Mechanism for the Desulfurization of L-Cysteine Catalyzed by the *nifS* Gene Product†

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ABSTRACT: The *nifS* gene product (NIFS) is a pyridoxal phosphate binding enzyme that catalyzes the desulfurization of L-cysteine to yield L-alanine and sulfur. In *Azotobacter vinelandii* this activity is required for the full activation of the nitrogenase component proteins. Because the nitrogenase component proteins, Fe protein and MoFe protein, both contain metalloclusters which are required for their respective activities, it is suggested that NIFS participates in the biosynthesis of the nitrogenase metalloclusters by providing the inorganic sulfur required for Fe–S core formation [Zheng, L., White, R. H., Cash, V. L., Jack, R. F., & Dean, D. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 2754–2758]. In the present study the mechanism for the desulfurization of L-cysteine catalyzed by NIFS was determined in the following ways. First, the substrate analogs, L-allylglycine and vinylglycine, were shown to irreversibly inactivate NIFS by formation of a γ-methylcystathionyl or cystathionyl residue, respectively, through nucleophilic attack by an active site cysteinyl residue on the corresponding analog–pyridoxal phosphate adduct. Second, this reactive cysteinyl residue, which is required for L-cysteine desulfurization activity, was identified as Cys^{325} by the specific alkylation of that residue and by site-directed mutagenesis experiments. Third, the formation of an enzyme-bound cysteinyl persulfide was identified as an intermediate in the NIFS-catalyzed reaction. Fourth, evidence was obtained for an enamine intermediate in the formation of L-alanine. All of these results support a mechanism for NIFS-catalyzed desulfurization of L-cysteine which involves formation of a substrate cysteine–pyridoxal phosphate ketimine adduct and subsequent nucleophilic attack by the thiolate anion of Cys^{325} on the sulfur of the substrate cysteine. These events result in formation of a protein-bound persulfide, which is the proposed sulfur donor in Fe–S core formation, and a pyridoxal phosphate-bound enamine which is ultimately released as L-alanine.

Nitrogenase is a two-component metalloenzyme that catalyzes the MgATP-dependent reduction of dinitrogen. During catalysis, an Fe protein component sequentially delivers single electrons to a MoFe protein component upon which the substrate reduction site is located [reviewed by Burgess (1990)]. A feature common to both the Fe protein and the MoFe protein is that they contain metalloclusters comprised of Fe–S cores (Georgiadis *et al.*, 1992; Kim & Rees, 1992). These metalloclusters are necessary for nitrogenase activity, and they appear to participate in various aspects of electron transfer or substrate reduction. In *Azotobacter vinelandii*, the genes that encode the nitrogenase structural components (*nifH*, Fe protein subunit; *nifDK*, MoFe protein subunits) are contiguous and arranged *nifHDK* (Brigle *et al.*, 1985). However, the primary translation products of the nitrogenase structural genes are not active. Rather, immature forms of the nitrogenase component proteins are activated through the formation and insertion of their complementary metalloclusters in processes that require the activities of a consortium of associated *nif*-specific gene products [see Dean and Jacobson (1992) for a review]. One such processing step must include the mobilization of the inorganic sulfide required for formation of the Fe–S cores. We have recently demonstrated (Zheng *et al.*, 1993) that the *A. vinelandii nifS* gene product, which is required for the full *in vivo* activation of both the Fe protein and the MoFe protein (Jacobson *et al.*, 1989), is a pyridoxal phosphate (PLP)[1] enzyme that catalyzes the desulfurization of L-cysteine to yield L-alanine and elemental sulfur. On the basis of these results we proposed that NIFS catalyzes the release of sulfur from L-cysteine in a pathway which ultimately provides the sulfide present in the metalloclusters of the nitrogenase component proteins. Because the catalytic activity of NIFS is extremely sensitive to alkylating reagents, such as *N*-ethylmaleimide, a reactive cysteine was proposed to be involved in catalysis. We also suggested that an intermediate step in Fe–S core assembly includes the formation of a NIFS-bound cysteinyl persulfide. Such a cysteinyl persulfide could be formed through nucleophilic attack by the active site cysteinyl thiolate on the cysteine–PLP ketimine adduct. In the present study we have tested this possible mechanism for the mobilization of sulfur by identifying the active site cysteinyl residue located on NIFS and by demonstrating the formation of an enzyme-bound cysteinyl persulfide at that site.

EXPERIMENTAL PROCEDURES

Materials. γ-Methylcystathionine, prepared by the condensation of L-allylglycine with L-cysteine catalyzed by cystathionine γ-synthase from *Escherichia coli* (Brzović *et al.*, 1990), was kindly supplied by Dr. Peter Brzović, Department of Biochemistry, University of California at Riverside. Homocystathionine, *S*-(4-amino-4-carboxybutyl)-cysteine,

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[1] Abbreviations: DTT, dithiothreitol; GC–MS, gas chromatography–mass spectroscopy; HPLC, high-pressure liquid chromatography; 1,5-I-AEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; NIFS, *nifS* gene product; PLP, pyridoxal phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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was synthesized in the following manner. Diethyl acetamidomalonate was condensed with 1,3-dibromopropane in ethanol containing sodium ethoxide to form 1-acetamido-1,1-bis(carboxyethoxy)-4-bromobutane. This product was then condensed with cysteine under basic conditions to generate *S*-(4-acetamido-4,4-dicarbethoxybutyl)cysteine, which was subsequently converted into the desired product after saponification with NaOH, decarboxylation, and hydrolysis with HCl.

