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# PURIFICATION OF A HIGH SPECIFIC ACTIVITY METHANE MONOOXYGENASE HYDROXYLASE COMPONENT FROM A TYPE II METHANOTROPH

Brian G. Fox and John D. Lipscomb[1]

Department of Biochemistry, Medical School, University of Minnesota, Minneapolis 55455

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**The purification of the hydroxylase component of a 3 component methane monooxygenase from the type II methanotroph *Methylosinus trichosporium* OB3b is reported. The enzyme (240 kDa) has an $(\alpha\beta\gamma)_2$ subunit structure as observed for hydroxylases isolated from other Type I and Type II methanotrophs, but it exhibits a 5 to 10 fold higher specific activity and is isolated in 2 to 10 fold higher yield. EPR and Mössbauer spectra of the hydroxylase show that it contains a coupled iron center containing an even number of iron atoms. The spectra are similar to those of proteins known to contain oxo-bridged binuclear iron centers. The presence of such a center is unprecedented in a monooxygenase and suggests that a novel mechanism is utilized.** 

The methane monooxygenase system (E.C. 1.14.13.25) of methanotrophic bacteria catalyzes the NAD(P)H dependent cleavage of $O_2$ and incorporation of one oxygen atom into methane to give methanol (1). This enzyme system can exist in either a soluble or a membrane bound form depending on growth conditions (2,3). The soluble MMO from the Type I methanotroph *Methylococcus capsulatus* (Bath) has been purified and shown to consist of 3 protein components. Component A, which appears to contain the active site for hydroxylation, is a 210 kDa protein ($(\alpha\beta\gamma)_2$, subunits 54, 42, and 17 kDa) that contains 2.3 mol Fe / mol protein and no acid labile sulfide or heme (4). Component B (15.7 kDa) contains no cofactors or metals and may serve a regulatory role (5). Component C (39 to 45 kDa) contains FAD and a [2Fe 2S] cluster, and functions as an NADH oxidoreductase (6,7). A remarkably similar MMO has been purified from the Type II methanotroph, *Methylobacterium species* CRL-26 (8,9). However, it was reported not to require a component B. We will refer to components A and C as the hydroxylase and reductase, respectively.

Although the purification of the hydroxylases from both Type I and Type II methanotrophs are reported to yield homogeneous proteins, the final specific activities, 72 and 208, respectively (4,8), account for only a small fraction of the *in vivo* methane turnover after the yield of the preparations is taken into account. Several problems are apparent in the previously reported purification protocols which may contribute to low final specific activity. These include lack of significant increases in specific activity beyond the first step of the multistep purification protocols, low overall yields, and poor stabilities of the purified hydroxylases. Such effects may derive from inherent properties of the hydroxylases and / or a requirement for stabilizing agents. Therefore, we have purified the hydroxylase from a different Type II methanotroph utilizing a new protocol which makes use of stabilizers successfully used in the

[1] To whom correspondence should be addressed.

Abbreviations: MMO, methane monooxygenase; MOPS, 3-(N-morpholino)propanesulfonic acid; ICPES, inductively coupled plasma emission spectroscopy

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purification of other oxygenases. We report here the purification of the hydroxylase from *Methylosinus trichosporium* OB3b to homogeneity. The procedure yields hydroxylase exhibiting 5 to 10 fold higher specific activity, 2 to 10 fold higher yield and significantly improved stability. Moreover, the selected bacterium reproducibly expresses the soluble form of MMO and can be cultured in high yield. The high yield of active hydroxylase facilitates spectroscopic investigations of the metal center structure, some of which are reported here.

## MATERIALS AND METHODS

**BACTERIAL CULTURE**- *M. trichosporium OB3b* was provided by Dr. R.S. Hanson (Univ. of Minnesota) and cultured as described by Cornish et al. (10). Cells used for the purification procedures were grown in a 14 liter New Brunswick fermentor in continuous culture. Cells used in Mössbauer studies were cultured on media containing 2 mg / liter of $^{57}Fe$. A typical yield of 20 to 28 g cell paste / liter was obtained.

