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Crystallization and Preliminary X-Ray Analysis of Salicylate Hydroxylase from *Pseudomonas putida* S-1

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Apo-salicylate hydroxylase from *Pseudomonas putida* S-1 has been crystallized by the dialysis method, using ammonium sulfate as the precipitant. The crystals belong to hexagonal space group $P6_2$ or $P6_4$ with unit cell dimensions of $a=b=142.8$ Å and $c=63.8$ Å, and diffract X-rays at higher than 3.5 Å resolution. A heavy-atom derivative has been prepared by soaking a crystal in an ammonium sulfate solution containing *p*-chloromercuriphenylsulfonate.

Key words: crystallization, *Pseudomonas putida*, salicylate hydroxylase, X-ray diffraction.

Salicylate hydroxylase [salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, decarboxylating), EC 1.14.13.1] from *Pseudomonas putida* S-1 catalyzes the decarboxylative hydroxylation of salicylate to catechol and CO_2, with a 1:1:1 stoichiometry (*1-3*). It is a flavin-dependent monooxygenase containing 1 mol of FAD per mol of enzyme with a molecular weight of 54,000 (*4-6*). The enzyme is a unique monooxygenase that catalyzes in that the hydroxylation with decarboxylation of salicylate or deformylation of salicylaldehyde (*7*). The crystal structure of a similar enzyme, *p*-hydroxybenzoate hydroxylase, has been reported and discussed regarding the catalytic mechanism (*8-10*). The amino acid sequence of the salicylate hydroxylase, however, has not been determined, and there has been no report about this enzyme. To understand the catalytic mechanism of the enzyme and to determine functional differences from *p*-hydroxybenzoate hydroxylase, it is important to determine the crystal structure of the enzyme. Here, we report on the crystallization and preliminary structural analysis of the apo-form of salicylate hydroxylase.

Holo-salicylate hydroxylase was purified from *P. putida* S-1 by the previously described method (*11*). Crystallization of the apo-enzyme was performed by dialyzing (at 4°C) a 20 μl solution containing 22.7 mg/ml holo-protein exhibiting a yellow color with 50 mM Tris-HCl buffer (pH 8.0) containing ammonium sulfate as a precipitant. The concentration of ammonium sulfate was increased from 0.5 to 1.6 M. The initial yellow color exhibited by the protein solution disappeared gradually and large crystals developed in 3 or 4 days with 1.6 M ammonium sulfate (Fig. 1). Since the crystals lost the yellow color and the spectrum of the enzyme when crystals were dissolved in a small amount of buffer solution showed no specific peak caused by FAD (Fig. 2), they were regarded as being of the apo-enzyme.

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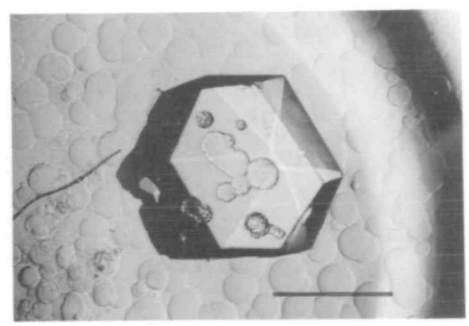

Fig. 1. **A crystal of salicylate hydroxylase from *P. putida* S-1.** The size is $0.8\times0.8\times0.4$ mm³. The scale bar represents 0.5 mm.

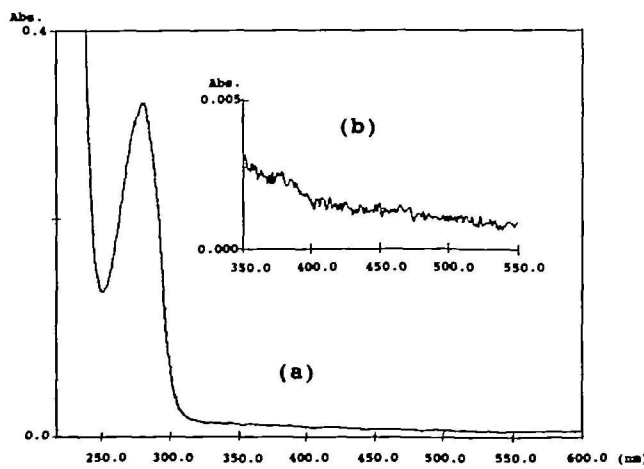

Fig. 2. **Spectra of salicylate hydroxylase from crystals.** Spectra of the enzyme derived from crystals just before (a) and after (b) X-ray diffraction measurements. (b) only shows the visible wavelength region with an expanded ordinate.

