Truncations at the NH$_2$ Terminus of Rhodanese Destabilize the Enzyme and Decrease Its Heterologous Expression*

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Rhodanese mutants containing sequential NH$_2$-terminal deletions were constructed to test the distinct contributions of this region of the protein to expression, folding, and stability. The results indicate that the first 11 residues are nonessential for folding to the active conformation, but they are necessary for attaining an active, stable structure when expressed in Escherichia coli. Rhodanese species with up to 9 residues deleted were expressed and purified. Kinetic parameters for the mutants were similar to those of the full-length enzyme. Compared with shorter truncations, mutants missing 7 or 9 residues were (a) increasingly inactivated by urea denaturation, (b) more susceptible to inactivation by dithiothreitol, (c) less able to be reactivated, and (d) less rapidly inactivated by incubation at 37 °C. Immunoprecipitation showed that mutants lacking 10–23 NH$_2$-terminal amino acids were expressed as inactive species of the expected size but were rapidly eliminated. Cell-free transcription/translation at 37 °C showed mutants deleted through residue 9 were enzymatically active, but they were inactive when deleted further, just as in vivo. However, at 30 °C in vitro, both Δ1-10 and Δ1-11 showed considerable activity. Truncations in the NH$_2$ terminus affect the chemical stability of the distantly located active site. Residues Ser-11 through Gly-22, which form the NH$_2$-proximal α-helix, contribute to folding to an active conformation, to resisting degradation during heterologous expression, and to chemical stability in vitro.

The enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) catalyzes in vitro the transfer of sulfur from thiosulfate to a number of acceptors including cyanide, lipoic acid, or dithiothreitol (1). The protein is imported into the matrix of the mitochondrion from its site of translation in the cytosol without proteolytic processing of its NH$_2$ terminus (2). Rhodanese has become an important model for studying issues related to the problems of protein folding (3, 4). This monomeric protein is folded into two similar independent, equal-sized domains. The NH$_2$-terminal sequence of rhodanese is located around position 17 with proline. The purified protein showed increased susceptibility to denaturation by urea and a decreased efficiency of refolding in vitro (12). However, the mutant protein was stable, active, and purifiable from Escherichia coli. The E17P mutant does not directly address the role of the NH$_2$-terminal segment in folding to a stable conformation during heterologous expression.

We expressed NH$_2$-terminal truncations of rhodanese in E. coli and in an E. coli-based, cell-free transcription/translation system to investigate contributions of the NH$_2$-terminal segment to folding, stability, and resistance to degradation during heterologous expression. E. coli is considered particularly appropriate for rhodanese, since the bacterial cytosol provides a folding environment similar to the mitochondrial matrix (13, 14), and many useful recombinant proteins are produced in E. coli.
Rhodanese Is Stabilized by Its NH$_2$-Terminal Sequence

TABLE I

| NH$_2$-terminal rhodanese truncations |
|--------------------------------------|
| (M) V H Q V L Y R A L V S T K W L A E S V R A G K . . . WT |
| M V L Y R A L V S T K W L A E S V R A G K . . . Δ 1–3 |
| M Y R A L V S T K W L A E S V R A G K . . . Δ 1–5 |
| "M' Y R A L V S T K W L A E S V R A G K . . . Δ 1–5 Y6A |
| (M) A L V S T K W L A E S V R A G K . . . Δ 1–7 |
| M V S T K W L A E S V R A G K . . . Δ 1–9 |
| "M' Y S T K W L A E S V R A G K . . . Δ 1–9 |
| M D T K W L A E S V R A G K . . . Δ 1–10 S11D |
| "M' T K W L A E S V R A G K . . . Δ 1–11 |
| M' D T K W L A E S V R A G K . . . Δ 1–11 T12D |
| "M' K W L A E S V R A G K . . . Δ 1–12 |
| M' A W L A E S V R A G K . . . Δ 1–12 K13A |
| "M' A E S V R A G K . . . Δ 1–15 |
| "M' V R A G K . . . Δ 1–18 |
| "M' A G K . . . Δ 1–20 |
| "M' Δ 1–23 |