**ENZYME ASSAYS**- Assays of MMO activity were performed by measuring propene oxide formation from propene using a Hewlett Packard 5700A Gas Chromatograph equipped with a Porapak Q column. For a given protein component, assays were performed such that the other two protein components, propene, and NADH were present in saturating concentrations. A standard assay contains hydroxylase, component B, and reductase in a total volume of 500 µl of 25 mM MOPS, pH 7.5 sealed in a 7 ml reaction vial. Propene was added to the gas headspace and the reaction was initiated by the addition of NADH. Assays were performed at 30°C. Reaction products were extracted with chloroform prior to gas chromatographic analysis. Quantification was done by the method of internal standardization using pentene as a standard. A typical assay is shown in Table II. Oxygen uptake measurements were made with a Clark type oxygen electrode as previously reported (11). The specific activity values calculated from oxygen uptake or from product formation agree within 10%.

**SEPARATION OF THE METHANE MONOOXYGENASE COMPONENTS**- All purification procedures were performed at 4°C. The cell paste (100 g) was suspended in 200 ml of 25 mM MOPS, pH 7.0, containing 200 µM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 2 mM cysteine (Buffer A). The cells were disintegrated by sonication. The sonicated suspension was centrifuged at 48000xg for 60 min. The supernatant was decanted, diluted with an additional 100 ml of buffer A, and adjusted to pH 7.0. The cell free extract was immediately loaded onto a fast flow DEAE Sepharose CL-6B column (40 mm x 250 mm) equilibrated with buffer A. The column was then washed with 600 ml of buffer A. All MMO components were completely adsorbed. The MMO components were eluted with a 2000 ml gradient of 0.0 to 0.40 M NaCl in buffer A. The hydroxylase eluted at 0.075 M NaCl. The two additional fractions required for reconstituted hydroxylase activity, component B and the reductase, eluted at 0.18 M NaCl and 0.27 M NaCl, respectively.

**PURIFICATION OF THE HYDROXYLASE**- The pooled fractions of the hydroxylase were immediately concentrated by ultrafiltration. The concentrated protein was applied to a Sephacryl S-300 column (25 mm x 950 mm) equilibrated in 50 mM MOPS, pH 7.0 containing 200 µM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2 mM cysteine, and 0.1 M NaCl. Fractions containing the hydroxylase were concentrated using ultrafiltration.

**OTHER METHODS**- Protein concentration was determined colorimetrically using dialyzed and lyophilized hydroxylase samples as standard (12). Iron was determined by complexation with 2,4,6 triphenyl triazine (13). Iron and other metals were also determined by ICPES. Inorganic sulfide was determined by the method of Beinert (14). Denaturing PAGE was done as previously reported (11).

**SPECTROSCOPY**- EPR spectra were recorded at X-band on a Varian E-109 spectrophotometer with an Oxford Instruments ESR-10 liquid helium cryostat. Temperature and g-value calibrations were as previously described (15). Mössbauer spectra were obtained and analyzed as previously described (11). The EPR spectrum of reduced uteroferrin was provided by Dr. L. Que (Univ. of Minnesota).

## RESULTS AND DISCUSSION

The methane monooxygenase system from *M. trichosporium* OB3b has been resolved into 3 protein components by ion exchange chromatography. The elution profile obtained during the DEAE fractionation of the

