or small oligomers. Active dimers can be isolated using gel filtration and can be demonstrated by cross-linking. We have shown that formation of heat-modified dimers or small oligomers is an essential prerequisite for the thermal aggregation. This is the first time that it has been demonstrated that rhodanese forms dimers and small oligomeric species during heat inactivation, and they are the basis for irreversible aggregation.

EXPERIMENTAL PROCEDURES

Materials

Reagents. 6-Iodoacetamidofluorescein (IAF)[1] and 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) were purchased from Molecular Probes Inc. (Eugene, OR). Bicinchonic acid (BCA) was purchased from Pierce (Rockford, IL). Sephacryl S-100-SR, bovine serum albumin (BSA), and 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 (*20*). Rhodanese was desalted on a G-50 column before use. Rhodanese concentrations were determined using a value of $A_{0.1\%,280nm} = 1.75$ (*21*) and by the BCA method (*22*).

Rhodanese Assay. Rhodanese activity was measured using a colorimetric method based on the absorbance at 460 nm of the complex formed between the reaction product, thiocyanate, and ferric ion (*21*).

GroEL and GroES Purification. GroES and GroEL were purified as described previously (*23, 24*). Protein concentrations were determined by the BCA method (*22*).

Rhodanese Modification (RHIAF). Rhodanese was modified by reaction of the active site cysteine residue (Cys 214) with IAF. The modification was performed as previously described (*14, 25*).

Heat Inactivation of Rhodanese. For heat inactivation at different temperatures, rhodanese was added either to 50 mM Tris-HCl, pH 7.8, containing 50 mM thiosulfate, 10 mM KCl, 10 mM $MgCl_2$, and 0.2 M β mercaptoethanol (folding buffer) or to 50 mM sodium phosphate, pH 7.6, or 50 mM Tris-HCl, pH 7.8, preincubated at the desired temperature. For activity measurements, aliquots were removed after specified times of incubation and assayed at 25 °C. For light scattering measurements at different temperatures (see below), the cuvette chamber was preequilibrated at the desired temperature. For kinetics of light scattering, rhodanese was added to temperature-equilibrated buffer. For binding to GroEL, 36 μg/mL rhodanese was incubated in the folding buffer preincubated at a specific temperature. Aliquots were removed at different times and added to 2.25 mg/mL GroEL preincubated at 25 °C and incubated at that temperature for an additional 60 min. For reactivation from the rhodanese−GroEL complex, first, 0.375 mg/mL GroES and then 20 mM ATP were added, and the incubation was continued for at least 1.5 h at 25 °C. To assay the successful reactivation, 100 μL of the incubation mix was added to 1 mL of rhodanese assay mix and incubated for 5 min. The assay reaction was stopped by the addition of formaldehyde. The percent reactivation was calculated based on the activity of native enzyme that had been subjected to the same incubation conditions at 25 °C.

Light Scattering. Light scattering measurements were performed using a Fluorolog-3 (Jobin Yvon-Spex) spectrofluorometer, equipped with a temperature-controlled cuvette chamber. Both excitation and emission wavelengths were set at 340 nm with 0.5 nm slit widths. All scattering values were corrected using appropriate buffer blanks. The reported scattering values are given in photon counts per second (CPS).

Fluorescence and Anisotropy Measurements. Anisotropy and fluorescence were measured using a L-formatted Fluorolog-3 (Jobin Yvon-Spex) spectrofluorometer, equipped with a temperature-controlled cuvette chamber. Fluorescence anisotropy (r) is defined by $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} are the fluorescence intensities of the vertically (VV) and horizontally (VH) polarized emission, when the sample is excited with vertically polarized light. G is a correction factor for instrument response to polarized light at a particular emission wavelength. This factor is defined as I_{HV}/I_{HH}, where I_{HV} and I_{HH} correspond to fluorescence intensities of vertically (HV) and horizontally polarized emission with horizontally (HH) polarized excitation. For anisotropy measurements of IAF bound to rhodanese (RHIAF), excitation was set at 492 nm, and emission was at 515 nm. The bandwidths for both were 1 nm. For bis-ANS binding studies, the excitation was maintained at 385, and the emission was followed at 490 nm with 1 nm bandpasses. For studies with the noncovalent probe bis-ANS, the ratio of rhodanese to bis-ANS was always maintained at 3:1 molar ratio.

Cross-Linking. Glutaraldehyde was used as a cross-linking reagent. The method used here was a modified protocol of Azem et al. (*26*); 0.1 mg/mL rhodanese in 50 mM sodium phosphate, pH 7.6, was incubated at 40 °C. Glutaraldehyde was added to it, after incubation for various times, to a final concentration of 2.5% and incubated at 40 °C for 4 min. The reaction was stopped by the addition of 50 mM Tris-HCl, pH 7.6, and 3% β-ME (final concentrations). The cross-linked samples were analyzed on SDS−PAGE (*27*). A 10.5% gel was run by adding sample buffer for final concentrations of 400 mM glycine, 50 mM Tris-HCl, 3% (w/v) SDS, 3% (v/v) β-ME, and 10% (v/v) glycerol. The gel was stained with silver nitrate (*28*). The band intensities corresponding to the 66 kDa band in each lane of a gel as in Figure 6 were scanned using the program Scion Image for Windows (IBM PC). The intensities were plotted against the time to measure the rate constant using the program Origin (Microcal software, Inc., Northampton, MA).

