HYDROXYLATION OF PHENYLALANINE BY PURIFIED PREPARATIONS OF ADRENAL AND BRAIN TYROSINE HYDROXYLASE

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Sympathetically innervated tissues and brain contain an L-tyrosine hydroxylase which catalyzes the initial step in the formation of norepinephrine from the dietary precursor, tyrosine (1). In studying the inhibition of purified adrenal tyrosine hydroxylase by phenylalanine, it has been found that the latter can also be hydroxylated by these preparations to yield first tyrosine and then dopa. The present report presents initial evidence which suggests that hydroxylation of phenylalanine is catalyzed by tyrosine hydroxylase itself and not by a specific phenylalanine hydroxylase similar to the liver enzyme (2).

MATERIALS AND METHODS

L-tyrosine 3,5-^{3}H (5600 μc/μmole), DL-meta tyrosine-^{3}H (27 μc/μmole), and L-phenylalanine-^{14}C (333 μc/μmole) were obtained from New England Nuclear Corporation; α-methyl-L-tyrosine from Merck Sharp and Dohme; 3-iodo-L-tyrosine from Aldrich Chemical Company and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) from Calbiochem.

Beef adrenal and dog brain tyrosine hydroxylase were purified by the method of Nagatsu *et al.* (1). With the adrenals an additional dialysis and ammonium sulfate precipitation were used. Rat liver phenylalanine hydroxylase was purified according to the method of Kaufman (3) up to the second ammonium sulfate precipitation stage.

The incubation mixture for the adrenal enzyme, unless otherwise stated, consisted of 0.1 μmole of L-tyrosine (1 x 10^5 cpm), or L-

phenylalanine (5×10^5 cpm), 100 μmoles mercaptoethanol, 200 μmoles of acetate buffer pH 6.0, 1 μmole of $DMPH_4$, in a final volume of 1 ml. The incubation mixture for the liver enzyme consisted of 200 μmole of phosphate buffer, pH as indicated, 1 μmole $DMPH_4$, 0.1 to 2 μmoles L-tyrosine or L-phenylalanine, as described, and water to 1.0 ml. Incubations were performed for 15 minutes at 37°.

The phenylalanine hydroxylase activities of adrenal and brain

Table 1: Chromatography of products formed from L-phenylalanine-^{14}C.

	R_f values	
	A	B
Authentic phenylalanine	0.58	0.75
Found in incubation mixture	0.58	0.75
Authentic p-tyrosine	0.44	0.48
Authentic m-tyrosine	0.45	0.48
Formed from phenylalanine	0.44	0.47
Authentic dopa	0.30	0.26
Formed from phenylalanine	0.29	0.25

Whatman # 3MM paper; development overnight.
Solvent A - n-butanol, acetic acid, water (25:6:25).
Solvent B - phenol, ethanol, water, ammonia (150:40:10:1).

preparations were assayed by the use of L-phenylalanine-^{14}C as substrate followed by isolation of the products by paper chromatography. Incubation mixtures were prepared for chromatography by deproteinizing and desalting as follows: 0.1 ml of 2 N HCl was added, followed by 1 ml ethanol and 10 ml acetone. The tube was centrifuged and the supernatant fluid removed and evaporated under a stream of nitrogen. The residue was defatted by washing the dry tube with 0.5 ml of benzene. Traces of the benzene were removed by evaporation and the residue was taken up in 50% ethanol containing 0.005 N HCl and applied to the paper. After development (solvent A - Table 1) the paper was dipped in acid ninhydrin and the areas corresponding to dopa, tyrosine and

phenylalanine were cut out and placed in counting vials with 2 ml of water. Ten ml of phosphor solution was added and the vials were placed in a scintillation spectrometer for radioassay. The recovery through the entire process was about 70%.

Tyrosine hydroxylase activity was assayed by following the release of tritium from L-tyrosine 3,5-^{3}H into water (4). Liver phenylalanine hydroxylase activity was followed by the fluorometric assay of tyrosine (5).

