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Biochemistry

Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis

(nitrogenase/FeS cores/pyridoxal phosphate/sulfur mobilization/cysteinyl persulfide)

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ABSTRACT **Biological nitrogen fixation is catalyzed by nitrogenase, a complex metalloenzyme composed of two separately purifiable component proteins encoded by the structural genes *nifH*, *nifD*, and *nifK*. Deletion of the *Azotobacter vinelandii nifS* gene lowers the activities of both nitrogenase component proteins. Because both nitrogenase component proteins have metallocluster prosthetic groups that are composed of iron- and sulfur-containing cores, this result indicated that the *nifS* gene product could be involved in the mobilization of the iron or sulfur required for metallocluster formation. In the present work, it is shown that NIFS is a pyridoxal phosphate-containing homodimer that catalyzes the formation of L-alanine and elemental sulfur by using L-cysteine as substrate. NIFS activity is extremely sensitive to thiol-specific alkylating reagents, which indicates the participation of a cysteinyl thiolate at the active site. Based on these results we propose that an enzyme-bound cysteinyl persulfide that requires the release of the sulfur from the substrate L-cysteine for its formation ultimately provides the inorganic sulfide required for nitrogenase metallocluster formation. The recent discovery of *nifS*-like genes in non-nitrogen-fixing organisms also raises the possibility that the reaction catalyzed by NIFS represents a universal mechanism that involves pyridoxal phosphate chemistry, in the mobilization of the sulfur required for metallocluster formation.**

Many proteins that have important electron transfer properties are known to contain nonheme metalloclusters that are composed mainly of an inorganic iron and sulfide core, but in some cases also contain another metal (e.g., Mo; ref. 1) or an organic constituent (e.g., homocitrate; ref. 2). Included among these is nitrogenase, which catalyzes the reduction of dinitrogen, an essential reaction in the global nitrogen cycle. Although such metalloclusters contained within the nitrogenase component proteins and metalloclusters present in other proteins having redox, catalytic, and regulatory properties are obviously critical to the functions of their respective polypeptides, to our knowledge, there is currently no information concerning the specific steps involved in the mobilization of the inorganic iron and sulfur required for metallocluster assembly.

In a previous study, we reported (3) that the *nifS* gene product (NIFS) from *Azotobacter vinelandii* is required for the full activation of the two metalloproteins that are the catalytic components of nitrogenase. The two component proteins of nitrogenase include an Fe protein {also called dinitrogenase reductase (4), a homodimer that contains an [4Fe-4S] cluster} and MoFe protein [also called dinitrogenase (4), an α_2-β_2 tetramer that contains two eight-Fe centers and two FeMo cofactors] (for a recent review of the composition and functions of the nitrogenase metalloclusters, see ref. 5). The reduction in both *A. vinelandii* nitrogenase component protein activities as a result of *nifS* deletion, which has also been reported for *Klebsiella pneumoniae* (6), could not be attributed to a regulatory effect (3). Thus, because both Fe protein and MoFe protein activities were affected by deletion of *nifS*, and the common feature of both component proteins is that they contain metalloclusters, we have considered that NIFS might be involved in the acquisition or mobilization of the inorganic Fe or sulfur required for metallocluster formation. This possibility is supported by the observation that cell pellets of diazotrophically grown *A. vinelandii nifS* mutants are pale tan rather than the characteristic dark brown of wild type. The dark color of diazotrophically grown wild-type *A. vinelandii* is attributed to the metalloclusters contained within the nitrogenase component proteins that form a substantial portion of the soluble protein fraction.

As a strategy to elucidate the function of the NIFS polypeptide, we chose to produce large amounts of it in *Escherichia coli* to facilitate its purification and biochemical characterization. In the present study, we have purified the NIFS protein, demonstrated that a specific reaction is catalyzed by NIFS, and proposed a biochemical function for NIFS activity in relation to metallocluster formation. In addition, we discuss the possibility that the reaction catalyzed by NIFS represents a universal mechanism for the mobilization of the sulfur required for metallocluster formation.