Gel Filtration. Gel filtration was performed to separate monomeric rhodanese and oligomer, formed during heat incubation. One hundred fifty microliters of 0.1 mg/mL rhodanese in 50 mM sodium phosphate, pH 7.6, was incubated at 40 °C for 60 min and loaded on a 15 mL (19 × 1 cm) Sephacryl S-100-SR column (fractionation range 1000−100 000), equilibrated with 50 mM sodium phosphate,

[1] Abbreviations: IAF, 6-iodoacetamidofluorescein; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; BCA, bicinchonic acid; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; BSA, bovine serum albumin; β-ME, 2-mercaptoethanol; RHIAF, IAF-modified rhodanese.

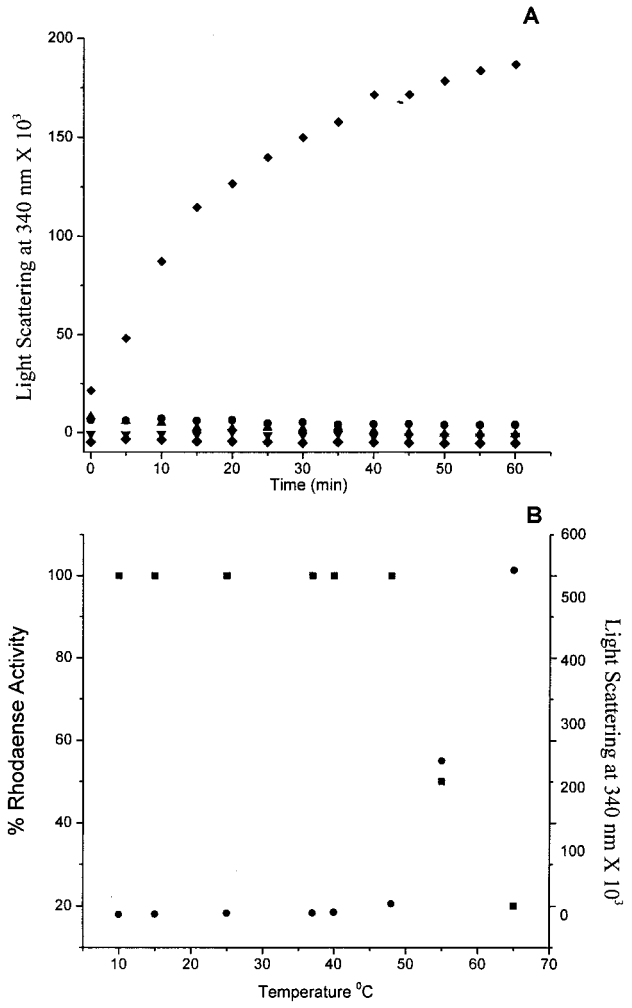

FIGURE 1: Aggregation of rhodanese at different temperatures. (A) The light scattering values of 36 μg/mL rhodanese in folding buffer (see Experimental Procedures) were measured at 5 (■), 15 (●), 25 (▲), 40 (▼), and 48 °C (◆). (B) 0.1 mg/mL rhodanese in 50 mM sodium phosphate, pH 7.6, was incubated at different temperatures for 90 min. The cuvette chamber was incubated at the corresponding temperature, and the light scattering values (●) were measured. The activity (■) was measured at 25 °C after 90 min incubation. The activity of 0.1 mg/mL rhodanese in 50 mM sodium phosphate, pH 7.6, incubated at 25 °C for 90 min, was taken as 100% active.

pH 7.6 at 37 °C. Then 100 μL fractions were collected and assayed for rhodanese concentration and activity. Two column volumes of buffer were used for the elution. The same experiment was done at 25 °C using 0.1 mg/mL rhodanese and 0.1 mg/mL BSA.

RESULTS

Effect of Thermal Incubation on the Activity and the Aggregated State of Rhodanese. Previously, it was shown (*19*) that rhodanese is protected from thermal inactivation at higher protein concentrations. It was hypothesized that rhodanese may form dimers and/or small oligomeric species that are resistant to thermal perturbation. Figure 1A shows the effect of temperature on the aggregation of 36 μg/mL rhodanese in folding buffer at different temperatures. There is no significant increase in light scattering up to 40 °C, but there is a large increase at 48 °C (◆). The protein remains fully active up to 40 °C even after 90 min incubation (data not shown). A similar trend has been found at higher protein concentration, e.g., when 0.5 mg/mL rhodanese was used. At this protein concentration, rhodanese remains fully active even at 48 °C with only a small increase in light scattering after 60 min incubation (data not shown). High concentrations of β-ME and thiosulfate in the folding buffer may help to reduce heat aggregation resulting from disulfide bridge formation. A similar effect has been observed in heat aggregation of β-globulin at high pH (*29*). Figure 1B shows the temperature dependence for the light scattering and activity of rhodanese incubated at the lower concentration of 0.1 mg/mL incubated for 90 min in 50 mM sodium phosphate, pH 7.6, which gives a similar profile as that observed in folding buffer. Though at 40 °C there is a small increase in the light scattering value (53 665 cps) compared to that at 25 °C (38 665 cps), the value is considerably smaller than that observed at 55 °C (2 425 405 cps) after 90 min incubation. The enzyme remains fully active up to 48 °C and loses activity beyond that. A similar profile has been found when 50 mM Tris-HCl, pH 7.6, is used (data not shown). The small increase in light scattering at ~40 °C may indicate that small oligomers are formed which are either active or form active monomer in the assay solution.

