Phenylalanine as substrate for tyrosine hydroxylase in bovine adrenal chromaffin cells

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Incubation of bovine chromaffin cells with L-[¹⁴C]phenylalanine resulted in label accumulation in catecholamines at about 30 % of the rate seen with L-tyrosine as precursor. Studies with purified tyrosine hydroxylase (EC 1.14.16.2) showed that the enzyme catalysed the hydroxylation of L-phenylalanine first to L-*p*-tyrosine and then to 3,4-dihydroxyphenylalanine (DOPA). No evidence for a significant involvement of an L-*m*-tyrosine intermediate in DOPA formation was found.

INTRODUCTION

The hydroxylation of phenylalanine by tyrosine 3-mono-oxygenase (tyrosine hydroxylase, EC 1.14.16.2) was reported as early as the 1960's with partially purified enzyme preparations from dog brain, bovine adrenal medulla and guinea-pig heart [1–4]. Direct injection of labelled phenylalanine into brains of rats resulted in the formation of labelled catecholamines [5]. In spite of these early observations, the possibility that phenylalanine could be a physiologically significant precursor for catecholamine biosynthesis in neurons and chromaffin cells has remained controversial, probably for the following reasons: (1) non-enzymic oxidation of phenylalanine to tyrosine in the presence of cofactor and dioxygen is known to occur [6,7]; (2) one of the products of the enzymic reaction catalysed by tyrosine hydroxylase was claimed to be *m*-tyrosine [8], which was found not to be a substrate for the enzyme [9]; and (3) highly purified preparations of tyrosine hydroxylase were reported not to catalyse phenylalanine hydroxylation [10,11]. In our laboratory, however, phenylalanine was found to be almost as good a substrate for highly purified tyrosine hydroxylase as tyrosine itself under certain experimental conditions *in vitro* [12]. In the present study we confirm and extend this finding, by showing that phenylalanine can also be utilized for the biosynthesis of catecholamines in isolated bovine adrenal chromaffin cells.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade and obtained from standard suppliers. Substrates and catalase were from Sigma Chemical Co. (6*R*)-L-erythro-Tetrahydropterin was from Dr. B. Schircks Laboratories (Jona, Switzerland). Radioisotopes were from Amersham. Tyrosine hydroxylase was purified from adrenal-medulla cytosol [13] and assayed as described previously [14].

Preparation of chromaffin cells

Bovine adrenal chromaffin cells were isolated essentially as described by Livett [15]. Yields were (5–10) × 10⁷ cells for four glands, and the viability was 90 % or more, as determined by the Trypan Blue exclusion method. The catecholamine content of the cell preparations was 185 ± 22 nmol/10⁶ cells, and the molar ratio of adrenaline to noradrenaline was 3.4 ± 0.8 (*n* = 6).

Incubation of chromaffin cells with [¹⁴C]phenylalanine and [³H]tyrosine

Biosynthesis of catecholamines was determined by incubation of chromaffin cells with [¹⁴C]phenylalanine or [³H]tyrosine at 37 °C. In time-course experiments, the reaction was stopped by addition of HClO₄ (final concn. 3 %, w/w) and cooling. The supernatants were neutralized to pH 3–5 with K₂CO₃, and the content of catecholamines was assayed by h.p.l.c. as described below. Further details of the incubation conditions are given in the Figure legends.

The hydroxylated products of phenylalanine and tyrosine were separated by h.p.l.c. on a strong cation exchanger (Whatman Partisphere SCX, 12.5 cm column, with a CX Guard pre-column) and quantified by fluorimetric detection [14], with excitation at 274 nm and emission at 304 nm for DOPA, tyrosine and *m*-tyrosine, and excitation at 258 nm and emission at 288 nm for phenylalanine. This latter pair of wavelengths was also used for assay of noradrenaline and adrenaline in cell extracts, because the catecholamines were eluted immediately after phenylalanine.

