NH$_2$-terminal Sequence Truncation Decreases the Stability of Bovine Rhodanese, Minimally Perturbs Its Crystal Structure, and Enhances Interaction with GroEL under Native Conditions*

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Richard J. Trevino‡, Francesca Gliguibich§, Rodolfo Berni, Michele Cianci§, John M. Chirgwin‡ **, Giuseppe Zanotti§, and Paul M. Horowitz‡ ‡‡

From the Departments of £Biochemistry and **Medicine-Endocrinology, the University of Texas Health Science Center, San Antonio, Texas 78284, the $Department of Organic Chemistry, University of Padova and Biopolymer Research Center, Consiglio Nazionale delle Ricerche, 35131 Padova, Italy, the ‡Institute of Pharmaceutical Chemistry, University of Milano, 20135 Milano, Italy, and the §§Institute of Biochemical Sciences, University of Parma, 43100 Parma, Italy

The NH$_2$-terminal sequence of rhodanese influences many of its properties, ranging from mitochondrial import to folding. Rhodanese truncated by >9 residues is degraded in Escherichia coli. Mutant enzymes with lesser truncations are recoverable and active, but they show altered active site reactivities (Trevino, R. J., Tsalkova, T., Drager, G., Hardesty, B., Chirgwin, J. M., and Horowitz, P. M. (1998) J. Biol. Chem. 273, 27841–27847), suggesting that the NH$_2$-terminal sequence stabilizes the overall structure. We tested aspects of the conformations of these shortened species. Intrinsic and probe fluorescence showed that truncation decreased stability and increased hydrophobic exposure, while near UV CD suggested altered tertiary structure. Under native conditions, truncated rhodanese bound to GroEL and was released and reactivated by adding ATP and GroES, suggesting equilibrium between native and non-native conformers. Furthermore, GroEL assisted folding of denatured mutants to the same extent as wild type, although at a reduced rate. X-ray crystallography showed that $\Delta$1–7 crystallized isomorphously with wild type in polyethyleneglycol, and the structure was highly conserved. Thus, the missing NH$_2$-terminal residues that contribute to global stability of the native structure in solution do not significantly alter contacts at the atomic level of the crystallized protein. The two-domain structure of rhodanese was not significantly altered by drastically different crystallization conditions or crystal packing suggesting rigidity of the native rhodanese domains and the stabilization of the interdomain interactions by the crystal environment. The results support a model in which loss of interactions near the rhodanese NH$_2$ terminus does not distort the folded native structure but does facilitate the transition in solution to a molten globule state, which among other things, can interact with molecular chaperones.

The enzyme rhodanese (thiosulfate:cyanide sulfuryltransferase, EC 2.8.1.1) catalyzes in vitro the transfer of sulfur from donors such as thiosulfate to a number of acceptors including cyanide (1), and it has become an important model for studying protein folding (2, 3). Rhodanese is monomeric (~32 kDa) and folded into two domains of very similar size and conformation, connected by a surface loop (4). The active site is located in the COOH-terminal domain, near the interdomain interface.

Rhodanese is encoded in the nucleus, translated in the cytosol, and imported into the mitochondrial matrix without NH$_2$-terminal proteolytic processing, except for the removal of the initiating methionine (5). The NH$_2$-terminal 23 amino acids have been implicated in rhodanese properties including its unassisted folding, interactions with molecular chaperones, and mitochondrial import. It has been suggested, for example, that the disposition of the NH$_2$-terminal sequence is important for allowing rhodanese to maintain the non-native conformation that is required for mitochondrial import (6). Although this sequence has been thought to contribute to the stability of the active structure of the enzyme (7, 8), its influence on the overall conformational potentials of rhodanese has never been studied directly. We have used here NH$_2$-terminal truncations to study the influence of this region on the structure and function of rhodanese.

The NH$_2$-terminal sequence of rhodanese is located primarily on the surface of the NH$_2$-terminal domain of the protein (9–11). The first 9 amino acids contribute to the hydrophobic interdomain interface. Residues Ser-11 to Gly-22 form an $\alpha$-helix that has been suggested to be critical for transport across mitochondrial membranes (12). The influence of the amino-terminal residues on the rhodanese activity is surprising, since all of the residues required for catalysis are on the COOH-terminal domain, and the active site Cys-247 is >18 Å from any of the first 23 NH$_2$-terminal amino acids.

Previous reports suggested that changes within the NH$_2$-terminal sequence altered the properties of the enzyme. A point mutation, E17P, within the NH$_2$-proximal $\alpha$-helix, facilitated urea denaturation and led to increased proteolytic susceptibility in vitro (8). When mutants of the enzyme with sequential NH$_2$-terminal deletions were expressed in Escherichia coli, the first 10 residues were found to be necessary for stable, heterologous expression, and longer truncations were rapidly degraded within the cells (7). Rhodanese mutants with up to 9 NH$_2$-terminal residues deleted were purified, and their enzyme kinetic parameters were found to be similar to those of the full-length enzyme. However, using activity as a criterion, mutants missing 7 or 9 residues were less resistant to urea per-
turbation and exhibited significantly altered active site reactivities.

The present work assessed the biophysical effects of the truncations on the overall stability of rhodanese. Urea denaturation transitions for enzymatically active rhodanese with truncations of 3, 7, or 9 NH$_2$-terminal amino acids were monitored using the fluorescence of tryptophan or the hydrophobic probe, bis-ANS.

Circular dichroism was used to compare secondary and tertiary structures under native conditions. Interactions with GroEL under native conditions were investigated to evaluate the influence of the truncations on the ability of rhodanese to adopt conformations that can be recognized by the molecular chaperone, GroEL. The data are consistent with the hypothesis that NH$_2$-terminal truncations between residues 4 and 9 produce a fully active enzyme with decreased global stability. The results are of particular interest, because the x-ray results show that the native states of all the species are virtually identical.

