

Tetrahydrobiopterin is synthesized from 6-pyruvoyl-tetrahydropterin by the human aldo-keto reductase AKR1 family members

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Abstract

Tetrahydrobiopterin (BH₄) is a cofactor for aromatic amino acid hydroxylases and nitric oxide synthase. The biosynthesis includes two reduction steps catalyzed by sepiapterin reductase. An intermediate, 6-pyruvoyltetrahydropterin (PPH₄) is reduced to 1'-oxo-2'-hydroxypropyl-tetrahydropterin (1'-OXPH₄) or 1'-hydroxy-2'-oxopropyl-tetrahydropterin (2'-OXPH₄), which is further converted to BH₄. However, patients with sepiapterin reductase deficiency show normal urinary excretion of pterins without hyperphenylalaninemia, suggesting that other enzymes catalyze the two reduction steps. In this study, the reductase activities for the tetrahydropterin intermediates were examined using several human recombinant enzymes belonging to the aldo-keto reductase (AKR) family and short-chain dehydrogenase/reductase (SDR) family. In the reduction of PPH₄ by AKR family enzymes, 2'-OXPH₄ was formed by 3 α -hydroxysteroid dehydrogenase type 2, whereas 1'-OXPH₄ was produced by aldose reductase, aldehyde reductase, and 20 α -hydroxysteroid dehydrogenase, and both 1'-OXPH₄ and 2'-OXPH₄ were detected as the major and minor products by 3 α -hydroxysteroid dehydrogenases (types 1 and 3). The activities of aldose reductase and 3 α -hydroxysteroid dehydrogenase type 2 (106 and 35 nmol/mg/min, respectively) were higher than those of the other enzymes (0.2–4.0 nmol/mg/min). Among the SDR family enzymes, monomeric carbonyl reductase exhibited low 1'-OXPH₄-forming activity of 5.0 nmol/mg/min, but L-xylulose reductase and peroxisomal tetrameric carbonyl reductase did not form any reduced product from PPH₄. Aldose reductase reduced 2'-OXPH₄ to BH₄, but the other enzymes were inactive towards both 2'-OXPH₄ and 1'-OXPH₄. These results indicate that the tetrahydropterin intermediates are natural substrates of the human AKR family enzymes and suggest a novel alternative pathway from PPH₄ to BH₄, in which 3 α -hydroxysteroid dehydrogenase type 2 and aldose reductase work in concert. © 2003 Elsevier Science (USA). All rights reserved.

Keywords: Tetrahydrobiopterin; Carbonyl reductase; Aldo-keto reductase; Sepiapterin reductase; Biosynthesis of tetrahydrobiopterin; Tetrahydrobiopterin deficiency; Sepiapterin reductase deficiency

Tetrahydrobiopterin is a cofactor for aromatic amino acid hydroxylases [1,2], which catalyze the initial steps in phenylalanine degradation in the liver and the rate-limiting steps in the biosynthesis of catecholamine and indoleamine neurotransmitters in the brain. BH₄¹ is also

required by nitric oxide synthase, which generates nitric oxide, a messenger molecule involved in various processes in many tissues [3,4].

The pathway of the de novo biosynthesis of BH₄ from GTP involves GTP cyclohydrolase I (EC 3.5.4.16),

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¹ Abbreviations used: BH₄, tetrahydrobiopterin; BH₂, dihydrobiopterin; PPH₄, 6-pyruvoyl-tetrahydropterin; 1'-OXPH₄, 1'-oxo-2'-hydroxy-

propyl-tetrahydropterin; 2'-OXPH₄, 1'-hydroxy-2'-oxopropyl-tetrahydropterin; SPR, sepiapterin reductase; AKR, aldo-keto reductase; SDR, short-chain dehydrogenase/reductase; NH₂TP, dihydroneopterin triphosphate; CSF, cerebrospinal fluid.

