Dihydropteridine Reductase: Implication on the Regulation of Catecholamine Biosynthesis
(adrenal/DOPA/tetrahydropteridine/tyrosine hydroxylase)

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ABSTRACT The low tissue concentrations of tetrahydrobiopterin, as well as the antagonism between the catecholamine feedback inhibition of tyrosine hydroxylase and the reduced cofactor concentrations, suggest that dihydropteridine reductase may play an important role in the regulation of catecholamine biosynthesis.

The interaction of the different components involved in the hydroxylation of tyrosine was studied in vitro in a complex system composed of tyrosine hydroxylase, dihydropteridine reductase, and the different cofactors. This system has several important characteristics: (a) the rate of dihydroxyphenylalanine formation can be controlled by the concentration of dihydropteridine reductase; (b) low concentrations of catecholamines (2 × 10⁻⁴ M) can produce a marked inhibition of tyrosine hydroxylase activity; and (c) the catecholamine feedback-inhibition of tyrosine hydroxylase can be antagonized by increasing concentrations of dihydropteridine reductase.

The properties of the in vitro tyrosine hydroxylase-dihydropteridine reductase system suggest that dihydropteridine reductase may have an important role in vitro in the determination of the rates of dihydroxyphenylalanine formation and on the effectiveness of the catecholamine feedback-inhibition of tyrosine hydroxylase activity.

The hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) is considered to be the rate-limiting step in catecholamine biosynthesis (1). This step, however, is quite complex and it is not known which of its components is actually the limiting factor in the overall reaction. Tyrosine hydroxylase requires molecular oxygen and a reduced pteridine as cofactors (2-4). In addition, a pteridine-reducing system is required to reduce the pteridine cofactor that is continuously oxidized during the reaction. Adrenal dihydropteridine reductase has been found in bovine adrenal glands (5). This enzyme reduces the pteridine cofactor oxidized in the hydroxylation of tyrosine to DOPA, allowing cyclic and catalytic use of the cofactor.

The effect of a tetrahydropteridine-generating system on the in vitro activity of tyrosine hydroxylase is reported in the present communication. In addition, since there are several indications from in vivo and in vitro experiments that tyrosine hydroxylase activity is controlled, at least in part, by a feedback end-product inhibition, the effects of catecholamines on the entire in vitro system were also studied.

MATERIALS AND METHODS

The reduced pteridine, 6,7-dimethyl-5,6,7,8-tetrahydropterine (DMPH₃), was purchased from Calbiochem. Biopterin was generously donated by Smith Kline and French Laboratories. [3,5-³H]Tyrosine (1000 Ci/mol) was purchased from Amersham Searle; it was purified by passage through a Dowex 50W-X4 K⁺ column, and the purified material was diluted to a specific activity of 10 Ci/mol Tris(hydroxymethyl) aminomethane (Trizma base), TPN⁺, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (crude powder from Baker's Yeast, 0.3-1.0 units/mg) were obtained from Sigma Chemical Co. Beef liver catalase, 2X crystallized, an aqueous suspension, 150,000 units/ml, was purchased from Worthington Biochemical Corp., and 2-mercaptoethanol was purchased from Eastman Organic Chemicals. All other chemicals were obtained from standard commercial sources. Deionized glass-distilled water was used for the preparation of all reagents and buffers.