The rhodanese structure has been studied in some detail, so that influences of NH$_2$-terminal truncations can be interpreted. Recently, the crystal structure has been refined to 1.36Å resolution (13). The sulfur-free and sulfur-substituted enzymes crystallized isomorphously. Chemical modifications of the catalytic cysteine led to limited changes of the protein structure, confined to the enzyme active site (11). In this work, we have compared the structure of rhodanese crystallized in a new orthorhombic crystal form with that of the monoclinic form, and we have determined the structure of an NH$_2$-terminal-truncated rhodanese (Δ1–7) in order to establish the role of NH$_2$-terminal amino acid residues 1–7 on the protein structure.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild type and mutant rhodanese species were purified as described previously (7) and stored as 2.5 M (NH$_4$)$_2$SO$_4$, pellets containing 5 mM Na$_2$S$_2$O$_3$ and 50 mM NaCl at –70°C. Some preparations for crystallography used molecular sieve chromatography as the last step of the purification (11). Prior to use, they were resuspended in 200 mM Na$_2$PO$_4$, 50 mM Na$_2$S$_2$O$_3$, 200 mM β-mercaptoethanol, pH 7.4. Procedures for the purification of GroEL/ES were modified for optimal yield and purity (14, 15), and these proteins were stored at –70°C in 50 mM Tris-HCl, 10% (v/v) glycerol, and 1 mM dithiothreitol. Urea was obtained from Bio-Rad Laboratories. Molecular Probes, Inc. supplied the bis-ANS. All other chemicals were analytical grade from Sigma.

**Protein and Activity Assays**—Rhodanese concentrations were determined using both A$_{290\text{nm}}$ = 1.75 (16) and micro-bicinchoninic acid (BCA) assays (Pierce). Activity was calculated from the absorbance at 460 nm of the complex formed between the reaction product, thiosulfate, and ferric ion (1). GroEL/ES concentrations were measured using the BCA assay.

**Crystallization of Wild Type and NH$_2$-terminal-truncated (Δ1–7) Rhodanese**—Single crystals of the sulfur-containing form of both wild type and NH$_2$-terminal-truncated (Δ1–7) rhodanese were grown at 4°C in sitting-drop vapor-diffusion experiments. Droplets (5 μl) containing protein at a concentration of approximately 4 mg/ml in the presence of 10% (w/v) PEG 6000, 50 mM Tris-HCl, 50 mM sodium acetate, 1 mM sodium thiosulfate, pH 7.3, were equilibrated with a reservoir solution (0.8 ml) containing 16% (w/v) PEG 6000, 50 mM Tris-HCl, 50 mM sodium acetate, 1 mM sodium thiosulfate, pH 7.3.

**X-ray Diffraction Data Collection and Processing**—The x-ray source for data collection was a Siemens M18XHF rotating anode generator, operating at 50 kV and 90 mA, with an apparent focus size of 0.3 × 3 mm. Copper radiation was selected by a graphite crystal monochromator. A 0.5-mm collimator was used. Data were measured on a HIGH STAR area detector system, mounted on a three-axis goniometer. The crystal was rotated 0.25° per frame. Two crystals for both wild type and Δ1–7 mutant were necessary to collect an entire set of diffraction data.

**Scaling and merging** were performed with the SAINT program (Siemens Industrial Automation). The wild type and mutant forms of the protein are isomorphous: the space group is P2$_1$2$_1$2. For wild type rhodanese the unit cell dimensions are: $a$ = 89.74 Å, $b$ = 72.35 Å, and $c$ = 39.07 Å; for the Δ1–7 mutant, $a$ = 90.69 Å, $b$ = 73.03 Å, and $c$ = 39.67 Å. Taking into account one molecule per asymmetric unit, this gives a V$_{M}$ of 1.9 Å$^3$/Da, which corresponds to a solvent content of 37%. Table IV gives the statistics for data collection and the percentage of reflections measured as a function of resolution for the two data sets.

**Structure Solution and Refinement**—The structure determination for wild type rhodanese in the orthorhombic crystal form was accomplished by means of the molecular replacement method, using the high resolution structure of sulfur-substituted rhodanese in the monoclinic crystal form as a starting model (13). The model, deprived of all solvent molecules, was positioned inside peaks close to the polar side chains. Water molecules with a B factor greater than 65 Å$^2$ were excluded or their position corrected. In a total of 26 cycles, the R factor was reduced to the final value of 0.166 ($R_{free}$ = 0.215). The final model consists of 2327 protein atoms and 63 solvent molecules.

For the mutant enzyme, whose crystals are isomorphous with the wild type protein, the atomic coordinates of the model from the previous refinement, deprived of solvent and of the first 7 amino acids, represented the initial model. The initial R factor was 0.36, which dropped, after several cycles of minimization, to 0.248 ($R_{free}$ = 0.327). The map calculated with coefficients $|F_{obs} - F_{calc}|$ clearly showed the absence of any electron density corresponding to amino acids from 1 to 7. A refinement procedure similar to that used for the wild type protein brought the R factor to the final value of 0.162 ($R_{free}$ = 0.219). The final model contained 2264 protein atoms and 194 solvent molecules. The maps were displayed on an IRIS 4D Graphics Workstation (Silicon-Graphics) using the program TOM.

**Gel Filtration Chromatography Analysis**—Each rhodanese species was separately loaded on a Superose 12 high performance gel filtration column connected to a fast protein liquid chromatography system with an absorbance detector (Pharmacia). The protein sample for each run was at a concentration of 1–7) rhodanese were grown at 4 °C in 10% (v/v) glycerol, and 1 mM dithiothreitol. Urea was separately loaded on a Superose 12 high performance gel filtration column connected to a fast protein liquid chromatography system with an absorbance detector (Pharmacia). The protein sample for each run was at a concentration of 250 μM and 200 μM β-mercaptoethanol, pH 7.6. A 200-μl aliquot was injected onto the column equilibrated with 200 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0. The flow rate was kept constant at 0.3 ml/min. The effluent was analyzed by enzymatic activity and absorbance at 280 nm.