6-pyruvoyl-tetrahydropterin (PPH₄) synthase (EC 4.6.1.10), and sepiapterin reductase (SPR, EC 1.1.1.153). SPR catalyzes the last step of biosynthesis, in which the diketo group on the side chain of PPH₄ is converted into the corresponding diol form in BH₄. PPH₄ is first reduced to 1'-hydroxy-2'-oxopropyl-tetrahydropterin (2'-OXPH₄) or 1'-oxo-2'-hydroxypropyl-tetrahydropterin (1'-OXPH₄), and then both tetrahydropterin intermediates are reduced to BH₄ [5–7]. The formation of 1'-OXPH₄ in the first reduction is catalyzed by PPH₄ reductase [8], which has been shown to be identical to aldose reductase in the human brain [9]. Aldose reductase belongs to the aldo-keto reductase (AKR) family [10] and has been named AKR1B1. A deficiency of BH₄ causes hyperphenylalaninemia, which leads to abnormal development of mammalian neonates. Three enzymatic defects that lead to BH₄ deficiency have been identified. The relevant enzymes are GTP cyclohydrolase I, PPH₄ synthase, and dihydropteridine reductase [11]. However, SPR deficiency has not been reported from patients of hyperphenylalaninemia, who can be detected by neonatal screening for phenylketonuria. In 2001, SPR deficiency was first discovered from a patient with progressive psychomotor retardation and dystonia. However, the patient shows normal urinary pterins without hyperphenylalaninemia [12–14]. These findings suggest that enzyme(s) other than SPR may be involved in the formation of BH₄ from PPH₄.

Park et al. [15] previously reported that human monomeric carbonyl reductase reduces PPH₄ to both 1'-OXPH₄ and 2'-OXPH₄, and AKR1B1 catalyzes the reduction of 2'-OXPH₄ to BH₄. They proposed an alternative pathway of BH₄ formation from PPH₄ through 2'-OXPH₄ catalyzed by the two enzymes. However, the 2'-OXPH₄-forming activity of monomeric carbonyl reductase is quite low compared to its 1'-OXPH₄-forming activity. We previously discovered two carbonyl reductases (CRI and CRII) that are involved in the formation of BH₄ from PPH₄ in the fat body of the *lemon* mutant and normal strain of silkworm, *Bombyx mori* [16–18]. The silkworm carbonyl reductases differ from SPR and human carbonyl reductase because CRI catalyzes the reduction of the 2'-keto group of both PPH₄ and 2'-OXPH₄ and CRII reduces the 1'-keto group of PPH₄ and 1'-OXPH₄ [18]. In addition, the enzymes, similar to silkworm CRI and CRII, exist in chicken tissues [unpublished data]. A feature common to the silkworm enzymes [18], human monomeric carbonyl reductase [19], SPR [20], and AKR1B1 [21] is their broad substrate specificity for various exogenous carbonyl compounds. Similar broad substrate specificity for carbonyl compounds is also observed for human aldehyde reductase (AKR1A1, EC 1.1.1.2) [19,21], 20 α -hydroxysteroid dehydrogenase (AKR1C1, EC 1.1.1.149) [22], and three isozymes (AKR1C2, AKR1C3, and AKR1C4) of 3 α -hydroxysteroid dehydrogenase (EC

Table 1
Human enzymes used in this study

Family	Enzyme	Nomenclature
AKR	Aldose reductase	AKR1B1
	Aldehyde reductase	AKR1A1
	20 α -hydroxysteroid dehydrogenase	AKR1C1
	3 α -hydroxysteroid dehydrogenase type 1	AKR1C4
	3 α -hydroxysteroid dehydrogenase type 2	AKR1C3
	3 α -hydroxysteroid dehydrogenase type 3	AKR1C2
	SDR	Monomeric carbonyl reductase
L-Xylulose reductase		
Peroxisomal tetrameric carbonyl reductase		

The enzymes belong to the AKR or SDR superfamily and the enzymes in the AKR family are named according to the nomenclature system [10].

1.1.1.213) [22–24] in the AKR family. Human monomeric carbonyl reductase and SPR belong to the short-chain dehydrogenase/reductase (SDR) superfamily [25], which includes human L-xylulose reductase [26] and peroxisomal tetrameric carbonyl reductase ([27], unpublished data) with high reductase activities for dicarbonyl compounds. In order to elucidate the SPR-unrelated BH₄ formation route from PPH₄ in humans, we have here examined the reductase activities of the above enzymes in the AKR1 subfamily and the SDR family (Table 1) towards PPH₄, 1'-OXPH₄, and 2'-OXPH₄ and compared them with those of human monomeric carbonyl reductase and AKR1B1. The data show that AKR1C3 efficiently catalyzes the reduction of PPH₄ to the intermediate metabolite, 2'-OXPH₄, which is reduced to BH₄ only by AKR1B1. This is the first report that shows PPH₄ as a new endogenous substrate of the AKR1 subfamily enzymes and suggests a novel BH₄ formation route from PPH₄ in which AKR1C3 and AKR1B1 work in concert.

