The presence of L-2,4-diaminobutyric acid decarboxylase activity in *Vibrio* species: a new biosynthetic pathway for 1,3-diaminopropane

(Pyridoxal 5’-phosphate-dependent enzyme; precursor of norspermidine; polyamine)

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1. SUMMARY

A new enzyme activity, which catalyzes decarboxylation of L-2,4-diaminobutyric acid (DABA) to yield 1,3-diaminopropane (DAP), has been found in dialyzed crude extracts prepared from *Vibrio alginolyticus*. The pH optimum for the activity was 8.0–8.5, and the enzyme showed a pyridoxal 5’-phosphate (PLP) requirement. Mg2+ caused about 30% stimulation in activity. The enzyme was active to only L-DABA among the diamino acids examined, and the $K_m$ value for L-DABA was 0.13 mM. Ammonium sulfate fractionation of a dialyzed crude extract followed by HPLC separation allowed us to conclude that this enzyme differed from the decarboxylase which occurs in *Vibrio* spp. to produce norspermidine (Nspd) for carboxynorspermidine (C-Nspd) having a moiety similar in structure to DABA. The same enzyme activity was detected in several other *Vibrio* species.

2. INTRODUCTION

In our recent paper [1], Nspd which is characteristically present in members of the genus *Vibrio* as a predominant triamine species [2,3] has been shown to be produced by a novel biosynthetic pathway which utilizes DAP and aspartic β-semialdehyde as an amine substrate and an aminopropyl group donor, respectively. In this enzyme system, putrescine served as a substrate to form spermidine (Spd), but the reaction rate was only about 7% of that for DAP. Moreover, activity of the aminopropyltransferase responsible for Spd biosynthesis was not detectable. These results were in accordance with the fact that at any stage of growth the intracellular content of Spd was much lower than that of Nspd [4]. Although DAP was detected in small amounts in some *Vibrio* species examined [3], the question was raised as to its origin since Spd is generally assumed to be the only known precursor of DAP in bacteria [5,6].

Here we present evidence that *Vibrio* spp. contain a new enzyme, termed DABA decarboxylase(DABA-DC), which specifically decarboxylates L-DABA to yield DAP.

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3. MATERIALS AND METHODS

3.1. Chemicals

PLP, l- and DL-DABA, DL-2,3-diaminoporporionic acid, bovine serum albumin (BSA) and DAP·2HCl were purchased from Sigma, St. Louis, MO, U.S.A. The other chemicals were reagent grade products available from commercial sources.

3.2. Bacterial strains and growth conditions

The bacterium mainly used as the cell source for enzymic studies was V. alginolyticus ATCC17749, which was grown at 37°C for 10 h in the liquid medium (MMOF medium; [2]) containing 0.5% NaCl. The other vibrios were grown in the same medium under the indicated conditions (see Table 2). The reference bacteria were grown for 6 h in the same medium as for V. alginolyticus. Each culture previously grown for 12 h in the same medium was used as the inoculum (2%).

3.3. Preparation of dialyzed crude extracts

All subsequent operations were carried out at 0–4°C. Cells harvested by centrifugation at 8500 x g for 20 min were suspended in 5 vols. of 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM PLP (buffer A) and then sonicated for a total of 5 min (15-s bursts at an interval of 20 s) using an Ultrasonic homogenizer (Nissei US-300, Tokyo, Japan). The sonicate was centrifuged at 40 000 x g for 30 min and the supernatant fluid was dialyzed overnight against two changes of 200 vols. of buffer A to provide a preparation termed the dialyzed crude extract. Unless otherwise stated, this preparation was used for the following enzymic studies.

