ELECTRON MICROSCOPIC STUDIES ON MICROCRYSTALS OF PARAHYDROXYBENZOATE HYDROXYLASE FROM *PSEUDOMONAS FLUORESCENS*

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1. Introduction

The enzyme parahydroxybenzoate hydroxylase (EC 1.14.13.2) has been isolated from three different *Pseudomonas* species [1–3]. It catalyzes the following reaction:

$$p\text{-OHB} + \text{NADPH} + O_2 + H^+ \longrightarrow 3,4\text{-di-OHB} + \text{NADP}^+ + H_2O.$$

The binding of *p*-OHB to the free enzyme induces a conformational change which facilitates the binding of NADPH to the enzyme [1–3].

The enzyme investigated in this study was isolated from *Pseudomonas fluorescens*. Its mol. wt. is 55 000 [4]. The enzyme is inhibited by halogen ions and the inhibition is competitive with respect to NADPH [5].

Microcrystals of the free enzyme are easy to prepare. We have therefore investigated such crystals by electron microscopy techniques in order to gather information concerning the arrangement of the molecules in the crystal. Electron microscopy and optical diffraction studies gave information about the shape and the packing of the molecules in the crystal.

Abbreviations: PHBH: *p*-hydroxybenzoate hydroxylase; *p*-OHB: *p*-hydroxybenzoate; 3,4-di-OHB: 3,4-dihydroxybenzoate.

2. Materials and methods

Large scale production of *Pseudomonas fluorescens*, cultured on the medium described by Howell et al. [1], were performed for us by Diosynth B.V., Oss, The Netherlands. The enzyme PHBH was isolated and purified according to Howell et al. [1]. The free enzyme was crystallized from ammonium sulfate solutions as described elsewhere [1]. Crystals of the enzyme–*p*-OHB complex were obtained in the following ways: (i) microcrystals were incubated in a 50% saturated ammonium sulfate solution in the presence of 0.5 M *p*-OHB; (ii) co-crystallization of the enzyme and *p*-OHB was as described elsewhere [6]. The crystal preparations were negatively stained using a one step droplet method. One droplet of the crystal suspension was diluted with 1–2 ml unbuffered 0.5% uranyl acetate solution [7]. The electron micrographs were taken at 80 kV and analyzed with an optical diffractometer [8].

3. Results and discussion

Negatively stained microcrystals obtained from free PHBH generally show the pattern illustrated in fig.1A. The corresponding optical diffraction pattern is given in fig.1B. The symmetry derived from this result is pgg [9] with $a = 13.4$ nm and $b = 8.6$ nm. Fig.2A

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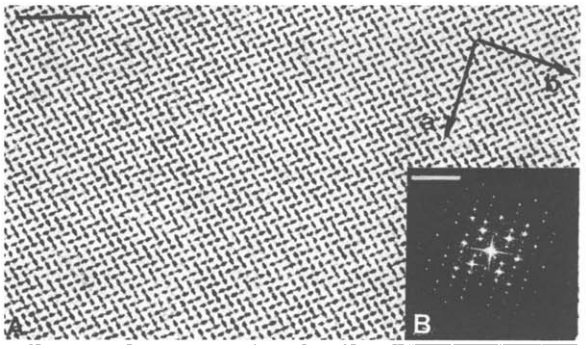

Fig.1. (A) Electron micrograph of a negatively stained crystal obtained from free PHBH. The direction of view is along the *c* axis. In the micrographs the arrows indicate the crystal axes, while the bar represents 50 nm. (B) Corresponding optical diffraction pattern. In the patterns the reciprocal axes are parallel to the corresponding crystal axes, the bar corresponding to 0.4 nm^{-1}.