Wild-type NIFS and NIFS–Ala^{325} were hyperexpressed in *E. coli* cells and purified as described before (Zheng *et al.*, 1993). The K_M was determined using the hydrogen sulfide assay described previously (Zheng *et al.*, 1993). The cysteine concentrations used were 0.2, 0.5, 1.0, 2.0, 4.0, and 9.0 mM. Because cysteine interferes with the hydrogen sulfide assay, separate sodium sulfide standard curves were respectively obtained in the presence of each cysteine concentration used to determine the K_M. It should be noted that NIFS activity is highly sensitive to pH and temperature, so the K_M observed might depend on the condition used. Ammonia was assayed according to the method of Dilworth *et al.* (1992). The reagents L-cysteine, allylglycine, vinylglycine, and *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-I-AEDANS) were freshly prepared in water and adjusted to pH 8.0, if necessary. All of the reactions described below were performed at ambient temperature unless specifically noted otherwise.

Isolation and Identification of the Protein-Bound γ-Methylcystathionine. Purified NIFS (5 mg, 110 nmol) was inactivated by incubation with 5 μmol of L-allylglycine in 1 mL of 50 mM Tris·HCl buffer, pH 8.0, for 2 h. After this treatment the excess unreacted amino acid analog was separated from the protein fraction by three cycles of centrifugal ultrafiltration using an Amicon Centricon 30 device. The isolated protein was then hydrolyzed under N_2 in 1 mL of 6 M HCl at 110 °C for 21 h in the presence of 5 μL of mercaptoethanol. After evaporation of the HCl, the resulting amino acids were dissolved in 0.5 mL of water, applied to a Dowex 50W-8X H+ column (1 × 0.6 cm), and eluted with an HCl gradient consisting of 3 mL of 1 M HCl, 0.5 mL of 2 M HCl, and finally 3 mL of 3 M HCl. Under this condition authentic γ-methylcystathionine was eluted in the 3 M HCl fraction. The amino acids present in the 3 M HCl eluant fraction were then converted into their *n*-butyl trifluoroacetyl derivatives as previously described (White & Rudolph, 1978) and were separated by gas chromatography–mass spectroscopy (GC–MS) on a DB-5 column (30 m × 0.32 mm, J&W Scientific Co.) programmed from 175 to 300 °C at 10 °C/min. Under these conditions the γ-methylcystathionine derivative eluted after 8.3 min. The mass spectra were obtained at 70 eV using a VG-70-70EHF mass spectrometer.

Isolation and Identification of the Protein-Bound Cystathionine. Vinylglycine-inactivated NIFS was prepared by incubation of 17 nmol of NIFS with two additions of 5 μmol of L-vinylglycine in a total volume of 1 mL of 50 mM Tris·HCl buffer, pH 8.0, for 8 h. NIFS-bound cystathionine was identified using the same procedures described above for the identification of γ-methylcystathionine. Authentic cystathionine was used as standard.

Separation and Identification of the Peptide Containing the Active Site Cysteinyl Residue. NIFS (120 μg, 2.7 nmol) was incubated with 6.5 nmol of the dansylated alkylating reagent 1,5-I-AEDANS for 12 h. After the excess of 1,5-I-AEDANS was removed by three cycles of ultrafiltration, as described above, NIFS was denatured in 1 mL of 4.8 M Gdn·HCl, 80 mM Tris·HCl, pH 8.0, and 50 mM dithiothreitol (DTT) buffer and the three remaining cysteinyl residues were alkylated with a 1.1 molar excess of iodoacetamide. The protein treated in this way was then extensively dialyzed against water and dried in a speed vacuum apparatus. The resulting pellet was dissolved in 0.5 mL of 100 mM $(NH_4)_2CO_3$ buffer, pH 7.7, and digested with 2.4 μg of trypsin for 18 h at 37 °C. The separation of the resulting peptides was by HPLC using an Axxion octadecylsilane column (5 μm, 4.6 × 250 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid in water. Elution was with a 20–40% gradient of 0.075% (v/v) trifluoroacetic acid in acetonitrile at a flow rate of 1 mL/min. The elution was monitored both by UV absorbance (220 nm) and by fluorescence (excitation at 340 nm; emission at 500 nm; Gorman *et al.*, 1987). The peak having the highest fluorescence was collected and sequenced on an Applied Biosystems protein sequencer (Model 477A) operated by the Virginia Tech sequencing facility.

Site-Directed Mutagenesis and Gene Replacement. Methods for site-directed mutagenesis and gene replacement were the same as previously described for the analysis of the *nifD* gene from *A. vinelandii* (Brigle *et al.*, 1987). The template used for mutagenesis was an *Xba*I fragment containing the entire *nifS* gene from pDB551 (Zheng *et al.*, 1993) cloned into the M13mp18 *Xba*I site. The codon for Cys^{325}, UGC, was changed to GCC. The oligonucleotide primer used for mutagenesis had the following sequence: 5'GGTTCGGCCGCCACCTCCGG3'.

