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EFFECTS OF STEREOCHEMICAL STRUCTURES OF TETRAHYDROBIOPTERIN ON TYROSINE HYDROXYLASE

YUKIKO NUMATA (SUDO) [a], TAKESHI KATO [a], TOSHIHARU NAGATSU [b], TAKASHI SUGIMOTO [c] and SADAO MATSUURA [c]

[a] *Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, Nagoya 464,* [b] *Laboratory of Cell Physiology, Department of Life Chemistry, Graduate School at Nagatsuda, Tokyo Institute of Technology, Tokyo 152, and* [c] *Department of Chemistry, College of General Education, Nagoya University, Nagoya 464 (Japan)*

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Summary

1. Four stereochemical isomers of tetrahydrobiopterin, i.e., 6-L-*erythro*-, 6-D-*erythro*-, 6-L-*threo*-, or 6-D-*threo*-1,2-dihydroxypropyltetrahydropterin, have been synthesized and used as cofactors for tyrosine hydroxylase (EC 1.14.18.-) purified from the soluble fraction of bovine adrenal medulla. The L-*erythro*- (the putative natural cofactor) and D-*threo* isomers showed a striking similarity in their cofactor activities for tyrosine hydroxylase; the remaining two isomeric tetrahydrobiopterins, D-*erythro* and L-*threo* isomers, also had very similar cofactor characteristics.

2. The K_m values of the L-*erythro* and D-*threo* isomers as cofactor were found to be dependent on their concentrations. When their concentrations were below 100 μM, the K_m values of the L-*erythro* and D-*threo* isomers were fairly low (about 20 μM). However, the K_m values were markedly higher (about 150 μM) at concentrations above 100 μM. The same kinetic behavior was also observed with the tetrahydrobiopterin prepared from a natural source (bullfrog). In contrast, the K_m value of the L-*threo* or D-*erythro* isomer was found to be independent of the concentration and remained constant throughout the concentration examined.

3. The K_m values of tyrosine did not show much difference (from 20 μM to 30 μM) with respect to the structure of the four isomeric cofactors. At high concentrations tyrosine inhibited the enzymatic reaction with any one of the four tetrahydrobiopterin cofactors.

4. Oxygen at high concentrations was also inhibitory with any one of the four stereochemical isomers as cofactor. Approximate K_m values for oxygen with the tetrahydrobiopterins as cofactor were 1—5%.

5. In contrast to the four isomers of tetrahydrobiopterin, when 6-methyltetrahydropterin or 6,7-dimethyltetrahydropterin was used as cofactor tyrosine

or oxygen did not inhibit the enzymatic reaction at high concentrations, and the K_m values toward the pterin cofactor, tyrosine, and oxygen were significantly higher than the K_m values with the tetrahydrobiopterins as cofactor.

Introduction

Various 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridines (5,6,7,8-tetrahydropterins) act as a cofactor of tyrosine hydroxylase (EC 1.14.16.-) [1—4]. 6-L-*Erythro*-1,2-dihydroxypropyltetrahydropterin [6-L-*erythro*-tetrahydrobiopterin], which is the natural cofactor of rat liver phenylalanine hydroxylase [5], is thought to be the natural cofactor also of tyrosine hydroxylase [6].

We have previously synthesized several tetrahydroneopterins which have a stereochemically isomeric trihydroxypropyl groups at the 6-position, and have studied their cofactor activities for tyrosine hydroxylase. It was found that all three stereoisomers of the tetrahydroneopterins (L-*erythro*, D-*erythro*, and D-*threo* forms) have similar cofactor characters. We used stereochemical isomers of tetrahydroneopterins in the previous study mainly because they can be synthesized more easily than the tetrahydrobiopterins.

In the present study, we synthesized four stereochemically isomeric tetrahydrobiopterins, the L-*erythro*, D-*erythro*, L-*threo*, and D-*threo* isomers. Of these isomers, the L-*erythro* isomer is the putative natural cofactor of the tyrosine hydroxylase. The kinetic properties of tyrosine hydroxylase purified from the soluble fraction of bovine adrenal medulla with each stereoisomer of tetrahydrobiopterin as cofactor were compared.

