FEBS Letters 523 (2002) 234–238 FEBS 26287

Escherichia coli 6-pyruvoyltetrahydropterin synthase ortholog encoded by *ygcM* has a new catalytic activity for conversion of sepiapterin to 7,8-dihydropterin

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Received 8 May 2002; revised 14 June 2002; accepted 15 June 2002

First published online 27 June 2002

Edited by Stuart Ferguson

Abstract The putative gene (*ygcM*) of *Escherichia coli* was verified in vitro to encode the ortholog of 6-pyruvoyltetrahydropterin synthase (PTPS). Unexpectedly, the enzyme was found to convert sepiapterin to 7,8-dihydropterin without any cofactors. The enzymatic product 7,8-dihydropterin was identified by HPLC and mass spectrometry analyses, suggesting a novel activity of the enzyme to cleave the C6 side chain of sepiapterin. The optimal activity occurred at pH 6.5–7.0. The reaction rate increased up to 3.2-fold at 60–80°C, reflecting the thermal stability of the enzyme. The reaction required no metal ion and was activated slightly by low concentrations (1–5 mM) of EDTA. The apparent K_m value for sepiapterin was determined as 0.92 mM and the V_{max} value was 151.3 nmol/min/mg. The new catalytic function of *E. coli* PTPS does not imply any physiological role, because sepiapterin is not an endogenous substrate of the organism. The same activity, however, was also detected in a PTPS ortholog of *Synechocystis* sp. PCC 6803 but not significant in *Drosophila* and human enzymes, suggesting that the activity may be prevalent in bacterial PTPS orthologs.

Key words: 6-Pyruvoyltetrahydropterin synthase; Tetrahydrobiopterin; Sepiapterin; 7,8-Dihydropterin; Overexpression; *Escherichia coli*

1. Introduction

6-Pyruvoyltetrahydropterin synthase (PTPS, EC 4.6.1.10) is an essential enzyme catalyzing the second step of tetrahydrobiopterin (BH4) biosynthesis [1–3]. BH4 is a well-known cofactor for aromatic amino acid hydroxylation and nitric oxide synthesis in higher animals [4]. The de novo synthesis of BH4 starts from GTP by GTP cyclohydrolase I (GTPCH, EC 3.5.4.16), yielding dihydroneopterin triphosphate (H2-NTP). H2-NTP is converted to 6-pyruvoyltetrahydropterin (PPH4) by PTPS and then to BH4 by sepiapterin reductase (SR, EC 1.1.1.153). In addition, aldose reductase (EC 1.1.1.21) catalyzes the reduction of the C2′-oxo group in PPH4 to yield 6-lactoyltetrahydropterin (LPH4) [5–7]. LPH4 is reduced further to BH4 by SR or is oxidized non-enzymatically to sepiapterin. In the salvage pathway of BH4, sepiapterin is converted to 7,8-dihydrobiopterin by SR and then to BH4 by dihydrofolate reductase.

BH4 is ubiquitous in eukaryotes including fungi but not in prokaryotes. There has been no report of BH4 synthesis in prokaryotes, except for some bacteria, such as cyanobacteria and *Chlorobium* sp., which produce glycosidic forms of BH4 [8–10]. BH4 synthesis in these organisms seems to follow the same biochemical steps established in higher animals, being catalyzed by the consecutive actions of GTPCH, PTPS, and SR [11]. Even in the other prokaryotes, which do not synthesize BH4, GTPCH is essential for the synthesis of tetrahydrofolate, whereas the other biosynthetic enzymes do not appear to be required. Nevertheless, the gene encoding PTPS was identified in the genome sequenced cyanobacterium *Synechocystis* sp. PCC 6803, which produces 6-hydroxymethylpterin glycoside [12]. Since 6-hydroxymethylpterin is an intermediate in tetrahydrofolate synthesis, PTPS was not expected to be present in this organism. However, a putative open reading frame (ORF) slr0078 was verified to encode PTPS and to be expressed under normal growth conditions [13], while no gene encoding SR has been identified. Subsequent sequencing of the genomes of several prokaryotes including *Escherichia coli* has also revealed genes putatively encoding PTPS orthologs. As these prokaryotes were not known to produce BH4, it was doubted whether the PTPS orthologs have any physiological function in the absence of SR. In order to answer this question, we investigated the putative function of the PTPS orthologs using *E. coli* as a model organism.

