Active Rhodanese Lacking Nonessential Sulphydryl Groups Contains an Unstable C-terminal Domain and Can Be Bound, Inactivated, and Reactivated by GroEL*

Reactivated by GroEL∗ an Unstable C-terminal Domain and Can Be Bound, Inactivated, and Active Rhodanese Lacking Nonessential Sulphydryl Groups Contains THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 3, Issue of January 17, pp. 1693–1699, 2003

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This work was supported by National Institutes of Health Grant GM 5177 and Welch Grant AQ 725 (to P. M. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Jesse Ybarra, Anusri Mitra Bhattacharyya, Markandeswar Panda, and Paul M. Horowitz‡ From the Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229-3900

Received for publication, July 26, 2002, and in revised form, November 11, 2002 Published, JBC Papers in Press, November 13, 2002, DOI 10.1074/jbc.M207574200

The mitochondrial enzyme rhodanese (EC 2.8.1.1) catalyzes the transfer of a sulfur atom from a sulfur donor, e.g. thiosulfate to an acceptor, e.g. cyanide. In the course of catalysis, the enzyme cycles between a form containing a transferred sulfur held as a persulfide (RSSH) on active site cysteine 247 and a form without the transferred sulfur (1). Rhodanese is a monomeric protein, and its crystal structure is known (2–4). The protein is folded into two domains that are independently folded but tightly coupled at the interdomain interface. The active site is in the C-terminal domain close to the interdomain interface, and domain separation is associated with inactivation. There are four cysteine residues at sequence positions 63, 247, 254, and 263, and all are reduced in the native enzyme. The persulfide-containing form of wild type rhodanese, WT-ES1, and rhodanese without the transferred sulfur, WT-E, have virtually identical crystal structures (2, 5, 6). Site-directed mutagenesis shows that Cys-247 is the only cysteine residue essential for rhodanese activity (7, 8). Although it is clear from the crystal structure that none of the cysteine residues in native rhodanese is in a favorable position to form disulfide bonds, Cys-247 always forms disulfide bonds with other cysteines in the C-terminal domain during oxidative inactivation, to which rhodanese is particularly sensitive. These disulfides can form from the native state during oxidative inactivation by reagents such as phenylglyoxal in a process that obviously requires a conformational change. In addition, single site oxidation at the active site can form a sulfenic acid or higher oxidation states. These oxidative events are the major reactions that result in incomplete reactivation of the enzyme after denaturation. The mutagenesis of the three nonessential cysteine residues as studied here (C63S, C254S, and C263S) to produce the species C3S removes the possibility of intramolecular disulfide bonds and makes the enzyme an important model for studies of folding.

Rhodanese has been one of the most studied substrates for assisted folding by the chaperonin GroEL. Using the wild type enzyme, rhodanese only binds easily to GroEL when it is presented to the chaperonin after it has been extensively unfolded. The native enzyme does not interact with GroEL (9).

The results presented here, based on studies using controlled proteolysis and mass spectrometry, suggest that the C-terminal domain of the persulfide-containing C3S (C3S-ES) is considerably less stable than WT-ES although the specific enzyme activities are the same. Although it is known that significant binding to GroEL requires forms of rhodanese that are unfolded further than states with folded domains, no specific information is known about these less folded forms. Because it was found that active C3S-ES is less stable than the wild type enzyme, we compared the properties of the form of C3S lacking transferred sulfur (C3S-E) and rhodanese (WT-E) with respect to GroEL binding. The results suggest that active C3S with an unstable C-terminal domain exists in equilibrium with the conformer(s), accessible from the native state that can interact with GroEL. This contrasts with the lack of binding observed

1 The abbreviations used are: WT-ES, sulfur-loaded rhodanese; C3S-ES, sulfur-loaded C3S; WT-E, rhodanese lacking transferred sulfur; C3S-E, C3S lacking transferred sulfur; β-ME, β-mercaptoethanol; C3S, rhodanese mutant with the substitutions C63S, C254S, C263S; HPLC, high performance liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
with the wild type enzyme. Thus, we have identified a particular region of C3S that is correlated with its decreased stability and increased binding to GroEL. These results present an opportunity for studying the conformational correlates required for substrate binding to GroEL.