Materials and methods

Chemicals and enzymes

BH₄ and sepiapterin were purchased from Dr. Schircks (Jona, Switzerland). Dihydroneopterin triphosphate (NH₂TP) was synthesized enzymatically from GTP by the method of Yoshioka et al. [28] using purified GTP cyclohydrolase I from chicken liver [29]. 1'-OXPH₄ and 2'-OXPH₄ standards were prepared as described previously [16]. Other chemicals were of analytical grade and obtained from commercial sources. The PPH₄ synthase was purified from chicken liver by the method of Takikawa et al. [30]. The recombinant AKR1C1, AKR1C2, AKR1C3, and AKR1C4 were

prepared and purified to homogeneity as previously described [23,24,31,32]. The cDNAs for AKR1A1 and AKR1B1 were amplified from a total RNA of human kidney (Stratagene) by reverse transcription-PCR using *Pfu* DNA polymerase (Stratagene) and following primers that annealed the 5'- and 3'-coding regions of the cDNAs for AKR1A1 and AKR1B1 [33]. The forward and reverse primers for the amplification of AKR1A1 cDNA were 5'-ATGGCGGCTTCCTGTGTT-3' and 5'-GTCTCAGTACGGGTCATTA-3', respectively, and the respective primers for the amplification of AKR1B1 cDNA were 5'-ATGGCAAGCCGTCTCCTG-3' and 5'-AGCTTCAAACCTTTCATGG-3'. The cDNAs for the two enzymes were inserted into pCR T7/TOPO expression plasmids using a pCR T7/CT-TOPO TA cloning kit (Invitrogen). The constructs were transformed into *Escherichia coli* BL21(DE3)pLysS (Stratagene). The *E. coli* cells were cultured in 1 liter of an LB medium containing ampicillin (50 µg/ml) and chloramphenicol (50 µg/ml) at 37 °C until the turbidity reached 0.4. Then, 1 mM isopropyl β-D-thiogalactopyranoside was added and the growth of the culture was continued for 6 h. The cell extracts were prepared as described previously [24]. The recombinant AKR1A1 and AKR1B1 were purified to homogeneity from the cell extracts as described previously [34]. Recombinant monomeric carbonyl reductase [35], L-xylulose reductase [26], and peroxisomal tetrameric carbonyl reductase ([27], unpublished data) were expressed in *E. coli* cells and purified to homogeneity. The recombinant human L-xylulose reductase and peroxisomal tetrameric carbonyl reductase showed 1-phenyl-1,2-propanedione reductase activities of 10 µmol/mg/min and 20 µmol/mg/min, respectively, at 25 °C and pH 7.0.

Assay of tetrahydropterin-producing activity

Analysis of tetrahydropterins was performed by HPLC with electrochemical detection, as described previously [16]. The standard reaction mixture contained the following components: a 50 mM potassium phosphate buffer (pH 7.0), 100 µM NADPH, 10 µl of a concentrated solution of PPH₄ synthase, 5 mM dithiothreitol, 8 mM MgCl₂, 14 µM NH₂TP, and an appropriate amount of one of the enzyme in the AKR1 subfamily and the SDR family in a final volume of 100 µl. The reaction mixture was flushed with N₂ gas, sealed, and incubated at 37 °C for 60 min in darkness. To test whether 2'-OXPH₄ is a substrate for an enzyme, the standard reaction mixture containing AKR1C3 as the enzyme was incubated for 40 min, and then 10 µl of the test enzyme was added to the reaction mixture. On the other hand, when 1'-OXPH₄ was tested as a substrate, an appropriate amount of AKR1C3 was added to the reaction mixture, which had been reacted with the other AKR1 subfamily enzyme (1A1, 1B1, 1C1,

1C2, or 1C4) in the standard reaction mixture. The reaction mixtures were again flushed with N₂ gas, sealed, and incubated at 37 °C for 40 min in darkness. Quantitative analysis of tetrahydropterins in the reaction mixture was conducted with BH₄ as a standard. To estimate the kinetic constants for PPH₄, the reactions were performed at three concentrations of NH₂TP. The concentration of PPH₄ formed in the incubation mixture without AKR1B1 and AKR1C3 was determined separately. The K_m and k_{cat} values were calculated by fitting the data with an Eadie–Hofstee plot.