coli host cells. Full-length rhodanese is stable and active when expressed in *E. coli* (15). We now report that the first 9 residues of the NH$_2$-terminus of rhodanese are nonessential for the activity or resistance to elimination of the protein expressed in *E. coli*. Deletions of 7 or 9 NH$_2$-terminal residues alter the chemical stability of the isolated protein. Deletions of residues 10 and beyond resulted in loss of activity when the protein was expressed in *E. coli* at 37 °C. These same mutant proteins were not degraded in the cell-free system, and they were synthesized in similar quantities without degradation at either 30 or 37 °C. At 37 °C, the synthesized rhodanese was inactive when 10 or more NH$_2$-terminal residues were removed. Expression at 30 °C, however, resulted in active protein even when 11 residues were deleted, showing that under conducive conditions rhodanese can fold to an active conformation even when the truncation extends into the first α-helix. In sum, the interactions involving the NH$_2$-terminal sequence of rhodanese can have global effects on the protein altering the reactivity of the active site.

EXPERIMENTAL PROCEDURES

Materials—All chemicals (analytical grade) and antibodies were obtained from Sigma. All media components were purchased from Life Technologies, Inc. H$_2$SO$_4$ and H$^{14}$C leucine were supplied by NEN Life Science Products. Polyclonal antibodies were previously described (16). Oligonucleotide primers were made by Genosys or Life Technologies, Inc. Restriction endonucleases were obtained from New England Biolabs. Chromatographic steps for purification of mutant proteins were performed using a Pharmacia fast protein liquid chromatography system.

Construction of Deletion Mutants—Polymerase chain reaction-based mutagenesis techniques were employed to make the NH$_2$-terminal truncation mutants (17, 18). Rhodanese numbering follows the originally determined protein sequence (1), which omits the initiator methionine (15). In the polymerase chain reaction used, we used the plasmid pCRM (15) as a template and designed primers flanking each respective truncation mutant, which added 5' Neol or PstI and 3' BamHI restriction enzyme sites. This enabled us to clone the polymerase chain reaction fragments (truncated rhodanese coding regions) into the polylinker of pTTQ19 (Amer sham). An Neol site was added between the XbaI and BamHI sites in the original pTTQ19 for mutants that required it. The same 3'-flanking primer was used in all reactions: 5'-GCC ATG GAC ACC AAG TGG CTG GCC TCT GCC CGG GCT; Δ1–11, 5'-GCC ATG CAG ATG ACC AAG TGG CTG GCC TAC GAG TCC GTG CGG TCT CGG CGG GTG; Δ1–11 T12D, 5'-GCC ATG ACC AAG TGG CTG GCC TCT GCC CGG GCT CGG CTG GCC CGT GCT CGG CGT; Δ1–12, 5'-GCC ATG CAG ATG AAG TGG CTG GCC TCT GCC GAG TCC GTG; Δ1–12 K13A, 5'-GCC ATG CTG TCG GCC TCT GCC CGG GCT GGT; Δ1–15, 5'-GCC ATG GCC TGCC GCC GCT GTG AAG ATG GGC GCC GCT; Δ1–20, 5'-GCC ATG GCC GTG AAG ATG GCC GCC CGG GCT GGT; Δ1–23, 5'-GCC GGA TCC TTA CGA CCT GCC CCC ATC CCC. This primer incorporated an additional *E. coli*-specific stop codon (TAA) to inhibit potential ribosomal read-through of the bovine rhodanese stop codon (TGA). The GI–23 mutant coding region was transferred from a previous construct (19) into pTTQ19. All deletions were verified by DNA cycle sequencing (20). Table I gives the amino acid sequences for the NH$_2$-terminal segments of all truncation mutants.

