Escherichia coli NifS-like Proteins Provide Selenium in the Pathway for the Biosynthesis of Selenophosphate*

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Selenophosphate synthetase (SPS), the selD gene product from Escherichia coli, catalyzes the biosynthesis of monoselenophosphate, AMP, and orthophosphate in a 1:1:1 ratio from selenide and ATP. Kinetic characterization revealed the $K_m$ value for selenide approached levels that are toxic to the cell. Our previous demonstration that a $Se^0$-generating system consisting of l-selenocysteine and the Azotobacter vinelandii NifS protein can replace selenide for selenophosphate biosynthesis in vitro suggested a mechanism whereby cells can overcome selenide toxicity. Recently, three E. coli NifS-like proteins, CsdB, CSD, and IscS, have been overexpressed and characterized. All three enzymes act on selenocysteine and cysteine to produce $Se^0$ and $S^0$, respectively. In the present study, we demonstrate the ability of each E. coli NifS-like protein to function as a selenium delivery protein for the in vitro biosynthesis of selenophosphate by E. coli wild-type SPS. Significantly, the SPS (C17S) mutant, which is inactive in the standard in vitro assay with selenide as substrate, was found to exhibit detectable activity in the presence of CsdB, CSD, or IscS and l-selenocysteine. Taken together, the ability of the NifS-like proteins to generate a selenium substrate for SPS and the activation of the SPS (C17S) mutant suggest a selenium delivery function for the proteins in vivo.

The highly reactive, reduced selenium compound monoselenophosphate is required for the insertion of selenium into sulfur-dependent enzymes (1) and seleno-tRNAs (2). The selD gene product from Escherichia coli catalyzes the biosynthesis of monoselenophosphate, AMP, and orthophosphate in a 1:1:1 ratio from selenide and ATP (Reaction 1).

$$\text{ATP} + \text{HSe}^- + \text{H}_2\text{O} \rightarrow \text{H}_2\text{SePO}_4^- + \text{AMP} + \text{P}_i$$

**REACTION 1**

Selenophosphate synthetase (SPS)$^1$ from E. coli (3) and the closely related enzyme from Haemophilus influenzae (4) have been characterized. Both enzymes are active in the presence of high levels of free selenide (1–5 mM) and dithiothreitol (8–10 mM) that are included in the in vitro assay system employed routinely. Under these conditions, the apparent $K_m$ values for ATP and selenide are 1 mM and 20 $\mu$M, respectively. Although the reactions are carried out under argon, any traces of contaminating oxygen tend to reduce the effective concentration of selenide. The standard use of high selenide levels in vitro is a partial solution of this problem.

The $K_m$ value determined for selenide in the in vitro system is far above the optimal concentration (0.1–1 $\mu$M) of selenium needed for the growth of various bacterial species and cultured mammalian cells. In fact, levels above 10 $\mu$M are toxic for many bacterial species. The fact that the high reactivity and thus the toxicity of selenide in biological systems is even greater than that of sulfide is apparent from the greater extent of ionization at physiological pH of H$_2$Se with a $pK_a$ of 3.89 as compared with that of H$_2$S with a $pK_a$ of 7.5. Rate acceleration by ionized selenols of disulfide reduction and disruption of protein structures or destabilization of coordinated metal ions from metallothionines (5) can occur if selenide concentrations are not maintained at low and optimal levels. Potential candidates for control of selenium levels are the specific l-selenocysteine-lyase enzymes that decompose selenocysteine to elemental selenium ($Se^0$) and alanine (6, 7) and a closely related enzyme d-selenocysteine-lyase (8). The attractive possibility that these lyases also may serve normally to deliver $Se^0$ to SPS was suggested by previous studies in which it was shown that l-selenocysteine and the Azotobacter vinelandii NifS protein effectively replaced the high level of free selenide in the in vitro SPS assay (9). In fact, even though the normal substrate for A. vinelandii NifS is cysteine (10, 11), the extent of utilization of selenocysteine in vitro was sufficient to promote a higher rate of selenophosphate formation by SPS than was observed with free selenide alone.

