Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 586-595

ENZYME-CATALYSED NON-OXIDATIVE DECARBOXYLATION OF AROMATIC ACIDS: I.PURIFICATION AND SPECTROSCOPIC PROPERTIES OF 2,3 DIHYDROXYBENZOIC ACID DECARBOXYLASE FROM ASPERGILLUS NIGER

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Received March 23, 1987

SUMMARY : In order to understand the molecular mechanism of non-oxidative decarboxylation of aromatic acids observed in microbial systems, 2,3 dihydroxybenzoic acid (DHBA) decarboxylase from Aspergillus niger was purified to homogeneity by affinity chromatography. The enzyme (Mr 120 kDa) had four identical subunits (28 kDa each) and was specific for DHBA. It had a pH optimum of 5.2 and K_m was 0.34mM. The decarboxylation did not require any cofactors, nor did the enzyme had any pyruvoyl group at the active site. The carboxyl group and hydroxyl group in the ortho-position were required for activity. The preliminary spectroscopic properties of the enzyme are also reported.

Decarboxylation reactions, either of oxidative or non-oxidative type is a cardinal step prior to the ring cleavage of benzoic acid and its derivatives(1). The non-oxidative decarboxylation of various aromatic acids have been shown both in bacterial (2-6) and fungal systems (7-10). The enzymes involved have not been purified but for 4,5 dihydroxyphthalate decarboxylase from pseudomonads (11,12) and 2,3 dihydroxybenzoic acid (DHBA) decarboxylase from yeast(13). However, none of the above reports have attempted to understand the molecular mechanism of enzymatic non-oxidative decarboxylation of the aromatic acids. An unusual feature of all these non-oxidative decarboxylases is the absence of pyridoxal phosphate(PLP), thiamine pyrophosphate (TPP) or a pyruvoyl residue (14) at the active site.

As an essential step towards the understanding of the molecular mechanism of decarboxylation, we have purified DHBA decarboxylase from Aspergillus niger to homogeneity. An earlier report (15) has only attempted a partial purification from the same source and hence do not contain significant information

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0006-291X/87 $1.50

relevant to understanding its mode of action. In this communication, we present the purification and preliminary reports of the spectroscopic properties of the enzyme.

MATERIALS AND METHODS

Chemicals : Anthranilic acid, Sepharose 4B, DEAE (diethylaminoethyl) - sephacel, protamine sulfate, sodium dodecyl sulfate (SDS) and the kit of molecular weight markers were purchased from Sigma Chemical Co. U.S.A. 2,3-dihydroxybenzoic acid (DHBA) was from Aldrich Co. U.S.A. and Koch-Light Laboratories, U.K. Other chemicals used were of analytical grade.

Organism and growth conditions:

Aspergillus niger maintained in our laboratory was used for the study. For enzyme preparation, it was grown at pH 5.5 on modified Byrde's medium (16) containing 0.5% glucose and 0.2% anthranilic acid; no other nitrogen source was provided. The fungus was grown at room temperature (28 ± 2°C) as stationary cultures for 30 h. The mycelia were then harvested and stored frozen at -20°C until use. The enzyme activity could be detected in the mycelia even after three weeks of storage.

Preparation of affinity chromatography:

Since salicylate was earlier reported to be a competitive inhibitor in the preliminary studies (15), it was used as a ligand for the preparation of an affinity matrix. CNBr - activated sepharose was converted to benzidyl derivative which was coupled to salicylate according to the method reported earlier (17). The affinity matrix was stored in 50 mM sodium phosphate buffer, pH 7.0, until use. Regeneration of the gel was achieved by washing it successively with 0.05 N NaOH and HCl, followed by a large amount of double distilled water.

Purification of DHBA decarboxylase :

All operations were carried out at 0-4°C. The centrifugations were done at 27,000Xg in a Sorvall RC-5B centrifuge.

About 30 g of the mycelia was macerated with an equal amount of acid washed glass powder in a pre-chilled mortar with a pestle for 20 min. It was suspended in 200 ml of 50 mM sodium phosphate buffer, pH 7.0 (buffer A). The homogenate was passed through double layered cheese cloth and centrifuged for 20 min. The supernatant (crude enzyme) was treated with protamine sulfate (1 mg/ml of extract) and stirred for 15 min. The precipitate was removed by centrifugation for 15 min and the supernatant was stirred with solid ammonium sulfate to obtain 50% saturation. The proteins precipitated after 20 min was collected by centrifugation and dissolved in minimum amount of buffer A, followed by dialysis against the same buffer. The ammonium sulfate fraction was loaded onto a DEAE-sephacel ion-exchange column (1.5 cm x 8 cm), which had been equilibrated with buffer A. The column was washed thoroughly with the same buffer and the elution of the enzyme was achieved by using a linear gradient of 50 mM to 200 mM sodium phosphate buffer, pH 7.0. The active

fractions were pooled and loaded onto affinity column (1.5 cm x 5 cm). It was washed with buffer A to remove all unbound proteins. The enzyme was then specifically eluted using 10 mM DHBA in buffer A. It was concentrated, extensively dialysed against the same buffer, and used for our studies.