Identification of the NIFS-Bound Persulfide. The procedure for the identification of a NIFS-bound persulfide is outlined in Figure 1. In a typical experiment 4.4 mg (100 nmol) of NIFS was incubated with or without 100 nmol of L-cysteine (substrate-treated enzyme, as in the left route, or untreated enzyme, as in the right route, respectively) in 50 mM Tris·HCl buffer, pH 8.0, for 10 s (step 1 in Figure 1) before 300 nmol of 1,5-I-AEDANS was added (step 2 in Figure 1). The reaction was allowed to proceed for 30 min before the excess of the 1,5-I-AEDANS was removed by washing three times with the above buffer using ultrafiltration. The protein samples were then brought to 2 mL with the buffer and equally divided. An aliquot of 1 M DTT was added to one sample to give a final concentration of 5 mM to reduce any disulfide bond formed between NIFS and the fluorescent compound (step 3 in Figure 1). The other half was not reduced and served as a control. After incubation for 30 min, small molecules were separated from protein by washing twice using a ultrafiltration Centricon 30 device. All of the samples were then brought to 2 mL with Tris·HCl buffer and the relative concentrations of the 1,5-I-AEDANS derivatives determined spectrofluorometrically. The *N*-(thioacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine produced by reductive cleavage of the disulfide adduct with DTT was identified by its quantitative methylation to *N*-[(methylthio)acetyl]-*N'*-(5–sulfo-1-naphthyl)ethylenediamine using excess methyl iodide in diluted aqueous ammonia and comparing the R_f of this methylated product with that of a synthetic sample of *N*-[(methylthio)-acetyl]-*N'*-(5-sulfo-1-naphthyl)ethylenediamine. The authentic sample of *N*-[(methylthio)acetyl]-*N'*-(5-sulfo-1-naphthyl)ethylenediamine was prepared by reacting 1,5-AEDANS with an excess of methylthiol in aqueous ammonia. In each case, the methylated products were separated from the salts by retention on a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA), the column was washed with water, and the compounds were eluted with 60% acetonitrile in water. The fluorescent compounds were then analyzed by TLC using

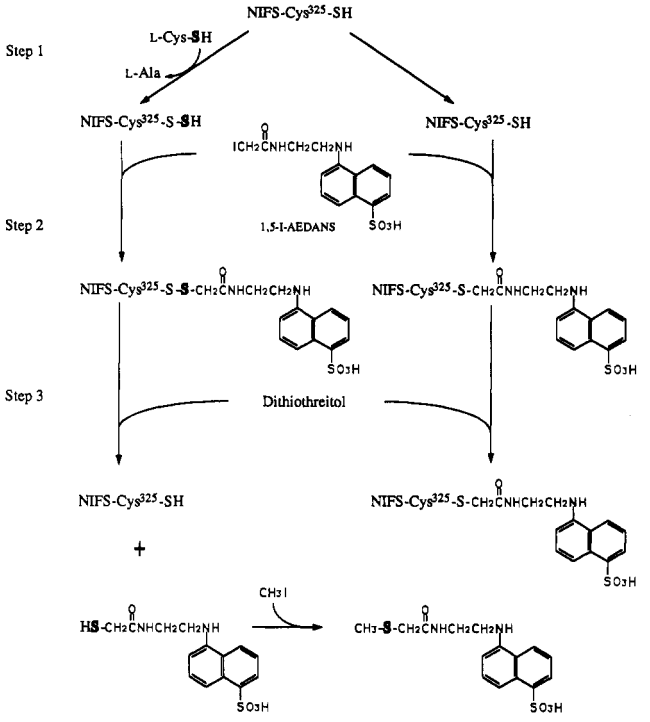


FIGURE 1: Scheme for the identification of a NIFS-bound persulfide. The left route shows the formation of a persulfide on NIFS when incubated in the presence of the substrate L-cysteine (step 1), the alkylation of this persulfide by 1,5-I-AEDANS (step 2), and the reduction of the disulfide bond between the NIFS and the fluorescent complex (step 3). The right route shows the control experiment in which the substrate cysteine was omitted. Under this condition 1,5-I-AEDANS reacts directly with Cys^{325} to form a stable enzyme-bound thioether and, thus, no fluorescent group should be released from the enzyme by reduction with DTT. The total fluorescence bound to NIFS at step 2 was approximately equal for both samples. Quantitation of the relative amounts of the fluorescent compound released in these schemes gave the following results: 81% of the total fluorescent compound bound to NIFS was released by DTT treatment in the left route, whereas only 1.4% of the fluorescent compound bound to NIFS was released by DTT treatment in the right route. The alkylation step used to identify the fluorescent compound released by DTT treatment is shown at the bottom of the scheme.

two different solvent systems, acetonitrile–water–formic acid (180:20:10 v/v/v) and acetonitrile. 1,5-I-AEDANS had R_f values of 0.63 in the first solvent system and 0.175 in the second, whereas the methylated product had R_f values of 0.59 in the first solvent system and 0.11 in the second. The compounds were readily visualized on the TLC plate as bright blue fluorescent bands when the plate was exposed to UV light.

NIFS Desulfuration Reaction in ^2H_2O. L-Alanine, L-cysteine, and Tris·HCl buffer were prepared in ^2H_2O and titrated to pH 8.0 using HCl or NaOH. NIFS was transferred to 50 mM Tris·HCl prepared in ^2H_2O and equilibrated by repetitive dilution and ultrafiltration using the deuterated buffer. Following this treatment the $^1H/^2H$ ratio was calculated to be less than 1/30. L-Alanine (20 μmol) or L-cysteine (1 μmol) was incubated with 0.5 mg of NIFS in a 1-mL reaction mixture containing 50 mM Tris·HCl buffer for 2 h. After the reaction, the amino acids were separated from NIFS by ultrafiltration and analyzed by GC–MS as described above in the analysis of γ-methylcystathionine.

