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# Crystallization and preliminary X-ray crystallographic analysis of 2-methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase from *Pseudomonas* sp. MA-1

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2-Methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase (MHPCO) catalyzes the conversion of an aromatic substrate, MHPC, to an aliphatic compound, α-(*N*-acetylaminomethylene)-succinic acid, and is involved in the degradation of vitamin $B_6$ by the soil bacterium *Pseudomonas* sp. MA-1. Using only FAD as a cofactor, MHPCO is unique in catalyzing hydroxylation and subsequent aromatic ring cleavage without requiring a metal-ion cofactor. Here, the crystallization of MHPCO is reported together with preliminary X-ray crystallographic data. An MHPCO crystal obtained by hanging-drop vapour diffusion diffracted X-rays to 2.25 Å resolution and belonged to the triclinic space group *P*1, with four molecules per asymmetric unit.

## 1. Introduction

2-Methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase (MHPCO; EC 1.14.12.4) is an FAD-containing enzyme involved in the degradation of vitamin $B_6$ (pyridoxine) by the soil bacterium *Pseudomonas* sp. MA-1 (Sparrow *et al.*, 1969). MHPCO catalyzes the conversion of its aromatic substrate, MHPC, into α-(*N*-acetylaminomethylene)-succinic acid (AAMS; Fig. 1). Previous mechanistic studies have shown that MHPCO belongs to the class of aromatic flavoprotein hydroxylase enzymes (Chaiyen, Ballou *et al.*, 1997; Chaiyen, Brissette *et al.*, 1997*a*,*b*,*c*; Chaiyen *et al.*, 2004). Enzymes in this class usually incorporate the hydroxyl group into phenolic substrates at the *ortho* position (Palfey *et al.*, 1995). MHPCO differs from other enzymes in the same class in recognizing a pyridyl derivative MHPC instead of a simple phenol. Nevertheless, studies have shown that the first part of the reaction is the hydroxylation of MHPC, which occurs *via* an electrophilic aromatic substitution mechanism, with the enzyme intermediate [C(4a)-hydroperoxy FAD] acting as an electrophile, similar to that found in other enzymes of the class (Chaiyen, Brissette *et al.*, 1997*b*; Chaiyen *et al.*, 2004). However, unlike other enzymes of the same class, MHPCO further catalyzes a ring cleavage of the hydroxylated MHPC, which apparently only requires the protein moiety (Chaiyen, Brissette *et al.*, 1997*b*). The ability of MHPCO to catalyze the conversion of an aromatic to an aliphatic compound using only the flavin cofactor is unique since the ring-cleavage reaction of aromatic compounds is typically catalyzed by dioxygenase enzymes, which generally require a metal ion (usually ferrous or ferric iron) as a cofactor (Hayaishi *et al.*, 1975).

The only other enzyme so far reported to catalyze a similar reaction to MHPCO is 5-pyridoxic acid oxygenase, an enzyme involved in the degradation of vitamin $B_6$ in *Arthrobacter* (Nelson & Snell, 1986).

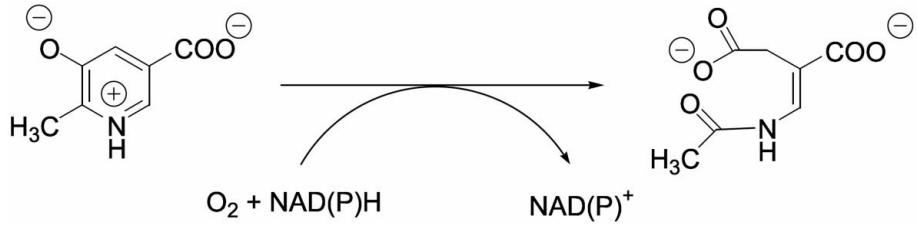


**Figure 1**
The reaction of 2-methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase (MHPCO). MHPCO catalyzes the oxygenation of MHPC to α-(*N*-acetylaminomethylene)-succinic acid (AAMS).

MHPCO has been found in the genome of *Mesorhizobium loti*, where it shows 97% amino-acid sequence identity to *Pseudomonas* MHPCO (Kaneko *et al.*, 2000). Other homologues of MHPCO have also been found in the quinoline-degradation pathway of *Arthrobacter ilicis* (Betz *et al.*, 2000) and in chromosome IV of *Arabidopsis thaliana* (Aubourg *et al.*, 1999). Based on sequence analysis, MHPCO is generally related to enzymes from the aromatic flavoprotein hydroxylase class with ∼12–16% amino-acid identity and to numerous hypothetical proteins derived from genomic studies with ∼25–40% identity (Altschul *et al.*, 1997).

