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Catalytic properties of selenophosphate synthetases: Comparison of the selenocysteine-containing enzyme from *Haemophilus influenzae* with the corresponding cysteine-containing enzyme from *Escherichia coli*

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ABSTRACT **The *selD* gene from *Haemophilus influenzae* has been overexpressed in *Escherichia coli*. The expressed protein was purified to homogeneity in a four-step procedure and then carboxymethylated by reaction with chloroacetate. N-terminal sequencing by Edman degradation identified residue 16 as carboxymethyl selenocysteine, which corresponded to the essential cysteine residue in the glycine-rich sequence of the *E. coli* selenophosphate synthetase. It would be expected that an ionized selenol of a selenocysteine in place of a catalytically essential cysteine residue would result in an enzyme with increased catalytic activity. To test this hypothesis we kinetically characterized the selenocysteine containing selenophosphate synthetase from *H. influenzae* and compared its catalytic activity to that of the cysteine containing selenophosphate synthetase from *E. coli*. Our characterization revealed the K_m values for the two substrates, selenide and ATP, were similar for both enzymes. However, the selenocysteine-containing enzyme did not exhibit the expected higher catalytic activity. Based on these results we suggest a role of selenocysteine in *H. influenzae* that is not catalytic.**

The insertion of selenocysteine into selenium-dependent proteins requires the products of four genes (*selA*, *selB*, *selC*, and *selD*) in prokaryotes (1–3). The 37-kDa *selD* gene product, selenophosphate synthetase, catalyzes the synthesis of selenophosphate from ATP and selenide. Inherent errors can be introduced in the determined K_m and specific activity values because of the extreme oxygen liability of selenide. Reproduction of kinetic values strongly depends on the rigorous exclusion of oxygen from all solutions. The homogenous *Escherichia coli* selenophosphate synthetase previously characterized under strictly anaerobic conditions had a determined specific activity of 83 nmol/min per mg (k_{cat} = 3 min^{-1}) (4). The K_m values for both substrates, ATP and selenide, were determined to be 900 μM and 7 μM, respectively. In the absence of selenide, selenophosphate synthetase catalyzes the slow hydrolysis of ATP to AMP and two orthophosphates (4).

The *E. coli* selenophosphate synthetase contains an essential cysteine, Cys-17, within the glycine-rich sequence, -Gly-Ala-Gly-Cys^{17}-Gly-Cys-Lys-Ile-. Replacement of Cys-17 with serine results in the complete loss of activity with ATP and selenide as substrates (5). Additionally, sequence analysis of the genomes of *Methanoccus jannaschii* (6), *Haemophilus influenzae* (7), mouse (8), and human (8) revealed their *selD* genes encode a selenocysteine residue corresponding to Cys-17 in the *E. coli* enzyme. A second human homolog also has been identified that contains a threonine substitution (9), and a homolog from *Drosophila* contains an arginine corresponding to Cys-17 in the *E. coli* enzyme (10). The *Drosophila selD* gene has been cloned and overexpressed in *E. coli*. The purified enzyme is unable to perform the selenide-dependent ATP hydrolysis reaction and the gene product fails to complement a *selD* lesion in *E. coli* (10). In contrast, the overexpressed threonine-containing human enzyme can weakly complement a *selD* lesion in *E. coli,* and transfection of the enzyme into mammalian cells results in an increased ^{75}Se-labeling of mammalian selenium-dependent deiodinase (9). Attempts to express the selenocysteine-containing selenophosphate synthetase proteins have been made with varying degrees of success. Expression of the mouse *selD* gene in the insect cell baculovirus system was very poor, accounting for 0.04% of total cellular protein (11). Expression of the *H. influenzae selD* in *E. coli* complemented a *selD* lesion in a mutant strain (12). Although overall protein expression levels were low, a functional enzyme containing selenium was obtained (12).

The variability of enzyme activity with substitutions in the glycine-rich sequence at the residue corresponding to Cys-17 in *E. coli* again supports the importance of this residue in catalysis. Previously it has been shown with formate dehydrogenase H from *E. coli* that a sulfur replacement for selenium resulted in more than two orders of magnitude reduction in the turnover number for the oxidation of formate. The marked effect of this substitution indicates the selenium of formate dehydrogenase is directly involved in formate oxidation (13). By analogy, it would be expected that if Cys-17 in the *E. coli* selenophosphte synthetase is catalytically involved in the biosynthesis of selenophosphate a substitution at this position with selenocysteine should result in an increased turnover number for selenophosphate formation. In this paper we report the cloning, overexpression, purification, and characterization of the *H. influenzae selD* in *E. coli* and compare its catalytic activity to the cysteine-containing enzyme from *E. coli*.

