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SUBSTRATE SPECIFICITY AND OTHER PROPERTIES OF DOPA DECARBOXYLASE FROM GUINEA PIG KIDNEYS

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Summary

DOPA decarboxylase (aromatic-L-amino-acid carboxy-lyase, EC 4.1.1.28) from guinea pig kidneys has been purified to a specific activity of 9370 or 330-fold. Efficient purification was possible by employing apolar interaction chromatography. The purified enzyme gives a single component on polyacrylamide gel electrophoresis and the absorption spectrum of the enzyme reveals two forms of binding of pyridoxal 5-phosphate. The pure enzyme decarboxylates L-DOPA, 5-hydroxytryptophan, *o*-tyrosine and *m*-tyrosine but it is inactive towards phenylalanine, tyrosine, tryptophan, histidine and 3-methoxyphenylalanine. The enzyme behaves as an undissociated enzyme but only towards 5-hydroxytryptophan. It behaves as an enzyme from which the coenzyme is partially dissociated when it attacks L-DOPA, *o*-tyrosine and *m*-tyrosine.

Introduction

The number of amino acids that are decarboxylated by mammalian DOPA decarboxylase (aromatic-L-amino-acid carboxy-lyase, EC 4.1.1.28) remains an undecided question. Lovenberg et al. [1] listed 13 aromatic amino acids as substrates for DOPA decarboxylase from guinea pig kidneys, but using a homogeneous enzyme from hog kidneys, Christenson et al. [2] reported activity only towards L-DOPA, 5-hydroxytryptophan, phenylalanine, tyrosine and to a lesser extent towards histidine.

Different results were obtained by Lancaster and Sourkes [3] who also used a homogeneous enzyme from hog kidneys. Of the substrates they tested, L-DOPA, 5-hydroxytryptophan, *o*-tyrosine and *m*-tyrosine were decarboxylated at substantial rates but erythro-3, 4-dihydroxyphenylalanine was

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decarboxylated at an insignificant rate and tyrosine was not decarboxylated. The substrate specificity of the enzyme from other mammalian sources also seems to be limited to L-DOPA, 5-hydroxytryptophan, *o*-tyrosine and *m*-tyrosine [4—6].

It is evident from the cited work that additional research into the substrate specificity of this important enzyme is needed in order to decide on two central points: (1) the number of amino acids that serve as substrates and (2) the number of enzymes involved. As shown in this report a homogeneous DOPA-decarboxylase from guinea pig kidneys did not decarboxylate phenylalanine, tyrosine, tryptophan, histidine or 3-methoxyphenylalanine. The relative rates at which L-DOPA, 5-hydroxytryptophan, *o*-tryrosine and *m*-tyrosine were decarboxylated remained constant throughout the purification procedure making it highly improbable that more than one enzyme is involved in their decarboxylation.

Materials and Methods

Materials. Frozen guinea pig kidneys were bought from Pel Freez Biologicals, Inc. and processed as soon as possible after arrival. The following compounds were bought from Sigma Chemicals, Co.: DL-*o*-tyrosine, DL-*m*-tyrosine, L-3,4-dihydroxyphenylalanine, L-5-hydroxytryptophan, L-tyrosine, L-phenylalanine, L-histidine, L-3-methoxyphenylalanine, iproniazid phosphate, picryl sulfonic acid and pyridoxal 5-phosphate. Benzene (used in the extraction step of the assay) was spectral grade and obtained from Mallinckrodt. Sepharose 4B and Sephadex were obtained from Pharmacia Fine Chemicals. Butyl-agarose and hexyl-agarose were prepared from Sephadex 4B activated with CNBr. The alkylation of the activated Sepharose 4B was accomplished by following the procedure of Shaltiel [7]. Agarose-*o*-tyrosine was prepared in a similar fashion except that *o*-tyrosine replaced the alkylamine.

