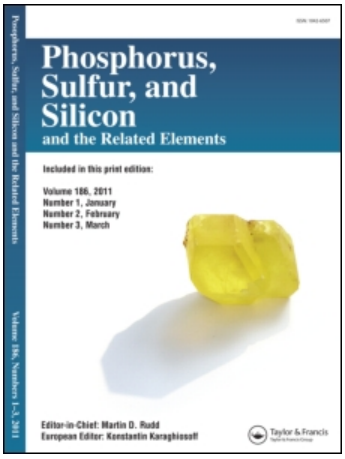

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SELENOPHOSPHATE: BIOLOGICAL ROLES AND STUDIES ON MECHANISM OF BIOSYNTHESIS

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Selenophosphate, H_3SePO_3, is the activated selenium donor compound required for the biosynthesis of selenocysteyl-tRNA^{Sec}, the precursor of specific selenocysteine residues in bacterial and mammalian selenoproteins, and for the formation of 2-selenouridine from 2-thiouridine in the anticodons of various bacterial tRNAs (1-4).
Selenophosphate synthetase, the 37 kDa product of the *Escherichia coli* selD gene (1), and various mutant forms produced by site directed mutagenesis have been characterized in some detail (5,6,7). Replacement of Cys-17 with serine or Lys-20 with glutamine resulted in loss of catalytic activity with ATP and selenide as substrates thus establishing the importance of these residues in the glycine rich sequence -His-Gly-Ala-Gly-Cys^{17}-Gly-Cys-Lys^{20}-Ile- (5,6). Catalytic activity was unaffected by replacement of His^{13} with asparagine or Cys^{19} with serine. The occurrence of selenophosphate synthetase in eukaryotes was indicated by the discovery that the -GCGCK- sequence motif is highly conserved in a developing mouse embryo gene and a human gene designated selD2 (8) and in another human gene, selD1 (9). Interestingly, the mouse embryo selD2 gene differed in that an in-frame TGA codon replaced the Cys^{17} codon in the -GCGCK- motif indicating that the gene product should contain selenocysteine and thus might be a more active catalyst. The Cys^{17}codon was replaced by a threonine codon in the human selD1 gene and recently, a related Drosophila selD1 gene termed selD-like (10) was reported to contain a -GRGCK- motif indicating replacement with arginine. Comparison of the published entire genome sequences of

Methanococcus jannashii (8,11), an Archae extreme thermophile, and *Haemophilus influenzae* (8,12) shows that selD genes are present in these organisms and in each a selenocysteine residue is predicted as a replacement of Cys^{17} in *E. coli* selenophosphate synthetase.

Previously, using antibodies directed to the wild type *E. coli* enzyme, strong cross reactivity was observed in immunoblot assays with a protein present in crude extracts of several rat tissues (13). This eukaryotic protein, enriched from rat brain, was similar in size and hydrophobicity to the *E. coli* enzyme. *Methanococcus vannielii*, an Archae that synthesizes several essential selenoenzymes and high levels of seleno-tRNAs is a particularly rich source of selenophosphate synthetase (13). Enzyme prepared from ^{75}Se-labeled cells did not contain radioactivity indicating absence of a selenocysteine residue. The sequence of the first 24 amino acid residues of the protein, following an N-terminal methionine, does not resemble the deduced N-terminal region sequences reported for other selD genes. Thus the selenophosphate synthetase enzymes from two related methane-producing organisms, *M. jannashii* and *M. vannielii*, may differ significantly in properties.

Catalytic activities of selenophosphate synthetase enzymes from different sources:

Selenophosphate (SeP) is formed from ATP and selenide by *E. coli* selenophosphate synthetase (2,3). In contrast, the protein products of the human selD1 gene* and the Drosophila selD1 gene (10), expressed in *E. coli*, failed to form SeP from selenide and ATP in the same *in vitro* assay system. In these enzymes either an -OH group of threonine or a N of arginine occurs in a location corresponding to the S of the essential Cys^{17} in the *E. coli* enzyme. In view of the fact that the function of the essential cysteine residue in the *E. coli* enzyme has not been determined, it is difficult to predict with any certainity the result of substitution of -OH or N for S at this position in the protein. Although replacement of Se in the form of selenocysteine for S is expected to result in greater catalytic activity, amounts of these putative selenoenzymes presently available are not sufficient for accurate evaluation. A mutated mouse embryo selD2 gene, in which cysteine was encoded in place of selenocysteine (14), when expressed in the insect cell bacculovirus system gave rise to a protein that exhibited detectable selenophosphate synthetase activity with selenide as substrate. The catalytic activity of the *E. coli* Cys^{17} enzyme with selenide as substrate is very low (about 80 nmoles/min/mg of protein) (Kcat = 3) which has led us to suspect that a more reactive form of selenium, presumably generated *in situ*, is the actual *in vivo* substrate.

Furthermore, in view of the fact that selenide is even more toxic than sulfide, only very low levels of this reduced inorganic selenium species would be expected to occur in cells and tissues.

Enzyme catalyzed reactions:

In the selenide-dependent reaction catalyzed *in vitro* by selenophosphate synthetase, SeP is derived from the γ-phosphoryl group of ATP and orthophosphate (Pi) is formed from the β–phosphoryl group (reaction 1; 2, 3, 15). Initially the very oxygen labile SeP product was

$$ATP + HSeNa = SeP + Pi + AMP \quad (1)$$

detected by ^{31}P NMR spectroscopy as a new resonance peak appearing at 26 ppm that was dependent on the presence of selenide in the reaction mixture (2). By comparison with authentic monoselenophosphate the enzyme product and the synthetic compound were shown to be identical (4).

