The NIFS Protein Can Function as a Selenide Delivery Protein in the Biosynthesis of Selenophosphate*

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The NIFS protein from Azobacter vinelandii is a pyridoxal phosphate-containing homodimer that catalyzes the formation of equimolar amounts of elemental sulfur and L-alanine from the substrate L-cysteine (Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2754–2758). A sulfur transfer role of NIFS in which the enzyme donates sulfur for iron sulfur center formation in nitrogenase was suggested. The fact that NIFS can also catalyze the decomposition of L-selenocysteine to elemental selenium and L-alanine suggested the possibility that this enzyme might serve as a selenide delivery protein for the in vitro biosynthesis of selenophosphate. In agreement with this hypothesis, we have shown that replacement of selenide with NIFS and L-selenocysteine in the in vitro selenophosphate synthetase assay results in an increased rate of formation of selenophosphate. These results thus support the view that a selenocysteine-specific enzyme similar to NIFS may be involved as an in vivo selenide delivery protein for selenophosphate biosynthesis. A kinetic characterization of the two NIFS catalyzed reactions carried out in the present study indicates that the enzyme favors L-cysteine as a substrate compared with its selenium analog. A specific activity for L-cysteine of 142 nmol/min/mg compared with 55 nmol/min/mg for L-selenocysteine was determined. This level of enzyme activity on the selenoamino acid substrate is adequate to deliver selenium to selenophosphate synthetase in the in vitro assay system described.

The insertion of selenium into Se-dependent enzymes and Se-tRNAs requires the formation of the highly reactive, reduced selenium donor compound monoselenophosphate (1). The Escherichia coli selD gene product, selenophosphate synthetase, catalyzes the synthesis of monoselenophosphate from selenide and ATP (Scheme 1) (2).

\[
\text{ATP} + \text{HSe}^- + \text{H}_2\text{O} \rightarrow \text{H}_2\text{SePO}_3^- + \text{P}_i + \text{AMP}
\]

\text{Scheme 1}

Selenophosphate synthetase from E. coli has been characterized previously (2). The enzyme has a determined specific activity of 83 nmol/min/mg (K_m = 3 min^{-1}). The initial K_m values reported for each substrate, ATP and selenide, were 0.9 mM and 46 µM, respectively. Selenide is extremely oxygen labile, so the kinetics were repeated using more stringent anaerobic conditions. Under these conditions, the K_m for selenide was calculated as 7.3 µM, which is still above the range of 0.1–1 µM which is the optimal concentration of selenite used in most growth media. Despite being below the K_m value for one essential enzyme in the pathway, this concentration of selenide is sufficient for the biosynthesis of selenium-containing proteins and tRNAs.

The E. coli selenophosphate synthetase contains an essential cysteine, Cys^17, within the glycine-rich sequence -Gly-Ala-Gly-Cys^17-Gly-Cys-Lys-Ile-. Replacement of Cys^17 with serine results in complete loss of activity with selenide and ATP as substrates (3). Additionally, both a human form of the enzyme that contains threonine (4) in place of the essential Cys^17 in the E. coli enzyme and a Drosophila enzyme that has an arginine replacement (5) are inactive in the in vitro selenophosphate synthetase assay with selenide and ATP as substrates. The observed low specific activity of the E. coli selenophosphate synthetase and the inactivity of human and Drosophila homologs suggests that an essential unidentified component is required for the formation of selenophosphate.

In an early attempt to understand the mechanism of the incorporation of selenocysteine into proteins, ³H-, ¹⁴C-, and ⁷⁵Se-labeled L-selenocysteine samples were prepared. These labeled compounds were added to media of Clostridium sticklandii cultures to determine whether they were capable of being directly incorporated into selenoprotein A of the glycine reductase complex. ⁷⁵Se derived from [⁷⁵Se]selenocysteine was incorporated more efficiently into selenoprotein A than H₂⁷⁶Se (generated by reduction of ⁷⁵SeO₃²⁻ by excess thiols in the incubation medium). There was no detectable incorporation of ¹⁴C or ³H into the selenocysteine amino acid of selenoprotein A (6). These results suggest an activated selenium derived from L-selenocysteine may be the optimal substrate for selenophosphate synthetase. Additionally, this suggests the involvement of a selenocysteine lyase protein in substrate formation.