MATERIALS AND METHODS

Reagents—SDS, acrylamide, and bisacrylamide were from Fisher. Bicinchoninic acid protein assay reagent was purchased from Pierce. Other chemicals were from Sigma. Sequencing grade, modified trypsin (reductively methylated) was from Promega (Madison, WI).

Rhodanese Purification—Recombinant bovine rhodanese was purified previously and stored at -70 °C as a crystalline suspension in 1.8 M ammonium sulfate containing 1 mM sodium thiosulfate (10). Rhodanese was desalted on a G-50 column before use. Rhodanese concentration was determined using a value of $A_{280} = 1.75$ (11).

Preparation of E Rhodanese—Rhodanese as prepared is in the sulfur-substituted form. The E form was prepared by treating the enzyme with a 5-fold molar excess of cyanide (12).

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 (11).

Construction and Isolation of C3S—The rhodanese gene (1.1 kbase) was cleaved between the XhoI and BamHI sites of Pet-11d. Cysteines (a63, b254, and c263) were sequentially mutated to serines using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The corresponding mutagenic oligos were (a) 5′-GGCTTACCGCTAGTCTCTATG-3′, (b) 5′-GAGGGCGATTGAGGAGCGGTGACACC-3′, and (c) 5′-ATCGGTTGCTGTGAGGCTGACCAGC-3′. BL21(D3) competent cells (Novagen, Madison, WI) were transformed with the mutant DNAs, and the DNA was purified by miniprep (Qiagen, Valencia, CA). DNA sequencing was performed by the Center For DNA Technology, University of Texas Health Science Center, San Antonio, TX. Once the first change was verified, that DNA was used for the next step of mutagenesis. The T7 universal primer was used to sequence into the 5′-end of the rhodanese insert. Likewise, a sequencing primer (5′-ACAGAGGGGAGCCCCATGAgGAGCCACCCAGCATGAA-3′) was used to sequence from the middle of the insert past the C terminus. There were no unintended changes evident in the primary sequence. C3S was purified essentially by the procedure used for WT with the exception that the cells were grown at 25 °C, and the purification steps were performed at 4 °C.

GroEL and GroES Purification—GroES2 and GroEL1 were purified as described previously (13, 14). Protein concentration was determined by the BCA method (15).

Protection of Rhodanese—Rhodanese (0.2 mg/ml) in 40 mM NH4HCO3, pH 7.5, was treated with trypsin (3.75% w/w) at 25 °C for the times indicated under “Results.” At each time, 2.5 μl of 200 μM phenylmethanesulfonyl fluoride and 5 μl of 4× SDS (0.25% Triton X-100, 1 M KCl, 1 mM MgCl2, pH 7.8) were added to the solution. The absorbance at 290 and 340 nm were measured for each data point and used to correct the fluorescence for the inner filter effect. The Stern-Volmer equation, $F/F_0 = 1 + K_{SV}[Q]$, was used for analysis. In this equation, $F$ and $F_0$ are the fluorescence in the absence and presence of quencher, respectively. $[Q]$ is the quencher concentration, and $K_{SV}$ is the Stern-Volmer constant, which correlates with the accessibility of the fluorescence for quenching (19).

Gel Filtration—Gel filtration was performed to separate monomeric WT-E and C3S-E and oligomers, formed during incubation. 150 μl of 3 mM (E), 10 mM (C3S), or 50 mM (C3S) rhodanese was incubated with 3.3×10-5 M KCN. After equilibration, 1 μl aliquots of a concentrated and fluorescence-free GroEL were added from a 217 mg/ml stock. The solution was then incubated at 25 °C for 60 min and loaded on a 15-ml (19 × 1 cm) Sephacryl S-100-SR column (fractionation range 1,000–100,000), equilibrated with 50 mM Tris-Cl, 10 mM KCl, 10 mM MgCl2, pH 7.8 at 25 °C. 100-μl fractions were collected and assayed for protein 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 (33 kDa) and 0.1 mg/ml bovine serum albumin (66 kDa) for the calibration.