Preparation of enzymes

Beef adrenal dihydropteridine reductase was prepared by a modification of the method of Kaufman for the sheep-liver enzyme (6). Frozen beef adrenal medullae were homogenized with 1.5 volumes of 0.03 M acetic acid for 1 min at 4°C in a Waring Blender at full speed. Another 1.5 volumes of 0.03 M acetic acid was added and the homogenization was continued for 0.5 min. The homogenate was centrifuged for 20 min at 10,000 × g. To each 100 ml of supernatant, 29.3 g of ammonium sulfate was added (to 50% saturation). The mixture was centrifuged for 20 min at 10,000 × g; the residue was discarded. Another 17.5 g of ammonium sulfate per 100 ml of ammonium sulfate-treated supernatant was added (to 80% saturation). Centrifugation was repeated and the supernatant was discarded. The precipitate was dissolved in 5% of the original homogenate supernatant volume with 0.01 M Tris-HCl, (pH 7.4) and dialyzed overnight against 100 volumes of the same buffer. The next morning, the buffer was changed and dialysis was continued for 8 hr. The dialyzed enzyme preparation was divided into small fractions and stored frozen. Dihydropteridine reductase is exclusively localized in the 100,000 × g supernatant fraction; all attempts to detect enzyme activity in the different particulate subcellular fractions have failed. An ammonium sulfate fraction of tyrosine hydroxylase was prepared by a modification of the method of Nagatsu et al. (2) as described by Wurzburger and Musacchio (7). Trypsin-treated tyrosine

Abbreviations: DMPH₃, 6,7-dimethyl-5,6,7,8-tetrahydropterine; Q-DMPH₃ quinonoid form of 6,7-dimethylpteridine; 7,8-DMPH₃, 6,7-dimethyl-7,8-dihydropterine, DOPA, 3,4-dihydroxyphenylalanine.
hydroxylase was prepared by the procedure of Petrack et al. (8). This trypsin-treated tyrosine hydroxylase was preferentially used because the tyrosine hydroxylase prepared by the procedure of Nagatsu et al. contains some dihydropteridine reductase activity, which obscures the effects of added dihydropteridine reductase. All experiments were performed at 4°C unless otherwise indicated.

**Preparation of pteridines**

6,7-Dimethyl-5,6,7,8-tetrahydropterine was oxidized to the quinonoid isomer dimethyldihydropterine (Q-DMPH₄) by a modification of the 2,6-dichlorophenolindophenol method described by Kaufman (9). DMPH₂, 10 μmol, was dissolved in 5 ml of water; immediately, 12 μmol of 2,6-dichlorophenolindophenol in 5 ml of water were added. Complete oxidation was instantaneous; the quinonoid dihydropteridine was extracted three times with 10–12 ml of ether to purify it from the dye. All traces of ether were removed with a stream of nitrogen. The length of time from the oxidation of DMPH₂ to the addition of the quinoid DMPH₄ to the incubation tubes was 4 min. It is critical to keep this time as short as possible since Q-DMPH₂ isomerizes very rapidly to the inactive 7,8-DMPH₂. The spectrum of the oxidized pteridine, immediately after preparation, indicated that the compound was substantially in the quinoid form (Fig. 1). The inactive dihydro form, 7,8-DMPH₄, was obtained from Q-DMPH₂ after several hours of incubation at room temperature. As shown in Fig. 1, a spectrum showed the pteridine to be completely converted to the 7,8 form. Dihydrobiprotein was prepared from biprotein with a modification of the method of Pohland et al. (10) as follows: 10 μmol of biprotein was dissolved in 1 ml of 1 N HCl containing 10 mg of platinum oxide. The solution was bubbled with hydrogen for 1 hr at 55°C and rapidly filtered through a Buchner funnel with a fritted disc. The pH of the filtrate was adjusted to 5.5 with 0.25 M Tris. Tetrahydrobiprotein was immediately oxidized to the quinonoid dihydro form by titration with a solution of 2,6-dichlorophenolindophenol in water (2.4 μmol/ml); the yield of tetrahydrobiprotein obtained by the hydrogenation procedure was measured by the amount of dichlorophenolindophenol used to oxidize it to the quinonoid dihydro form. The dihydrobiprotein formed was rapidly prepared free from the dye according to the above procedure and used immediately.