Fluorescence Homotransfer Suggests the Formation of Rhodanese Dimer and/or Higher Order Oligomers at Higher Temperature. Fluorescence homotransfer is a powerful method with which to assay the formation of protein oligomers (*30*, *31*). When fluorescently labeled monomers associate, their fluorophores become close, and energy transfer among them can occur (homoenergy transfer). This process is marked by a decrease in the fluorescence anisotropy of the labels. Fluorescein is quite sensitive to this effect. During fluorescence homotransfer, a decrease in the anisotropy of fluorophore is observed with increase in its concentration (*32*). Figure 2A shows the concentration-dependent loss of anisotropy of IAF-labeled rhodanese (RHIAF) at 40 °C. Each curve represents a different concentration of RHIAF with enough unlabeled added rhodanese to keep the total protein concentration at 36 μg/mL. At lower RHIAF concentration, e.g., at 1.8 μg/mL (highest curve, Figure 2A), there is a very small drop in anisotropy after 90 min of incubation at 40 °C ($r_{520} = 0.17$ from 0.18). There is progressive loss of anisotropy as the RHIAF concentration is increased (Figure 2A). The chance of forming an oligomer containing multiple RHIAF molecules increases as the fraction of the labeled monomer increases and the anisotropy decreases (lower curves in Figure 2A). Thus, with 36 μg/mL RHIAF, r_{520} decrease to 0.09 from 0.15, after 90 min incubation at 40 °C. This concentration-dependent loss of anisotropy due to fluorescence homotransfer is consistent with the formation of dimers or small oligomers at 40 °C. Similar concentration-dependent homoenergy transfer has observed with other oligomeric proteins (*30*, *31*).

If 0.5 mg/mL unlabeled rhodanese is added to samples prior to incubation at 40 °C, only a small increase in anisotropy is actually observed ($r_{520} = 0.16$ from 0.15); 36 μg/mL RHIAF alone and in the presence of 0.5 mg/mL rhodanese does not show any significant increase in light scattering as the temperature increases from 10 to 40 °C (Table 1). This indicates that either dimers or small oligomers are formed that do not scatter light significantly. The same experiment was done in the presence of 1 mg/mL BSA

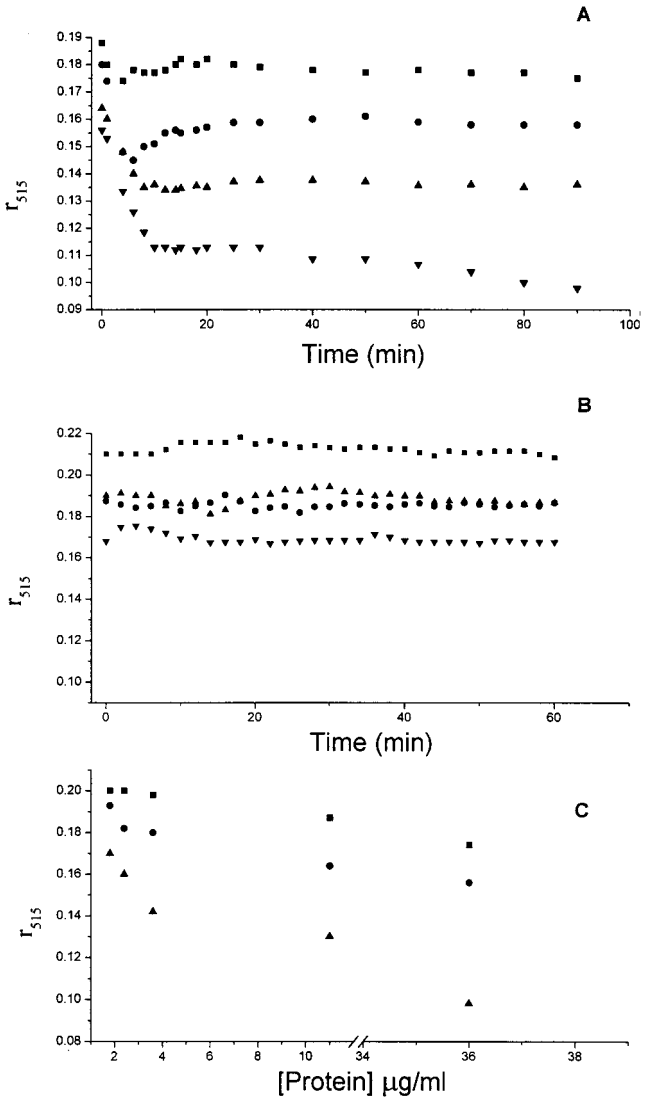

FIGURE 2: Measurement of fluorescence anisotropy of RHIAF at different temperatures and at different concentrations. (A) Anisotropy of RHIAF in folding buffer was measured at 40 °C. The protein concentrations were (■) 1.8 μg/mL RHIAF + 34.2 μg/mL rhodanese, (●) 3.6 μg/mL RHIAF + 32.4 μg/mL rhodanese, (▲) 11 μg/mL RHIAF + 25 μg/mL rhodanese, and (▼) 36 μg/mL RHIAF. (B) Anisotropy of RHIAF in folding buffer was measured at 5 °C. The protein concentrations were (■) 1.8 μg/mL RHIAF + 34.2 μg/mL rhodanese, (●) 3.6 μg/mL RHIAF + 32.4 μg/mL rhodanese, (▲) 11 μg/mL RHIAF + 25 μg/mL rhodanese, and (▼) 36 μg/mL RHIAF. (C) RHIAF at different concentrations in folding buffer was incubated at (■) 5, (●) 25, and (▲) 40 °C for 90 min.