Expression in *E. coli*—The constructed vectors were used to transform a BL21 strain of *E. coli* (Novagen), which is lon protein-defective but degrades aberrantly folded proteins (21–25). No mutant had an NH$_2$-terminal amino acid residue associated with rapid degradation in *E. coli* (26–29). Transformed cells were grown to mid-log phase in M9ZB medium (1% tryptone (w/v), 0.5% yeast extract (w/v), 85 mM NaCl, 20 mM NH$_4$Cl, 2 mM KH$_2$PO$_4$, 4 mM Na$_2$HPO$_4$, 0.2% glucose (w/v), 1 mM MgSO$_4$, 50 μg/ml ampicillin, 35 μg/ml chloramphenicol) and induced to express rhodanese with 1 mM D-thiogalactopyranoside. Activity was measured 2 h after induction.

Immunoprecipitation of Expressed, Radiolabeled Proteins—After a 2 h induction period, samples were centrifuged and the medium decanted. The cell pellets were resuspended and grown in radioactive M9 minimal medium (20 μCi/ml H$_2$SO$_4$, 40 mM Na$_2$HPO$_4$, 20 mM KH$_2$PO$_4$, 10 mM NaCl, 20 mM NH$_4$Cl, 0.2% glucose (w/v), 0.1 mM CaCl$_2$).

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MBS, 4-morpholinenethanesulfonic acid; DTTr, dithiothreitol.
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50 μg/ml ampicillin, 35 μg/ml chloramphenicol, 150 μg/ml rifampicin, pH 7.4) for 15 min. The samples were then switched to nonradioactive M9 medium containing 2 mM MgSO₄. Parallel samples without radio-labeling were used for activity determinations.

At time points after the addition of nonradioactive medium, 1-ml aliquots were harvested and the supernatants decanted. The pellets were resuspended in 100 μl of Laemmli sample buffer (34) and placed in a boiling water bath for 5 min. Samples were then centrifuged to pellet insoluble debris, and the supernatants were immunoprecipitated at 4 °C by adding 900 μl of immunoprecipitation buffer (100 mM KCl, 100 mM MgCl₂, 1% Triton X-100 (v/v), 0.5% SDS (w/v)). Polyclonal antibodies raised against wild-type rhodanese was added (1:1000 dilution) and after 18 h the samples were briefly centrifuged. The pellets were discarded, and the supernatants were mixed with 100 μl of a 50% (v/v) suspension of protein A-Sepharose (Sigma) equilibrated with immunoprecipitation buffer. After 2 h of incubation with the protein A-Sepharose, the samples were centrifuged, and the pellets were washed with 1 ml of immunoprecipitation buffer three separate times. The washed pellets were resuspended in 30 μl of Laemmli sample buffer, incubated 5 min in a boiling water bath, and centrifuged. The pellets were discarded, and the supernatants were subjected to SDS-PAGE. The gels were fixed, treated with En′hance (Dupont), dried, and exposed to autoradiographic film (Kodak) or analyzed with a PhosphorImager (Molecular Dynamics). Signal intensities were quantified using NIH Image software or ImageQuant software (Molecular Dynamics).

Mutant Protein Purification—Purification protocols for Δ1–3, Δ1–7, and Δ1–9 mutant proteins were based on the method described previously to purify recombinant rhodanese (35). The procedure was unchanged up to the point of dialysis in low ionic strength buffer of the resuspended pellet from the final (NH₄)₂SO₄ precipitations of the cellular lysates. During dialysis, the mutant proteins formed precipitates that contained the enzyme activity. These precipitates were extracted with 1.5 mM (NH₄)₂SO₄, 5 mM Na₂S₂O₃, 5 mM NaC₂H₃O₂, 10 mM MES, 1 mM EDTA, pH 5.0, and applied to a hydrophobic interaction column (Pharmacia CL-4B phenyl Sepharose) equilibrated with the extraction buffer. Elution of the active mutant proteins was performed by adding a 0–100% gradient with an increase of 1.5% of the elution buffer for every milliliter added to the column. SDS-PAGE revealed significant contamination of the eluted active fractions, which were combined and dialyzed in the above elution buffer prior to loading onto a dye-binding column (Bio-Rad Affi-Gel Blue) equilibrated in the same buffer (36). Elution of the active mutant proteins was achieved by adding 1.5 mM NaCl, 5 mM Na₂S₂O₃, 5 mM NaC₂H₃O₂, 10 mM MES, 1 mM EDTA, pH 5.0, to the previous gradient conditions. The Δ1–3 and Δ1–7 mutant proteins were free of contaminating proteins as judged by an SDS-PAGE gel stained with AgNO₃. Δ1–9 still contained three to four lower molecular weight proteins, which were not detected in a Western blot using polyclonal antibodies raised against wild-type rhodanese. The active mutant proteins were precipitated with 2.5 M (NH₄)₂SO₄ and were stored at −70 °C.