3.4. Enzyme assay method

DABA-DC was routinely assayed by measuring the formation of DAP from l-DABA. The assay mixture in a total volume of 1 ml contained 100 mM Tris–HCl buffer (pH 8.25), 7.5 mM l-DABA, 0.1 mM PLP, 20 mM MgSO₄, 1 mg BSA and the enzyme protein (0.05–0.25 mg). The blank contained all of the ingredients except that the substrate had been replaced with 0.2 ml of distilled water. Reaction was usually initiated by adding the substrate. After a 30 min incubation at 37°C with occasional shaking, reaction was stopped by addition of 0.4 ml of 20% HClO₄. An internal standard (1,6-diaminoxynohexane) was added to the mixture and this solution was treated for preparation of the N-ethylxynocarbonyl (EOC) derivatives of the amines as in [1], except that after derivatization the reaction mixture was shaken with 0.1 ml of 60% NaOH for 1 min in order to eliminate extra peaks derived from the substrate. The resultant derivatives were analyzed by GLC with a nitrogen-selective detector (Shimadzu FTD-8) as in [1], except that the column temperature was programmed from 170°C to 275°C at 6°C/min. The amount of DAP formed was obtained from the peak height ratio relative to the internal standard. Under the standard assay conditions, DABA-DC activity was a linear function of both incubation time (10–80 min) and enzyme protein (0.05–0.25 mg). The blank values were negligible.

3.5. Ammonium sulfate fractionation and HPLC analysis

Ammonium sulfate was added with stirring at 0°C to the dialyzed crude extract, and the precipitate between 30% and 60% saturation was collected by centrifugation at 20 000 x g for 20 min. The precipitate was dissolved in a suitable amount of buffer A and dialyzed overnight against two changes of 100 vols. of the same buffer. The dialyzed solution was clarified by filtering through a Milllex-GS (0.22 μm, Millipore) and an aliquot corresponding to 20 mg protein was injected into a Pharmacia Fast Protein Liquid Chromatography (FPLC) system (Uppsala, Sweden) equipped with a Mono Q anion exchange column (HR 5/5, 50 x 5 mm I.D.). The analytical conditions were as follows: mobile phase, 20 mM Tris–HCl (pH 7.5); flow rate, 0.5 ml/min; a linear gradient of 0–1 M NaCl started 16 min after sample injection; fractions of 5 ml were collected at room temperature (20–23°C). The activity assay was carried out for 0.5 ml of each eluate without removal of NaCl, since NaCl up to 0.5 M in the assay mixture had no effect on the activity.

3.6. Other methods

Protein content was determined as in [7] using BSA as the standard. Prior to determination, the
samples from HPLC containing NaCl were dialyzed against buffer A. Gas chromatography-mass spectrometry (GC–MS) was performed as in [8].

4. RESULTS AND DISCUSSION

The present GLC method was capable of separating and quantifying the diamines as shown in Fig. 1A, and the minimum amount of dAP formed enzymatically that could be quantified was 50 pmol. Fig. 1 shows the formation of DAP on incubation of a dialyzed crude extract with L-DABA. A peak with the same retention time as that of the authentic N-EOC derivative of DAP was clearly detected. A DAP peak was not observed in a blank. The DAP peak in Fig. 1B was analyzed by GC–MS. The spectrum obtained was identical with that observed from the authentic standard. The amine formed was also identified as DAP by TLC after dansylation [9] (results not shown). The evolution of CO₂, another reaction product, was confirmed qualitatively using a Warburg apparatus.

The pH-optimum for enzyme activity was found to be 8.0–8.5 in 100 mM Tris–HCl buffer, the activity falling off more steeply at the lower pH values (Fig. 2). The enzyme was most active at 45°C, but unstable at that temperature; 18% and 55% of the activity were lost by 30-min and 2-h incubations at 45°C, respectively. There was no loss of the activity when the enzyme was incubated at 37°C for up to 90 min. The enzyme at low concentrations (< 0.1 mg protein/ml) was stabilized by addition of BSA.