Table 1: Light Scattering Value of RHIAF[a]

protein concentration	10 °C	15 °C	25 °C	40 °C
36 μg/mL RHIAF	43756	44961	42245	40718
36 μg/mL RHIAF + 0.5 mg/mL rhodanese			57456	80780

[a] 36 μg/mL RHIAF in folding buffer alone and in the presence of 0.5 mg/mL rhodanese was incubated at different temperatures for 90 min, and the light scattering values were measured at that temperature. Both the excitation and emission wavelengths were set at 340 nm with 0.5 nm band-passes.

instead of unlabeled rhodanese, and it did not show any decrease in anisotropy during incubation at 40 °C (data not shown). However, once those species are formed from 36 μg/mL RHIAF, the subsequent addition of 0.5 mg/mL unlabeled rhodanese and incubation at 40 °C for an additional 120 min recover only a small amount of anisotropy (r_{520} = 0.1 from 0.09). Further, 10- or 20-fold dilution of 36 μg/mL RHIAF after incubation at 40 °C for 90 min does not increase the anisotropy value (data not shown). Thus, oligomers formed at 40 °C have slow dissociation rates and, in this sense, appear to be stable. In Figure 2A, the anisotropy values at "0" time indicate values at 25 °C. Even at 25 °C, there is some decrease in anisotropy value as the labeled protein concentration increases. To check whether dimer or higher order oligomers can be formed at lower temperature, the same experiment as described in Figure 2A has been done both at 5 °C and at 25 °C. Figure 2B show the effect of concentration on the anisotropy at 5 °C. There is a very small change in anisotropy as the labeled protein concentration increases (compare with change in anisotropy values at 40 °C in Figure 2A). The absence of energy homotransfer at 5 °C indicates the absence of formation of oligomeric species. Figure 2C shows the effect of protein concentration on the anisotropy at different temperatures after 90 min incubation. The anisotropy at 5 °C (■) is virtually constant. Relatively greater changes in anisotropy are observed at 25 °C (●), as concentration is increased. This suggests that rhodanese may form intermediate(s) at higher temperature that is (are) prone to form dimers or small oligomers. These experiments indicate that small oligomer formation is a temperature-dependent phenomenon.

GroEL Binds the Thermally Perturbed Species, but Not the Dimer or Small Oligomeric Species. Figure 3 shows the effect of GroEL on the anisotropy of RHIAF. When 36 μg/mL RHIAF in folding buffer is incubated at 40 °C, there is drop in anisotropy as incubation proceeds as shown in Figure 2. If GroEL (1.5:1 molar ratio of $GroEL_{14}$ to rhodanese) is added after allowing incubation to proceed for various times (▼ in Figure 3A), the anisotropy values are similar to those in the absence of GroEL (▲). But if GroEL is added to the labeled protein prior to the incubation, the anisotropy values remain high (r_{520} = 0.17) and close to those observed at 5 °C (r_{520} = 0.174) (● in Figure 3A). So there is no fluorescence homotransfer observed in the presence of GroEL, provided it is present prior to the incubation. Figure 3B shows a similar effect of GroEL with 2.4 and 3.6 μg/mL. At both protein concentrations, there is a time-dependent drop in anisotropy as observed in Figure 2A (2.4 μg/mL = ■; 3.6 μg/mL = ▲). In the presence of GroEL, there is only a very small change in anisotropy if the GroEL is added before the incubation (2.4 = ▼; 3.6 = ●), and the values are close to those observed at 5 °C (Figure 2C). This clearly suggests that GroEL can capture the thermally perturbed monomeric species, and therefore prevent the formation of dimers or small oligomers that lead to a decrease in anisotropy. In addition, the rhodanese oligomers must be very stable, since, after their formation, GroEL addition does not lead to increase in anisotropy.

Addition of GroES and ATP Can Release Active Rhodanese from Complexes of GroEL with Captured Thermally Perturbed Species. Figure 4 shows the activity of 36 μg/mL rhodanese in folding buffer at 40 °C. Rhodanese alone (■) is fully active throughout the incubation. When GroEL is present, rhodanese loses activity as the incubation at 40 °C proceeds (●), consistent with the formation of a complex. This is a stable complex as there is no difference in activities