In this study, we cloned and overexpressed the putative *E. coli* gene (*ygcM*) of the PTPS ortholog to identify the enzymatic activity to synthesize PPH4 from H2-NTP. In order to investigate a putative metabolic function of the enzyme we intended to create *E. coli* transformants overproducing PPH4 and BH4 individually by coexpression of *E. coli* PTPS with GTPCH or GTPCH/SR. *Synechocystis* sp. PCC 6803 GTPCH and mouse SR were employed in the experiment simply because their ORF clones were available and also identified to produce functional proteins [13,14]. In the course of analyzing the transformants, we found that *E. coli* PTPS has a novel activity of bleaching the yellow color of sepiapter-

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Abbreviations: BH4, tetrahydrobiopterin; GTPCH, GTP cyclohydrolase I; H2-NTP, dihydroneopterin triphosphate; LPH4, 6-lactoyltetrahydropterin; PPH4, 6-pyruvoyltetrahydropterin; PTPS, 6-pyruvoyltetrahydropterin synthase; SR, sepiapterin reductase

0014-5793/02/$22.00
PII: S0014-5793(02)02997-6

in, giving rise to 7,8-dihydropterin. As the activity was so far unknown in our knowledge in any enzymes, we characterized the enzymatic properties and here report the results.

2. Materials and methods

2.1. Construction of plasmids

The putative *E. coli* PTPS gene (*ygcM*) was amplified by PCR from genomic DNA using a primer pair, CATATGATGTCCACCACGTTAT (forward) and GGATCCTAACATAGCGTTACTTAA (reverse) containing restriction sites (underlined) for *Nde*I and *Bam*HI, respectively. The DNA was cloned into the pGEM-T vector (Promega) and subsequently cloned as an *Nde*I/*Bam*HI restriction fragment into pET-28a (pET-ePTPS). The overexpression plasmids of *Synechocystis* sp. PCC 6803 GTPCH (pET-cGTPCH), *Synechocystis* sp. PCC 6803 PTPS (pET-cPTPS), mouse SR (pET-mSR), *Drosophila* PTPS (pET-dPTPS), and human PTPS (pET-hPTPS) were prepared previously [13–16]. In order to coexpress *E. coli* PTPS and *Synechocystis* sp. PCC 6803 GTPCH, a *Bgl*II/*Hin*dIII fragment of pET-cGTPCH was introduced into the *Bam*HI/*Hin*dIII site of pET-ePTPS plasmid, giving rise to pET-cGTPCH/ePTPS. The plasmid pET-cGTPCH/ePTPS was digested with *Bam*HI/*Hin*dIII and then ligated with the *Bgl*II/*Hin*dIII fragment of pET-mSR to generate pET-cGTPCH/ePTPS/mSR plasmid. The plasmids were transformed into *E. coli* BL21(DE3) (Novagen). DNA sequences were confirmed by sequencing.

2.2. Expression and purification of recombinant proteins

The transformed cell was grown at 37°C in LB+Amp broth with vigorous shaking. Overexpression was induced by 50 μM IPTG when A_{600} reached 0.6. The cells were allowed to continue to grow overnight and harvested by centrifugation. The cells were washed and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole), and disrupted by sonication. The crude extract obtained after centrifugation was applied to a column of Ni-NTA gel (Qiagen) and purified by following the instructions in the product manual. His-tag protein was eluted with 100 mM EDTA in the lysis buffer. The purified protein was dialyzed against 10 mM PIPES (pH 7.5) neutralized by NaOH, mixed with glycerol to a final concentration of 10%, and stored at −70°C until use.

2.3. Activity assay

PTPS activity to catalyze the synthesis of PPH4 from H2-NTP was assayed in the absence or presence of SR [17,18]. The reaction mixture consisted of 100 mM Tris–HCl (pH 7.5), 0.1 mM H2-NTP, 10 mM $MgCl_2$, 10 mM dithiothreitol, and aliquots of enzyme in a final volume of 50 μl. In a coupled assay with SR, the mixture was supplemented with 0.2 mM NADPH and 5 μg of recombinant mouse SR. The mixture was incubated for 1 h at 37°C and mixed with an equal volume of an acidic iodine solution (2% KI and 1% I_2 in 1 N HCl) for 1 h in the dark. After centrifugation, the supernatant was reduced with ascorbic acid and subjected to HPLC. H2-NTP was prepared from GTP by incubation with recombinant *Synechocystis* sp. PCC 6803 GTPCH [13] according to the published method [19].