In the absence of selenide and at high enzyme concentration (160 μM) selenophosphate synthetase catalyzes a slow partial reaction in which ATP is converted quantitatively to AMP and 2 Pi (7, 15). In the overall reaction with 3-5 μM enzyme as catalyst, AMP is a competitive inhibitor of ATP (Ki = 170 uM) but neither SeP nor Pi is effective as an inhibitor suggesting a multistep reaction mechanism. Based on these and related observations, an enzyme-pyrophosphate intermediate, analogous to the enzyme-bound imidazole-pyrophosphate derivative of a histidine residue in pyruvate phosphate dikinase, was postulated initially. This seemed to be supported by data of labeling experiments using [λ^{32}P]ATP and [8-^{14}C]ATP, under enzyme turnover conditions, in which it was found that 0.6 equivalent of the γ-phosphoryl group of ATP and 0.4 equivalent of [^{14}C] identified as labeled AMP remained bound to the enzyme (16). However, when [β^{32}P]ATP was substituted for the γ-labeled ATP no radioactivity was recovered with the enzyme showing that no pyrophosphoryl-enzyme derivative was present.

Subsequently, using the positional isotope exchange procedure, evidence was obtained supporting the formation of a phosphorylated enzyme intermediate (17). These experiments utilized [γ-$^{18}O_4$]ATP and monitored the rate of enzyme-catalyzed migration of ^{18}O from the β,γ-bridge position of ATP to the β-nonbridge position in the absence of selenide. The observed rate of this exchange reaction was catalytically competent. In this case the migration of isotope occurs as a result of initial cleavage of the β-γ phosphoryl bond of ATP to form a covalent enzyme-phosphoryl group and bound ADP. Rotation of the β-phosphoryl group followed by

reversal of the cleavage reaction accounts for the isotope exchange. In parallel experiments with [β-$^{18}O_4$]ATP no exchange was detected providing evidence that no pyrophosphorylated enzyme intermediate had formed.

Experimental evidence in support of the formation of a phosphorylated enzyme intermediate in the initial reaction with ATP came from exchange experiments with [8-^{14}C]ADP or [8-^{14}C]AMP and unlabeled ATP carried out with high enzyme levels (37 μM) in the absence of selenide (15). Under these conditions a very slow exchange of labeled ADP with ATP was observed, and a significant amount of AMP was produced. After a 24 hour incubation, only 11% of the radiolabel was found in the ATP, while the AMP product contained 32% of the radiolabel from the added [^{14}C]ADP. ^{31}P NMR analysis of products of reactions carried out in $H_2{}^{18}O$ showed that hydrolysis of enzyme-bound ADP by solvent water produced [^{18}O]Pi and unlabeled AMP. It is possible that the extremely slow (0.027 nmol/min/mg) enzyme-dependent ADP-ATP exchange reaction is due to weak binding of free ADP and/or a slow off rate of enzyme-bound ADP. For example, ADP has never been detected as a free intermediate in either the partial or the overall enzyme-catalyzed reaction and the inhibitory effect of added ADP, in concentrations equivalent to substrate ATP levels, on the overall reaction is neglegible (7). The reaction mechanism predicted by the data obtained thus far in our studies involves formation of an enzyme-phosphoryl intermediate which is attacked by selenide to form the selenophosphate product. The enzyme-bound ADP intermediate appears to be hydrolyzed by a solvent water molecule to form the AMP and Pi products. This is in agreement with the observations that exchange of [^{14}C]AMP with ATP was insignificant and could be attributed to incomplete removal of the last trace of contaminating adenylate kinase from the enzyme preparation used. Prior to rechromatography using various HPLC procedures, trace amounts of highly active adenylate kinase contaminated selenophosphate synthetase preparations that by the usual criteria appeared to be homogeneous (7, 15). Thus the enzyme preparations employed for positional isotope exchange measurements and for ADP-ATP exchange or ADP to AMP conversion were rigorously freed of adenylate kinase before use. This was especially critical since these types of experiments required the use of very high enzyme levels.

Biological roles of selenophosphate:

The role of SeP in the biosynthesis of seleno-tRNAs involves a substitution of sulfur in a 2-thiouridine residue in the anticodons of certain bacterial tRNAs with selenium derived from SeP (2, 18, 19). Although the

mechanism of this substitution reaction has not been elucidated, attack of SeP at carbon-2 of the 2-thiouridine is postulated to produce an intermediate which can decompose to give 2-selenouridine and thiophosphate (19).

Details of the biosynthesis in prokaryotes of selenocysteyl-tRNA^{Sec}, anticodon UCA, from the precursor seryl-tRNA involves a β-elimination of the hydroxyl group of the esterified serine by selenocysteine synthase followed by addition of selenium across the double bond of the resulting aminoacrylyl-tRNA intermediate (20). For this reaction the biological selenium donor is selenophosphate. The nature of the intermediate steps of this process in eukaryotes has not been elucidated. However, it is clearly established that a selenocysteyl-tRNA^{Sec} in both prokaryotes and many eukaryotes is required for the specific cotranslational incorporation of selenocysteine in selenoenzymes.

The ability of selenophosphate to phosphorylate various alcohols and amines (21) in aqueous solutions under anaerobic conditions by an apparently dissociative mechanism calls attention to the possibility that this reactive compound may serve as a phosphorylating agent in biological systems. The hydrophobicity of the selenium atom that is bonded directly to the phosphorus of selenophosphate might aid in directing this phosphorylating agent to specific targets in cells.

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