NH₂ Terminus Analysis—The amino-terminal sequence of each purified, mutant protein was determined using an Applied Biosystems 477A Sequence coupled to an ABI 120 HPLC Analyzer using a reversed phase C18 PTH column.

Enzyme Kinetic Determinations—Absorbance at 280 nm of 1.75 cm⁻¹ at 1 mg/ml was used to determine concentrations of the rhodanese species that were purified (up to Δ1–9) (37). These purified mutants (up to Δ1–9) that were analyzed retained all tryptophan residues found in the wild type. Activity was determined using a standard rhodanese assay (33). The formation of SCN⁻ was determined as ferric thiocyanate with a molar extinction coefficient at 480 nm of 4.2 mmol⁻¹ cm⁻¹. For determination of Kᵣ, the concentration of thiourea was varied between 1 and 10 mM, and the data were fit to the equation \( V = V_{max} \times \frac{[\text{thiourea}]}{[\text{thiourea}]+K_r} \), where \( V \) is the observed reaction velocity and \( V_{max} \) is the maximum velocity in μmol of thiocyanate min⁻¹ mg⁻¹. No significant enzymatic activity was detected in cell lysates of induced control samples transformed with empty vector.

Inactivation and Reactivation of Mutant Enzymes—Spontaneous inactivation caused by oxidation of the active site cysteine or dithiothreitol (DTT)-induced inactivation (38, 39) was performed separately with 1.5 μM protein in 50 mM Tris-HCl, pH 7.8. Rates of spontaneous inactivation were determined by comparing activities of samples at 37 °C with parallel control samples at 23 °C. DTT inactivation was performed by adding 15 mM DTT to the samples and incubating for 30 min at 37 °C and comparing with samples without DTT. Reactivation of DTT-inactivated samples was measured by adding 50 mM Na₂S₂O₃, 10 mM MES, 1 mM EDTA, pH 5.0, to a 0–100% gradient with an increase of 1.5% of the elution buffer for every milliliter added to the column. SDS-PAGE revealed significant contamination of the eluted active fractions, which were combined and dialyzed in the above elution buffer prior to loading onto a dye-binding column (Bio-Rad Affi-Gel Blue) equilibrated in the same buffer (36). Elution of the active mutant proteins was achieved by adding 1.5 mM NaCl, 5 mM Na₂S₂O₃, 5 mM NaC₂H₃O₂, 10 mM MES, 1 mM EDTA, pH 5.0, to the previous gradient conditions. The Δ1–3 and Δ1–7 mutant proteins were free of contaminating proteins as judged by an SDS-PAGE gel stained with AgNO₃. Δ1–9 still contained three to four lower molecular weight proteins, which were not detected in a Western blot using polyclonal antibodies raised against wild-type rhodanese. The active mutant proteins were precipitated with 2.5 M (NH₄)₂SO₄ and were stored at −70 °C.

RESULTS

Expression in E. coli—Activities in cell lysates are shown in Table II. Deletions through 9 amino acid residues from the NH₂-terminus of rhodanese resulted in levels of activity similar to the wild-type control. Deletions past 9 residues resulted in no detectable activity compared with the negative control at 37 °C. Fig. 1 shows a plot of activity as a function of time at 37 °C for

| Sample | Expression at 37 °C | Expression at 30 °C |
|--------|---------------------|---------------------|
| Wild type | 152 ± 15 | 31 ± 3 |
| Δ1–5 | 147 ± 13 | 28 ± 1 |
| Δ1–5 Y6A | 166 ± 24 | 26 ± 2 |
| Δ1–7 | 136 ± 14 | 45 ± 4 |
| Δ1–9 | 138 ± 26 | 59 ± 4 |
| Δ1–10 | 7 ± 1 | 11 ± 1 |
| Mock | 11 ± 2 | 6 ± 1 |