MATERIALS AND METHODS

Materials. The following chemicals were purchased from the indicated sources. [8-^{14}C]ATP (49 mCi/mmol) was from ICN. Na_2ATP was from Boehringer Mannheim and Na selenite from Sigma. *H. influenzae* clone GHICN38 containing the *selD* gene was purchased from The Institute for Genomic Research, Gaithersburg, MD. *Taq* polymerase and dNTPs were purchased from Perkin–Elmer. Synthetic oligonucleotides were purchased from Biosynthesis, Lewisville, TX. TOPO TA cloning kit was from Invitrogen, pET3a expression vector was from Novagen. All other chemicals and supplies were the highest grade available.

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Cloning and Overexpression of *H. influenzae selD* in *E. coli*. An expression vector containing the *H. influenae selD* gene was prepared via PCR. Synthetic primers were constructed to amplify the *selD* gene from the GHICN38 genomic clone and were designed to add a *Nde*I restriction site at the 5′ end and a *Bam*HI site at the 3′ end of the gene to facilitate cloning. The PCR was performed on a Techne Genius thermocycler. The reaction for amplification was as follows: after an initial denaturation at 94°C for 5 min, 30 cycles were performed with a denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, and polymerization at 72°C for 2 min. After the 30 cycles were complete a final polymerization step at 72°C for 10 min was performed. The 1,053-bp PCR product was visualized on a 0.8% agarose gel and ligated directly into the TOPO TA cloning vector. The gene was cut from the TOPO vector with *Nde*I and *Bam*HI and purified on a 0.8% agarose gel with the Boehringer Mannheim agarose gel DNA extraction kit. The purified gene was ligated directly into pET3a cut with *Nde*I and *Bam*HI. Ligated vectors were transformed into *E. coli* BL21 cells.

Overexpression and Purification of *H. influenzae selD*. BL21 cells containing pET3a*HIselD* were grown aerobically in a 10-liter fermentor with vigorous stirring in Luria–Bertani medium supplemented with 0.3% glucose, 1 μM selenite, and 10 μM molybdate until A_{600} reached 0.6. At that time agitation was stopped and lactose was added to a final concentration of 0.4% and cells were left to induce *selD* anaerobically for 2.5 hr. Cells were harvested, frozen, and stored at −80°C.

Nineteen grams of frozen cells together with 0.5 g of [^{75}Se]HI *selD* were thawed in 50 ml of 50 mM Tricine·KOH, pH 7.8/1 mM Mgtitriplex/2 mM DTT (buffer A). Cells were disrupted by sonication and cell debris was centrifuged at 16,000 rpm for 30 min. Ammonium sulfate was added to the cell supernatant to 45% of saturation, and the protein precipitate was resuspended and dialyzed against buffer A and 1 M ammonium sulfate. Dialyzed protein was applied directly to a phenyl Sepharose column and washed with a 120-ml gradient of 1 M to 0 M ammonium sulfate in buffer A. Selenophosphate synthetase was eluted at the end of the gradient by the addition of 40% ethylene glycol in buffer A. Fractions containing *selD* were pooled and dialyzed against buffer A. Dialyzed protein was added directly to DEAE Sepharose and a 400-ml gradient of 0 M to 0.35 M KCl in buffer A was applied to the column. Fractions containing selenophosphate synthetase were pooled and dialyzed against buffer A and 1 M ammonium sulfate. Dialyzed protein was applied to butyl Sepharose and a 400-ml gradient of 1.0 M to 0 M ammonium sulfate in buffer A was applied to the column. Fractions containing selenophosphate synthetase were pooled, concentrated, frozen, and stored at −80°C until needed.

Carboxymethylation of *H. influenzae selD*. *H. influenzae selD* (100 μg) in 160 μl of 100 mM Tricine·KOH (pH 7.8) was denatured by mixing with 91 mg of guanidine-hydrochloride in a 1.9-ml microcentrifuge tube. The solution was sparged with N_2 for 20 min at room temperature, then 15 μl of 100 mM DTT was added and the reaction mixture was incubated for an additional 40 min at room temperature under N_2. For alkylation a 33-μl aliquot of 250 mM chloroacetic acid was added, and the solution (pH 7.0) was incubated in the dark at room temperature for 40 min. To quench the reaction, 15 μl of 1 M DTT was added. The modified protein then was used for Edman degradation and N-terminal sequencing.

