THE ROLE OF TETRAHYDROPTERIDINES IN THE ENZYMATIC CONVERSION OF TYROSINE TO 3,4-DIHYDROXYPHENYLALANINE

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The first step in the sequence of reactions leading to norepinephrine synthesis in the adrenal medulla is the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) (Hagen, 1956; Goodall and Kirshner, 1957). This reaction has recently been demonstrated in mitochondrial preparations from brain and adrenal medulla (Nagatsu, et al., 1964a). With a soluble enzyme from the latter source, it has been shown that DOPA formation is stimulated by the addition of tetrahydrofolate and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine (Nagatsu, et al., 1964b), the same reduced pteridines that have previously been shown to be involved in phenylalanine hydroxylation (Kaufman, 1959).

We have been studying the conversion of tyrosine to DOPA with an enzyme partially purified from bovine adrenal tissue and have independently confirmed the stimulation of the reaction by tetrahydropteridines (Kaufman, 1964). Since an example is known where tetrahydropteridines stimulate an enzyme-catalyzed reaction by protecting the enzyme from inactivation (Zannoni, et al., 1963), we were particularly interested in determining the mechanism by which the pteridine stimulates DOPA

formation in the tyrosine-hydroxylating system.

The present communication presents evidence in support of the idea that tetrahydropteridines function as specific coenzymes in the adrenal tyrosine-hydroxylating system, in the same way as they do in the phenylalanine-hydroxylating system. Materials and Methods: L-tyrosine-u-^{14}C (154 uc/μmole) was obtained from Schwarz Bioresearch Inc.[1] (1-Methyl-2-phenyl)-ethylhydrazine hydrochloride (J.B. 516), a monoamine oxidase inhibitor, was a product of Lakeside Laboratories. N-Methyl-N-(2-hydroxybenzyl)-hydrazinium oxalate (N.S.D. 1039), a decarboxylase inhibitor, was donated by Dr. R. Levine. Dihydrofolate reductase (DHF reductase) was a gift from Dr. B. Kaufman. The reduced pteridines, glucose dehydrogenase, rat liver cofactor (dihydrobiopterin), and sheep liver enzyme (dihydropteridine reductase) were prepared by published methods (Kaufman, 1959; Kaufman and Levenberg, 1959; Kaufman, 1963). Aminopterin was obtained from Lederle Laboratories, a Division of American Cyanamid Co. Aluminum oxide was purchased from M. Woelm Eschwege and treated according to the method of Anton and Sayre (1962). Hydroxylapatite was purchased from the Clarkson Chemical Company, Inc. S. faecalis, tyrosine decarboxylase (also active on DOPA) was obtained from the Worthington Biochemical Corporation.

Fresh beef adrenal glands were homogenized in 3 volumes of 0.25 M sucrose. The homogenate was centrifuged at 700 x g for 15 minutes, and centrifuged at 100,000 x g for 1 hour. The enzyme remaining in the 100,000 x g supernatant fluid was

[1] The tyrosine-^{14}C contained about 0.2% catechols as measured by the procedure of Anton and Sayre. Treatment of the tyrosine-^{14}C with alumina according to the above procedure followed by chromatography of the tyrosine-^{14}C on Dowex-50 (H^+) removed most of the interfering substances.

partially purified by ammonium sulfate precipitation, treatment with hydroxylapatite, and a second ammonium sulfate precipitation. The experiments reported in this paper were performed with enzyme purified through the second ammonium sulfate step.

The standard assay system contained the following components in μmoles unless stated: potassium phosphate, pH 6.4, 200; TPNH, 0.5; DPNH, 0.5; D-glucose, 125; L-tyrosine-^{14}C, 0.3; glucose dehydrogenase in excess; sheep liver enzyme purified through the first ammonium sulfate step (Kaufman and Levenberg, 1959) was used except where noted. The reduced pteridines were added as specified in the Tables. Routine assays during enzyme purification also contained 2 μmoles each of the inhibitors NSD 1039 and JB 516. These compounds were unnecessary in experiments with tyrosine hydroxylase purified through the second ammonium sulfate step. The reaction mixture (final volume, 2 ml) was incubated at 35°. The reaction was terminated by the addition of 1.5 ml of 1.1 N perchloric acid. A 2 ml aliquot was used to assay for catechols according to the method of Anton and Sayre (1962), modified (Kopin, personal communication) by the use of alumina columns (0.6 x 3 cm) for the separation of DOPA from tyrosine. The alumina columns were washed twice with 20 ml of water and then eluted with 10 ml of 0.04 N perchloric acid. A 3 ml aliquot of the perchloric acid eluate containing the ^{14}C-DOPA was added to 10 ml of Bray's solution (Bray, 1960) and the radioactivity determined in a liquid scintillation spectrometer. Boiled enzyme controls were included and corrections applied when required.

Results: Characterization of the product of the enzymatic reaction as DOPA is based on the following criteria: 1. It

migrated with the same R_f as DOPA in several solvents. 2. After treatment with the decarboxylase from S. faecalis, the product migrated with the same R_f as DOPamine. 3. A mixture of the radioactive product with authentic L-DOPA maintained a constant specific activity on repeated recrystallization.

