Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7710–7713, August 1995
Biochemistry

Selenophosphate synthetase: Detection in extracts of rat tissues by immunoblot assay and partial purification of the enzyme from the archaean *Methanococcus vannielii*

(mammalian selenophosphate synthetase)

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Contributed by Thressa C. Stadtman, May 22, 1995

ABSTRACT **In *Escherichia coli* and *Salmonella typhimurium* it has been shown that selenophosphate serves as the selenium donor for the conversion of seryl-tRNA to selenocysteyl-tRNA and for the synthesis of 2-selenouridine, a modified nucleoside present in tRNAs. Although selenocysteyl-tRNA also is formed in eukaryotes and is used for the specific insertion of selenocysteine into proteins, the precise mechanism of its biosynthesis from seryl-tRNA in these systems is not known. Because selenophosphate is extremely oxygen labile and difficult to identify in biological systems, we used an immunological approach to detect the possible presence of selenophosphate synthetase in mammalian tissues. With antibodies elicited to *E. coli* selenophosphate synthetase the enzyme was detected in extracts of rat brain, liver, kidney, and lung by immunoblotting. Especially high levels were detected in *Methanococcus vannielii*, a member of the domain Archeae, and the enzyme was partially purified from this source. It seems likely that the use of selenophosphate as a selenium donor is widespread in biological systems.**

It is now established that the UGA codon directs the specific incorporation of selenocysteine into proteins. The selenocysteine to be inserted is synthesized on a specific tRNA, anticodon UCA, from a serine molecule attached to the tRNA in ester linkage. In prokaryotes the seryl-tRNA is converted to 2,3-aminoacrylyl-tRNA by a pyridoxal phosphate-dependent enzyme (1), and then a reactive selenium donor compound formed from selenide and ATP by another enzyme, the *selD* gene product, adds to the double bond to form selenocysteyl-tRNA (2, 3). The reactive selenium compound also is required for a very different type of biological reaction, the conversion of a 2-thiouridine residue in the anticodons of certain tRNAs to a 2-selenouridine residue (4–6). In this case a substitution rather than an addition reaction appears to be involved (7). By using ^{31}P NMR spectroscopy it was established that the labile selenium donor compound is a selenophosphate (6), and by comparison with an authentic compound the labile compound was identified as monoselenophosphate (8). The enzyme that forms this compound is termed selenophosphate synthetase. The weaker covalent bond strength of the phosphorus–selenium bond in selenophosphate, as compared, for example, with the analogous phosphorus–sulfur bond in thiophosphate, which in turn is ≈30 kcal/mol weaker than the phosphorus–oxygen bond in orthophosphate (9), may account for the effectiveness of selenophosphate as a selenium donor.

In eukaryotes the intermediate steps in the synthesis of selenocysteyl-tRNA from the precursor seryl-tRNA and the identity of the selenium donor have not been established. Nevertheless it seemed likely from the chemical point of view that selenophosphate would be the compound of choice to serve as a key intermediate in the eukaryotic process.

As one approach to implicate selenophosphate in eukaryotic metabolism, antibodies elicited to selenophosphate synthetase isolated from an overproducing strain of *Escherichia coli* were used in immunoblot assays as a screening procedure. We report here the presence of readily detectable amounts of immunoreactive protein in brain, kidney, liver, and lung of rat. Even higher amounts were found in extracts of *Methanococcus vannielii*, a member of the domain Archaea related to both prokaryotes and eukaryotes, and the enzyme was purified from this source.

MATERIALS AND METHODS

Materials. [8-^{14}C]ATP (44.9 mCi/mmol; 1 Ci = 37 GBq) was purchased from DuPont/NEN and [^{75}Se]selenous acid (1000 Ci/mmol) was purchased from the Research Reactor Facility, University of Missouri, Columbia. Q Sepharose Fast Flow, butyl-Sepharose 4 Fast Flow, butyl-Sepharose 4B, and phenyl-Sepharose CL-4B were from Pharmacia, and cellulose polyethylenimine plastic-backed TLC sheets were from J. T. Baker. Polyacrylamide (12%) gel and an immunoblot module were obtained from NOVEX (San Diego). Goat anti-rabbit immunoglobin G(H+L) conjugated to alkaline phosphatase and a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase substrate system were purchased from Kirkegaard & Perry Laboratories. Poly(vinylidene difluoride) membranes (Immobilon-P) were from Millipore.