**Enzyme assay**

Dihydropteridine reductase activity was detected indirectly by the use of a tyrosine hydroxylase system that requires a reduced pteridine to hydroxylate tyrosine to dihydroxyphenylalanine (DOPA). This system was found to be more efficient if a TPNH-generating system was included. The complete assay system for dihydropteridine reductase contained the

**Table 1. Effect of different components on dihydropteridine reductase activity**

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.20</td>
</tr>
<tr>
<td>Dihydropteridine reductase</td>
<td>0.17</td>
</tr>
<tr>
<td>G-6-PO₄ dehydrogenase</td>
<td>3.18</td>
</tr>
<tr>
<td>G-6-PO₄</td>
<td>0.54</td>
</tr>
<tr>
<td>TPN⁺</td>
<td>0.22</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>11.21</td>
</tr>
<tr>
<td>MgCl₂; Fe⁺⁺ added</td>
<td>10.23</td>
</tr>
<tr>
<td>Catalase, MgCl₂; Fe⁺⁺ added</td>
<td>4.73</td>
</tr>
</tbody>
</table>

Dihydropteridine reductase (2.8 mg of protein) was assayed with the use of trypsin-treated tyrosine hydroxylase. MgCl₂, which is required in a purified TPNH-generating system, was added in a final concentration of 10 mmol/liter. The blank contained no dihydropteridine reductase or tyrosine hydroxylase, and its activity was equivalent to 0.94 nmol. The results are the average of two determinations.

**Fig. 1. Comparison of the absorption spectra of Q-DMPH₂ and 7,8-DMPH₂.** The spectrum of Q-DMPH₂ (0.05 μmol/ml), −−−, was determined in 0.1 M Tris-HCl (pH 6.1), 3 min after preparation by the dye-oxidation procedure. The spectrum of 7,8-DMPH₂ (0.05 μmol/ml), −−−−, also determined in 0.1 M Tris-HCl (pH 6.1), was obtained after a preparation of Q-DMPH₂ had been allowed to stand for 24 hr at room temperature.

**Fig. 2. Effect of epinephrine (Epi) and dihydropteridine reductase (DHPR) on tyrosine hydroxylase activity.** Different amounts of dihydropteridine reductase (28 μg of protein/ml) were assayed with the use of trypsin-treated tyrosine hydroxylase and Q-DMPH₂ to a final concentration of 0.1 μmol/ml; epinephrine, 2 × 10⁻⁴ M, was added to each tube before preincubation. Controls contained all components except epinephrine; blanks contained no dihydropteridine reductase or tyrosine hydroxylase. The results are expressed as nmol of DOPA formed in 30 min and are the average of two determinations.
following components: 0.1 ml of 1 M Tris-HCl (pH 6.1); [3,5,4-H]-tyrosine, 0.1 μmol; ferrous ammonium sulfate, 0.5 μmol; beef-liver catalase, 60 units; TPN+, 0.01 μmol; D-glucose-6-phosphate, 5.0 μmol; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.035 units; beef-adrenal dihydropteridine reductase, 0.28–2.8 mg of protein; ammonium sulfate fraction of tyrosine hydroxylase, 6.6 mg of protein or trypsin-treated tyrosine hydroxylase, 0.95 mg of protein; 1-epinephrine bitartrate and pteridine cofactors as indicated in the tables and figures; water was added to 1 ml. Blanks were usually obtained by omission of tyrosine hydroxylase and dihydropteridine reductase, but in some of our preliminary investigations, blanks were also included without substrate (Q-DMPH2) or with 7,8-DMPH2 in order to rule out any false positive results that could have been produced by tyrosine aminotransferase (EC 2.6.1.5) or by tetrahydrofolate dehydrogenase (EC 1.5.1.3). Incubation tubes were kept in an ice bath, while the different components were added. They were then incubated for 4 min at 37°C; during this interval, the quinonoid form of the pteridine cofactors was prepared. The reaction was started by the addition of the appropriate pteridine. The tubes were incubated for 30 min at the same temperature and the reaction was stopped by the addition of 0.1 ml of 30% trichloroacetic acid. The hydroxylation of tyrosine to DOPA was measured by the method of Nagatsu et al. (11) with modifications described by Wurzburger and Musacchio (7).

