Sepiapterin reductase producing L-threo-dihydrobiopterin from *Chlorobium tepidum*

Seung-Hyun CHO, Jong-Uk NA, Hwan YOUN, Cheol-Sang HWANG, Chang-Hun LEE and Sa-Ouk KANG¹

Laboratory of Biophysics, Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Republic of Korea

A novel type of NADPH-dependent sepiapterin reductase, which catalysed uniquely the reduction of sepiapterin to L-threo-dihydrobiopterin, was purified 533-fold from the cytosolic fraction of *Chlorobium tepidum*, with an overall yield of 3%. The native enzyme had a molecular mass of 55 kDa and SDS/PAGE revealed that the enzyme consists of two subunits with a molecular mass of 26 kDa. The enzyme was optimally active at pH 8.8 and 50 °C. Apparent K_m values for sepiapterin and NADPH were 21 and 6.2 μ M, respectively, and the k_{cat} value was 5.0 s⁻¹. Diacetyl could also serve as a substrate, with a K_m of 4.0 mM. The

inhibitory effects of *N*-acetylserotonin, *N*-acetyldopamine and melatonin were very weak. The K_i value of *N*-acetyldopamine was measured as 400 μ M. The N-terminal amino acid sequence was revealed as Met-Lys-His-Ile-Leu-Leu-Ile-Thr-Gly-Ala-Xaa-Lys-Lys-Ile-Xaa-Arg-Ala-Ile-Ala-Leu-Glu-Xaa-Ala-Arg-Xaa-Xaa-Xaa-His-His-His-, which shared relatively high sequence similarity with other sepiapterin reductases.

Key words: L-threo-biopterin, tepidopterin, tetrahydrobiopterin.

INTRODUCTION

L-erythro-Tetrahydrobiopterin is the coenzyme for the aromatic amino acid hydroxylases, such as phenylalanine hydroxylase, tryptophan hydroxylase and tyrosine hydroxylase in mammals [1]. The *de novo* biosynthesis of L-erythro-tetrahydrobiopterin from GTP proceeds through three main enzymic steps, GTP cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase in the cells and tissues of mammals [1–4]. The involvement of 6-pyruvoyl tetrahydropterin reductase, aldose reductase or carbonyl reductase in the biosynthetic pathway remains controversial [5,6]. Sepiapterin reductase catalyses the two-step reduction of 6-pyruvoyl tetrahydropterin via 1'-hydroxy-2'-oxopropyl tetrahydropterin, and also the one-step reduction of 6-lactoyl tetrahydropterin, catalysed by 6-pyruvoyl tetrahydropterin reductase, to L-erythro-tetrahydrobiopterin in some tissues [4,5]. Moreover, in salvage pathway, sepiapterin reductase catalyses the reduction of sepiapterin to L-erythro-dihydrobiopterin, which is further reduced to L-erythro-tetrahydrobiopterin by dihydrofolate reductase [1].

In *Drosophila melanogaster*, the three enzymes were purified and characterized, and the biosynthetic pathway was found to be similar to that of the mammalian system, although the necessity of the involvement of 6-pyruvoyl tetrahydropterin reductase was still proposed [7–9]. There was a report that the biosynthesis of L-erythro-tetrahydrobiopterin from 6-pyruvoyl tetrahydropterin was mediated by a new tetrahydrobiopterin-synthesizing enzyme in the *lemon* mutant of the silkworm *Bombyx mori*, although it was catalysed by sepiapterin reductase in normal silkworm [10].

In some micro-organisms, even though L-erythro-biopterin was detected, the biosynthesis of its tetrahydro form was not well elucidated [11,12]. The three enzymes involved in the *de novo* biosynthesis of L-erythro-tetrahydrobiopterin were detected and characterized in *Euglena gracilis*, *Phycomyces blakesleeanus* and *Neurospora crassa* [12].