MATERIALS AND METHODS

Hyperproduction and Purification of NIFS. Hyperproduction of NIFS in *E. coli* was accomplished by constructing a *nifS* gene cartridge *in vitro* and cloning this cartridge into the pT7-7 plasmid such that *nifS* gene expression is controlled by the T7 transcriptional and translational regulatory elements (7). *E. coli* cells [BL21(DE3)] containing the *nifS* expression plasmid were grown in 500-ml batches in Luria broth containing ampicillin (100 mg/liter) at 30°C and NIFS production was induced when cells reached ≈160 Klett units (red filter) by the addition of lactose to 1% final concentration. After the addition of lactose, cells were cultured for an additional 2 h. After the induction period, cells were harvested by centrifugation and stored at −80°C until used.

For purification of NIFS, cell pellets were thawed in 25 mM Tris·HCl (pH 7.4) at 2.5 ml of buffer per g of cell pellet. After disruption by sonication, cells were centrifuged at 15,000 rpm for 20 min in a Beckman Ty35 rotor. Solid streptomycin sulfate was added to the crude extract supernatant, which was then incubated on ice for 15 min. The resulting precipitate was removed by centrifugation at 13,000 rpm in a Sorvall GSA rotor. The supernatant from the streptomycin sulfate-treated sample was then fractionated at ice temperature by using solid ammonium sulfate. NIFS

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Abbreviations: PLP, pyridoxal 5-phosphate; GC/MS, gas chromatography/mass spectrometry; DTT, dithiothreitol.

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precipitated between 25% and 45% ammonium sulfate saturation and this fraction was collected by centrifugation as above and resuspended in buffer (≈20 ml of buffer was added per g of pellet). The sample was then loaded onto a 3 cm × 15 cm Mono Q Sepharose column by using a peristaltic pump and fractionated using a 600-ml 0.1 M–0.6 M NaCl gradient controlled by FPLC pumps. NIFS was eluted between 0.38 M and 0.45 M NaCl. The fractions containing NIFS were pooled and dialyzed at 4°C overnight against 0.5 M ammonium sulfate. The dialyzed sample was then loaded onto a 3 cm × 15 cm phenyl-Superose column as described above and eluted with a 500-ml decreasing 0.5 M–0.0 M ammonium sulfate gradient. NIFS was eluted at the end of the gradient. All fractions containing NIFS were pooled, frozen, and stored in liquid nitrogen or at −80°C until used. The protein determination was by a biuret reaction (8). The N-terminal sequence analysis of isolated NIFS was performed using an Applied Biosystems model 477A protein sequencer operated by the Virginia Tech sequencing facility. Spectroscopic analysis of NIFS was performed using a Beckman DU-70 spectrophotometer.

Determination of the Molecular Weight of Native and Denatured NIFS. The molecular weight of NIFS monomer was determined by gel electrophoresis of the isolated protein under denaturing conditions as described by Laemmli (9). The native molecular weight was determined by gel-filtration chromatography using a 1 cm × 30 cm Superose 12 FPLC column. The buffer for native molecular weight determination was 50 mM Tris·HCl, pH 7.4/200 mM KCl/10 mM $MgCl_2$. A 0.3 ml/min flow rate was maintained using an FPLC pump.

