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Identification of the genes encoding enzymes involved in the early biosynthetic pathway of pteridines in *Synechocystis* sp. PCC 6803

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Abstract

The biosynthetic pathway for the pteridine moiety of cyanopterine, as well as tetrahydrobiopterine, has been investigated in *Synechocystis* sp. PCC 6803. Open reading frames slr0426, slr1626, slr0078 and sll0330 of the organism putatively encoding GTP cyclohydrolase I, dihydroneopterine aldolase, 6-pyruvoyltetrahydropterine synthase and sepiapterine reductase, respectively, have been cloned into T7-based vectors for expression in *Escherichia coli*. The recombinant proteins have been purified to homogeneity and demonstrated to possess expected genuine activities except that of sll0330. Our result is the first direct evidence for the functional assignment of the open reading frames in *Synechocystis* sp. PCC 6803. Furthermore, the 6-pyruvoyltetrahydropterine synthase gene is demonstrated for the first time in prokaryotes. Based on the result, biosynthesis of cyanopterine is discussed.

Keywords: *Synechocystis* sp. PCC 6803; GTP cyclohydrolase I; 6-Pyruvoyltetrahydropterine synthase; Dihydroneopterine aldolase; Heterologous overexpression; Cyanopterine

1. Introduction

Unconjugated pteridines are ubiquitous in nature and function as pigments as well as biochemically important cofactors involving oxidative hydroxylation reactions [1]. However, the biological functions of pteridine glycosides, which have been known for many years [2,3] and found only in prokaryotes [4–9], are still unknown. Recently, we identified a new pteridine glycoside, cyanopterine, in the photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803 [9]. Cyanopterine has a chemical structure of 6-(1-(4-*O*-methyl-α-D-glucuronyl)-(1,6)-(β-D-galactosyloxy)methylpterine. It exists in the tetrahydro form in vivo and is produced constitutively in large amounts, comparable to chlorophyll a, suggesting that it has some essential function in *Synechocystis* [9].

Cyanopterine contains 6-hydroxymethylpterine as the pterine moiety, whereas most other pteridine glycosides have the structures of biopterine glycosides [3,5–8] probably derived from tetrahydrobiopterine

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(BH4). Sugar transferases are responsible for the transfer of sugars to the pteridines, as demonstrated recently in the green sulfur bacterium *Chlorobium limicola* [10]. The biosynthesis of the dihydro form of 6-hydroxymethylpterine and BH4 are well-established in various organisms. In the first step of their biosynthesis, they share a common enzyme, GTP cyclohydrolase I (GTPCH: EC 3.5.4.16). The enzyme catalyzes GTP to dihydroneopterine triphosphate (H_2-NTP), which is then bifurcated to 6-hydroxymethyldihydropterine and BH4. Dephosphorylated by unknown phosphatase(s) and subsequently cleaved by dihydroneopterine aldolase (DA: EC 4.1.2.25), H_2-NTP is converted to 6-hydroxymethyldihydropterine. On the other hand, two consecutive actions of 6-pyruvoyltetrahydropterine synthase (PTPS: EC 4.6.1.10) and sepiapterine reductase (SR: EC 1.1.1.153) complete the synthesis of BH4.

As a first step to study the biosynthesis and function of cyanopterine, we investigated genes of *Synechocystis* putatively encoding the enzymes described above, GTPCH, DA, PTPS and SR. The determined genome sequence of the organism [11] permitted us to find high homologs. In this study, we report the identification of them by obtaining purified recombinant proteins after heterologous expression of the putative genes in *Escherichia coli*.

2. Materials and methods

2.1. Culture conditions

Synechocystis sp. PCC 6803 cells were grown photoautotrophically at 30°C in medium C under continuous white light (100–120 μmol m^{-2} s^{-1}) while bubbling with air. Cultures in the exponential growth phase were used for experiments. *E. coli* XL-1 blue and BL21/pLysS were cultivated at 37°C in LB medium

2.2. Preparation of protein extract from Synechocystis and gel permeation chromatography

Harvested cells were suspended in 10 mM Pipes (pH 7.5), 1 mM PMSF (4 ml g^{-1} wet weight) and were disrupted by sonication. The homogenate was centrifuged for 30 min at 15 000×*g* to discard the precipitate. Saturated ammonium sulfate solution was added to the supernatant to a final concentration of 70%. The centrifuged precipitate was dissolved in 10 mM Pipes (pH 7.5) and desalted for an assay of enzyme activities through Sephadex G-25 pre-equilibrated with the same buffer.