The activity to convert sepiapterin to 7,8-dihydropterin was measured by disappearance of the yellow color at 420 nm. Unless stated otherwise, all reactions were performed in the following conditions (standard assay conditions). The reaction mixture contained 50 mM PIPES, pH 7.5, 2.88 mM sepiapterin, and enzyme (17 μg for *E. coli* PTPS) in a total volume of 50 μl. The reaction was performed at 37°C for 20 min; linearity of the reaction was observed up to 60 min. An equal volume of cold 10% trichloroacetic acid was added to stop the reaction and, after 10 min on ice, the mixture was centrifuged at 10 000×*g* for 10 min. The supernatant was diluted 10-fold with water and measured for remaining sepiapterin. In order to normalize the quenching effect of trichloroacetic acid on spectral absorption of sepiapterin at 420 nm, every assay was accompanied by a blank reaction without enzyme, especially when using different quantities of sepiapterin for kinetic analysis. The concentration of sepiapterin was calculated from the molar extinction coefficient, 10.4 mM^{-1} cm^{-1} at 420 nm [20].

2.4. HPLC analysis of pteridines

HPLC was performed on a Kontron Model 430 equipped with a Rheodyne loop of 20 μl, an Inertsil ODS-3 C18 column (5 μm, 150×2.3 mm, GL Science, Japan), and a HP Model 1046A fluorescence detector (350/450 nm, excitation/emission). Pteridines were eluted isocratically with 10 mM sodium phosphate (pH 6.0) at a flow rate of 1.2 ml min^{-1} and monitored at 350/450 nm (excitation/emission) as described previously [21]. Pteridine compounds were purchased from Schirck's Lab (http://www.schircks.com). In order to isolate pteridine compounds chromatography was performed in the same conditions as described above except using water as a mobile phase.

2.5. Miscellaneous methods

ESI(+) mass spectrometry of pteridine compounds was performed with Mariner (Perseptive Biosystem, USA) in the Korea Basic Science Institute (Taejon, South Korea). SDS–PAGE analysis of proteins was performed on denaturing conditions with a 12.5% polyacrylamide gel. Protein was measured by the Bradford method using bovine serum albumin as a standard.

3. Results

3.1. Identification of PPH4 synthesis by E. coli PTPS

A homology search of PTPS against the finished genome sequence of *E. coli* [22] revealed a putative protein encoded by the unidentified gene *ygcM*. The translated protein consisted of 121 amino acid residues which shared 26.3%, 27.8%, and 44.2% sequence identity with the respective PTPSs of human (accession number JC1405), rat (A39499), and *Synechocystis* sp. PCC 6803 (ORF slr0078) as shown in the multiple alignment (Fig. 1). Also conserved were most of the critical residues for catalysis, positioning the pterin ring, and coordinating with zinc ion (Fig. 1), which were originally suggested from the crystal structure of rat PTPS [23]. In order to determine whether the putative *E. coli* gene encodes PTPS, the gene was amplified by PCR from genomic DNA and cloned into the pET-28a expression vector. The recombinant protein was overexpressed, purified (Fig. 2B), and assayed for its activity to synthesize PPH4 from H2-NTP. The assay was performed in the absence or presence of mouse recombinant SR and analyzed by HPLC after iodine oxidation. The enzymatic products were eluted at the position corresponding to pterin in the absence of SR (Fig. 2AII, upper) and biopterin in the coupled assay with SR (Fig. 2AII, lower), supporting that the enzymatic product of *E. coli* PTPS was PPH4. Therefore, it was concluded that *ygcM* encodes a PTPS ortholog of *E. coli*. The specific activity was determined to be approx. 8.7% of the human enzyme (Table 1).

3.2. Analysis of pteridine compounds produced by coexpression transformants

We created an *E. coli* transformant carrying the coexpression plasmid, pET-cGTPCH/ePTPS, expecting that expression

Table 1
Comparative activity analysis of recombinant PTPSs

Organism	Specific activity (nmol/min/mg)	
	PTPS	SSCR
E. coli	1.13 ± 0.04	99.36 ± 4.87
Synechocystis sp. PCC 6803	0.39 ± 0.01	97.67 ± 0.61
Drosophila	8.91 ± 0.20	0.34 ± 0.21
Human	13.03 ± 0.33	0.23 ± 0.01

The PTPS activity was measured by the synthesis of BH4 in the coupled assay with SR. The SSCR activity was measured in the standard assay conditions as described in Section 2. The mean values were determined from three independent experiments.