Isolation and Analysis of the Chromophore Present in NIFS. Purified NIFS protein was dialyzed for 4 h against water at room temperature, and trifluoroacetic acid was added to the resulting solution to 0.2 M. This acidification resulted in the precipitation of the protein as a yellow solid, which changed to a white solid in 10–20 sec as the chromophore protonated and dissociated from the protein. The protein precipitate was removed by centrifugation. Based on the absorbance at 400 nm of a portion of the extracted sample, recorded at neutral pH, the recovery of the cofactor was >90%. The sample was then purified by HPLC using a Whatman Partisil PXS ODS column and a linear gradient of 0–50% (vol/vol) methanol over a 10-min period at a flow rate of 1 ml/min. After its isolation, the chromophore extracted from NIFS was analyzed and identified as pyridoxal 5-phosphate (PLP) by NMR, UV/visible spectroscopy, and gas chromatography/mass spectrometry (GC/MS) analysis as described in *Results*. Phosphate analysis of the acid-hydrolyzed chromophore was conducted using the classical phosphomolybdate method (10). The phosphate released by acid hydrolysis was also identified by GC/MS analysis of the trimethylsilyl derivative. For GC/MS analysis, the HPLC-purified cofactor was hydrolyzed with 6 M HCl for 3 h and, after evaporation of the HCl, the hydrolysis products were converted into trimethylsilyl derivatives by reaction with an equal mixture of *N,O*-bis(trimethylsilyl)acetamide and pyridine for 10 min at 100°C. GC/MS of the resulting derivatized sample was performed using a Durabond column (0.32 mm × 30 mm, J & W Scientific, Rancho Cordova, CA) programmed from 100 to 300°C at 10°C/min. The amount of PLP contained in NIFS was determined using the phenylhydrazine method of Wada and Snell (11). Purified samples of NIFS were assayed directly without deproteinating the samples. Weighted samples of authentic PLP were used as standards.

Assays for NIFS Activity. All NIFS reactions were performed with 0.5 mM L-cysteine in 20 mM Tris·HCl (pH 8.0). Dithiothreitol (DTT, 1 mM) was added to the samples where indicated. For product analysis, NIFS (2 mg/ml) was used and elemental sulfur, hydrogen sulfide, pyruvate, and alanine were quantitated after the reaction mixture was incubated at ambient temperature for 2.5 h (see Table 2).

The elemental sulfur produced was determined by a modification of the cyanolysis method described by Wood (12). Samples (0.50 ml) were mixed with 0.4 ml of 1 M NH_4OH, 3.6 ml of water, and 0.5 ml of 0.5 M KCN, incubated at ambient temperature for 20 min, and then heated at 65°C for 1 min. After the samples were cooled to ambient temperature, 1.0 ml of Goldstein's reagent (12) was added, precipitates were removed by centrifugation, and the absorbance was read at 460 nm.

For pyruvate and alanine assays, the reaction mixtures were loaded onto Amicon Centricons and the products were separated from NIFS by ultrafiltration. Two 0.25-ml aliquots of product were then used for pyruvate assays. The samples were brought to 1.0 ml with water before 0.25 ml of 1.5 M Tris·HCl (pH 9.3), 0.25 ml of 0.58 mM NADH, and 20 units of lactate dehydrogenase were added. Oxidation of NADH was recorded by following the decrease in absorbance at 340 nm compared to a standard curve.

Alanine was quantitated by its deamination to pyruvate, which was then assayed as described above. The NIFS reaction product (0.3 ml) obtained from filtration was incubated at 37°C for 3 h with 30 μl of 100 mM 2-oxoglutarate and 0.3 unit of L-alanine aminotransferase. Two 0.125-ml aliquots were then used for pyruvate assay.

Sulfide formation was quantitated as described by Siegel (13) except that the reaction mixture was prepared in a 1-ml volume by using 2-ml serum vials sealed with Parafilm-wrapped rubber stoppers. The reaction was terminated by injecting 0.1 ml of 0.02 M *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl and 0.1 ml of 0.03 M $FeCl_3$ in 1.2 M HCl into the vials. After color developed for 20 min and precipitates were removed by centrifugation, the absorbance at 650 nm was recorded and the sulfide concentration was calculated based on a Na_2S standard curve.

Inhibition of NIFS Activity. Purified NIFS [1.7 nmol in 1 ml of 20 mM potassium phosphate (pH 7.0)] was incubated with 0–1.7 nmol of *N*-ethylmaleimide for 3 h at ambient temperature. DTT and substrate cysteine were then added to 1 mM and 0.5 mM, respectively, to initiate the NIFS reaction. NIFS activity was based on the formation of hydrogen sulfide as described above.