**Sedimentation Velocity Analysis**—Each rhodanese species was examined separately by sedimentation velocity ultracentrifugation using a Beckman XLA analytical centrifuge. The rhodanese concentration was 0.5 mg/ml for each analysis. The temperature was maintained at 25°C. The rotor speed was 55,000 rpm and absorbance at 280 nm was monitored during at least 20 scans of the sedimenting boundary. Data were collected and analyzed with the UltraScan program developed by B. Demeler, Department of Biochemistry, University of Texas Health Science Center at San Antonio. This analysis is based on the method of van Holde and Weisheit (17). All data were corrected for buffer density and viscosity.

Urea Unfolding of Rhodanese Proteins Monitored by Tryptophan Fluorescence and bis-ANS Fluorescence—Wild type rhodanese or mutant protein (50 μg/ml) was denatured at 0–8 μM urea in a buffer containing 50 mM Na$_2$S$_2$O$_3$, 50 mM Tris-HCl, and 200 mM β-mercaptoethanol, pH 7.8, for 24 h at 25°C. Samples were then excited at 280 nm and emission spectra were scanned from 300 to 400 nm using an SLM 48000C spectrofluorometer. Fluorescence is a sensitive measure of rhodanese conformation, and all the species retained the 8 tryptophans found in the wild type protein (Trp-14, Trp-35, Trp-112, Trp-113, Trp-177, Trp-275, Trp-278, and Trp-287). A Perkin-Elmer LS-50B spectrophotometer was used to monitor fluorescence at 360 nm after excitation at 399 nm for bis-ANS fluorescence (18, 19). 30 μM bis-ANS was added to each sample immediately prior to excitation. Intensities were corrected with buffer blanks without protein. All analyses were performed at 25°C.

Urea denaturation was performed under conditions known to produce transitions reflecting reversible unfolding (3). The intrinsic
tryptophan fluorescence intensities as a function of urea concentration were fit to a model for a two-state transition, \([N \text{ (native)}] \cdot [D \text{ (denatured)}] \). Values for the free energy of unfolding, \(\Delta G_p\), at a given urea concentration were calculated from the equilibrium constant, \(K_p\), using,

\[K_p = \frac{e^{-\Delta G_p/RT}}{f_{pH}f_{m}} = \frac{(y - y_p)(y_0 - y)}{y_0^2}
\]  

(Eq. 1)

where \(f\) is fraction of protein and \(y\) is measured fluorescent emission intensities. Linear extrapolations of these values to \([\text{urea}] = 0\) by the method of Pace were used to estimate the conformational stability of the protein, \(\Delta G_p^{H_2O}\) (20).

Circular Dichroism—CD spectra were scanned at 25 °C in a 0.1-cm path length cell from 260 to 340 nm for near UV data and from 180 to 280 nm for far UV data using a Jasco J500C spectropolarimeter. All samples were in 50 mM Na2S2O3, 50 mM Tris-HCl, and 200 mM b-mercaptoethanol, pH 7.8. CD measurements for far UV-CD measurements were at 7–8 μM, and 50 to 75 μM for near UV-CD measurements. CD data were calculated in terms of mean residue ellipticity (θ) at a specified wavelength using a mean residue weight of 115 (21, 22).

Capture/Release of Native Rhodanese Species by GroEL/ES—Rhodanese is enzymatically inactive when it interacts with GroEL, and capture of rhodanese is defined in this study as the loss of enzymatic activity that occurs in the presence of GroEL compared with parallel controls without GroEL. Each rhodanese species, at a final concentration of 150 nm, was added to a buffer containing 2.5 μM GroEL, 10 mM MgCl2, 10 mM KCl, 2 mM ATP, 200 mM b-mercaptoethanol, 50 mM Na2S2O3, 50 mM Tris-HCl, pH 7.8, at 23 °C. Rhodanese activities for each sample were recorded hourly using 30-μl aliquots assayed for 10 min. These activities were normalized to parallel control samples under the same conditions, but without GroEL. To release bound rhodanese, 5 μM GroES and 5 mM ATP were added to all samples after incubation for 90 h in the above buffer conditions. After an additional 25 h, 2 mM more fresh ATP was added to all samples. Release of rhodanese was monitored by measuring regain of activity. To prevent microbial growth over the long incubation times, all buffers were filtered through 0.22-μm filters and/or by autoclaving. The samples were incubated in sterile microcentrifuge tubes.

GroEL/ES-assisted Refolding of Urea-unfolded Rhodanese—All protein samples were denatured for 4 h at 25 °C at 90 μg/ml in 8 M urea containing 50 mM Na2S2O3, 50 mM Tris-HCl, and 200 mM b-mercaptoethanol, pH 7.8. Refolding was performed by diluting to a protein concentration of 3.6 μg/ml into the buffer above without urea, additionally containing 10 mM MgCl2, 10 mM KCl, 2 mM ATP, 2.5 μM GroEL, and 5 μM GroES. Rhodanese assays were performed at specific time points during refolding by diluting 25 μl of the sample into the assay reaction mixture and stopping the reaction after 15 min.

RESULTS

Wild Type and Mutant Forms of Rhodanese Are Homogenous and Monomeric

Rhodanese species were analyzed for aggregation that may occur due to truncation of the NH2 terminus. Gel filtration behavior of mutants and wild type were identical (not shown). A single peak with the same shape and same enclosed area eluted at the same volume expected for monomeric rhodanese, independent of the species used. Mutant and wild type proteins gave virtually identical sedimentation velocity results. Each protein behaved as a single, pure component when the data were analyzed by the method of van Holde and Weichert (17), in that there was little variation in the integral distribution of s20,w values at all boundary fractions scanned. The s20,w values for the native proteins were as follows: wild type = 2.97 ± 0.09 S, Δ1–7 = 2.88 ± 0.10 S, and Δ1–9 = 2.83 ± 0.07 S. No evidence of large aggregates or oligomers (dimer, trimer, etc.) was detected for any species.