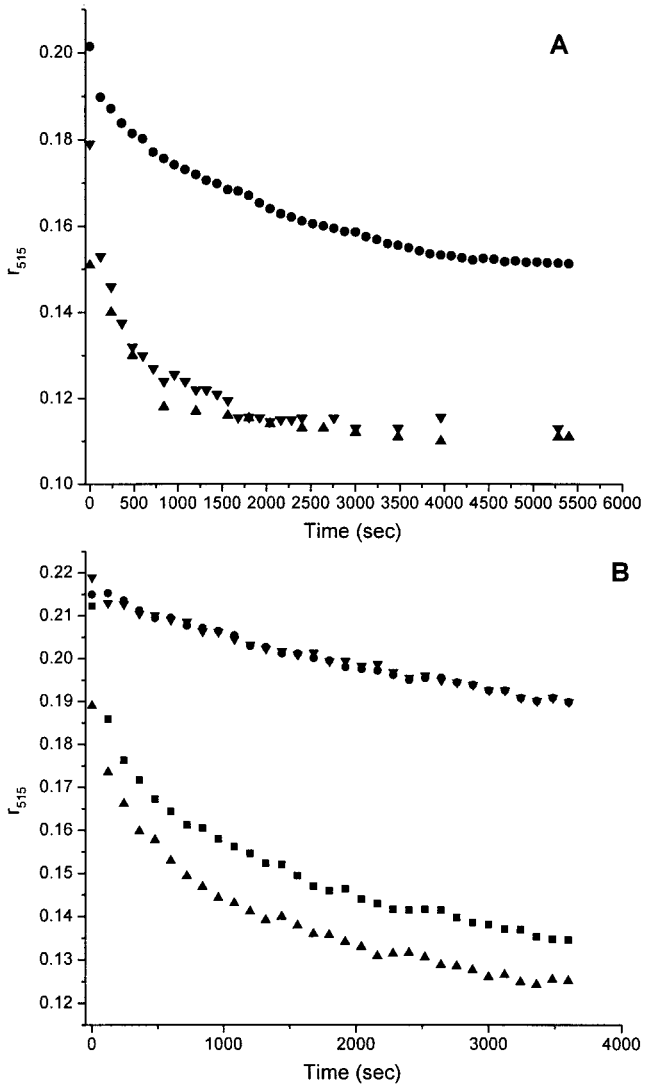

FIGURE 3: Anisotropy measurement of RHIAF in the presence of GroEL. (A) (●) 36 μg/mL rhodanese in folding buffer containing 1.5 mg/mL GroEL was incubated at 40 °C, and the anisotropy was measured at the different times. (▼) 36 μg/mL rhodanese in folding buffer was incubated at 40 °C for the indicated times. Then, 2.25 mg/mL GroEL was added and incubated at 25 °C for 60 min, and the anisotropy was measured at 25 °C. (▲) Same experiment as described in (▼) was done without addition of GroEL. (B) The anisotropy values of 2.4 μg/mL (■) and 3.6 μg/mL (▲) RHIAF in folding buffer were measured at 40 °C. The same experiments were done at 40 °C with 2.4 μg/mL (●) and 3.6 μg/mL (▼) RHIAF in folding buffer containing 0.1 mg/mL GroEL and 0.15 mg/mL GroEL, respectively.

whether the sample is assayed immediately (data not shown), after 120 min of incubation at 25 °C (●), or after 18 h (data not shown). When GroES alone is added to the complex, no activity is recovered (data not shown). But when both GroES and ATP are added to the complex, 80% of the activity is recovered (▼). The 18 h incubated complex also releases active rhodanese to a similar extent as that obtained after 120 min (data not shown). If GroEL is added after the incubation at 40 °C, there is no decrease in rhodanese activity (▲). These data clearly show that GroEL is not able to capture the associated species once they are formed, and those species are active in the assay.

Rhodanese Dimers or Small Oligomers Formed at 40 °C Are Not Associated with the Exposure of Hydrophobic Sites. Figure 5A shows the bis-ANS binding to rhodanese (1:3

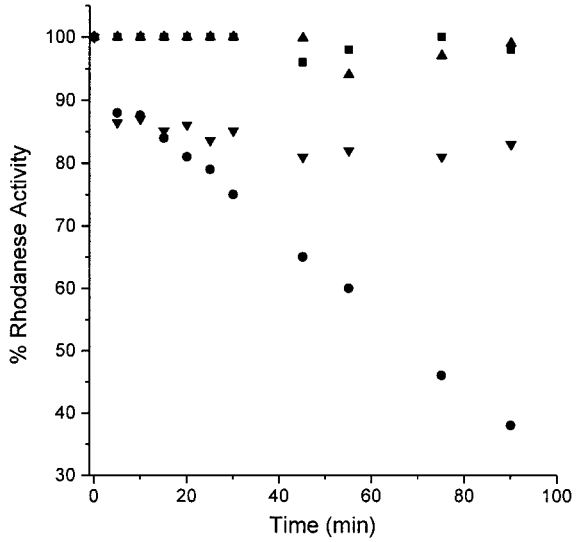

FIGURE 4: Activity of rhodanese at 40 °C under different conditions. (■) 36 μg/mL rhodanese in folding buffer was incubated at 40 °C, and the activity was measured as a function of time. (▲) 36 μg/mL rhodanese in folding buffer was incubated at 40 °C for the indicated times, and 2.25 mg/mL GroEL was added and kept at 25 °C for 120 min before the activity was measured at 25 °C. (●) 36 μg/mL rhodanese in folding buffer containing 2.25 mg/mL GroEL was incubated at 40 °C, and the activity at 25 °C was measured at the indicated times. (▼) 36 μg/mL rhodanese in folding buffer containing 2.25 mg/mL GroEL was incubated at 40 °C for a definite period of time. 0.375 mg/mL GroES and 20 mM ATP were added and incubated at 25 °C for 90 min. The activity of 36 μg/mL rhodanese in folding buffer incubated at 25 °C for identical lengths of time as described above was taken as 100%.