From 1960 to the present, there have been many reports that L-erythro-biopterin analogues are found in many micro-

organisms and in human urine. Many glycosides containing L-erythro-biopterin are found in cyanobacteria [11,13] and the green sulphur bacterium, *Chlorobium limicola* f. *thiosulphatophilum* NCIB 8327 [14]. D-threo-Biopterin (dictyopterin) has been reported in *Dictyostelium discoideum* [15], L-threo-biopterin in human urine [16], and glycosides containing L-threo-biopterin in the cyanobacterium *Aphanizomenon flos-aquae* [17]. In the biosynthesis of glycosides containing L-erythro-tetrahydrobiopterin, the three main enzymes of the biosynthesis of L-erythro-tetrahydrobiopterin and the enzyme transferring *N*-acetylglucosamine to L-erythro-tetrahydrobiopterin were found in *C. limicola* f. *thiosulphatophilum* NCIB 8327 [18]. GTP cyclohydrolase I has been reported to be involved in the synthesis of tetrahydrodictyopterin in *D. discoideum* [19]. A non-enzymic transformation of L-erythro-dihydrobiopterin by a mechanism analogous to keto-enol tautomerism was postulated for the formation of L-threo-dihydrobiopterin in human urine [16].

We recently reported 1-*O*-(L-threo-tetrahydrobiopterin-2'-yl)- β -*N*-acetylglucosamine (tetrahydrotepidopterin), a glycoside containing L-threo-tetrahydrobiopterin, as a natural component of a thermophilic green sulphur bacterium, *C. tepidum* [20]. There have been many reports on the enzymic conversion of the L-erythro-1',2'-dihydroxypropyl moiety in the biosynthesis of L-erythro-tetrahydrobiopterin, as described above. However, there has been no report on that of the L-threo-1',2'-dihydroxypropyl moiety in the biosynthesis of glycoside containing L-threo-tetrahydrobiopterin, such as tetrahydrotepidopterin. In the present study, we report the properties of a novel type of sepiapterin reductase from *C. tepidum*, which catalyses uniquely the reduction of sepiapterin to L-threo-dihydrobiopterin.

MATERIALS AND METHODS

Micro-organisms and culture conditions

C. tepidum, kindly donated by R. Sirevag (University of Oslo, Oslo, Norway), was grown photo-autotrophically under the light

Abbreviations used: IC₅₀, inhibitor concentration producing 50% inhibition; tetrahydrotepidopterin, 1-*O*-(L-threo-tetrahydrobiopterin-2'-yl)- β -*N*-acetylglucosamine.

¹ To whom correspondence should be addressed (e-mail kangsaou@plaza.snu.ac.kr).

intensity of 330 lux for 4–5 days at 45 °C. The cells were cultivated according to the method proposed by Pfennig and Truper [21], with some modifications of the concentrations of sulphide and bicarbonate.

Chemicals

Sepiapterin, D-threo-biopterin (dictyopterin), L-erythro-dihydrobiopterin and L-threo-dihydrobiopterin were purchased from Schricks Laboratories, Jona, Switzerland; *N*-acetylserotonin was from Aldrich; and pterin, L-erythro-biopterin, Red Sepharose CL-6B, DEAE-Sepharose CL-6B, Sephacryl S-300, diacetyl, phenylglyoxal, methylglyoxal, menadione, *N*-acetyldopamine, platinum oxide, D-phenylalanine and melatonin were from Sigma. Mono-Q, and molecular-mass markers for SDS/PAGE and for gel-permeation chromatography were from Amersham Pharmacia Biotech; and sodium ascorbate was from Merck. All other chemicals used were of the highest quality generally available.

L-threo-Biopterin and tepidopterin were prepared according to the method proposed in our previous report [20]. The tetrahydro forms of D-threo-biopterin, L-threo-biopterin and tepidopterin were obtained by reduction with hydrogen gas catalysed by platinum oxide, according to the method proposed by Kaufman [22]. Dihydro forms were prepared by oxidizing tetrahydro forms for 24 h at 4 °C in air, according to the method proposed by Davis et al. [23].