The conversion of tyrosine to DOPA catalyzed by the partially purified adrenal enzyme is completely dependent on a reduced pteridine (Table I). This requirement appears to be

Table I. Requirement for Reduced Pteridine for DOPA Formation

Experiment No.	Pteridine added	mμmoles added	DOPA formed mμmoles
1	6-methyltetrahydropteridine	50	68.20
	6,7-dimethyltetrahydropteridine	50	30.62
	Reduced biopterin	4.6	24.88
	None		0
2	Tetrahydrofolate	1.9	0.05
	6-methyltetrahydropteridine	1.6	0.96
	Reduced biopterin	2.3	5.70
	Rat liver cofactor	1.6	1.78
	None		0

The assay system is described in the "Methods". In experiment 1 highly purified sheep liver enzyme (0.78 mg protein) and tyrosine hydroxylase (11.6 mg protein) were used. In experiment 2 the crude sheep liver enzyme (4.10 mg protein) and a different tyrosine hydroxylase preparation (11.8 mg protein) were used. The incubation was 3 hours in both experiments. All pteridines were 2-amino-4-hydroxy compounds.

specific. The following compounds have been tested as possible substitutes for the reduced pteridine and found to be completely inactive at a final concentration of 0.1 mM: ascorbate, cysteine, GSH, $FeCl_2$, $FeCl_2$ and GSH, flavin mononucleotide, and FAD. In addition, DOPA (0.05-2.5 mM) was unable to substitute for the pteridine. The inactivity of DOPA distinguishes this enzyme system from tyrosinase (Pomerantz, 1964).

The structural requirements for the active pteridine are similar in this system and in the phenylalanine hydroxyl-

ating system (Kaufman, 1959; Kaufman and Levenberg, 1959). Thus, in both systems, at low pteridine concentrations, the order of activity from lowest to the highest is: tetrahydrofolate, the dimethyl compound, the monomethyl compound and reduced biopterin. (Table I). The rat liver cofactor is somewhat less active than reduced biopterin.[2]

Reduced biopterin functions catalytically, 4 to 5 times as much DOPA being formed as there was reduced biopterin present.

The results summarized in Table II show that DOPA formation exhibits the same requirements as does the enzymatic conversion of phenylalanine to tyrosine. The reaction requires reduced pyridine nucleotide (expt 1), TPNH being more active than DPNH (expt 2). In addition, sheep liver enzyme (expts 1, 4) and oxygen (expt 3) are necessary. In the presence of the purified rat liver cofactor, DHF reductase[3] stimulates the reaction (expt 4) and aminopterin, an inhibitor of this enzyme and a potent inhibitor of phenylalanine-hydroxylation (Kaufman, 1963) inhibits the conversion of tyrosine to DOPA.

Discussion: One of the goals of this study was to determine the mechanism by which tetrahydropteridines stimulate the conversion of tyrosine to DOPA in the adrenal enzyme system. The results obtained strongly support the conclusion that reduced pteridines function as coenzymes in this hydroxylation reaction in the same

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[2]It has been found that the chemical reduction of biopterin leads to a variable mixture of the dihydro and tetrahydro derivatives. The presence of large amounts of the tetrahydro compound in the preparation used in this experiment probably accounts for the difference in activity between the chemically reduced biopterin and the cofactor.

[3]The purified DHF reductase preparation contains detectable sheep liver enzyme activity (S. Kaufman, unpublished observation). This slight contamination probably accounts for the relatively small stimulation by added sheep liver enzyme in this experiment.

Table II. Dependencies for DOPA Formation

Experiment No.	Component omitted	DOPA formed mμmoles
1	None	23.44
	TPNH, DPNH	1.75
	TPN, DPN added instead of TPNH, DPNH	1.17
	Sheep liver enzyme	6.29
	6-methyltetrahydropteridine	0.12
2	None	3.63
	TPNH	1.82
	DPNH	3.44
	TPNH, DPNH	0.20
3	None	17.32
	Oxygen	0.11
4	None	1.83
	Dihydrofolate reductase + Sheep liver enzyme	0
	Dihydrofolate reductase	0.62
	Sheep liver enzyme	1.25
	Aminopterin added	0

The conditions were the same as described in "Methods" with the following exceptions. In experiment 1, the 6-methylpteridine (50 mμmoles), a tyrosine hydroxylase preparation containing 2.9 mg protein per ml and L-tyrosine-^{14}C (125 mμmoles) were used. In experiments 2 and 4, rat liver cofactor (3.64 mμmoles) was used. In experiment 3, the 6-methylpteridine (40 mμmoles) was used. In experiments, 1, 2 and 3, crude sheep liver enzyme (4.10 mg protein) was used. In experiment 4, a highly purified fraction of sheep liver enzyme (S. Kaufman, unpublished procedure), essentially free of DHF reductase, was used. In experiments 2, 3, and 4, a tyrosine hydroxylase preparation containing 11.6 mg protein per ml was used. The final concentration of aminopterin was 5×10^{-7} M. All incubations were for 3 hours.

way as they do in the enzymatic conversion of phenylalanine to tyrosine. The conclusion is based on the following findings: 1. The requirement for a reduced pteridine is specific. 2. The reduced pteridine functions catalytically. 3. The reduced pteridine is utilized during the hydroxylation reaction, as indicated by the requirement for TPNH and the sheep liver enzyme.

Based on analogy with the phenylalanine-hydroxylating

system, the conversion of tyrosine to DOPA may be formulated as shown in equations 1 and 2 where XH_4 stands for tetrahydro and XH_2 for dihydropteridine.

$$\text{tyrosine} + XH_4 + O_2 \xrightarrow[\text{hydroxylase}]{\text{tyrosine}} \text{DOPA} + H_2O + XH_2 \quad (1)$$

$$XH_2 + \text{TPNH} + H^+ \xrightarrow[\text{enzyme}]{\text{sheep liver}} XH_4 + \text{TPN}^+$$

When the rat liver cofactor is used, the system requires, in addition, DHF reductase, which in the presence of TPNH, catalyzes the initial reduction of the cofactor to the tetrahydro form.

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