Analysis of biopterin

The nonenzymatic formation of sepiapterin from 1'-OXPH₄ in the above reaction mixtures was examined by determining it as biopterin as described below. 1'-OXPH₄ (8 µM) was initially produced by the incubation with PPH₄ and AKR1B1 in the absence of dithiothreitol, and the reaction mixture was kept at 37 °C for 2 h in darkness. Since human monomeric carbonyl reductase reduces sepiapterin to dihydrobiopterin (BH₂) [15], the enzyme (2 µg) was added to the reaction mixture and further incubated at 37 °C for 40 min. The reaction was stopped by the addition of 10 µl of 20% trichloroacetic acid solution and 20 µl of iodine solution (1% I₂, 2% KI). After allowing the mixture to stand for 30 min at room temperature in darkness, excess iodine was reduced by the addition of 10 µl of 2% ascorbic acid solution [36], and the mixture was centrifuged at 15,000g for 5 min. The amount of biopterin in the resulting supernatant was measured by HPLC with fluorometric detection, as previously described [16].

Results

The PPH₄ reducing activities of the enzymes of the AKR1 subfamily were first analyzed by HPLC. AKR1B1 reduced PPH₄ to 1'-OXPH₄, as described previously [15]. Representative HPLC elution patterns of the products by AKR1C1 and AKR1C2 are shown in Figs. 1A and B; AKR1A1 and AKR1C1 also formed only 1'-OXPH₄, but AKR1C2 and AKR1C4 reduced PPH₄ to both 1'-OXPH₄ and 2'-OXPH₄ as the major and minor products, respectively. In contrast, AKR1C3 produced only 2'-OXPH₄ (Fig. 1C) from PPH₄. The specific activity of AKR1C3 was lower than that of AKR1B1 but more than 4-fold higher than the values of the other enzymes (Table 2). The K_m and k_{cat} values of AKR1B1 were 5.4 µM and 6.2 min⁻¹, respectively, and the respective values of AKR1C3 were 6.8 µM and 2.1 min⁻¹.

In the reactions using SDR family enzymes as PPH₄ reductases, human monomeric carbonyl reductase formed only one product, 1'-OXPH₄, but its specific