RESULTS

Allylglycine and Vinylglycine Are Suicide Inhibitors of NIFS-Catalyzed Cysteine Desulfurization Activity. Olefinic

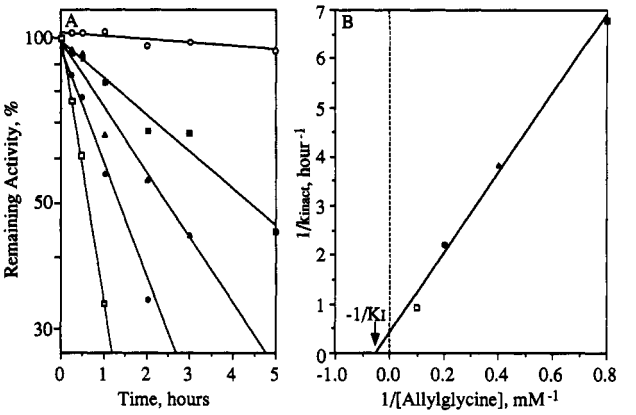
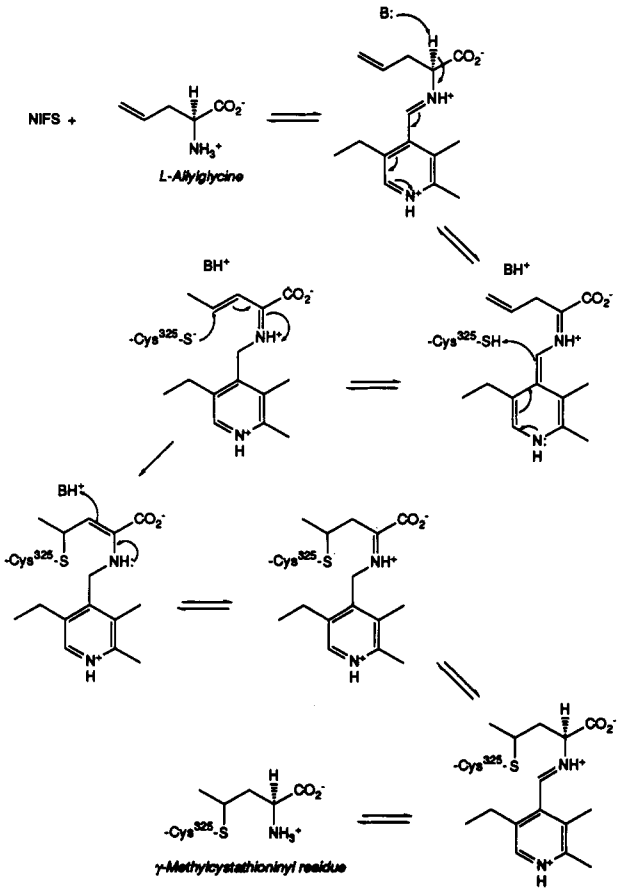


FIGURE 2: Inhibition of NIFS activity by L-allylglycine. (A) Time-dependent loss of NIFS activity by incubation with no (○), 1.25 mM (■), 2.5 mM (▲), 5.0 mM (●) and 10 mM (□) allylglycine. (B) Double-reciprocal plots of the observed rate constant vs the concentration of allylglycine using the data in panel A. $k_{inact} = \ln 2/T_{1/2}$, where $T_{1/2}$ is the half-life of the NIFS activity under a certain concentration of allylglycine.

amino acids are often used as mechanism-based suicide inhibitors of PLP enzymes (Cooper, 1983; Walsh, 1986). In this study L-allylglycine and L-vinylglycine were used to investigate the mechanism of NIFS-catalyzed cysteine desulfurization. Incubation of NIFS with allylglycine resulted in a time-dependent loss of cysteine desulfurase activity (Figure 2A). Such inhibition was not reversed by addition of the substrate L-cysteine, nor was it reversed by addition of PLP or DTT. Furthermore, extensive dialysis of the allylglycine-inhibited enzyme did not result in its reactivation. DL-Allylglycine, at a concentration twice as great, inactivated NIFS-catalyzed cysteine desulfurase activity at the same rate as L-allylglycine. These results indicate that allylglycine inhibition of NIFS activity requires an enzymatic reaction that is specific for the L-form of the olefinic amino acid which is consistent with the requirement of L-cysteine as substrate in the desulfurization reaction (Zheng *et al.*, 1993). A kinetic analysis showed that NIFS inactivation by allylglycine is a pseudo-first-order reaction (Figure 2A) having an apparent K_I of 20 mM (Figure 2B). In contrast, the apparent K_M for L-cysteine is about 75 μM.

To test whether or not NIFS can use L-allylglycine as substrate to catalyze formation of a product, we searched for product formation following the co-incubation of NIFS and L-allylglycine. On the basis of thin-layer chromatography using ninhydrin detection, such incubation of NIFS and L-allylglycine did not lead to formation of a product that could be distinguished from the substrate L-allylglycine. Because allylglycine irreversibly inactivates NIFS, it seemed more likely that a protein-bound covalent adduct was formed during the inactivation. Two possible routes for allylglycine inactivation of NIFS can be considered. One route could involve a mechanism-based suicide inactivation in which the enzyme tautomerizes the terminal double bond of allylglycine followed by nucleophilic attack by the proposed active site cysteinyl at the γ-carbon (Scheme 1). This mechanism has precedence in cystathionine γ-synthase which is able to catalyze the condensation of a PLP–allylglycine adduct and L-cysteine to form γ-methylcystathionine (Brzović *et al.*, 1990). It should be noted here that the protonation of the quinonoid form of PLP and the deprotonation of the reactive cysteine are not necessarily coupled as shown in Scheme 1 but are presented in this way for convenience. Also, the L-structure of the final product shown in Scheme 1 was not determined experimentally

Scheme 1: Proposed Mechanism of NIFS Inhibition by L-Allylglycine



but was inferred from the fact that L-alanine is the product of the NIFS-catalyzed cysteine desulfuration reaction (Zheng *et al.*, 1993). An alternative possibility is that the inactivation is not mechanism based but involves a simple addition reaction which occurs by nucleophilic attack at the δ-carbon to form homocystathionine. Of the two possible mechanisms, the former was confirmed by the identification of γ-methylcystathionine as one of the amino acids produced by the acid hydrolysis of the allylglycine inactivated enzyme (Figure 3). γ-Methylcystathionine was identified in the acid hydrolysate of the L-allylglycine-treated enzyme by comparing the gas chromatographic retention time and the mass spectra of the *n*-butyl trifluoroacetyl derivative of isolated and known compounds (Figure 3). The known compounds used in the analysis were γ-methylcystathionine and homocystathionine, the corresponding products expected by attack at either the γ- or δ-carbon of allylglycine, respectively, and these were readily distinguished by their mass spectroscopic fragmentation pattern (Figure 3) and gas chromatographic retention times (data not shown).

