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Effects of stereochemical structures of tetrahydropterins on tyrosine hydroxylase

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5,6,7,8-Tetrahydropteridine (tetrahydropterin) requires the presence of 2-amino and 4-hydroxy groups to act as a cofactor of tyrosine hydroxylase. The 6-alkyl group also affects the cofactor activity of tetrahydropterins; tetrahydrobiopterin (6-L-(*erythro*) dihydroxypropyltetrahydropterin) has been found to be the most active cofactor [1–3], and is supposed to be the natural cofactor of tyrosine hydroxylase [4]. These results suggest that the L-(*erythro*) dihydroxypropyl side chain at the 6-position of the pteridine ring may be stereochemically optimal for the cofactor of tyrosine hydroxylase. However, the effect of the stereochemical structure of the 6-substituents on the cofactor activity has not been determined. We have synthesized several tetrahydropterins with stereochemically isomeric trihydroxypropyl groups at the 6-position, and have compared their cofactor activities and K_m values for tyrosine hydroxylase. We used stereochemical isomers of trihydroxypropyltetrahydropterins mainly because they can be synthesized easier than those of dihydroxypropyltetrahydropterin (tetrahydrobiopterin). In addition we synthesized and examined two stereochemical isomers of tri- and tetra- hydroxybutyltetrahydropterins in order to see the effect of the length of the 6-alkyl side chain on the cofactor activity. Since most of previously published studies were carried out using 6,7-dimethyltetrahydropterin as a cofactor, this compound was used for reference. Also, tetrahydrobiopterin was examined as a putative natural cofactor of tyrosine hydroxylase.

6,7-Dimethyltetrahydropterin was purchased from CalBiochem. The following pterins were synthesized by a modification of the method of Viscontini [5]: 6-L-(*erythro*)dihydroxypropylpterin (biopterin), 6-L-(*erythro*)trihydroxypropylpterin, 6-D-(*erythro*)trihydroxypropylpterin, 6-D-(*threo*)trihydroxypropylpterin, 6-D-(*arabo*)tetrahydroxybutylpterin, and 6-L-(*arabo*)-trihydroxybutylpterin. The structures of these stereochemical isomers are shown in Fig. 1. The corresponding 5,6,7,8-tetrahydro derivative of each pterin was prepared by catalytic hydrogenation in 0·1 N HCl over platinum oxide catalyst [6]. The reduction was followed by examining the ultraviolet absorption spectrum. After the completion of reduction, the catalyst was removed by filtration, and the filtrate containing tetrahydropterin was stored at $-20°$ in the dark. Tetrahydropterins stored in this way were found to be stable for at least 3 months, when examined both by ultraviolet absorption spectra and by the cofactor activity for tyrosine hydroxylase.

The molar concentration of 6,7-dimethyltetrahydropterin was estimated from the extinction coefficient of 6-methyltetrahydropterin, $16{\cdot}0 \times 10^3\ M^{-1}, cm^{-1}$ at 265 nm in 0·1 N HCl, as reported by Whiteley and Huennekens [7]. The molar concentrations of other tetrahydropterins were estimated from the extinction coefficient of $18 \times 10^3\ M^{-1}, cm^{-1}$ at 264nm in 1·5 N HCl, which was determined for L-(*erythro*) tetrahydrobiopterin.

Tyrosine hydroxylase was purified from bovine adrenal medulla by a procedure similar to that described by Joh *et al.* [8]. Bovine adrenal medulla was homogenized in 4 vol of 20 mM potassium phosphate buffer, pH 6·5, and the soluble fraction was isolated after centrifuging at 800 *g* for 15 min, and then at 40,000 *g* for 60 min. The enzyme in the supernatant was precipitated by adding solid ammonium sulfate to 80% saturation. After centrifugation, the precipitate was dissolved in the buffer, and the solution was dialyzed against the same buffer containing 5% ammonium sulfate. The dialyzed solution was then chromatographed on a Sephadex G-200 column equilibrated with 20 mM potassium phosphate buffer, pH 6·5, containing 5% ammonium sulfate, and the same buffer was used for elution. The most active fractions were combined and used as the enzyme. The specific activity increased about 5-fold after the Sephadex G-200 step.

