NOTE

Enzymatic Conversion of 1-Hydroxy-2-naphthoate in Phenanthrene-grown *Aeromonas* sp. S45P1

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In the previous paper,¹⁾ it was described that *Aeromonas* sp. S45P1 which was adapted to phenanthrene degraded the hydrocarbon via 1-hydroxy-2-naphthoate (1H2NA) and *o*-phthalate (OPA). Enzyme(s) converting 1H2NA to 2-carboxybenzaldehyde (2CBAL) and a dehydrogenase oxidizing 2CBAL to OPA in the presence of NAD were formed in the phenanthrene-grown cells. *Aeromonas* sp. S45P1 was found to metabolize phenanthrene through a catabolic pathway differing from that in a *Pseudomonas* isolated by Evans *et al.*²⁾ In the latter microorganism, it was suggested that the 1H2NA produced from phenanthrene was oxidized to 1,2-naphthalenediol which was then catabolized through the degradative pathway of naphthalene.

In the present investigation, the properties of the enzyme(s) catalyzing the conversion of 1H2NA to 2CBAL were examined in the *Aeromonas*. Cultivation of the cells in phenanthrene-mineral solution, preparation of the cell-free extract and spectrophotometrical assay of the activity degrading 1H2NA were performed as described in the previous paper.¹⁾ The crude enzyme solution prepared from the cell-free extract with 40 to 70% saturation of ammonium sulfate was applied to a DEAE-cellulose column equilibrated with 10 mM phosphate buffer (P-buffer), pH 7.1. After the column was washed with the same buffer, elution was carried out successively with 30 mM P-buffer, pH 7.1, 1% NaCl- and 2% NaCl-containing 30 mM P-buffers, pH 7.1. As the fractions which were eluted merely with 30 mM P-buffer showed the activity, they were collected and examined for the enzymatic properties. The enzyme preparation was 24.3 times as pure as the cell-free extract.

The cell-free extract and the ammonium sulfate-treated preparation were able to degrade 1H2NA without other supplement, but they almost completely lost their activity in 20 hr at 4°C. Although the loss was hardly prevented by the addition of EDTA (10 mM), acetone (10%, v/v), mercaptoethanol (1 mM) or ethanol (10%, v/v), ferrous sulfate (1 mM) completely prevented the loss of activity for more than 10 days.

FIG. 1. Effect of Ferrous Ion on the Degradation of 1H2NA.

The reaction mixture consisted of enzyme solution containing 1.4 mg protein, 600 nmol of FeSO_4 and 600 nmol of 1H2NA in 3.0 ml of 10 mM phosphate buffer, pH 7.1. Reaction was carried out in a cuvette with 1 cm light-path at 25°C.

A: $[\text{Fe} + \text{E}] + \text{S}$, B: $[\text{S} + \text{Fe}] + \text{E}$, C: $\text{E} + \text{S}$, D: $[\text{S} + \text{E}] + \text{Fe}$, E: $\text{S} + \text{Fe}$. Abbreviations, E: the enzyme, S: 1-hydroxy-2-naphthoate (1H2NA), Fe: ferrous sulfate. Brackets mean the preincubation for 1 min.

Furthermore, the activity was restored by preincubation of the above inactivated enzyme with ferrous ion (0.6 mM) for about 1 min. As the preparation obtained by the DEAE-cellulose column chromatography was always inactive, preincubation with ferrous ion was required for activation (Fig. 1). Among other metallic ions and reducing agents such as L-ascorbate, reduced glutathione, L-cysteine and sodium borohydride, only the borohydride gave the activation effect. The enzyme(s) did not require FAD, FMN, NADH and/or NADPH as cofactor in addition to ferrous ion. The enzyme(s) was inhibited by the preincubation with Cu^{2+} , Hg^{2+} , Ag^{+} , *o*-phenanthroline, α, α' -dipyridyl, monoiodoacetate or *p*-chloromercuribenzoate. Consequently, it was assumed that a SH-enzyme was responsible for the conversion of 1H2NA.

The degradation of 1H2NA by the enzyme preparation was spectrophotometrically examined. As illustrated in Fig. 2, the spectrum of 1H2NA changed immediately to that having a maximal absorption at 300 nm which further changed with the lapse of the reaction time. The final spectrum showed maximal absorptions at 293 and 252 nm corresponding to those of 2CBAL. It was manometrically observed that during the reaction oxygen was consumed, but carbon dioxide did not evolve (Fig. 3). Spectra of substances such as 2-hydroxy-1-naphthoate, 2-hydroxy-3-naphthoate, salicylate, gentisate and homogentisate did not

FIG. 2. Changes in Spectra during the Degradation of 1H2NA.

The reaction condition was the same as in Fig. 1 except for use of 0.8 mg protein of the enzyme solution. A and H represent the spectra of 1H2NA and 2CBAL, respectively. B, C, D, E, F and G represent the spectra of products from 1H2NA after incubations for 0.2, 3.0, 5.0, 8.0, 11.0 and 30 min, respectively.

FIG. 3. Oxygen Consumption and Carbon Dioxide Evolution during the Degradation of 1H2NA.

The incubation was carried out in Warburg's vessel. The composition of the reaction mixture was the same as in Fig. 1, except for use of ammonium sulfate-treated preparation as the enzyme.

O₂-uptake: ●—●, with Fe²⁺; ○—○, without Fe²⁺.
CO₂-evolution: △—△, with Fe²⁺.

FIG. 4. The Proposed Scheme of Conversion of 1H2NA to 2CBAL in *Aeromonas* sp. S45P1.

⇒ in *Aeromonas* sp. S45P1, → in pseudomonads isolated by Evans *et al.*⁽²⁾ and Davis *et al.*⁽⁶⁾ → in *m*-cresol-grown pseudomonad isolated by Hopper *et al.*⁽⁵⁾

change under the same condition as above. But a slight change was observed in the spectrum of 1,4-dihydroxy-2-naphthoate.

From these results, our enzyme preparation was considered to contain three enzymes, *i.e.* a ferrous ion-dependent oxygenase which catalyzed the cleavage of the aromatic ring of 1H2NA at the bond between C-1 and C-2 and was similar to gentisate oxidase,⁽³⁾ homogentisate oxidase⁽⁴⁾ and 1,4-dihydroxy-2-naphthoate oxygenase,⁽⁵⁾ a hydratase which catalyzed the addition of a water molecule to the bond between C-3 and C-4 in the formed side chain and an aldolase which splitted the side chain to give 2CBAL and pyruvate (Fig. 4). The spectrum having a maximal absorption at 300 nm during the degradation of 1H2NA seemed to be attributed to one of the intermediates from 1H2NA to 2CBAL. More than one enzyme are considered to take part in the conversion of 1H2NA to 2CBAL, but separation of them is not yet successful. Electrophoresis of the enzyme preparation on polyacrylamide gel showed several bands of protein. The mechanism of the conversion appeared to be similar to that of 1,2-naphthalenediol to salicylaldehyde in naphthalene degradation by a soil *Pseudomonad* isolated by Davis *et al.*⁽⁶⁾

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