Purification of *E. coli* Selenophosphate Synthetase. Selenophosphate synthetase (*selD* gene product) was purified from *E. coli* strain BL21(DE3) transformed with a plasmid containing a wild-type *selD* gene (10) as described (11).

Preparation of Selenophosphate Synthetase Antibody. Purified selenophosphate synthetase was supplied to Rockland (Gilbertsville, PA) and used to elicit polyclonal antibodies in rabbits. The antiserum was purchased from the producer.

Preparation of Crude Extracts of Rat Tissues. Tissues and organs excised from a rat immediately after killing were frozen in liquid nitrogen and stored at −80°C before use. Frozen tissue was thawed in buffer containing 100 mM Tricine·KOH (pH 7.2), 0.1 mM EDTA, and 2 mM dithiothreitol, rinsed several times, and homogenized in the same buffer, 1:3 (wt/vol) using a hand-held homogenizer (8–10 strokes). After centrifugation at 300,000 × *g* for 15 min the supernatant fraction was separated and used as crude extract.

Bacterial Extracts. Sonic extracts were prepared from frozen cells of *M. vannielii* (12) and *Salmonella typhimurium* (13).

Selenophosphate synthetase immunoblot assays were done by using the rabbit antiserum, goat anti-rabbit immunoglob-

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ulin G(H+L) conjugated to alkaline phosphatase, and a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase detection system according to established procedures.

Selenophosphate Synthetase Activity Assay. Reaction mixtures (100 μl) containing 100 mM Tricine·KOH (pH 7.2), 1.5 mM [^{14}C]ATP (0.25 μCi), 3 mM $MgCl_2$, 10 mM dithiothreitol, 1.5 mM NaSeH, and an appropriate amount of enzyme were incubated at 37°C under argon. The NaSeH was prepared as described by Veres *et al.* (11). The reactions were terminated by addition of $HClO_4$, and aliquots of the supernatant solutions, after neutralization with KOH, were chromatographed on polyethylenimine-cellulose thin layer sheets developed in 1.0 M LiCl or in 0.5 M potassium phosphate, pH 3.5. Radioactivity in the AMP spots was measured by liquid scintillation spectroscopy.

RESULTS

The rabbit polyclonal antibodies elicited to *E. coli* selenophosphate synthetase were used in immunoblot assays to screen crude extracts of various species of bacteria. An extract of *S. typhimurium*, an organism known to express the *selD* gene, was included as a control. As shown in Fig. 1, the antibody reacted with a 37-kDa protein band from the extract of wild-type *S. typhimurium* (lane 3), whereas the corresponding protein was not detected in an extract of the *Salmonella sel*A1 mutant strain (lane 4). There is no obvious explanation for the absence of detectable antigen in extracts of the mutant. The mutation is a defect in the selenophosphate synthetase gene itself, as evidenced by restoration of enzyme activity upon complementation with the normal gene from *E. coli* (14). Because the structure of the mutated gene has not been determined, the precise nature of the defect and its effect on protein expression are unknown. A protein with similar electrophoretic mobility that gave a very strong positive reaction with the antibody was detected in an extract of *M. vannielii* (Fig. 1, lane 2). We had selected this organism as a possible source of selenophosphate synthetase because it contains high levels of two selenocysteine-containing enzymes, a formate dehydrogenase and a hydrogenase and, in addition, ≈20% of the tRNAs of the cell contain selenium in the form of 2-selenouridine derivatives (12). In organisms such as *E. coli* and *Salmonella* strains, it has been shown that biosynthesis of both types of macromolecules