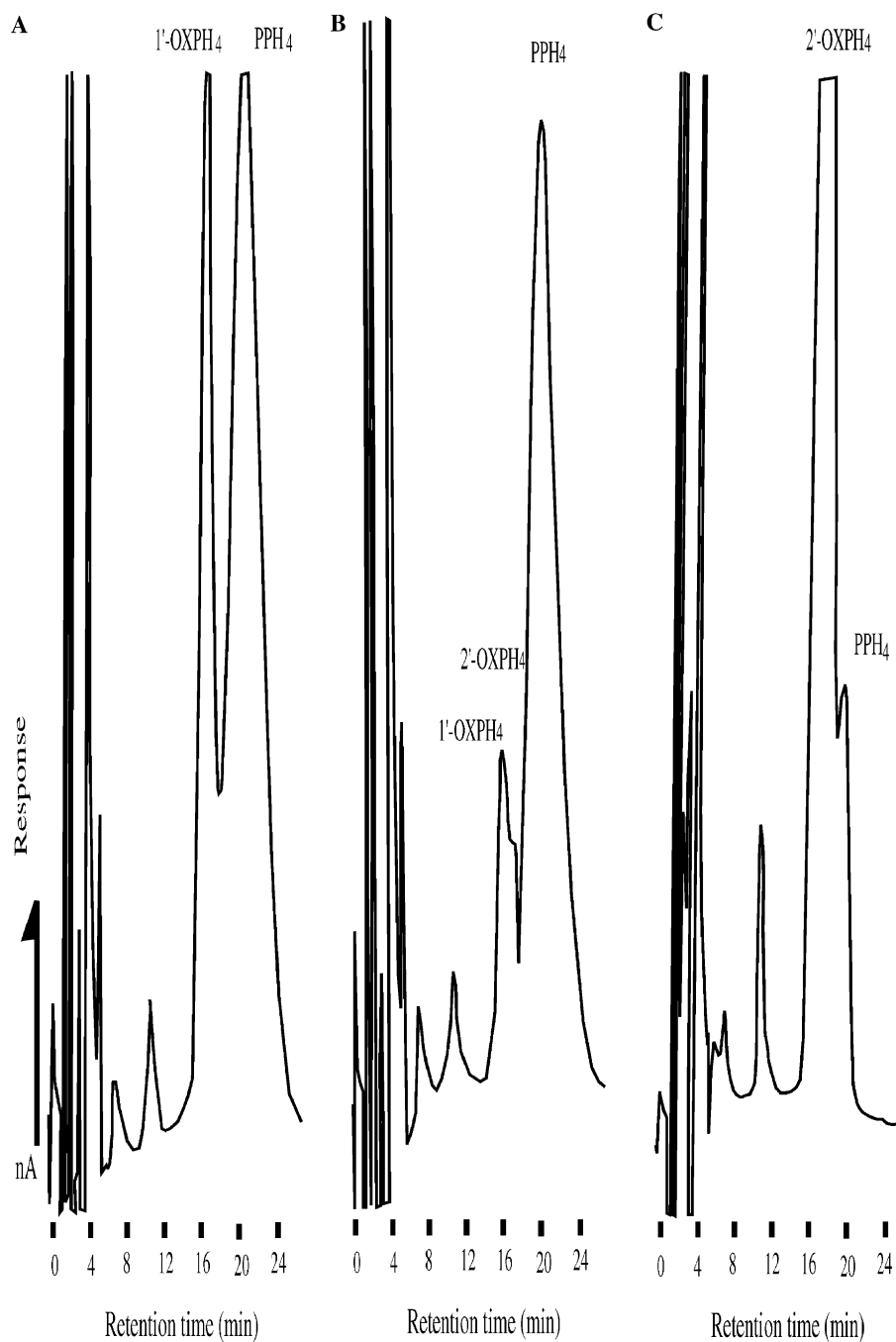


Fig. 1. HPLC analysis of products formed from PPH₄ reduction by AKR1C1, AKR1C2, and AKR1C3. AKR1C1 (3 μg in A), AKR1C2 (3 μg in B), or AKR1C3 (1 μg in C) was added to the reaction mixture containing 14 μM NH₂TP, PPH₄ synthase, 50 mM potassium phosphate buffer, pH 7.0, 8 mM MgCl₂, 5 mM dithiothreitol, and 100 μM NADPH. It was flushed with N₂ gas, sealed, and incubated for 60 min at 37°C in darkness. The products and substrate were analyzed by HPLC with electrochemical detection.

activity was much lower than those of AKR1B1 and AKR1C3 (Table 2). No reduced product of PPH₄ was observed in the reaction mixtures containing human L-xylulose reductase and peroxisomal tetrameric carbonyl reductase.

We examined the reactivity of the enzymes towards 1'-OXPH₄ and 2'-OXPH₄. Because AKR1C3 reduced specifically the 1'-keto group of PPH₄, the reactivity for

2'-OXPH₄ was assayed by analyzing BH₄ formation in the reaction mixture that was incubated for 40 min after adding one of the other enzymes to the reaction mixture which had been reacted with AKR1C3 in the standard mixture containing PPH₄ as the substrate. BH₄ was formed by AKR1B1 (Fig. 2), but not by AKR1A1, AKR1C1, AKR1C2, AKR1C4, and human monomeric carbonyl reductase. The reactivity for 1'-OXPH₄ was

Table 2
PPH₄ reductase activities of the AKR1 subfamily enzymes and human monomeric carbonyl reductase

Enzyme	PPH ₄ (μ M)	Specific activity (nmol/mg protein/min)	
		1'-OXPH ₄ formed	2'-OXPH ₄ formed
AKR1B1	2.2	48	n.d.
	5.0	82	n.d.
	9.6	106	n.d.
AKR1C3	2.2	n.d.	16
	5.0	n.d.	21
	9.6	n.d.	35
AKR1C1	5.0	4.0	n.d.
AKR1C2	5.0	1.5	0.4
AKR1C4	5.0	0.7	0.2
AKR1A1	5.0	0.2	n.d.
Carbonyl reductase	5.0	5.0	n.d.

The value represents mean of at least three determinations. n.d., not detected.

