# Identification of a Phosphorylated Enzyme Intermediate in the Catalytic Mechanism for Selenophosphate Synthetase

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*Received April 4, 1997*

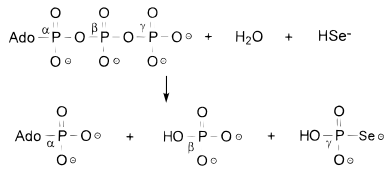
Selenophosphate synthetase (SPS) from *Escherichia coli* catalyzes the formation of the reactive selenium donor, selenophosphate. Selenophosphate is required for the biosynthesis of selenocysteyl-tRNA$^{SEC}$, the precursor of specific selenocysteine residues in bacterial and mammalian selenoproteins,[1−6] and for the formation of 2-selenouridine from 2-thiouridine in the anticodons of various bacterial tRNA molecules.[1−3,7] The biological importance of selenophosphate was emphasized by the discovery that two well-known eukaryotic enzymes, type I iodothyronine 5′-deiodinase[8] and mammalian thioredoxin reductase,[9] are selenocysteine-containing proteins. Activities of the deiodinase, which forms the active thyroid hormone, and thioredoxin reductase, which reduces protein disulfide groups, are essential for normal embryonic development, DNA biosynthesis, redox-regulation of certain transcription factors, regeneration of several antioxidants, and cellular redox control.

ATP and selenide are converted stoichiometrically to selenophosphate, orthophosphate, and AMP by *E. coli* SPS, and in the absence of selenide the enzyme catalyzes a slow conversion of ATP to AMP and two orthophosphates.

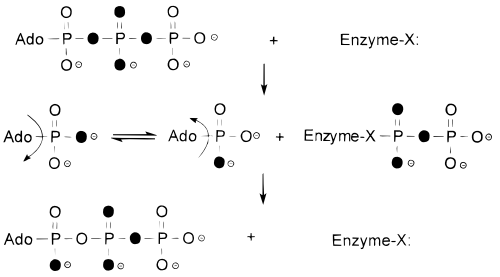
$$\text{ATP} + \text{selenide} + \text{H}_2\text{O} \rightarrow \text{selenophosphate} + \text{P}_\text{i} + \text{AMP} \quad (1)$$

Mechanistic studies have shown that the phosphoryl moiety of selenophosphate is derived from the $\gamma$-phosphoryl group of ATP, whereas the orthophosphate product is derived from the $\beta$-phosphoryl group as illustrated in Scheme 1.[2,10] The possibility was considered[2] that the chemical mechanism might be similar to that of pyruvate phosphate dikinase (PPDK) which has been shown to involve the initial formation of a covalent pyrophosphoryl−enzyme intermediate that is then converted to a phosphoryl−enzyme intermediate and pyrophosphate by reaction with $P_i$.[2] By analogy, the initial reaction of SPS with ATP has been proposed to form a pyrophosphoryl−enzyme intermediate (eq 2), which subsequently reacts with selenide to form selenophosphate and a phosphoryl−enzyme intermediate (eq 3). Hydrolysis of the latter complex then regenerates the active form of the enzyme (eq 4). Two amino acid residues, Cys-17 and Lys-20, have been shown to be essential for the overall catalytic activity.[12,13]

**Scheme 1**



**Scheme 2**



$$\text{E}-\text{X}: + \text{ATP} \rightarrow \text{E}-\text{X}-\text{PP}_\text{i} + \text{AMP} \quad (2)$$

$$\text{E}-\text{X}-\text{PP}_\text{i} + \text{H}_2\text{Se} \rightarrow \text{E}-\text{X}-\text{P}_\text{i} + \text{Se-P}_\text{i} \quad (3)$$

$$\text{E}-\text{X}-\text{P}_\text{i} + \text{H}_2\text{O} \rightarrow \text{E}-\text{X}: + \text{P}_\text{i} \quad (4)$$

It has been postulated that Cys-17 acts as the active site nucleophile in the catalytic mechanism proposed above.[12] However, despite numerous attempts using a variety of mechanistic techniques, no direct experimental evidence has been found for the putative pyrophosphoryl−enzyme intermediate.[14] The positional isotope exchange technique of Midelfort and Rose has now been applied to the reaction catalyzed by SPS, and in this paper, we demonstrate for the first time, direct experimental evidence for the existence of a covalent intermediate formed in the absence of any added selenide.[15]