Effect of H_2O_2 on the Catalytic Activity of Selenophosphate Synthetase Enzymes. Selenophosphate synthetase (30 μM) in 100 mM Tricine·KOH (pH 7.8) was incubated with the indicated concentration of H_2O_2 for 30 min at room temperature. Mixtures then were applied on PD10 columns (Pharmacia) equilibrated with Tricine·KOH, pH 7.8/2 mM DTT/6 mM $MgCl_2$/20 mM KCl. Equilibrated enzyme solutions were used directly to measure selenophosphate synthetase activity. Activity of H_2O_2-treated enzymes is expressed as the percent [^{14}C]AMP produced compared with amount produced by unmodified protein in 30 min under standard assay conditions.

Enzyme Assays. Selenophosphate synthetase assays with selenide as substrate were performed as described (4). NIFS and selenophosphate synthetase assays were performed as described (14). All assays were performed anaerobically under argon as gas phase.

RESULTS

Overexpression and Purification of *H. influenzae selD* in *E. coli*. Our expression vector containing the *H. influenzae selD* gene weakly expressed a recombinant protein. The low expression level resulted in the inability to visualize a Coomassie blue-stained protein band at its expected size when crude cell extracts were analyzed by SDS/PAGE. However, labeling with ^{75}Se allowed detection by PhosphorImager analysis of a protein that migrated on an SDS/PAGE gel as a 38-kDa species. The recombinant protein was purified to homogeneity by a four-step purification procedure consisting of ammonium sulfate fractionation, phenyl Sepharose chromatography, DEAE-Sepharose chromatography, and butyl-Sepharose chromatography.

The purified ^{75}Se-labeled protein was carboxymethylated with chloroacetate and subjected to N-terminal sequencing by automated Edman degradation. Sequence analysis of the first 20 amino acid residues (Fig. 1) showed that residue 16 eluted as carboxymethyl [^{75}Se]selenocysteine.

Catalytic Activity of the Recombinant *H. influenzae* Selenophosphate Synthetase. Sequence alignments of the *E. coli* and *H. influenzae* selenophosphate synthetase proteins show both proteins share a high percentage of sequence identity (Fig. 2). Of particular interest are the N-terminal regions of both proteins. The glycine-rich sequence of the *E. coli* protein contains the essential cysteine at position 17. In the corresponding sequence of the *H. influenzae* protein a selenocysteine residue, Secys-16, aligns with the *E. coli* Cys-17. If Cys-17 has a direct catalytic role in the biosynthesis of selenophosphate it would be expected that replacement with selenocysteine would increase the catalytic activity of the enzyme. Instead the determined specific activity was slightly lower as compared with the *E. coli* enzyme (Table 1). Despite the fact that the K_m value for selenide is difficult to reproduce, because of its extreme oxygen lability, the determined K_m value for selenide from the *H. influenzae* enzyme was very close to the

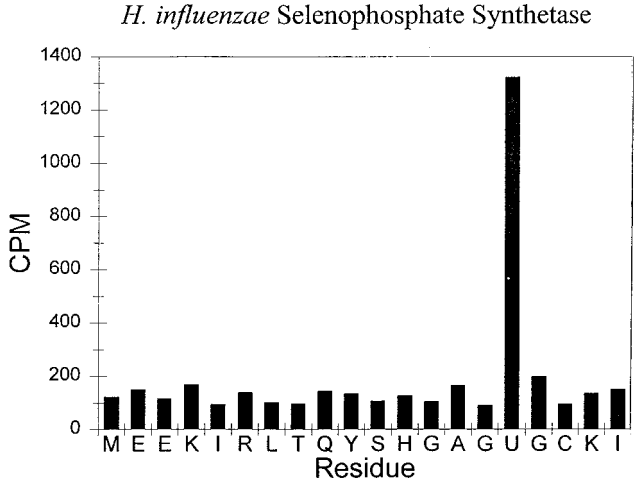

FIG. 1. N-terminal sequence as determined by automated Edman degradation of residues 1–20 of ^{75}Se-labeled *H. influenzae* selenophosphate synthetase. The ^{75}Se that eluted in cycle 16, labeled U, was present in carboxymethyl [^{75}Se]selenocysteine.