Materials and Methods

6,7-Dimethyltetrahydropterin and 6-methyltetrahydropterin were purchased from Calbiochem. The isomeric biopterins, 6-L-*erythro*-biopterin, 6-D-*erythro*-biopterin, 6-L-*threo*biopterin, and 6-D-*threo*biopterin were synthesized by the method of Sugimoto and Matsuura [7]. This method involves the condensation of 2,4,5-triamino-6-hydroxypyrimidine with the respective 5-deoxypentose phenylhydrazones followed by oxidation with hydrogen peroxide in the presence of ferricyanide and potassium iodide. The only by-product in this method was unsubstituted pterin which was easily separated during purification and no 7-isomer of biopterin was detected. The purity of the biopterins was confirmed by their ultraviolet spectra [8], R_f values, and by permanganate oxidation; the oxidation of the biopterins by potassium permanganate gave only 6-carboxypterin and no isomeric 7-carboxypterin was detected. Authentic L-*erythro*-biopterin isolated from bullfrog [9] was kindly supplied by Prof. M. Tsusue of Kitasato University. 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 [10]. The reduction was followed by examining the ultraviolet absorbance spectrum. After completion of the reduction, the catalyst was removed by filtration, and the filtrate containing tetrahydrobiopterin was sealed without air and stored at −20°C in the dark. The structures of these tetrahydrobiopterin isomers are shown in Fig. 1. The molar concentrations of

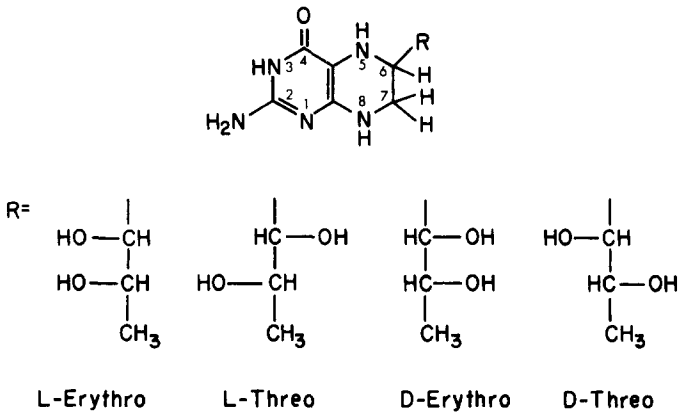


Fig. 1. Structures of stereochemical isomers of tetrahydrobiopterin.

6,7-dimethyltetrahydropterin and 6-methyltetrahydropterin were estimated based on the extinction coefficient of $16.0 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 265 nm in 0.1 M HCl, as reported by Whiteley and Huennekens [11]. The molar concentrations of the tetrahydrobiopterins were estimated based on the extinction coefficient of $18 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 264 nm in 1.5 M HCl, which was determined for 6-L-*erythro*tetrahydrobiopterin.

Tyrosine hydroxylase was purified from bovine adrenal medulla by a procedure similar to that described by Joh et al. [12]. Bovine adrenal medulla was homogenized in 5 vols. of 0.25 M sucrose, and the soluble fraction was isolated by centrifugation at 100 000 $\times g$ for 60 min. The enzyme in the supernatant was precipitated by adding solid ammonium sulfate until the concentration reached 25—40% saturation. After centrifugation, the precipitate was dissolved in 20 mM potassium phosphate buffer, pH 6.5, and then chromatographed on a Sephadex G-200 column equilibrated with 20 mM potassium phosphate buffer, pH 6.5, containing 2% ammonium sulfate. The same buffer was used for elution, and the most active fractions were combined and used as the enzyme.

Tyrosine hydroxylase activity was measured by estimating the formation of [^{14}C]dopa 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 containing 0.07 μCi L-[U-^{14}C]-tyrosine (483 mCi/mmol) and water to make up a total volume of 0.5 ml. For controls, water was added instead of enzyme. Boiled enzyme controls were also carried out, and both control values were similar (about 350 cpm). The reaction began with the addition of tyrosine to obtain K_m values for the tetrahydrobiopterin and for tyrosine or with the addition of enzyme to obtain K_m values for oxygen, and continued for 15 min at 30° C in a metabolic shaker in air or in an atmosphere containing various concentrations of oxygen. The dopa formed was isolated using an alumina column and determined using a liquid scintillation spectrometer. The K_m values and maximal velocities (V) were

determined from Lineweaver-Burk plots [15] on a FACOM-230 computer using Wilkinson's program [16].

Results

(A) The K_m values of the stereochemical tetrahydrobiopterin isomers

The K_m values of each stereoisomeric tetrahydrobiopterin cofactor itself were obtained by using 100 μM tyrosine as substrate in air (20.9% oxygen) (Table I).

