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EFFECTS OF STRUCTURES OF TETRAHYDROPTERIN COFACTORS ON TYROSINE HYDROXYLASE

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Summary

Two pairs of stereochemical isomers of new tetrahydropterin cofactors for tyrosine hydroxylase, i.e., 6-L-hydroxyethyl- and 6-D-hydroxyethyltetrahydropterins, and 6-L-dihydroxyethyl- and 6-D-dihydroxyethyltetrahydropterins, have been synthesized. By using these new stereochemical isomers as well as 6-methyl- and 6-hydroxymethyltetrahydropterins, the effects of the structure of the 6-substituent of tetrahydropterin cofactors on tyrosine hydroxylase purified from the soluble fraction of bovine adrenal medulla have been examined. The K_m value of tyrosine with 6-L-hydroxyethyl- or 6-L-dihydroxyethyltetrahydropterin as cofactor was significantly lower than that obtained with 6-D-hydroxyethyl- or 6-D-dihydroxyethyltetrahydropterin, indicating a profound effect of the configuration at C-1′ (the carbon of the side-chain nearest to the pteridine ring) on the K_m values of the substrate. In addition, tyrosine was inhibitory at a high concentration only with L-isomers. The K_m values for the tetrahydropterin cofactors were also found to be structure-dependent: 6-L(and D)-hydroxyethyltetrahydropterins or 6-methyltetrahydropterin exhibited significantly lower K_m values than the 6-L(and D)-dihydroxyethyl or 6-hydroxymethyl analogues, respectively. This result clearly shows that the K_m values are influenced more by the presence of a hydroxyl group at the terminal carbon of the 6-substituent than by the C-1′ configuration, as long as the lengths of the carbon chain of the substituents are the same.

Introduction

The 5,6,7,8-tetrahydro derivatives of various 2-amino-4-hydroxypteridines (5,6,7,8-tetrahydropterins) with an alkyl or polyhydroxyalkyl substituent at the 6-position act as a cofactor for tyrosine hydroxylase [1—4]. The cofactor activities of the pterins vary depending upon the structure of the side-chain; and L-*erythro*-tetrahydrobiopterin, which has an L-*erythro*-1,2-dihydroxypropyl side chain and is thought to be the natural cofactor of tyrosine hydroxylase, was found to have the most active cofactor properties among the various pterins examined [1—5]. Recently we synthesized the four stereochemical isomers of biopterin, i.e., 6-L-*erythro*-, 6-D-*erythro*-, 6-L-*threo*- and 6-D-*threo*-1,2-dihydroxypropylpterins [6], and examined the cofactor activities of the 5,6,7,8-tetrahydro derivatives of these stereochemical isomers for tyrosine hydroxylase activity [5]. The L-*erythro* and D-*threo* isomers, both of which have the same configuration at C-1′ (the carbon nearest to the pteridine ring) of the side-chain, exhibited similar cofactor characteristics. On the other hand, the D-*erythro* and L-*threo* isomers behaved like the pseudocofactors such as 6-methyl- and 6,7-dimethyltetrahydropterins, and were less active than the former pairs. They have the same but reversed C-1′ configuration compared to the others. These results suggest that the cofactor activities are controlled to some extent by the configuration at C-1′ of the side-chain [5]. To test this possibility, we have synthesized several tetrahydropterins having a side-chain at the 6-position with an asymmetric carbon only at the C-1′ position, and have examined their cofactor characteristics for tyrosine hydroxylase. 6-L-1′-Hydroxyethyl-, 6-D-1′-hydroxyethyl-, 6-L-1′,2′-dihydroxyethyl and 6-D-1′,2′-dihydroxyethyltetrahydropterins have been examined for this purpose. The examination of the cofactor characteristics has been extended to 6-methyl- and 6-hydroxymethyltetrahydropterins to see the effects of a hydroxyl group at the terminal carbon of the side-chain at the 6-position.

Materials and Methods

Two pairs of the isomeric pterins, 6-L(and D)-1′-hydroxyethylpterins and 6-L-(and D)-1′,2′-dihydroxyethylpterins, were synthesized by the method of Sugimoto and Matsuura [7]. 6-Methylpterin [8] and 6-hydroxymethylpterin [9] were also synthesized in our laboratory according to known methods. The purity of the pterins was confirmed by their ultraviolet spectra, by paper chromatography and by permanganate oxidation to the known pterin-6-carboxylic acid [10]. The corresponding 5,6,7,8-tetrahydro derivative of each pterin was prepared by catalytic hydrogenation in 0.1 M HCl using platinum oxide as catalyst [11]. The reduction was followed by examining the ultraviolet absorption spectrum. After completion of the reduction, the catalyst was removed by filtration, and the filtrate containing tetrahydropterin was sealed without air and stored at —20°C in the dark. The structures of these tetrahydropterins and of four stereochemical isomers of tetrahydrobiopterin are shown in Fig. 1. The molar concentrations of these tetrahydropterins were estimated, based on the extinction coefficient of 18 500 $M^{-1} \cdot cm^{-1}$ at 264 nm in 2 M HCl.