Tyrosine hydroxylase activity was measured by estimating the formation of [^{14}C]dopa from L-[^{14}C]tyrosine, as described previously [9]. The incubation mixture contained 0·2 M sodium acetate buffer pH 6·0, 1 mM $FeSO_4$, enzyme, 0·1 M mercaptoethanol, 1·0 mM (or at various concentrations for kinetic studies) tetrahydropterin in 0·1 N HCl, 0·1 mM (or

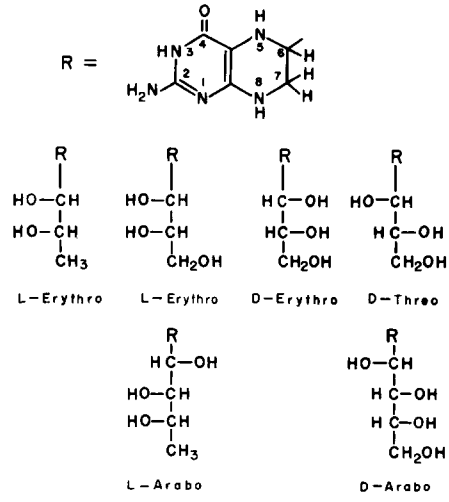

Fig. 1. Structures of stereochemical isomers of tetrahydropterins.

Table 1. Relative cofactor activity and K_m values of tetrahydropterins and of tyrosine with each tetrahydropterin as cofactor for tyrosine hydroxylase

Tetrahydropterin	Relative activity* (%)	K_m (M)	
		Pterin	Tyrosine
6,7-Dimethyltetrahydropterin	57	$1{\cdot}8 \times 10^{-4}$	$6{\cdot}7 \times 10^{-5}$
L-(*erythro*)Dihydroxypropyltetrahydropterin	100	$8{\cdot}8 \times 10^{-5}$	$7{\cdot}5 \times 10^{-6}$
L-(*erythro*)Trihydroxypropyltetrahydropterin	68	$2{\cdot}1 \times 10^{-4}$	$2{\cdot}9 \times 10^{-5}$
D-(*erythro*)Trihydroxypropyltetrahydropterin	53	$4{\cdot}1 \times 10^{-4}$	$4{\cdot}8 \times 10^{-5}$
D-(*threo*)Trihydroxypropyltetrahydropterin	107	$2{\cdot}2 \times 10^{-4}$	$3{\cdot}0 \times 10^{-5}$
D-(*arabo*)Tetrahydroxybutyltetrahydropterin	16	$8{\cdot}8 \times 10^{-4}$	$2{\cdot}5 \times 10^{-4}$
L-(*arabo*)Trihydroxybutyltetrahydropterin	29	$1{\cdot}2 \times 10^{-3}$	$1{\cdot}0 \times 10^{-4}$

* Relative activity is based on tetrahydrobiopterin. The activity was assayed in the presence of $1{\cdot}0 \times 10^{-4}$ M of tyrosine as substrate and $1{\cdot}0 \times 10^{-3}$ M tetrahydropterin as a cofactor. K_m value of each tetrahydropterin cofactor or that of tyrosine was obtained by a Lineweaver–Burk plot using $1{\cdot}0 \times 10^{-4}$ M tyrosine or $1{\cdot}0 \times 10^{-3}$ M tetrahydropterin. Each value represents the mean of duplicate determinations.

at various concentrations for kinetic studies) L-tyrosine containing 0·5 μCi L-[U-^{14}C]tyrosine (483 mCi/m-mole) and H_2O to make up a total incubation volume of 0·5 ml. The reaction was started by the addition of tyrosine, and was continued for 15 min at 30° in a metabolic shaker. The Dopa formed was isolated by an alumina column and its concentration was measured using a liquid scintillation spectrometer [9].

