Sepiapterin reductases from *Chlorobium* tepidum and *Chlorobium* limicola catalyze the synthesis of L-threo-tetrahydrobiopterin from 6-pyruvoyltetrahydropterin

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**Abstract**

The ORF sequences of the gene encoding sepiapterin reductase were cloned from the genomic DNAs of *Chlorobium* tepidum and *Chlorobium* limicola, which are known to produce L-threo- and L-erythro-tetrahydrobiopterin (BH4)-N-acetylglucosamine, respectively. The deduced amino acid sequence of *C. limicola* consists of 241 residues, while *C. tepidum* SR has three residues more at the C-terminal. The overall protein sequence identity was 87.7%. Both recombinant proteins generated from *Escherichia coli* were identified to catalyze reduction of diketo compound 6-pyruvoyltetrahydropterin to L-threo-BH4. This result suggests that *C. limicola* needs an additional enzyme for L-erythro-BH4 synthesis to yield its glycoside. The catalytic activity of *Chlorobium* SRs also supports the previously proposed mechanism of two consecutive reductions of C1’ carbonyl group of 6-pyruvoyltetrahydropterin via isomerization reaction.

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1. **Introduction**

Sepiapterin reductase (SR; EC 1.1.1.153) catalyzes the last step of the de novo synthesis of tetrahydrobiopterin (BH4), which is the well-known cofactor for aromatic amino acid hydroxylation and nitric oxide synthesis in higher animals [1]. BH4 is also ubiquitous as glycosidic forms in particular groups of bacteria, such as cyanobacteria [2,3] and anaerobic photosynthetic bacteria *Chlorobium* species [4,5]. Although the function remained unclear [6,7], there were suggestions that pteridine glycosides in cyanobacteria provide protection from UV-light [8], perhaps by photostabilization of photosynthetic pigments [9].

The BH4 synthesis starts from GTP by GTP cyclohydrolase I (GTPCH; EC 3.5.4.16). The product dihydronopterin triphosphate (H2–NTP) is converted to the 6-pyruvoyltetrahydropterin (PPH4) by 6-pyruvoyltetrahydropterin synthase (PTPS; EC 4.2.3.12). SR reduces the diketo group in C6-side chain of PPH4 [10]. The biochemical data and crystallographic analysis proposed that SR carries out two consecutive steps of reduction at the C1’ carbonyl function separated by an isomerization reaction to produce L-erythro-BH4 [11–13]. Although L-erythro-BH4 (6R-(1’R,2’S)-5,6,7,8-BH4) is common in nature, other stereoisomers (D-threo-, 6R-(1’R,2’R); L-threo-, 6R-(1’S,2’S)-) were found in nature. D-Threo form (dictyopterin) was...
identified in *Dictyostelium discoideum* [14]. A glycosylated L-threo-stereoisomer was isolated from *Chlorobium tepidum* (tepidopterin; L-threo-BH4-N-acetylgulcosamine) [5], while another species *C. limicola* was found to produce L-erythro-BH4-N-acetylgulcosamine (limipterin) [4]. *Dictyostelium* SR was identified to catalyze the synthesis of L-erythro-BH4, remaining D-threo-BH4 synthesis unsolved [15]. On the other hand, the purified SR from *C. tepidum* was demonstrated in vitro to generate L-threo-dihydrobipterin from sepiapterin, an in vitro substrate of SR having a C1 carbonyl group [16]. However, the activity with in vivo substrate PPH4 was not demonstrated and, furthermore, partially purified SR from *C. limicola* was shown to involve in the synthesis of L-erythro-BH4 from PPH4 [6]. These ambiguous results provoked us two questions of whether *C. tepidum* and *C. limicola* SRs, which were presumed to share a high protein similarity, would really produce different isomers of BH4 from PPH4 and, if it were true, how they would catalyze the synthesis. In order to answer these questions, by taking advantage of the completed genome sequence of *C. tepidum* [17], we cloned SR genes from *C. tepidum* and *C. limicola* by PCR amplification. The recombinant proteins heterologously overexpressed in *Escherichia coli* were purified and characterized for the enzymatic products.