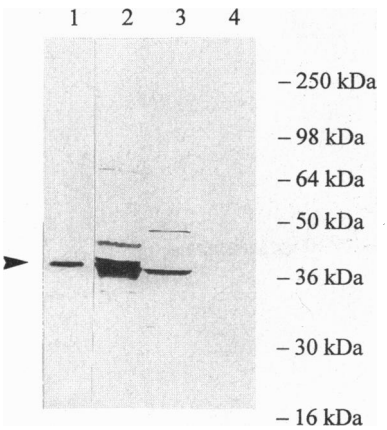

FIG. 1. Detection of selenophosphate synthetase in crude bacterial extracts by immunoblotting. Aliquots of crude extracts containing ≈50 μg of protein were fractionated by SDS/12% PAGE, electrotransferred to a poly(vinylidene difluoride) membrane, and immunoblotted with rabbit polyclonal antibodies elicited to *E. coli* selenophosphate synthetase. Lanes: 1, reference sample of *E. coli* selenophosphate synthetase; 2, *M. vannielii*; 3, *S. typhimurium*, wild type; 4, *S. typhimurium*, *sel*A1 mutant.

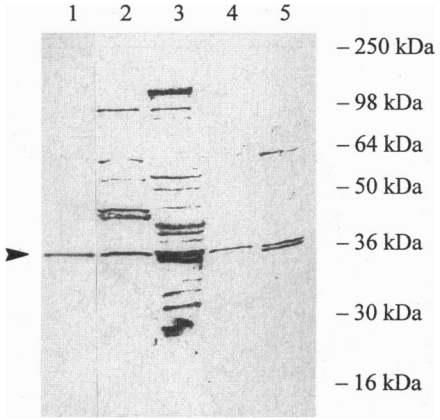

FIG. 2. Detection of selenophosphate synthetase in extracts of rat tissues by immunoblotting. Rat tissue extracts, prepared as described, were analyzed as described in the legend to Fig. 1. About 50 μg of crude extract protein was applied to each lane. Lanes: 1, reference sample of *E. coli* selenophosphate synthetase; 2, brain; 3, liver; 4, lung; and 5, kidney.

depends on the availability of selenophosphate as a selenium donor (14).

Detection of the synthetase in *M. vannielii*, a typical member of the Archaea, which are related to eukaryotes as well as to prokaryotes, suggested that the enzyme might also occur in eukaryotes. Fig. 2 shows that this is indeed the case. Application of the immunoblot assay to extracts of various tissues from rat established the presence of a protein of ≈37 kDa that reacted with the selenophosphate synthetase antibody. On the basis of a comparison of the intensity of the stained protein bands, rat brain (lane 2), liver (lane 3), lung (lane 4), and kidney (lane 5) appeared to contain the highest enzyme levels. Testis, uterus, spleen, and heart contained lower, but clearly detectable, levels (data not shown). The immunoreactive protein in rat brain extracts proved to be hydrophobic and could be enriched by chromatography on butyl-Sepharose 4B. In a typical experiment brain extract adjusted to 1 M in ammonium

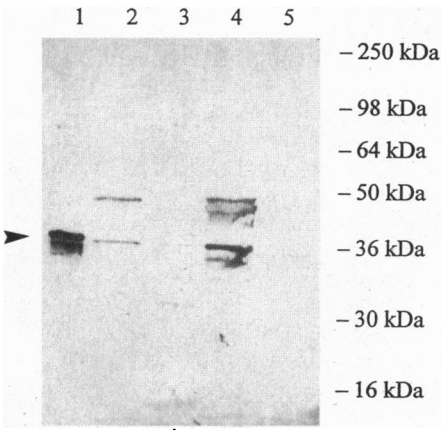

FIG. 3. Immunoblot of rat brain extract fractions separated by chromatography on butyl-Sepharose. Rat brain extract (2.5 ml), prepared as described, was adjusted to 1 M in ammonium sulfate and applied to a 2-ml butyl-Sepharose 4B column equilibrated with 0.1 M Tricine·KOH, pH 7.2/0.1 mM EDTA/2 mM dithiothreitol (buffer A) containing 1 M ammonium sulfate. Proteins were eluted stepwise with buffer A containing 1 M, 0.5 M, and 0 ammonium sulfate, 10 ml each. Fractions, 1 ml, were monitored for protein by using the Bio-Rad protein assay. Protein peak fractions were pooled, and after dialysis against buffer A, aliquots containing 20 μg of protein were analyzed as described in the legend to Fig. 1. Lanes: 1, reference sample of *E. coli* selenophosphate synthetase; 2, crude extract; 3, 1 M ammonium sulfate eluate; 4, 0.5 M ammonium sulfate eluate; 5, buffer A eluate.