Assay of the enzyme :

DHBA decarboxylase was assayed as follows:
The typical assay mixture (1 ml) contained 50mM sodium acetate buffer pH 5.2, 2 mM DHBA and the enzyme. It was incubated at room temperature (28 ± 2°C) for 15 min and the reaction was terminated with 1 ml of 15% of trichloro acetic acid. The precipitated protein was removed by centrifugation and the supernatant was diluted to 6 ml with double distilled water. The decrease in the optical density was monitored at 316 nm where only DHBA absorbs. The reaction product catechol was also estimated at its absorbance maximum at 276 nm after extracting the mixture with ethyl acetate under mild alkaline conditions. As catechol dioxygenase which acts on catechol, got partially co-purified till step 3, assays were done following the disappearance of the substrate.

Identification of the reaction product :

After arresting the reaction mixture with 1N HCl, it was repeatedly extracted with peroxide-free diethyl ether. The ether layers were pooled, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was dissolved in methanol and a small aliquot (5 μl) was subjected to High Performance Liquid Chromatography (HPLC) on a Lichrosorb RP18 (10 μm) column preceded by a guard column. The eluent was a mixture of methanol : acetic acid : water [60 : 1 : 40 (v/v)]. The absorbance was monitored at 270 nm to detect the disappearance of the substrate and appearance of the product. Similar procedure was followed for the standards (DHBA and catechol).

Other methods :

Polyacrylamide gel electrophoresis (PAGE) (18) of the enzyme and SDS-PAGE (19) was done using standard markers, α-lactalbumin (14 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa) and Glyceraldehyde-3-phosphate dehydrogenase (36 kDa). Protein was estimated using bovine serum albumin as the standard (20). The moleculear weight of the native protein was determined on a Biogel P-150 column (21).

The ultraviolet absorption spectra were recorded in a Hitachi 557 double beam double wavelength spectrophotometer. The fluorescence and circular dichroism (CD) spectra were recorded using a Hitachi 650 flurescence spectrophotometer and Jasco J-20C spectropolarimeter (attached with a Jasco DP 500 N data processor) respectively. Each CD spectrum reported here is an average of two runs.

RESULTS AND DISCUSSIONS

This is the first report of the isolation of a DHBA decarboxylase, from *A. niger* to homogeneity. As an improvement over the earlier reports (15), the specific activity of the

Table 1

Purification of DHBA decarboxylase from Aspergillus niger

Step	Total protein (mg)	Total activity (Units)[a]	Specific activity (Units/mg)	Fold purification	Percent recovery
Crude	440	352	0.80	-	100
protamine sulfate precipitation	320	352	1.1	1.4	100
Ammonium sulfate fractionation (0-50%)	78	452	5.80	7.3	128
DEAE-sephacel chromatography	18	160	8.9	11.2	45
Affinity chromatography	7.6	121	15.9	19.9	35

[a] Expressed as μmoles of DHBA disappeared per min

enzyme in the crude preparations was about 30 times more (Table 1); this might be due to low concentration of glucose used in the growth medium and high concentration of anthranilate which also served as the sole nitrogen source in our study.

The enzyme was purified using an affinity chromatography with salicylate as the ligand; the elution profile is shown in figure 1. The final preparation was homogeneous as determined by PAGE (inset of figure 1) and had a M_r of 120 kDa (as determined from Biogel. P-150 column). The SDS-PAGE gave a single band of

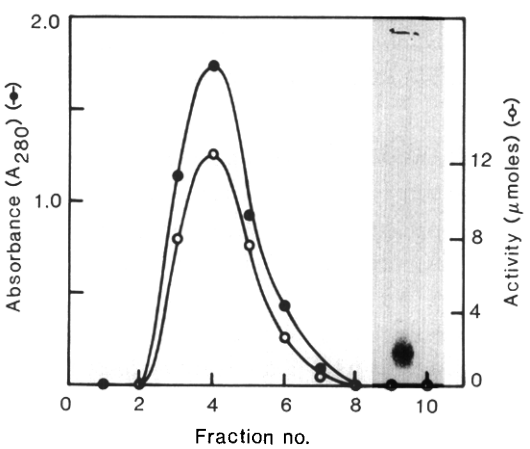

Figure 1. Elution profile of DHBA decarboxylase on salicylate coupled benzidyl sepharose column. The enzyme after DEAE-sephacel chromatograghy was loaded onto the column in buffer A and eluted with 10mM DHBA in the same buffer (Details given under "Materials and Methods").