molar ratio of bis-ANS:rhodanese). There is more bis-ANS binding at 25 °C (●) than at 40 °C (■), which indicates that hydrophobic sites are less available at 40 °C. Therefore, the domain-separated monomers are not significantly present at 40 °C, as those have more exposed hydrophobic sites (*33*, *34*). For example, during urea-induced unfolding, intense bis-ANS fluorescence is observed at 3−4 M urea (*33*). At this concentration, domain separation starts, which is followed by global unfolding as the urea concentration is raised further (*34*, *35*). Less bis-ANS binding at 40 °C suggests that species formed after thermal perturbation do not have exposed hydrophobic sites. Thus, the dimers or smaller order oligomers formed at 40 °C may protect the interdomain hydrophobic regions by association at these sites. Figure 5B shows the change in anisotropy of 1 μM bis-ANS, as a function of incubation at 25° (●) or 40 °C (■) with 3 μM rhodanese. The samples were first incubated with bis-ANS at 25 °C and then transferred to either 25 °C (●) or 40 °C (■) for measurement. There is no change in emission maxima at either temperature, which indicates that there is no change in quantum yield or lifetime since these quantities are correlated for this probe. Thus, increases in anisotropy reflect increases in the rotational correlation time of the bound probe and, therefore, the size of the protein. The bis-ANS anisotropy is higher at 40 °C (■). This is consistent with the idea that dimers or small oligomers are formed since those have higher molecular volumes than monomers and hence higher anisotropies. The light scattering values of rhodanese in the presence or absence of bis-ANS are very close at both 25 °C and 40 °C (data not shown). This indicates that no large oligomers are formed in the presence of bis-ANS at 40 °C.

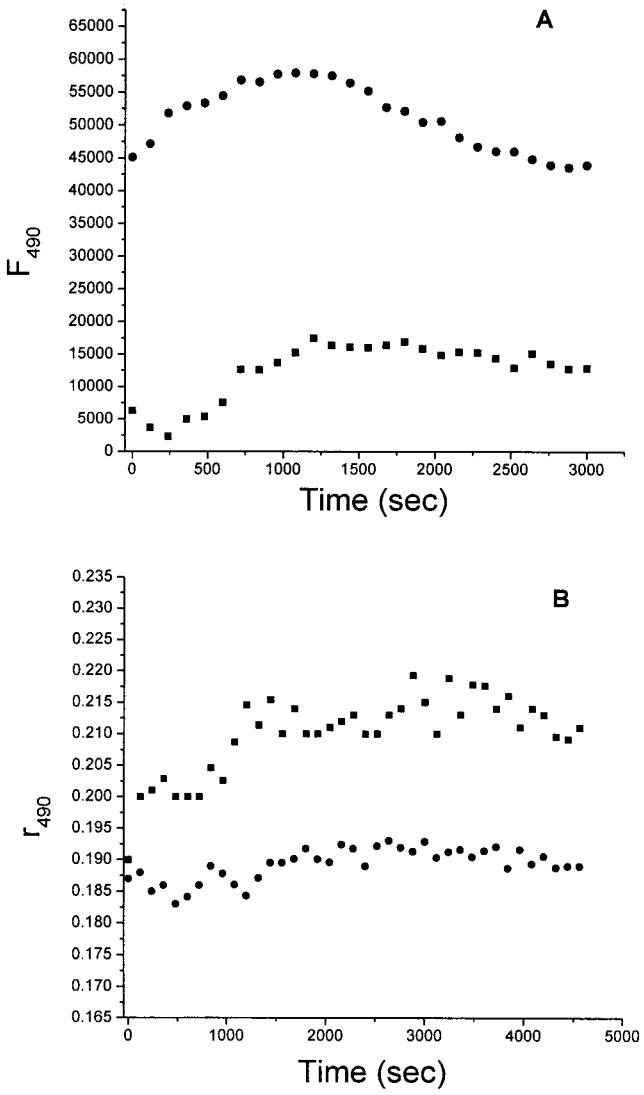

FIGURE 5: Binding of bis-ANS to rhodanese at 40 °C. (A) 3 μM rhodanese in folding buffer containing 1 μM bis-ANS was incubated at 40 °C (■) and at 25 °C (●), and fluorescence was measured as a function of time. (B) The same experiment as described above was done, and the anisotropy was measured as a function of time at 40 °C (■) and at 25 °C (●).

Dimer Can Be Detected by Glutaraldehyde Cross-Linking. Figure 6 shows SDS−PAGE of glutaraldehyde cross-linked rhodanese at 40 °C; 0.1 mg/mL rhodanese in 50 mM sodium phosphate has been used for the cross-linking as there is no significant increase in light scattering at 40 °C under these conditions (Figure 1B) so that no large oligomers are formed. The gel shows the time-dependent formation of dimer as incubation proceeds at 40 °C. Dimer is observed even after 5 min of incubation. The band that forms below the un-cross-linked band (see band at $t = 0$) represents intramolecularly cross-linked rhodanese (*35*, *36*). This is consistent with the data observed in Figure 2A, where even at 36 μg/mL RHIAF shows a sharp decrease in anisotropy by 5 min. As incubation proceeds, more and more dimers are formed. No higher order cross-linked species are observed up to 60 min. This is also consistent with the observation that there is no substantial change in light scattering (Figure 1) at 40 °C. The kinetic analysis of the gel (as in Figure 6) yields a rate constant for dimerization of 0.00277 ± 0.00013/s.