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E.coli              MSENSIRLTQYSHGAGCGCKISPKVLETILHSEQAKFVDPNLLVGNETRD
                    : :  :::::::::::: :::::::::: ::::::  :: ::::.::::: :
H.influenzae        M-EEKIRLTQYSHGAGUGCKISPKVLGTILHSELEKFYDPNLIVGNETAD

E.coli              DAAVYDLGNGTSVISTTDFFMPIVDNPFDFGRIAATNAISDIFAMGGKPI
                    :::::::::::: .:::::::::::::: ::::::::::::::::::::::::
H.influenzae        DAAVYDLGNGTAIISTTDFFMPIVDDPFDFGRIAATNAISDIFAMGGKPI

E.coli              MAIAILGWPINKLSPEIAREVTEGGRYACRQAGIALAGGHSIDAPEPIFG
                    : ::::::.: : :  : :    .:::.:: :::::::::::::: ::::::
H.influenzae        MGIAILGFPTNVLPAEVAQKIVDGGRFACHQAGIALAGGHSIDSPEPIFG

E.coli              LAVTGIVPTERVKKNSTAQAGCKLFLTKPLGIGVLTTAEKKSLLKPEHQG
                    :::::.. ::.::.:  :  ::::. ::::::: ::::::: :::::::
H.influenzae        LAVTGVIDTEKVKRNASAKSGCKLYMTKPLGIGILTTAEKKGKLKPEHQG

E.coli              LATEVMCRMNIAGASFANIEGVKAMTDVTGFGLLGHLSEMCQGAGVQARV
                    ::: .::.::  :  : ...:: :::::::::::::: : : :    : :
H.influenzae        LATAAMCQMNSIGSQFSQVDGVTAMTDVTGFGLLGHLIEICEGSNLSAVV

E.coli              DYEAIPKLPGVEEYIKLGAVPGGTERNFASYGHLMGEMPREVRDLLCDPQ
                      . :  : :: .::  : ::::: ::: ::::  :    :   .:::::
H.influenzae        FSDKIKTLDGVKDYIAQGCVPGGTGRNFDSYGHKVGILTEEQKAILCDPQ

E.coli              TSGGLLLAVMPEAENEVKATAAEFGIELTIGELVPARGGRAMVEIR
                    :::::: ::      . :    : . ::.:     .  .     :
H.influenzae        TSGGLLVAVELNSVQTVIDIAKDAGIDLYEVGKLKPKSESDIVVEVK
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FIG. 2. Sequence alignment of *E. coli* selenophosphate synthetase with *H. influenzae* selenophosphate synthetase. Identical residues are identified by a colon (:), similar residues are identified by a period (.).

determined value for the *E. coli* enzyme under identical conditions. The determined K_m values for ATP also were in close agreement for both proteins (Table 1).

Treatment of Selenophosphate Synthetases with H_2O_2. Treatment of both the *E. coli* and *H. influenzae* selenophosphate synthetase enzymes with increasing concentrations of H_2O_2 caused a concentration-dependent rate of inactivation. H_2O_2 inactivated both enzymes at a similar rate, showing no preference of inactivation of the selenium over the cysteine-containing enzyme (Table 2).

***H. influenzae* Activity in the Presence of a Selenide Delivery Protein.** We previously have demonstrated with the *E. coli* enzyme that the rate of selenophosphate biosynthesis is increased in the *in vitro* assay when the substrate selenide is replaced with L-selenocysteine and the NIFS protein (14). We performed coupled assays in which the NIFS protein served as a selenide delivery system for the *H. influenzae* selenophosphate synthetase and determined the rate of selenophosphate biosynthesis by monitoring the hydrolysis of ATP to AMP. As shown in Fig. 3, *H. influenzae* selenophosphate synthetase also is active in the presence of the NIFS selenide delivery system and the amount of AMP formed increased as the molar ratio of NIFS to selenophosphate synthetase varied from 1 to 6.