The Lineweaver-Burk plots for the L-*erythro*- or D-*threo*tetrahydrobiopterin were very similar. Interestingly, for both isomers two different K_m values were obtained depending on whether the concentrations were lower or higher than 100 μM. At low concentrations (less than 100 μM) a relatively low K_m value of about 20 μM was observed, whereas at higher concentrations (higher than 100 μM) the K_m value was much higher and registered about 150 μM (Fig. 2). An essentially similar kinetic behavior was also observed with L-*erythro*tetrahydrobiopterin prepared from the natural L-*erythro*biopterin isolated from bullfrog skin; two K_m values were obtained depending on whether the concentration was above or below 100 μM, and the values were the same as with L-*erythro* isomer within experimental errors.

In contrast, the L-*threo* or D-*erythro* isomer gave a single K_m value of about 50 μM regardless of concentrations (Fig. 3).

6-Methyltetrahydropterin or 6,7-dimethyltetrahydropterin also gave a single K_m value, but the values (approx. 100 μM) were much higher than the tetrahydrobiopterin values.

TABLE I

K_m VALUES OF TETRAHYDROBIOPTERINS, TYROSINE, AND OXYGEN WITH EACH TETRAHYDROPTERIN AS COFACTOR OF TYROSINE HYDROXYLASE

Tyrosine hydroxylase activity was assayed as descibed in Methods with each tetrahydropterin as cofactor. K_m values of each tetrahydropterin and of tyrosine were determined from Lineweaver-Burk plots using $1.0 \cdot 10^{-4}$ M tyrosine or $1.0 \cdot 10^{-3}$ M tetrahydropterins in air on a FACOM-230 computer using Wilkinson's program [14] and expressed as mean ± standard error of mean. V values were also determined on a computer from Lineweaver-Burk plots using various concentrations of tyrosine with $1.0 \cdot 10^{-3}$ M of each tetrahydropterin in air. Apparent K_m for oxygen was obtained from Michaelis-Menten curves using $1.0 \cdot 10^{-4}$ M tyrosine and $1.0 \cdot 10^{-3}$ M tetrahydropterin under various oxygen concentration.

Tetrahydropterin	K_m for tetrahydropterin (μM)	K_m for tyrosine (μM)	K_m for O_2 (%)	V (nmol/min per mg protein)
L-*erythro* tetrahydrobiopterin		21 ± 3	1.5	11.73 ± 0.77
100 μM>	19 ± 4			
100 μM<	142 ± 27			
D-*threo* tetrahydrobiopterin		22 ± 4	1.0	10.78 ± 0.90
100 μM>	21 ± 4			
100 μM<	148 ± 32			
L-*threo* tetrahydrobiopterin	58 ± 6	35 ± 2	4.4	8.15 ± 0.23
D-*erythro* tetrahydrobiopterin	37 ± 5	33 ± 2	2.7	6.14 ± 0.13
6-methyltetrahydropterin	103 ± 6	52 ± 5	6.2	13.9 ± 0.43
6,7-dimethyltetrahydropterin	87 ± 6	90 ± 8	15.7	9.60 ± 0.34