Fig. 1. Multiple alignments of the amino acid sequences of PTPS proteins. Conserved sequences are shaded at four levels using GeneDoc software and the residues suggested for catalysis (+), positioning of the pterin ring (!), and coordinating with zinc ion (*) are indicated.

of both cyanobacterial GTPCH and *E. coli* PTPS might generate high cellular concentrations of PPH4 and would in turn cause some phenotypic change(s) in the transformant. In contrast to non-transformed cells which produce no pteridine compounds in the medium, the transformant produced considerable amounts of a pteridine compound (Figs. 2AIII and 3). However, the transformant exhibited no obvious growth advantages under various stress conditions, such as UV light, metal ions, ascorbate, and hydrogen peroxide (data not shown). The pteridine compound in the medium was analyzed by HPLC (Fig. 2AIII). Without iodine oxidation a small peak appeared at the retention time corresponding to pterin (Fig. 2AIII, upper) but the peak increased several-fold after iodine oxidation (Fig. 2AIII, lower). The peak fraction isolated from the oxidized sample was further analyzed by mass spectrometry to confirm the molecular mass of pterin (data not shown). These data indicated that the transformant probably produced 7,8-dihydropterin a portion of which was oxidized to pterin in the medium. Finally, the soluble fraction of the sonicated transformant cells was analyzed by SDS–PAGE (Fig. 2B) in order to identify overexpression of the recombinant proteins. The recombinant GTPCH and PTPS were clearly seen as dominant bands, which migrated at the same rates as their purified counterparts shown in the parallel lanes. It therefore was suggested that the recombinant *Synechocystis* GTPCH and *E. coli* PTPS in the transformant contributed to the production of 7,8-dihydropterin.

The above result did not resolve the question of whether the 7,8-dihydropterin originated from PPH4, which was expected to occur by the recombinant proteins. In order to answer this question, we created another *E. coli* transformant of pET-cGTPCH/ePTPS/mSR, harboring an additional ORF for mouse SR. We expected production of BH4 from the transformant, if PPH4 were synthesized by *E. coli* PTPS. In contrast to our expectation, the transformant expressed barely detectable amounts of mouse SR and thus did not produce any form of biopterin but 7,8-dihydropterin (data not shown). In the course of analyzing SR expression in the transformant we found an enzymatic activity bleaching the yellow color of sepiapterin in the absence of NADPH. The enzymatic product analyzed by HPLC after iodine oxidation was pterin (see below). As this activity has never been found in any enzymes, we initially suspected the presence of a novel enzyme, which might have been induced to cope with increased synthesis of PPH4 in the transformant and also responsible for 7,8-dihydropterin production from PPH4. In order to identify the putative protein we performed purification of the protein from the transformant using conventional chromatographies and FPLC, but recombinant *E. coli* PTPS was what we finally purified to homogeneity (data not shown). The recombinant enzyme purified previously by metal affinity chromatography was also confirmed to have the same activity. These results strongly supported that *E. coli* PTPS is responsible for the bleaching activity.

3.3. Identification of 7,8-dihydropterin catalyzed from sepiapterin

The reaction mixture of sepiapterin with *E. coli* PTPS exhibited fluorescence under UV light, indicating conversion of sepiapterin to another pteridine compound. The time course of the incubation analyzed by absorption spectra clearly showed a gradual decrease of the sepiapterin peak at 420 nm with the concomitant increase of a new peak at 320 nm (data not shown). Aliquots of the reaction mixture were also