Assay. The procedure consisted of converting the substrate and the resulting amine to their corresponding trinitrophenyl derivatives by treatment with picryl sulfonate and selectively extracting the amine derivative with benzene. The method described here is essentially the same as that reported by Streffer [8] except that we changed the concentration of picryl sulfonate from 3.4 mM to 70 mM as used by Okuyama and Sakate [9] for the nitrophenylation of proteins.

The standard incubation mixture (0.6 ml) consisted of 0.2 mM substrate, 0.4 mM pyridoxal phosphate, 0.1 ml enzyme solution and 1 mM iproniazid phosphate all made up in 0.1 M potassium phosphate buffer (pH 7.1). After incubation at 37°C for 15 min, the reaction was terminated by heating in boiling water. All assays were run in triplicate except during the purification procedure where duplicate assays were adequate. A unit of enzyme is defined as nmol product formed/min per mg protein.

Results

Purification procedure

The final procedure adopted after a number of trials included a major innovation: the use of hydrophobic interaction chromatography. We investi-

gated other chromatographic methods but none gave us as good results as chromatography on butyl-agarose and hexyl-agarose. All procedures described, if not otherwise stated, were conducted at 4°C.

(1) Preparation of extracts. To 250 g frozen guinea pig kidneys were added 600 ml ice-cold 0.1 M potassium phosphate buffer (pH 7.1)/1 mM iproniazid phosphate. The kidneys were blended briefly in the Waring blendor and then homogenized in a glass homogenizer. The resulting broken cell suspension was centrifuged in a refrigerated centrifuge at 9000 × *g* for about 1 h. The supernatant was filtered through several layers of glass wool to remove suspended lipids and the filtrate saved for the next step.

(2) Further fractionation. The precipitate sedimentaing between 30 and 50% saturation of $(NH_4)_2SO_4$ at pH 7.1 was prepared and dissolved in 50 ml 0.1 M potassium phosphate buffer (pH 7.1) and applied to an 80 × 2.5 cm column of Sephadex-G50 equilibrated with 0.1 M potassium phosphate buffer (pH 7.1). The column was eluted using the same buffer and 20-ml fractions were collected. Samples from alternate fractions were removed and assayed for activity. All the active fractions were pooled and the pool assayed for decarboxylase activity against L-DOPA, *o*-tyrosine, *m*-tyrosine and 5-hydroxytryptophan. To the pooled fractions pyridoxal 5-phosphate was added to a final concentration of 0.4 mM and the entire solution lyophilized. At this stage the lyophilized material could be stored at −20°C for several weeks with no more than 10—15% loss of activity.

(3) Chromatography on butyl-agarose. The lyophilized material from the previous step was dissolved in distilled water and applied to a 3 × 50 cm column of butyl-agarose. The column was eluted with 0.05 M potassium phosphate buffer (pH 7.1); (15-ml fractions). The active fractions were pooled and a sample assayed for activity.

(4) Chromatography on DEAE-Sephadex. The active fractions from step 3 were applied to a 40 × 3 cm column of DEAE-Sephadex A-50 and the column eluted with a linear gradient of 20—200 mM potassium phosphate buffer (pH 7.1); (15-ml fractions). A sample of the pooled active fractions was assayed again and pyridoxal 5-phosphate was added to 0.4 M and the solution rapidly lyophilized.

(5) Chromatography on hexyl-agarose. The lyophilized material from the previous step was dissolved in distilled water and applied to a 12 × 2 cm column of hexyl-agarose. The column was eluted with 0.5 mM potassium phosphate buffer (pH 7.1); (15-ml fractions). The active fractions were pooled and a sample assayed. At this stage the specific activity of the enzyme was 7900 units/mg or 282 times greater than in the original extract. (see Table I, for a summary of the purification procedure). After reaching this stage of purification, pyridoxal phosphate was added and the solution lyophylized and stored at −20°C.

In all chromatographic steps, enzymatic activity emerged from the columns as single, sometimes broad but symmetrical, peaks.

