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RAT STRIATAL SYNAPTOSOMES AS A MODEL SYSTEM FOR STUDYING THE INHIBITION OF DIHYDROPTERIDINE REDUCTASE ACTIVITY

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The distribution of dihydropteridine reductase between soluble and particulate fractions in synaptosomes parallels that of lactate dehydrogenase, but not monoamine oxidase. K_i and I_{50} values for inhibitors obtained with the enzyme-rich P_2 fraction and its twice-washed fraction (P_2W_2) were essentially the same, and were similar to those obtained with highly purified human liver enzyme. Dihydropteridine reductase inhibitory potency of multi-ring compounds containing a catechol-moiety was greater than that of single ring catecholic compounds, which in turn was greater than that of *p*-hydroxy-phenolic compounds. The P_2 fraction of rat striatal synaptosomal preparations may serve as a convenient source of dihydropteridine reductase for studying the inhibition of this enzyme.

KEY WORDS: Rat striatal synaptosomes, dihydropteridine reductase, tetrahydrobiopterin.

INTRODUCTION

Dihydropteridine reductase (E.C. 1.6.99.7) (NADH: 6,7-dihydropteridine oxidoreductase) catalyzes the reduction of quinonoid dihydrobiopterin (qBH$_2$) to L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH$_4$)[1]. The hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine during the synthesis of dopamine and l-norepinephrine, and the hydroxylation of L-tryptophan to L-5-hydroxytryptophan during the synthesis of serotonin all require BH$_4$[2]. Dihydropteridine reductase regulates the rate of BH$_4$ regeneration, thereby maintaining a catalytic pool of this essential cofactor in mammalian brain for the activities of tyrosine 3-hydroxylase (E.C. 1.14.16.2) and tryptophan 5-hydroxylase (E.C. 1.14.16.4) during neurotransmitter biosynthesis. Dihydropteridine reductase is inhibited by catechol- and *p*-hydroxyphenyl-containing compounds (see reference 3 for review). Inhibition of the activity of this enzyme interrupts the recycling of the biopterin cofactor and could cause affected mammals to compensate by increasing the more energy costly BH$_4$ synthetic pathway. Defects of dihydropteridine reductase cause a variant form of hyperphenylalaninemia in

Abbreviations: BH$_4$, L-*erythro*-5,6,7,8-Tetrahydrobiopterin; qBH$_2$, Quinonoid dihydrobiopterin; DMPH$_4$, 2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine; qDMPH$_2$, Quinonoid 2-amino-6,7-dimethyl-4-hydroxydihydropteridine.

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humans[4,5]. Studies on the inhibition of dihydropteridine reductase bears physiological significance on the neurotransmitter synthesis in mammals.

Rat striatal synaptosomal preparations have been used as a model system for studying the regulation of tyrosine 3-hydroxylase and its effect on catecholamine synthesis[6–10], because synaptosomes contain the enzymes and cofactors necessary for dopamine synthesis from L-tyrosine[11]. Likewise, rat striatal synaptosomes may serve as a model system for examining the regulation of dihydropteridine reductase activity. This paper describes the inhibition of rat striatal synaptosomal dihydropteridine reductase by exogenous and endogenous catecholic compounds.

MATERIALS AND METHODS

Materials

The following compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI): dopamine (3,4-dihydroxyphenylethylamine) hydrochloride, DL-3,4-dihydroxymandelic acid, *p*-hydroxyphenylpyruvic acid, and 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine ($DMPH_4$) hydrochloride. The following compounds were purchased from Sigma Chemical Co. (St Louis, MO): 17β-estradiol (1,3,5(10)-estratriene-3,17β-diol), estrone (3-hydroxy-1,3,5(10)-estratriene-17-one), 2-hydroxyestrone, sodium pyruvate, benzylamine (phenylmethylamine), horseradish peroxidase and NADH. L-Tyrosine was obtained from Pharmacia P-L Biochemicals Inc. (Milwaukee, WI) and (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide from Research Biochemicals Inc. (Wayland, MA). Nomifensine maleate (Merital, HOE 984, 8-amino-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline) and 4′-hydroxy-nomifensine maleate were generously supplied by Hoechst-Roussel Pharmaceuticals Inc. (Somerville, NJ). (±)-Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) hydrobromide and higenamine (norcoclaurine) hydrobromide were generously donated by Dr Robert V. Smith, Washington State University (Pullman, WA); tetrahydropapaveroline hydrobromide and (±)-1-(3′,4′-dihydroxyphenyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide by Dr A. Brossi, National Institutes of Health (Bethesda, MD). Solutions of these compounds were prepared fresh in Tris-HCl buffer (0.05 M), pH 6.8, and degassed immediately prior to use. [Methylene-^{14}C]-benzylamine hydrochloride (14 mCi/mmol) was obtained from ICN Pharmaceuticals Inc. (Irvine, CA).