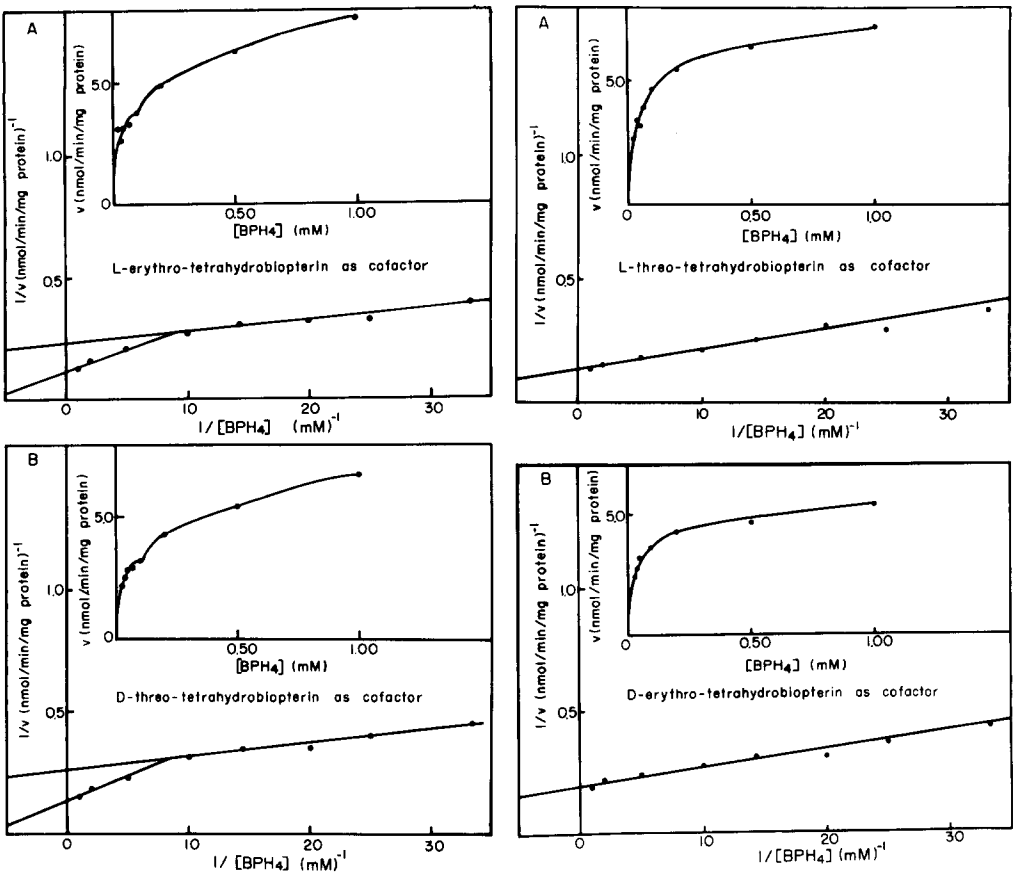


Fig. 2. Lineweaver-Burk and Michaelis-Menten plots illustrating the effect of L-*erythro* isomer (A) and D-*threo* isomer (B) of tetrahydrobiopterins on the rate of dopa formation by tyrosine hydroxylase. Incubation was carried out in air (oxygen concentration, 20.9%) using 100 μM tyrosine.

Fig. 3. Lineweaver-Burk and Michaelis-Menten plots illustrating the effect of L-*threo* isomer (A) and D-*erythro* isomer (B) of tetrahydrobiopterins on the rate of dopa formation by tyrosine hydroxylase. Incubation was carried out in air (oxygen concentration, 20.9%) using 100 μM tyrosine.

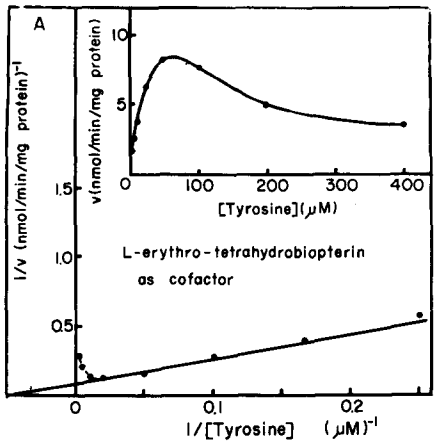
(B) The K_m value of tyrosine with the stereochemical tetrahydrobiopterin isomers as cofactor

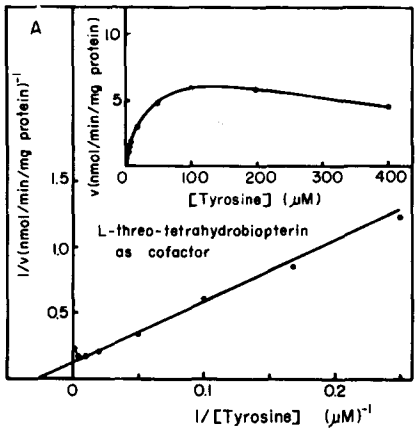
The K_m values of tyrosine were measured in air (20.9% oxygen) with each tetrahydrobiopterin cofactor at 1 mM and the values were obtained from the Lineweaver-Burk plots (Table I).