The pattern of inhibition by L-vinylglycine on cysteine desulfurase activity catalyzed by NIFS was more complicated because vinylglycine serves as both a substrate and a suicide inhibitor. In our initial experiments, approximately equimolar amounts of vinylglycine and NIFS were co-incubated, which resulted in deamination of vinylglycine to yield ammonia and an α-keto compound, most likely α-ketobutyrate. Thus, NIFS and cystathionine γ-synthase share another feature in that both are able to catalyze deamination of vinylglycine (Brzović *et al.*, 1990). Under these conditions there was no apparent inactivation of the NIFS-catalyzed cysteine desulfurase activity. In contrast, NIFS lost all cysteine desulfurase activity

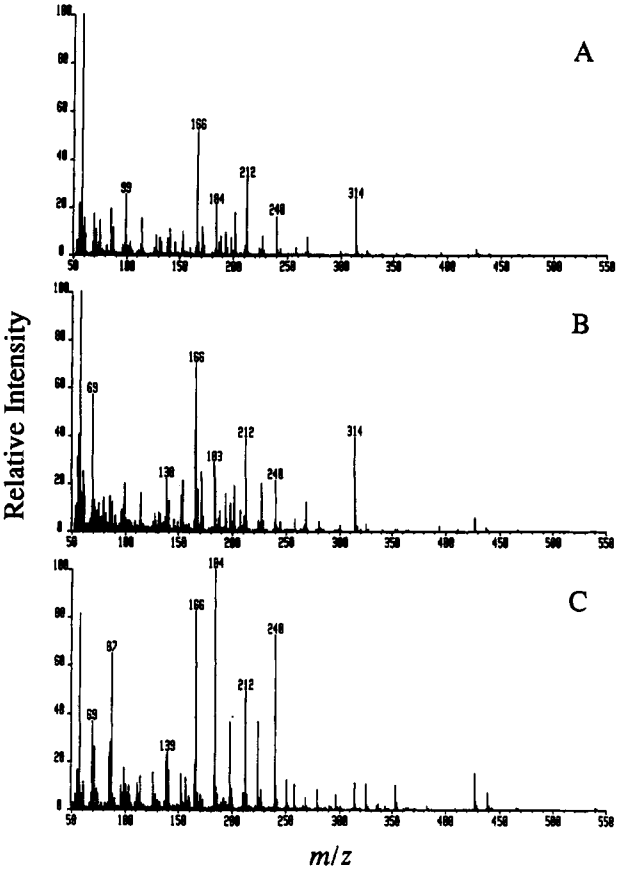
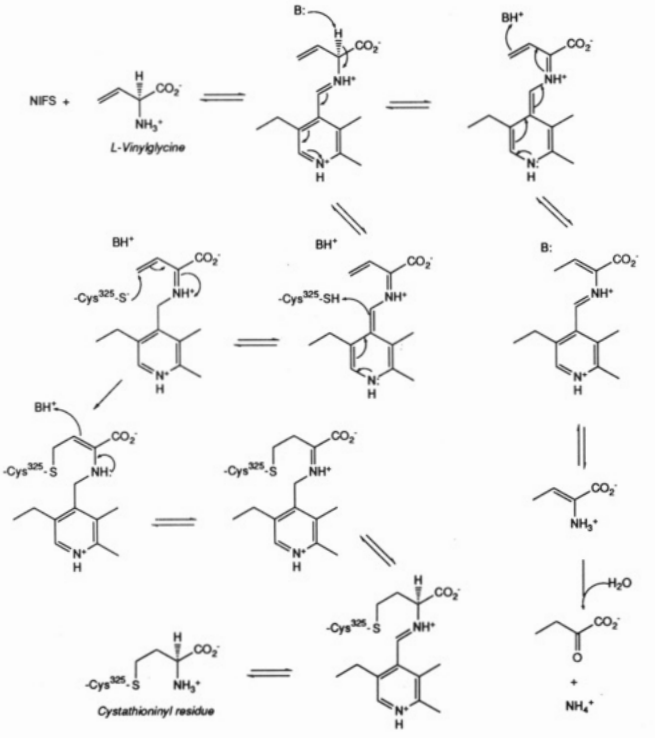


FIGURE 3: 70-eV mass spectra of *n*-butyl trifluoroacetyl derivatized samples. (A) The complex isolated from hydrolysis of L-allylglycine inactivated NIFS; (B) γ-methylcystathionine; (C) homocystathionine. The preparation of the samples is described under Experimental Procedures.

when incubated with a large molar excess of vinylglycine. Using the same methods decribed above for the allylglycine-inactivated enzyme, it was shown that vinylglycine inactivation occurred through formation of a cystathionyl residue. The vinylglycine deamination reaction catalyzed by NIFS apparently does not require participation of the reactive cysteinyl residue because allylglycine-treated NIFS, vinylglycine-treated NIFS, and an altered form of NIFS that has an alanyl substitution for the active site cysteinyl residue (discussed below) were all able to catalyze the deamination reaction at rates comparable to that of the native enzyme. The mechanisms for the deamination of L-vinylglycine catalyzed by NIFS and the vinylglycine-directed suicide inactivation of NIFS cysteine desulfurase activity are presented in Scheme 2.