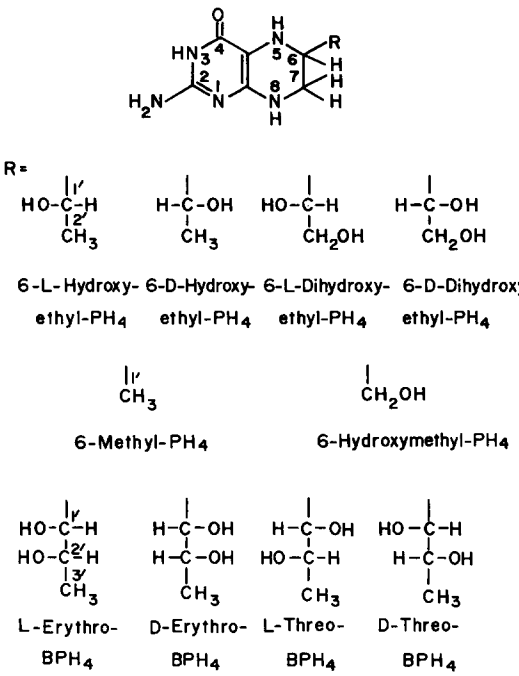

Fig. 1. Structures of tetrahydropterins. PH_4, tetrahydropterin; BPH_4, tetrahydrobiopterin.

Tyrosine hydroxylase was purified from bovine adrenal medulla by ammonium sulfate fractionation and Sephadex G-200 column chromatography, as described previously [5].

Tyrosine hydroxylase activity was measured by estimating the formation of dihydroxyphenylalanine from L-tyrosine by a newly-established fluorimetric method [12]. In this method, interfering substances for the fluorescence assay of dihydroxyphenylalanine are rapidly removed by a double-column procedure fitted together sequentially (the first column of Amberlite CG-50 and the second column of Al_2O_3), at 4°C. Thus, a concentration as low as 1 nmol of dihydroxyphenylalanine can be measured simply by using the native fluorescence (excitation at 280 nm and emission at 320 nm). In some experiments, tyrosine hydroxylase activity was also measured by estimating the formation of [^{14}C]dihydroxyphenylalanine from L-[U-^{14}C]tyrosine [13,14]. The incubation mixture contained 0.2 M sodium acetate buffer (to obtain the final pH of 6.0), 1 mM $FeSO_4$, the enzyme, 0.1 M mercaptoethanol, 1.0 mM (or at various concentrations for kinetic studies) tetrahydropterin in 0.1 M HCl, 0.1 mM (or at various concentrations for kinetic studies) L-tyrosine (including 0.07 μCi L-[U-^{14}C]tyrosine (483 Ci/mol) for radioassay). D-Tyrosine was used in controls for the fluorimetry and in enzyme incubation for the radioassay. The reaction began with the addition of tyrosine. The K_m values and maximal velocities (V) were determined from Lineweaver-Burk plots [15] using Wilkinson's program [16].

Results

K_m value for tyrosine with each tetrahydropterin as cofactor

The K_m value for tyrosine was measured in air (20.9% O_2) with each tetrahydropterin cofactor at a concentration of 1 mM (but 6-L-hydroxyethyl- and 6-D-hydroxyethyltetrahydropterins at 0.6 mM) and the value was obtained from the Lineweaver-Burk plots (Table I). As shown in Fig. 2, 6-L-hydroxyethyl- and 6-L-dihydroxyethyltetrahydropterins, both of which have the same L-configuration at the C-1′ position of the side-chain, revealed similar Michaelis-Menten and Lineweaver-Burk plots; tyrosine was inhibitory at concentrations higher than 100 μM, and the K_m values for tyrosine were approx. 15 μM and 20 μM, respectively. In contrast, 6-D-hydroxyethyl- and 6-D-dihydroxyethyltetrahydropterins, both of which have the same but reversed D-configuration, were similar to each other but very different from the L-isomers as cofactors; tyrosine was not inhibitory at high concentrations up to 400 μM, and the K_m values for tyrosine were high at about 40 μM and 60 μM, respectively. Thus, 6-L-hydroxyethyltetrahydropterin as cofactor gave the lowest K_m value for tyrosine, followed by 6-L-dihydroxyethyl-, 6-D-hydroxyethyl- and 6-D-dihydroxyethyltetrahydropterins in an increasing order of the K_m values for tyrosine. 6-Methyl- and 6-hydroxymethyltetrahydropterins were found to be similar to 6-D-mono- and dihydroxyethyltetrahydropterins as cofactors, and gave the K_m values for tyrosine of approx. 40 μM and 30 μM, respectively.