(6) Sucrose gradient centrifugation. An aliquot of the enzyme from step 5 was dissolved in 0.1 M phosphate buffer (pH 7.1) and 0.5 ml of the solution applied to a 5—20% sucrose gradient and centrifuged for 5 h at 50 000 rev./min 0.5-ml fractions were collected using an Auto Densi Flow (Buchler Instruments

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

Substrate; *o*-tyrosine.

Step	Volume (ml)	Total protein (mg)	Total activity units	Specific activity	Recovery (%)
Kidney extract	540	9500	266000	28	—
$(NH_4)_2SO_4$ and Sephadex G-50	230	2400	235000	98	88
Butyl-agarose	250	230	172000	750	65
DEAE-Sephadex	350	50	130000	2600	49
Hexyl-agarose	140	6.8	53700	7900	20
Sucrose gradient		2.5	23375	9350	

Div.). Two protein peaks were detected: a large less-dense peak containing 80% of the enzymatic activity and a very small denser peak containing the remaining activity (Fig. 1). The ratio of DOPA-decarboxylase activity to 5-hydroxytryptophan decarboxylase activity was identical in both peaks. We could not explain why the enzyme splits into two components except that it might aggregate in sucrose solution. In this step additional unwanted protein was removed and the specific activity at this point was 9350 units/mg, which represents a 334-fold purification of the enzyme in the original extract.

Properties of DOPA decarboxylase

Purity. The enzyme represented by the major component of step 6 appears on polyacrylamide gel electrophoresis as a single component. A single component appears at various concentrations of the enzyme and at different pH values. Because the amount of protein available from the more dense peak was too small, we were unable to determined whether it too appears as a single component on polyacrylamide gel electrophoresis. Additional evidence for a single enzyme was obtained by subjecting the purified enzyme to affinity chromatography on Sepharose-*o*-tyrosine. The two activities emerged from the

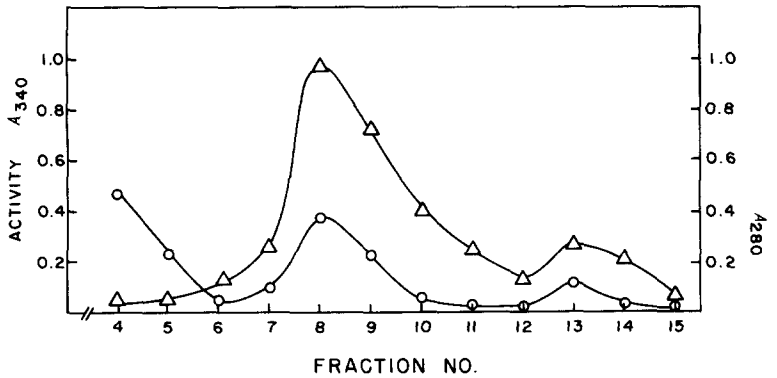

Fig. 1. Sucrose gradient centrifugation of enzyme purified to step 5. ○, protein concentration; △, enzyme activity. High 280 values in fractions 1—4 are mainly due to excess pyridoxal 5-phosphate and to some protein.

TABLE II

SUBSTRATE SPECIFICITY OF DOPA DECARBOXYLASE

For comparison, the activities of an extract from guinea pig kidney (Step 1) are also presented.

Substrate	Specific activity	
	Kidney extract	Pure enzyme
o-Tyrosine	28	9370
L-DOPA	23	6900
m-Tyrosine	10	4950
5-Hydroxytryptophan	6	2200

column as a single peak and without change in their activity ratio.

Substrate specificity. The enzyme was assayed with most aromatic amino acids known to occur in mammalina tissues and also with *o*-tyrosine, *m*-tyrosine and 3-methoxyphenylalanine. Blanks consistently gave readings of 0.020 ± 0.005 at 340 nm; for comparison, absorbance readings with L-DOPA as substrate were around 0.400—0.600. The amino acids decarboxylated by the purified enzyme are listed in Table II. Only L-DOPA and 5-hydroxytryptophan, of the naturally occurring amino acids were decarboxylated. L-Phenylalanine, L-tyrosine, L-tryptophan, L-histidine and 3-methoxyphenylalanine did not act as substrates. Readings with all of the above were the same as with the blanks.