Enzyme assay

The standard assay mixture was composed of 45 μ M sepiapterin/50 μ M NADPH, and an aliquot of enzyme in 50 mM Tris/HCl buffer (pH 8.8). The reaction was carried out for 10 min at 45 °C. The product was oxidized acidically and then assayed according to the method proposed previously [20]. Alternatively, the reduction of sepiapterin was measured spectrophotometrically by the decrease in the absorbance at 420 nm ($\epsilon = 1.04 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [24], or the oxidation of NADPH by the decrease in the absorbance at 340 nm ($\epsilon = 6.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Enzyme activity (1 unit) was defined as the amount of enzyme that produced 1 μ mol of L-threo-dihydrobiopterin/min or 1 μ mol of NADP⁺/min.

Identification of the product of enzymic reaction

To identify the oxidation product prepared previously, the comparison with the standard pterin compounds was made in retention time according to the method proposed previously [20]. To determine the enantiomeric configuration of the oxidation product, ligand-exchange chromatography was adopted using a reversed-phase column with a mobile phase containing D-phenylalanine as the chiral modifier and CuSO₄ as the source of metal ion, according to the method proposed by Klein [25]. The oxidation–reduction states of the product of enzymic reaction were determined according to the method proposed by Fukushima and Nixon [26].

Determination of the protein concentration

The protein concentration was determined according to the methods proposed by Bradford [27], using BSA as a reference protein.

Enzyme purification

Cultured cells (70 g wet weight) were harvested by centrifugation at 20000 *g* for 15 min, and suspended in 350 ml of 20 mM

Tris/HCl buffer (pH 8.8; buffer A). The suspension was sonicated with Microson XL-2000 (Heat Systems Ultrasonics), and the cell debris and membrane fractions were removed by ultracentrifugation at 100000 *g* for 1 h. Ammonium sulphate fractionation was added to the supernatant and the precipitate of 0–60 % was collected. It was resuspended in 100 ml of buffer A and dialysed for 15 h against the same buffer. The dialysed solution was applied to a DEAE-Sepharose CL-6B column (2.5 × 30 cm) equilibrated previously with buffer A. After the column was washed with 0.15 M NaCl in buffer A, the enzyme was eluted with a linear concentration gradient of 0.15–0.4 M NaCl in buffer A. The active fractions were concentrated by ultrafiltration using an Amicon PM10 membrane and loaded on to a Sephacryl S-300 column (1.5 × 120 cm). The active fractions were also combined and chromatographed by a preparative HPLC system (Waters) with a Protein-Pak DEAE 5PW column (2.15 × 15 cm; Waters) equilibrated with 20 mM Tris/HCl buffer (pH 7.7; buffer B). After the column was washed with 0.15 M NaCl in buffer B, the enzyme was eluted with a linear concentration gradient of 0.15–0.4 M NaCl in buffer B. The active fractions were concentrated, desalted and applied to a Red Sepharose CL-6B column (0.5 × 7 cm) equilibrated with buffer A. The washing step with 0.8 M NaCl in buffer A was followed by eluting the column in the constant concentration of 1.2 M NaCl in buffer A and desalting the active fractions. They were further purified by a FPLC system with a Mono-Q column (0.5 × 5 cm; Amersham Pharmacia Biotech) equilibrated with 20 mM Tris/HCl buffer (pH 7.4, buffer C). The enzyme was eluted with a linear concentration gradient of 0.15–0.37 M NaCl in buffer C. After the combined active fractions were concentrated, the buffer system was exchanged with buffer A by ultrafiltration and the active fractions were stored at –70 °C.

Determination of molecular mass

The molecular mass of the purified enzyme was determined by gel-permeation chromatography operated by a FPLC system equipped with a Superdex 200 HR column (1 × 30 cm; Amersham Pharmacia Biotech). The column was equilibrated with buffer A, and then calibrated with ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). The partition coefficient (K_{av}) was calculated according to the method proposed by Laurent and Killander [28]. For the determination of the molecular mass of the subunit, SDS/PAGE was performed with a linear concentration gradient of 5–20 % polyacrylamide according to the method proposed by Arcus [29]. As molecular-mass markers, myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soya bean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa) were used.