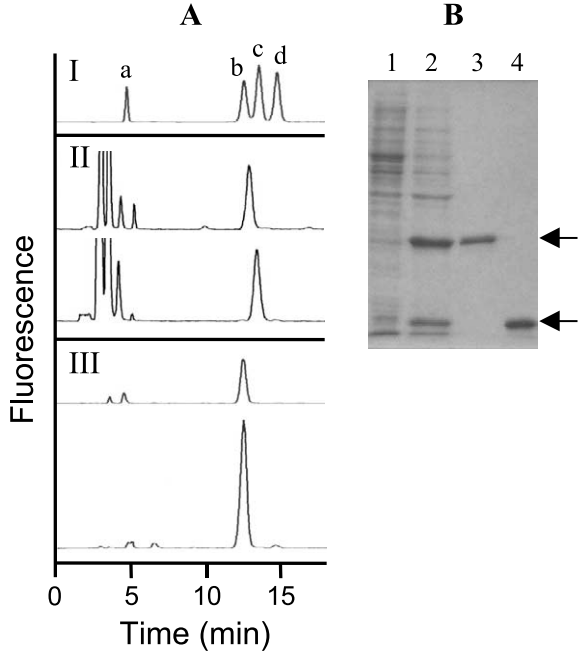

Fig. 2. Identification of pteridines and recombinant proteins. A: HPLC of the enzymatic products of *E. coli* PTPS and the pteridine product of the cGTPCH/ePTPS transformant. AII: The purified recombinant *E. coli* PTPS was assayed using H2-NTP as a substrate. After iodine oxidation, the enzymatic product was pterin in the absence of SR (upper) or biopterin in the presence of SR (lower). AIII: The transformant medium was centrifuged to discard cell precipitate and then injected for analysis before (upper) and after (lower) iodine oxidation. AI: Standard pteridines were neopterin (a), pterin (b), biopterin (c), and 6-hydroxymethylpterin (d). Pteridines were separated on a C18 column isocratically with 10 mM sodium phosphate, pH 6.0, at a flow rate of 1.2 ml/min. Fluorescence was at 350/450 nm (em/ex). B: SDS–PAGE analysis of the recombinant proteins in crude extract of the pET-cGTPCH/ePTPS transformant. Lane 1, BL21; lane 2, transformant; lane 3, recombinant *Synechocystis* GTPCH; lane 4, recombinant *E. coli* PTPS.

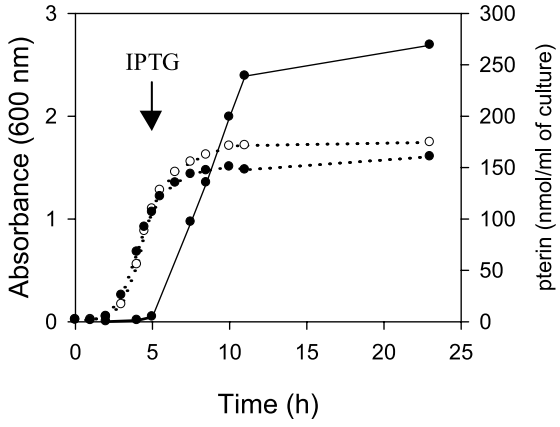

Fig. 3. Quantitative analysis of pterin production by the cGTPCH/ePTPS transformant. Cell growth was measured at 600 nm (dashed lines). Total pterin in the medium was measured by HPLC after iodine oxidation (solid line). The non-transformed BL21 is marked by open circles and the transformant by closed circles. The non-transformed cells did not produce pterin in measurable amounts.

analyzed by HPLC (Fig. 4). Direct injection of the reaction mixtures exhibited a small fluorescent peak eluting at the position corresponding to pterin and increasing with incubation times (Fig. 4A). When the same reaction mixtures were injected after iodine oxidation, the pterin peak increased approx. 16-fold (Fig. 4B). These results suggested that the enzymatic product was the dihydro form of pterin. The pterin peak isolated from the reaction mixture without iodine oxidation was further analyzed by ESI(+) mass spectrometry to show two major spectrum peaks corresponding to the molecular masses of 163.1 and 165.1, which coincide exactly with the respective values of pterin and 7,8-dihydropterin (data not