Spectrophotometry
Spectra of DMPH4, Q-DMPH2, and 7,8-DMPH2 were obtained with a Beckman DB-G grating spectrophotometer, with an attached Beckman potentiometric recorder. Absorbance of DMPH4 was measured in a Beckman DU-2 spectrophotometer.

RESULTS
General properties of the enzyme system
The different components of the tyrosine hydroxylase–dihydropteridine reductase system were all adjusted to obtain maximal rates of DOPA formation in the presence of dihydropteridine reductase and minimal rates without the enzyme. The concentration of TPNH used in these experiments is 10-fold lower than the concentration used previously (5), in order to reduce the amount of Q-DMPH2 that is non-enzymatically reduced to DMPH2 by high concentrations of TPNH. The low level of TPNH (1 × 10–4 M) was kept constant with a TPNH-generating system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The results in Tables 1 and 2 indicate that the synthesis of DOPA is dependent on the presence of dihydropteridine reductase. The results described in Table 1 also demonstrate that the production of DOPA is dependent on TPNH; in the absence of TPN+ or glucose-6-phosphate, there is no synthesis of DOPA. The production of DOPA was markedly reduced by omission of glucose-6-phosphate dehydrogenase in the experiment described in Table 1. However, the requirements for glucose-6-phosphate dehydrogenase are variable with the different enzyme preparations, probably because of the presence of different amounts of glucose-6-phosphate dehydrogenase in the tyrosine hydroxylase or in the dihydropteridine reductase preparations. The TPNH-generating system does not require the addition of MgCl2, probably because some traces of metals are already present in the enzyme preparations. The dihydropteridine reductase prepared as indicated in Methods was found free of TPN+ reducing properties in the absence of a TPNH-generating system. In contrast, the enzyme prepared with the method previously described (5) could reduce nucleotides without an added nucleotide-reducing system.

The production of DOPA in the tyrosine hydroxylase–dihydropteridine reductase system is nearly linear with time for 30 min, but the rate of DOPA production is slightly slower during the first few minutes of the incubation, probably due to the low initial concentrations of DMPH4.

As is demonstrated in Table 2, dihydropteridine reductase can reduce only the quinonoid form of DMHP2. The 7,8-DMPH2 isomer is not reduced to DMHP2, as indicated by its inability to sustain the hydroxylation of tyrosine to DOPA. Moreover, the dihydropteridine reductase prepared as indicated in Methods is free of tetrahydrofolate reductase activity, as demonstrated by the incapacity of the enzyme to reduce 7,8-DMPH2, and by the lack of effect of methotrexate on the production of DOPA (Table 2).

Effects of catecholamines
The effects of epinephrine were tested on a complete system containing various amounts of dihydropteridine reductase. As shown in Fig. 2, a marked inhibition of DOPA formation is produced by an epinephrine concentration of 2 × 10–5 M. It is also evident that, within certain limits, the epinephrine inhibition can be antagonized by increasing concentrations of dihydropteridine reductase. Similar results are obtained when the quinonoid form of dihydrobipterin is used instead of Q-DMPH2.

The inhibition of tyrosine hydroxylase by catecholamines has been described as competitive with DMHP4 (12, 13); as indicated in Fig. 3, this inhibition is also competitive with the natural cofactor, tetrahydrobipterin. It was suggested, however, that catecholamines could react directly with the pteridine cofactor and produce the same kinetic changes as if there was a competition for a tyrosine hydroxylase site (14). In order to investigate this possibility, the effects of epinephrine on the spectrum of DMHP4 were studied under conditions similar to those at which the competition between epinephrine and DMHP4 is demonstrated. The results illustrated in Fig. 4 demonstrate that the addition of epinephrine does not produce any change in the spectrum of DMHP4 that would indicate oxidation of the tetrahydropteridine. In a similar

| Table 2. Activity of dihydropteridine reductase with Q-DMPH2 and 7,8-DMPH2, and the effect of methotrexate |
|---------------------------------------------------------------|-----------------|-----------------|
| Q-DMPH2 | 7,8-DMPH2 |
| Complete system | 18.00 | 0.47 |
| Methotrexate 10–4 M added | 16.55 | 0.22 |
| No dihydropteridine reductase | 1.52 | 0.09 |