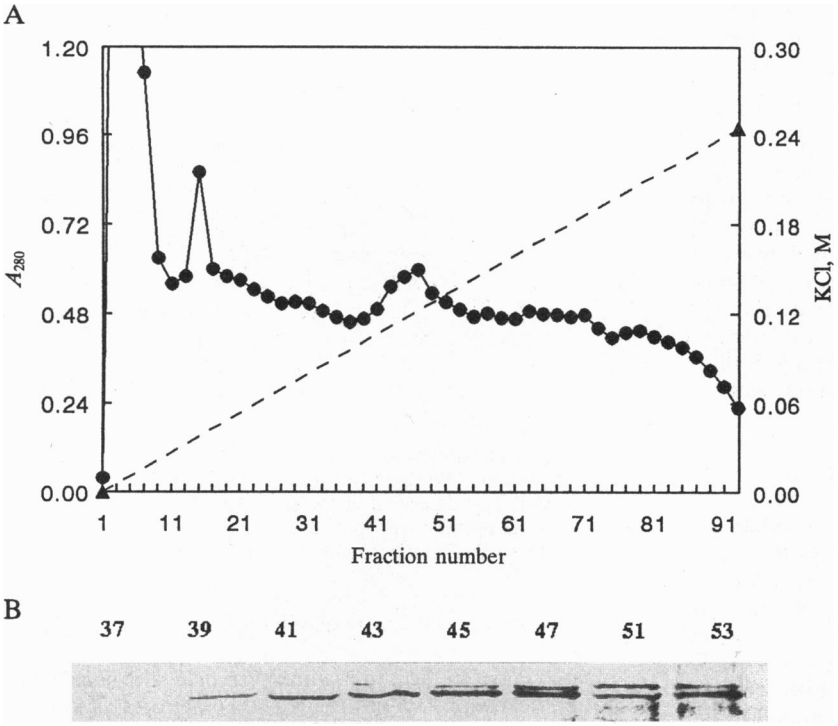

FIG. 4. Chromatography of *M. vannielii* crude extract on DEAE-Sephadex. A sonic extract prepared from frozen *M. vannielii* cells (15 g), after centrifugation to remove cell debris, was adjusted to 55% of saturation with ammonium sulfate. The precipitated protein resuspended in 0.1 M Tricine·KOH, pH 7.2, containing 2 mM dithiothreitol and 0.1 mM EDTA (buffer A) was desalted on a Sephadex G-25 column equilibrated with buffer A and applied to a DEAE-Sepharose Fast Flow anion-exchange column (2.5 × 17 cm) equilibrated with buffer A. Proteins were eluted with a linear gradient of 0–0.3 M KCl in buffer A (1600 ml), and 16-ml fractions were collected. (*A*) Protein determined by absorbency at 280 nm (●) and the KCl gradient (▲). (*B*) Proteins in fractions from the DEAE column were separated on a SDS/12% PAGE gel and immunoblotted as described. Numbers above bands indicate fraction numbers.

sulfate was applied to a butyl-Sepharose 4B column, and the column was washed with buffers containing 1 M and 0.5 M ammonium sulfate. The immunoreactive protein adsorbed to the column and was eluted with 0.5 M ammonium sulfate (Fig. 3).

Purification of Selenophosphate Synthetase from *M. vannielii*. A purification procedure developed for isolation of selenophosphate synthetase from an overproducing strain of *E. coli* (11) was used in preliminary experiments to obtain the enzyme from extracts of the strictly anaerobic methane-producing organism. A crude sonic extract was fractionated by addition of ammonium sulfate to 60% of saturation, and the precipitated protein was desalted and chromatographed on a DEAE-Sepharose Fast Flow column developed with a linear gradient of KCl (0–0.3 M). A protein peak that emerged in fractions 39–55 in the profile (Fig. 4*A*) contained selenophosphate synthetase as determined by immunoblot assay of these fractions (Fig. 4*B*). Aliquots (20 μl) of the peak fractions were assayed for selenide-dependent formation of AMP from ATP as described in *Materials and Methods*. In reaction mixtures incubated for 35 min under argon the enzyme activities of fractions 39, 41, 45, 47, and 53 were 4.2, 5.0, 11.2, 8.6, and 7.5 nmol of AMP/min per mg of protein, respectively. In the absence of selenide the formation of AMP by these enzyme samples was negligible.

