Note

A non-radioactive assay for selenophosphate synthetase activity using recombinant pyruvate pyrophosphate dikinase from *Thermus thermophilus* HB8

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Biosynthesis of selenocysteine-containing proteins requires monoselenophosphate, a selenium-donor intermediate generated by selenophosphate synthetase (Sephs). A non-radioactive assay was developed as an alternative to the standard [8-14C] AMP-quantifying assay. The product, AMP, was measured using a recombinant pyruvate pyrophosphate dikinase from *Thermus thermophilus* HB8. The *K*ₘ and *k*_cat for Sephs2-Sec60Cys were determined to be 26 μM and 0.352 min⁻¹, respectively.

**Key words:** selenophosphate synthetase; monoselenophosphate; pyruvate pyrophosphate dikinase; *Thermus thermophilus* HB8; non-RI method

A highly oxygen-sensitive metabolite, monoselenophosphate (HSe-PO₃⁻), is required as the substrate for selenocysteine synthetase, which generates L-seLENophosphate; pyruvate pyrophosphate dikinase

AMP formation via the following scheme:

\[\text{AMP} + \text{PEP} + \text{P} - \text{Pi} \rightarrow \text{ATP} + \text{Pyruvate} + \text{Pi} \]

Pyruvate + NADH + H⁺ → Lactate + NAD⁺

In this enzyme-coupled reaction system above, AMP quickly recycles to ATP, and therefore, it is maintained at the original concentration. The absorption at 340 nm decreases upon the consumption of NADH. In the reference reaction (lacking selenide), the futile hydrolysis of ATP into AMP by Sephs is measured, and subtracted as the background. The scheme for this reaction is:

\[\text{HSe}^- + \text{P} - \text{Pi} + \text{PEP} + \text{NADH} + \text{H}^+ \rightarrow \text{SeP} + 2\text{Pi} + \text{Lactate} + \text{NAD}^+ \]

The high sensitivity of LDH to dithiothreitol (DTT), the reducing reagent for maintaining selenide, was a key bottleneck in coupling the three enzyme activities. LDH activity diminished in the assay solution containing even 5 mM DTT but it was tolerant to 2-mercaptoethanol (2ME) from 0 to 40 mM.

Human *Seps2* (GenBank access GL:119572631) was amplified from a human lung cDNA library (TaKaRa) using a pair of primers (5’-TACCATGCGG-GAACCTCGGGAG3’ and 5’-TACCATGCGG-GAACCTCGGGAG3’) and KOD FX polymerase (Toyobo, Osaka, Japan). The thermal cycler was measurement of [8-14C] AMP, after a TLC procedure to separate it from the substrate, [8-14C] ATP. The non-radioactive assay noted from the literature employs AMP deaminase. In this enzyme-coupled assay, Sephs activity is indirectly measured by quantifying the ammonium (which is released by the deamination of AMP) by L-glutamate dehydrogenase. However, this procedure requires the enzyme preparations to be free of other interfering enzymatic activities, which liberate significant amounts of ammonia. In the present study, a recombinant pyruvate pyrophosphate dikinase (PPDK) from *Thermus thermophilus* HB8 was used to measure AMP formation via the following scheme:

\[\text{AMP} + \text{PEP} + \text{P} - \text{Pi} \rightarrow \text{ATP} + \text{Pyruvate} + \text{Pi} \]

Pyruvate + NADH + H⁺ → Lactate + NAD⁺

The three reaction products are formed in a 1:1:1 ratio, and the γ-phosphoryl group of ATP is liberated as the product, SeP. Direct measurement of SeP for routine enzyme assay is impractical because the compound is highly oxygen-labile. The standard assay method for Sephs activity relies on the relative ease of
Cell debris was removed by centrifugation at 8000 × g (Kubota Insonator 201 M, Kubota, Tokyo, Japan) for 20 min, and ultracentrifugation at 10,500 rpm for 1 h. For enzyme induction, 1.0 mM IPTG was added and incubated at 30 °C for further 20 h. Cells were harvested by centrifugation, suspended in 10 mL of 50 mM potassium phosphate buffer, pH 7, and disrupted by ultrasonication (Kubota Insonator 201 M, Kubota, Tokyo, Japan). Cell debris was removed by centrifugation at 8000 × g for 20 min, and ultracentrifugation at 10,500 × g for 1 h (Hitachi Himac CS100GX). Approximately 1 mg of C-terminal (His)_6-tagged Sephs2-U60C was obtained by Ni-NTA agarose affinity chromatography.