The complete assay system was the same as that indicated under Materials and Methods, with the use of trypsin-treated tyrosine hydroxylase; the pteridine cofactors Q-DMPH2 and 7,8-DMPH2 were prepared as described, and each at a final concentration of 0.1 μmol/ml, was tested for cofactor activity. Activity was also determined with each cofactor in the presence of methotrexate (1 × 10–4 M). Controls contained all components for the assay except dihydropteridine reductase. Blank activity, determined without dihydropteridine reductase and tyrosine hydroxylase, was equivalent to 1.0 nmol. The results are the average of two determinations.
experiment not illustrated here, the rate of oxidation of DMPH$_4$ was followed by measurement of the rate of decrease in absorbance at 302 nm; this rate was found to be identical before and after the addition of epinephrine. As can be observed in Fig. 4, the magnitude of these changes is negligible if it is considered that the competition experiments between epinephrine and DMPH$_4$ on tyrosine hydroxylase activity are finished 9 min after the addition of epinephrine. In addition, it should be noted that, due to optical interferences, the spectrophotometric measurements on the effects of epinephrine on DMPH$_4$ were performed with a mercaptoethanol concentration that is 10-fold lower than that used for the determinations of tyrosine hydroxylase activity. Therefore, even slower changes may be expected with the higher mercaptoethanol concentrations used in the enzymatic studies.

**DISCUSSION**

The production of DOPA in the tyrosine hydroxylase-dihydropteridine reductase system described is completely dependent on both enzymes and their substrates and cofactors. Ferrous iron and catalase are not essential components, but they stimulate the formation of DOPA; ferrous iron has been described to be a requirement for tyrosine hydroxylase activity (2, 8). However, Shiman et al. (15) have recently indicated that the stimulatory effects of ferrous iron may be due to its ability to decompose H$_2$O$_2$; this phenomenon may also explain why we have been unable to observe the stimulatory effects of iron in the presence of catalase. Catalase has a marked stimulatory effect on this system; its mechanism of action is unknown, but is presumably related to its ability to decompose H$_2$O$_2$. Catalase protects liver phenylalanine hydroxylase from inactivation during incubation (16); it is conceivable that it may also protect one or both enzymes in our system, and thereby increase the formation of DOPA.

After a short lag period, the production of DOPA is linear for at least 30 min. The initial lag period is expected because (a) tyrosine hydroxylase itself shows an initial lag period when it is not preincubated with DMPH$_4$ and (b) the production of DOPA can start only after enough DMPH$_4$ is formed to serve as cofactor for tyrosine hydroxylase.

Adrenal-medulla dihydropteridine reductase can reduce the quinonoid forms of DMPH$_4$ and dihydrobiopterin as previously demonstrated (5); this enzyme is different from tetrahydrofolate dehydrogenase (EC 1.5.1.3), as indicated by its inability to reduce 7,8-DMPH$_4$ and by its resistance to methotrexate inhibition. All the characteristics of adrenal dihydropteridine reductase examined indicate that the enzyme is similar to the liver dihydropteridine reductase described by Kaufman (6).

The production of DOPA is the system described is, within certain limits, proportional to the activity of dihydropteridine reductase. This assay for dihydropteridine reductase can be used to quantitate the enzyme activity, providing precautions are taken to keep the results within the proportional range.

Adrenal dihydropteridine reductase is found only in the soluble fraction of the homogenate (as described in Methods) and tyrosine hydroxylase is a cytoplasmic enzyme (17, 18, 7). Obviously, the location of both enzymes has important implications regarding the regulation of catecholamine synthesis: the amine pool that regulates tyrosine hydroxylase activity should necessarily be located in the cell cytoplasm. Weiner and Selvaratnam (19) and Kopin et al. (20) have shown that in adrenergic nerves, tyrosine hydroxylase activity is regulated by a cytoplasmic catecholamine pool. Therefore,
there is evidence obtained from different sympathetic structures by different methods that indicates that the feedback control of catecholamine biosynthesis takes place in the cytoplasm of adrenergic structures.