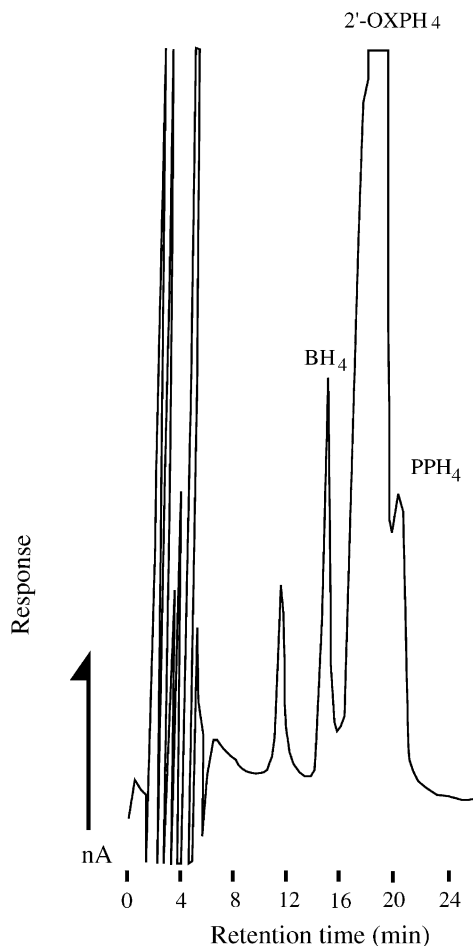


Fig. 2. Production of BH₄ from 2'-OXPH₄ by AKR1B1. The reaction containing AKR1C3 as the enzyme was performed as described in the legend of Fig. 1. Then AKR1B1 (1 μ g) was added to the reaction mixture, which was again flushed with N₂ gas, sealed, and further incubated for 40 min at 37 °C in darkness. The products and substrate were analyzed by HPLC with electrochemical detection.

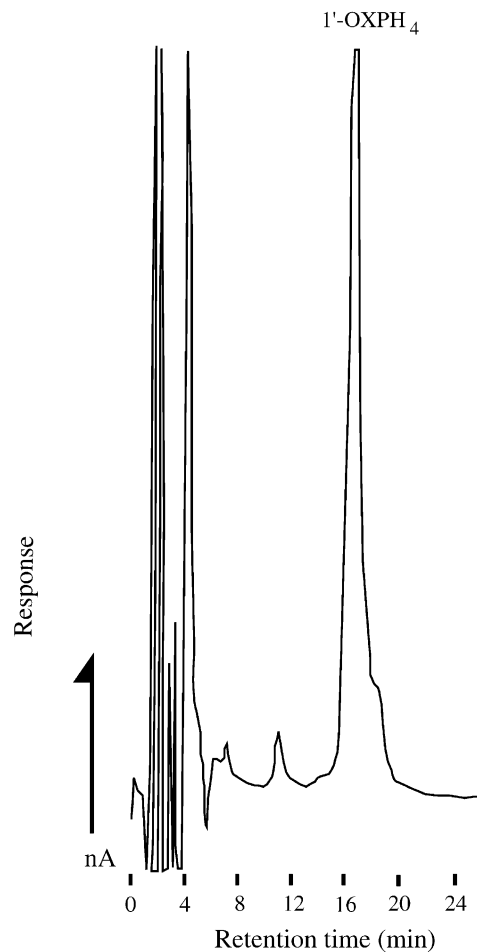


Fig. 3. HPLC analysis of products formed from 1'-OXPH₄ by AKR1C3. The reaction containing AKR1B1 as the enzyme was performed as described in the legend of Fig. 1. Then AKR1C3 (1 μ g) was added to the reaction mixture, which was incubated and subjected to HPLC analysis as described in the legend of Fig. 2. No BH₄ was detected in the reaction mixture.

similarly examined by adding the enzymes to the reaction mixture that had been incubated with PPH₄ and AKR1B1. However, no BH₄ formation was observed, as shown by the representative HPLC pattern of the reaction products by AKR1C3 in Fig. 3. These results indicated that, although AKR1B1 is able to reduce both PPH₄ and 2'-OXPH₄, the other enzymes accept the diketone form of PPH₄ but not its metabolites, 1'-OXPH₄ and 2'-OXPH₄, as the substrate.

Since 1'-OXPH₄ has been suggested to be nonenzymatically converted into sepiapterin [13], sepiapterin in the incubation mixture containing PPH₄ and AKR1B1 was determined by its derivation to biopterin. No sepiapterin was detected in the incubation mixture, and only a small amount (0.7 pmol) of it was formed when the mixture containing 800 pmol 1'-OXPH₄ was further kept at 37 °C for 2 h in darkness. The result suggests that the rate of nonenzymatic formation of sepiapterin from 1'-OXPH₄ is quite slow.