2. Materials and methods

2.1. Genomic DNAs

The genomic DNAs of *C. limicola* f. thiosulfatophilum NCIB 8327 and *C. tepidum* were provided by Prof. Yim and Prof. Kang (School of Biological Science, Seoul National University, Seoul, Korea), respectively.

2.2. Molecular gene cloning

The ORF sequence of *C. tepidum* SR gene (CT 0809), which was available from the completed genome sequence [17], was amplified by using a PCR primer pair (forward: *CATATGAAACATATACTCTCTG*, reverse; *GGATCCCTACAGATCTCCAGCGGT*) designed from the determined sequence of *C. limicola* SR gene. The amplified ORF DNA, tailed with 5′-NdeI sequence and 3′-BamHI restriction sites, was cloned into the pGEM-T vector (Promega) and then subsequently subcloned as a NdeI-BamHI restriction fragment into the pET-28a expression vector (Novagen) to generate His-tagged versions of the proteins. PCR amplifications was performed with *Pfu* polymerase (Promega) in 1× reaction buffer, 200 μM dNTPs, 0.2 μM each of primer pairs, and templates, under the following conditions: 4 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, 50–60 °C depending on the primer pairs for 1 min, and 72 °C for 1 min, and a final DNA polymerization at 72 °C for 7 min.

2.3. Overexpression and purification of recombinant proteins

The PCR amplified ORF sequences were cloned into the pGEM-T vector and sequenced. *E. coli* strain BL21(DE3)/pLysS was transformed with the pET-28b plasmid harboring the PCR products. The transformed cells were induced to overexpress the cloned gene with 0.5 mM IPTG and continued to grow overnight. The harvested cells were washed by lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole), resuspended in the same buffer, and disrupted by sonication. The crude extract obtained after centrifugation was applied to a column of Ni–NTA gel (QIA-GEN) and purified according to the product manual. The recombinant SR protein was eluted with 250 mM imidazole in the lysis buffer. The purified protein was dialyzed against 10 mM PIPES (pH 7.5) and assayed. SDS–PAGE was performed with a 12.5% polyacrylamide gel under reducing condition.

2.4. Activity assays

Sepiapterin reductase activity with sepiapterin was assayed in 50 μl reaction volume of 100 mM sodium phosphate, pH 5.5, 0.2 mM NADPH, 0.1 mM sepiapterin, and enzyme solution for 30 min at 37 °C. The
assay with in vivo substrate PPH4 was performed in a coupled assay with PTPS as described previously [19]. The reaction mixture consisted of 50 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 10 mM dithiothreitol, 0.2 mM NADPH, aliquots of H2–NTP mixture, 10 μg of purified human recombinant PTPS [20], and 2 μg of SR. The reaction was carried out for 60 min at 42 °C. The reaction mixture was oxidized by addition of an equal volume of acidic iodine solution (2% KI/1% I2 in 1 N HCl) for 1 h in the dark. After centrifugation, the supernatant was mixed with ascorbic acid and subjected to HPLC [19]. H2–NTP was prepared from GTP by incubation with recombinant Synechocystis GTPCH purified previously [21]. Pteridine compounds were purchased from Dr. B. Schircks Lab (Jona, Switzerland). L-Threo-biopterin was a kind donation of Prof. Kang in Seoul National University. HPLC was performed with a Kontron Model 430 system. Samples were applied on an Inertsil ODS-3 C18 column (5 μm, 150 × 2.3 mm, GL Sci., Japan) equilibrated with 10 mM potassium phosphate buffer (pH 6.0), eluted isocratically at a flow rate of 1.2 ml/min, and monitored at 350/450 nm (excitation/emission) by using a fluorescence detector (HP Model 1046A). Chiral chromatography was performed in a mobile phase of 4 mM cupric sulfate and 8 mM d-phenylalanine [22].