Conformational Stability, \(\Delta G_p^{H_2O}\), Is Diminished in Rhodanese Mutants

We investigated the conformational properties of Δ1–3, Δ1–7, and Δ1–9. The kinetic properties of these species were reported previously and found to be very similar (7). The stability of each species, \(\Delta G_p^{H_2O}\), was derived from denaturation curves by extrapolating the measured \(\Delta G\) values to \([\text{urea}] = 0\). Tryptophan fluorescence was used to monitor unfolding as described under “Experimental Procedures.” \(\Delta G_p\) is then plotted against urea concentration. Linear regression extrapolation of the plots to 0 M urea estimates conformational stability of the native structure (20).

![Graphs showing the effect of urea concentration on the stability of rhodanese mutants](image-url)
Lability of Truncated Rhodanese

Biophysical characteristics of purified rhodanese mutants

Conformational stabilities were extrapolated for native proteins, see Fig. 1B and detailed description under “Experimental Procedures” and Ref. 20. The standard deviations for the conformational stabilities at 0 M urea were given at 95% confidence interval data plotted for the linear regression extrapolations. Urea denaturation transition midpoints were calculated from the nonlinear curve fit function: y = (a - dv1 + (x/c)^d) + e; where c = U_{1/2} (urea denaturation transition midpoint), x = [urea], y = ΔG_{mol}, a = asymptotic maximum, b = slope function, and d = asymptotic minimum. Standard deviations for transition midpoints are from at least 20 iterations of the fit function. Spectral maxima for the native forms of the proteins are taken from the original three trials ± standard deviations.

| Sample               | Conformational stability, ΔG^0 mol | Urea denaturation transition midpoints, U_{1/2} | Spectral maxima for native forms a |
|----------------------|-----------------------------------|-----------------------------------------------|-----------------------------------|
| Wild type            |                                   |                                               |                                   |
| Δ1–3                 | 6.2 ± 0.15                        | 3.98 ± 0.02                                   | 334 ± 1.0                          |
| Δ1–7                 | 6.3 ± 0.1                         | 4.02 ± 0.03                                   | 334 ± 0.6                          |
| Δ1–9                 | 5.2 ± 0.4                         | 3.27 ± 0.02                                   | 337 ± 1.0                          |
|                      | 4.2 ± 0.2                         | 2.74 ± 0.03                                   | 336 ± 0.6                          |

S.D. = (a) = three separate experiments performed for each sample (urea denaturations for conformational stability and transition midpoints, spectrofluorimetric analyses for spectral maxima) and results were calculated from these trials and averaged ± S.D.

mol, U_{1/2} = 2.74 ± 0.03 M urea).

As seen in Fig. 1a and Table I, there were measurable red shifts of the fluorescence maxima for Δ1–7 in the absence of urea (336–337 nm) compared with the maximum for wild type or Δ1–3 (334 nm) suggesting increased solvent exposure of tryptophans in the native structures of the Δ1–7 mutant.

Hydrophobic Surfaces Are More Easily Exposed in the Rhodanese Truncation Mutants Compared with Wild Type

The fluorescence enhancement when the molecule bis-ANS binds to hydrophobic surfaces was used to compare hydrophobic exposure in the truncation mutants during urea-induced unfolding.

Fig. 2 shows the bis-ANS fluorescence as a function of the urea concentration. The increase in intensity starting at low urea concentrations has been suggested to reflect the formation of folding intermediates with exposed hydrophobic surfaces. At higher urea concentrations, the protein begins to unfold further, and the organized surfaces are disrupted, accompanied by a decrease in fluorescence intensity. The fluorescence intensity reaches a maximum at an intermediate point between the opposing tendencies of formation of the folding intermediates and their subsequent unfolding. Fig. 2 shows that increased truncation facilitates hydrophobic surface exposure at low urea concentrations. The parameters characterizing these transitions are given in Table II. Δ1–7 and Δ1–9 showed more bis-ANS fluorescence in the absence of urea, suggesting greater hydrophobic exposure in the unperturbed structures of these mutants; the results are in keeping with increasing hydrophobic exposure with increasing truncation.

Δ1–9 Rhodanese Exhibits an Altered Near UV-CD Signal

The mutants and wild type proteins were analyzed by UV-CD. Spectra of the wild type protein were in agreement with those described previously (26). The spectra for the mutants and wild type were virtually identical in the far UV. Table III compares the ellipticities for the proteins at 210 and 292 nm.

Far UV-CD molar ellipticities at 210 nm for all mutants were not significantly different from wild type, $-7 \times 10^{-3}$ deg cm$^{-2}$ dmol$^{-1}$, suggesting that the secondary structures are not significantly different.

The molar ellipticities at 292 nm, $\theta_{292}$, were used to compare the tertiary structures in the vicinity of the Trp residues (Table III). Molar ellipticities were the same for wild type, Δ1–3, and Δ1–7 (55 to 60 deg cm$^{-2}$ dmol$^{-1}$). However, $\theta_{292}$ for Δ1–9 was considerably smaller than for the other species. This smaller value of $\theta_{292} = 39.7$ deg cm$^{-2}$ dmol$^{-1}$ is similar to that seen for structurally perturbed wild type protein. Thus, wild type in 3 M urea had a $\theta_{292}$ of 48.5 deg cm$^{-2}$ dmol$^{-1}$, in 4 M urea, $\theta_{292}$ = 33.4 deg cm$^{-2}$ dmol$^{-1}$, and in 6 M urea, $\theta_{292}$ = 9.2 deg cm$^{-2}$ dmol$^{-1}$. The decreased molar ellipticity seen for Δ1–9 suggests an altered tertiary structure similar to urea-perturbed wild type.