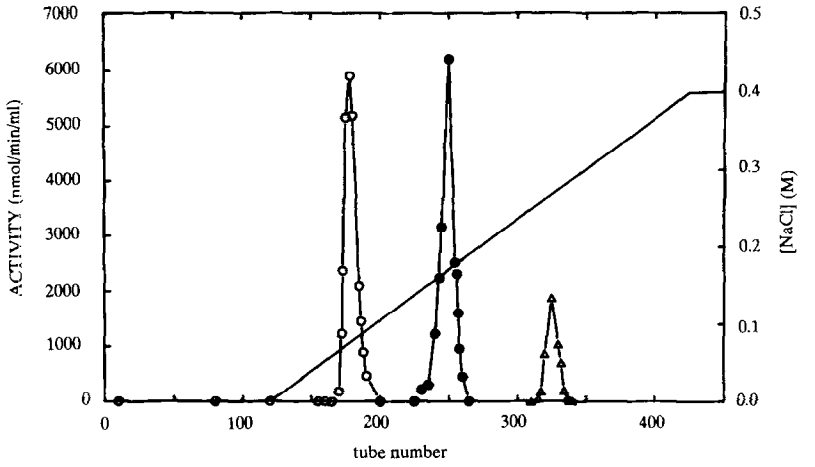



**Figure 1.** Separation of the methane monooxygenase components using DEAE Sepharose CL-6B. Column conditions: 2000 ml gradient from 0.0 M NaCl to 0.4 M NaCl in Buffer A begun at tube 120, 8 ml / tube, linear flow rate 15 cm / h. The peaks shown represent the elution of ( O ) hydroxylase, ( ● ) component B, and ( Δ ) reductase activity when assayed for the production of propene oxide from propene at 30°C in the presence of saturating amounts of the other components. The progress of the salt gradient is indicated by the solid line.

cell free extract is shown in Fig. 1. All 3 components are required for rapid hydroxylation activity upon reconstitution. These components appear to be analogous to the hydroxylase, component B, and reductase purified from *M. capsulatus* (Bath). However, when purified through the single gradient ion exchange step reported here in the presence of stabilizers, the hydroxylase exhibits an increase in specific activity greater than observed in the complete preparation of hydroxylases from *M. capsulatus* (Bath) or *M. species* CRL-26. Furthermore, the gradient elution gives 25 and 80 fold increases in the specific activities of the component B and reductase, respectively, as compared with the 4 fold and 6 to 15 fold increases observed using the previous batch elution procedures (5,6,9). The protocol reported here results in the complete resolution of the components and apparently homogeneous hydroxylase. Complete purification schemes for the component B and reductase are currently being developed.

The simple and rapid purification procedure of the hydroxylase is summarized in Table I. The purified enzyme is homogeneous as judged by repeated gel filtration, denaturing PAGE, or ultracentrifugation. It exhibits a turnover number of 3.1 $sec^{-1}$ for propene and 2.6 $sec^{-1}$ for methane. No optical spectrum in the visible region is observed for the protein, and no inorganic sulfide is detected. Calibrated gel filtration and ultracentrifugation experiments indicate that the hydroxylase is a single component with a native molecular weight of 240 kDa. Denaturing PAGE experiments indicate that the hydroxylase is composed of 3 subunits ($M_r$ 54, 43 and 23 kDa), suggesting an $(\alpha\beta\gamma)_2$ structure. The subunit structure and the native molecular weight of the hydroxylase are similar to those reported for the hydroxylases isolated from other methanotrophs (4,8). However, the specific activity of the hydroxylase isolated

**Table I**
**Purification of the methane monooxygenase hydroxylase**

| Step | Vol. ml | Protein mg/ml | Units/ ml[a] | Units nmol/min | Recovery % | Specific Activity | Fold Purification |
|---|---|---|---|---|---|---|---|
| Cell free extract[b] | 315 | 15.8 | 2055 | 647000 | 100 | 130 | 1.0 |
| DEAE Sepharose | 120 | 10.2 | 3500 | 420000 | 65 | 343 | 2.6 |
| Sephacryl S-300 | 61 | 5.0 | 3820 | 233000 | 36 | 764 | 5.9 |

[a] A unit is defined as the production of 1 nmol propene oxide per minute.
[b] from 100 g *Methylosinus trichosporium* OB3b cell paste.