Table 1. Determined kinetic constants for the selenophosphate synthetase enzymes

Selenophosphate synthetase	K_m ATP, mM	K_m selenide, μM	Specific activity, nmol/min per mg
E. coli	0.9	20	29
H. influenzae	1.3	25	16

Reactions were performed anaerobically at 37°C. Reaction mixtures (0.1 ml) contained 100 mM Tricine·KOH (pH 7.8), 2 mM DTT, 20 mM KCl, 4 mM $MgCl_2$, 5 μM selenophosphate synthetase, and the appropriate concentration of substrate.

Selenocysteine in the absence of NIFS was not used as a selenium source and thus could not replace free selenide.

DISCUSSION

The enzymatic mechanism whereby selenophosphate synthetase catalyzes the biosynthesis of selenophosphate has yet to be determined. Although several homologs of the enzyme from other organisms have been identified, until now the only extensively studied enzyme was from *E. coli*. The identification of an essential cysteine residue, Cys-17, in the N-terminal glycine-rich region of the protein has for several years led to the assumption that this residue is catalytically essential, perhaps behaving as a nucleophile in the hydrolysis of ATP. Recent positional isotope exchange experiments have demonstrated that an enzyme phosphoryl-intermediate is formed during catalysis (15). However, repeated attempts to trap an

Table 2. Effect of H_2O_2 on the catalytic activity of selenophosphate synthetase enzymes

$[H_2O_2]$, mM	*E. coli* selenophosphate synthetase activity, %	*H. influenzae* selenophosphate synthetase activity, %
0	100	100
2.5	71	63
5	56	47
10	38	46

Reaction mixtures contained 100 mM Tricine·KOH (pH 7.8), 2 mM DTT, 6 mM $MgCl_2$, 20 mM KCl, 30 μM selenophosphate synthetase, and the indicated concentration of H_2O_2. Samples were incubated at room temperature for 30 min. Reactions were quenched by applying the mixtures to PD10 columns equilibrated in buffer A. Equilibrated protein was used directly in the selenophosphate synthetase assay (4).

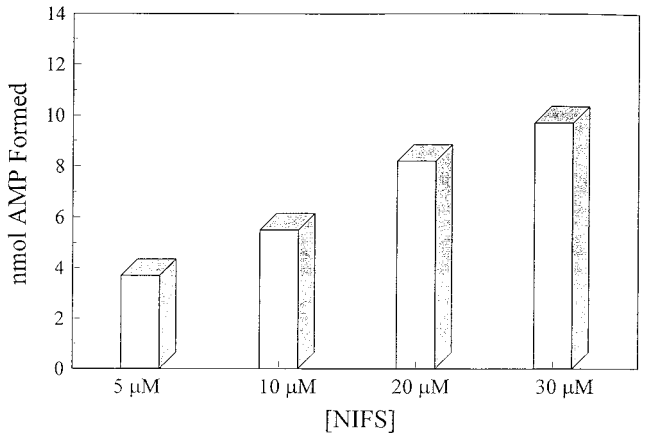

FIG. 3. NIFS and *H. influenzae* selenophosphate synthetase coupled assays. Assays were performed anaerobically at 37°C in 100 mM Tricine·KOH (pH 8.0), 2 mM DTT, 20 mM KCl, 4 mM $MgCl_2$, 2 mM ATP, 0.2 μCi [^{14}C]ATP, 2 mM L-selenocysteine, 5 μM selenophosphate synthetase, and the indicated concentration of NIFS.

enzyme thiophosphate intermediate have failed, suggesting that Cys-17 may not be the nucleophile. The sequence alignment of the *E. coli* selenophosphate synthetase with selenophosphate synthetase proteins from other organisms shows some variability at the residue corresponding to Cys-17 (Fig. 4), again offering some support for catalytic relevance. Of particular interest were the homologs from mouse (8), human (8), *M. jannaschii* (6), and *H. influenzae* (7) because the *selD* gene from these organisms encodes a selenocysteine corresponding to Cys-17 in the *E. coli* enzyme. The replacement of an essential cysteine residue with selenocysteine in an enzyme would be expected to result in increased catalytic activity. Although sulfur and selenium are chemically similar and they can undergo many of the same reactions; organo selenols are generally more reactive. The selenol of free selenocysteine has a pKa of 5.2 compared with the pKa of the thiol of free cysteine of ≥8. Hence, at physiological pH a selenol group usually would become fully ionized as compared with a thiol that would be mainly protonated (13). Previously, attempts made in our laboratory to obtain significant levels of the murine selenophosphate synthetase protein by using the baculovirus expression system were unsuccessful. Although the corresponding cysteine mutant of mouse selenophosphate synthetase was expressed efficiently there was only negligible expression of the wild-type selenocysteine-containing enzyme. Thus, direct comparison of the activities of these enzymes activities was not possible.