N-terminal amino acid sequence analysis

For analysing N-terminal amino acid sequence, enzyme was subjected to Tricine/SDS/PAGE. After electrophoresis, electrotransfer of proteins was performed by the method proposed by Towbin et al. [30]. N-terminal chemical deblocking of the modified forms was carried out on dried samples by incubation at 60 °C for 30 min in trifluoroacetic acid vapour according to the method proposed by Hirano et al. [31]. The N-terminal amino acid sequence of this protein was determined with a Procise Protein Sequencing System (Applied Biosystems).

RESULTS AND DISCUSSION

Identification of the product of enzymic reaction

When the product of enzymic reaction was oxidized acidically and this oxidation product was analysed by HPLC, a peak was found at a similar retention time to that of L-threo-biopterin, compared with the retention times of reference compounds 6-carboxypterin (Figure 1A, peak a), L-threo-neopterin (Figure 1A, peak b), L-erythro-biopterin (Figure 1A, peak c) and L-threo-biopterin (Figure 1A, peak d). In a spiking test with L-threo-biopterin they were co-eluted with a retention time of 8.6 min (Figure 1B). Under the above-mentioned conditions,

Figure 1 HPLC analysis of L-threo-biopterin formation

(A) Reference compounds: a, 6-carboxypterin; b, L-threo-neopterin; c, L-erythro-biopterin; and d, L-threo-biopterin. (B) The acidically oxidized product of the reaction mixture of sesipapterin and NADPH by ammonium sulphate fractionation (0–60%) of cell crude extract plus L-threo-biopterin. (C) Reference compounds: a, L-threo-biopterin; and b, D-threo-biopterin. (D) The acidically oxidized product prepared from reaction with purified enzyme plus L-threo-biopterin. The analysis of (A) and (B) was performed according to the method proposed by Cho et al. [20] and that of (C) and (D) by Klein [25].

A

B

C

Figure 2 Structure of tetrahydrotepidopterin and the reduction catalysed by sesipapterin reductase

The structure of tetrahydrotepidopterin (A) containing moieties of L-threo-1',2'-dihydroxypropyl and N-acetylglucosamine was proposed in our previous study [20]. The reduction of sesipapterin by NADPH catalysed by sesipapterin reductase produces L-erythro-dihydrobiopterin (B) in mammalian systems, but L-threo-dihydrobiopterin (C) in *C. tepidum*.

D-threo-biopterin, L-threo-biopterin and the oxidation product were not separated. These results indicate that the oxidation product is one of the enantiomers of threo-biopterin. This analysis was followed by ligand-exchange chromatography for the determination of enantiomeric configuration of the oxidation product. As shown in Figure 1(C), L-threo-biopterin (Figure 1C, peak a) and D-threo-biopterin (Figure 1C, peak b) were separated. When the oxidation product was analysed, a peak at a retention time similar to L-threo-biopterin was found and, in a spiking test with L-threo-biopterin, the co-elution was identified with the retention time of 35.3 min (Figure 1D). These data indicate that the oxidation product was L-threo-biopterin. When the oxidation products, under acidic and alkaline conditions, and the non-oxidized product were analysed by HPLC, they gave identical retention times. However, in the comparison of fluorescence intensities, both oxidation products, which had identical intensities, gave higher intensities than the non-oxidized product (results not shown). According to the result of Fukushima and Nixon [26], the above data show that the oxidation–reduction state of the enzymic product was the dihydro form. Therefore, the product of the enzymic reaction was L-threo-dihydrobiopterin. This is consistent with the facts that sesipapterin used as a substrate is in the L-configuration and that tetrahydrotepidopterin (Figure 2A) exists as a natural constituent of *C. tepidum* [20].