The A. vinelandii NifS protein and the selenocysteine-lyases are pyridoxal 5’-phosphate-dependent enzymes that catalyze the elimination of sulfur from l-cysteine and selenium from l-selenocysteine (10, 11). Because in vivo concentrations of sulfur-containing compounds are a thousand-fold or more greater than their selenium analogs, proteins such as NifS presumably bind and metabolize l-cysteine preferentially. Thus, lyase proteins that are highly specific for l-selenocysteine as substrate may function as the actual selenium delivery proteins in vivo. In E. coli three NifS-like proteins were identified recently (12). All three proteins resemble A. vinelandii NifS in amino acid sequences and catalytic properties. Sequence alignments also revealed similarities between the N-terminal region amino acid sequence of the pig liver selenocysteine-lyase (6). Characterization of the three E. coli NifS-like
proteins CsdB, CSD, and IscS revealed considerable differences in the degree of discrimination between l-cysteine and l-selenocysteine as substrates (12). The CsdB protein, although not entirely specific for the seleno-amino acid, substrate was 290 times more active on l-selenocysteine than on l-cysteine. This protein thus appears to be the E. coli counterpart of the mammalian selenocysteine-lyase.

In an attempt to identify residues that are essential for catalysis in the SPS enzyme, mutagenesis experiments have been performed (13, 14). Certain amino acids in a glycine-rich potential ATP-binding site in the N-terminal region of the protein were selected as targets. Mutagenesis of cysteine 17, located in the potential ATP-binding site, to serine resulted in the complete loss of detectable SPS activity in both the in vitro selenide-dependent assay and the in vivo complementation of a selD lesion in E. coli strain MBO8 (13). The inability to detect SPS catalytic activity of the cysteine 17 mutant enzyme and the failure of this mutated enzyme to catalyze a positional isotope exchange reaction between the γ-phosphoryl group and the β-phosphoryl group of ATP supported a catalytic role for cysteine 17. However, a catalytic role was contradicted by the finding that substitution of selenocysteine for cysteine at position 17, which occurs normally in H. influenzae SPS, fails to elicit an increase in catalytic activity (4). In the present work, we report the ability of the CsdB, CSD, and IscS proteins to function both in vitro and in vivo as selenium delivery proteins to the E. coli wild-type SPS and also to SPS (C17S) mutant.

EXPERIMENTAL PROCEDURES

Materials

L-Selenocystine was synthesized (15) and converted to l-selenocysteine as reported (6). [8-14C]ATP was purchased from ICN Biomedical Research Products, Costa Mesa, CA. [75Se]Selenite was purchased from The University of Missouri Research Reactor Facility, Columbia, MO. The lyase proteins CsdB, CSD, and IscS were purified as described by Mihara et al. (12). SPS was purified by the procedure of Veres et al. (3).

Methods

Complementation of the selD Mutant MBO8—E. coli strain MBO8 (16, 17) transformed with plasmids containing either the SPS gene or the SPS (C17S) mutant gene was grown anaerobically at 37 °C overnight in Luria broth containing 0.5% glucose. Cultures were observed after 24–48 h for the production of H2, a product of an active formate hydrogen-lyase (18). Synthesis of the selenocystine-containing FDH2* depends on the availability of selenophosphate.

In parallel experiments, MBO8 cultures containing both plasmids were streaked out on Luria medium plus glucose agar plates and incubated anaerobically for 48 h at 37 °C. After growth, all individual colonies were colorless. The plates were then overlaid with agar containing benzyl viologen, an electron acceptor for active FDH2*, that turns blue when reduced. Thus, any colonies appearing blue in color provide a test for the presence of FDH2*. (75Se)Selenocystine-labeled FDH—Single colonies of MBO8, MBO8/SPS, and MBO8/SPS (C17S) were grown anaerobically in Luria broth + 20 μCi [75Se]Selenite at 37 °C for 24 h. Following growth, cells were harvested, resuspended in 100 mM potassium phosphate buffer, pH 7.2, and sonicated. Supernatants were analyzed for the presence of the selenocystine-containing FDH2* by SDS-polyacrylamide gel electrophoresis and PhosphorImager detection of radioactivity.