Selenocysteine lyase enzymes have been isolated from both bacteria (7) and pig liver (8). This family of enzymes catalyzes the exclusive decomposition of L-selenocysteine to L-alanine and elemental selenium. They are very specific for their selenoamino acid substrate and do not utilize the sulfur analog L-cysteine as a substrate. Recently, Mihara et al. (9) have found that the N-terminal amino acid sequence of the pig liver selenocysteine lyase is similar to the amino acid sequence of the cysteine desulfurase protein NIFS.

The nifs gene product (NIFS) from the diazotrophic bacterium Azotobacter vinelandii has been previously isolated and characterized. The protein has been identified as a pyridoxal phosphate-containing homodimer that catalyzes the formation of equimolar amounts of elemental sulfur and L-alanine from the substrate, L-cysteine (10). It was reported that NIFS is also able to catalyze the removal of selenium from selenocysteine; a mechanism similar to the L-cysteine reaction was postulated (11).
In this work, we report the characterization of the NIFS catalyzed reaction with L-selenocysteine as substrate. We also report the use of NIFS and L-selenocysteine as a selenode­livery system in the selenophosphate synthetase assay.

**EXPERIMENTAL PROCEDURES**

**Materials**

*A. vinelandii* NIFS protein and pDB551 (*nifs* gene under control of the T7 promoter) were gifts from Dennis Dean, Virginia Polytechnic Institute. All buffers and reagents were prepared from the highest grade chemicals available.

**Methods**

Overexpression and Purification of NIFS—*A. vinelandii* NIFS was overexpressed in E. coli cells and purified by a procedure as described previously, with slight modifications (10). BL21 cells transformed with pDB551 were grown in a 10-liter fermentor containing ampicillin (50 mg/ml) at 37 °C to an A600 of 0.6. Cells were induced by the addition of lactose to a concentration of 0.4%. After 2.5 h, cells were harvested by centrifugation, frozen in liquid N2, and stored at −80 °C until needed.

Preparation of Cell Extract—20 grams of E. coli BL21 cells containing overexpressed NIFS were thawed in 50 ml of 25 mM Tris-HCl (pH 7.4). Extract was prepared by passing the cell suspension once through a French pressure cell. Cell debris was removed by centrifugation at 15,000 rpm for 20 min. Streptomycin sulfate was added to the supernatant to a final concentration of 15%. After 15 min, the precipitate was 15,000 rpm for 20 min. Streptomycin sulfate was added to the supernatant to a final concentration of 15%. After 15 min, the precipitate was sedi­mented by centrifugation at 15,000 rpm for 20 min. Ammonium sulfate was added to the supernatant to a final concentration of 45% of saturation, and the sedi­mented and stored at −80 °C until needed.

Preparation of Cell Extract—20 grams of E. coli BL21 cells containing overexpressed NIFS were thawed in 50 ml of 25 mM Tris-HCl (pH 7.4). Extract was prepared by passing the cell suspension once through a French pressure cell. Cell debris was removed by centrifugation at 15,000 rpm for 20 min. Streptomycin sulfate was added to the supernatant to a final concentration of 15%. After 15 min, the precipitate was sedimented by centrifugation at 15,000 rpm for 20 min. Ammonium sulfate was added to the supernatant to a final concentration of 45% of saturation, and the protein precipitate was resuspended in 10 ml of 25 mM Tris-HCl (pH 7.4).

**DEAE Chromatography:**—The redissolved ammonium sulfate precipi­tate was desalted on PD10 columns equilibrated with 25 mM Tris-HCl (pH 7.4), and the resulting protein solution was applied to a DEAE-Sepharose Fast Flow anion-exchange column (2.5 × 20 cm) equilibrated with 25 mM Tris-HCl (pH 7.4). Proteins were eluted with a linear gradient of 0.1–0.6 M NaCl in 25 mM Tris-HCl (pH 7.4) at a flow rate of 4 ml/min. Fractions (8 ml) were collected and analyzed by SDS-poly­acrylamide gel electrophoresis (12) for the presence of NIFS, a 44-kDa protein. Fractions containing NIFS were pooled and dialyzed overnight against 25 mM Tris-HCl (pH 7.4) in 0.5 M ammonium sulfate.