The reduction product of sesipapterin generated by sesipapterin reductase using NADPH has been known generally as L-erythro-

Table 1 Purification of sepiapterin reductase from *C. tepidum*

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Ammonium sulphate precipitation	3130	197	0.06	100	1
DEAE-Sepharose chromatography	400	131	0.33	66.5	5.5
Sephacryl S-300 chromatography	266	118	0.44	59.9	7.3
DEAE 5PW chromatography	40	65	1.63	33.0	27.2
Red Sepharose chromatography	1	30	30.0	15.2	500.0
Mono-Q chromatography	0.2	6	31.50	3.0	533.3

dihydrobiopterin in mammalian systems [1] (shown in Figure 2B). The conversion of L-erythro-dihydrobiopterin into L-threo-dihydrobiopterin by chemical tautomerism was postulated for explaining the existence of L-threo-dihydrobiopterin in human urine [16]. In *Escherichia coli*, D-erythro-dihydrobiopterin triphosphate 2'-epimerase, which converts D-erythro-dihydrobiopterin triphosphate into L-threo-dihydrobiopterin triphosphate, was reported [32]. Thus for explaining the formation of L-threo-dihydrobiopterin in *C. tepidum*, chemical tautomerism or the presence of an epimerase, such as D-erythro-dihydrobiopterin triphosphate 2'-epimerase, may be proposed. In addition, the interconversion during the oxidation of the enzymic reaction product under acidic conditions may be also proposed. However, in the analysis of the oxidation product described above, L-erythro-biopterin was not detected and, in the analysis of the oxidation product of L-erythro-dihydrobiopterin, only L-erythro-biopterin was detected (results not shown). Thus it can be demonstrated that the L-threo-1',2'-dihydroxypropyl moiety of L-threo-dihydrobiopterin is formed by sepiapterin reductase from *C. tepidum* using NADPH, as shown in Figure 2(C). Up to now, all the sepiapterin reductases catalyse the reduction of sepiapterin to L-erythro-dihydrobiopterin [1]. Therefore, this

enzyme isolated from *C. tepidum* can be referred to as a novel type of sepiapterin reductase.

Purification and molecular properties of sepiapterin reductase

Sepiapterin reductase was purified 533-fold from the cytosolic fraction of *C. tepidum*, with a recovery of 3% relative to the preparation of ammonium sulphate precipitation, as summarized in Table 1. The preparation of the purified enzyme gave a single band on SDS/PAGE (12% gel; Figure 3A).

The apparent molecular mass of the purified enzyme was determined to be 55 kDa by gel-permeation chromatography (Figure 3B). On the other hand, when the enzyme was subjected to gradient SDS/PAGE, a single band of 26 kDa was found (Figure 3C), indicating that the enzyme is composed of two identical subunits. Sepiapterin reductase is composed of two identical subunits in human, rat and mouse, and the molecular masses of the subunits in these organisms range from 27.9 to 28.1 kDa, as deduced from cDNA sequences [33]. Therefore, the molecular mass of sepiapterin reductase from *C. tepidum* is similar to those of mammalian sepiapterin reductases.

Effects of pH and temperature

In sodium citrate buffer, from pH 3.0 to 6.0 (Figure 4A, line a), in potassium phosphate buffer, from pH 5.8 to 8.0 (Figure 4A, line b), and in Tris/HCl buffer, from pH 7.0 to 8.9 (Figure 4A, line c), the activity of sepiapterin reductase was increased as the pH increased. As the pH value was increased in glycine/NaOH buffer, from pH 8.6 to 10.6 (Figure 4A, line d), the activity of the enzyme was decreased. Thus the optimal pH for its activity was determined to be around 8.8. Although the stability of the enzyme disappeared in sodium citrate buffer (Figure 4B, line a) and potassium phosphate buffer (Figure 4B, line b) for 24 h at 4 °C, it increased continuously in Tris/HCl buffer (Figure 4B, line c) and in glycine/NaOH buffer (Figure 4B, line d). The sepiapterin reductases from horse liver, rat erythrocytes and human liver were reported to have optimal activities at pH