CD Measurements—CD spectra were scanned at 25 °C from 250 to 200 nm for far UV data using an OLIS DSM 16 UV-visible CD spectropolarimeter (On Line Instrument Systems, Inc., Bogart, GA). All samples were in 50 mM NaCl, 10 mM Tris-Cl, pH 7.8 (A), with a flow rate of 0.5 ml/min. The path length of the cell was 0.1 cm. Data were collected at 0.3 nm intervals. Appropriate blanks were subtracted from the observed spectra. CD data were calculated in terms of molar ellipticity [θ] (20–22) at each specified wavelength using a protein molecular weight of 33,000. The molar ellipticity [θ] is expressed as degrees cm2 dmol−1.

Fluorescence Titration of GroEL with WT-E and C3S-E—The equilibrium binding of E forms of both WT and C3S rhodanese was studied using a fluorescence titration method described in the literature (23). The reaction conditions were similar to the experiments described for the inactivation studies except that the solutions contained 1 mM ADP instead of 3 mM ATP (see legend for Fig. 9). A Fluorolog-3 (Jobin Yvon-Apex) spectropho-

trometer was used. The excitation was at 295 nm (path length 5 mm, band pass 5 nm), and emission was recorded in the range of 300–450 nm (path length 2.5 mm, band pass 5 nm). In a 1-ml cuvette containing either WT or C3S (1 mM), the E form of rhodanese was formed by adding 5 μM RCN. After equilibration, 1–5-μl aliquots of a concentrated and fluorescence-free GroEL were added to the sample. All solutions were equilibrated for another 5 min before the emission spectra were recorded. The titration was continued until the final concentration of GroEL reached about 5 μM. In an identical experiment the scattering contributions from GroEL alone were studied by titrating it into the buffer without rhodanese. The overlaid emission scans, after the subtraction of GroEL contribution, showed no contribution to the scattering for both WT-E and C3S-E. These fluorescence intensity changes (ΔF) at different GroEL concentrations could be fitted to a binding model(s) to obtain the equilibrium binding constant, $K_B$ (23). Further details on the models used and their limitations are presented in the literature (23).
RESULTS

Expression and Purification of Active, Soluble C3S Requires Lowered Temperatures—When expression of C3S was attempted using growth conditions (37 °C) suitable for wild type rhodanese, very little enzyme was detected in the extracts. Almost all of the enzyme was contained in the pellets as demonstrated by SDS gels (data not shown), and attempts to solubilize and activate this enzyme were unsuccessful. When the cells were grown at 25 °C, a large amount of active enzyme could be recovered in the extracts. Attempts to purify this enzyme using procedures at room temperature as for the wild type enzyme led to enzyme inactivation, and very little active purified C3S was recovered. Significant recoveries of C3S were achieved when the purification steps were carried out at 4 °C. The purified C3S had a specific activity of 760 IU/mg and was, in this regard, very similar to the wild type protein.

C3S Is Less Stable Than Wild Type Rhodanese—The growth and purification conditions suggested that C3S is less stable than the wild type protein. This expectation is strengthened by the results shown in Fig. 1, where the effect of urea on the activities of WT and C3S is compared. The activity of C3S is very sensitive to urea, and there is a decrease in activity with even the smallest addition of urea. The C3S loses half its activity at ~1.75 M urea, whereas WT loses half its activity at ~4.3 M urea.

Comparison of Secondary Structures of C3S and Wild Type Rhodanese—Fig. 2 shows the circular dichroism spectra for C3S and WT rhodanese. As can be observed, there are only small differences in these spectra. Thus, although there are significant differences in the apparent stabilities of these proteins, their secondary structures are identical within experimental error.