X-ray Structures of Wild Type and Δ1–7 Show Virtually Identical Structures That Are Not Perturbed by the Crystal Environments

Overall Structures for Wild Type Rhodanese and Δ1–7 Crystallized in the Orthorhombic Crystal Form—A schematic representation of the two domain structure of rhodanese is shown in Fig. 3. The overall structure of the wild type enzyme, crystallized in the orthorhombic crystal form using PEG 6000 as precipitant, is very similar to the structure of the monoclinic form obtained in the presence of ammonium sulfate (data not shown): the root mean square deviation between the Cα positions of the two models is 0.47 Å (Table IV for x-ray statistics). Root mean square differences greater than 1 Å can be observed only at positions 3 and 292, i.e., at the NH$_2$- and COOH-terminal ends, which are usually flexible areas in proteins, and in the region from amino acid residue 194 to 199, a loop exposed to the solvent and involved in intermolecular contacts in the orthorhombic crystal. Finally, helix B (residues 42–50) is also slightly shifted as compared with its position in the monoclinic crystal form. Thus, drastically different crystalization conditions and a different crystal packing do not affect significantly the protein structure. The molecular model of the Δ1–7 mutant, which crystallized isomorphously with the wild type enzyme in the presence of PEG 6000, compares very well with that of the wild type protein (Fig. 4): excluding the first seven amino acids, the Cα of the remaining portion of the two molecules superimpose with a root mean square deviation of 0.23 Å. Appreciable differences can be observed only at the COOH terminus. These findings clearly indicate that the lack of the enzyme NH$_2$ terminus up to residue 7 is not critical for protein folding, in the sense that native conformation can be achieved by the NH$_2$-terminal truncated enzyme. A detailed
The protein surface, establishes several interactions: O4-NH2 of the enzyme shows that the short sequence 1–7, which is located at the active site are shown as corresponding elements of secondary structure in domains I and II are read using the program MOLSCRIPT.

inspection of the region of the NH2 terminus in the wild type enzyme shows that the short sequence 1–7, which is located at the protein surface, establishes several interactions: O4-NH2 of the enzyme shows that the short sequence 1–7, which is located at the active site are shown as corresponding elements of secondary structure in domains I and II are read using the program MOLSCRIPT.

TABLE II

Urea denaturation transition parameters for rhodanese mutants monitored by bis-ANS fluorescence

| Sample          | Point of 1/2 rise to maximum fluorescence intensity* | Point of maximum fluorescence intensity* | Point of 1/2 fall to maximum fluorescence intensity* | Fluorescence intensity at 0 M urea* | Maximum fluorescence intensity* |
|-----------------|------------------------------------------------------|------------------------------------------|------------------------------------------------------|------------------------------------|---------------------------------|
|                 | 0 M urea                                             | 4.4                                     | 4.4                                                  | 0.25                               | 3.5                             |
| Wild type       | 3.4                                                  | 3.7                                     | 3.7                                                  | 0.22                               | 3.5                             |
| Δ1–3            | 3.3                                                  | 3.7                                     | 3.7                                                  | 0.22                               | 3.5                             |
| Δ1–7            | 2.1                                                  | 3.7                                     | 3.7                                                  | 0.22                               | 3.5                             |
| Δ1–9            | 1.9                                                  | 3.0                                     | 3.0                                                  | 0.8                                | 5.4                             |

*a (n) = three separate urea denaturation experiments performed for each sample. The data are averages of these trials.

TABLE III

Molar ellipticities of rhodanese mutants at far (210 nm) and near (292 nm) UV-CD wavelengths

The specified wavelengths were chosen based on characteristic maxima for elliptically polarized light seen for all samples scanned in the UV spectrum. All samples are corrected for the buffer, 50 mM sodium phosphate, pH 7.6. Protein concentrations were determined using A292 readings and BCA assays and ranged from 7 to 8 μM in the θ292 readings and 50–75 μM in the θ210 readings. See “Experimental Procedures” for details.

| Sample          | Molar ellipticity | θ292 | θ210 |
|-----------------|-------------------|------|------|
| Wild type       | -6.83 ± 0.29*     | 54.7 ± 3.4 | 54.7 ± 3.4 |
| Wild type in 2 M urea | 53.8            |          |      |
| Wild type in 3 M urea | 48.5            |          |      |
| Wild type in 4 M urea | 33.4            |          |      |
| Wild type in 6 M urea | 9.2             |          |      |
| Δ1–3            | -6.93 ± 0.15*     | 60.5 ± 5.6 | 60.5 ± 5.6 |
| Δ1–7            | -6.86 ± 0.27*     | 54.5 ± 5.5 | 54.5 ± 5.5 |
| Δ1–9            | -7.06 ± 0.05*     | 39.7 ± 5.7 | 39.7 ± 5.7 |

* Sample trials (n) = 6 ± S.D.
 b Sample trials (n) = 12 ± S.D.
 c Control sample for urea denaturation of wild type monitored only at θ292.

deg · cm² · dmol⁻¹

FIG. 3. Schematic drawing of the folding of rhodanese. Corresponding elements of secondary structure in domains I and II are denoted by capital letters. The sulfur atoms of the persulfide group at the active site are shown as black spheres. The drawing was obtained using the program MOLSCRIPT.

relative to the Δ1–7 mutant (see above and Ref. 7). The space previously occupied by residues 1–7 is not filled with ordered solvent molecules in the crystal of the mutant.