RESULTS

Purification of NIFS. Hyperproduction of NIFS was accomplished in *E. coli*. Accumulation of NIFS in crude extracts of BL21(DE3) cells harboring the *nifS* expression plasmid was demonstrated by denaturing gel electrophoresis (Fig. 1, lane B). This analysis showed the accumulation of a soluble polypeptide having a molecular weight of ≈44,000 that was not produced in control cells that harbored the parental plasmid pT7-7 (data not shown). Such results are in good agreement with the predicted molecular weight for

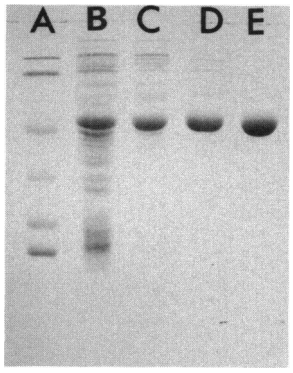

FIG. 1. Polyacrylamide gel electrophoretic analysis of the stages of NIFS purification. Lanes: A, molecular weight standards that include phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme; B, crude extract; C, ammonium sulfate fraction; D, Mono Q Sepharose fraction; E, phenyl-Superose fraction.

NIFS deduced from gene sequence data (14). Cell pellets of the *E. coli* strain that hyperproduced NIFS had a characteristic yellow color, indicating that NIFS contained a chromogenic prosthetic group. This assumption was, in fact, correct and provided a convenient means for visually following the purification of NIFS. NIFS was purified to homogeneity in a four-step procedure that included streptomycin sulfate fractionation, ammonium sulfate fractionation, Mono Q Sepharose column chromatography, and phenyl-Superose column chromatography (Table 1). Purity of the sample was confirmed by gel electrophoresis (Fig. 1, lane E) and automated N-terminal sequence analysis. The final yield from the purification, based on the desulfurization of L-cysteine to give hydrogen sulfide, was 39%, and the specific activity of purified NIFS was 89.4 nmol of hydrogen sulfide produced per min per mg of protein (Table 1). The identity of isolated NIFS was confirmed by comparison of the N-terminal sequence determined from the isolated protein (Ala-Asp-Val-Tyr-Leu) to the predicted N-terminal sequence deduced from the gene sequence (14). The N-terminal methionine was not present in the isolated NIFS. Native molecular weight determination of NIFS was accomplished by gel-filtration chromatography using a Superose 12 FPLC column. Based on comparison to the migration of proteins having known molecular weights, native NIFS was shown to be a homodimer having a molecular weight of ≈87,500 (Fig. 2).

Identification of PLP as the Cofactor in NIFS. The isolated chromophore extracted from NIFS was readily retained when a solution of the compound, at neutral pH, was passed through a Q-Sepharose column but was not retained under the same conditions when passed through a CM-Sepharose column, indicating that the compound was anionic. That a portion of the negative charge resulted from the presence of a phosphate ester in the molecule was confirmed by cleavage of the sample by acid phosphatase. Identity of phosphate in the sample was also confirmed by the analysis of phosphate released from the sample by acid hydrolysis. The ultraviolet absorbance spectra of the sample was unaltered after acid hydrolysis, indicating that the phosphate ester was isolated from the chromophore of the molecule.

The purified cofactor dissolved in 0.05 M HCl had absorbance maxima at 230 (s, shoulder), 253, 293, and 324 nm with relative intensities of 0.41, 0.24, 0.52, and 0.51, respectively. The same concentration of cofactor, dissolved in 0.1 M NaOH, had absorbance maxima at 225 (s), 254, 304, and 388 nm with relative intensities of 1.0, 0.34, 0.19, and 0.03, respectively. Changes in the UV spectra of the compound with pH (data not shown) demonstrated that the chromophore showed both acidic and basic pKa values.