Accessibility of Tryptophan Residues in WT-E and C3S-E toward Collisional Quenching—Fig. 3 shows the acrylamide quenching of C3S-E and WT-E. The Stern-Volmer constants (KSV) are 1.073 ± 0.02 m⁻¹ and 1.481 ± 0.04 m⁻¹, respectively. The higher Stern-Volmer constant indicates that the tryptophan residues in C3S-E are more exposed than those in WT-E. There is no significant curvature over the acrylamide concentration range used, which suggests that there is no conformational change induced by acrylamide that is associated with changes in tryptophan exposure. These data are consistent with the view that C3S-E is less stable than WT-E.

Tryptic Digestion of C3S Leads to a Resistant Fragment Not Observed with Wild Type Rhodanese—Figs. 4 and 5 show the comparison between the time courses of digestion of WT and C3S proteins, respectively, with trypsin. Panel A shows that the WT enzyme in the presence of thiosulfate is not susceptible to digestion at the level of trypsin and the conditions that are used. Panel B shows that C3S, in the presence of thiosulfate, is not significantly digested by trypsin. WT enzyme in the absence of thiosulfate is similarly unaffected (panel C). In contrast to these results, when C3S is treated with trypsin in the absence of thiosulfate (Fig. 5) there is a progressive digestion of the parent protein with the appearance of a band at an apparent molecular weight of 21,000 that remains as an apparently stable product under these conditions. The amino acid sequence of the daughter band (Fig. 5, band B) was determined as discussed under “Materials and Methods.” The sequence corresponded to the N-terminal region of rhodanese, confirming that the clip occurred in the C-terminal domain. This result is in agreement with the sequence of C3S bands identified by mass spectra (Fig. 7).

Fig. 6 shows the activity of C3S in the absence of thiosulfate and in the presence and absence of trypsin. In the absence of trypsin (upper curve, solid circles), the activity is stable for at least 30 min. However, with trypsin, the activity falls in a time course that is compatible with the digestion pattern seen on the SDS gels (lower curve: solid triangles indicate density of parent band; solid squares indicate rhodanese activity). This indicates that C3S is stable in the absence of trypsin and that the activity...
loss follows digestion rather than the digestion following inactivation of the protein to a susceptible form.

**Mass Spectrometry Indicates That the C-terminal Domain of C3S Is Proteolytically Susceptible**—In-gel digestion, peptide extraction, and mass spectrometry were performed as described under “Materials and Methods” on the 21-kDa daughter band formed by trypsin digestion as in Fig. 5. Fig. 7 shows the amino acid sequences of fragments that are detected from the parent band and the daughter band after digestion. The total molecular weight of the C3S daughter fragment from mass spectrometry was determined to be 20,783, which is consistent with the migration of the daughter fragment in the SDS gels. Fragments were observed from both bands that correspond to peptides in the N-terminal domain. No fragments were detected in the daughter band beyond residue Arg-182, although large C-terminal fragments were detected from the parent band. The single band from WT with or without thiosulfate displayed the same pattern as the parent band from C3S. It is clear that the stable daughter band that remains on the gel in Fig. 5, band B, results from cleavages within the C-terminal domain. Thus, the digestion occurs within the C-terminal domain of C3S in the absence of thiosulfate. These results suggest that the C-terminal domain of C3S is less stable than the corresponding region of WT.

**Binding of Sulfur-loaded Forms of C3S (C3S-ES) and Rho- danese (WT-ES) to GroEL**—Fig. 8, panel A, shows the activity of C3S-ES alone and in the presence of GroEL in folding buffer. The activity of C3S-ES remains unchanged at least up to 4 h at 25 °C (Fig. 8, panel A, filled squares). However, the enzyme is stable up to 1 h in the presence of GroEL. After that, there is a small but significant inactivation (~15%) in the presence of GroEL (Fig. 8, panel A, solid circles). This loss in activity may be due to the fact that in dilute solution, less ordered C3S-ES equilibrated slowly to a population of conformers, some of which can interact with GroEL, and the unfolding activity of GroEL makes the protein inactive. In the absence of GroEL, these conformers are in equilibrium with the native form and show 100% activity in the assay mixture. Fig. 8, panel B, shows the same experiment with WT-ES. Both in the absence and presence of GroEL, the enzyme remains 100% active, which...
Peptides (Bold, underlined) from in-gel digestion of SDS-gel bands of C3S after limited tryptic digestion in the absence of thiosulfate *