TABLE I. **Statistics of the diffraction measurements.**

	Native	Hg-derivative
No. of crystals used	2	1
Resolution (Å)	3.5	3.5
No. of measured reflections	127,632	63,775
No. of unique reflections	9,490	9,319
Completeness (%)	99.8	98.9
R_{merge} (%)	9.8	9.0
R_{iso} (%)	—	9.6

$R_{merge}=\Sigma(|I(hi)-\langle I(h)\rangle|)/\Sigma(I(h))$. $R_{iso}=\Sigma(|F_{PH}-F_P|)/\Sigma(|F_P|)$.

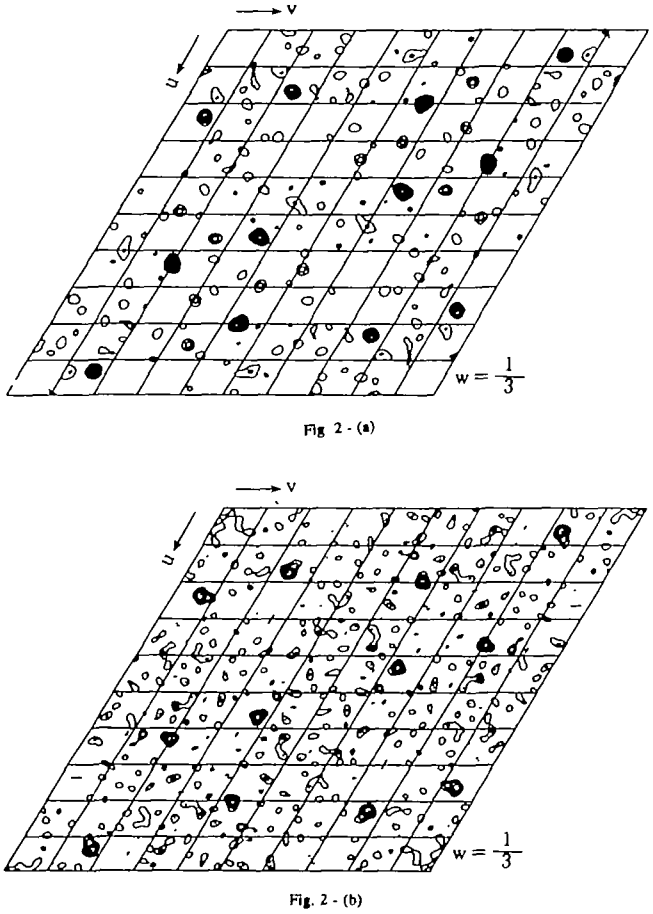

Fig. 3. **The Harker sections at $w=1/3$ of the isomorphous (a) and anomalous (b) difference Patterson functions for the mercury derivative.** Intensity data from 15 to 5 Å resolution are included. The peaks are consistent with a heavy-atom binding site at $x=0.563$, $y=0.363$, $z=0$, or its enantiomer corresponding to $P6_2$.

A single crystal was sealed in a glass capillary tube together with a trace amount of the mother liquor. X-ray diffraction experiments were performed at about 15°C using nickel-filtered CuKα radiation. The diffraction pattern indicates that the crystal belongs to the hexagonal system and that the space group is $P6_2$ or $P6_4$. The unit cell parameters are $a=b=142.8$ Å, and $c=63.8$ Å. The crystals diffracted at higher than 3.5 Å resolution. The density of the crystal was determined to be 1.17 g/cm^3 by the linear gradient method with an *m*-xylene and bromobenzene system. These results indicate that one molecule is present in an asymmetric unit and that the V_m value is 3.48 Å^3/Da (*12*). We collected diffraction data up to 3.5 Å resolution by the oscillation method using a MAC Science DIP-2000 diffractometer equipped with imaging plate detectors.

Heavy-atom derivatives were prepared by the soaking method. Each heavy-atom reagent was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 2.0 M ammonium sulfate. The intensities of native and derivative crystals were determined with the program, DENZO (*13*).

The isomorphous difference Patterson function with coefficients $(F_{PH}-F_P)^2$ and anomalous difference Patterson function with $(F_{PH}^+-F_{PH}^-)^2$ were calculated for each of the derivative crystals. In the above formula, F_{PH} and F_P are the structure amplitudes for a heavy-atom derivative crystal and the native crystal, respectively, and F_{PH}^+ and F_{PH}^- represent $F_{PH}(h\,k\,l)$ and $F_{PH}(-h\,-k\,-l)$, respectively. Although various heavy-atom reagents caused large intensity changes compared with in the native crystal, the peaks in most of the difference Patterson maps were not interpretable except for in the case of the following one derivative. The results of the intensity measurements for the native crystal and a derivative crystal soaked in a 5 mM *p*-chloromercuriphenyl sulfonate solution for 24 h are given in Table I. The isomorphous difference Patterson map (Fig. 3a), as well as the anomalous difference Patterson map (Fig. 3b), shows one major heavy-atom binding site in an asymmetric unit. Peptide model building of the enzyme by use of a Fourier electron density map and the crystallization of holo-enzyme molecules reconstituted by the addition of FAD are now in progress.

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