^{1}H NMR (400 Mz) of the HPLC-purified cofactor in ^2H_2O acidified with CF_3COO^2H showed resonances [expressed below as ppm (coupling pattern), proton identification] for two compounds in the ratio of ≈4:1. The most abundant compound had resonances at 2.63 (s, singlet), CH_3; 5.23 (d, doublet), CH_2—OP; 6.53 (s), $^2HOCHO^2H$; and 8.21 (s), aromatic H. The minor compound had resonances at 2.69 (s), CH_3; 5.38 (d), CH_2—OP; 8.32 (s), aromatic H; and 10.48 (s), CHO. The chemical shifts of the protons present in the minor compound were similar to those previously reported for PLP recorded at neutral pH (15). The shifts for the major compound were about the same as observed in the minor compound with the exception that the resonance at 10.48 had shifted to 6.53. Thus, we concluded that the major compound was the hydrated form of PLP. The lack of rapid exchange between the pyridoxal aldehyde and its hydrate, required for their detection as separate resonances, has been reported (15).

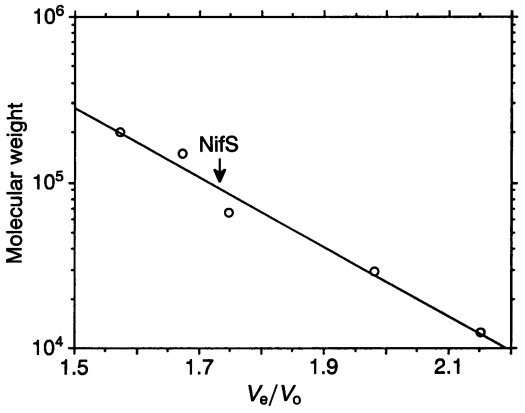

FIG. 2. Native molecular weight determination of NIFS. The void volume (V_o) was identified by blue dextran. Circles are proteins used as molecular weight markers, as follows: cytochrome *c*, M_r 12,400; carbonic anhydrase, M_r 29,000; bovine serum albumin, M_r 66,000; alcohol dehydrogenase, M_r 150,000; and β-amylase, M_r 200,000. The arrow indicates the migration position of native NIFS.

The GC/MS of the trimethylsilyl (TMS) derivative of the products resulting from the acid hydrolysis of the cofactor showed the presence of $(TMS)_3$phosphate and a peak identified as the $(TMS)_2$ derivative of the hemiacetal of pyridoxal (15).

All of these data are consistent with the assignment of the cofactor isolated from NIFS as being PLP. This structural assignment was confirmed by comparison of the extracted cofactor to an authentic sample of PLP. Both compounds had the same retention times on HPLC and TLC. Quantitation of the PLP content in NIFS showed 0.8 mol of PLP per NIFS monomer.

Identification of the Reaction Catalyzed by NIFS. PLP enzymes catalyze a diverse group of elimination and replacement reactions involving amino acids or their derivatives. To test whether or not NIFS catalyzed such a reaction using amino acids as substrate, we individually added each of the 20 L-amino acids to separate samples of NIFS and looked for changes in the visible spectra of the protein. Only L-cysteine was found to effect a change in the visible spectra, with the major absorbance peak at 392 nm changing to 416 nm (Fig. 3). Also, a new peak at 370 nm was formed upon addition of

Table 1. Purification of NIFS

Step	Total protein, mg	Total activity, nmol of H_2S per min	Specific activity, nmol of H_2S per min per mg	Yield, %
Crude extract	3550	40,500	11.4	100
Streptomycin supernatant	2250	37,800	16.8	93
25% ammonium sulfate supernatant	2250	36,000	16.0	89
45% ammonium sulfate precipitate	1110	26,300	23.7	65
Mono Q Sepharose	432	26,000	60.1	64
Phenyl-Superose	176	15,800	89.4	39