**C3S Parent Band**

MVKQVLRAVSTKLVLAESVRAGKVGQLISLAASWSYSPDTREKKE
YLRKHYQPGSPFDFIESESRDEASYFVSNLPSAGFADYGSGSLGIDNDTHVV
YGDDGFYAFRVMNPVGHKRVTSELQGRNFNLKKEHPTSERSEPS
PAIPKATLNSLLKTYEYQVLENSKKEQFVLQDSRAQRYLGTQEPDPADAVL
DGRHIGSVNMPFPMFLTGEDFGIESPEHLRAFEEKVDLTKPL1ATCRKG
VTASHACALAYLSKPDVSAYCGSWFPHRFAPTPWVSQGGK

**C3S Daughter Band**

MVKQVLRAVSTKLVLAESVRAGKVGQLISLAASWSYSPDTREKKE
YLRKHYQPGSPFDFIESESRDEASYFVSNLPSAGFADYGSGSLGIDNDTHVV
YGDDGFYAFRVMNPVGHKRVTSELQGRNFNLKKEHPTSERSEPS
PAIPKATLNSLLKTYEYQVLENSKKEQFVLQDSRAQRYLGTQEPDPADAVL
DGRHIGSVNMPFPMFLTGEDFGIESPEHLRAFEEKVDLTKPL1ATCRKG
VTASHACALAYLSKPDVSAYCGSWFPHRFAPTPWVSQGGK

* The sequences are based on that of WT with serines substituted at positions 63, 254 and 255.

**Fig. 7.** Sequence of the proteolytic products of C3S by Mass Spectrometry. The amino acid sequences of C3S parent band and C3S daughter band after limited proteolysis by trypsin. Sample preparation, acquisition of mass spectra, and sequence analysis are discussed under “Materials and Methods.” Underlined portions of the sequences represent fragments detected by mass spectrometry.

**Fig. 8.** Inactivation of C3S-ES and WT-ES in the presence and absence of GroEL. 0.1 μM (A) C3S-ES and 0.1 μM WT-ES (B) were incubated in 50 mM Tris-Cl, 10 mM KCl, 10 mM MgCl₂, pH 7.8, containing 0.5 μM KCN in the absence (●) and presence (○) of 0.2 μM GroEL at 25 °C.

Fig. 9 shows the same experiment as done in Fig. 8 with C3S-E and WT-E in folding buffer. Unlike C3S-ES and WT-ES, the E forms of both the proteins are unstable in solution and lose activity with time. Fig. 9, panel A, shows that C3S-E inactivates in solution with a rate constant of 0.08 ± 0.008 min⁻¹ (t₁/₂ = 8.7 min). But the rate of inactivation becomes more than 2 times faster in the presence of GroEL, with a rate constant of 0.18 ± 0.004 min⁻¹ (t₁/₂ = 3.85 min). The faster rate of inactivation in the presence of GroEL suggests that C3S-E may form a less ordered conformer(s) that can be captured by GroEL. Fig. 9, panel B, shows that WT-E is also unstable in solution. However, in this case both in the presence and absence of GroEL it inactivates with a similar rate as C3S-E, with a rate constant of 0.18 ± 0.004 min⁻¹ (t₁/₂ = 9.9 min). The observation that the rate of inactivation both in the presence and absence of GroEL are virtually identical suggests that during inactivation WT-E does not form any conformation that can be captured by GroEL. The inactivation of C3S-E and WT-E may be due to intermolecular (for both C3S-E and WT-E) and intramolecular (for WT-E) disulfide formation. However, the SDS-PAGE results (without β-ME) of the inactivated C3S-E and WT-E without GroEL show only a single band corresponding to a 33-kDa molecular mass (data not shown). Similar experiments with both C3S-E and WT-E were done where excess cyanide was removed by gel filtration, and they showed a similar profile. Attempts to isolate oligomeric species from the above samples by gel permeation chromatography also yielded only monomers for both C3S-E and WT-E (data not shown). These data indicate that the change in conformation leading to inactivation is not due to any intermolecular covalent modification of either C3S-E or WT-E.