Preparation of Brain Synaptosomes

Rat striatal synaptosomes were prepared by the method of Gray and Whittaker[12] with slight modification[13]. Male Sprague-Dawley rats (220–280 g) were decapitated and their striatal tissue dissected out and homogenized in sucrose solution (0.32 M), pH 7.4, (1 ml/rat) with a motor-driven teflon pestle-glass homogenizer (0.13 to 1.8 mm clearance). Striatal homogenates were centrifuged at 1000 × g for 15 min. The resulting supernatant was centrifuged at 17 500 × g for 20 min to sediment the P_2 fraction, which was washed twice by homogenization with sucrose solution (0.32 M), pH 7.4, (10 ml for the first wash and 5 ml for the second wash) followed by centrifugation (17 500 × g for 20 min). The pellet thus obtained was designated as the P_2W_2 fraction. P_2 or P_2W_2 were suspended in 15 volumes (w/v) of a buffered medium containing Tris-HCl (50 mM), pH 7.4, NaCl (125 mM), KCl (5 mM), $CaCl_2$ (1 mM), $MgCl_2$

(1 mM), and glucose (10 mM) (physiological Tris). These synaptosomal preparations were stored at 4°C and were stable for at least 24 h. Protein in the synaptosomal preparation was measured by the method of Lowry *et al.*[14] after freezing synaptosomes at − 20°C overnight and then thawing. Dihydropteridine reductase activity in synaptosomal preparations was assayed at 50 μM each of $DMPH_4$ and NADH or at 10 μM BH_4 and 50 μM NADH. Kinetic studies indicated that dihydropteridine reductase in the P_2 fraction of rat striatal synaptosomes had K_m values of 17 μM for qDMPH$_2$ and 10 μM for NADH. The K_m values of the enzyme in the P_2W_2 fraction of rat striatal synaptosomes were 4 μM for qBH$_2$ and 9 μM for NADH.

Human Liver Dihydropteridine Reductase

The enzyme was purified by ammonium sulphate precipitation, sequential column chromatography on DEAE-Sephacel, Matrex Gel Blue A, and hydroxyapatite, and high performance liquid chromatography, according to the procedures described previously[15,16]. The enzyme preparation used in this study was form B, which had a specific activity of 100 U/mg protein.

Determination of Enzyme Activities

Dihydropteridine reductase activity was determined spectrophotometrically by measuring the rate of disappearance of NADH at 340 nm at 25°C, according to the method of Nielsen *et al.*[17]. The quinonoid $DMPH_2$ was generated *in situ* by the peroxidase-catalyzed oxidation of $DMPH_4$ in the presence of H_2O_2.

Lactate dehydrogenase activity was determined spectrophotometrically in the presence of sodium pyruvate (0.2 mM) and NADH (50 μM) in Tris-HCl buffer (0.05 M), pH 7.4, by measuring the rate of disappearance of NADH at 340 nm at 25°C.

Monoamine oxidase activities were measured radiochemically by using labeled benzylamine as substrate, according to the method of Wurtman and Alexrod[18]. Synaptosomal preparations (5–20 μg protein) were incubated with [^{14}C]-benzylamine (2 mM, specific activity = 3 μCi/μmol) in potassium phosphate buffer (0.05 M), pH 7.4, (final volume 0.015 ml) at 37°C for 30 min. The reaction mixture was adjusted to 1.2 N HCl with 6 N HCl and the radioactive benzaldehyde was extracted into toluene (0.12 ml). After centrifugation (Eppendorf model 5413 centrifuge, 5 min), the radioactivity of the supernatant (0.06 ml) in Hydrocount (2 ml, J.T. Baker Chemicals B.V., Deventer, Holland) was counted on a Beckman LS 6800 Liquid Scintillation System.