Figure 3 SDS/PAGE and estimation of the molecular mass of sepiapterin reductase purified from *C. tepidum*

SDS/PAGE (A) of the purified enzyme (lane 1) and molecular-mass markers (lane 2). Molecular masses were estimated by gel-permeation chromatography (B) and by electrophoresis on a polyacrylamide gradient gel (C). Molecular-mass markers (in kDa) used in (A) were phosphorylase b (a, 97.4), BSA (b, 66.2), monomeric aldolase (c, 39.2), triose phosphate isomerase (d, 26.6), soya bean trypsin inhibitor (e, 21.5) and lysozyme (f, 14.4); in (B) were ferritin (a, 440), tetrameric aldolase (b, 158), ovalbumin (c, 43) and ribonuclease A (d, 13.7); and in (C) were myosin (a, 200), β -galactosidase (b, 116), phosphorylase b (c), BSA (d), ovalbumin (e, 43), carbonic anhydrase (f, 31), soya bean trypsin inhibitor (g), lysozyme (h) and aprotinin (i, 6.5).

Figure 4 Effects of pH on the activity and stability of sespiapterin reductase

(A) The pH dependence of the activity of sespiapterin reductase was measured at the pH values indicated. Sodium citrate buffer (50 mM), pH 3.0–6.0 (a), 50 mM potassium phosphate buffer, pH 5.8–8.0 (b), 50 mM Tris/HCl buffer, pH 7.0–8.9 (c), and 50 mM glycine/NaOH buffer, pH 8.6–10.6 (d), were used. (B) The stability of sespiapterin reductase was investigated after incubating the enzyme with the defined pH buffers described in (A) at 4 °C for 24 h prior to assay.

5.5–6.1 [24,34,35], a more acidic pH range than the optimal pH range in *C. tepidum*. This may be due to the different reduction mechanisms of the enzymes between *C. tepidum* and mammals.

The enzyme showed its highest activity at about 50 °C and its activity decreased slowly above this temperature (Figure 5A). From the linear range of the Arrhenius plot over 30–50 °C, the activation energy of the enzymic reaction was calculated to be 8.36 kcal/mol. The enzyme was relatively stable for 2 h at 25 °C (Figure 5B, line a) and 50 °C (Figure 5B, line b); however, the activity was lost at 70 °C in 20 min (Figure 5B, line c). The stability and the activity of this enzyme at relatively high temperatures might be ascribed to the fact that the optimal temperature for the growth of this organism is 45 °C.

Substrate specificity

Kinetic parameters of the enzyme were determined from a Lineweaver–Burk plot of the two substrates, sespiapterin and NADPH (Table 2A). The K_m values for sespiapterin and NADPH

Figure 5 Effects of temperature on the activity and stability of sespiapterin reductase

(A) Changes of the enzyme activity were measured at the indicated temperature (T) using 50 mM Tris/HCl buffer (pH 8.8). (B) Relative activity was assayed with the same buffer at 45 °C, after incubation at 25 °C (a), 50 °C (b) and 70 °C (c) for the indicated times.

Table 2 Kinetic parameters for the reaction of sespiapterin reductase (A) and substrate specificity of the reverse reaction of sespiapterin reductase (B)

K_m for NADPH was determined with 45 μ M sespiapterin as another substrate. In (B), the concentrations of substrates and NADP were 0.1 mM.

(A)			
Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Sespiapterin	5	2.1×10^{-2}	2.4×10^2
NADPH		6.2×10^{-3}	
Diacetyl	33	4.0	8.3
(B)			
Substrate	Relative activity (%)		
L-Threo-dihydrobiopterin	100		
D-Threo-dihydrobiopterin	45		
L-Erythro-dihydrobiopterin	5		
L-Threo-dihydrobiopterin	45		
Dihydrotepidiopterin	15		

Table 3 Inhibition of sepiapterin reductase from *C. tepidum*

All inhibitors were dissolved in 20% aqueous methanol and the concentrations were 340 μ M.