K_m value of each tetrahydropterin as cofactor

The K_m of each tetrahydropterin cofactor itself was determined in the pres-

TABLE I

V AND K_m VALUES OF TETRAHYDROPTERINS AND TYROSINE WITH EACH TETRAHYDROPTERIN AS COFACTOR OF TYROSINE HYDROXYLASE

Tyrosine hydroxylase activity was assayed with each tetrahydropterin as cofactor. K_m values of each tetrahydropterin and of tyrosine were determined from Lineweaver-Burk plots using 0.1 mM tyrosine or 1 mM tetrahydropterin [or 0.6 mM 6-L(and D)-hydroxyethyltetrahydropterin] in air using Wilkinson's program and expressed as mean ± S.E. V values were determined from Lineweaver-Burk plots using various concentrations of tyrosine with 1 mM of each tetrahydropterin [or 0.6 mM 6-L(and D)-hydroxyethyltetrahydropterin], or using various concentrations of each tetrahydropterin with 0.1 mM tyrosine. Dihydroxyphenylalanine formed from tyrosine was assayed fluorimetrically [12], except the values in parentheses which were determined by radioassay [13,14] using L-[^{14}C]tyrosine as substrate.

Tetrahydropterin	K_m for tyrosine (μM)	K_m for tetrahydropterin (μM)	V (nmol/min per mg protein)	
			Tyrosine	Tetrahydropterin
6-L-Hydroxyethyltetrahydropterin	14 ± 0.4	57 ± 5	5.5 ± 0.1	5.9 ± 0.4
6-D-Hydroxyethyltetrahydropterin	40 ± 5	89 ± 6	6.1 ± 0.2	5.8 ± 0.1
6-L-Dihydroxyethyltetrahydropterin	22 ± 1 (25 ± 2)	218 ± 16 (225 ± 35)	7.6 ± 0.1	7.3 ± 0.1
6-D-Dihydroxyethyltetrahydropterin	64 ± 12 (73 ± 6)	234 ± 10 (176 ± 39)	5.4 ± 0.3	4.1 ± 0.1
6-Methyltetrahydropterin	38 ± 3	115 ± 9 (120 ± 11)	13.7 ± 0.3	10.7 ± 0.2
6-Hydroxymethyltetrahydropterin	28 ± 3	230 ± 22 (224 ± 25)	8.3 ± 0.2	9.8 ± 0.3

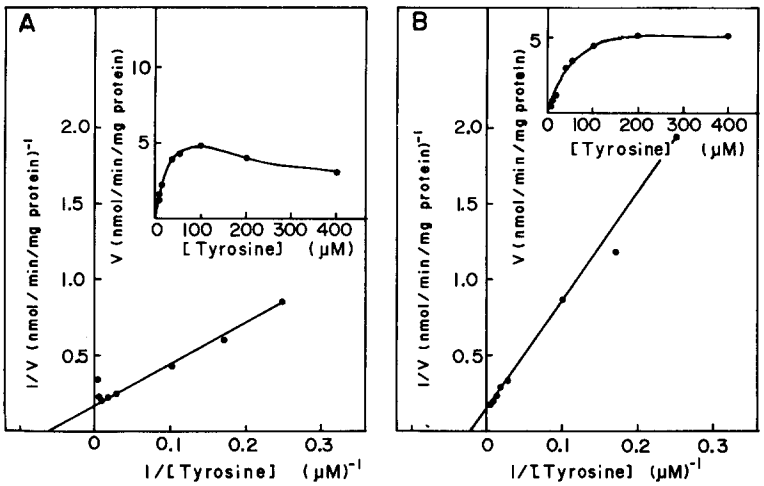

Fig. 2. Lineweaver-Burk and Michealis-Menten plots illustrating the effect of tyrosine with: (A) 6-L-hydroxyethyl- and (B) 6-D-hydroxyethyltetrahydropterin, as cofactor, on the rate of dihydroxyphenylalanine formation by tyrosine hydroxylase. Incubation was done in air (20.9% oxygen) using 0.6 mM of each tetrahydropterin as cofactor.

ence of 100 μM tyrosine as substrate in air (Fig. 2 and Table I). We previously reported that L-*erythro*- and D-*threo*-tetrahydrobiopterin, both of which have the same L-configuration at C-1′ of the side-chain as 6-L-hydroxyethyl- and 6-L-dihydroxyethyltetrahydropterins, showed non-Michaelis-Menten kinetics and two K_m values depending on the concentrations of the tetrahydrobiopterin [5]. However, either 6-L-hydroxyethyl- or 6-L-dihydroxyethylpterins showed classical Michaelis-Menten kinetics and gave a single K_m value.