All enzyme activities were expressed in units (U). One U was defined as the amount of enzyme that catalyzed the formation of one μmol product per min at 25°C.

Dihydropteridine Reductase Inhibition

The reaction mixture in the presence of inhibitors contained 50 μmol of Tris, approximately 7 mU of dihydropteridine reductase and the indicated amount of each test compound in a volume of 0.6 ml. Blanks contained inhibitors but no enzyme. The reaction mixture in the absence of inhibitors contained Tris buffer and enzyme, and the blanks contained no inhibitor and no enzyme. After incubation for 10 min at 25°C, $DMPH_4$, peroxidase, H_2O_2, and NADH were added to each reaction mixture to a final volume of 1 ml. The reaction rates were monitored for 2–4 min by recording

the decrease in absorbance at 340 nm with a Gilford model 250 spectrophotometer equipped with a model 6051 recorder. Methods for obtaining the dissociation constants of the enzyme-inhibitor complexes (K_i values) and the inhibitor concentrations that give 50% inhibition of the enzyme activity (I_{50} values) have been described previously[19].

RESULTS

Table I represents a typical profile of the 3 selected enzymes in rat striatal synaptosomal fractions. Activities of dihydropteridine reductase along with a soluble enzyme, lactate dehydrogenase, and one membrane enzyme, monoamine oxidase, were found in all tissue, cellular, and subcellular fractions. The distribution of dihydropteridine reductase activities parallels that of lactate dehydrogenase, but not monoamine oxidase, indicating that this enzyme is located primarily in the cytoplasm. Rat striatum contains approximately 3, 29, and 0.05 U/mg protein for dihydropteridine reductase, lactate dehydrogenase, and monoamine oxidase, respectively (data not shown). The P_2 fraction of the synaptosomal preparation contained about 25% of the total dihydropteridine reductase activity. When the P_2 fraction was washed twice, about 50% of the protein but only 6% of dihydropteridine reductase activity was lost. Consequently, the specific activity of this enzyme was enhanced about 70% in the P_2W_2 fraction. Specific activities of the two marker enzymes, lactate dehydrogenase and monoamine oxidase, also were enhanced 80–87% in the P_2W_2 fraction. Subcellular fractionation of the P_2W_2 synaptosomes gave approximately 60% of dihydropteridine reductase and lactate dehydrogenase activities in the soluble fractions, but no monoamine oxidase activity (data not shown). However, all monoamine oxidase activity was found in the membrane fraction.

The effect of storage at 4°C on the activities of dihydropteridine reductase, lactate

TABLE I

Activities of dihydropteridine reductase (DHPR), lactate dehydrogenase (LDH), and monoamine oxidase (MAO) in rat striatal synaptosomal fractions. The P_2W_2 fraction was frozen (−20°C) and thawed (25°C) three times to lyse synaptosomes, and then centrifuged (30,000 × g, 20 min). The supernatant was saved and the pellet resuspended in physiological Tris, and then centrifuged again. This supernatant was combined with the previous one to form the soluble P_2W_2 fraction. The pellet was resuspended in physiological Tris to yield the membrane P_2W_2 fraction. All enzyme activities were the mean ± S.D. of at least three determinations from a group of 6 rats. One mU is the amount of enzyme which catalyzes the conversion of one nmol substrate per min at 25°C. Two-tailed t-tests were used to analyze the differences in means between the P_2 and P_2W_2 fractions.

Synaptosomal fraction	Total Protein (mg)	Specific activity (mU/mg)		
		DHPR	LDH	MAO
Crude homogenate	30.2	91.3 ± 3.3	962 ± 62	1.69 ± 0.07
P_2-fraction	13.5	51.3 ± 3.4	437 ± 31	1.06 ± 0.17
Twice-washed P_2 (P_2W_2)	7.4	87.7 ± 1.2[a]	793 ± 40[a]	1.98 ± 0.21[a]
P_2W_2-membrane	3.6	47.7 ± 0.5	391 ± 21	2.71 ± 0.26
P_2W_2-soluble	1.1	262.7 ± 0.9	2,057 ± 138	0

[a] $p < 0.001$

dehydrogenase, and monoamine oxidase in rat striatal synaptosomal preparations are presented in Table II. Activities of all three enzymes in P_2 and P_2W_2 remained unchanged for at least 24 h.