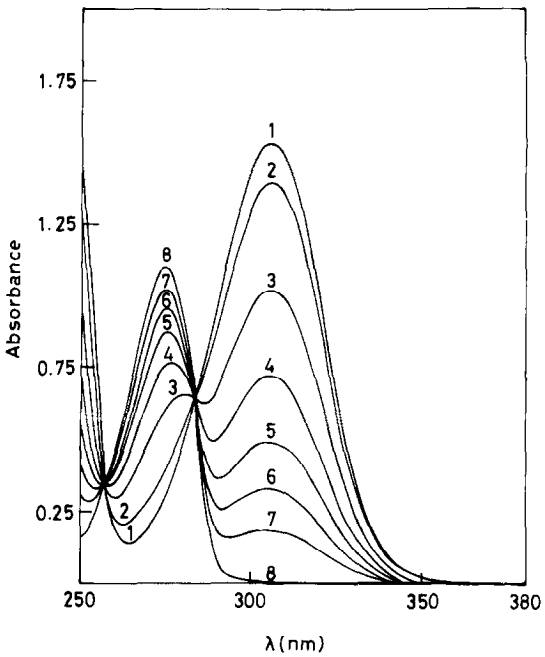

Figure 2. UV absorption spectral changes of DHBA in the absence (spectrum No. 1) and presence (spectrum No. 2-8) of the enzyme. The sample cuvette contained 0.5mM DHBA in 50mM sodium acetate buffer pH 5.2 at 28° C and the enzyme (4μg/ml, if added). In the case of the spectra recorded in the presence of the enzyme, the reference cuvette also contained an equal amount of the enzyme. The spectra were recorded at various time intervals, as follows, after the addition of the enzyme; - 2(1.5min), 3(4.5min), 4(7.5min),5(10.5min), 6(13.5min), 7(16.5min) and 8(30min).

M_r 28 kDa indicating the native protein to be a tetramer of identical subunits. The apparent increase in the fold purification might be due to the removal of dialysable inhibitors which was also observed by others (13,15). The enzyme isolated from the yeast had a M_r of 65 kDa and was dimer with identical subunits (13).

The action of the purified enzyme upon the substrate (DHBA) was monitored spectrophotometrically (figure 2). It is clear from the figure that there is a progressive time-dependent decrease in the concentration of DHBA, concurrent with an increase in the concentration of the product as apparant from the appearance of a new peak at 276 mM. The spectrum of the product (No. 8 in the figure) resembles that of catechol. The identification of the product as catechol was also indicated from TLC and specific color reactions. It was further confirmed by the HPLC analysis of the reaction product (figure 3). The HPLC profiles after 10 min and 35 min show that there is an increase in the area of the peak corresponding to catechol (retension time 7.1 min) along with the decrease in the area of the peak where DHBA comes off (retension

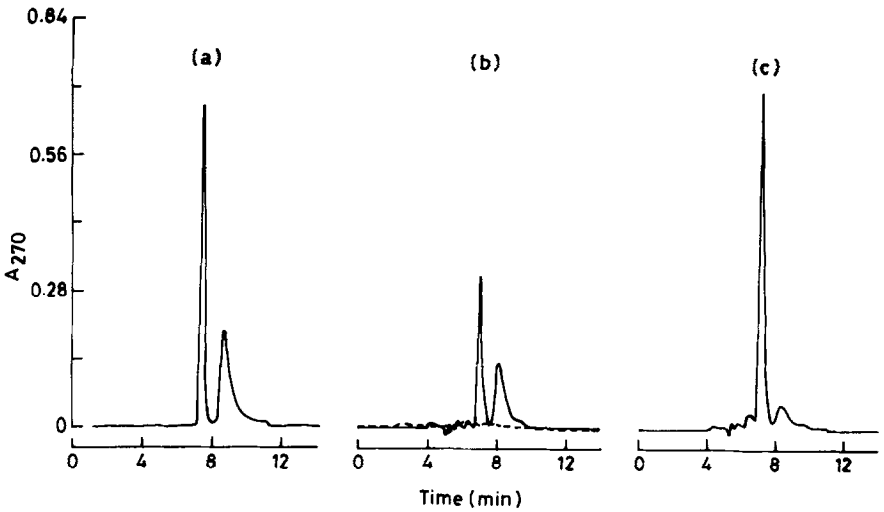

Figure 3. HPLC profiles for the action of enzyme upon the substrate DHBA (Details given under "Materials and Methods") (a) Standard materials - Substrate DHBA (retention time 8.2min) and product catechol (retention time 7.1min). Enzyme reation terminated (b) after 10min and (c) after 35min, of incubation.

time 8.2 min). The HPLC profiles also suggest the absence of any significant side reaction.