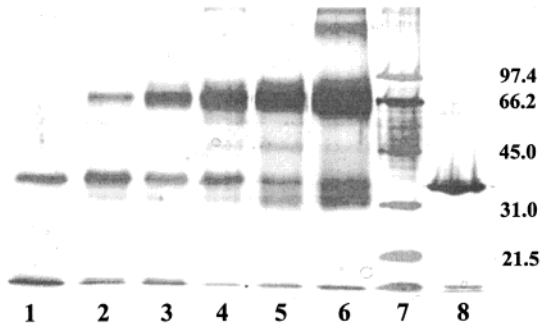

FIGURE 6: Cross-linking of rhodanese at 40° C. 0.1 mg/mL rhodanese in 50 mM sodium phosphate, pH 7.6, was incubated at 40 °C, and the cross-linking by glutaraldehyde at 40 °C was done after the indicated times as described under Experimental Procedures. The lanes contained the cross-linked product after (1) 0, (2) 5, (3) 10, (4) 15, (5) 30, or (6) 60 min incubation at 40 °C. Lane 7 represents molecular mass standards, and lane 8 contains rhodanese. The molecular mass standard contained myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

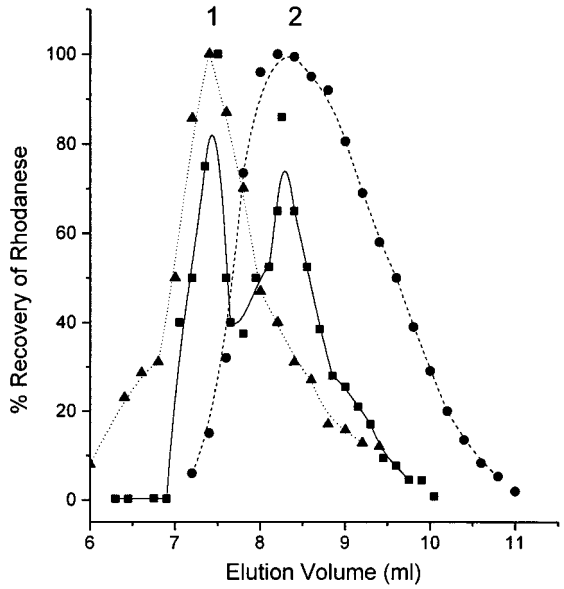

FIGURE 7: Separation of rhodanese species by gel filtration. (—) 0.1 mg/mL rhodanese in 50 mM sodium phosphate, pH 7.6, was incubated at 40 °C for 45 min and loaded on a 15 mL (19 × 1 cm) S-100 column, preequilibrated with 50 mM sodium phosphate, pH 7.6, 40 °C. Protein was eluted from the column as described under Experimental Procedure. BCA and activity assays were measured for each fraction. (- - -) The same experiment as described above was done, where both the incubation and elution were performed at 25 °C. (···) 0.1 mg/mL BSA was loaded on the same column, protein was eluted with 50 mM sodium phosphate, pH 7.6, 25 °C, and BCA assay esd determined for each fraction. To normalize, the maximum activity of rhodanese was taken as 100%. For BSA, the maximum concentration of protein eluted was taken as 100%.

Detection of Dimer by Gel Filtration. From the above-mentioned data, it is evident that rhodanese forms a thermally perturbed intermediate that is prone to form dimers or small oligomeric species at 40 °C. If these are stable species, then one can expect to fractionate them on a gel filtration column. Figure 7 shows the activity profile of rhodanese fractions as a function of elution volume at 37 °C; 0.1 mg/mL rhodanese in 50 mM sodium phosphate, pH 7.6, is first incubated at 40 °C for 45 min and then loaded on the S-100 column at

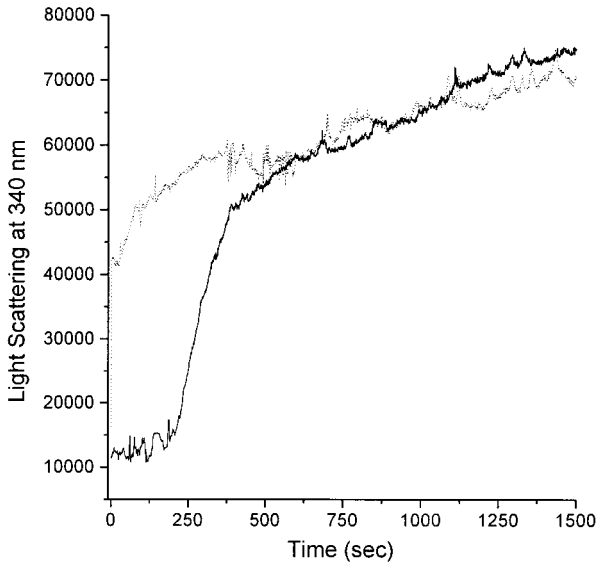

FIGURE 8: Formation of aggregates from monomeric and dimeric rhodanese. 0.1 mg/mL rhodanese in folding buffer was incubated at either 25 °C (—) or 40 °C (•••) for 90 min. The incubated samples were transferred to a cuvette preheated at 50 °C. Then, light scattering of both the samples was measured at 50 °C. The "0" time indicates the light scattering values at either 25 or 40 °C.

37 °C. Elution is also done at 37 °C. No protein is found in the void volume. Two active rhodanese peaks are observed. The first active peak (1) at 40 °C elutes at a similar elution volume as BSA elutes at 25 °C. The molecular mass of BSA is 66 kDa, which is the dimeric molecular mass of rhodanese (monomer molecular mass is 33 kDa). The second active protein peak (2) at 40 °C overlaps with the rhodanese eluted at 25 °C. At 25 °C, rhodanese remains predominantly as monomeric protein with molecular mass 33 kDa. This profile clearly shows that at 40 °C, rhodanese forms stable dimer. Either the dimer itself is active or it can be dissociated to active monomer in the assay buffer, as the dimeric peak is 100% active. The total protein eluted as peaks 1 and 2 accounts for more than 95% of the protein loaded onto the column. Thus, there is no large aggregate formed under this incubation condition.