Gel permeation chromatography was performed in a column (2.5×42 cm) of Sephadex G-100. Ammonium sulfate precipitate prepared from 10 g of frozen cells was applied on the column pre-equilibrated with 20 mM Tris-HCl, pH 7.5. The column was chromatographed with the same buffer at a flow rate of 20 ml h^{-1}. Fractions of 2 ml were collected and assayed for protein and enzyme activities.

2.3. Assay of enzyme activities

Enzyme activities were assayed according to the methods as described by Maier and Ninnemann [12] with minor modifications. The GTPCH assay was performed in 100 mM Tris-HCl (pH 7.5), 5 mM EDTA, 50 mM KCl, 2 mM DTT, 0.2 mM GTP and enzyme solution in a total volume of 50 μl for 1 h at 37°C. The reaction mixture was oxidized by addition of 30 μl of acidic iodine solution (2% KI/1% I_2 in 1 N HCl) for 10 min in the dark. After centrifugation, the supernatant was partially neutralized with 15 μl of 1 N NaOH and the excess iodine was reduced by 10 μl 5% ascorbic acid. The amount of produced neopterine phosphates was quantified by HPLC analysis. Neopterine cleaved from the neopterine phosphates by alkaline phosphatase was compared for the quantitation [13]. The PTPS assay was performed in 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 8 μM H_2-NTP, 2 mM DTT, containing the enzyme solution in a total volume of 50 μl for 1 h at 37°C. The reaction mixture was oxidized with 100 μl of acidic iodine solution for 1 h in the dark. After centrifugation, the excess iodine in the supernatant was reduced by 50 μl 5% ascorbic acid for HPLC. The assay for DA was the same as for PTPS, except that 20 μM of dihydroneopterine was used instead of H_2-NTP. The SR assay was performed in 100 mM Tris-HCl (pH 7.5), 0.1 mM NADPH, 50 mM sepiapterine and enzyme solution in a total volume of 50 μl for 1 h at 37°C. The mixture was oxidized by 30 μl of acidic iodine sol-

ution for 10 min in the dark and analyzed for biopterine by HPLC after centrifugation and reduction by 10 μl 5% ascorbic acid. Phosphatase activity was assayed with 8 μM H_2-NTP as a substrate following the method for DA.

The products were separated by HPLC and the amounts were calculated by comparison with external pteridine standards (Dr B. Schircks Lab, Switzerland). H_2-NTP was prepared fresh from GTP by the recombinant *Synechocystis* GTPCH purified in this study. The reaction mixture containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM EDTA, 2 mM dithiothreitol, 3 mM GTP and enzyme solution was incubated overnight at 42°C and used directly for the enzyme assay in aliquots according to the concentration of the product. HPLC was performed on a Kontron Model 430 equipped with a Rheodyne loop of 20 μl, an Inertsil ODS-3 C_{18} column (5 μm, 150×2.3 mm, GL Sci., Japan) and a HP Model 1046A fluorescence detector (350 nm excitation, 450 nm emission). Aliquots of reaction mixtures were injected into the column in 10 mM Na_2HPO_4, pH 6.0, at a flow rate of 1.2 ml min^{-1}.

2.4. SDS-PAGE and protein determination

SDS-PAGE was performed in a mini-gel (Bio-Rad) according to the method of Laemmli [14]. Protein concentrations were measured by Bradford reagent [15] using bovine serum albumin as a standard.

2.5. Construction of overexpression vectors

Overexpression of the putative open reading frames (ORFs) was accomplished using the T7 RNA polymerase expression system. The ORFs were amplified by PCR, based on the available *Synechocystis* genomic sequence [11]. The forward primers were designed to contain *Nde*I restriction sites (underlined) at the translation start sites. The reverse primers were designed to contain *Bam*H1 restriction sites (underlined) downstream from the end of each ORF. Primer sequences were slr0426 (CATATGAACACCGGTAAATTA and GGATCCTCGATGGGTTTTAGATAA), sll0330 (CATATGAATTTACTCAACAAAA and GGATCCTAGGTAGACAATCCTAT), slr0078 (CATATGTGGATTATTTATAAGG and GGATCCAATTTGTTCGGTTAAATC) and slr1626 (CATATGGCAATTTTTGACG and GGATCCCATGGTTAACACCTT). PCR was performed with total DNA of *Synechocystis* PCC 6803 as described elsewhere and the products of expected sizes were cloned into the T-vector (pCR2.1-TOPO, Invitrogen). The genes were then subsequently subcloned as *Nde*I-*Bam*HI restriction fragments into the T7 polymerase expression vector pET-15b (Novagen). Cloning of the genes in correct reading frames was confirmed by DNA sequencing.