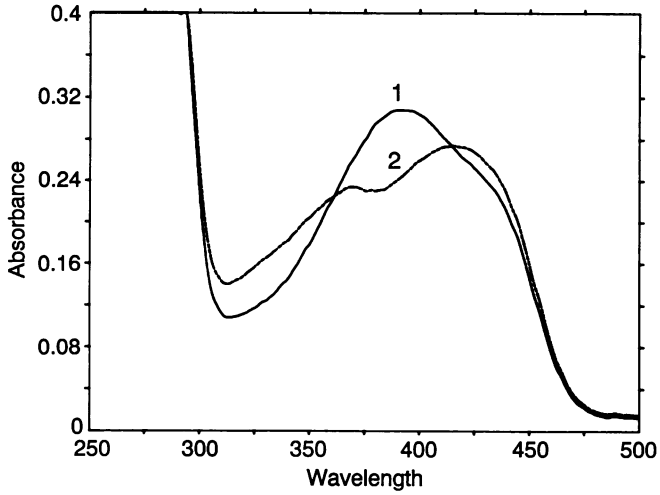

FIG. 3. Spectra of NIFS. Spectroscopic analysis was performed in a 1-ml cell containing purified NIFS at 4 mg/ml. Curves: 1, no addition; 2, in the presence of 0.5 mM L-cysteine.

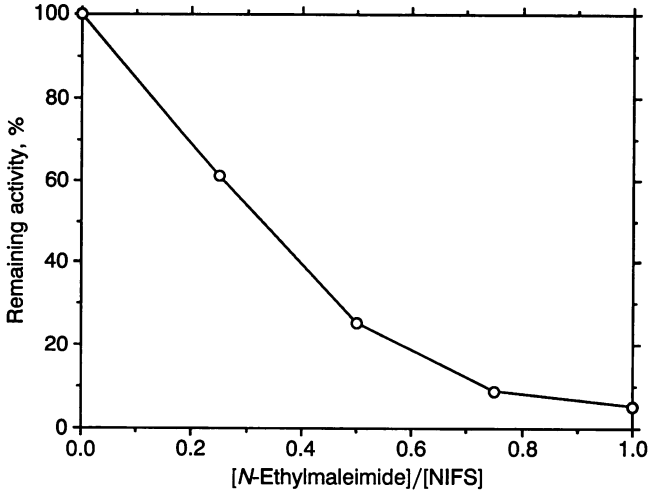

FIG. 4. Effect of *N*-ethylmaleimide on NIFS activity. Circles indicate the remaining NIFS activity, expressed as the percentage of that without inhibitor.

L-cysteine. The addition of D-cysteine had no effect on the spectrum. Incubation of L-cysteine with NIFS for 10 min or longer also resulted in formation of a cloudy precipitate of elemental sulfur. Table 2 shows the result of an analysis of the products formed from L-cysteine by NIFS. These results demonstrate that, in the absence of a reductant, NIFS desulfurates L-cysteine with the concomitant formation of elemental sulfur and L-alanine as the products. In the presence of dithiothreitol, the products were hydrogen sulfide, most likely produced by chemical reduction of the sulfur, and L-alanine.

Effect of Thiol-Specific Alkylating Reagents on NIFS Activity. Based on the consideration of potential mechanisms for desulfurization of L-cysteine catalyzed by NIFS and the possible physiological function for such activity (discussed below), it was of interest to determine whether a cysteinyl residue was involved in catalysis. To test this possibility, NIFS activity was assayed after its preincubation in the presence of thiol-specific alkylating reagents. The activity of NIFS was dramatically inhibited by preincubation of the protein with equimolar concentrations of *p*-chloromercuribenzoic acid, iodoacetamide, or *N*-ethylmaleimide. Fig. 4 shows that NIFS activity could be quantitatively inhibited by equimolar amounts of *N*-ethylmaleimide.