The Active Site—The active sites for the monoclinic and orthorhombic crystal forms of wild type sulfur-substituted rhodanese and for the orthorhombic form of the Δ1–7 sulfur-substituted mutant are highly preserved, especially with regard to protein atoms. The persulfide group at Cys-247 in the sulfur-substituted form of the enzyme is substantially stabilized by a number of hydrogen bonding interactions between both sulfur atoms and neighboring groups at the active site, as established for the monoclinic crystal form (4). Virtually the same hydrogen bonding interactions involving the persulfide group are present in the orthorhombic form of wild type and Δ1–7 mutant rhodanese. Despite the two drastically different crystallization media for the monoclinic and orthorhombic crystal forms, solvent molecules at the active site also show a remarkable conservation. In a previous crystallographic study with monoclinic crystals, the presence of a sulfate ion in close proximity to the positive charges of Arg-186 and Lys-249 residues at the enzyme active site was demonstrated (27). When the mother liquid of the monoclinic crystals of rhodanese containing ammonium sulfate, was exchanged with a cryo-protectant solution devoid of ammonium sulfate and containing PEG 6000 as precipitant (13), the density accounting for the sulfate ion at the active site was replaced by a peak of lower intensity, attributable to a water molecule. This observation is consistent with the finding of a substantial reactivation of the crystalline enzyme inhibited by sulfate, upon replacement of ammonium sulfate with PEG 6000 as precipitant in the crystal mother liquid. Solvent molecules lying in a sphere of radius 10 Å from the S(2) bound to the catalytic cysteine are represented in Fig. 5 and Table V which compares wild type rhodanese and Δ1–7 mutant crystallized in the orthorhombic form. A solvent molecule (O362) is present, at a short distance from S6, in both wild type and mutant proteins and is also present in the monoclinic form (Trp-581 in Fig. 2 in Ref. 13). Such a conserved water molecule might be involved in the catalytic reaction. Six additional water molecules are present in similar positions in both orthorhombic wild type and mutant rhodanese (see the Table V and Fig. 5), forming a network of hydrogen bonds among them and with protein atoms. A very similar situation is observed for the monoclinic crystal form transferred to a cryo-protectant solution devoid of ammonium sulfate (13): only one additional solvent molecule is visible at the active site, despite the significantly higher resolution of that data.

The Cation-binding Site—The presence of a cation-binding site was postulated some time ago (28) and observed in a previous crystallographic study (27) on the basis of a difference
Fourier map from rhodanese crystals soaked in a medium in which ammonium sulfate was replaced by sodium thiosulfate. The cluster of peaks and holes close to Asp-272 were interpreted as due to a Na$^+$ ion, which substituted for an ammonium ion previously present. In both structures of wild type and D1–7 mutant a solvent molecule in this position shows a six-donor coordination: five oxygens and a sulfur atom surround the central atom with a bipyramidal geometry, as shown in Fig. 6 and Table VI. Interatomic distances between oxygens and the cation are in the range 2.48–2.65 Å, expected for this kind of coordination, while distances between cation and the sulfur atom of Met-73 are 3.30–3.17 Å, respectively, for wild type and D1–7 mutant, accounting for the larger radius of the sulfur atom. The peak in the electron density map, although higher than that expected for a water molecule, is not very high (4$s$ in 2$u_F$ obs$^2F_{calc}u$ map and 10$s$ in the $u_F$ obs$^2F_{calc}u$ map): it could correspond to a small cation, like Na$^+$ or K$^+$, present in the buffers used for protein purification and crystallization. The cation site is about 10 Å from the transferred sulfur atom that is bound to Cys-247 and not expected to affect the catalytic activity, in accordance with the finding that cations are not required for catalytic activity (29).

The Molecular Chaperone GroEL Can Slowly Capture D1–9 Rhodanese

GroEL associates with non-native protein conformations by hydrophobic interactions to inhibit off-pathway misfolding of proteins. It does not interact with native proteins (30). Rhodanese mutants were captured by GroEL (17-fold molar excess) in the presence of ADP at room temperature (23 °C) over long periods of time (up to 67 h with precautions to maintain sterile conditions as described under “Experimental Procedures”). Fig. 7 shows a gradual loss of rhodanese activity that is taken to represent binding of the protein to GroEL. This binding correlates with the length of truncation. D1–9 displayed almost complete inactivation at 67 h. The percent inactivations of the samples at 67 h were as follows: wild type, 8.1 ± 3.4%; D1–3, 26.7 ± 4.6%; D1–7, 39.4 ± 1.7%; and D1–9, 86.3 ± 3.3%. Loss of activity in identical controls without GroEL over the same time period was wild type = 4.3 ± 6.9%, D1–3 = 5.3 ± 6.1%, D1–7 = 6.8 ± 0.5%, and D1–9 = 10.8 ± 7.4%. These slight decreases in activity due to factors other than GroEL capture do not substantially alter the conclusions that can be drawn from the data in Fig. 7.

| Table IV | Statistics for x-ray data collection and percentage of reflections measured as a function of resolution |
|----------|------------------------------------------------------------------------------------------|
|          | Wild type | D1–7 mutant |
|          | No. of reflections | Independent reflections | Internal R factor | No. of reflections | Independent reflections | Internal R factor |
| A        | Wild type | D1–7 mutant |
| 60,362   | 10,362    | 11.08        | 109,730      | 13,716    | 10.4         |

| Resolution interval | Wild type | D1–7 mutant |
|---------------------|-----------|-------------|
|                     | Independent reflections | % | Independent reflections | % |
| 00–4.4              | 1,822     | 96.8        | 1875       | 99.6    |
| 4.4–3.49            | 1,707     | 95.8        | 1785       | 99.8    |
| 3.49–3.05           | 1,689     | 97.3        | 1739       | 99.8    |
| 3.05–2.77           | 1,682     | 97.0        | 1736       | 99.9    |
| 2.77–2.57           | 1,667     | 96.4        | 1730       | 99.9    |
| 2.57–2.42           | 1,569     | 91.1        | 1723       | 99.9    |
| 2.42–2.30           | 226       | 13.4        | 1694       | 99.3    |
| 2.30–2.20           | 1299      | 77.5        |            |         |

* Total number of measured reflections, number of independent reflections and internal R factor (defined as $R = \sqrt{\sum(\text{obs} - \text{calc})^2 / \sum\text{obs}^2}$, where $\text{obs}$ is the intensity observed in the i$\text{th}$ source and $\text{calc}$ the intensity in the j$\text{th}$ source).