2.6. Purification of recombinant proteins

E. coli strain BL21(DE3)/pLysS was transformed with the pET-15b plasmids harboring PCR products. Overexpression was induced by the addition of IPTG to a final concentration of 0.4 mM. The cells were allowed to grow for more than 3 h and harvested by centrifugation. The cells were washed by suspension in 50 mM Tris-HCl (pH 8.0), suspended in the same buffer and disrupted by sonication. The overexpressed His-tag fusion protein in the crude extract was purified by chromatography on a column of Ni-NTA gel (QIAGEN). The overexpressed protein of slr1626, however, was obtained from the insoluble precipitate. After cell disruption, most of the expressed protein was discovered in the precipitate. The precipitate after cell disruption was dissolved in 50 mM Tris-HCl (pH 8.0), sonicated briefly and centrifuged to discard supernatant. The washing steps were repeated twice. The final precipitate was dissolved in 0.1 M acetic acid plus 1% Triton X-100, sonicated and centrifuged to save the supernatant. The supernatant dialyzed against 10 mM Tris-HCl (pH 8.0) contained 0.072 mg ml^{-1} of pure active enzyme and was used for the assay in this report.

3. Results and discussion

3.1. Heterologous expression of the putative genes in E. coli

The most homologous ORFs were searched in the translated genome sequence of *Synechocystis* for the enzymes involved in the biosynthesis of unconjugated pteridines. The homologs were slr0426, sll0330, slr0078 and slr1626, putatively encoding

GTPCH, SR, PTPS and DA, respectively. The slr0426 was already annotated as the gene for GTPCH (*folE*) in the Cyanobase. The deduced sequence of 234 residues of slr0426 was 63.3% identical with rat GTPCH. The sll0330 product of 259 residues was annotated as the 3-ketoacyl-acyl carrier protein reductase in the Cyanobase but still shared 34% identity with rat SR. On the other hand, the gene products of slr0078 and slr1626 were unspecified. However, the slr0078 product of 129 residues showed 38% sequence similarity with human PTPS and the slr1626 product of 150 residues displayed 36.7% similarity with *E. coli* DA.

In order to verify their genuine enzyme activities, the ORFs were amplified from the genomic DNA by PCR and cloned in pET-15b expression vectors. The expression vector of slr0426 was constructed to start from an ATG that is 12 amino acid residues below the annotated translation start codon GTG. The recombinant proteins were obtained to apparent homogeneities as shown in Fig. 1. The molecular masses of the subunits are in good accordance with those deduced from the amino acid compositions including His-tag region, which are 27 522 (slr0426), 30 448 (sll0330), 16 633 (slr0078) and 18 642 (slr1626), respectively.

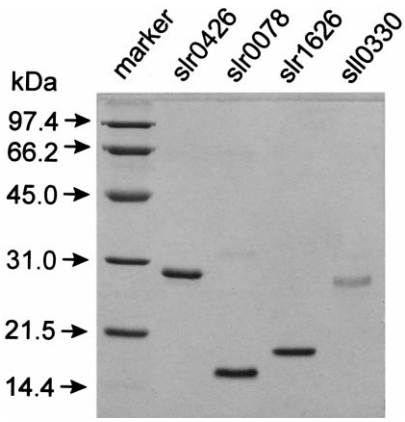

Fig. 1. SDS-PAGE of the purified proteins from *E. coli* strains containing the *Synechocystis* genes. Purified His-tag recombinant proteins were analyzed on a 12.5% SDS-polyacrylamide gel. The molecular masses of the protein markers (Bio-Rad low range) used are depicted to the left.