The cofactor activity and K_m value (with $1{\cdot}0 \times 10^{-4}$ M tyrosine as substrate) of each tetrahydropterin for tyrosine hydroxylase, and K_m value of tyrosine in the presence of each tetrahydropterin ($1{\cdot}0 \times 10^{-3}$ M) as a cofactor are shown in Table 1.

Among the three pairs of stereochemical isomers of trihydroxypropyltetrahydropterins, the D-*erythro* isomer showed activity similar to that of the L-*erythro* isomer which is expected to have the same stereochemical structure as the putative natural cofactor, L-(*erythro*)dihydroxypropyltetrahydropterin (tetrahydrobiopterin). The D-*threo* isomer gave a significantly higher activity than the L-*erythro* and D-*erythro* isomers. These results indicate that the L-*erythro* structure, as in tetrahydrobiopterin, is not essential for cofactor activity.

Both D-(*arabo*)tetrahydroxybutyltetrahydropterin and L-(*arabo*)trihydroxybutyltetrahydropterin gave significantly lower cofactor activities than other tetrahydropterins. This suggests that the optimum length of the carbon chain of the 6-alkyl group is from one to three, and that the cofactor activity of 6-alkyltetrahydropterin decreases when the number of carbon atoms is increased to four.

In the presence of $1{\cdot}0 \times 10^{-4}$ M tyrosine as substrate, all the tetrahydropterins had similar K_m values ($1{-}4 \times 10^{-4}$ M), except tri- or tetra- hydroxybutyltetrahydropterins (about 1×10^{-3} M). Among these tetrahydropterins, tetrahydrobiopterin had the lowest K_m value, but the difference was small. K_m values did not differ significantly among the three isomeric trihydroxypropyltetrahydropterins. These results suggest that the stereochemical structures have little effect on the affinity of a tetrahydropterin for tyrosine hydroxylase, and that a 6-alkyl tetrahydropterin with 4 carbons has a significantly lower affinity.

In contrast to the similar K_m values of various tetrahydropterins, the K_m values of tyrosine differed strikingly with each tetrahydropterin cofactor. Tetrahydrobiopterin gave the lowest K_m value of tyrosine ($7{\cdot}5 \times 10^{-6}$ M), indicating the promotion of the affinity of tyrosine for the enzyme. The K_m value is similar to that reported by Kaufman for particulate enzyme from bovine adrenal medulla (4×10^{-6} M) [4]. The three stereochemical isomers of trihydroxypropyltetrahydropterins also gave low K_m values ($3{-}5 \times 10^{-5}$ M), and the difference in stereochemical structures did not affect the K_m values of tyrosine significantly. In contrast, tri- and tetra-hydroxybutyltetrahydropterins gave much higher K_m values of tyrosine ($1{-}2 \times 10^{-4}$ M). This suggests that the polyhydroxybutyl side chain of a tetrahydropterin cofactor may interfere with the binding of tyrosine with the enzyme.

In previous experiments [3], tetrahydrobiopterin gave a lower K_m value ($2{\cdot}0 \times 10^{-5}$ M) than in the present study ($8{\cdot}8 \times 10^{-5}$ M). The reason is not clear; in the present study, however, the enzyme preparation had a higher purity, and synthetic biopterin was used. It is tempting to speculate that there is a substance which increases the affinity of tyrosine hydroxylase toward tetrahydrobiopterin in the crude enzyme preparation, such as phenylalanine hydroxylase stimulating protein for phenylalanine hydroxylase reported by Haung and Kaufman [10]. Removal of such a substance from the enzyme during Sephadex G-200 chromatography would make the K_m value of tetrahydrobiopterin high.