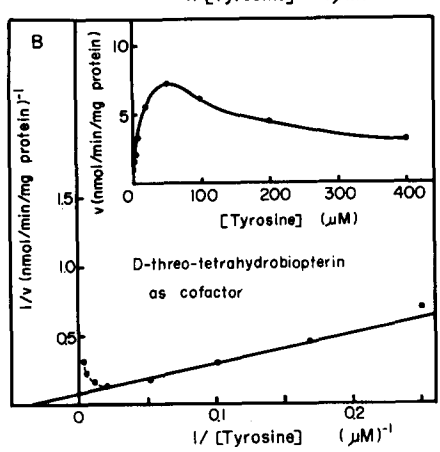
L-*Erythro*-tetrahydrobiopterin gave Michaelis-Menten plots and Lineweaver-Burk plots similar to those of the D-*threo* isomer (Fig. 4). Tyrosine was inhibitory at a concentration higher than 50 μM. The apparent K_m values of tyrosine were about 20 μM.

The L-*threo*- and D-*erythro*tetrahydrobiopterin isomers also gave similar Michaelis-Menten plots and Lineweaver-Burk plots, but tyrosine was inhibitory only at concentrations higher than 200 μM. The apparent K_m values were about 30 μM (Fig. 5).

With 6-methyltetrahydropterin or 6,7-dimethyltetrahydropterin as cofactor,







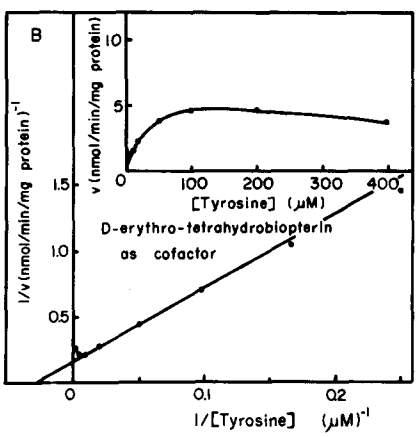


Fig. 4. Lineweaver-Burk and Michaelis-Menten plots illustrating the effect of tyrosine with L-*erythro* isomer (A) and D-*threo* isomer (B) of tetrahydrobiopterins as cofactor on the rate of dopa formation by tyrosine hydroxylase. Incubation was carried out in air (oxygen concentration, 20.9%) using 1 mM tetrahydrobiopterin as cofactor.

Fig. 5. Lineweaver-Burk and Michaelis-Menten plots illustrating the effect of tyrosine with L-*threo* isomer (A) and D-*erythro* isomer (B) of tetrahydrobiopterins as cofactor on the rate of dopa formation by tyrosine hydroxylase. Incubation was carried out in air (oxygen concentration, 20.9%) using 1 mM tetrahydrobiopterin as cofactor.

tyrosine was not inhibitory at high concentrations, up to 400 μM, and the K_m value toward tyrosine was higher than the K_m when the tetrahydrobiopterins were present as cofactor.

(C) The K_m value of oxygen with the stereochemical tetrahydrobiopterin isomers as cofactor

Michaelis-Menten curves toward oxygen concentrations with the tetrahydrobiopterin isomers, 6-methyltetrahydropterin and 6,7-dimethyltetrahydropterin as cofactor and $1.0 \cdot 10^{-4}$ M tyrosine as substrate are shown in Fig. 6, and the apparent K_m values in Table I.

When the L-*erythro*- or D-*threo*tetrahydrobiopterin isomer was present as cofactor, oxygen became inhibitory when its concentrations exceeded 2.5%.

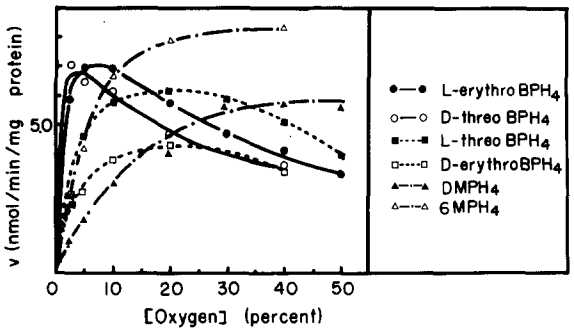


Fig. 6. Michaelis-Menten plots illustrating the effect of oxygen with each tetrahydropterin as cofactor on the rate of dopa formation from tyrosine. Incubation was carried out under various oxygen concentrations using 1 mM tetrahydropterin as cofactor and 100 μM tyrosine. ●———●, L-*erythro* tetrahydrobiopterin; ○———○, D-*threo*-tetrahydrobiopterin; ■ . . . . . ■, L-*threo*-tetrahydrobiopterin; □ . . . . . □, D-*erythro*-tetrahydrobiopterin; ▲- · - · - ·▲, 6,7-dimethyltetrahydropterin; and △- · - · - ·△, 6-methyltetrahydropterin.