6-L-Hydroxyethyl- and 6-D-hydroxyethyltetrahydropterins gave lower K_m values than the corresponding dihydroxyethyl analogues, suggesting that the hydroxyl group at the terminal carbon of the side-chain increased the K_m value for the tetrahydropterin. In accordance with this assumption, 6-methyltetrahydropterin also gave a lower K_m value than 6-hydroxymethyltetrahydropterin. However, the K_m values of pterins were not so much different between 6-L-hydroxyethyl- and 6-D-hydroxyethyltetrahydropterins, and between 6-L-dihydroxyethyl- and 6-D-dihydroxyethyltetrahydropterins.

Maximal velocity (V) of tyrosine hydroxylase with each tetrahydropterin as cofactor

The apparent V values with each tetrahydropterin as cofactor at 1 mM and in air (20.9% O_2) extrapolated to an infinite concentration of tyrosine, and that with 0.1 mM tyrosine and in air extrapolated to an infinite concentration of each tetrahydropterin cofactor, were obtained from the Lineweaver-Burk plots (Table I). V values were similar with 6-L-hydroxyethyl- and 6-D-hydroxyethyltetrahydropterins as cofactor. However, 6-L-dihydroxyethyltetrahydropterin gave a higher V value than 6-D-dihydroxyethyltetrahydropterin.

Discussion

6-L-Hydroxyethyl- and 6-L-dihydroxyethyltetrahydropterins, both of which have the same L-configuration at C-1′ of the side-chain as the putative natural cofactor L-*erythro*-tetrahydrobiopterin, were found to exhibit similar cofactor characteristics; tyrosine was inhibitory at high concentrations and had low K_m values in the presence of these cofactors. In contrast, in the presence of 6-D-hydroxyethyl- and 6-D-dihydroxyethyltetrahydropterin as cofactors, tyrosine was not inhibitory up to 400 μM and had higher K_m values. These results agree with our previous findings that among the four stereochemical isomers of tetrahydrobiopterin, the L-*erythro* and D-*threo* isomers exhibited similar and more active cofactor characteristics as compared with the D-*erythro* and L-*threo* isomers [5]. L-*erythro* and D-*threo* isomers have the same L-configuration at the C-1′ position of the side-chain, whereas D-*erythro* and L-*threo* isomers have the same, though reversed, C-1′ configuration. The present results support our previously reported conclusion that the K_m values for tyrosine are controlled to a fair extent by the configuration at C-1′ of the side-chain and the configuration at C-1′ of L-*erythro*-tetrahydrobiopterin may be favourable for the cofactor activity.

Interestingly, the present results also show the profound effect on the cofactor characteristics by a hydroxyl group at the terminal carbon of the side chain. In general, as long as the lengths of the carbon chains are the same, those tetrahydropterins possessing a methyl group at the end of the side-chain exhibited much lower K_m values for the cofactor than those having a hydroxymethyl group. 6-L-Dihydroxyethyl- or 6-D-dihydroxyethyltetrahydropterin, and 6-hydroxymethyltetrahydropterin had higher K_m values than 6-Lhydroxyethyl- or 6-D-hydroxyethyltetrahydropterin, and 6-methyltetrahydropterin, respectively (Table I). Thus, the K_m value for tetrahydropterin is increased when a hydroxyl group is introduced into the terminal methyl group of the side-chain. The same tendency was also observed with 6-L-*erythro*-1′,2′-dihydroxypropyl- and 6-L-*erythro*-1′,2′,3′-trihydroxypropyltetrahydropterins, where the former exhibited a lower K_m value than the latter [4,5],

Since the reduction of the pterin to the tetrahydro derivative introduces another center of asymmetry at position 6, the chemical reduction should produce a mixture of enantiomers of diastereoisomers. Each of such stereochemical isomers of tetrahydropterin may have a different K_m value. In fact, we have reported that, as to the configuration at position 6, the 'natural' (6*R*) L-*erythro*-tetrahydrobiopterin exhibited the same, but more intense, cofactor characteristics as those shown by a mixture of the (6*R*) and (6*S*) diastereoisomers produced by catalytic hydrogenation over platinum [17]. We have tried to separate the diastereoisomers of 6-L-dihydroxyethyl-, 6-D-hydroxyethyl-, 6-L-dihydroxyethyl- or 6-D-dihydroxyethyltetrahydropterins by high performance liquid chromatography by the method of Bailey and Ayling [18], but none of the diastereoisomers could be separated efficiently. The CD spectrum of each reduction showed a feeble signal, indicating that the relative amounts of the diastereoisomers are nearly equal. This may suggest that the characteristic cofactor properties observed in this study with a mixture of diastereoisomers are mainly due to the diastereoisomers having the 'natural' configuration at position 6.

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