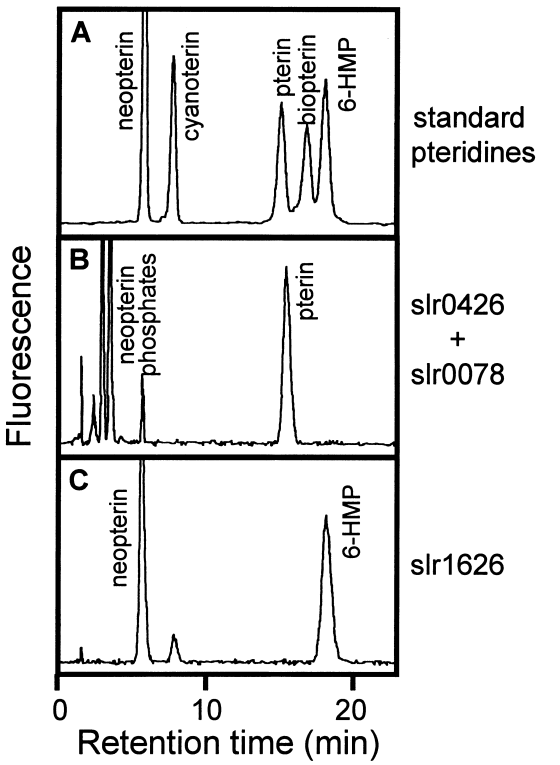

Fig. 2. HPLC identification of enzymatic products formed by the purified recombinant proteins. A, standard pteridines; B, reaction products of GTP by combined activities of slr0426 and slr0078 gene products; C, reaction product of dihydroneopterine by recombinant protein of slr1626. The reaction products were oxidized by iodine and separated by chromatography on a C_{18} column in 10 mM Na_2HPO_4, pH 6.0, at a flow rate of 1.2 ml min^{-1}.

3.2. Enzyme activities of the recombinant proteins

The purified recombinant proteins and cell-free extracts of *E. coli* and *Synechocystis* as well were assayed for the expected enzyme activities (Table 1). Except SR, enzyme activities of GTPCH, PTPS and DA were exhibited well in the recombinant proteins of putative ORFs, slr0426, slr0078 and slr1626, respectively. Although 6-hydroxymethylpterine was observed, instead of pterine, in the PTPS assay of the cell-free extracts from both non-transformed *E. coli* and *Synechocystis*, it could be explained by the presence of a high phosphatase activity (discussed later in more detail). The enzymatic products of the recombinant proteins were identified by HPLC

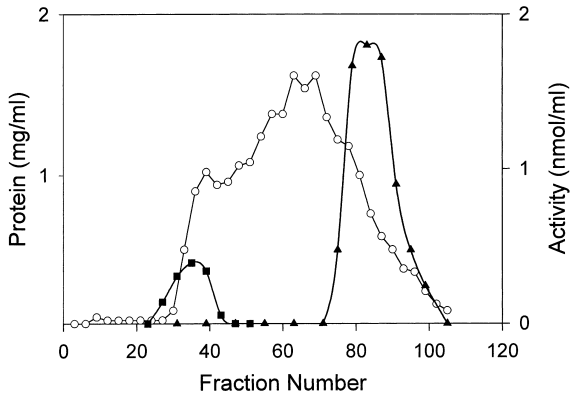

Fig. 3. Enzyme activities of *Synechocystis* PTPS and phosphatase separated on a column of Sephadex G-100. Ammonium sulfate precipitate of *Synechocystis* prepared from 10 g of frozen cells was chromatographed on a column (2.5×42 cm) of Sephadex G-100. Fractions of 2 ml were assayed for proteins (○) and enzyme activities (PTPS, ■; phosphatase, ▲). Enzymatic activities were expressed as nmol product per 6 h (PTPS) or 1 h (phosphatase), respectively, formed in 1 ml of reaction mixture under assay conditions as described in Section 2.