L-*m*-Tyrosine was isolated from the racemic mixture by using a Bakerbond Crownpak CR chiral h.p.l.c. column (Daicel Chemical Industries, Tokyo, Japan) with HClO₄ (1.63 g/l) as the mobile phase, neutralized with K₂CO₃ and concentrated under reduced pressure.

RESULTS

Biosynthesis of catecholamines in chromaffin cells

The chromatographic systems used to separate and quantify the products in the catecholamine-biosynthetic pathway are shown in Fig. 1. Adrenal chromaffin cells incubated with [³H]tyrosine incorporated label into noradrenaline and adrenaline almost in parallel (Fig. 2*a*). The amount of adrenaline formed after 60 min was 97 pmol/10⁶ cells (average of two determinations), a value comparable with that reported by Haycock *et al.* [16]. In contrast, the levels of labelled DOPA and dopamine were very low.

When the cells were incubated with [¹⁴C]phenylalanine, a linear increase in [¹⁴C]tyrosine was observed, but with a pronounced lag phase in the labelling of catecholamines (Fig. 2*b*). However, after 15–30 min, the rate of adrenaline labelling was about 50 % of that observed with [³H]tyrosine as the

Abbreviation used: DOPA, 3,4-dihydroxyphenylalanine.

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Fig. 1. Chromatographic separation of catecholamines and intermediates in their biosynthesis

Standards were dissolved in 10 mM-sodium acetate buffer, pH 3.5, and separated by h.p.l.c. on a strong cationic exchanger (see the Materials and methods section). (a) L-DOPA, L-tyrosine and L-m-tyrosine (60, 10 and 6 μ M respectively) were first eluted with 10 mM-sodium acetate buffer, pH 3.5, and detected fluorimetrically (excitation at 274 nm and emission at 304 nm). (b) L-Phenylalanine, noradrenaline and adrenaline (100, 200 and 200 μ M respectively) were subsequently eluted from the same column with 0.4 M-sodium acetate buffer, pH 3.5, and detected with excitation at 258 nm and emission at 288 nm. (c) and (d). The same standards as above were added to a neutralized HClO_4 extract of bovine adrenal chromaffin cells and eluted consecutively with 10 mM-sodium acetate (c) and 0.4 M-sodium acetate (d). The large increases in noradrenaline and adrenaline represent endogenous catecholamines of the chromaffin cells.

substrate. In a series of experiments, the accumulation of label into adrenaline was estimated to be 23.5 ± 4.8 pmol/ 10^6 cells (mean $\pm$ S.D., $n = 3$) after 60 min. No significant levels of L-m-tyrosine or DOPA were observed (results not shown). For both

Fig. 2. Biosynthesis of catecholamines in bovine adrenal chromaffin cells

(a) Chromaffin cells (9×10^6 cells/ml) were incubated at 37 $^{\circ}\text{C}$ in a medium consisting of 150 mM-NaCl, 5 mM-KCl, 25 mM-Hepes, 2.2 mM- CaCl_2 , 0.8 mM- MgCl_2 and 5 mM-glucose, with shaking in air; 100 μCi of L-[3,5- ^3H]tyrosine with 100 μM -L-tyrosine was added at zero time. Samples (0.5 ml) of the incubation mixture were removed at the times noted and added to 0.5 ml of ice-cold medium. The cells were centrifuged at 1000 g for 30 s and then resuspended in 0.5 ml of 3% (w/w) HClO_4 . Quantification of the labelled noradrenaline ($\blacksquare$) and adrenaline ($\bullet$) was corrected for the loss of half of the label as a result of hydroxylation in the 3-position. (b) Cells were incubated exactly as described in (a), except that 7.5 μCi of [^{14}C]phenylalanine with 100 μM -phenylalanine was used as substrate and the extracts were assayed for L-p-[^{14}C]tyrosine ($\circ$) and [^{14}C]adrenaline ($\bullet$) as described in the Materials and methods section. Less than 10% of the radioactivity was recovered in the cell pellets; up to 1500 c.p.m. was found in the fractions with the highest labelling. Each point represents the mean value of duplicate determinations in three experiments.

substrates, the steady-state labelling of dopamine in chromaffin cells was found to be very low (results not shown) [17].