Table III summarizes the kinetic constants derived from the inhibition of human liver and rat striatal dihydropteridine reductase by 16 compounds. The results indi-

TABLE II

Effect of storage at 4°C on the activities of dihydropteridine reductase (DHPR), lactate dehydrogenase (LDH), and monoamine oxidase (MAO) in rat striatal synaptosomes. The P_2 and P_2W_2 fractions of rat striatal synaptosomes were suspended in physiological Tris at protein concentrations of 1.3–1.5 mg/ml. They were stored at 4°C overnight and activities of DHPR, LDH, and MAO determined. Each value was the mean ± S.D. of at least three determinations and was expressed in mU/mg.

Preparation	Day	DHPR	LDH	MAO
P_2	0	51.3 ± 3.4	437.0 ± 31.1	1.06 ± 0.17
	1	50.7 ± 4.2	458.1 ± 18.0	1.18 ± 0.03
P_2W_2	0	87.7 ± 1.2	792.7 ± 40.1	1.98 ± 0.21
	1	92.0 ± 7.1	817.9 ± 29.4	1.89 ± 0.19

TABLE III

K_i and I_{50} values of phenolic and catechol-containing compounds as inhibitors of dihydropteridine reductase. Human liver enzyme (6.6 mU or 17 ng protein) or rat striatal synaptosomes (6.5 mU or 100 μg P_2 protein and 7.0 mU or 82 μg P_2W_2 protein) were incubated with at least 6 concentrations of each compound for 10 min at 25°C. Residual enzyme activity was assayed spectrophotometrically at 50 μM each of $DMPH_4$ and NADH in Tris-HCl buffer (0.05 M), pH 7.4. K_i and I_{50} values were obtained according to the previously published methods[20]. All values were expressed in μM. Coefficients of linear correlation: 0.9289 (N = 15) between I_{50} values obtained with P_2 synaptosomal and human liver enzyme; 0.9745 (N = 15) between I_{50} values obtained with P_2W_2 synaptosomal and human liver enzyme; 0.9874 (N = 15) between I_{50} values obtained with P_2 and P_2W_2 synaptosomal enzyme; 0.9718 (N = 13) between I_{50} and K_i values obtained with human liver enzyme.

	P_2		P_2W_2		Human liver	
Compound	I_{50}	K_i	I_{50}	K_i	I_{50}	K_i
L-Tyrosine	590.0		464.0		440.0[a]	250.0[b]
p-Hydroxyphenylpyruvic acid	2.4		3.2		3.3[b]	3.7[b]
Dopamine	24.0[c]		10.5	13.0[c]	20.0[a]	14.0[a]
DL-3,4-Dihydroxy-mandelic acid	13.0[c]	17.0[c]	12.0		8.5[c]	9.0[c]
(±)-Salsolinol	56.0		45.0		52.0	90.0[d]
Higenamine	5.0		2.6	3.1	2.6	1.5[d]
(±)-1-(3′,4′-Dihydroxy-phenyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	10.1		9.6		8.9	8.0
(±)-Tetrahydro-papaveroline	1.6		2.0		2.0	0.5
R-(-)-Apomorphine	1.2[e]	1.2[e]	0.9[e]		2.0[e]	2.2[e]
(±)-2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene		5.1	4.6	5.7	4.9	
17β-Estradiol	2.2		4.0		5.7[f]	3.5[f]
Estrone	4.5		5.3		5.4	2.1
2-Hydroxyestrone	1.5		1.8	1.5	3.3[f]	4.6[f]
Nomifensine	192.0[g]		230.0		345.0[g]	
4′-Hydroxynomifensine	35.0[g]	44.0[g]	22.0		45.0[g]	35.0[g]

[a–g] For comparison, a–g represents data taken from references 19, 20, 3, 16, 13, 21, and 22, respectively.