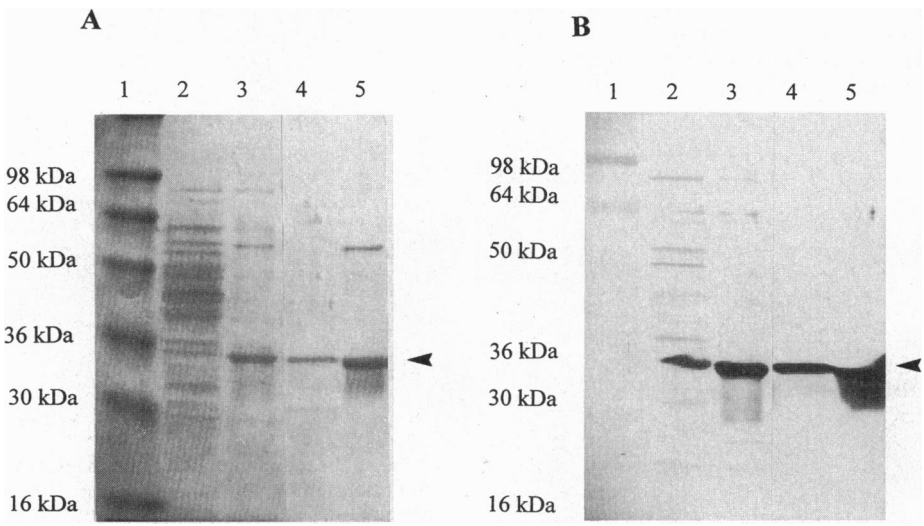

FIG. 5. Enrichment of *M. vannielii* selenophosphate synthetase. (*A*) Proteins separated by SDS/PAGE on 12% gel and stained with Coomassie blue. (*B*) Proteins separated as in *A* and immunoblotted after transfer to an Immobilon-P membrane. Lanes: 1, protein standards; 2, pooled selenophosphate synthetase fractions from Sephacryl S-200; 3, chromatography of the pooled Sephacryl S-200 fractions shown in lanes 2 on butyl-Sepharose 4 Fast Flow using a decreasing linear gradient of 0.5–0 M ammonium sulfate in Tricine·KOH, pH 7.5, buffer. Selenophosphate synthetase was eluted near the end of the gradient, and a peak fraction was analyzed; 4, in a similar experiment to the above, additional protein remaining on the butyl-Sepharose column was eluted with buffer and buffer/10% (vol/vol) ethylene glycol; 5, protein further purified by chromatography of the selenophosphate synthetase peak fractions (lane 3) from butyl-Sepharose 4 Fast Flow on Q Sepharose Fast Flow. The fraction shown was eluted from the strong anion exchanger with 1 M NaCl. Proteins in lanes 2–5 were concentrated on Centricon 10 microconcentrators before application to gels. Arrowheads, selenophosphate synthetase.

Table 1. Comparison of amino acid sequences of *E. coli* and *M. vannielii* selenophosphate synthetases

	1	5	10	15	20	25
E.c.	Met-Ser-Glu-Asn-Ser-Ile-Arg-Leu-Thr-Gln-Tyr-Ser-His-Gly-Ala-Gly-Cys-Gly-Cys-Lys-Ile-Ser-Pro-Lys-Val-					
M.v.	Met-Phe-Lys-Ala-Thr- ? -Asn-Ser-Arg-Asp-Phe-Lys-Lys-Val-Ile-Asn-Ala-Thr-Ser-Asn-Leu-Val-Asp-Glu-(Ile)-					

After SDS/12% PAGE separation and staining with Coomassie blue the selenophosphate synthetase band was cut from the gel and extracted with buffer containing 100 mM Tris·Cl (pH 8.5) and 0.1% SDS. The clarified solution was applied directly to a sequencing column, and the amino acid sequence of the protein was determined in house using a Hewlett–Packard model G100A protein sequencer. The unidentified residue in position 6 may be cysteine, which is not detected in the program used. *E.c.*, *E. coli*; *M.v.*, *M. vanniellii*.