*The following mutants tested in this work were not significantly different from the mock-transformed control: Δ1–11, Δ1–11 T12D, Δ1–12, Δ1–12 K13A, Δ1–15, Δ1–18, Δ1–20, Δ1–33.*

**Fig. 1.** Activities of stable rhodanese truncation mutants expressed in E. coli. Cell lysates were assayed for activity over a period of 4 h after the 2-h induction as described under “Experimental Procedures.” Activity averages of at least three determinations at 0, 5, 15, 30, 60, and 240 min are plotted. Standard deviations were not significant. Error bars are omitted for clarity.
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Expression in the Cell-free System—Wild-type and selected truncation mutants were expressed at 37 or 30 °C in a cell-free, coupled transcription/translation system derived from E. coli. At 37 °C, activity (Table III) was undetectable when 10 or more residues were deleted, which was similar to results obtained when these mutants were expressed in E. coli. At 30 °C, activity was detected with up to 11 residues deleted. All samples contained similar amounts of protein, and little or no degradation of mutant proteins was observed (data not shown).

Purification of Rhodanese Truncation Mutants—Mutant proteins not eliminated in E. coli were purified to homogeneity to compare their catalytic characteristics. The purification method for the wild-type enzyme required some modification, since the mutant proteins tended to precipitate at lower (NH₄)₂SO₄ concentrations than wild-type rhodanese. Both hydrophobic interaction chromatography and dye-binding affinity chromatography were needed to purify Δ1–3 and Δ1–7, whereas wild type could be purified to apparent homogeneity using only cation exchange chromatography. Purification of Δ1–9 required all three chromatographic steps. As seen in Table IV, the final yields were greatest for wild type and Δ1–3. The percent recoveries for Δ1–7 and Δ1–9 were significantly less, with Δ1–9 being the most difficult to separate from contaminating proteins.

Kinetics and Stability Measurements of Purified Truncation Mutants—Table IV shows that the $K_m$ values and specific activities for the purified mutants did not differ significantly from wild type. The concentrations of urea representing the denaturation transition midpoints ($U_{1/2}$) shown in Fig. 4 were the same for wild type and Δ1–3 (each 3.6 M). However, for Δ1–7 ($U_{1/2} = 2.9$ M) and Δ1–9 ($U_{1/2} = 2.45$ M) there was a progressive shift to lower urea concentrations. Values for the free energy of unfolding estimated for each protein at 0 M urea buffer conditions ($ΔG^\ddagger$) and the measure of dependence of $ΔG$ on urea concentration (m) were significantly lower for Δ1–9 and Δ1–7 than for wild type and Δ1–3 (See Table IV).

Inactivation and Reactivation of Purified Truncation Mutants—Although DTT is a reductant, at low DTT and low protein concentrations, it has the paradoxical effect of inactivating rhodanese by a complex series of reactions at the active site that include oxidation (38, 39). This has been ascribed to...
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The facts that 1) reduced DTT is analogous to the dithiol acceptor substrate, dihydrolipoic acid, and it can remove the persulfide sulfur from the rhodanese active site to produce a labile form of the enzyme; 2) the autoxidation of DTT leads to formation of partially reduced reactive oxygen species such as hydrogen peroxide, which can oxidize the active site of rhodanese, thereby inactivating the enzyme through a series of states that become progressively refractory to reduction; and 3) DTT, through its resemblance to dihydrolipoic acid, can specifically interact with the active site and form a stabilized disulfide-bonded adduct. Fig. 5 displays the percent activity of rhodanese as a function of DTT concentration. At the lowest DTT concentration of 20 μM, 50% of wild type was inactivated, whereas no more than 10% of any of the mutants was inactivated. The remaining 50% of wild-type activity was more resistant to inactivation, but complete inactivation was achieved at 15 mM DTT (data not shown). Relative to this resistant phase of the wild-type inactivation, the mutants were less sensitive with Δ1–3 being more sensitive than Δ1–7 and Δ1–9, which were almost equal. The relative sensitivity of the proteins can be defined in terms of the DTT concentration giving 50% inactivation. These were as follows: wild type (low sensitivity response), 7.5 mM; Δ1–3, 1.3 mM; Δ1–7 and Δ1–9, 0.25 mM.