The addition of 0.1 mM of the cholinergic agonist carbachol increased the incorporation of label into catecholamines 2–3-fold

Fig. 3. Effect of carbachol and α -methyltyrosine on catecholamine biosynthesis from phenylalanine

The incubations were either carried out as described in the legend to Fig. 2 (●), or in the presence of 0.1 mM-carbachol (○), or in the presence of 20 μ M- α -methyltyrosine (▲). Each point represents the mean for the sum of noradrenaline and adrenaline formed ($\pm$ S.D. for end points) of triplicate determinations in two experiments.

with both phenylalanine (Fig. 3) and tyrosine (results not shown) as the substrate. Formation of labelled hydroxylated products did not occur in experiments with boiled cells (results not shown) or with fresh cells incubated with 20 μ M- α -methyltyrosine (Fig. 3).

Studies on purified tyrosine hydroxylase

Since phenylalanine hydroxylase has not been demonstrated in adrenal medulla [18], we conclude that the hydroxylation of phenylalanine in these cells is catalysed by tyrosine hydroxylase. Studies on the isolated purified enzyme confirmed this conclusion (Fig. 4a). Thus, when the enzyme was incubated with 0.2 mM-phenylalanine, catalase and cofactor, and the incubation mixture was analysed for products as a function of time, DOPA, L-*p*-tyrosine and a small amount of L-*m*-tyrosine were formed (Fig. 4a). Control incubations without added enzyme contained no hydroxylated products. The apparent K_m value for L-phenylalanine was 0.06 mM (0.04 mM for L-*p*-tyrosine) and the V was 1.05 times that with L-*p*-tyrosine as the substrate.

Hydroxylation of L-*m*-tyrosine

When DL-*m*-tyrosine was used as substrate, significant amounts of DOPA were formed (Fig. 4b). Since the possibility remained that *m*-tyrosine was a preferred substrate for tyrosine hydroxylase because it did not accumulate to a significant degree in cells, the D- and L-isomers were isolated and studied as substrates. D-*m*-Tyrosine was not hydroxylated by the enzyme, whereas L-*m*-tyrosine was slowly converted into DOPA, with an apparent K_m of about 0.8 mM at pH 7.2.

DISCUSSION

The studies reported here show that phenylalanine can be converted into catecholamines in intact bovine adrenal chromaffin cells, by a hydroxylation reaction catalysed by tyrosine hydroxylase, since phenylalanine hydroxylase has not been found in this tissue [18]. The ability of tyrosine hydroxylase

Fig. 4. Hydroxylation of L-phenylalanine and L-*m*-tyrosine catalysed by isolated bovine tyrosine hydroxylase

The enzyme activity was assayed by incubation of 0.2 mM substrate with 1 mM-(6*R*)-(L)-erythro-tetrahydrobiopterin, 0.1 mg of catalase/ml, 1 mM-dithiothreitol, 20 mM-Hepes, pH 7.2, and 16 μ g of purified enzyme protein/ml. Then 0.1 ml samples were added to an equal volume of 6% HClO₄ on ice. K₂CO₃-neutralized supernatants were analysed by h.p.l.c. as described in the Materials and methods section. (a) L-Phenylalanine (200 μ M) was used as substrate and the products measured were L-*m*-tyrosine (▲), L-*p*-tyrosine (○) and L-DOPA (▲). (b) DL-*m*-Tyrosine (200 μ M) was used as substrate and the measured product was L-DOPA (▲).

to hydroxylate phenylalanine has been reported previously [1–5], but for some reason the results on partially purified tyrosine hydroxylase [1–4] have not been widely accepted, perhaps because of reports on non-enzymic conversion of phenylalanine into tyrosine under certain reaction conditions [6,7], as well as enzymically catalysed formation of some *m*-tyrosine, which was shown not to be a substrate for the enzyme [8]. Reports on tyrosine hydroxylase isolated from rat pheochromocytoma (PC12) cells by two separate groups claimed that phenylalanine was not hydroxylated by their enzyme preparations [10,11], even under conditions in which 'one turnover event per enzyme molecule could be detected' [10].

