PRELIMINARY NOTES

BBA 61201

Beef adrenal medulla dihydropteridine reductase

A pteridine reducing system had been found in the beef adrenal gland which is capable of reducing some pteridines that function as cofactors for tyrosine hydroxylase[1]; the dihydropteridine reductase system may be an important regulatory component in the biosynthesis of catecholamines, since the reduced pteridines competitively antagonize the end-product inhibition of tyrosine hydroxylase by catecholamines[2].

Dihydropteridine reductase activity was detected indirectly by using a tyrosine hydroxylase system that requires a reduced pteridine to hydroxylate tyrosine to DOPA. The hydroxylation of tyrosine to DOPA was measured by the method of NAGATSU *et al.*[3]. The assay system for dihydropteridine reductase contained the following components: 100 μmoles Tris–maleate–KOH buffer (pH 5.7); 0.4 μmole L-[3,5-$^3H_2$]tyrosine (specific activity, 10 μC/μmole); 0.5 μmole ferrous ammonium sulfate; 0.1 μmole NADPH; tyrosine hydroxylase, about 3–4 mg of protein; dihydropteridine reductase, 3–4 mg of protein; final volume was 1 ml. The type and amount of pteridine used is described in Tables I and II. The reaction is linear during the first 8–10 min, but incubations have been carried on for longer periods in order to obtain more product and to demonstrate that the amount of DOPA formed is larger than the amount of reduced pteridine available. Incubations were performed in open test tubes at 37° with constant shaking.

Preparation of enzymes. Tyrosine hydroxylase was prepared from the 100 000 × *g* supernatant of beef adrenal medulla as described by NAGATSU *et al.*[4], but the adrenal medullae were homogenized in 2 vol. of isotonic KCl rather than in sucrose. The supernatant fluid obtained after the precipitation of the fraction containing the tyrosine hydroxylase was measured and was taken to 60% $(NH_4)_2SO_4$ saturation by the addition of 12 g of $(NH_4)_2SO_4$ per 100 ml. This fraction was centrifuged as before and the precipitate was discarded. The resultant supernatant was measured and 12.9 g of $(NH_4)_2SO_4$ per 100 ml were slowly added to obtain an 80% saturation. The precipitate containing the dihydropteridine reductase was separated by centrifugation at 14 000 × *g* during 30 min and it was dissolved with a volume of 0.005 M Tris–HCl buffer (pH 7.0) equivalent to 0.03 the 100 000 × *g* supernatant volume. The dissolved dihydropteridine reductase fraction was dialyzed twice against 100 vol. of 0.005 M Tris–HCl buffer (pH 6.7) during 8–12 h, divided in small fractions and stored frozen.

Substrates. 6,7-Dimethyl-5,6,7,8-tetrahydropterine was purchased from Calbiochem. The quinonoid isomer of oxidized dimethyltetrahydropterine (dimethyldihydropterine) was prepared by the 2,6-dichlorophenolindophenol method described by KAUFMAN[5], and its spectrum indicated that the compound was substantially in the quinonoid form. The phenylalanine hydroxylase rat liver cofactor was prepared as described by KAUFMAN[6] without including the Dowex-1 step. Tetrahydrobiopterin was prepared by catalytic hydrogenation of biopterin with the method described by

TABLE I

EFFECT OF DIHYDROPTERIDINE REDUCTASE ON [^{14}C]DOPA FORMATION

Samples were incubated for 1 h at 37° with 10 nmoles of dimethyltetrahydropterine. All other components as described in the text with the exception that 0.1 μmole of uniformly ^{14}C-labeled tyrosine with the specific activity of 5.5 mC/mmole was used. [^{14}C]DOPA was determined according to the method of WHITBY *et al.*[8] for catechols; the results are the average of two experiments.

Addition	*([^{14}C]DOPA formed (nmoles/h)*
Control	1.94
+ dihydropteridine reductase	24.90

O'DELL *et al.*[7]. The crude adrenal cofactor was prepared by methods to be published[9].

Properties of the enzyme. The conditions of the enzymatic assay are such that the amount of reduced pteridines is the limiting factor in the formation of DOPA. The formation of DOPA is, under this condition and within certain limits, proportional to the amount of dihydropteridine reductase added. The reductase activity is non-dialyzable and it is destroyed by heating at 80° for 1 min.