Recombinant PPDK was also expressed as a C-terminal (His)_6-tagged protein from the expression vector, pET28a-ThiPPDK; the ORF was amplified from a pET11a-PPDK provided from RIKEN, and ligated to the NcoI-XhoI restriction enzymes and ligated into pET28a. His-tagged human Sephs2, although there have been no reports on kinetics of mammalian Sephs to compare with those of the Cys-substituted enzyme. PPDK has been earlier employed in an enzyme-coupled assay, for the indirect quantification of an oxygen-labile compound formed in milieu.

Selenide was prepared by the reduction of sodium selenite by 2ME; an aliquot of 10 μL aqueous solution of NaSeO₃ (0.04–0.4 mM) and 90 μL of 100 mM 2ME (contained in glass vial sealed with rubber septum) was flushed with a stream of Ar gas for 2 min through a syringe needle. The solution of 2ME was transferred to the selenite bottle using a syringe and flushed with a stream of Ar for 2 min. This was used for assays within a day.

Reaction mixture (400 μL) contained 50 mM HEPES-KOH, pH 7.0, 10 mM KCl, 5 mM MgSO₄, 1.0 mM sodium pyrophosphate, 0.75 mM PEP, 0.1 mM NADH, 0.05 mM ATP, 20 μg PPDK, 50 μM (114 μg) Sephs2-U60C, 10 U LDH, and 0.01–0.1 mM NaSeH. The reaction mixture without selenide and ATP was once flushed with a stream of Ar gas in the septum-sealed vial, mixed with freshly prepared selenide. It was subsequently transferred to a quartz vial and sealed from air by overlaying 50 μL of mineral oil (ABI, Tokyo, Japan). The reaction was initiated by the addition of ATP, and the time-dependent decrease in absorbance at 340 nm was monitored for 10 min. The rate of NADH depletion detected in the control without selenide was subtracted as the background.

The enzyme-coupled assay gave steady-state kinetics for the C-terminally 6x His-tagged Sephs2-U60C (Fig. 1). The Michaelis constant (K_M) and specific activity (k_cat) values were obtained by regression analysis (R² = 0.96) as 26 μM and 0.35 min⁻¹, respectively.

Fig. 1. Double reciprocal plot of the PPDK catalytic rate vs. selenide concentration at fixed concentration of ATP.

Using [8-¹⁴C] ATP assay, E. coli SelD had shown K_M and k_cat values of 20 μM and 1.07 min⁻¹ respectively. The same values for Haemophilus influenzae SelD were 25 μM and 0.53 min⁻¹. The findings suggest that the substitution of active site Cys with Cys did not significantly perturb the catalytic function of the 6x His-tagged human Sephs2, although there have been no reports on kinetics of mammalian Sephs to compare with those of the Cys-substituted enzyme.

PPDK has been earlier employed in an enzyme-coupled assay. Therein, pyrophosphate was determined for accurate L-Met determination in human plasma. The work employed the enzyme from Thermoproteus tenax. Herein, we demonstrate that the homolog form of T. thermophilus HB8 can also be used for enzyme-coupled AMP assay, for the indirect quantification of an oxygen-labile compound formed in milieu.

Author contributions
Saho Kamada, Takahiro Okugochi, Kaori Asano, Ryuta Tobe, and Hisaaki Mihara carried out the experiments. Michiko Nemoto, Kenji Inagaki, and Takashi Tamura prepared the manuscript.

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References