Phenyl-Sepharose Chromatography—Freshly dialyzed NIFS was ap­plied to a phenyl-Sepharose CL-4B column equilibrated with the same buffer. Proteins were eluted with a linear gradient of 0.5–0 M am­monium sulfate in 25 mM Tris-HCl (pH 7.4) (500 ml). NIFS was eluted in fractions at the end of the gradient. These fractions were pooled, dia­lyzed, concentrated, and stored at −80 °C.

**Assays for NIFS Activity**—All NIFS assays were performed anaero­bically in a reaction mixture containing 50 mM Tricine-KOH (pH 8.0), 4 mM DTT, 8 mM MgCl2, 50 mM KCl, 0.1 mM Mgtriptilex, and 2 μM NIFS. Activity with L-cysteine was determined by monitoring sulfide produc­tion from cysteine. Sulfide was measured by its conversion to methylene blue in 0.02 M N,N-dimethyl-p-phenylenediamine sulfate in 7.2 N HCl, and 0.03 M FeCl3 in 1.2 N HCl. After color development, absorbance was measured at 650 nm, and sulfide concentration was determined based on a standard Na2S curve.

The coupled selenophosphate synthetase-NIFS protein assay was performed in the same reaction mixture described above with the fol­lowing modifications: 2 mM ATP, 0.2 μM [8-3H]ATP, 10 μM selenophos­phate synthetase, 5 μM NIFS were additionally added, and reaction mixtures were incubated at 37 °C. After 30 min, the reactions were terminated by the addition of 1.2 N HClO4 followed by neutralization with KOH. The nucleotides in the supernatant solutions were sepa­rated chromatographically on cellulose-polyethyleneimine thin layer sheets developed in 1.0 M formic acid and 0.5 M LiCl. Nucleotide spots identified by UV quenching were cut out, and radioactivity was measured by liquid scintillation spectroscopy.

**35S NMR Spectroscopy**—To detect reaction products of the coupled reaction between NIFS and selenophosphate synthetase, a coupled reaction was performed in an NMR tube under nitrogen for 3 h at 37 °C. The reaction mixture (1 ml) contained 50 mM Tricine-KOH (pH 8.0), 4 mM DTT, 8 mM MgCl2, 50 mM KCl, 0.1 mM Mgtriptilex, and 10 μM selenophosphate synthetase, and 10 μM L-selenocysteine. The NMR spectra were obtained on a Bruker 500 MHz at 37 °C as described previously (1). The catalytic properties of the enzyme were determined from the reaction rate in the presence of L-cysteine as a substrate.

**RESULTS**

**Catalytic Properties and Substrate Specificity**—As previously reported, NIFS is a pyridoxal phosphate-containing enzyme that catalyzes the formation of L-alanine and sulfur from L-cysteine as substrate (10). To confirm that NIFS can also use L-selenocysteine as substrate and to compare enzyme activities on the two amino acid substrates, two types of assays were carried out. NIFS cysteine desulfurase activity was determined by monitoring the production of H2Se in the methylene blue assay. Cysteine desulfurase assays were performed in the presence of DTT to keep the sulfur product reduced to H2S. The kinetic constants observed for this reaction are reported in Table 1. A specific activity of 142 nmol/min/mg was determined for the NIFS reaction with L-cysteine, as well as a kcat of 6 min−1 and a Vmax of 2.4 nmol/min. A Km for the substrate L-cysteine could not be determined because, at substrate concentrations as low as 10 μM, the velocity was still maximal (Fig. 1).

In our assays, NIFS catalyzed the elimination of selenium from L-selenocysteine as monitored by the reaction of H2Se with lead acetate (8). The velocity of the NIFS catalyzed reaction with L-selenocysteine as the substrate exhibited a sub-
Selenophosphate Synthetase Assays Performed with L-selenocysteine—

The synthesis of monoselenophosphate from selenide and ATP (2), additional reaction products include AMP and orthophosphate.