Inhibitor	Relative activity (%)	K_i (μ M)
Control	100	—
<i>N</i> -Acetylserotonin	97.6	—
<i>N</i> -Acetyldopamine	79.5	400*
Melatonin	96.1	—

* Taken from a Lineweaver–Burk plot for sepiapterin as a function of *N*-acetyldopamine concentration. Slopes were calculated and the K_i value was determined from replotting the slopes against *N*-acetyldopamine concentration.

were 21 and 6.2 μ M, respectively, and the respective k_{cat} and k_{cat}/K_m values for sepiapterin were 5.0 s⁻¹ and 2.4 $\times$ 10² s⁻¹·mM⁻¹. The enzyme from *C. tepidum* had similar K_m values to its counterparts from mammalian sources [24,34,35]; however, it did not show any activity with NADH, unlike the sepiapterin reductase of rat erythrocytes [36]. According to a report [36], sepiapterin reductase from rat erythrocytes had carbonyl reductase activity. When the reducibility of diacetyl, phenylglyoxal, methylglyoxal and menadione by the enzyme was examined, diacetyl and phenylglyoxal could be reduced, although the activity of the latter was very weak. As shown in Table 2(A), k_{cat} , K_m and k_{cat}/K_m values for diacetyl were 33 s⁻¹, 4.0 mM and 8.3 s⁻¹·mM⁻¹, respectively. k_{cat} and K_m values towards diacetyl were 6.6 and 190.5 times higher than those towards sepiapterin, respectively. From this comparison, it is concluded that sepiapterin is a more specific substrate for the enzyme than diacetyl. The effects of dihydropterin compounds and NADP on the rate of the reverse reaction of the enzyme are shown in Table 2(B). As expected, the enzyme showed the highest activity towards L-threo-dihydrobiopterin. The enzyme also readily oxidized D-threo-dihydrobiopterin and L-threo-dihydroneopterin. In contrast, L-erythro-dihydrobiopterin and dihydrotepidopterin played little role in serving as a substrate. These data also imply that the L-threo-1',2'-dihydroxypropyl moiety may be formed by

the enzyme before sugar transfer in the biosynthesis of tetra-hydrotepidopterin.

Inhibitors

The effects of *N*-acetylserotonin, *N*-acetyldopamine and melatonin on the activity of sepiapterin reductase were examined. This was because *N*-acetylserotonin has been reported to be a strong inhibitor of the enzyme from rat erythrocytes (the concentration of inhibitor that produces a 50% inhibition, IC₅₀, 0.6 μ M), stimulated mononuclear blood cells, amniotic fibroblasts (IC₅₀ 2 μ M) and bovine adrenal medullary (K_i 0.12 μ M) [37,38]. *N*-Acetyldopamine from bovine adrenal medullary has also been shown to inhibit the enzyme (K_i 0.4 μ M) [38], and melatonin from bovine adrenal medullary has also been shown as a weak inhibitor (K_i 30 μ M) [38]. As shown in Table 3, very weak inhibitory effects were observed in the three compounds. Even the K_i value of *N*-acetyldopamine, which showed the strongest inhibitory effect among the examined inhibitors, was calculated to be 400 μ M. The effective inhibitors against sepiapterin reductases from mammalian system did not show effective inhibition against the enzyme from *C. tepidum*. The inhibition pattern in *C. tepidum* was also different from that of the enzyme from *D. melanogaster* (K_i of *N*-acetyldopamine, 40 μ M; K_i of *N*-acetylserotonin, 127 μ M) [39].

Spectral properties

The UV-visible absorption spectrum of the purified enzyme was recorded with a UV-1601PC spectrophotometer (Shimadzu) in 20 mM Tris/HCl (pH 8.8). It showed maximum absorbance at 278 nm. Thus like the enzyme reported in rat erythrocytes [36] the enzyme does not contain a UV-visible light-absorbing prosthetic group.