* Independent reflections as a function of resolution and percentages of reflection measured.
Rhodanese-GroEL complexes formed by addition of denatured wild type enzyme can be dissociated with regain of activity by addition of GroES and ATP (31). In Fig. 8, the slow capture experiments were extended to 90 h followed by addition of GroES and ATP. The 0-h time point is the activity of each sample after the 90-h capture period compared with controls without GroEL. Wild type activity increased 26% from 65.8 ± 1.5% to 92.1 ± 3.1% after 1 h of incubation with GroES and ATP. The 92.1 ± 3.1% of wild type activity was near the averaged maximum activity recovered, 94.4 ± 2.6% at 42 h. At the same time Δ1–3 also had a 26% recovery (53.7 ± 0.6% to 80.4 ± 1.6%), but Δ1–7 and Δ1–9 each had only a 10% recovery of activity (49.0 ± 0.3 to 60.1 ± 4.0% and 9.8 ± 1.1 to 20.9 ± 1.6%, respectively). 24 h later Δ1–3 released another 10% (80.4 ± 4.1% to 90.8 ± 3.8%) to near its averaged maximum recoverable activity of 92.1 ± 3.6%, and Δ1–7 and Δ1–9 each recovered another ~15% activity (60.1 ± 4.0 to 75.5 ± 3.0% and 20.9 ± 1.6% to 38.4 ± 3.5%, respectively). Addition of fresh ATP released another 10% of Δ1–7 activity and 30% of Δ1–9 activity to their respective maximum recoverable activities of 85.7 ± 4.1 and 72.9 ± 3.4%.

Although the binding of Δ1–9 is quite slow under native conditions, Fig. 9 shows that GroEL can assist similarly the refolding of denatured wild type rhodanese and the truncation mutants Δ1–3, Δ1–7, and Δ1–9 in the standard assay for GroEL function. The extent of refolding is approximately the same for all species, and, although the rates of refolding appear to be somewhat slower the longer the truncation, the process occurs within a similar time. Thus, while the wild type protein completed folding within 5 min, the mutant Δ1–9 was only 50% folded at that time.

**DISCUSSION**

Rhodanese truncation mutants lacking the first 7 or 9 NH₂-terminal residues were previously shown to have virtually indistinguishable catalytic constants, but changes in structural integrity were suggested by subtle differences in the reactivities of the active site Cys-247 (7). The present studies show that these species are less stable to urea-induced unfolding than the wild type enzyme. Extrapolation of urea unfolding data show decreases in the free energies of stabilization for Δ1–7 and Δ1–9 relative to wild type rhodanese of 1 and 2 kcal/mol, respectively. Even in the absence of perturbation, the intrinsic fluorescence wavelength maxima of these mutants were slightly red-shifted. This increased tryptophan exposure may be due to partial uncovering of Trp-112 and Trp-113, which are located just below the sequence Arg-7 to Leu-9 in the wild type protein. These suggestions are supported by the x-ray structure of Δ1–7, which is virtually identical to that of the wild type protein with the exception of the missing residues. The crystal data suggest a high degree of rigidity of sulfur-substituted

**TABLE V**

| Wild type | Δ1–7 mutant |
|-----------|-------------|
| 362 O-S5 247 | 3.24 | 3.57 |
| 316 O-O1 252 | 2.81 | 2.74 |
| 316 O-332 O | 3.65 | 2.95 |
| 339 O-Na 186 | 3.48 | 3.25 |
| 362 O-339 O | 2.67 | 2.94 |
| 362 O-334 O | 2.90 | 2.82 |
| 334 O-OH 107 | 2.98 | 2.95 |
| O 363-Na 249 | 2.98 | 3.00 |
| 332 O-Na 186 | 2.96 | 3.33 |
| 339 O-361 O | 2.79 | 2.82 |

**FIG. 5.** View of a portion of the active site of wild type rhodanese (a) and Δ1–7 mutant (b), with the solvent molecules in the active site cavity. Symbols for atoms are: C, O, N, and S.

**TABLE V**

| Distances (Å) from protein to solvent atoms |
|-------------------------------------------|
| Wild type | Δ1–7 mutant |
|-----------|-------------|
| 362 O-S5 247 | 3.24 | 3.57 |
| 316 O-O1 252 | 2.81 | 2.74 |
| 316 O-332 O | 3.65 | 2.95 |
| 339 O-Na 186 | 3.48 | 3.25 |
| 362 O-339 O | 2.67 | 2.94 |
| 362 O-334 O | 2.90 | 2.82 |
| 334 O-OH 107 | 2.98 | 2.95 |
| O 363-Na 249 | 2.98 | 3.00 |
| 332 O-Na 186 | 2.96 | 3.33 |
| 339 O-361 O | 2.79 | 2.82 |
rhodanese in its native conformation. In particular, the two-domain structure of rhodanese is not significantly affected by drastically different crystallization conditions and crystal packing or by the lack of seven NH₂-terminal residues. The extent of conservation is very high for the active site region, with regard to both protein atoms and solvent molecules surrounding the active center. It is likely that the initial structural changes that occur on urea perturbation involve perturbation of the interdomain interface and separation of the domains. This would be inhibited in the crystal. This interpretation would reconcile the x-ray information and the numerous solution studies of rhodanese.