Enzyme Assays—All assays were performed anaerobically three to six times and averaged for each reported value. The coupled SPS-lyase reaction mixtures contained 50 mM Tricine-KOH, pH 8.0, 8 mM MgCl2, 20 mM KCl, 50 mM dithiothreitol, 200 μM pyridoxal 5'-phosphate, 2 mM ATP, 10 mM l-selenocysteine, 10 mM SPS, 0.2 μM [8-14C]ATP, and the indicated concentration of added lyase. Reaction mixtures were incubated at 37 °C for 30 min, terminated by the addition of 1.2 N HCl, and analyzed as described in Methods, with KOH added to provide Se0, S0, or Se2 respectively. One of the proteins, CsdB, utilizes selenocysteine more effectively than cysteine, indicating a more specific role for Se2 generation. To test the relative abilities of the three NiS-like proteins from E. coli to provide Se2 to SPS, assays were performed with L-selenocysteine and CsdB or CSD, or IscS in place of free selenide. SPS catalytic activity was supported in the presence of each NiS-like protein (Fig. 1A). The determined activity of SPS was directly related to the concentration of lyase used in each assay. When lyase concentrations were increased above 0.025 μM, the amount of AMP generated by SPS was slightly higher than AMP formed in assays using 1.5 mM free selenide (data not shown). However, by lyase concentrations above 0.05 μM, the amount of selenide generated was much higher (Fig. 1B) as compared with the amount of AMP generated by SPS. (In the absence of an acceptor (SPS) and in free solution the product of the lyase reaction is referred to as selenide.) The inability to detect increased SPS activity in the presence of higher concentrations of lyase indicates SPS is saturated with selenium. SPS may have an intrinsic catalytic role because of a possible rate-limiting step in the reaction mechanism. The rate-limiting step may occur after selenium binding and could include bound ADP hydrolysis (20), product release (AMP) or an enzyme conformational change.

In Vivo Selenophosphate Synthetase Assay—An in vivo test to detect activity of the formate hydrogen-lyase complex in E. coli depends on the evolution of gas (H2) from formate generated during anaerobic growth on glucose (18). This test was later extended to detect in vivo activity of the selD gene (13, 14). The selD gene product, SPS, forms the reactive selenium donor compound, selenophosphate, which is required for the production of selenocystyl-tRNA22 (21). The synthesis of an active selenocysteine containing formate dehydrogenase depends on the availability of selenocysteine-containing tRNA. A more sensitive in vivo test, devised for the detection of redox-active enzymes such as formate dehydrogenase, involves the ability of the enzyme to reduce benzyl viologen to a blue color when agar containing the dye was overlaid on bacterial colonies grown anaerobically on glucose agar plates. E. coli MBO8, a selD mutant strain, cannot make selenophosphate and lacks an active selenocysteine-containing formate dehydrogenase. Therefore, MBO8 fails to produce H2 when grown in glucose media (16, 17) and is unable to reduce benzyl viologen, resulting in colonies that turn blue.

Introduction of a plasmid bearing a wild-type selD gene into MBO8 complements the selD lesion and allows the synthesis of an active selenocysteine containing formate dehydrogenase that can be detected in both in vivo assays (Data not shown). The SPS (C17S) mutation failed to complement the selD lesion in MBO8 as judged by lack of visible H2 production from glucose during
anaerobic growth (13, 14). The purified mutant enzyme was also unable
to catalyze the formation of selenophosphate from 1.5 mM selenide and
ATP in the usual in vitro assay (13, 14). To re-evaluate the activity of
the SPS (C17S) mutant, MBO8 cells were transformed with a plasmid
bearing the mutant gene. In the benzyl viologen overlay assay the SPS
(C17S) mutant gene was able to complement the selD lesion, and
transformed MBO8 colonies turned blue in color (data not shown).
Although reduction of benzyl viologen is a more sensitive test for FDHH
activity than production of H₂ gas in glucose media, the extent of blue
color development is more qualitative than quantitative. Nevertheless,
the benzyl viologen assay is a rapid and selective assay for determining
formate dehydrogenase activity (19). MBO8 colonies bearing either the
wild-type SPS or the mutant SPS (C17S) gene turned blue in the assay.