Binding of pyridoxal 5-phosphate. A portion of the enzyme was dialyzed against 0.1 M potassium phosphate buffer (pH 7.1) for 24 h and assayed for activity with and without 0.4 mM pyridoxal 5-phosphate. The data indicate that the addition of pyridoxal 5-phosphate increases enzymatic activity towards L-DOPA, *o*-tyrosine and *m*-tyrosine but not towards 5-hydroxytryptophan (Table III).

The enzyme was also dialyzed for 16 h against 0.02 M cysteine followed by 6 h dialysis against 0.1 M phosphate buffer and then assayed with and without 0.4 M pyridoxal 5-phosphate. The addition of pyridoxal 5-phosphate increased the activity against L-DOPA by 65% and by 25% against 5-hydroxytryptophan.

In other experiments we measured the concentration of pyridoxal 5-phosphate above which no further increase in enzymatic activity towards L-DOPA

TABLE III

EFFECT OF PYRIDOXAL 5-PHOSPHATE ON DOPA DECARBOXYLASE ACTIVITY

Enzyme dialyzed for 24 h and assayed in the absence and presence of 0.4 mM pyridoxal 5-phosphate (PLP).

0.2 mM substrate	Specific activity	
	With PLP	Without PLP
o-Tyrosine	5300	4525
L-DOPA	3850	2610
m-Tyrosine	2800	2500
5-Hydroxytryptophan	1190	1180

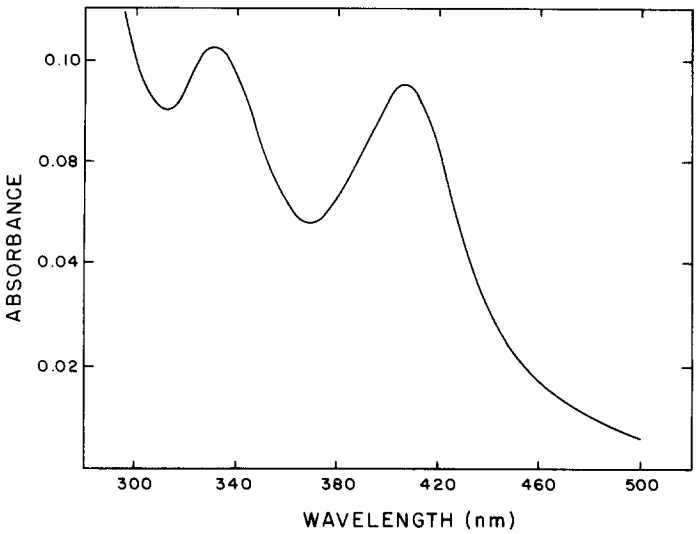

Fig. 2. Absorption spectrum of 1.6 mg/m purified DOPA-decarboxylase in 0.1 M potassium phosphate buffer (pH 7.1).

could be observed. That concentration was found to be $1 \cdot 10^{-6}$ M which is about the same as that measured by Werle and Aures [10] for a partially purified enzyme from guinea pig kidneys.

Absorption spectrum. The enzyme dialyzed against 0.1 M phosphate buffer (pH 7.1) was concentrated to 1.6 mg/ml and the absorption spectrum determined in a Cary 118 spectrophotometer. The cell was kept at 4°C by circulating cold water. The spectrum was measured from 300 to 500 nm. As shown in Fig. 2, the spectrum has two absorption maxima, one at 330 nm and another at 405 nm, which is very similar to the spectrum of a purified DOPA decarboxylase from hog kidneys [11]. Upon addition of $NaBH_4$, only the 405 nm peak was eliminated as it is with several other pyridoxal enzymes.

We had hoped to determined whether borohydride reduction causes a selective loss of activity, but the results were inconcluside due to some denaturation of the enzyme.