N-terminal amino acid sequence

The first attempt to determine the N-terminal amino acid sequence failed. Therefore, the procedure of deblocking the N-terminal amino acid was carried out according to the method proposed by Hirano et al. [31]. The N-terminal amino acid sequence of sepiapterin reductase purified from *C. tepidum* is shown in Figure 6, together with the amino acid sequences near

Figure 6 The alignment of amino acid sequences near the N-termini of sepiapterin reductases from various sources

The N-terminal amino acid sequence of sepiapterin reductase from *C. tepidum* is aligned with the amino acid sequences from near the N-termini of sepiapterin reductases from *Homo sapiens*, *Mus musculus* and *Rattus norvegicus* [33], and of putative sepiapterin reductases from *Fugu rubripes* [40] and *Bacillus subtilis* [41]. The numbers at the beginning and end of each sequence are the residue numbers in the full protein sequence. X represents undetermined residues. Below the sequences: . represents amino acid residues with similarity, and * shows residues with identity.

the N-termini of sepiapterin reductases and putative enzymes from several organisms [33,40,41]. From the comparison of the amino acid sequence from *C. tepidum* with those from other organisms, the identity and similarity were calculated to be 23 and 43 %, respectively, in the case of *Homo sapiens*, 27 and 43 % for *Mus musculus*, 23 and 40 % for *Rattus norvegicus*, 27 and 47 % for *Fugu rubripes*, and 34 and 48 % in the case of *Bacillus subtilis* [33,40,41]. These data show that the amino acid sequence from *C. tepidum* has relatively high sequence similarity with those from other organisms. Sepiapterin reductase generally belongs to the short-chain dehydrogenases/reductases family, of which the representative conserved sequences are -Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly- as an NAD(P)-binding site in the N-terminal region and -Tyr-Xaa-Xaa-Xaa-Lys- as a catalytic site [42]. However, in the N-terminal amino acid sequence of the enzyme from *C. tepidum*, the fifth residue of the conserved sequence, glycine, is replaced by lysine, although in others it is well conserved (shown in Figure 6).

The N-terminal amino acid sequence from *C. tepidum* has the highest similarity with that of meso-2,3-butanediol dehydrogenase from *Klebsiella pneumoniae*, which also belongs to the short-chain dehydrogenases/reductases family [42]. This sequence connectivity might explain the activity of diacetyl reduction of this enzyme.

Conclusions

In this study we have demonstrated that, in *C. tepidum*, L-threo-dihydrobiopterin was produced by catalytic activity of sepiapterin reductase. Therefore, this enzyme may be involved in the third step of the *de novo* biosynthesis of tetrahydrotepidopterin, although the first step (GTP cyclohydrolase I), second step (6-pyruvoyl tetrahydropterin synthase) and the step transferring N-acetylglucosamine to the L-threo-tetrahydrobiopterin remain to be elucidated and confirmed. 6-Pyruvoyl tetrahydropterin, 1'-hydroxy-2'-oxopropyl tetrahydropterin and 6-lactoyl tetrahydropterin are proposed as substrates *in vivo* for sepiapterin reductase in the *de novo* biosynthesis of L-erythro-tetrahydrobiopterin in mammalian systems [4–6]. Although, in this study, we have examined on the optical configuration of the 1',2'-dihydroxypropyl moiety of biopterin using sepiapterin as a substrate, we did not examine the substrate *in vivo*. Thus the substrate *in vivo* for this enzyme in the *de novo* biosynthetic pathway of tetrahydrotepidopterin should be examined in *C. tepidum*.

In addition to the optical properties of the product formed by sepiapterin reductase in *C. tepidum*, the enzyme had optimal activity at a more alkaline pH and a higher temperature than other sepiapterin reductases. The strong inhibitors of mammalian sepiapterin reductases had little effect on this enzyme. In this enzyme, lysine was found in place of glycine, an amino acid residue conserved in the NAD(P)-binding region of short-chain dehydrogenases/reductases, including sepiapterin reductase [42]. With all these points taken into consideration, the enzyme from *C. tepidum* should be viewed as a new type of sepiapterin reductase.

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