To compare the in vivo activities of wild-type SPS and SPS (C17S),
MBO8 cells containing plasmids bearing either gene were grown anaer-
obically in the presence of 75SeO₃²⁻. After growth, cell extracts were
prepared and examined for the presence of [⁷⁵Se]selenocysteine con-
taining FDHH (Fig. 2). MBO8 cells alone are unable to incorporate
selenocysteine into FDHH. However, the selD lesion in MBO8 cells can
be complemented either by a plasmid bearing the SPS gene or a plasmid
bearing the SPS (C17S) mutant gene, thus restoring incorporation of
selenocysteine into FDHH. Quantitation of the amount of radioactivity
in FDHH in response to the two gene products (Fig. 2) revealed that the
level of selenophosphate synthesis by the SPS (C17S) gene product was
sufficient to allow incorporation of 44% as much ⁷⁵Se into FDHH as
observed with the wild-type SPS. The fact that the activity of the SPS
(C17S) mutant could be detected in vivo is indicative of an essential
component present in E. coli that is absent in our in vitro assay con-
taining purified SPS and free selenide.

**Fig. 1.** Selenophosphate synthetase- and lyase-coupled assays. Assays
were performed as described under “Experimental Procedures.” A, SPS ATP hy-
drolysis activity was measured in the presence of the indicated concentration of
lyase proteins CsdB, CSD, and IscS. B, selenocysteine-lyase activity assays of the
NifS-like proteins were performed as described under “Experimental Procedures.”
L-Selenocysteine-lyase activity was measured in the presence of the indicated
concentrations of CsdB, CSD, and IscS proteins.

**Fig. 2.** Complementation of the MBO8 selD lesion. E. coli strain
MBO8 containing an inactive selD gene was transformed with plasmids
bearing the SPS gene or the SPS (C17S) mutant gene. After anaerobic
growth in Luria broth containing ⁷⁵SeO₃²⁻, cell lysates were subjected to
SDS-polyacrylamide gel electrophoresis and examined for radioactivity
by PhosphorImager analysis. The amount of ⁷⁵Se incorporated in FDHH is
shown.
ability to detect catalytic activity of the SPS (C17S) mutant further supports the involvement of a lyase protein in selenophosphate biosynthesis and provides additional evidence that cysteine-17 of SPS does not have a direct catalytic role.

Biological roles of NifS-like proteins include the mobilization of sulfur [S] from cysteine to a specific protein acceptor. The mobilized sulfur can be distributed throughout the cell for iron-sulfur cluster assembly. Such a process is not possible with their selenium analogs. For example, cysteine-17 of SPS does not have a direct catalytic role.

FIG. 4. Proposed pathway for the delivery of Se⁰ for selenophosphate biosynthesis.

The generation of Se⁰ by the NifS-like proteins reflects an additional role in vivo in which these enzymes participate as components of a selenophosphate delivery system for the biosynthesis of selenoproteins and selenium modification of RNA nucleosides (Fig. 4). The chemical similarity between selenium and sulfur allows selenium to enter bacterial metabolism via the cysteine biosynthetic pathway where it can be incorporated into free selenocysteine (39). Free selenocysteine can be inserted into proteins nonspecifically in place of cysteine, or a NifS-like protein can utilize it to generate selenium for SPS. The biological relevance of the NifS-like proteins in selenophosphate biosynthesis is particularly evident in the case of the SPS (C17S) mutant. Catalytic activity of this mutated enzyme could be detected in the presence of CsdB, CSD, or IscS (Fig. 3). In the in vitro coupled assays, all three E. coli NifS-like proteins were able to function well as selenium delivery proteins with SPS. However, in vivo concentrations of sulfur-containing compounds are much higher compared with their selenium analogs. For the E. coli NifS-like proteins to be effective selenium delivery proteins under normal conditions additional proteins may be involved to assist in the discrimination between cysteine and selenocysteine. Future work will be focused on identifying additional cellular components and proteins that participate in selenium metabolism and selenophosphate biosynthesis. Clearly, generation of Se⁰ at or in proximity to SPS may be the mechanism whereby the essential substrate of the enzyme is made available and the obstacle of free selenide toxicity is avoided.

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