Large Inactive Aggregates Can Be Formed from Small Active Oligomeric Species. Figure 8 represents light scattering results that show the kinetics of formation of large aggregate at 50 °C from both predominantly monomeric and oligomeric rhodanese. Rhodanese (0.1 mg/mL) is incubated at 25 or 40 °C for 90 min. Then samples are immediately brought to 50 °C, and light scattering is measured. There is an immediate large increase in the light scattering value with the 40 °C (•••) sample, whereas there is a lag phase of about 200 s with sample preincubated at 25 °C (—). As the incubations are continued, both samples give almost identical final values of the light scattering. After 1000 s of incubation, the samples are inactive in both cases (data not shown). These results are consistent with a picture in which small associated species are formed at 40 °C that can rapidly form large aggregates when the temperature is raised to 50 °C, while directly jumping the temperature to 50 °C from 25 °C requires the formation and buildup of these smaller intermediates before there can be formation of the large aggregates. An immediate increase in light scattering is also observed when rhodanese (0.1 mg/mL) is incubated at 40 °C for 90 min and kept at 25 °C for 15 min and then light scattering is measured at 50 °C. These results show that large inactive aggregates are formed from the small active oligomers.

DISCUSSION

This study has addressed early events in the paradoxical effect of high temperature on rhodanese in which higher protein concentrations apparently protect the enzyme against thermal inactivation and aggregation. Rhodanese inactivates irreversibly and precipitates readily at 50 °C (*16*). Reversibility can be observed when rhodanese is covalently coupled to an insoluble support or in the presence of GroEL, thus preventing aggregation (*16*). In the present study, we have shown the nature of early processes that precede irreversible thermal inactivation of rhodanese. Small active dimers are formed during heat incubation. The species are not disulfide-linked species, since disulfide-linked misfolded conformers are inactive (*25*). A Similar observation is found with aspartate aminotransferase (*18*). Heat inactivation involving disulfide bond formation is also observed with β-lactoglobulin (*37*−*40*).

Fluorescence homotransfer is a valuable tool for measuring protein structure (*41*), and oligomerization (*31*). The concentration-dependent loss in anisotropy of RHIAF due to energy homotransfer supports the formation of associated species. The temperature-dependent homotransfer, and hence association, suggests that the association may occur from an intermediate formed during incubation at higher temperature. Similar behavior has been observed in the case of aspartate aminotransferase from *Sulfolobus solfataricus* (*42*). The associated species formed in this study are apparently very stable as they cannot be dissociated easily nor can they be exchanged with unlabeled rhodanese. Increase in anisotropy of bound bis-ANS at higher temperature supports the formation of small oligomeric species. Cross-linking of homooligomeric proteins with bifunctional reagents has been extensively used for the determination of the oligomeric state of the proteins (*26*), and the present results clearly indicate the predominant formation of dimeric species during heat incubation.

These species occur under conditions where there is no evidence of significant large aggregates by light scattering. The fractionation of heat-incubated rhodanese by the gel filtration column with no detectable oligomers also supports the predominant formation of dimeric species. These data are supported by the small increase in light scattering. Similar observations are found with β-lactoglobulin (*29*).

It appears that the dimers and small oligomers are formed from an intermediate that can be recognized by GroEL to form a stable complex. The unfoldase activity of GroEL unfolds rhodanese intermediate and forms a stable reactivable comlex. Similar unfoldase activity of GroEL is observed with barnase (*43*). Importantly, the data indicate that once the small oligomeric species are formed from the heat-modified intermediates, GroEL cannot capture those. A similar effect has been observed with aspartate aminotransferase (*18*).

Hydrophobic interactions are important factors for protein association and aggregation. The X-ray structure of rhodanese (*44*, *45*) shows that the two independently folded domains into which it is folded are strongly associated by hydrophobic

interactions, and the active site cysteine is in the interdomain interface. Any concerted disruption in the interdomain interface would expose the hydrophobic surfaces, and the enzyme will lose activity. Less bis-ANS binding during heat incubation indicates that the formation of heat-modified oligomers actually protects the hydrophobic surfaces. No loss in activity also indicates that the interdomain interface remains relatively unperturbed during heat incubation. These results are consistent with a picture in which surfaces that were complementary in the monomer interact with their counterparts in a second monomer to form a dimer. Previously it has been reported that rhodanese binds more ANS at 40 °C than that at 25 °C (*19*). This may be due to the fact that authors have used 10-fold excess of ANS over protein which may induce some additional structural changes as has been pointed out in other systems (*46*).

The absence of lag phase and the faster kinetics during aggregation of rhodanese that is preincubated at high temperatures clearly indicate that the dimers or small oligomers are on-pathway intermediates for the heat-induced aggregation. A similar observation has been reported for β-lactoglubulin at high pH (*29*).

Based on these data, a model can be suggested for the aggregation of rhodanese: active monomer → active intermediates → stable active dimer and/or small oligomers → large, inactive aggregates.

Therefore, during thermal inactivation, rhodanese adopts a heat-modified but still enzymatically active conformation, that is prone to form dimer and/or small oligomers. These species are the structural precursors of irreversible aggregation, which leads to large inactive species.

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