RESULTS

To identify the products formed from L-phenylalanine-^{14}C by tyrosine hydroxylase preparations deproteinized incubation mixtures were subjected to chromatography on paper using several solvents. As shown in Table 1, two products appeared, tyrosine and dopa. The enzymatically formed dopa was further characterized by its ability to absorb onto alumina at pH 8.4 and be eluted by dilute acetic acid. The product also yielded the fluorescent trihydroxyindole product typical of dopa on oxidation with iodine and rearrangement in alkali (6).

The tyrosine could have been the meta or para isomer since either may be an intermediate in dopa formation and chromatography did not affect a separation. However, the formation of dopa indicated that much of the tyrosine was the para form since m-tyrosine is not oxidized by tyrosine hydroxylase (1). On addition of carrier p-tyrosine to the enzymatically formed tyrosine eluted from chromatograms, it was possible to achieve constant specific activity over several recrystallizations (Table 2). Had the product contained appreciable quantities of m-tyrosine-^{14}C the carrier p-tyrosine would have continually lost radioactivity, as in the control.

The time course of the reaction is shown in Fig. 1. Tyrosine appears first and phenylalanine oxidation is linear for 15 minutes then falls off markedly. For this reason, incubations in subsequent

Table 2: Repeated recrystallizations of carrier p-tyrosine added to enzymatically formed tyrosine.

Crystallizations	Specific activity of carrier p-tyrosine added to:		
	Authentic p-tyrosine-^{3}H	Enzymatically formed tyrosine-^{14}C	Authentic m-tyrosine-^{3}H
		cpm/mg	
0 (calculated)	5,250	800	715
2	4,180	653	10.2
4	4,290	620	2.4
6	4,490	621	5.5

To 30 mg samples of p-tyrosine were added 157,500 cpm of p-tyrosine-^{3}H, 21,400 cpm of m-tyrosine-^{3}H and 24,000 cpm of tyrosine isolated from an incubation mixture of L-phenylalanine-^{14}C and adrenal enzyme. For each recrystallization step the tyrosine was dissolved in a minimal volume of 0.1 N HCl, the pH adjusted to 6.0 by adding 0.1 N NaOH and 0.1 ml of 1 M acetate buffer and the sample cooled in an ice bath. The crystals were isolated by filtration, redissolved and small aliquots taken for fluorescence and radioassay. The remainder was then recrystallized.

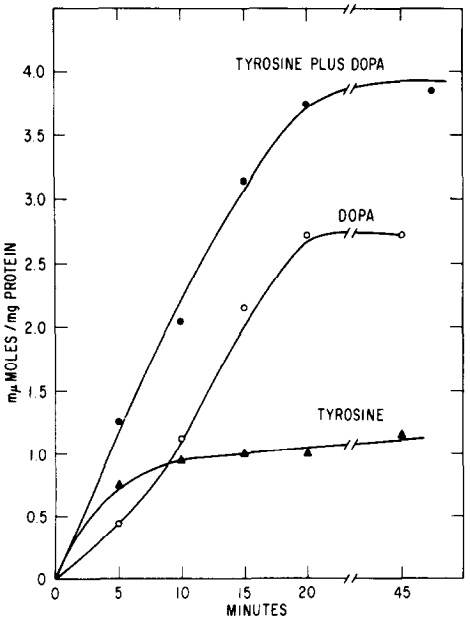

Fig. 1. Formation of tyrosine and dopa from phenylalanine by purified adrenal tyrosine hydroxylase. Incubation mixtures contained 100 mμmoles of L-phenylalanine and 7 mg of enzyme protein.

studies were for 15 minutes. Dopa was the end product of the reaction. In a typical incubation using 7 mg of adrenal enzyme 19.8 mμmoles of phenylalanine disappeared and 6.0 mμmoles of tyrosine and 13.9 mμmoles of dopa were formed.