The DHBA decarboxylase had a pH optimum of 5.2. From the assay mentioned under materials and methods, the K_m was calculated to be 0.34 mM, which is higher than the value (37 μM) reported for the same enzyme from yeast (13). The K_m was determined also by a continuous assay from the initial slope of the decrease in absorbance at 306 nm and the value (0.4 mM) is in excellent agreement with the value determined by the other method of assay. From pH - dependent variation of K_m, the ionisation constant, pKa, for the amino acid involved in catalysis was determined to be 6.2 which corresponds to the pKa of histidyl residue in proteins. This might indicate the possible role of histidine, a potential electron acceptor and donor.

Unlike the broad substrate specificity exhibited by the yeast enzyme (13), as well as other partially purified decarboxylases (10,12), our enzyme preparation was unable to use salicylate, m-hydroxybenzoate, gentisate and protocatechuate under a variety of conditions.

In order to understand the structural requirements necessary for interaction with the enzyme, compounds (listed in table 2) were used as substrate and also as possible inhibitors of the enzyme. None of the compounds could be decarboxylated by the enzyme. It is apparent from the table that benzoic acid derivatives with a hydroxyl group at the ortho-position to the carboxyl group are potent inhibitors and hence the enzyme could

Table 2

Inhibition of DHBA decarboxylase by substrate analogs

Compound(2mM)[a]	Inhibition(%)
None	0
Salicylate(1mM)	90
Anthranilate(1mM)	90
2,3 Dihydroxybenzaldehyde	80
2,4 dihydroxybenzate	40
3-Hydroxyanthranilate	50
3-Hydroxybenzoate	10
3,4 Dihydroxybenzoate	10
Benzoic acid	9
4-Hydroxybenzoate	0

[a] Number in the parenthesis denotes the concentrations of the compounds.

be purified using o-hydroxybenzoate (salicylate) as the ligand for affinity matrix. 3-Hydroxybenzoate, and 3,4 dihydroxybenzoate which closely resemble the substrate were not inhibitors suggesting that the hydroxyl group at C-3 carbon of the benzene ring of the substrate might not be involved in catalysis. This is further supported by the observation that the enzyme failed to bind to the affinity matrix with m-hydroxybenzoate as ligand. These results indicated that an -OH group at the ortho-position to -COOH (as in DHBA) is, probably, essential for the activity and the presence of one more -OH group at C-3 might facilitate the release of CO_2. It is worth noting that the preliminary results with the crude enzymes from other sources also showed that all groups on the aromatic ring might not be required for the catalysis (2,8,10,12).

The UV absorption spectrum of the enzyme, (figure 4a) is characteristic of a simple protein where the peak at 280 nm with a shoulder at 290 nm, has originated from $\pi \rightarrow \pi^*$ transition of side chains of aromatic amino acids. The absence of absorbance in the visible region indicated the absence of any prosthetic group such as PLP. Fluorescence excitation spectrum (figure 4b) also indicated a shoulder at 290 nm. A comparitive study of the emission spectra (figure 4b) corresponding to the excitation wavelengths of 280nm and 290 nm suggest that the major part of emission intensity at 340 nm is contributed by the absorption at 290 nm, the region where the side chain of typtophan contributes. The absence of a major contribution by tyrosine residues is indicated from the relative insensitivity of the emission intensity when the pH was changed from 4 to 11.0. The CD spectrum of the protein in the far and near UV region is shown in figure 4c and 4d respectively. The CD spectrum in the far UV region is characteristic of α-helix structure with troughs at 209