DISCUSSION

Results described in this report show that NIFS is a PLP-containing homodimer that specifically catalyzes the desulfurization of L-cysteine to yield equimolar amounts of L-alanine and elemental sulfur. Our interpretation of these findings is that NIFS catalyzes the release of sulfur from L-cysteine; the sulfur then becomes the sulfide present in the nitrogenase component protein metalloclusters. This suggestion is supported by previous work that showed that the sulfide present in iron–sulfur proteins from *E. coli* likely originates from cysteine sulfur (16). It also provides an explanation for the presence of a *nif*-specific serine acetyltransferase that, in *A. vinelandii*, is located in the same transcriptional unit as the *nifS* gene (14, 17). Formation of *O*-acetyl-L-serine is the step that is feedback-regulated in L-cysteine biosynthesis (18) and, thus, the presence of a serine acetyltransferase under *nif* gene regulation provides the cell a way to meet the increased demand for the L-cysteine that is required for metallocluster cluster formation under nitrogen fixing conditions.

Table 2. Desulfurization of L-cysteine catalyzed by NIFS

Reaction conditions	Products, % cysteine substrate		
	Sulfur	Sulfide	Alanine
L-Cysteine + NIFS	94.7	2.1	80.6
L-Cysteine + DTT + NIFS	8.0	96.5	92.8

Each reaction was performed in 2 or 4 ml of a mixture containing 0.5 mM L-cysteine in 20 mM Tris·HCl (pH 8.0). DTT (1 mM) was added to the reaction mixture where indicated. Reactions were initiated by addition of purified NIFS to 2 mg/ml. The reaction was incubated at ambient temperature for 2.5 h. In the absence of DTT, a small amount of pyruvate was also found as product and was subtracted from the alanine value shown in the table. There was no sulfur, hydrogen sulfide, or alanine produced in control experiments where either the enzyme or L-cysteine was omitted from the reaction mixture.

The route to the formation of sulfur from L-cysteine catalyzed by NIFS may have some similarities to the mechanism proposed for selenocysteine β-lyase, which is also a PLP enzyme and catalyzes the formation of L-alanine and Se by using L-selenocysteine as substrate (19). However, in contrast to the mechanism suggested for the elimination of Se from selenocysteine, we propose a different mechanism for the elimination of sulfur from L-cysteine catalyzed by NIFS. Namely, we suggest that after the cysteine-PLP Schiff's base adduct is formed, a nucleophilic attack occurs by an enzyme thiolate anion at the sulfur of the substrate cysteine. This results in repulsion of the stabilized C-3 anion of alanine and formation of an enzyme-bound cysteinyl persulfide, which in turn is the likely candidate for acting as the sulfur donor during metallocluster formation. In the present experiments, where the suitable acceptors for the activated sulfur are not available, namely, iron or the appropriate apoprotein, the cysteinyl persulfide decomposes to elemental sulfur (20), or, in the presence of a reductant, is reduced to hydrogen sulfide. Evidence for the presence of a highly reactive cysteinyl thiolate group on NIFS that could be involved in the formation of the cysteinyl persulfide during catalysis was obtained by titrating the enzyme with *N*-ethylmaleimide (Fig. 4), which, when present at an equimolar concentration with NIFS, resulted in nearly complete inactivation of NIFS.

Formation of protein-bound cysteinyl persulfides has precedence in thiosulfate sulfurtransferase (rhodanese), which has also been suggested to have a role in metallocluster formation (21). However, rhodanese does not use PLP chemistry and has not been shown to have a specific role in metallocluster formation. Indeed, Sandberg *et al.* (22) have argued against a physiological role for rhodanese in metal-

locluster formation because rhodanese has not been detected in appreciable levels in certain bacteria known to contain nonheme iron sulfur proteins. Moreover, any enzyme that catalyzes formation of sulfide could be expected to assist reconstitution of apoproteins *in vitro* because such reconstitution occurs spontaneously, provided that sufficient sulfide and iron are available (22). In contrast to rhodanese, a specific physiological role for NIFS in mobilizing the sulfur required for nitrogenase metallocluster formation is indicated because NIFS is required for maturation of both the nitrogenase component proteins and because it uses L-cysteine, a known sulfur source for nonheme metalloclusters in *E. coli* (16), as substrate.