We therefore cloned and overexpressed selenophosphate synthetase from *H. influenzea*. Expression of the enzyme in *E. coli* produced a protein that contained selenocysteine in the position corresponding to Cys-17 as determined by Edman degradation. Previous expression of this protein in *E. coli* produced a catalytically active enzyme that *in vivo* was able to complement an *E. coli selD* lesion (12). Our kinetic characterization of the *H. influenzae* selenophosphate synthetase revealed that, instead of an enzyme with the expected greater catalytic activity, the selenocysteine-containing enzyme was slightly lower in activity than the cysteine-containing *E. coli* homolog. Moreover, our determined K_m values for ATP and selenide were similar for both proteins.

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HuSPS2  ...ALGLSPSWRLTGFSGMKGUGCKVPQEALLKKLLAG...
MoSPS2  ...TLGFSPSWRLTSFSGMKGUGCKVPQETLLKLLEGL...
HuSPS1  ...SYELDKSFRLTRFTELKGTGCKVPQDVLQKLLESL...
EcSPS      MSENSIRLTQYSHGAGCGCKISPKVLETILHHS...
HiSPS       MEEKIRLTQYSHGAGUGCKISPKVLGTILHSE...
MjSPS     MERGNEKIKLTELVKLHGUACKLPSTELEFLVKGI...
DMSPS  ...AHDLDASFRLTRFADLKGRGCKVPQDVLSKLVSAL...
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FIG. 4. Partial amino acid sequences of selenophosphate homologs from (Hu) human, (Mo) mouse, (Ec) *E. coli*, (Hi) *H. influenzae*, (Mj) *M. jannaschii*, and (Dm) *Drosophila melanogaster*. Residues corresponding to Cys-17 are identified in bold (U = selenocysteine).

The inability of the *H. influenzae* selenophosphate synthetase to exhibit an increased rate of selenide-dependent ATP hydrolysis sheds light on the role of selenocysteine in this protein. Previously, it was assumed, based on mutagenesis studies, that Cys-17 in the *E. coli* enzyme is catalytically essential. In a similar study of pseudouridine synthase I it was demonstrated that cysteine residues were important in maintaining the active site structure of the enzyme and were possibly involved in the catalytic mechanism (16). Initially, each of three cysteine residues of this protein were mutated to serine and two of the mutants were inactive, suggesting a direct catalytic function for cysteine. Additional mutants were constructed later in which each cysteine was replaced with alanine as well as a triple mutant in which all three cysteines were mutated to alanine. All alanine mutants, including the triple mutant, exhibited near wild-type activity. These results provided direct chemical evidence against a catalytic function for a cysteine residue in the rearrangement of uridine to pseudouridine by pseudouridine synthetase I. Based on a consideration of the properties of both sets of mutants it can be seen that although cysteine and serine are structurally similar they differ in polarity. The sulfhydryl group of cysteine is relatively nonpolar compared with the hydroxyl side chain of serine. If cysteine occupies a hydrophobic environment within the protein, its replacement with serine could disturb the structure of the enzyme, resulting in a loss of catalytic activity, whereas replacement of cysteine with the nonpolar amino acid alanine would not cause a loss in catalytic activity. Thus, by analogy we could suggest that not only is cysteine inessential catalytically in selenophosphate synthetase but rather its substitution with serine results in the alteration of a hydrophobic region of the enzyme's structure, which indirectly affects enzyme activity.

As shown in the present work, the replacement of cysteine by selenocysteine does not result in an increase in the rate of catalysis. Moreover, when Cys-17 of the *E. coli* enzyme was changed to serine, by site-directed mutagenesis, the result was complete loss of catalytic activity. These results taken together suggest a role for selenocysteine of *H. influenzae* that is not catalytic. If the seleno amino acid is located in a hydrophobic region of the enzyme then the nonpolar nature of the selenium side chain could be essential in maintaining this hydrophobicity. In the *E. coli* enzyme Cys-17 would have a similar role. Additional mutagenesis experiments in which Cys-17 is replaced with other hydrophobic residues such as alanine need to be performed to fully understand the role of the Se and S residues in the conserved glycine-rich region of the proteins.

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