To date, only two crystal structures of aromatic flavoprotein hydroxylases, those of *p*-hydroxybenzoate hydroxylase (PHBH; Wierenga *et al.*, 1979) and phenol hydroxylase (Enroth *et al.*, 1998), have been determined. Structural and kinetic studies have allowed these two enzymes, especially PHBH, to become the best understood of the class (Palfey *et al.*, 1995; Palfey & Massey, 1998). Some detailed catalytic properties of PHBH, such as the movement of the flavin upon substrate binding (Gatti *et al.*, 1994), were originally identified by structural analysis. Therefore, structural study of *Pseudomonas* MHPCO should also reveal information necessary for in-depth understanding of the reaction mechanism and is especially needed in order to understand the ring-cleavage reaction and the specificity for a pyridyl substrate, features that are not found in the reactions of PHBH or phenol hydroxylase. Therefore, an attempt is being made to determine the three-dimensional structure of *Pseudomonas* sp. MA-1 MHPCO and here we report crystallization conditions and preliminary X-ray crystallographic analysis.

## 2. Methods

### 2.1. Protein purification and crystallization

MHPCO from *Pseudomonas* sp. MA-1 was purified using the previously published protocol (Chaiyen, Brissette *et al.*, 1997*a*). The purity of the protein was >95% judging with 12.5% SDS–PAGE. The protein concentration was determined by measuring the absorbance at wavelength 452 nm, employing a molar extinction coefficient of 13 110 $M^{-1}$ cm$^{-1}$ (Kishore & Snell, 1981). The purified protein was concentrated to 20–40 mg ml$^{-1}$ by centrifugation at 3500*g* for 20 min at 277 K using an Amicon Ultra-4 (Millipore) membrane with 10 kDa cutoff. Prior to crystallization experiments, the concentrated protein was filtered using an Ultrafree-MC 0.22 µm (Millipore) filter by centrifugation at 10 000*g* for 5 min. The initial crystallization conditions of MHPCO were screened with the microbatch method using 60-well minitray plates (NUNC; Chayen *et al.*, 1992; D'Arcy *et al.*, 1996). Each droplet was prepared by mixing 1 µl protein solution and 1 µl precipitant solution under approximately 6 ml of mineral oil containing vitamin E (Babimild Baby oil; Chitnumsub *et al.*, 2004). An initial screen for crystallization conditions was set up using a combination of polyethylene glycol 4000 (PEG 4000), salts and buffers (pH 4.6–8.5) with a protein concentration of about 10 mg ml$^{-1}$. The crystallization conditions producing crystals were subsequently optimized by the hanging-drop vapour-diffusion method (McPherson, 1999) using standard Greiner plates. All crystallization experiments were carried out at 295 K.

### 2.2. X-ray data collection

X-ray diffraction data were collected at the Center for Excellence in Protein Structure and Function (CPSF), Faculty of Science, Mahidol University, Thailand. X-ray radiation was generated by a Rigaku RU-H3R rotating-anode X-ray generator (Cu $K\alpha$; $\lambda$ = 1.5418 Å) operating at 50 kV and 100 mA and equipped with Osmic Confocal Maxflux multi-layer optics and a 0.3 mm collimator. The crystals were flash-frozen in a nitrogen stream (100 K) generated using an X-Stream 2000 low-temperature system (Rigaku/MSC). X-ray diffraction patterns were recorded on an R-AXIS IV$^{++}$ image-plate system (Rigaku/MSC). All data were processed and scaled with the *CrystalClear*/*d*TREK* program suite (Pflugrath, 1999). The data-collection statistics are summarized in Table 1.

**Table 1**
Data-collection statistics for MHPCO crystals complexed with FAD.

Values in parentheses correspond to the highest resolution shell.

| | |
|---|---|
| Space group | *P*1 |
| Unit-cell parameters | |
| *a* (Å) | 80.6 |
| *b* (Å) | 86.8 |
| *c* (Å) | 86.5 |
| $\alpha$ (°) | 103.2 |
| $\beta$ (°) | 102.7 |
| $\gamma$ (°) | 106.9 |
| Unit-cell volume (Å$^3$) | 536246 |
| Resolution limits (Å) | 41.33–2.25 (2.28–2.25) |
| No. of observed reflections | 218513 |
| No. of unique reflections | 180861 |
| Completeness (%) | 91.7 (84.9) |
| Multiplicity | 1.20 (1.23) |
| $R_{\rm merge}$† (%) | 7.2 (28.8) |
| $\langle I/\sigma(I)\rangle$ | 5.5 (1.4) |
| $V_{\rm M}$ (Å$^3$ Da$^{-1}$) | 3.2 |
| Solvent content (%) | 61.2 |
| No. of molecules per AU | 4 |

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i$ is the intensity of the $i$th measurement of an equivalent reflection with indices *hkl*.