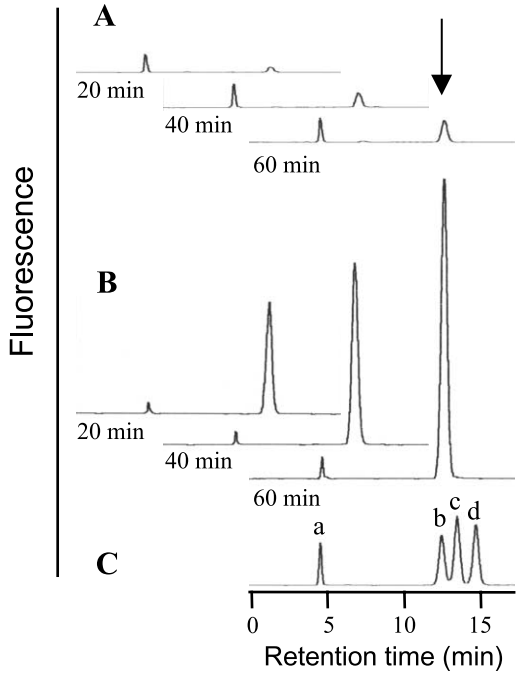

Fig. 4. HPLC identification of the enzymatic product from sepiapterin. Sepiapterin was incubated with *E. coli* PTPS for the indicated times and then subjected to HPLC before (A) and after (B) iodine oxidation. C: A chromatogram of authentic pteridines, which are also shown in Fig. 2. HPLC conditions are described in Fig. 2.

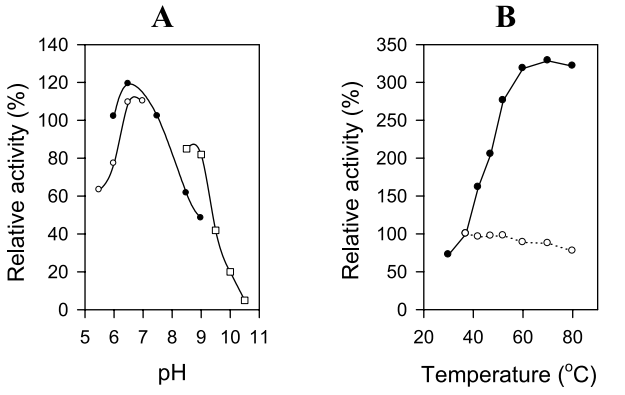

Fig. 5. Effects of pH and temperature on SSCR activity. A: SSCR activity was measured in 0.05 M sodium phosphate (○; pH 5.5–7.0), Tris–HCl (●; pH 6.0–9.0), and glycine–NaOH (□; pH 8.5–10.5). B: Temperature effect on the activity (●) and enzyme stability (○). Reactions were carried out at 37°C in the standard assay conditions at the indicated temperatures. For thermal stability analysis proteins were preincubated for 10 min at the indicated temperatures and then assayed for the remaining activities in the standard conditions.

shown). Therefore, it was concluded that *E. coli* PTPS converts sepiapterin to 7,8-dihydropterin, presumably cleaving the C6 side chain of sepiapterin to yield 7,8-dihydropterin. We tentatively named the activity sepiapterin side chain releasing (SSCR) activity.

3.4. Enzymatic properties for SSCR activity

The optimum activity was found at pH 6.5–7.0 in 50 mM Tris–HCl (Fig. 5A). The activity was inhibited 40–100% by divalent metal ions (10 mM); the order of inhibition was $Mg^{2+} < Mn^{2+} < Ca^{2+} < Ni^{2+} < Zn^{2+} < Co^{2+} < Cu^{2+}$. EDTA stimulated the activity 110–120% at 1–5 mM but inhibited it above 10 mM. The SSCR activity was increased 3.2-fold at 60–80°C under the standard assay conditions (Fig. 5B). The enzyme retained 76% of the activity after 10 min of incubation at 80°C, indicating high thermal stability of the protein (Fig. 5B). The K_m value determined for sepiapterin was 0.92° ± 0.18 mM and the V_{max} was 151.3° ± 15.8 nmol/min/mg (Table 2).

In order to examine whether SSCR activity is unique to bacterial PTPS orthologs, the activity was determined in the purified recombinant PTPSs of *Synechocystis* sp. PCC 6803, *Drosophila*, and human, and compared with their activities for PPH4 synthesis (Table 1). The cyanobacterial enzyme exhibited high activity comparable to *E. coli* enzyme, while *Drosophila* and human enzymes showed barely detectable activities corresponding to 0.34% and 0.23%, respectively, of the *E. coli* enzyme. In contrast, the rates of PPH4 synthesis by bacterial enzymes were less than 10% of the higher animal enzymes.

Table 2
Kinetic parameters of SSCR activity by *E. coli* PTPS

K_m (mM)	V_{max} (nmol/min/mg)	Turnover number (s^{-1})
0.92 ± 0.18	151.3 ± 15.8	0.04

The kinetic parameters were determined in the standard assay conditions using various concentrations of sepiapterin. The mean values were determined from three independent experiments.