as shown in Fig. 2. Using GTP as the sole substrate in the mixed assay of the recombinant proteins of slr0426 and slr0078, their combined activities were expected to generate 6-pyruvoyltetrahydropterine as well as H_2-NTP. GTPCH activity of the slr0426 product would have produced H_2-NTP, which was then subsequently converted to 6-pyruvoyltetrahydropterine by PTPS activity of the slr0078 product. Upon acidic iodine oxidation, the remaining H_2-NTP was oxidized to neopterine phosphates and the 6-pyruvoyltetrahydropterine was oxidized and cleaved to pterine [16], as shown in Fig. 2B. In another mixed assay supplemented with recombinant mouse SR, biopterine was observed (data not shown), strongly supporting that *Synechocystis* PTPS is catalytically indistinguishable from mammalian enzymes. The DA activity of slr1626 was demonstrated with dihydroneopterine to produce 6-hydroxymethyldihydropterine, which was then oxidized by iodine (Fig. 2C). The small peak eluting after neopterine is presumed to be monapterine, because DA is known to catalyze epimerization of dihydroneopterine to dihydromonapterine [17]. These results clearly indicate that GTPCH, PTPS and DA in *Synechocystis* sp. PCC 6803 are encoded by slr0426, slr0078 and slr1626, respectively. On the other hand, SR activity was totally absent in the recombinant protein of sll0330 with either sepiapterine or in vivo substrate, 6-pyruvoyltetrahydropterine, which was provided by a mixed assay with PTPS. SR activity was also not detected in the cell-free extract of *Synechocystis*, highly indicating that SR does not exist in *Synechocystis* sp. PCC 6803. This result may explain why cyanopterine contains a structure of 6-hydroxymethylpterine, in contrast to more ubiquitous biopterine glycosides that should depend on SR activity.

3.3. Identification of PTPS expression in *Synechocystis*

The production of 6-hydroxymethylpterine observed in the assay of PTPS with cell-free extract of *Synechocystis* was presumed to mean either that PTPS is not expressed at all or, even expressed, it may not compete with phosphatase(s) in using the

Table 1
Enzyme activities of the heterologously overexpressed genes

Assay for enzyme activity	Crude extract of *E. coli*		Purified recombinant protein	Ammonium sulfate fraction of *Synechocystis* PCC 6803
	Control	Overexpressed (cloned ORFs)		
GTPCH	6.2	307.9 (slr0426)	19814.0	2.42
PTPS	(6.2)[a]	5952.8 (slr0078)	35123.8	(3.93)[a]
SR	n.d.[b]	n.d. (sll0330)	n.d.	n.d.
DA	34.8	1097.2 (slr1626)	103922.5	5.91

Enzyme activities were assayed and are expressed as pmol min^{-1} product formed per mg protein under assay conditions as described in Section 2. The BL21/pLysS cell harboring no plasmid was used as a control.

[a]The reaction product was 6-hydroxymethylpterine.

[b]Not detected in measurable amounts.