Discussion

Park et al. [15] reported that AKR1B1 catalyzes the NADPH-linked reduction of the 2'-keto group of both PPH₄ and 2'-OXPH₄ to 1'-OXPH₄ and BH₄, respectively, and monomeric carbonyl reductase of the human brain reduces PPH₄ to both 1'-OXPH₄ and 2'-OXPH₄ in the presence of NADPH. The present results are consistent with a previous report for AKR1B1 but not with those for monomeric carbonyl reductase. We expected that a small amount of 2'-OXPH₄ would be formed, as in the cases of AKR1C2 and AKR1C4 (Fig. 1B and Table 2), but only 1'-OXPH₄ was detected as the reaction product of PPH₄ by the recombinant monomeric carbonyl reductase despite the employment of a recombinant enzyme that showed 3-fold higher specific activity than that of the enzyme purified from the human brain [15]. Human monomeric carbonyl reductase is known to reduce either of the two keto groups of

dicarbonyl substrates. For example, the enzyme forms only 3-hydroxyisatin from a small dicarbonyl substrate, isatin [37]. Additionally, the reduction of 1'-OXPH₄ or 2'-OXPH₄ by the enzyme is not observed in previous [15] and present studies. Thus, monomeric carbonyl reductase probably reduces specifically the 2'-keto group of PPH₄.

The present study has also revealed that, in addition to AKR1B1, the other members of the AKR1 subfamily reduce PPH₄. Among the enzymes, AKR1C3 showed high PPH₄ reductase activity and produced 2'-OXPH₄. AKR1C3 was originally identified as an isozyme (type 2) of 3 α -hydroxysteroid dehydrogenase, but its substrate specificity is different from those of the other 3 α -hydroxysteroid dehydrogenase isozymes, AKR1C2 and AKR1C4, as it has been reported to exhibit high 17 β -hydroxysteroid dehydrogenase [38–40] and prostaglandin F synthase [23] activities. The high reactivity and region specificity in the PPH₄ reduction are also