3. Results and discussion

3.1. Molecular cloning and sequence comparison of chlorobium SRs

The annotated C. tepidum SR gene (CT 0609) encodes a protein of 244 amino acid residues [17], which contains N-terminal sequence coinciding with the previously determined from the purified enzyme [16]. The cloned C. limicola SR gene contained a complete ORF sequence of 726 nucleotides (GenBank accession number AY742215), which showed 69.7% identity with that of C. tepidum SR gene. The deduced protein of C. limicola SR gene consisted of 241 amino acid residues sharing 87.7% identity with C. tepidum enzyme (Fig. 1), suggesting that both enzymes would catalyze the synthesis of same isomeric products. Alignment of both SR protein sequences with mouse SR revealed well-conserved residues for substrate and cofactor binding as well as for catalysis (Fig. 1). Particularly, the catalytic triads (Ser158, Tyr171, and Lys175, according to the numbering in mouse SR) are conserved. However, both Chlorobium SRs are shorter than mammalian proteins in their C-terminal regions and are missing Asp258, which was identified to be important for anchoring the pterin moiety of sepiapterin in mouse SR [13], probably implying a different or more flexible substrate binding in Chlorobium SRs.

3.2. Enzyme activities of chlorobium SRs

To confirm the enzymatic activities in vitro the ORF sequences amplified by PCR from the genomic DNA were cloned in a T7-based vector pET-28b in correct reading frames and then heterologously overexpressed in E. coli. The soluble recombinant proteins harboring N-terminal His-tag sequence were purified to homogeneity by Ni–NTA column (data not shown). The purified recombinant proteins were assayed with in vivo substrate PPH4 as well as in vitro substrate sepiapterin. The iodine oxidized enzymatic products were identified by HPLC (Fig. 2; Only the results with C. limicola SR are shown, because both SR products generated absolutely identical chromatograms). The enzymatic product of sepiapterin was eluted as a single peak corresponding to threo-biopterin position (Fig. 2(A1)). Although the stereospecificity

Fig. 1. Alignment of the amino acid sequences of Chlorobium and mouse SR proteins. The displayed sequences were aligned using ClustalW. The central residues for substrate binding and catalysis are indicated by an arrow. Residues involved in cofactor binding are marked with a plus (+) sign.
of the product was not proved in our experiment, the unique synthesis of L-threo-dihydrobiopterin was already demonstrated in *C. tepidum* SR [16]. Therefore it was likely that *C. limicola* SR performs the same stereospecific catalysis like *C. tepidum* SR to generate L-threo-dihydrobiopterin from sepiapterin. Considering this stereospecific reduction of 1' carbonyl group of sepiapterin, the *Chlorobium* SRs were presumed to synthesize L-threo- or D-erythro-BH4 but not D-threo- or L-erythro-BH4 from the *in vivo* substrate PPH4. As there had been no report of D-erythro-BH4 production in *C. limicola* or *C. tepidum*, it was theoretically convincing to expect that *Chlorobium* SRs may produce L-threo-BH4 from PPH4. In order to confirm it experimentally the recombinant SR proteins were assayed together with PTPS using H2–NTP as a substrate. HPLC analysis of the reaction mixtures showed a peak of threo-type biopterin (Fig. 2(AII)) together with a pterin peak, which is the oxidized derivative of the remaining substrate PPH4, but no peak corresponding to erythro-type biopterin. The stereospecificity was finally verified by chiral chromatography [22] of the same enzymatic product (Fig. 2(BII)), which exhibited a peak corresponding to L-threo-biopterin (The peak was further confirmed by spiking injection with the standard; data not shown). It therefore concludes that both *C. tepidum* and *C. limicola* SRs, which share high structural similarity, catalyze the synthesis of L-threo-BH4 from the *in vivo* substrate PPH4. This result, on the other hand, strongly suggests that there may be another enzyme in *C. limicola* to catalyze the conversion of L-threo-BH4 to L-erythro form to yield its glycoside, L-erythro-BH4-N-acetylglucosamine. It is also interesting to speculate on the catalytic mechanism of the bacterial SRs, because they catalyze the synthesis of threo-stereoisomers. Although another stereoisomer D-threo-BH4 is known in *Dictyostelium*, its SR was identified to catalyze the synthesis of L-erythro-BH4 [15]. In order to produce the stereoisomer, it seems inevitable to follow the catalytic mechanism of two consecutive reductions of C1' carbonyl group via isomerization reaction [13]. Another possibility of directly reducing both 1' and 2' carbonyl groups in opposite stereospecificities looks improbable, because, if it were to happen, NADPH may be positioned at the opposite side of the substrate for the second hydride transfer to the C2' carbonyl group. In order to prove this speculation the crystal structure of *C. tepidum* SR is currently being examined.

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**References**


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