When the L-*threo*- or D-*erythro*tetrahydrobiopterin isomer was present as cofactor, oxygen was also inhibitory at concentrations higher than 20%.

When 6-methyltetrahydropterin or 6,7-dimethyltetrahydropterin was used as cofactor, oxygen was not inhibitory even at 40% concentration.

(D) Maximal velocities (V) of tyrosine hydroxylase with the stereochemical tetrahydrobiopterin isomers

The *V* values with the steroechemical tetrahydrobiopterin isomers as cofactor at 1 mM and in air were determined from the Lineweaver-Burk plot using various concentrations of tyrosine (Table I). Among the four isomeric tetrahydrobiopterins the *V* value was highest with the L-*erythro* isomer and decreased in the following order: L-*erythro*, D-*threo*, L-*threo*, and D-*erythro* tetrahydrobiopterin.

Discussion

Among the four stereochemical isomers of tetrahydrobiopterin, the L-*erythro* isomer and D-*threo* isomers showed similar kinetic properties: (1) they showed two distinct K_m values for tetrahydrobiopterin itself, depending on concentration; (2) tyrosine, the substrate, at concentrations higher than 50 μM was inhibitory; (3) oxygen at concentrations higher than 2.5% was inhibitory. On the other hand, the kinetic properties of the L-*threo* and D-*erythro* isomers were similar: (1) they showed a single K_m value for tetrahydrobiopterin itself; (2) tyrosine was inhibitory, but only at concentrations higher than 200 μM; (3) oxygen was inhibitory, but only at concentrations higher than 20%.

Inhibition by tyrosine or oxygen at high concentrations in the presence of L-*erythro*-tetrahydrobiopterin as cofactor had been first reported by Kaufman et al. [17,18].

In contrast to the tetrahydrobiopterins, neither tyrosine nor oxygen was inhibitory when 6-methyltetrahydropterin or 6,7-dimethyltetrahydropterin was used as cofactor. This agrees with the Kaufman's results [19].

It is tempting to speculate that either the L-*erythro* or the D-*threo* isomer of tetrahydrobiopterin may be the natural cofactor, since among the four stereochemical isomers they have the lowest K_m values, the lowest K_m value toward tyrosine, the lowest K_m value toward oxygen, and the highest V. Since L-*erythro*tetrahydrobiopterin is the natural cofactor of phenylalanine hydroxylase, the L-*erythro* isomer could also be the natural cofactor of tyrosine hydroxylase.

The configurations of the dihydroxypropyl group at C-1′ (the carbon nearest to the pteridine ring) are exactly the same in the L-*erythro* and D-*threo*-tetrahydrobiopterins; the D-*erythro* and L-*threo* isomers also have the same configuration at C-1′, though in reverse compared to the other two isomers. It is notable that the cofactor characteristics are extensively controlled by the configuration at C-1′. Our results suggest that configuration at C-1′ such as in the L-*erythro* or D-*threo* isomer facilitates the binding of tyrosine and oxygen to tyrosine hydroxylase.

The reason why the two distinct K_m values of chemically reduced L-*erythro*-tetrahydrobiopterin could not be detected in our previous studies [3,4] may be due to the fact that more precise kinetic analysis was carried out in the present study.

The explanation of the presence of two distinct K_m values toward L-*erythro*- or D-*threo*-tetrahydrobiopterin is required.

The alteration of the K_m values of L-*erythro*- or D-*threo*tetrahydrobiopterins by the concentration could be due to the conformational change of tyrosine hydroxylase depending upon the concentrations of the tetrahydropterin cofactor or due to the presence of two forms of tyrosine hydroxylase in the soluble fraction of bovine adrenal medulla. If the latter is the case, these two forms of tyrosine hydroxylase can be differentiated only by using the L-*erythro*- or D-*threo*tetrahydrobiopterin as cofactor of tyrosine hydroxylase, since other stereochemical tetrahydrobiopterin isomers gave a single K_m value toward the tetrahydropterin itself. However, we have not yet succeeded in isolating two forms of tyrosine hydroxylase with a low K_m and a high K_m toward the L-*erythro*- or D-*threo*tetrahydrobiopterin. Since the chemical reduction of the pterin to the tetrahydroderivatives introduces another center of asymmetry at position 6, each pair of stereochemical isomers of tetrahydrobiopterin may have different K_m values. This possibility may be examined by reducing the pterin enzymatically.

Acknowledgements

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