The positional isotope exchange (PIX) technique of Midelfort and Rose has been used to probe for the existence and kinetic competence of transient intermediates in enzyme-catalyzed reactions.[15] In the mechanism presented above, SPS catalyzes the cleavage at the $\beta$-phosphoryl group of ATP to form a $\beta,\gamma$-pyrophosphoryl−enzyme intermediate in the absence of selenide (eq 2). If [$\beta$-$^{18}O_4$ ]ATP were to be used as the nucleotide substrate for SPS, then a positional isotope exchange from the $\alpha,\beta$-bridge oxygen-18 of [$\beta$-$^{18}O_4$]ATP into the $\alpha$-nonbridge position would provide direct experimental evidence for a pyrophosphoryl−enzyme intermediate. This PIX reaction is illustrated in Scheme 2. Similarly, experimental evidence for an alternative $\gamma$-phosphoryl−enzyme intermediate would be demonstrated by positional isotope exchange from the $\beta,\gamma$-bridge oxygen-18 of [$\gamma$-$^{18}O_4$]ATP into the $\beta$-nonbridge position as illustrated in Scheme 3.

Selenophosphate synthetase was purified as described previously with some modifications.[2] The purified enzyme was concentrated and desalted, and the activity determined.[2] The enzyme was also assayed for adenylate kinase contamination.[16] The labeled nucleotides, [$\beta$-$^{18}O_4$]ADP and [$\gamma$-$^{18}O_4$]ATP, were synthesized as described by Moffatt and Khorana from the

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S0002-7863(97)01074-3 CCC: $14.00 

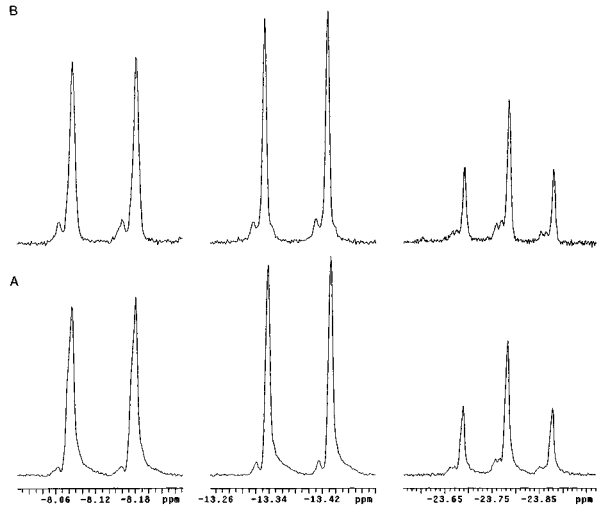



**Figure 1.** $^{31}$P NMR spectra of [$\beta$-$^{18}O_4$]ATP (A) prior to addition of enzyme and (B) 24 h after addition of SPS at 37 °C, pH 7.2. The spectra were obtained on a Varian Unity 500 utilizing a 5 mm broad-band probe. The $^{31}$P spectra were referenced to an external standard of inorganic phosphate. The α-, $\beta$-, and $\gamma$-phosphoryl groups of ATP resonate at approximately −13.2, −23.6, and −8.1 ppm, respectively.

**Scheme 3**

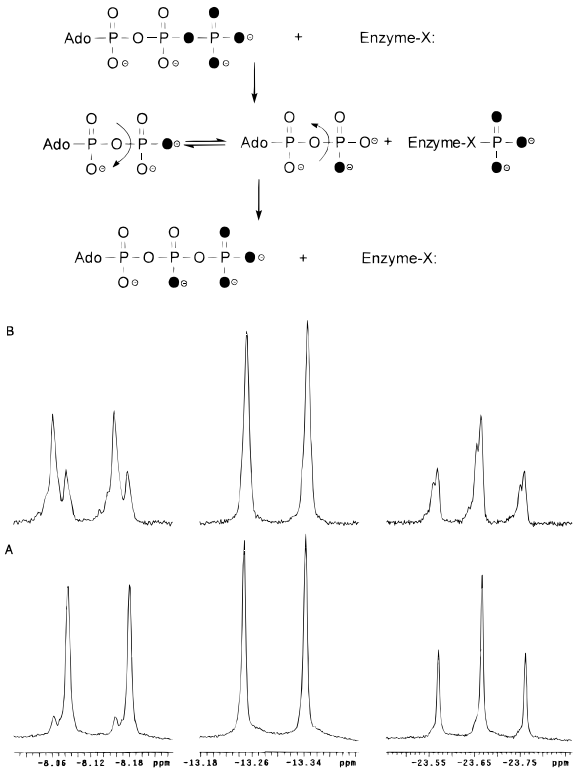
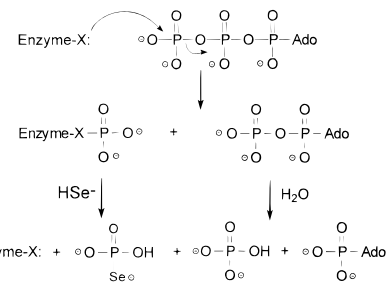