The results with bis-ANS further support these conclusions. The urea dependence of the bis-ANS fluorescence with wild type rhodanese has been reported (19). Initial intensities are related to the hydrophobic exposure on the unperturbed enzyme. The increasing intensities at the lower urea concentrations have been ascribed to the formation of a folding intermediate. The decreasing intensities as the urea is increased further represent the unfolding of these intermediates. These opposing phenomena give rise to transitions of the type observed in Fig. 2. The present results can be interpreted in terms of this model. Even in the absence of perturbation, there is slightly more hydrophobic surface accessible on Δ1–7 and Δ1–9 than on the wild type protein. Fig. 2 shows that there are greater differences in the rising phases of the intensities than in the falling phases. This would indicate that the longer truncations make it easier to form intermediates that bind bis-ANS.

This latter conclusion is supported by the observation that the

FIG. 6. View of the cation-binding site in the crystal of wild type rhodanese, showing the distorted bipyramidal coordination of the cation. Atom symbols as in Fig. 5 and Table V. A very similar situation is present in the crystal of Δ1–7 mutant.

TABLE VI

Distances of protein atoms from the cation (Å)

| Protein atom | Wild type | Δ1–7 mutant |
|--------------|-----------|-------------|
| O Glu-71     | 2.62      | 2.58        |
| O Val-72     | 2.52      | 2.58        |
| Sδ Met-73    | 3.30      | 3.17        |
| O Lys-249    | 2.58      | 2.60        |
| O Asp-272    | 2.51      | 2.48        |
| O Asp-272    | 2.51      | 2.48        |

FIG. 7. GroEL slow capture of rhodanese mutant proteins. Interaction of GroEL with rhodanese mutants led to inactivation of enzymatic catalysis followed over time. Activity is given as percent of an identical parallel control sample containing no GroEL. All data points are averages of three separate determinations with standard deviations. Loss of activity in control samples without GroEL for the entire time span of the experiment was not significant.

FIG. 8. GroES slow release of GroEL-captured rhodanese mutant proteins. Release of GroEL-captured rhodanese mutants by addition of GroES and ATP resulted in reactivation followed over time. Activity is given as percent of an identical parallel control sample containing no GroEL as detailed under “Experimental Procedures.” The 0-h time point gives percent activity after a 90-h incubation at 23 °C compared with parallel controls without GroEL. All data points are averages of three separate determinations with standard deviations. The experiments were stopped before loss of activity in control samples without GroEL became significant.
decrease in molar ellipticity for rhodanese structures to adopt conformations that could be detected. These changes occurred while there was no noticeable change in the secondary structures as reported by the far UV-CD. These changes occurred while there was no noticeable change in the secondary structures as reported by the far UV-CD.

These observations are consistent with the x-ray structure that shows the truncations uncover a hydrophobic cluster that lies below residues Arg-7 to Leu-9. The small increases in hydrophobic accessibility on the unperturbed truncation mutants are not sufficient to cause association of rhodanese molecules as demonstrated by ultracentrifugation and gel filtration, which demonstrate that each of the proteins used in this study is homogeneous and monomeric.

Circular dichroism studies provide additional evidence for changes in the tertiary structures induced by the truncations. The near UV-CD molar ellipticities (Table III) show that the tertiary interactions around the tryptophan residues of Δ1–9 are similar to a partially unfolded wild-type structure observed in a urea concentration between 3 and 4 M. This substantial decrease in molar ellipticity for Δ1–9 in the near UV reports a significant alteration in the environments formed by the tertiary fold of the protein at least some of the tryptophans.

The x-ray structure shows a complex set of interactions among the aromatic residues in rhodanese, a number of which could be expected to respond to the truncations described here. Thus, Tyr-6 is close to Tyr-261, which in turn is close to Trp-112 and Trp-113. In addition, Arg-6 is close to Trp-14. Thus, these interactions are expected to be altered in addition to uncovering Trp-112 and -115 by the truncations described here.

The molecular chaperone GroEL binds tightly to folding intermediates of rhodanese, but it does not interact with native wild type. Differences in stability of the species can be translated into differences in the equilibrium, in the absence of perturbation, between native and partially folded or denatured protein conformers. Thus, the ability of GroEL to capture rhodanese was used to detect differences in the ability of native rhodanese structures to adopt conformations that could be recognized. More than 85% of Δ1–9 was captured by GroEL under conditions where less than 11% of wild type was captured. The Δ1–7 mutant could also be bound by GroEL, but it is less able to be bound than Δ1–9. The slow rates of association presumably follow from the fact that, even though the intermediates are more accessible from the native state, they are present at low concentrations at equilibrium. These findings lend additional support to the view that the NH2-terminal sequence of rhodanese makes a measurable contribution to the conformational stability of the entire protein. The results with GroEL refolding of rhodanese show that the yields are equal when the various mutants are used, but the rates are somewhat slower. This would follow, because the less stable mutants which more easily form intermediates that can bind to GroEL would be expected to have lower off-rates from the complex and faster recapture rates. The suggestion that rhodanese bound to GroEL has been studied and suggested to be in the form of a molten globule state in which the individual domains are folded but not properly associated, to give a structure with the characteristics of a molten globule (32). This is consistent with the suggestions above that perturbations induce separation of domains that remain largely folded.

The present results provide evidence that NH2-terminal truncation in rhodanese leads to decreased conformational stability. This structural lability extends previous findings that truncated rhodanese species can fold to fully active enzymes that have altered in vivo survivability after expression and differences in active site reactivities. All the results are consistent with a model in which a folding intermediate of rhodanese consists of independently folded domains that are not optimally associated leaving easily exposed hydrophobic surfaces. The formation of this intermediate would be favored by NH2-terminal truncation. The separation of these domains requires perturbation under normal solution conditions, and it would be inhibited by contacts in the crystal. As an example of the biological consequences of the effects we have described, the labilization of the rhodanese structure by removal of the influence of its NH2-terminal sequence suggests that the interaction of this sequence with the mitochondrial import machinery could similarly loosen the protein and help maintain the protein in an import competent conformation required for the successful compartmentation of this enzyme.

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