Although the deletion of *nifS* lowers the activity of both nitrogenase component proteins, it does not completely eliminate the activity of either one. One explanation for this observation could be that the iron–sulfur cores required for nitrogenase metallocluster assembly could form spontaneously, albeit at a low level, in the absence of NIFS. This possibility is supported by previous work concerning the *in vitro* reconstitution of apoferredoxins using iron and sulfide (23). However, our inability to stimulate the diazotrophic growth of *nifS* mutants by increasing the sulfur and iron supplement to the growth medium does not support the idea of spontaneous iron–sulfur core assembly. An alternative explanation is that NIFS represents a general class of enzymes that use PLP chemistry and L-cysteine as substrate in a universal first step in the mobilization of sulfur for metallocluster formation. Namely, in the absence of NIFS, other NIFS-like enzymes involved in sequestering the sulfur for other metalloproteins might supplant the NIFS function. The identification of genes present in nonnitrogen fixing organisms that encode polypeptides having extraordinary sequence identity when compared to NIFS provides considerable support for this idea. For example, such genes have been identified from *Lactobacillus delbrueckii* (24), *Bacillus subtilis* (25), *Saccharomyces cerevisiae* (24), and *Candida maltosa* (24). Of particular relevance is the high degree of sequence identity among this class of polypeptides concentrated around the lysinyl and cysteinyl residues we have targeted as providing the probable PLP-binding and active-site cysteinyl residues, respectively (Fig. 5). Although the specific functions of the *nifS*-like genes from these organisms are not yet established, Leong-Morgenthaler *et al.* (24) have independently suggested that a common thread among such apparently different metabolic processes could involve redox functions.

	202 ⇓	325 ⇓
Av	...IHMLSLCGHKLHAPKGVG...	...IAASSGSACTSGSLEPS...
An	...IDMLTISGHKIHAPKGIG...	...ICASSGSACTSGSLEPS...
Kp	...IDMLSCSAHKFHGPKGVG...	...IAASSGSACTSGSLEPS...
Rs	...IDMLSLSAHKFHGPKGVG...	...ICVSSGAACASGAMEPS...
Sc	...IDLLSISSHKIYGPKGIG...	...IALSSGSACTSASLEPS...
Ld	...VDMMSFSSHKFHGPRGIG...	...IYISTTSACASKKADEA...
Bs	...VDALSMSSHKIHGPKGVG...	...ICISTGSACSAGYHGPS...

FIG. 5. Sequence comparison of NIFS with other *nifS*-like gene products. Av, *A. vinelandii* NIFS (26); An, *Anabaena* NIFS (27); Kp, *Klebsiella pneumoniae* NIFS (26); Rs, *Rhodobacter sphaeroides* NIFS (28); Sc, *Saccharomyces cerevisiae nifS*-like YCL17C gene product (24); Ld, *Lactobacillus delbrueckii nifS*-like gene product (24); Bs, *Bacillus subtilus nifS*-like gene product that is involved in NAD biosynthesis (25). Numbers refer to the *A. vinelandii* sequence. Lys-202 is considered to be the probable PLP-binding residue, based on comparison to certain other known sequences of PLP-binding proteins (for example, see ref. 29), and Cys-325 is targeted as the probable active-site cysteinyl because it is the only conserved cysteinyl residue among all known NIFS sequences from diazotrophic organisms. Strictly conserved amino acids are underlined.

In summary, NIFS has been shown to catalyze the desulfurization of L-cysteine to form L-alanine and elemental sulfur. Based on these results, consideration that elimination of NIFS function affects both nitrogenase component protein activities, and the recent discovery of *nifS*-like genes in nonnitrogen fixing organisms, we propose that NIFS activity represents a universal mechanism that uses PLP chemistry in the activation of the sulfur required for metallocluster formation.

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