The ability of the sulfur-donor substrate, Na₂S₂O₃, to reactivate the DTT-inactivated enzymes was studied. Fig. 6 displays the time dependence of the reactivation of rhodanese species that had been completely inactivated by DTT. Wild type and Δ1–3 could be reactivated by Na₂S₂O₃ to 55–60% activity, whereas Δ1–7 was reactivated to 40%. On the other hand, Δ1–9 had a maximum reactivation of only 6%, suggesting that the active site of Δ1–9 was able to be more highly oxidized, e.g., to a sulfenic acid, so that thiosulfate reactivation was less effective. It is interesting that the maximum observed reactivation was only to a level that was observed for the less sensitive inactivation component of the wild-type protein.

Spontaneous inactivation at elevated temperature reflects reactivity at the active site of rhodanese (41, 42). The spontaneous inactivation of the mutant and wild-type enzymes is shown in Fig. 7. Wild-type samples were inactivated in 2 h, whereas for Δ1–3, inactivation took 6 h. Δ1–7 and Δ1–9 were incompletely inactivated even after 6 h at 37 °C. Thus, the mutants were much less sensitive to oxidation than the wild-type protein.

### Discussion

It has been speculated that the amino-terminal 23 residues of mitochondrial rhodanese contribute to the global stability of the folded protein. In one study, for example, an E17P mutant was active but less stable than wild-type rhodanese, a result that was suggested to be due to destabilization of the α-helix proximal to the amino terminus (12). In another study, limited tryptic digestion could produce an active rhodanese in which the peptide composed of the NH₂-terminal 45 residues was non-covalently bound. After denaturation, this form of the enzyme was not able to refold under conditions appropriate for reactivation of the native enzyme. These results were taken to indicate that portions of the NH₂ terminus are important for the folding of the enzyme to an active form (43). The present studies used site-directed deletions to extend these earlier suggestions and to investigate effects including (a) the role of the NH₂-terminal region of rhodanese in the expression of active enzyme, i.e., its resistance to elimination in vivo; (b) the effect on the enzyme kinetic parameters of active mutants; and (c) the stabilities of mutants that could be purified.

Active rhodanese was expressed with deletions of up to the first nine residues. The crystal structure of wild-type rhodanese shows that these residues are situated parallel to, and form part of, the bi-domain interface, and they are the only residues out of the first 23 that interact hydrophobically with the carboxyl domain. These residues participate in a hydrophobic cluster that includes the following interactions: His-2 with Pro-266 and Asp-267; Leu-5 with Tyr-261, Leu-262, Gly-264, and Pro-266; Tyr-6 with Tyr-261; Ala-8 with Leu-258, Tyr-261, and Leu-262; and Leu-9 with Glu-222, Leu-258, and Leu-262. Hydrogen bonding of the first 9 residues is primarily with other residues of the NH₂ domain. Tyr-6 is the only one of these that H-bonds to the carboxyl domain, and it makes two H bonds to Tyr-261. Thus, it could be expected that these residues may be important for acquisition or maintenance of the active enzyme. Interestingly, the present results show that these residues are non-essential for the enzymatically active conformation during heterologous expression in E. coli or for the development of the kinetic parameters that characterize the wild-type enzyme. However, the results do indicate that deletions through residue

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**Table III**

| Sample | Expression at 37 °C | Expression at 30 °C |
|--------|---------------------|---------------------|
|        | μmol min⁻¹ mg⁻¹  | μmol min⁻¹ mg⁻¹  |
| Wild type | 874 ± 205  | 226 ± 34  |
| Δ 1–3 | 892 ± 171  | ND  |
| Δ 1–5 | 700 ± 192  | ND  |
| Δ 1–9 | 1316 ± 366 | ND  |
| Δ 1–10 | 0  | 225 ± 45  |
| Δ 1–11 | 0  | 86 ± 18  |
| Δ 1–15 | 0  | 0  |