A several-fold increase in the formation of DOPA (Table I) is obtained when dihydropteridine reductase is added to incubation mixtures containing limiting amounts of dimethyltetrahydropterine and no protective agent (*i.e.* mercaptoethanol); dihydropteridine reductase can, therefore, reduce the spontaneously oxidized pteridine, and since the amount of product obtained is larger than the total pteridine added, it can also reduce the pteridine oxidized by tyrosine hydroxylase. Adrenal dihydropteridine reductase can reduce the quinonoid form of dimethyldihydropterine prepared by the dichlorophenolindophenol oxidation (Table II, Expt. 3). The crude rat-liver phenylalanine hydroxylase cofactor can also be reduced, as it is indicated in Table II (Expt. 1), by the increase in DOPA production upon the addition of the dihydropteridine reductase. Since some DOPA was formed in this experiment, even in the absence of NADPH or dihydrobiopterine reductase, a second experiment was

TABLE II

EFFECT OF DIHYDROPTERIDINE REDUCTASE ON SEVERAL SUBSTRATES THAT CAN FUNCTION AS TYROSINE HYDROXYLASE COFACTORS

Assays were carried out as described in the text. The substrates used were the following: *Expt. 1:* 0.2 ml of rat liver cofactor; *Expt. 2:* 0.2 ml of oxidized rat liver cofactor; *Expt. 3:* 10 nmoles dimethyldihydropterine (quinonoid form); *Expt. 4:* tetrahydrobiopterin (10 nmoles of reduced biopterin were added, but since hydrogenation yields are variable, the actual amount is not known). The results are the average of two determinations. These experiments are not indicative of the relative effectiveness of the different substrates.

System	*[^{3}H]Hydroxyl formed (nmoles/h)*				
	Expt. 1	*Expt. 2*	*Expt. 3*	*Expt. 4*	*Expt. 5*
Complete	10.95	3.27	9.46	13.86	15.56
− dihydropteridine reductase	4.60	0.11	1.34	5.86	1.68
− substrate	0.85	0.34	0.00	—	0.00
− NADPH	6.90	0.35	0.30	—	0.69

performed in which the rat-liver extract was treated with dichlorophenolindophenol until it lost all its reducing capacity. As shown in Expt. 2, no appreciable activity was detected in the absence of NADPH or dihydropteridine reductase (Expts. 1 and 2 are not quantitatively comparable since the liver cofactor was diluted by the oxidation procedure). The crude adrenal cofactor oxidized by the same procedure is a substrate for the reductase and requires NADPH, as it is shown in Table II, Expt. 5.

Dihydropteridine reductase requires NADPH or NADH for activity; NADPH is more effective than NADH, but the relative effectiveness varies with the different enzyme preparations. It was found that the reaction could also be stimulated by the oxidized nucleotides (NAD^+ and $NADP^+$); this phenomenon was explained when it was found that dihydropteridine reductase preparations in the presence of Fe^{2+} will reduce $NADP^+$. Several other compounds were found inactive as cofactors: α-NAD^+, NMN, 2'-AMP, 3'-AMP, ADP and ATP.

Dihydropteridine reductase does not reduce the oxidized form of the pteridines (*e.g.* 2-amino-4-hydroxy-6,7-dimethylpteridine, biopterin), and it is not inhibited by aminopterin and methotrexate (up to 0.1 mM); therefore, this enzyme is presumable different from tetrahydrofolate dehydrogenase (EC 1.5.1.3).

Adrenal medulla dihydropteridine reductase is probably an essential component of the catecholamine biosynthetic mechanism because it is able to regenerate the cofactor for tyrosine hydroxylase. Dihydropteridine reductase may also have an important role in the regulation of catecholamine synthesis, since the feedback inhibition produced by catecholamines on tyrosine hydroxylase is competitive with respect to tetrahydropteridines[2]. Therefore, high dihydropteridine reductase activity may antagonize the catecholamine feedback inhibition, while low levels of reductase activity may sensitize the system to inhibition by small concentrations of catecholamines.

This work was supported by U.S. Public Service Grant AM HE 13128-04.

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Received July 31st, 1969