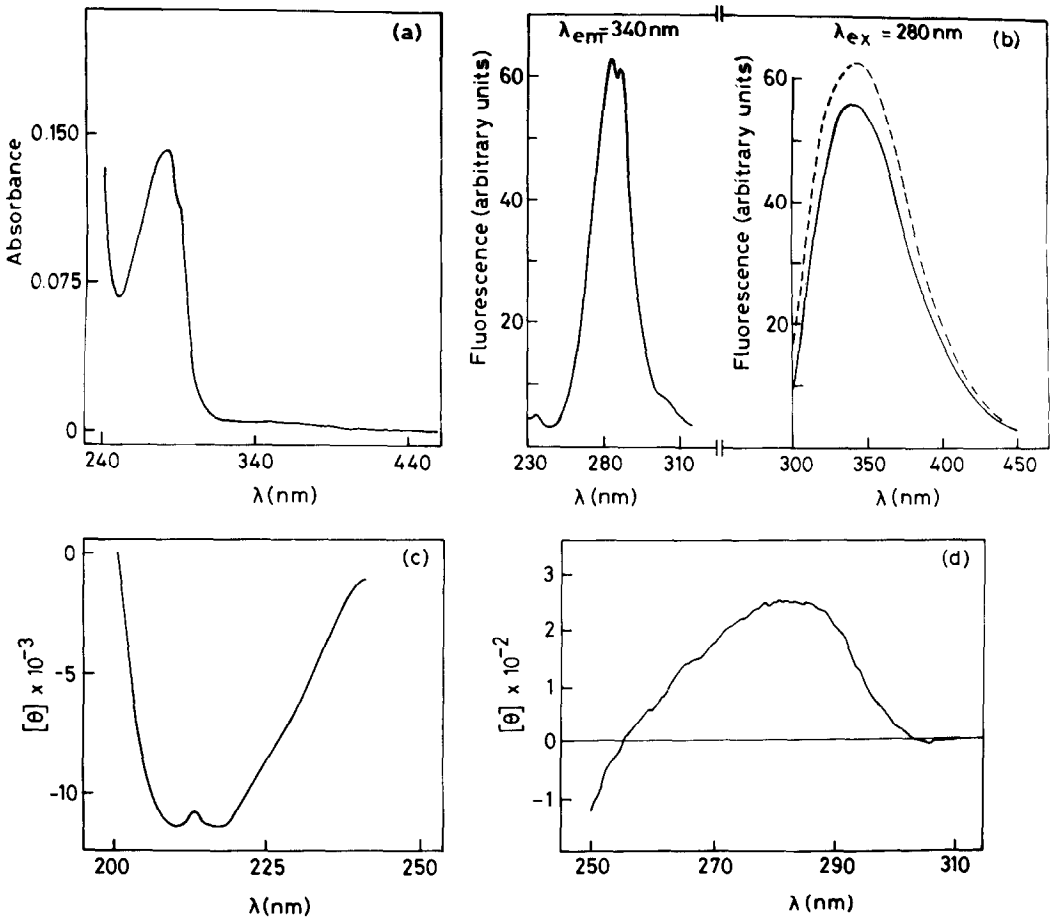

Figure 4. Spectral properties of DHBA decarboxylase in 50mM sodium acetate buffer pH 5.2 (28°C).
(a) The UV visible spectrum of the enzyme (75μg/ml) (b) The fluorescence excitation and emission spectra (27μg/ml). For the excitation spectrum, emission was fixed at 340nm. The excitation was fixed at 280nm & emission spectrum was recorded (solid line). The dotted line represents the emission spectrum upon excitation at 290nm. (c) Far UV-CD spectrum. The enzyme (90μg/ml) was taken in a cuvette of 0.1cm path length. (d) Near UV-CD spectrum. The enzyme (120μg/ml) was taken in a cuvette of 5cm path length. The molar ellipticity was calculated from the relation :

$$[\Theta] = \frac{\Theta}{c.l} \times 100$$

where [Θ] = molar ellipticity, Θ = observed ellipticity, c = concentration of the protein in moles/litre, calculated using a mean residue weight of 115, l = 0.1 cm (for far-UV spectrum) and 5 cm (for near-UV spectrum).

nm and 219 nm (22). The near UV CD spectrum showed a broad band around 280 nm; which might be contributed from tryptophan and/or tyrosine residues (23).

An interesting feature of this enzyme, as revealed from the CD studies, indicates that there is no significant change in the backbone structure upon 6 M urea denaturation, (figure not

shown), which otherwise results in complete loss of activity. In analogy to other cases such as creative kinase, it might be suggested that the active site of this decarboxylase is situated in such a region that is more sensitive to denaturants than the enzyme molecule as a whole (24). It is worth noting that there was a decrease in the intensity of CD band at 280 nm in near UV region upon urea treatment.

The substrate is an aromatic acid; one might, therefore, conjencture that an aromatic amino acid residue might play a role in catalysis. In such a case, the spectroscopic properties reported here would be a potential tool to probe into the molecular mechanism of the decarboxylation. Detail studies to understand the mechanism are in progress and will be reported elsewhere.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Prof. N.Appaji Rao for his helpful suggestions and discussions. One of us, (A.V.K) is a recepient of a senior research fellowship of the Department of Atomic Energy, Government of India.

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