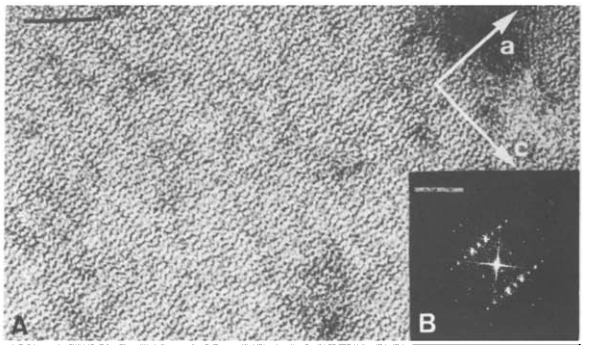

Fig.2. (A) Electron micrograph of a negatively stained PHBH crystal. The direction of view is along the *b* axis. (B) Corresponding optical diffraction pattern.

shows a different view of the crystal. The corresponding optical diffraction pattern is shown in fig.2B. The observed pmg symmetry [9] indicates that the projection is along the *b* axis with $a = 13.4$ nm and $c = 4.5$ nm. From the plane groups deduced from fig.1 and 2 we arrive at the three-dimensional space group $P2_12_12$ [9]. The number of molecules per unit cell (which must be a multiple of 4 in this space group) clearly is 4. Employing the equations of Matthews [10] it was calculated that the crystal volume per unit of protein molecular weight, V_M, is 2.36 $Å^3$/dalton and that the solvent content of the crystals is approx. 48%.

The staining pattern gives rise to pseudo dimers in both views (figs. 1 and 2) and can be clearly seen in fig.1. The pseudo dimers are well ordered giving rise to a 'fish-bone' like arrangement of the molecules (fig.1). The dimensions of the (monomeric) molecule, estimated from the crystal parameters, are 6, 6 and 4.5 nm, respectively. The size of the molecules measured on electron micrographs of single PHBH molecules (fig.3) is 8.5 nm. This discrepancy between the two sets of data is due to the fact that by the latter method one is looking at exclusion of stain in negatively stained preparations which yields too large values by the measurements of single molecules [11]. The single molecules exhibit circular to square profiles as is seen in fig.3.

No change of the crystal structure could be observed when the crystals of the free enzyme were soaked in *p*-OHB or NaCl. This indicates that under the experimental conditions employed *p*-OHB and NaCl probably did not bind to the crystalline enzyme. The electron microscopic results obtained with microcrystals of the enzyme-substrate complex were not very reliable because these crystals are not plate-like and do not lay on the grid with a crystallographic axis parallel to the electron beam. While this work was in progress it was possible to obtain crystals of the enzyme–*p*-OHB complex suitable for X-ray studies [6]. Therefore, we have not attempted to overcome the above mentioned problem. However, all efforts failed [6] to obtain crystals of the free enzyme suitable for X-ray studies.

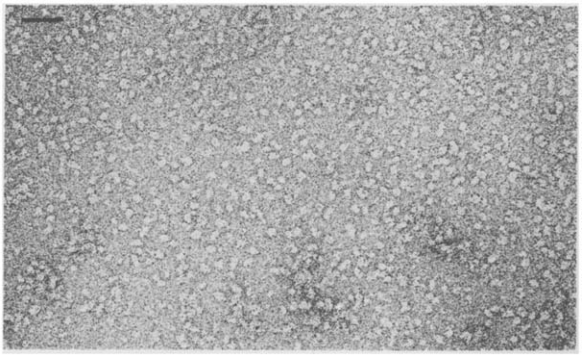

Fig.3. Electron micrograph of single PHBH molecules, negatively stained.

There are some remarkable differences in the physical parameters of the crystals of the free and *p*-OHB complexed enzyme. First, the crystal volume per unit of protein molecular weight of the free enzyme is larger than that of the *p*-OHB-complexed enzyme (2.36 $Å^3$/dalton vs 2·10 $Å^3$/dalton). Second, the solvent content of the crystal of the *p*-OHB complex is 42%, whereas that of the free enzyme is 48%. This result cannot be caused by shrinkage of the crystals when preparing them for electron microscopy as shrinkage would increase these differences. These results thus indicate that the crystals of the *p*-OHB complex are more tightly packed than are those of the free enzyme. These data support other results which indicated that the enzyme undergoes a conformational change upon formation of the *p*-OHB complex [1,4].

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