In a modified isolation procedure in which crude extract from ^{75}Se-labeled *M. vannielii* cells was passed over a short column of fibrous DEAE-cellulose the enzyme was about equally distributed between a fraction that was eluted with 0.25 M potassium phosphate, pH 7.2, and the subsequent fraction that was stripped from the column with 1 M NaCl. The latter fraction consisting of acidic components such as ferredoxin and [^{75}Se]tRNAs was concentrated with ammonium sulfate and subjected to molecular sieve chromatography on a Sephacryl S-200 column. Fractions containing selenophosphate synthetase also contained ^{75}Se-labeled tRNAs as a major contaminant. Butyl-Sepharose 4 Fast Flow was selected as an effective means of separating the hydrophilic ^{75}Se-labeled tRNAs from the hydrophobic selenophosphate synthetase. By stepwise elution of protein adsorbed to this matrix with decreased concentrations of ammonium sulfate the enzyme was completely separated from tRNAs, and the last fraction that emerged when buffer alone and buffer/10% (vol/vol) ethylene glycol were applied contained highly purified selenophosphate synthetase (Fig. 5, lanes 4). The protein bands corresponding to the immunoreactive protein bands in various enzyme preparations did not contain detectable ^{75}Se as determined by PhosphorImager analysis, indicating that the selenophosphate synthetase isolated from *M. vannielii* is not a selenoprotein.

A partial amino acid sequence starting from the amino terminus of the *M. vannielii* enzyme was obtained by analysis of selenophosphate synthetase bands extracted from SDS/PAGE gels. These protein bands were derived from the preparation shown in lanes 5 of Fig. 5. As shown in Table 1 the *M. vannielii* selenophosphate synthetase sequence differs from that of the *E. coli* enzyme over the first 25 amino acid residues. In the case of the *E. coli* enzyme the Cys-17 and the Lys-20 residues are essential for catalytic activity (15).

DISCUSSION

In view of the established role of selenophosphate as selenium donor for selenoprotein biosynthesis in prokaryotes it seemed likely that this reactive compound would have a more widespread use in nature. In eukaryotes it is known that selenocysteyl-tRNA also is required for the specific incorporation of selenocysteine in proteins, although details of its formation from seryl-tRNA have not been elucidated. Selenophosphate could be expected to be an effective selenium donor whether the intermediate is *O*-phosphoseryl-tRNA, for which there is circumstantial evidence (16), or 2,3-aminoacrylyl-tRNA as in *E. coli* (1, 2). Evidence that selenophosphate is available as a selenium donor in mammals is provided by the immunological studies reported here, showing the presence of selenophosphate synthetase in numerous tissues of the rat. The ease of detection in crude enzyme preparations by immunoblotting indicates wide distribution of the enzyme in significant levels. Furthermore, the recent preliminary report of Low *et al.* (17) describing the cloning and sequencing of the human homologue of the *E. coli selD* gene together with the demonstration that expression of this gene regulates the synthesis of ^{75}Se-labeled 5′-deiodinase provides direct evidence for a role of selenophosphate in eukaryotes. The presence of selenophosphate synthetase in all of the eight different rat tissues examined in the present study indicates that the extremely oxygen-labile selenium donor compound is synthesized *in situ* where it is to be used. Otherwise, for transport from one tissue to another, the compound would need to be protected from oxidation by some type of adduct formation.

Amino acid sequence analysis of the first 25 residues of the N-terminal region of *M. vannielii* selenophosphate synthetase revealed a lack of homology with that of the *E. coli* enzyme, deduced from the *selD* gene sequence (10), and also that of the human enzyme, deduced from the sequence of the human *selD* gene homologue (S. Low and M. Berry, personal communication). Selenophosphate synthetase from rat brain and also from *M. vannielii* is similar in size and in hydrophobicity to the *E. coli* enzyme, as judged by electrophoretic mobilities and affinity for hydrophobic chromatographic matrices. Detailed information on the catalytic properties of these enzymes awaits the availability of larger amounts of purified proteins.

We thank Michael Nauman for amino acid sequence analyses and Christina McLauchlan for preparation of the manuscript.

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