When selenide is replaced with L-selenocysteine, selenophosphate is not formed, as determined by the lack of ATP hydrolysis (Table II). However, replacement of selenide in the assay with L-selenocysteine in the presence of added NIFS protein results in the formation of AMP (Fig. 4). The amount of AMP formed is dependent on the concentration of NIFS added. In the coupled assay, when concentrations of NIFS are lower than the selenophosphate synthetase concentration, AMP production is lower as compared with the assay using selenide. However, if the concentration of NIFS is equal to or double the concentration of selenophosphate synthetase in the assay may activate the enzyme system. When selenophosphate synthetase is present, the amount of H₂Se produced is increased by 35%. The rate of elimination of selenium from L-selenocysteine increases, as well, from 6.1 to 9.5 nmol/min (Fig. 5). The presence of the selenophosphate synthetase in the assay may activate NIFS toward L-selenocysteine. In contrast, in standard selenophosphate synthetase assays performed, with the addition of NIFS, using H₂Se as substrate, no increase in either
the rate or the amount of AMP product formed was observed (data not shown).

**Analysis of Reaction Products by \(^{31}\text{P} \text{NMR}**—To directly observe the products generated by selenophosphate synthetase in the presence of NIFS and L-selenocysteine, reaction products were analyzed by \(^{31}\text{P} \text{NMR}\. As shown in Fig. 6, products formed in the coupled selenophosphate synthetase and NIFS assay from ATP and L-selenocysteine produced \(^{31}\text{P} \text{chemical resonances expected for AMP, inorganic phosphate, and selenophosphate}. Additional spectra were obtained for NIFS + ATP + L-selenocysteine as well as selenophosphate synthetase + L-selenocysteine + ATP. Both reactions did not produce the expected \(^{31}\text{P} \text{chemical resonance for selenophosphate} (\text{data not shown}).

**DISCUSSION**

Because of the chemical similarity between selenium and sulfur, enzymes that metabolize sulfur compounds frequently demonstrate similar activities with the selenium analogs of their natural substrates (9). It has been shown in recent studies with cysteine sulfinate desulfinase isolated from *E. coli* that the enzyme exhibits both selenocysteine lyase and cysteine desulfurase activities (9). Moreover, previous work on the *A. vinelandii* NIFS protein demonstrated that NIFS can act on both L-cysteine and L-selenocysteine (10). In the present paper, we characterized in more detail the enzymatic decomposition of L-selenocysteine by the NIFS protein.

Our studies confirm the work by Zheng *et al.*, 1993 showing that the protein can utilize both L-cysteine and L-selenocysteine as substrates (10). Our kinetic values indicate the enzyme favors L-cysteine as a substrate over its selenium analog (Table I). This is not surprising because NIFS has been shown to have a biologically significant role in the biosynthesis of Fe-S clusters (11). A specific activity for L-cysteine of 142 nmol/min/mg, compared with 55 nmol/min/mg for L-selenocysteine was deter-

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**FIG. 5.** Activation of selenocysteine lyase activity of NIFS in the presence of selenophosphate synthetase. Selenocysteine lyase activity of NIFS was measured as described under "Experimental Procedures" in the presence and absence of selenophosphate synthetase.

**FIG. 6.** \(^{31}\text{P} \text{NMR spectrum of selenophosphate synthetase reaction mixture containing NIFS and selenocysteine in place of selenide}\. To detect the products of the coupled reaction between NIFS and selenophosphate synthetase, a reaction was carried out in an NMR tube under nitrogen at 37 °C for 3 h. The reaction mixture (1 ml) contained 50 mM Tricine-KOH (pH 8.0), 4 mM DTT, 8 mM MgCl\(_2\), 50 mM KCl, 0.1 mM Mgtitriplex, 2 mM ATP, 2 mM L-selenocysteine, 10 \(\mu\)M selenophosphate synthetase, and 10 \(\mu\)M NIFS.
mined. An interesting aspect of our characterization of NIFS was that even at L-cysteine concentrations as low as 10 μM, the rate of the cysteine desulfurase activity remains at the V_max. The independence of the determined velocity of the cysteine desulfurase activity on substrate concentration, indicating a very high desulfurase rate, makes the determination of K_m more difficult. Our determined specific activity as well as the inability to determine a K_m for L-cysteine differs from the previous results (10, 11) in which a specific activity of 70 nmol/min/mg and a K_m of 75 μM for the substrate L-cysteine were reported. These investigators suggest NIFS activity is highly sensitive to both pH and temperature, which affects the determined K_m and specific activity values (11).