Discussion

The substrate specificity of mammalian DOPA-decarboxylase has been the subject of some controversy for a long time. What is in contention is whether the enzyme attacks phenylalanine, tyrosine, tryptophan and histidine. It should be commented that the decarboxylation of tyrosine and histidine by a hog kidney enzyme was reported to be about 300 and 3000, respectively, times slower than of L-DOPA [2] and that Corgier and Pacheco [6] did not detect decarboxylation of tryptophan, tyrosine or histidine by a partially purified preparation from rat brain even though they used a radiometric assay. Organ or species differences has been ruled out by immunological evidence [12].

We did not detect enzymatic activity towards any of these four amino acids even when the enzyme concentration was doubled. In this regard, we should

mention that using a crude preparation from guinea pig kidneys, we could detect CO_2 evolution from radioactive phenylalanine and tyrosine but the rates of evolution were extremely slow, erratic and not proportional to enzyme concentration [13].

Most of the evidence now indicates that the action of DOPA-decarboxylase in mammalian tissues is limited to L-DOPA and 5-hydroxytryptophan. Histidine is decarboxylated by a specific histidine decarboxylase [14,15].

The action of mammalian DOPA-decarboxylase on *o*-tyrosine and *m*-tyrosine, neither of which occur in mammalian tissues, could be explained on the basis that they share some structural features in common with L-DOPA. It appears that the enzyme attacks hydroxyphenylalanines with an OH group in position 2 or 3 but not in position 4. Neither 3-methoxyphenylalanine nor tyrosine were substrates. Similarly, 3-methoxy-4-hydroxyphenylalanine has already been shown not to be a substrate for DOPA-decarboxylase [16].

How 5-hydroxytryptophan fits into this scheme is difficult to say. The weight of evidence is that it is decarboxylated by DOPA-decarboxylase [12] and that the two substrates are competitive inhibitors of one another [8]. The possibility that two enzymes might be involved in the decarboxylation of L-DOPA and 5-hydroxtryptophan in rat brain has been suggested [17] but no conclusive experimental evidence supports this view. The enzyme from guinea pig kidney could not be resolved into two activities, and because the relative rates of decarboxylation of the four substrates did not change at any time, we can assume that a single protein is responsible for the four activities.

The behavior of our enzyme upon addition of pyridoxal 5-phosphate is worthy of comment. Addition of pyridoxal 5-phosphate to the dialyzed enzyme had no effect on the rate of decarboxylation of 5-hydroxytryptophan but it significantly increased the rates of decarboxylation of L-DOPA, *o*-tyrosine and *m*-tyrosine. This effect has been observed before with all or some of the above substrates [3,5,17,18]. One interpretation is that the observed need for additional coenzyme when L—DOPA is the substrate is due to competition of L-DOPA for the coenzyme. The competition could result from the property of L-DOPA to react nonenzymatically with pyridoxal 5-phosphate to a corresponding tetrahydroisoquinoline [19]. The same reaction occurs with *m*-tyrosine, but not with *o*-tyrosine or 5-hydroxytryptophan. If this interpretation were correct, one would expect that the rate of decarboxylation of *o*-tyrosine should not increase upon addition of pyridoxal phosphate, however, this is not borne out by the data of Table III. Another interpretation is that DOPA-decarboxylase has two binding sites, an interesting possibility that needs to be further explored. However, at the moment the only evidence available is presumptive [20].

Pyridoxal 5-phosphate is bound firmly to DOPA-decarboxylase: bound pyridoxal phosphate is found after dialysis for 24 h against phosphate buffer. The absorption spectrum of DOPA-decarboxylase reveals two maxima, each corresponding to a presumptive binding mode (Fig. 2). The peak at 405 nm, common in several pyridoxal enzymes, probably corresponds to a protonated Schiff's base and the peak at 330 nm is probably due to a substituted aldimine or a Schiff's base in an apolar medium [11,21].

In summary, it seem that the effect of pyridoxal 5-phosphate on DOPA-decarboxylase is not yet elucidated. What is clear is that the enzyme behaves as an undissociated enzyme with 5-hydroxytryptophan and as a partially dissociated enzyme with L-DOPA, *m*-tyrosine and *o*-tyrosine.

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