However, in our laboratory the bovine adrenal enzyme purified to homogeneity and with a specific activity of more than 400 nmol of DOPA/min per mg of protein was found to hydroxylate L-

phenylalanine and form the cofactor product 4a-hydroxytetrahydropterin almost as efficiently as with L-*p*-tyrosine [12]. With the longer incubation periods used in the present study, the subsequent hydroxylation of the tyrosine formed from phenylalanine to DOPA was also observed (Fig. 4). Furthermore, tyrosine hydroxylase isolated from rat pheochromocytoma also hydroxylated L-phenylalanine to L-*p*-tyrosine and DOPA in our standard incubation system (K. K. Andersson, unpublished work). Catalase was included in the assay to prevent the non-enzymic conversion of phenylalanine into tyrosine, which occurs in the presence of cofactor and dioxygen [6,7]; product formation was completely dependent on the presence of enzyme.

That the hydroxylation of phenylalanine is not a reaction limited to the highly purified enzyme, e.g. owing to a change in substrate specificity as a result of its isolation, is clear from the present studies on intact bovine adrenal chromaffin cells. L-[¹⁴C]Phenylalanine was incorporated into catecholamines at a rate which was about one-third of that with L-tyrosine. The increased cellular levels of labelled L-tyrosine (Fig. 2a), but not of L-DOPA (results not shown), are consistent with the role of tyrosine hydroxylase as the rate-limiting enzyme in catecholamine biosynthesis. In fact, the hydroxylation of two consecutive substrates, L-phenylalanine and L-tyrosine, by the rate-limiting enzyme in a pathway would decrease the overall flux, as shown by the accumulation of tyrosine, although the substrates tyrosine and phenylalanine have comparable kinetic constants in the tyrosine hydroxylase-catalysed reaction. The tyrosine accumulation itself could also inhibit the flux directly, since the hydroxylase is known to be inhibited by high concentrations of its substrate [19]. Thus we have confirmed the observations by Ikeda *et al.* [2], reporting that formation of DOPA, but not of tyrosine, from phenylalanine was inhibited by high substrate concentrations. The stimulatory effect of carbachol on the rate of catecholamine labelling is probably due to activation of tyrosine hydroxylase by phosphorylation. Using labelled tyrosine as the substrate, Haycock *et al.* [16] have similarly shown that tyrosine hydroxylase in chromaffin cells is activated (and phosphorylated) by stimulation with acetylcholine.

Physiological implications

The physiological significance or biological advantage of the ability of tyrosine hydroxylase to use these two substrates is not obvious from these mechanisms which seem to operate in hyperphenylalaninaemia. Thus phenylalanine in high concentrations as seen in hyperphenylalaninaemia probably acts as a competitive substrate for tyrosine hydroxylase, with the net result of decreasing the availability of the enzyme for tyrosine and decreasing the rate of catecholamine biosynthesis. However, it has been suggested that the intraneuronal hydroxylation of phenylalanine by tyrosine hydroxylase is important for normal

protein synthesis [20]. That tyrosine hydroxylase can provide all the tyrosine needed for cell growth is illustrated by the ability of certain cell lines expressing that enzyme to grow in tyrosine-free media [21]. In fact, the capacity to grow in tyrosine-free medium has recently been used as a criterion for successful transfection of human tyrosine hydroxylase cDNA into various cell lines [22].

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