cate that K_i and I_{50} values obtained with rat striatal synaptosomal preparations are essentially the same as those obtained with highly purified human liver dihydropteridine reductase. Coefficients of correlation were 0.9289 ($N = 15$) between I_{50} values obtained with P_2 synaptosomal and human liver enzyme; 0.9745 ($N = 15$) between I_{50} values obtained with P_2W_2 synaptosomal and human liver enzyme; 0.9874 ($N = 15$) between I_{50} values obtained with P_2 and P_2W_2 synaptosomal enzyme; and 0.9718 ($N = 13$) between I_{50} and K_i values obtained with human liver enzyme. The linear correlation between the K_i and I_{50} values suggests that the type of inhibition is noncompetitive[23], which is supported by Lineweaver–Burk plots of the inhibition kinetics of the enzyme by each compound (data not shown). These results are consistent with those previously published[3,13,16,19–22,24,25].

The results presented in Table III also indicate that multi-ring compounds containing a catechol moiety, such as higenamine, tetrahydropapaveroline, apomorphine, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene, and 2-hydroxyestrone, are in general more potent inhibitors of dihydropteridine reductase than single ring catechol-containing compounds, such as dopamine and 3,4-dihydroxymandelic acid, and that catecholic compounds are in turn more potent inhibitors of the enzyme than the *p*-hydroxyphenolic compounds, such as tyrosine, estrone, and 4′-hydroxynomifensine.

DISCUSSION

BH_4 is the obligatory cofactor for aromatic amino acid hydroxylases during biogenic amine synthesis[2]. When BH_4 is oxidized by tyrosine and tryptophan hydroxylases to the inactive dihydro-form, it is spontaneously regenerated by dihydropteridine reductase. BH_4 is synthesized from GTP by an as yet incompletely defined pathway initiated by GTP cyclohydrolase I (GTP 7,8-8,9-dihydrolase, EC 3.5.4.16)[26]. Therefore, intracellular BH_4 levels are governed by two enzymes–GTP cyclohydrolase I, which regulates its rate of formation, and dihydropteridine reductase, which regulates its rate of regeneration. The normal functioning of these two enzymes maintains a catalytic pool of BH_4, and thereby contributes to the regulation of the *in vivo* activities of aromatic amino acid hydroxylases. Both enzymes appear to be essential, however, because a defect in either can impair neurotransmitter biosynthesis, and can result in neurological and/or psychiatric disturbances[4,5,27,28].

Rat striatum is rich in dihydropteridine reductase, which is found mainly in the cytoplasm. Synaptosomal preparations yield approximately 25% of the total enzyme activity in rat striatum and are stable overnight. These qualities make P_2 or P_2W_2 a convenient source of dihydropteridine reductase for study of the activation, inhibition, and regulation of activity in the presence of other enzymes and cofactors that are involved in neurotransmitter synthesis.

Inhibition kinetic constants of enzyme determined with P_2 or P_2W_2 from rat striatal synaptosomal preparations are essentially the same as those obtained with highly purified human liver preparations. These similarities suggest that the molecular structure and properties of the enzyme in both species are related. Therefore, information collected by rat striatal synaptosomal dihydropteridine reductase may be interchangeable for human liver enzyme.

Although the P_2W_2 fraction has a dihydropteridine reductase specific activity that is 70% higher than that of the P_2 fraction, the I_{50} and K_i values are essentially the

same. Besides this the P_2 fraction appears to be more stable at 4°C than P_2W_2 fraction (data not shown) so that for practical reasons the P_2 fraction can be used as an enzyme source for studying dihydropteridine reductase.

Tetrahydroisoquinolines, such as higenamine, tetrahydropapaveroline, 1-(3′,4′-dihydroxyphenyl)-6,7-dihydroxy-1,2,3,6-tetrahydroisoquinoline, and salsolinol, contain a dopamine moiety and are found to be potent inhibitors of dihydropteridine reductase. This group of compounds, along with other catechol-containing compounds, such as catecholamines and their metabolites,[3,20,29,30] catechol estrogens[19], aporphines[13], and 3′,4′-dihydroxy-1,2,3,6-tetrahydropyridines[24,25], therefore represent a class of catecholoids that can inhibit dihydropteridine reductase.

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