4. Discussion

We demonstrated in vitro that the *E. coli* gene (*ygcM*) encodes a PTPS ortholog. Furthermore, the protein was verified to have another new catalytic function to convert sepiapterin to 7,8-dihydropterin (named SSCR activity). To our knowledge, this activity has never been found in any enzymes including PTPS, which has been studied extensively in mammals as an essential enzyme for BH4 synthesis.

Sepiapterin has the chemical structure of 6-L-lactoyl-7,8-dihydropterin. Our result clearly showed that 7,8-dihydropterin is one of the enzymatic products (Fig. 3). It therefore suggests that SSCR activity may be accomplished through cleavage of the C–C bond linking the lactoyl side chain at C6 of 7,8-dihydropterin. Based on the proposed mechanism for PPH4 synthesis [23] and the conserved amino acid residues in *E. coli* PTPS (Fig. 1), we speculate that a nucleophilic attack of residue Cys27 occurs on C1′ carbon of sepiapterin. The Cys27 residue in *E. coli* PTPS corresponds to rat Cys42, which was suggested to abstract protons from the side chain C2′ and C3′ carbons of H2-NTP for PPH4 synthesis. However, the detailed catalysis remains unclear, because we did not succeed in identifying the released side chain. A clear difference between PTPS and SSCR activities by *E. coli* enzyme is that the former required Mg^{2+} and reducing agents (data not shown), while the latter did not. The role of Mg^{2+} and reducing agents was also well established in higher animal enzymes [18,23–25].

The production of BH4 in a coupled assay of *E. coli* PTPS with mouse SR (Fig. 1) provides unequivocal evidence that *E. coli* PTPS catalyzes the synthesis of PPH4. However, it remains a question whether the catalytic activity is exerted in vivo, because the pET-cGTPCH/ePTPS transformant produced 7,8-dihydropterin (Fig. 3). It differs from our previous result that sepiapterin was a dominant product from the transformant coexpressing cyanobacterial GTPCH with human PTPS [16]. It was suggested that the PPH4 synthesized by human PTPS was converted to 6-LPH4 by endogenous aldose reductase(s) in *E. coli* and then oxidized non-enzymatically to sepiapterin [16]. In contrast to human PTPS, the *E. coli* enzyme may not be competitive enough in vivo to prevail over the dephosphorylation of H2-NTP, which is a prerequisite for folate synthesis [26]. In addition, we found that dihydroneopterin is converted to 7,8-dihydropterin by *E. coli* PTPS; the specific activity was determined to be 1.51 nmol/min/mg (data not shown), which corresponds to 1.55% of the value for sepiapterin (Table 1). Therefore, it seems plausible to postulate that H2-NTP might have been dephosphorylated to dihydroneopterin by non-specific phosphatase(s) and then converted to 7,8-dihydropterin by SSCR activity of *E. coli* PTPS. Of course, this suggestion does not rationalize a physiological role of SSCR activity, because none of the enzymatic substrates or products has a known role in *E. coli*. Nevertheless, the above in vivo results may be a clear demonstration that the *E. coli* PTPS ortholog has a different physiological role from the human enzyme, probably signifying SSCR activity rather than PTPS activity in the *E. coli* enzyme.

Synechocystis PTPS [13] was determined to have SSCR activity comparable to the *E. coli* enzyme, while the *Drosophila* and human enzymes showed negligible activities (Table 1). This result suggests strongly that SSCR activity may be more common and significant in bacterial PTPS orthologs, which are found in a majority of prokaryotic genome sequences. The COGs (Clusters of Orthologous Groups of proteins) website (http://www.ncbi.nlm.nih.gov/COG/) currently shows 33 bacterial PTPS orthologs assigned from 44 finished microbial genomes. This ubiquitous presence of putative PTPS orthologs in bacteria, which are devoid of SR and thus do not produce BH4, may further imply a functional role of the enzyme. If the *E. coli* PTPS ortholog does not exert PPH4 synthesis activity in vivo, SSCR activity may provide a clue for understanding the presence of PTPS orthologs in bacteria.

Acknowledgements: This work was supported by Grant R02-2000-00168 from the Korea Science and Engineering Foundation.

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