Identification of the Reactive Cysteinyl Residue in NIFS. From the above experiments and our previous results (Zheng *et al.*, 1993) it is known that NIFS has a reactive cysteinyl residue that is necessary for cysteine desulfurase activity. Blocking of a reactive thiolate group located on NIFS by using either a thiol-specific alkylating reagent or an olefinic amino acid analog results in irreversible inactivation of NIFS activity. Like *N*-ethylmaleimide, 1,5-I-AEDANS is able to react stoichiometrically with NIFS to effect greater than 90% loss of enzymatic activity (results not shown). Thus, the fluorescent property of 1,5-I-AEDANS was used to identify the reactive cysteinyl residue located on NIFS. In these experiments native NIFS was first alkylated by treatment with 1,5-I-AEDANS and subsequently denatured, alkylated with iodoacetamide, and digested with trypsin. After digestion, the resultant peptides were separated by reversed phase HPLC

Scheme 2: Proposed Mechanism of NIFS Inhibition by L-Vinylglycine



and the elution position of the dansylated peptide was determined by simultaneously monitoring the HPLC profile at 220 nm and the fluorescence emission at 500 nm (Figure 4). The major fluorescent peptide peak was eluted at ~25 min, and this fraction was collected and its sequence determined by automated amino acid sequence analysis. In this way the dansylated peptide was shown to have the N-terminal sequence Val-Gly-Ile-Ala-Ala-Ser-Ser-Gly-Ser-Ala-X-Thr, where the Val residue and the unidentified residue (X) respectively correspond to the Val^{315} and Cys^{325} positions in the NIFS primary sequence. On the basis of the primary sequence of NIFS deduced from the gene sequence (Beynon *et al.*, 1987; see Figure 4, top panel), formation of a peptide having this N-terminal sequence upon tryptic digestion of NIFS was expected. Also, Cys^{325} is the only cysteinyl residue expected to be present in this peptide.

That the hyperreactive Cys^{325} is, in fact, located at the active site was also shown by a site-directed mutagenesis experiment in which the Cys^{325} codon was substituted by an alanine codon. When this mutation was transferred to the *A. vinelandii* chromosome, it conferred the same diazotrophic growth characteristics previously reported for *nifS* deletion mutants (Jacobson *et al.*, 1989). In addition, the purified NIFS–Ala^{325} protein was shown to be inactive in the cysteine desulfurase assay but active in the vinylglycine deamination reaction. In another series of experiments NIFS, vinylglycine-treated NIFS, allylglycine-treated NIFS, and NIFS–Ala^{325} were individually incubated with 1,5-I-AEDANS. The NIFS proteins treated in this way were then separated from the excess of the fluorescent alkylating reagent by SDS–PAGE and analyzed by fluorography and Coomassie staining. The results of these experiments (Figure 5) show that substitution of an alanyl residue for Cys^{325} eliminates reactivity of NIFS toward alkylation. There are three other cysteinyl residues within the NIFS primary sequence (Beynon *et al.*, 1987), and therefore, these results confirm that Cys^{325} provides the only reactive thiolate in the native protein and agree well with the

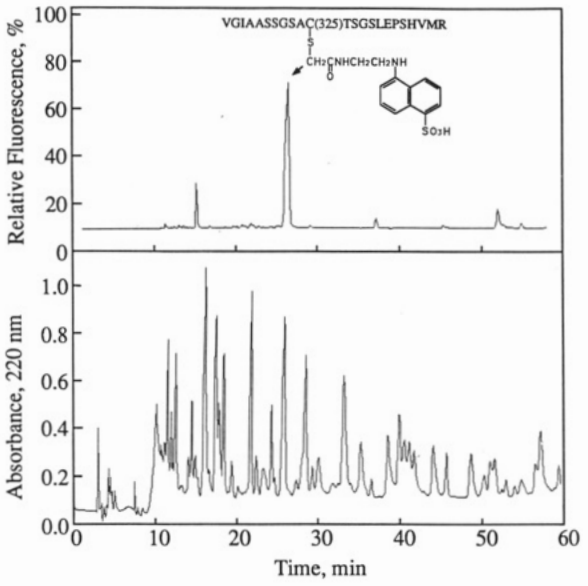


FIGURE 4: Reversed phase HPLC profiles of the tryptic digests of NIFS. Native NIFS (120 μg) was labeled with 1,5-I-AEDANS, then denatured, alkylated, digested with trypsin, and separated by HPLC with a gradient from 20 to 40% acetonitrile in water (v/v) from 5 to 55 min. The elution was monitored at 220 nm and fluorescence emission at 500 nm. The sequence of the peak with highest fluorescence was identified by automated amino acid sequencing. The structure of the peptide–1,5-I-AEDANS adduct is as shown in the upper panel. A time course of trypsin digestion revealed that the leftward fluorescent peak in the upper panel respresents a product of incomplete digestion.