**Reactivation of Inactivated C3S-E and WT-E by the GroEL Chaperonin System**

Binding to GroEL of C3S Lacking Transferred Sulfur (C3S-E) and Rhodanese Lacking Transferred Sulfur (WT-E)—
Rhodanese Mutant with Unstable Domain Interacts with GroEL

TABLE I
Chaperonin-assisted reactivation of WT-E and C3S-E after inactivation in solution

| Sample                  | C3S-E | C3S-E incubated with GroEL | WT-E | WT-E incubated with GroEL |
|-------------------------|-------|----------------------------|------|---------------------------|
| Incubated in folding buffer for 45 min | 0.2   | 3                           | 8    | 2                         |
| β-ME and thiouosphate were added to the above sample | 11    | 11.4                        | 16   | 19                        |
| GroEL/GroES/ATP 100 were added to the above sample | 70.4  | 72                          | 65.5 |                           |

further to get maximum inactivation, and then their reactivation was studied. Table I (first line) shows that all the incubated samples were almost inactive. When both thiouosphate (50 mM) and β-ME (0.2 mM) were added to these inactivated samples, only a small amount of reactivation (~10–20%) was observed from all samples. The small reactivation in the presence of high concentrations of reductant also shows that disulfide bond formation was not the predominant factor for inactivation. When GroEL/GroES/ATP were added to these samples, significant reactivation was observed with all the samples. GroEL could capture inactivated C3S-E and reactivate to 100% in the presence of GroES and ATP (Table I). This is the highest reactivation of inactivated rhodanese that has been observed with this enzyme. Reactivation to ~70.4% was also observed with C3S-E, where the inactivation was done in the presence of GroEL. Similar results were obtained with WT-E, where 72% reactivation was noted. The complete reactivation of C3S-E indicates that it can form a conformer in solution that is capable of more productive binding to GroEL. This very high reactivation clearly shows that inactivation is primarily due to non-covalent conformational change in the protein.

GroEL binding of the E forms of rhodanese was investigated using titration of GroEL into a fixed amount WT-E and C3S-E using fluorescence ("Materials and Methods"). The aim was to understand if the better reactivation of C3S-E relative to WT-E could be due to the increase in the population of specific sites that have higher affinity for the GroEL protein. The decrease in ΔF upon the increase in GroEL concentrations follows a binding curve in the case of WT-E (data not shown) with a binding constant (Kd) of 0.20 ± 0.01 μM. The results for C3S did not follow a binding curve in the GroEL concentrations studied (data not shown). Therefore, contrary to the better inactivation of C3S-E than WT-E in the presence of GroEL (Fig. 9), the fluorescence titration apparently showed the reverse. However, it must be noted that the fluorescence changes were not significant and probably are not better than the results obtained from activity studies (Fig. 9).

DISCUSSION

One of the major side reactions that limits the folding of denatured rhodanese is the formation of intramolecular disulfide bonds involving the active site cysteine residue and the nonessential sulfhydryl groups (25, 26). According to the x-ray structure, the cysteines are not close enough to form these disulfides. This implies flexibility in solution that would permit the observed disulfide formation. We find in this study that the removal of the three nonessential cysteine residues (cysteines 63, 254, and 263) to form C3S makes rhodanese less stable in its C-terminal domain, although the specific activities at 25 °C and the average secondary structures reported by CD are the same for the mutant and WT. In addition, C3S is protected against proteolysis by the presence of the substrate, thiosulfate.