**Table IV**

| Protein sample | % Yield of activity | Kₘ | Specific Activity | U₁/₂ | ΔG°¹⁄₂O | m value |
|----------------|-------------------|----|------------------|-------|---------|---------|
|                |                   | μM | μmol min⁻¹ mg⁻¹ | μ of area | kcal mol⁻¹ | kcal mol⁻¹ s⁻¹ |
| Wild type      | 25                | 12.4 ± 3.6 | 732 ± 63 | 3.6 | 12.8 ± 2.2 | 3.50 ± 0.35 |
| Δ 1–3          | 28                | 6.4 ± 2.6 | 702 ± 56 | 3.6 | 11.6 ± 0.7 | 3.22 ± 0.11 |
| Δ 1–7          | 15                | 31.8 ± 17 | 767 ± 69 | 2.9 | 7.7 ± 0.3 | 2.66 ± 0.11 |
| Δ 1–9          | 8                 | 8.9 ± 1.2 | 778 ± 43 | 2.45 | 6.5 ± 0.4 | 2.65 ± 0.16 |
9 may have pronounced effects on global stability of rhodanese.

The crystal structure shows that residues 11–22 form an amphipathic α-helix that interacts hydrophobically solely with the NH₂-terminal domain. In our experiments, truncations through Val-10 and beyond to Lys-23 resulted in mutant proteins that were rapidly degraded when expressed in E. coli.

Deletion of the residues immediately preceding the start of the helix at S11 may destabilize the N-capping sequence region of the α-helix, which is oriented perpendicular to the bi-domain interface with the N-cap region closest to, and forming part of, the interface. Other interactions made by the α-helix are with residues in the region spanning from Ser-124 to His-138, which are also located predominately at the bi-domain interface region. Thus, it is likely that changes in the NH₂-terminal sequence can perturb the global structure not only through effects on the NH₂-terminal domain but also through effects on the bi-domain interface. That these effects cannot occur by direct interaction is suggested by the fact that Val-10 is >18 Å from the active site sulphhydryl group.

All mutant proteins with deletions through Lys-23 were detected by immunoprecipitation as proteins of the predicted sizes (e.g., Fig. 2), indicating that removal of these residues did not result in cotranslational degradation in E. coli. Further, the protocol included solubilizing conditions so that it was clear that the mutants were degraded and not transferred into inclusion bodies. When degradation occurred, no discrete intermediates were observed. At 37 °C, deletions beyond residue 9 produced inactive enzyme both in vitro and in vivo, which was rapidly degraded in host cells. On the other hand, in the cell-free system, similar amounts of translated rhodanese, including Δ1–15, were observed in all experiments, indicating that lack of activity is a consequence of failure to fold completely and not due to degradation. Expression in the cell-free system at 30 °C showed that at least two more NH₂-terminal
residues, Val-10 and to a lesser extent Ser-11, could be removed and still permit synthesis of active enzyme. These two mutants were inactive when expressed at 37 °C in the cell-free system; yet, when they were expressed at 30 °C and then brought to 37 °C for 45 min, the proteins retained activity. This implies that truncation influences folding to the active conformation rather than stability of the native state when the protein is expressed in E. coli. The present study suggests that if there is insufficient NH2-terminal sequence of rhodanese to perform these multiple functions may require prior formation of the Ser-11–Gly-22 helix. These inactivations are particularly noteworthy since their extent correlates with activity. The ability of the NH2-terminal sequence left after truncation to aid in stabilization, the native state when the protein has adopted a near-native conformation (12). The NH2-terminal sequence can modulate the unassisted folding, global stability, and resistance to degradation in addition to any function in import. The stability of the native state when the protein is expressed in E. coli may be essential for mitochondrial import. The ability of the NH2-terminal sequence left after truncation to aid in stabilization, the native state when the protein has adopted a near-native conformation (12).

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