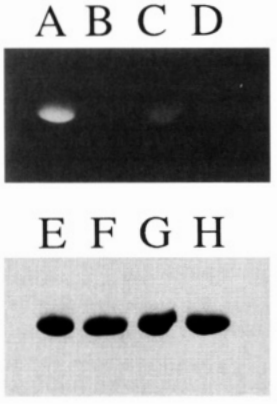
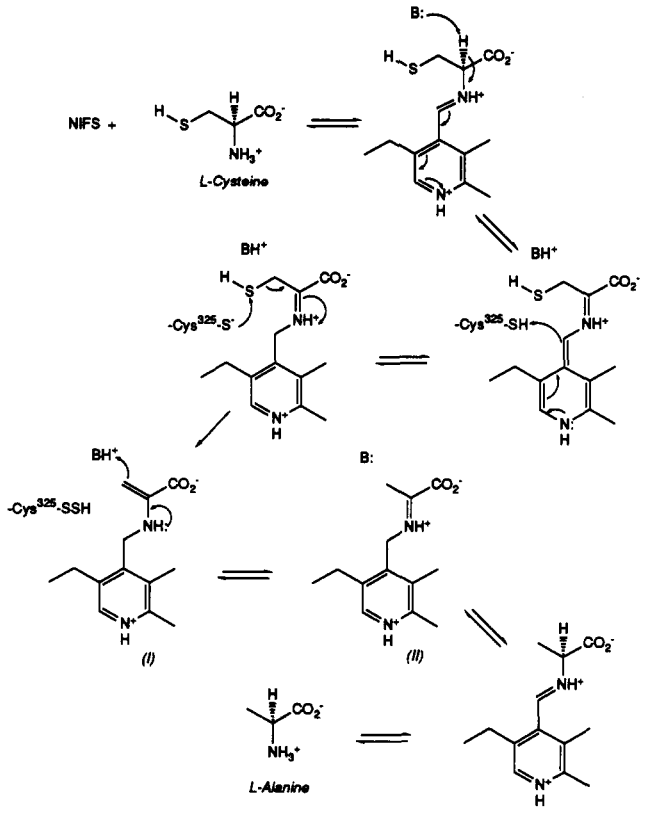


FIGURE 5: SDS–PAGE of modified NIFSs. NIFS, vinylglycine-inactivated NIFS, allylglycine-inactivated NIFS, or NIFS-Ala^{325} (20 μg each) was incubated with an equal molar amount of 1,5-I-AEDANS in 20 μL of 50 mM Tris·HCl buffer, pH 8.0, at ambient temperature for 60 min. The samples were then loaded onto an SDS–PAGE. After electrophoresis, the gel was visualized first by fluorography and then by Coomassie staining. Lanes A, B, C, and D, fluorography of NIFS, vinylglycine-treated NIFS, allylglycine-treated NIFS, and NIFS-Ala^{325}, respectively. Lanes E, F, G, and H, Coomassie stain of the same samples as shown in lanes A, B, C, and D, respectively.

observation that NIFS activity can be quantitatively inhibited by treatment with an equimolar amount of an alkylating reagent (Zheng *et al.*, 1993). Also, when an excess of 1,5-I-AEDANS was reacted with NIFS and the unreacted alkylating reagent subsequently removed by dialysis, it was found, on the basis of the extinction coefficient of cysteine-treated 1,5-I-AEDANS, that 1,5-I-AEDANS reacted with NIFS monomers at a 1.1 to 1 molar ratio. Finally, neither vinylglycine- nor allylglycine-treated NIFS could be effectively labeled with the fluorescent alkylating reagent when compared to alkylation of the native enzyme. This result indicates that Cys^{325} provides the thiolate which reacts with the respective

Scheme 3: Proposed Mechanism of NIFS Desulfuration Reaction



enzyme-bound analog adducts to form either a γ-methylcystathionyl or a cystathionyl residue.

Persulfide Formation Is an Intermediate in NIFS-Catalyzed Desulfuration of L-*Cysteine.* In Scheme 3 a mechanism for NIFS is proposed in which L-cysteine desulfurization occurs by nucleophilic attack of the active site Cys^{325} thiolate on the substrate cysteine PLP adduct. If correct, this mechanism predicts the formation of an enzyme-bound persulfide as an intermediate in the reaction. We therefore tested for the formation of such a persulfide during NIFS catalysis by reacting the substrate-treated enzyme with the alkylating reagent 1,5-I-AEDANS and asking if a disulfide linkage was formed from an enzyme-bound persulfide and the alkylating reagent. In a separate control experiment the untreated enzyme was also reacted with 1,5-I-AEDANS to form the stable thioether derivative. The rationale and results of these experiments are presented in Figure 1. The results show that reaction of 1,5-I-AEDANS with the substrate-treated form of NIFS results in formation of a DTT-reducible disulfide bond in more than 80% of the enzyme. The fluorescent species released from the substrate-treated enzyme by reducing this disulfide bond was shown to be *N*-(thioacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine. In contrast, the fluorescent NIFS derivative obtained by reacting the untreated enzyme with 1,5-I-AEDANS could not be released by treatment with DTT.

Evidence for the Reversible Formation of an Enamine Intermediate during L-*Cysteine Desulfurization Catalyzed by NIFS.* To further characterize the mechanism by which NIFS catalyzes the desulfurization of L-cysteine, the reaction was carried out in the presence of ^2H_2O and the deuterium incorporated into the reactants and products was determined by GC–MS analysis of their *n*-butyl trifluoroacetyl derivatives. Under these reaction conditions it was found that alanine generated by desulfurization of L-cysteine was completely deuterated. Thus, during the formation of the alanine product, the α- and β-hydrogens are all readily exchanged. The observed complete exchange of the β-hydrogens can be explained by the rapid equilibration of the enamine form of the product (compound **I**, Scheme 3) with the ketimine form of the product (compound **II**, Scheme 3) before rearrangement and separation of the alanine from the enzyme. It was observed that the α-hydrogen of all the cysteine remaining in the reaction mixture had been exchanged as well. Thus, it appears that the slow step in the reaction involves displacement of the sulfur from the bound cysteine.