The formation of tyrosine from phenylalanine posed the question whether tyrosine hydroxylase preparations contained a separate phenylalanine hydroxylase, perhaps comparable to the liver enzyme, or whether the phenylalanine and tyrosine activities were catalyzed by one and the same enzyme. The relative activities of purified preparations from adrenal medulla, brain and liver are shown in Table 3. It is apparent that the liver preparation does not contain tyrosine hydroxylase activity. Ratios of tyrosine to phenylalanine hydroxylating activity in the adrenal and brain preparations were comparable. Table 3 also shows the effects of two inhibitors of tyrosine hydroxylase. These inhibited adrenal tyrosine and phenylalanine activity markedly and to the same extent, but had no effect on the liver enzyme.

The adrenal phenylalanine hydroxylase activity also resembled brain

Table 3: Hydroxylation of phenylalanine and tyrosine by adrenal, brain and liver preparations and effect of inhibitors.

Tissue	Amino Acid	Amount oxidized	Inhibited by A	Inhibited by B
		mμmoles/mg/hr	per cent	
Adrenal	L-tyrosine	108.0	53	86
	L-phenylalanine	5.5	72	93
Brain	L-tyrosine	22.8	50	87
	L-phenylalanine	1.4	-	-
Liver	L-tyrosine	0	-	-
	L-phenylalanine	546.0	0	0

The concentrations of tyrosine and phenylalanine were 10^{-4} M in all experiments. Adrenal and brain enzymes were incubated at pH 6.0; the liver enzyme at pH 6.8. Inhibitor A = α-methyl-tyrosine (2.5×10^{-5} M); B = 3-iodotyrosine (1×10^{-5} M).

and adrenal tyrosine hydroxylase in its pH optimum and differed considerably from the liver enzyme (Table 4).

In many other respects, the adrenal phenylalanine hydroxylase activity differs from the liver enzyme. The liver enzyme is inhibited by high concentrations of substrate, the adrenal enzyme is not. The K_m for the liver enzyme is greater than 10^{-3} M, that of the adrenal enzyme is about 3×10^{-4} M for phenylalanine and 5×10^{-5} M for tyrosine. Phenylalanine inhibits the oxidation of tyrosine by the adrenal enzyme in a competitive fashion (K_i about 10^{-4} M). When tyrosine is added as an inhibitor it is rapidly metabolized. Nevertheless, high concentrations of tyrosine ($> 10^{-4}$ M) inhibit oxidation of phenylalanine by the adrenal enzyme.

Table 4: Effect of pH on hydroxylation activity.

pH	Adrenal enzyme		Brain enzyme	Liver enzyme	
	Tyrosine	Phenyl-alanine	Tyrosine	Phenyl-alanine	Tyrosine
6.0	100	100	100	40	0
6.8	46	56	45	91	-
7.4	22	19	25	100	-

The values represent per cent of maximal activity.

DISCUSSION

These studies demonstrate that tissues other than liver convert phenylalanine to tyrosine. The phenylalanine hydroxylating activity in adrenal and brain is much lower than that of liver, but it is not due to small amounts of the "liver" enzyme in these other tissues. The presence of extra-hepatic phenylalanine hydroxylating activity may explain the conversion of phenylalanine-^{14}C to tyrosine-^{14}C (about 5% of normal) by patients with phenylketonuria (7). Whether the huge amounts of phenylalanine, which are found in phenylketonuria, inhibit norepinephrine formation by competing with tyrosine is not clear since

phenylalanine also gives rise to dopa. If the residual phenylalanine hydroxylating activity in phenylketonurics is due to sympathetic tyrosine hydroxylase, such patients may provide a means for studying sympathetic tyrosine hydroxylase in man in vivo.

Hydroxylation of phenylalanine by tyrosine hydroxylase is also of interest from the standpoint of the enzyme mechanism. If the same enzyme catalyzes both reactions, as appears to be the case, then this represents one of the few instances where one enzyme catalyzes two similar consecutive steps. Free tyrosine appears during incubations with phenylalanine and is initially present in higher concentration than dopa. Since the K_m for tyrosine is lower and the V_{max} much higher, it is surprising that phenylalanine is not directly converted to dopa. One explanation is that the enzyme-tyrosine complex dissociates before conversion to dopa. Studies on the sequential hydroxylation of phenylalanine to p-tyrosine to dopa, should help in elucidating mechanisms of enzymatic hydroxylation.

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