The x-ray structure of WT rhodanese permits us to speculate as to the relation between the mutations prepared here and the consequences that are observed. Rhodanese is folded into two separate globular domains (N-terminal domain consisting of residues 1–142 and C-terminal domain consisting of residues 159–293) that are tightly coupled at an interdomain interface by numerous hydrophobic interactions. The domains are covalently connected by a tether consisting of a long loop of residues 143–158 that interacts with the N-terminal domain. The two domains are of nearly equal size, and they have very similar conformations. Thus, it is reasonable that the N-terminal domain is more stable than the C-terminal domain due to the additional interactions with the tether. The active site Cys-247 that holds the sulfur transferred from thiosulfate is in the interdomain region, and it has been shown that the activity of the enzyme is sensitive to conditions that disrupt the interdomain interactions. Numerous attempts at preparing individual domains have been unsuccessful, presumably due to the extensive hydrophobic surfaces that would be exposed on the individual domains.

Of the nonessential sulfhydryl groups, Cys-254 has been shown to be the most influential at affecting the structure and function of rhodanese (3, 4, 6). This is reasonable since Cys-63 is in the N-terminal domain, and the main effects in C3S are in the C-terminal domain. Cys-263 has been shown to have smaller effects, which is reasonable since it is close to the surface of the protein. Helices from each of the domains (D’ helix in the C-terminal domain, residues 251–264, and the D helix in the N-terminal domain, residues 107–119) cross each other at Cys-254, which appears to interact with Trp-113 and Pro-109 from the N-terminal domain. A number of residues from the D and D’ helices interact hydrophobically to stabilize the interdomain interface. Thus, the mutation at Cys-254 can affect the interdomain surfaces or their interactions. Changes in this contact region can affect the domain structure and permit the proteolytic clip that occurs within the C-terminal domain to leave a stable fragment that ends at Arg-182.

The proteolysis is interesting. Arg-182, the final cleavage point, is protected from proteolytic access by a long turn in the structure consisting of residues 189–211 (4). Movement of this turn would permit proteolytic access, and the unstructured nature of the turn would make changes in its orientation very difficult to detect by CD. Interestingly, Arg-186 separates this loop from bond 182–183. Arg-186 is the cationic binding site for the substrate thiosulfate. Thus, thiosulfate binding can easily be envisioned as stabilizing the structure or covering Arg-182, thus preventing proteolytic access.

GroEL does not bind native rhodanese in the form containing transferred sulfur, as studied by activity and direct measurement of complex formation (27, 28). It has been established that for productive binding to GroEL and its reactivation in the presence of GroES/ATP, rhodanese domains must be unfolded (28). The rhodanese form containing transferred sulfur, WT-E, does not bind to GroEL, as there is no loss in activity in the presence of GroEL. The sulfur-loaded form of mutant rho-
of GroEL/GroES/ATP to inactivated samples leads to a maximum intermediates formed during inactivation. The addition of GroEL/GroES/ATP to the absence of substrates (29, 30) and pre-β-lactamase (31), where starting with the native protein, stable complexes can be formed with GroEL. Mitochondrial aspartate aminotransferase forms a modified but catalytically active conformation that can be bound to GroEL (18). Although the sulfur-loaded forms of both rhodanese and C3S are stable in solution, the forms lacking transferred sulfur inactivate readily. The inactivation may be due to the formation of inter- and intramolecular disulfide-bonded species. The addition of high concentrations of rhodanese, C3S-ES, shows the same specific activity as WT-ES and remains fully active in solution. However, it shows slow but significant inactivation in the presence of GroEL. These data are consistent with the view that C3S-ES has a less stable structure than WT-ES and, thus, is able to exist in an equilibrium with an intermediate(s) that can bind to GroEL. Similar observations have been made with dihydrofolate reductase inactivation chromatography. All these data rule out the possibility of starting with the native protein, stable complexes can be formed with GroEL. Importantly, the differential stability of C3S is induced by the binding of the substrate, thiosulfate. These studies will provide a means to identify and characterize the binding determinants on protein targets for GroEL.

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