In summary the following conclusions may be drawn. First, the stereochemical structures of the 6-alkyl side chain of a tetrahydropterin have little effect on the cofactor activity or on the K_m value of the pterin cofactor or tyrosine. Second, the 6-polyhydroxybutyl group markedly decreases the cofactor activity and markedly increases the K_m value of the pterin cofactor or tyrosine, and therefore may hinder the pterin itself and tyrosine from binding to the enzyme. Third, tetrahydrobiopterin has a relatively low K_m value, and gives the lowest K_m value toward tyrosine and rather high cofactor activity. These properties favour the view that biopterin may be the natural

cofactor of tyrosine hydroxylase. However, since all the three stereoisomers of trihydroxypropyltetrahydropterin have similar characters as cofactor, it may be necessary to compare the properties as cofactor with the stereochemical isomers of tetrahydrobiopterin.

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SHORT COMMUNICATION

The influence of some acetylcholine-receptor activating and blocking agents on the esteratic site of acetylcholinesterase

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The sulfonylation of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) by methanesulfonyl fluoride is greatly accelerated by tetraethylammonium[1]. Tetraethylammonium changes the reactivity of the esteratic site of the enzyme, probably by changing the structure of the site[2]. On the other hand, the structure of tetraethylammonium is similar to that of the quaternary ammonium part of acetylcholine, which is the natural activator of acetylcholine-receptor; the receptor is supposed to undergo a structural change under the influence of an activator. Thus, the supposed change in the structure of the esteratic site of acetylcholinesterase under the influence of tetraethylammonium points to a similarity between the active site of the enzyme and that of the receptor. In order to obtain more data concerning this similarity the present investigation on the influence of some receptor activating and blocking agents on the esteratic site of acetylcholinesterase was undertaken. Experiments on the methanesulfonylation of acetylcholinesterase from the electric organ of the fish *Electrophorus electricus* in the presence of some typical activating and blocking agents of the acetylcholine-receptor from the same organ were performed and compared with those performed in the absence of these agents.

Methods and materials

The methanesulfonylation of acetylcholinesterase follows the scheme[3, 4]

$$EH + I \xrightarrow{k_a} E' + HF$$

with EH, the esteratic site of the enzyme; I, the inhibitor methanesulfonyl fluoride; E', the sulfonylated esteratic site; HF, hydrogen fluoride; and k_a, the second order rate constant for sulfonylation. The effect of an agent on sulfonylation is reflected in k_a and the corresponding thermodynamic quantities ΔH^+, ΔS^+, and ΔG^+. From these data conclusions can be drawn about the influence of the agent on the structure of the esteratic site. For our purpose typical activating and blocking agents of the acetylcholine-receptor from the electric organ of *E. electricus* were chosen: decamethonium, carbamycholine, D-tubocurarine, and gallamine.

The rate of the methanesulfonylation of acetylcholinesterase was studied in the presence of each of these agents at 5° and 25°, pH 8·4, ionic strength 0·2. The concentration of decamethonium ranged from 2 to 200 μM, of carbamylcholine from 10 to 500 μM, of D-tubocurarine from 2 to 200 μM, and of gallamine from 10 μM to 10 mM. The second order rate constant for sulfonylation in the presence of a given agent at a given concentration was determined as previously described[2]. From the dependence of the second order rate constant on the concentration of the agent, the maximum value of this constant was extrapolated and used for the calculation of the thermodynamic quantities for activation; this calculation was done as already described[2].

The enzyme preparation was acetylcholinesterase from the electric organ of *E. electricus*, Worthington, ECHP 1 JA, 1·079 units/mg. Methanesulfonyl fluoride, for chemical purposes, was obtained from Eastman Organic Chemicals; decamethonim iodide, pure, from Koch-Light Lab.; carbamylcholine chloride, for investigation use, from Mann Research Lab.; and gallamine, Lot No. 1570, from Specia.

Results and discussion

The results are summarized in Table 1. It can be seen that decamethonium accelerates the methanesulfonylation of acetylcholinesterase at 25° by about 28 times. This is in agreement with Suszkiw's result[5], considering the differences in methods and experimental conditions.