Although some activity was observed in a PLP-minus assay system with a dialyzed crude extract, an absolute PLP requirement could be demonstrated with the enzyme preparation obtained from HPLC. Carboxymethoxylamine, an inhibitor of PLP dependent enzymes, caused a 50% reduction in activity at 0.5 mM.

About 30% stimulation in activity was observed on addition of Mg²⁺ at 20 mM to the assay mixture. Other divalent cations, Cu²⁺, Zn²⁺, Pb²⁺, Sn²⁺, Mn²⁺, Fe²⁺ and Co²⁺, were potently inhibitory at a concentration of 20 mM; Ca²⁺ had no effect at the same concentration.

N-Ethylmaleimide, a thiol inhibitor, did not

![Fig. 1. Gas chromatograms showing enzymic formation of DAP from L-DABA. (A) Standard, Each peak represents 20 pmol of amine; (B) sample; (C) blank. Protein (0.1 mg) of a dialyzed crude extract from V. alginolyticus was used in (B) and (C). Peaks: (1) ethylenediamine; (2) DAP; (3) putrescine; (4) cadaverine; (IS) 1,6-diaminohexane.](https://academic.oup.com/femsle/article-abstract/35/2-3/289/578618)

![Fig. 2. Determination of optimum pH for the activity of DABA-DC. Protein (0.1 mg) of a dialyzed crude extract from V. alginolyticus was assayed for the activity at various pH values. O, 100 mM Tris-HCl buffer, pH 7.5–9; ●, Clark-Lubs buffer, pH 8–10, containing equimolar KCl and H₃BO₃ (100 mM) pH adjusted with NaOH.](https://academic.oup.com/femsle/article-abstract/35/2-3/289/578618)
inhibit the reaction even at 20 mM, being in accordance with the fact that no significant difference in activity was found on addition of di-thiothreitol to the assay mixture.

The $K_m$ value for L-DABA was estimated to be 0.13 mM (Fig. 3). In order to clarify whether the enzyme is active to D-isomer, the $K_m$ value for DL-DABA was determined since D-isomer was commercially unavailable. The $K_m$ value for DL-DABA was found to be 0.30 mM, about twice that for L-isomer, with the almost same $V_{\text{max}}$ value (Fig. 3). This at least suggests that the enzyme will not decarboxylate D-isomer and be optically specific.

Since DABA is structurally similar to C-Nspd[\(\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_2\text{CH(NH}_2)\text{COOH}\)] which is decarboxylated by a Vibrio enzyme (C-Nspd-DC) to yield Nspd[1], there was a possibility that decarboxylation activity for L-DABA might be due to the enzyme. However, a preliminary experiment demonstrated that these enzyme activities were separable from each other by ammonium sulfate fractionation, DABA-DC and C-Nspd-DC being recovered in 30-50% and 50-60% saturation, respectively. HPLC performed with a fraction of the 30-60% saturation demonstrated more directly their nonidentity. As shown in Table 1, the highest DABA-DC activity was observed in fraction 7 with a specific activity of 23-fold higher than that of the crude extract, whereas C-Nspd-DC activity was detected mainly in fraction 6 and slightly in fraction 7 (results not shown). These results indicate that two different proteins which carry out each decarboxylation are present in V. alginolyticus. In addition, any decarboxylation activity for DL-2,3-diaminopropionic acid, L-ornithine and L-lysine was not detectable in fraction 7. These diamino acids did not exhibit any inhibitory effect on the enzyme activity at a concentration of 7.5 mM.

The same enzyme activity was found in all Vibrio strains tested except Vibrio costicola that does not contain Nspd [3] (Table 2). No DABA-DC activity was detected in Photobacterium phosphoreum strain 404, Aeromonas hydrophila ATCC19570, Plesiomonas shigelloides ATCC-14029, Escherichia coli B, Salmonella typhimurium NCTC74 or Bacillus subtilis IFO3215.