The marked differences between the rates of synthesis of catecholamines in vivo with the rate of synthesis of DOPA in vitro suggest that tyrosine hydroxylase itself is not the rate-liming factor in catecholamine biosynthesis. It is difficult to compare in vivo with in vitro activities of tyrosine hydroxylase, but some idea of the differences can be obtained by comparing the rate of catecholamine synthesis in rat brain in vivo with the rate of DOPA formation obtained with equivalent amounts of rat-brain tyrosine hydroxylase. The highest rates of brain catecholamine synthesis that we found in the literature (1.5-3.2 nmol/g per hr, ref. 21) are at least 40- to 80-fold lower than rates of tyrosine hydroxylase obtained in vitro under optimal conditions, and with an equivalent amount of enzyme (120 nmol/g per hr; Wurzburger and Musacchio, unpublished observation). These marked differences in the synthesis rates are most likely due to the high tetrahydropteridine cofactor concentration used for the in vitro experiments. The tissue content of the pteridine cofactor in the adrenal medulla is 0.5-2.0 \( \mu\)g/g (22), and in the brain it has been estimated as 0.75 \( \mu\)g/g (23). If we assume that the pteridine cofactor is uniformly distributed in the adrenal medullary cells, the cofactor concentrations can be calculated to be between 2 \( \times 10^{-4}\) M and 1 \( \times 10^{-3}\) M. These concentrations are 100- to 500-fold lower than those used for the in vitro assays. The concentration of cofactor in the brain adrenergic structures cannot be even be roughly calculated, since the relative volume of these structures and the distribution of the cofactor are unknown. There is additional evidence that the concentration of pteridine cofactor, at least under certain conditions, may be rate limiting. Côté et al. (24) reported that the addition of tetrahydrofolic acid or bipterin to chick sympathetic chain cultures significantly increases their catecholamine content.

The concentrations of tyrosine and O\(_2\) used in these experiments are slightly higher than their in vivo concentrations, but since these concentrations are in the range of their respective Michaelis constants, no major increases in synthesis rates can be attributed to the slightly higher concentrations used in vitro. All these considerations indicate that the marked differences in the activity of tyrosine hydroxylase in vivo and in vitro could be explained by the different cofactor concentrations.

Our in vitro experiments indicate that dihydropteridine reductase can increase the activity of tyrosine hydroxylase and it can antagonize the inhibitory effects of catecholamines. The mechanism by which dihydropteridine reductase antagonizes the catecholamine inhibition is most likely due to an increased concentration of reduced pteridine cofactor. The competitive nature of the antagonism between catecholamines and the reduced pteridine cofactor analog DMPH4 was described by Udenfriend et al. (13). It has recently been suggested that this competition may be due to a direct chemical oxidation of the cofactor to an inactive form, instead of a true competition for enzyme sites (14). Our experiments, however, rule out the possibility of a direct interaction between cofactor and catecholamines. In addition, we have demonstrated that the natural cofactor for adrenal tyrosine hydroxylase, tetrahydrobiopterin, also inhibits competitively the inhibitory action of catecholamines on tyrosine hydroxylase.

**CONCLUSIONS**

In vitro experiments indicate that, under certain conditions, the rate of tyrosine hydroxylase activity can be controlled by dihydropteridine reductase, and that this enzyme can antagonize the catecholamine inhibition of tyrosine hydroxylase. Since the concentration of the natural cofactor seems to be rate limiting in vivo, it is suggested that the activity of dihydropteridine reductase may be an important factor in the determination of the in vivo rates of catecholamine synthesis and of the effectiveness of the catecholamine feedback inhibition.

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