**Figure 2.** $^{31}$P NMR spectra of [$\gamma$-$^{18}O_4$]ATP (A) prior to addition of enzyme and (B) 24 h after addition of SPS at 37 °C, pH 7.2.

respective morpholidate nucleotide precursor and $H_3P^{18}O_4$.[17,18] The labeled [$\beta$-$^{18}O_4$]ATP was synthesized enzymatically from the [$\beta$-$^{18}O_4$]ADP using 11 units of pyruvate kinase, 7.5 mM PEP, 50 mM KCl, 6 mM $MgCl_2$, and 50 mM HEPES, pH 7.5. Both [$\beta$-$^{18}O_4$]ATP and [$\gamma$-$^{18}O_4$]ATP showed greater than 97% incorporation of oxygen-18 as determined by $^{31}$P NMR as illustrated in Figures 1A and 2A, respectively.

The PIX reactions of SPS with either [$\beta$-$^{18}O_4$]ATP or [$\gamma$-$^{18}O_4$]-ATP were conducted in the absence of selenide at 37 °C for

**Scheme 4**




either 2 or 24 h. The reaction mixtures (0.5 mL) contained 100 mM Bis-Tris propane, pH 7.2, 2 mM DTT, 20 mM $MgCl_2$, 10 mM [$\beta$-$^{18}O_4$]ATP or [$\gamma$-$^{18}O_4$]ATP, and 0.6 mg SPS. The reactions were quenched by the addition of EDTA to give a final concentration of 150 mM EDTA and analyzed by $^{31}$P NMR on a Varian Unity 500 spectrometer utilizing a 5 mm broad-band probe operating at a frequency of 202 MHz.[19]

When SPS was incubated with [$\beta$-$^{18}O_4$]ATP for 24 h in the absence of added selenide, no positional isotope exchange between the oxygen-18 label at the α,$\beta$-bridge position and the two oxygen-16 labels at the α-nonbridge positions was detected (Scheme 2 and Figure 1B). However, incubation of the enzyme in the presence of [$\gamma$-$^{18}O_4$]ATP for 24 h in the absence of added selenide resulted in the complete scrambling of the oxygen-18 label in the $\beta$,$\gamma$-bridge position with the two oxygen-16 labels at the $\beta$-nonbridge positions (Scheme 3 and Figure 2B). These results clearly indicate that the initial attack by the enzyme nucleophile is directly with the $\gamma$-phosphoryl group of ATP rather than with the $\beta$-phosphoryl group.

For cleavage at the $\gamma$-phosphoryl group to be considered kinetically competent, the rate of positional isotope exchange, $V_{ex}$, must be similar to the overall catalytic rate.[20] When the reaction mixture was incubated for 24 h, the positional isotope scrambling reached the complete isotopic equilibrium of 0.67. In a 2 h incubation, the fraction of positional isotope scrambling was reduced to 0.4.[20] From this value, a positional isotope exchange rate, $V_{ex}$, of 34 nmol/min/mg of protein was calculated. The reported specific activity of SPS is 83 nmol/min/mg. Therefore, the rate of cleavage at the $\gamma$-phosphoryl group appears to be kinetically competent, and thus, these results are consistent with a catalytic mechanism where the first step in the reaction mechanism is a nucleophilic attack on the $\gamma$-phosphoryl group of ATP forming a phosphoryl−enzyme intermediate as presented in Scheme 4. The phosphoryl−enzyme intermediate then undergoes a nucleophilic attack by selenide to form selenophosphate and free enzyme. Since no ADP is detected as a final product, it must undergo subsequent hydrolysis to form AMP and $P_i$.

The PIX reactions reported here provide no evidence for a pyrophosphoryl−enzyme intermediate in the absence of added selenide. If the initial cleavage of ATP were to occur at the $\beta$-phosphoryl group to form a pyrophosphorylated intermediate, then the bound AMP would be unable to undergo any torsional rotation. Therefore, even though the overall stoichiometry of the SPS reaction is similar to that of PPDK, the chemical mechanisms do not appear to be similar. In summary, the catalysis of positional isotope exchange reactions by selenophosphate synthetase has clearly demonstrated that the $\gamma$-phosphoryl group of the substrate ATP is cleaved in a kinetically competent reaction to form a phosphoryl−enzyme intermediate in the absence of the second substrate, selenide.

**Acknowledgment.** This work was support in part by the National Institutes of Health (GM33894).

JA971074M

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