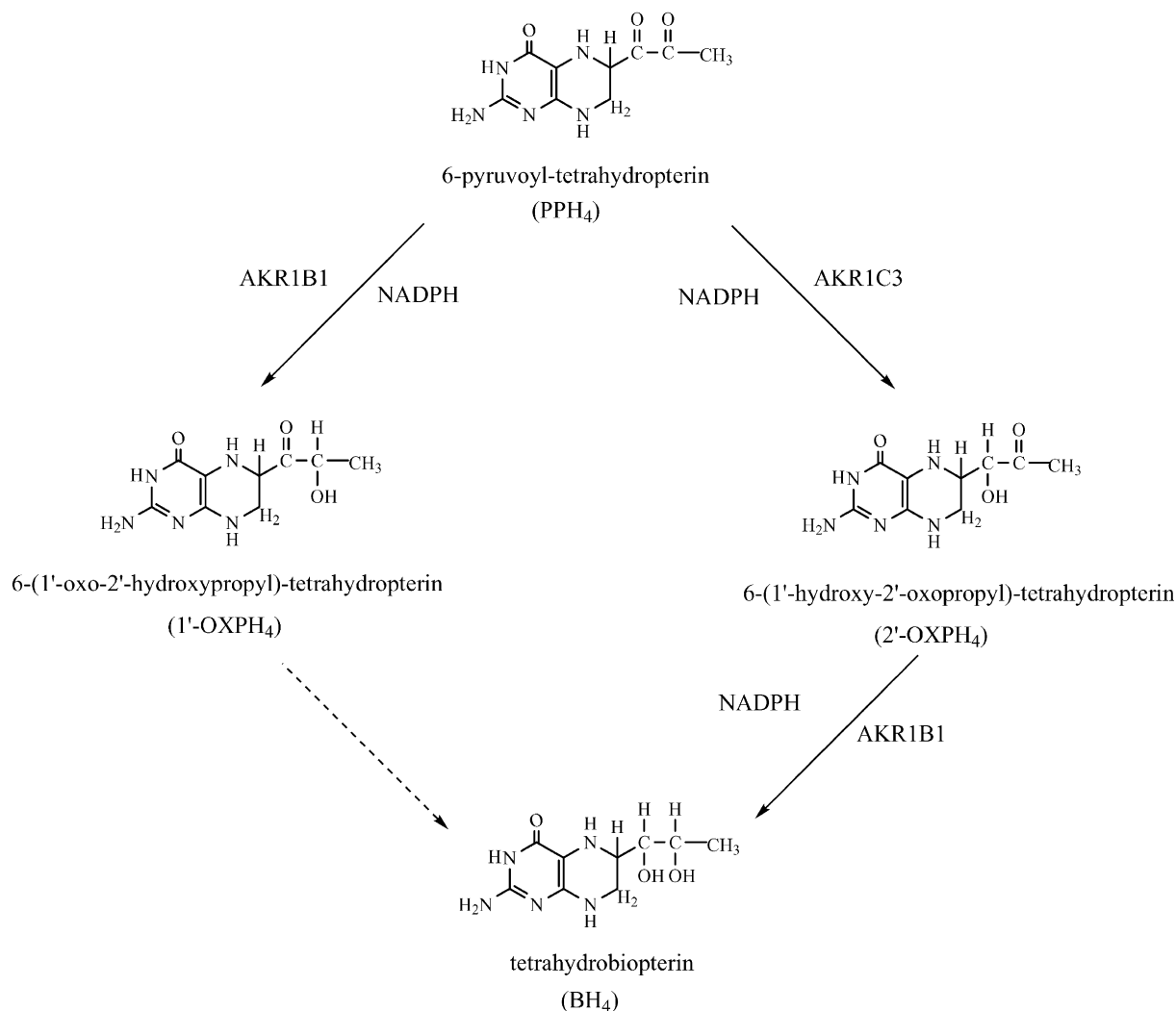


Fig. 4. A novel biosynthetic pathway of BH₄ from PPH₄ by AKR1C3 and AKR1B1.

additional properties unique for AKR1C3. Although the kinetic constants of the enzyme for PPH₄ may not be accurate because of the instability of the substrate, the predicted K_m and k_{cat} values are comparable with the values for the known steroidal and prostaglandin substrates [23,38–40]. Therefore, PPH₄ may be a novel endogenous substrate for this enzyme.

AKR1C3 is an additional enzyme to the previously known SPR and silkworm CRII that reduce the 1'-keto group of PPH₄. Furthermore, we showed the formation of BH₄ by the addition of AKR1B1 to the reaction mixture containing AKR1C3 and PPH₄, suggesting a novel alternative synthetic pathway of BH₄ from PPH₄ in the absence of SPR (Fig. 4). The mRNAs for AKR1C3 [40] and AKR1B1 [41] are expressed in many human tissues. However, the expression level of AKR1C3 mRNA in the brain was very low compared to those in other tissues, such as those in the liver, lung, prostate, and mammary gland, and the role of the novel synthetic pathway in the brain remains unknown.

SPR deficiency can be diagnosed only by investigation of pterin metabolites in cerebrospinal fluid (CSF), in which the neopterin level is normal but those of bi-apterin and BH₂ are high [13]. Furthermore, patients with SPR deficiency display normal urinary pterins without hyperphenylalaninemia. In order to explain these facts of SPR deficiency, Blau et al. [13] proposed a BH₄ biosynthetic salvage pathway, such as PPH₄ → 1'-OXPH₄ → sepiapterin → BH₂ → BH₄. In this pathway, 1'-OXPH₄ is produced from PPH₄ by aldose reductase (AKR1B1) and is nonenzymatically converted into sepiapterin, which is then reduced to BH₂ by carbonyl reductase. The final reduction of BH₂ to BH₄ is catalyzed by dihydrofolate reductase (DHFR, EC 1.5.1.3) [12–14]. The activity of DHFR in the brain is approximately 10-fold lower than that in the liver [42], suggesting that the reduction step is slow. This is also supported by a recent finding that the content of sepiapterin elevates in CSF of a patient with SPR deficiency [43]. However, the present result indicated that the rate of nonenzymatic conversion of 1'-OXPH₄ into sepiapterin is very slow, which suggests that it may be very difficult for sepiapterin formation from 1'-OXPH₄ to proceed in the proposed pathway.

The present discovery of the new synthetic pathway of BH₄ from PPH₄ by the combination of AKR1C3 and AKR1B1 probably proceeds more rapidly than the above salvage pathway containing a nonenzymatic step. In addition, this pathway provides a reasonable explanation for the characterization of SPR deficiency, such as abnormal CSF metabolites and normal urinary pterins without hyperphenylalaninemia. In many tissues of patients with SPR deficiency, BH₄ can be produced from PPH₄ by AKR1C3 and AKR1B1, leading to normal urinary pterins without hyperphenylalaninemia. However, the amount of the formation of BH₄ in the

brain is insufficient to exhibit its physiological function because of the low expression of AKR1C3 mRNA [40]. For this reason, a patient with SPR deficiency may show progressive psychomotor retardation and dystonia. In contrast to the low expression of AKR1C3, AKR1B1 is expressed at a high level in the brain [41] and produces 1'-OXPH₄ from PPH₄. The accumulation of 1'-OXPH₄ in the brain of a patient with SPR deficiency may produce abnormal CSF metabolites. Further studies on regional distribution of AKR1B1 and AKR1C3 and their co-localization in brain cells will be necessary to clarify the significance of this alternative BH₄ biosynthetic pathway.

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