An attempt was made to examine whether Spd oxidation activity, which leads to the formation of DAP and $\Delta^1$-pyrroline, was present in Vibrio. The crude supernatant fluids and the ammonium sulfate precipitates (0-40% and 40-60% saturation) prepared from V. alginolyticus demonstrated more directly their nonidentity. As shown in Table 1, the highest DABA-DC activity was observed in fraction 7 with a specific activity of 23-fold higher than that of the crude extract, whereas C-Nspd-DC activity was detected mainly in fraction 6 and slightly in fraction 7 (results not shown). These results indicate that two different proteins which carry out each decarboxylation are present in V. alginolyticus. In addition, any decarboxylation activity for DL-2,3-diaminopropionic acid, L-ornithine and L-lysine was not detectable in fraction 7. These diamino acids did not exhibit any inhibitory effect on the enzyme activity at a concentration of 7.5 mM.

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Table 1
Partial purification of DABA-DC from V. alginolyticus

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific activity (U/mg protein)</th>
<th>U/ml (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed crude extract</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate (30-60%)</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>HPLC b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 6</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>29.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Fraction 8</td>
<td>4.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>0.2</td>
<td>0.03</td>
</tr>
</tbody>
</table>

a Before assay, this was dialyzed overnight against buffer A.

b Protein (20 mg) of the dialyzed ammonium sulfate precipitate (total units: 36.0) was injected into a Pharmacia FPLC system. The analytical conditions are described in MATERIALS AND METHODS.
Table 2
Distribution of DABA-DC activity in *Vibrio* species

All strains were grown in MMOF medium under the indicated conditions and the enzyme activity was measured with the dialyzed crude extract (0.1–0.25 mg protein).

<table>
<thead>
<tr>
<th>Strain</th>
<th>nmol DAP formed</th>
<th>Growth conditions (NaC1, incubation time)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. alginolyticus</em> ATCC17749</td>
<td>63.2–73.5</td>
<td>0.5%, 6 h</td>
</tr>
<tr>
<td></td>
<td>76.8–111.3</td>
<td>0.5%, 10 h</td>
</tr>
<tr>
<td></td>
<td>21.5</td>
<td>2%, 6 h</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> AQ3627</td>
<td>42.6</td>
<td>2%, 6 h</td>
</tr>
<tr>
<td><em>V. cholerae</em> NCTC4716 (non-O1)</td>
<td>66.5</td>
<td>0.5%, 6 h</td>
</tr>
<tr>
<td><em>V. proteolyticus</em> ATCC15338</td>
<td>21.8</td>
<td>2%, 8 h</td>
</tr>
<tr>
<td><em>V. anguillarum</em> NCMB6</td>
<td>83.9</td>
<td>2%, 4.5 h</td>
</tr>
<tr>
<td><em>V. costicola</em> NCMB701</td>
<td>&lt; 0.2</td>
<td>5%, 6 h</td>
</tr>
</tbody>
</table>

*V. parahaemolyticus* according to the procedure in [10] and [11] were used as enzyme sources. The enzyme activities in these preparations were assessed by assaying DAP and Δ¹-pyrroline with the present GLC method and the usual colorimetric method, respectively. The incubation was carried out under the same conditions as those in [10] and [11]. However, neither DAP nor Δ¹-pyrroline was formed from Spd in these experiments.

It is evident from the results presented here that *Vibrio* spp. contain DABA-DC. This is, to our knowledge, the first evidence for the occurrence of DABA-DC in organisms. There are reports concerning the metabolism of this amino acid in rat [12], plant [13] and bacterium [14], but neither of them has mentioned its metabolism via decarboxylation. We failed to demonstrate the natural occurrence of DABA in *Vibrio* spp. However, it may be possible that this amino acid exists only transiently in *Vibrio* spp. due to its instantaneous conversion in vivo into DAP or other metabolites. The occurrence of DABA-DC in *Vibrio* spp. to form DAP, a precursor of Nspd, is consistent with the observation that no activity of Spd oxidation was detected.

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REFERENCES