It was also found that the α-hydrogen and all three β-hydrogens of a small fraction of L-alanine became deuterated when NIFS was incubated with L-alanine in ^2H_2O. Thus, exchange occurred at all four positions or not at all. This result indicates that L-alanine reacts slowly with the enzyme but once the alanine–PLP adduct is formed there is rapid exchange of all four hydrogens. The mechanism for the rapid exchange of the β-hydrogens is most likely explained as discussed above for the rapid equilbration of compounds **I** and **II** shown in Scheme 3. The exchange of the α-hydrogen would occur during the rearrangement of compound **II** to alanine. These results are similar to those of Babu and Johnston, who reported the complete exchange of the α- and β-hydrogens of alanine catalyzed by glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase (Babu & Johnston, 1976). Our results and the observation that millimolar amounts of L-alanine do not significantly inhibit NIFS-catalyzed cysteine desulfurase activity thus reflect a rather slow binding/dissociation of L-alanine with NIFS relative to formation of the enamine intermediate.

DISCUSSION

We have previously shown that the product of the *nifS* gene is an L-cysteine desulfurase which catalyzes the removal of cysteine sulfur to form L-alanine and elemental sulfur (Zheng *et al.*, 1993). Our current hypothesis is that the reaction catalyzed by NIFS represents a step in the formation of the Fe–S cores contained within the nitrogenase component proteins. In particular, we have suggested that NIFS catalyzes formation of an enzyme-bound persulfide which is the active species for providing the inorganic sulfide necessary for Fe–S cluster biosynthesis. In Scheme 3 a model that describes the proposed mechanism for the formation of an enzyme-bound persulfide using PLP chemistry and cysteine substrate is shown. The salient and novel feature of the model is nucleophilic attack by an active site cysteinyl thiolate anion on the sulfur of a cysteine–PLP adduct. This nucleophilic attack results in formation of a cysteinyl persulfide and an enamine derivative of alanine. In the present study, three basic features predicted by such a mechanism were experimentally confirmed: An essential active site cysteinyl thiolate (Cys^{325}) was identified, formation of an enzyme-bound persulfide was demonstrated, and indirect evidence for formation of an enamine intermediate during L-alanine formation was obtained. Furthermore, derivatization of the active site cysteinyl residue by incubation of NIFS with the mechanism-based inhibitors, allylglycine or vinylglycine, to form an enzyme-bound γ-methylcystathionyl or cystathionyl residue, respectively, clearly demonstrates that a cysteinyl thiolate is poised for nucleophilic attack at the appropriate substrate position (see Schemes 1–3). The observation that treatment of NIFS with L-allylglycine or L-vinylglycine specifically blocks alkylation of the Cys^{325} residue and the results of site-directed mutagenesis experiments, which show that Cys^{325} is required for cysteine desulfurase activity, also support this conclusion.

To our knowledge the cleavage of L-cysteine to yield L-alanine and sulfur by the mechanism presented in Scheme 3 represents a PLP-based biochemical reaction not previously described. It involves cleavage of a C–S bond by nucleophilic attack of a substrate cysteine sulfur by a protein cysteinyl anion with the expulsion of a stabilized β-carbanion of alanine. Although cleavage of a C–S bond by an attacking thiolate anion has not been described for biological systems, there is chemical precedence for such a reaction. One example is the thioalkylation of a thiocyanate to generate an unsymmetrical disulfide (Wijers *et al.*, 1969). This reaction proceeds by the nucleophilic attack of a thiolate anion at the sulfur of thiocyanate with the consequent expulsion of cyanide as the leaving group. Another example is the formation of dimethyl disulfide from the reaction of an α-methylthio ester with methyl thiolate (Trost, 1974). In this reaction the ester-stabilized C-2 anion facilitates the reaction.

The mechanism proposed here for NIFS-catalyzed cysteine desulfurization is different from that proposed for the analogous reaction catalyzed by selenocysteine β-lyase, which cleaves L-selenocysteine to yield L-alanine and elemental selenium (Esaki *et al.*, 1982, 1985). The most significant difference is that an active site cysteinyl is required for elimination of sulfur from L-cysteine in the NIFS-catalyzed reaction and an enzyme-bound persulfide is an intermediate in that process. In contrast, Esaki *et al.* have proposed a two-base mechanism analogous to aspartate β-decarboxylase (Chang *et al.*, 1982) in which selenium is directly released in elemental form from selenocysteine. Another difference is that NIFS is able to catalyze both the desulfurization of L-cysteine and removal of selenium from selenocysteine, whereas selenocysteine β-lyase is reported to be specific for selenocysteine. Although the NIFS-catalyzed elimination of selenium from selenocysteine has not been characterized in detail, this reaction is probably mechanistically similar to the cysteine desulfurase reaction because Cys^{325} is required for both activities. It is unlikely that elimination of selenium from selenocysteine catalyzed by NIFS is a physiologically relevant reaction, however, because Se is not required for biological nitrogen fixation.

In summary, the requirement of NIFS for the full activation of the nitrogenase component proteins, both of which require the formation of Fe–S cores for their respective activities, and the cysteine desulfurase activity exhibited by NIFS indicate a role for NIFS in the activation of the inorganic sulfide required for Fe–S core formation. Results of the present work which demonstrate the formation of an enzyme-bound persulfide as an intermediate in the desulfurization of L-cysteine provide evidence for this idea. It is of particular interest that NIFS-like proteins have also been recently identified in non-nitrogen fixing organisms (Sun & Setlow, 1993; Kolman & Söll, 1993). These findings, and the strong sequence conservation among NIFS and NIFS-like proteins in the region surrounding and including the active site cysteinyl residue identified for NIFS (Zheng *et al.*, 1993), suggest that the mechanism determined here for NIFS activity could represent a global mechanism for the mobilization of the inorganic sulfide required for Fe–S cluster biosynthesis.

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