## 3. Results and discussion

Many optimization experiments were systematically set up by varying the PEG 4000 and salt concentrations, buffers in the pH range 4.6–8.5 and also the volume ratios of protein *versus* precipitant and the drop size using the hanging-drop vapour-diffusion method. Crystals of MHPCO were obtained from conditions containing 15–20%(*w*/*v*) PEG 4000 and 0.1 *M* trisodium citrate in the pH range 5.0–6.0. Droplets consisting of 3 µl protein solution (10 mg ml$^{-1}$ and 3 µ*M* FAD in 50 m*M* MOPS pH 7.0, 0.3 m*M* EDTA and 1 m*M* DTT) were mixed with 1 µl reservoir solution and equilibrated against 500 µl reservoir solution. The bright yellow colour indicated the presence of oxidized flavin in the crystals. A crystal suitable for X-ray data

**Figure 2**
Crystals of *Pseudomonas* sp. MA-1 MHPCO photographed under polarized light. The MHPCO crystals were grown in 17%(*w*/*v*) PEG 4000, 0.1 *M* trisodium citrate pH 5.4. The crystals appeared after 1 d and reached approximate dimensions of 0.3 × 0.2 × 0.15 mm in 2–3 d.

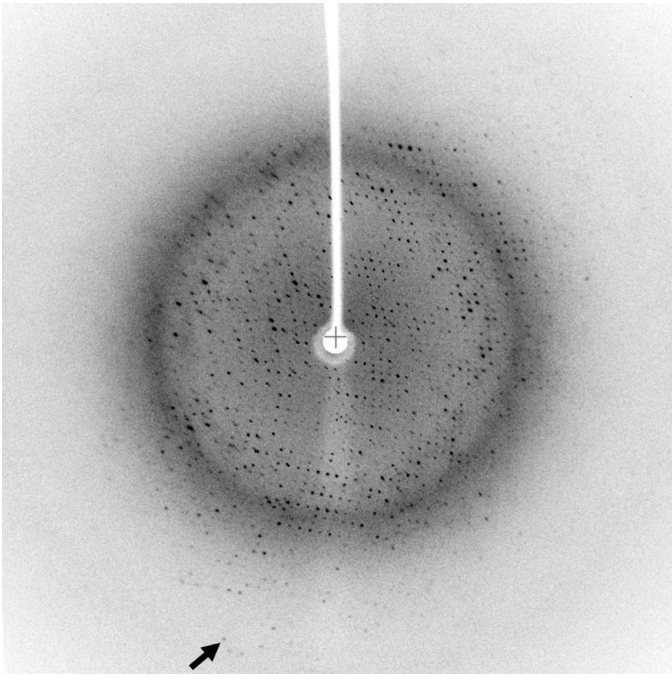

**Figure 3**
A 1°-rotation photograph showing the X-ray diffraction pattern of an MHPCO crystal. The arrow indicates a reflection of 2.3 Å resolution.

collection was obtained from a hanging drop containing 17%(*w*/*v*) PEG 4000, 0.1 *M* trisodium citrate buffer pH 5.4 (Fig. 2).

The MHPCO crystal was quickly transferred into 10 µl cryoprotectant solution containing 15%(*v*/*v*) glycerol, 17%(*w*/*v*) PEG 4000 and 0.1 *M* trisodium citrate pH 5.4 and soaked for 5 s before being flash-frozen in a nitrogen stream at 100 K. Using a 180 mm crystal-to-detector distance, the data were collected to an effective resolution of 2.25 Å using a rotation angle of 1.0° and an exposure time of 630 s per image. The diffraction images (Fig. 3) were processed by a semi-automated protocol using the *DPS* algorithm (Steller *et al.*, 1997) and least-squares fitting (Andrews & Bernstein, 1988; Paciorek & Bonin, 1992). The crystal belonged to the triclinic *P*1 space group, with the unit-cell parameters and data-collection statistics summarized in Table 1. It should be noted that the data could not be indexed with any other lattice characters with appreciable least-squares fitting. Self-rotation solutions calculated with the *AMoRe* and *MOLREP* programs (Navaza, 1994; Vagin & Teplyakov, 1997; Collaborative Computational Project, Number 4, 1994) suggested the presence of 222 symmetry. However, none of the twofold axes lie parallel to any of the unit-cell axes, suggesting that the 222 symmetry is non-crystallographic. The structure of MHPCO is being determined by the molecular-replacement method using *p*-hydroxybenzoate hydroxylase (PHBH) as a search model (Schreuder *et al.*, 1989).

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