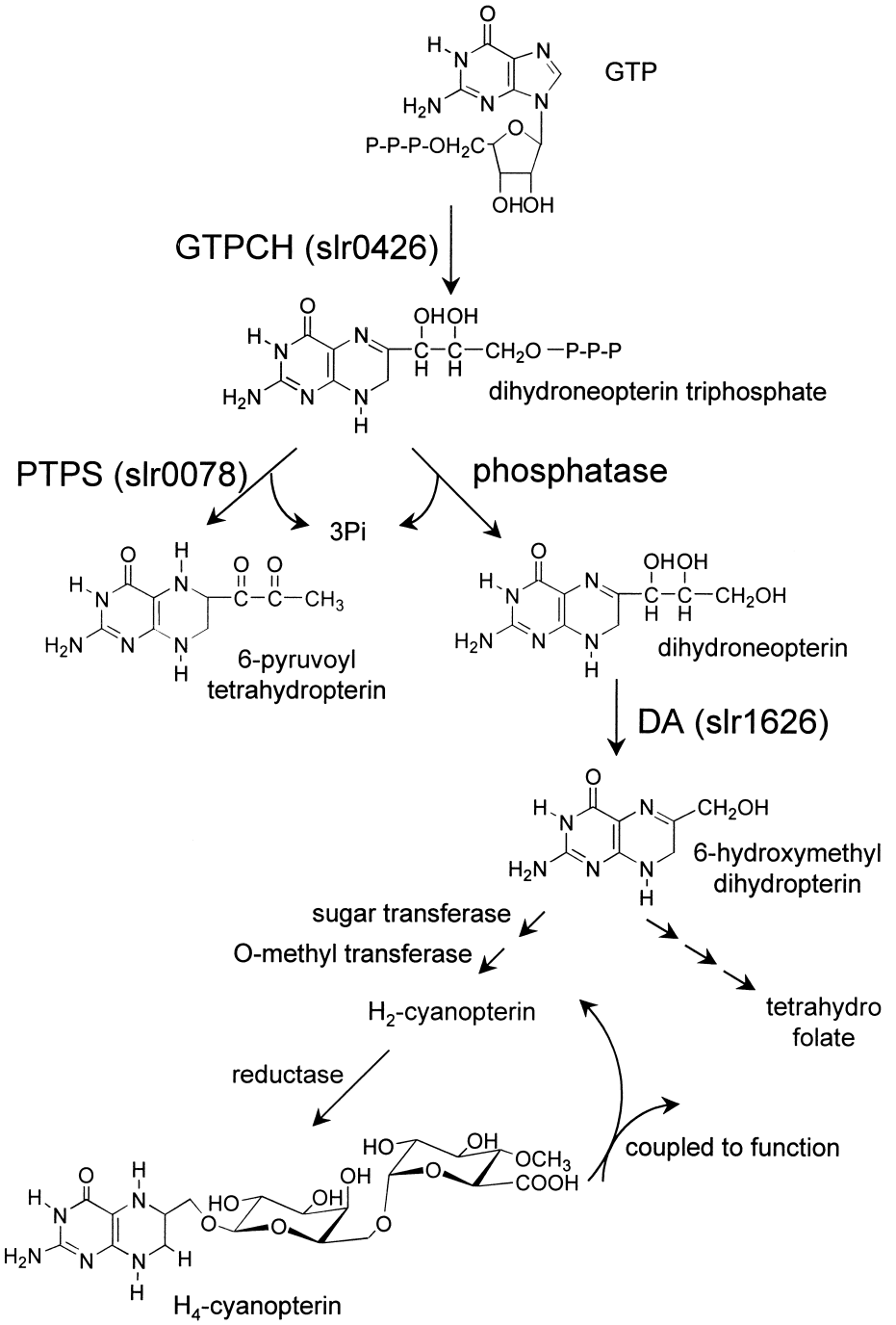

Fig. 4. Proposed biosynthesis of H_4-cyanopterine in *Synechocystis* sp. PCC 6803. The exact order of the putative enzymes in the steps from 6-hydroxymethyldihydropterine to H_4-cyanopterine has not been established yet.

identical substrate, H_2-NTP. When the purified PTPS was mixed with increasing amounts of the ammonium sulfate fraction of *Synechocystis*, the amount of pterine became decreased with a concomitant increase of 6-hydroxymethylpterine (data not shown), indicating the presence of a high phosphatase activity to H_2-NTP. In order to separate PTPS from a possible phosphatase(s) in *Synechocystis* cell-free extract, ammonium sulfate precipitate was chromatographed on Sephadex G-100 (Fig. 3). The PTPS was eluted as a well-isolated peak from that of phosphatase. The activity peak of PTPS eluting at the void volume supports the polymeric state of homohexamer as observed in animal enzymes. Therefore, it is certain that PTPS is expressed in *Synechocystis*, yet it remains another question why PTPS is in the absence of SR. In addition, an active peak of phosphatase on H_2-NTP emerging on the trailing side of the protein peak is quite interesting, because there has been no report on the involvement of a specific phosphatase in the step except a pyrophosphatase that was known in *E. coli* to produce dihydroneopterine monophosphate [18]. A preliminary experiment (data not shown) showed that the fractions of the active peak were not active on *p*-nitrophenylphosphate. The presence of a specific phosphatase on H_2-NTP, which should be defined in the future, may assist the high cellular production of cyanopterine in *Synechocystis*.

3.4. Proposed biosynthetic pathway of H_4-cyanopterine

Our results are summarized in the proposed pathway for H_4-cyanopterine (Fig. 4). The biosynthesis of 6-hydroxymethyldihydropterine and 6-pyruvoyltetrahydropterine has been well-established in other organisms. However, our result presents the first direct evidence for the functional assignment of the ORFs slr0426, slr1626 and slr0078 in *Synechocystis* sp. PCC 6803. Furthermore, the PTPS gene is identified for the first time in prokaryotes. The branched pathway from the 6-hydroxymethyldihydropterine to the functional form of cyanopterine is supposed to be catalyzed by at least three enzymes, sugar transferase, *O*-methyltransferase and reductase. The putative cyanopterine reductase seems to be involved in the final step, because it could constitute the simple regeneration step. Considering the functional mechanism and regeneration of the tetrahydro form of compounds, such as tetrahydrofolate and BH4, there may occur a turnover of H_4-cyanopterine to H_2-cyanopterine or vice versa.

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