Based on the studies of Zheng et al. (11) and Flint (14) with the E. coli NIFS protein, it was proposed that in the NIFS catalyzed reaction a sulfhydryl group, from an active site cysteine, acts as a nucleophile in the mobilization of sulfur from the L-cysteine substrate. Flint suggests that after sulfur is transferred to NIFS, creating a persulfide on the active site cysteine, catalysis stops or slows down until this sulfane sulfur is removed by an uncharacterized pathway. This would account for the relatively low turnover number for the enzyme. When assays are performed in the presence of DTT, as in this report, the rate-limiting sulfane sulfur undergoes nucleophilic displacement by DTT forming H_2S (14). Although the formation of a perselenide (R-S-Se^-) in the NIFS reaction with l-selenocysteine has not been investigated in the present study, Zheng et al. 1994 (11) have proposed that the mechanism of selenocysteine lysis by NIFS is mechanistically similar to the cysteine desulfurase reaction. This is suggested by the requirement of Cys325 that was determined to be required for both activities (11) (Scheme 2).

Because of the high reactivity of selenide within a biological system, the overall cellular concentrations must remain relatively low. The availability of selenide delivery proteins could effectively raise the concentration of selenide in a localized region of the cell where it could be rapidly metabolized by a second enzyme. Although concentrations of free selenide may be low, selenium is present in cells at higher concentrations in other less reactive forms such as selenomethionine. For instance, bacteria, plants, and yeast have been found to synthesize the selenium-containing amino acid selenomethionine. The presence of selenium in place of the sulfur of methionine offers no altered biological function. Selenomethionine is abundant in plants, and when consumed organisms can utilize this form as readily as the natural occurring amino acid methionine by incorporating it freely into proteins (15). In addition to becoming incorporated into cellular proteins, selenomethionine can be catabolized to selenocysteine by the transsulfuration pathway (16). Selenocysteine can then be metabolized by the pyridoxal phosphate-dependent selenocysteine lyase proteins to release elemental selenium. In the presence of reducing compounds, elemental selenium is reduced to selenide. This could provide an effective mechanism to maintain concentrations of selenide within the cell.

In addition to the formation of selenide from L-selenocysteine, the nonenzymatic reaction of selenite with thiols, such as glutathione or cysteine, is believed to be a significant pathway for the incorporation of selenium into living cells. The reduction to elemental selenium requires the combination of thiols to selenite in a 4:1 stoichiometric ratio. Further reduction to selenide requires two additional equivalents of thiols (17).

Our results clearly show the use of a selenide delivery protein can increase the turnover rate of an enzyme which utilizes selenide as a substrate (Figs. 4 and 6). The exact mechanism responsible for the increase in rate is not yet known. Possibilities include higher local concentrations of selenide in the vicinity of selenophosphate synthetase. Perhaps both proteins interact in solution, allowing direct transfer of selenide product from NIFS to selenophosphate synthetase. The precise chemical form of selenium produced in the reaction by NIFS may be an important determinant. Selenide can exist in several forms analogous to sulfane sulfur species, depending on the reaction conditions used. Thus, the product formed from NIFS may be a more optimal substrate for selenophosphate synthetase. Future experiments are now being planned to answer these questions.

The essential role of NIFS in the pathway for the biosynthesis...
sis of FeS clusters cannot be disputed as one of its primary biological roles \textit{in vivo}. In the \textit{in vitro} coupled assay, NIFS additionally can catalyze the elimination of selenium from L-selenocysteine as well as function as a selenide delivery protein. However, \textit{in vivo} concentrations of sulfur containing compounds are on the order of a thousand times greater than their selenium analogs (18), and thus proteins such as NIFS will preferentially bind to and metabolize L-cysteine over the corresponding selenium analog L-selenocysteine. Hence, for effective selenide delivery systems to operate, enzymes which are specific for selenide compounds are required. Among such possible enzymes are selenocysteine lyases that have been identified in several organisms. Although kinetic characterization of these enzymes reveals they have relatively high $K_m$ values \textit{in vitro} for their selenium containing substrates, the assay conditions used may not represent true cellular conditions where the $K_m$ may actually be lower. These enzymes may play an important role in the regulation of selenium availability in biological systems. Thus their ability \textit{in vitro} to metabolize selenocysteine to alanine and selenium or selenide suggests that, \textit{in vivo}, they may function as selenium delivery proteins analogous to sulfur delivery enzymes.

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