from *M. trichosporium* OB3b using the present procedure is 5 to 10 times higher than previously reported for other, apparently pure, hydroxylase preparations. Since it is unlikely that a specific activity increase of this magnitude is due solely to increases in protein homogeneity, stabilizing agents which effect the retention of iron by the hydroxylase are probably a major contributing factor. A combination of the molecular properties of the hydroxylase and the effect of the stabilizing agents also appears to significantly increase the stability of the protein. For example, our hydroxylase retains 80 % of its original specific activity in cell free extracts after 24 hr at 5°C while we have observed that the hydroxylase from *M. capsulatus* (Bath) loses 80 % of its activity in 4 hr at the same temperature. Purification of our hydroxylase using the previously reported protocols results in low specific activity enzyme, suggesting that the observed high specific activity is not a unique feature of the hydroxylase from *M. trichosporium* OB3b.

From measurements of whole cell methane oxidation rates, we estimate the specific activity of the hydroxylase should be ~800 nmol / min / mg to account for the *in vivo* growth rate of the organism. Therefore, unlike previous preparations, the specific activity of the hydroxylase purified here (~750 nmol / min / mg) is comparable to that expected from the whole cell methane oxidation activity of methanotrophic bacteria. The observed 5.9 fold purification suggests that approximately 17 % of the soluble cell protein is hydroxylase. This is consistent with the amount of hydroxylase estimated from denaturing PAGE of the cell free extract.

The observation that efficient substrate hydroxylation requires all 3 components of the MMO system (Table II) is the first conclusive demonstration that Type II methanotrophs utilize a 3 component MMO analogous to that observed in the Type I organisms. Component B appears to be absolutely required; in its absence the hydroxylation rate is decreased 99%. The specific requirement for component B in the *M. trichosporium* OB3b and *M. capsulatus* (Bath) monooxygenase systems is highly suggestive that, contrary to published reports (8), a component B also exists in the MMO system from the closely related Type II organism *M. species* CRL-26. Since very small amounts of the B protein are required to stimulate the hydroxylation reaction, and since the *M. species* CRL- 26 hydroxylase was purified without a molecular sizing step, it is possible that the preparations contained low amounts of component B. Purification of the MMO from *M. trichosporium* OB3b using the batch procedure described for *M. species* CRL-26 fails to resolve the hydroxylase and component B.

One previous attempt to purify the MMO system from *M. trichosporium* OB3b has been reported by Tonge *et al.* (16). The system was reported to consist of a 46 kDa copper protein, a cytochrome and a protein factor. When purified, this system required ascorbate as a reductant. Since the current preparation lacks copper and heme, does not utilize ascorbate as a reductant and consists of components with different molecular weights, it is probably unrelated to the previously reported system. The proteins purified by Tonge *et al.* may be associated with the membrane

**Table II**
**Protein requirements for hydroxylation activity**

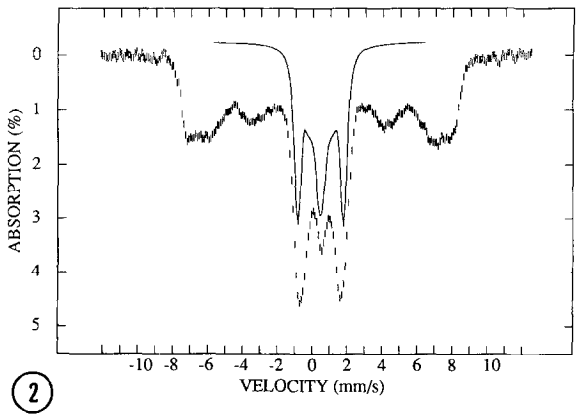
| Components Added | Oxygen Consumed (nmol/min) | Propene Oxide Produced (nmol/min) | Specific Activity (nmol/min/mg) |
|---|---|---|---|
| hydroxylase | 0 | 0 | 0 |
| component B | 0 | 0 | - |
| reductase | 4 | 0 | - |
| component B, reductase | 4 | 0 | - |
| hydroxylase, component B | 0 | 0 | 0 |
| hydroxylase, reductase | 10 | 2 | 5 |
| all components | 340 | 330 | 764 |

**Assays contained 1.8 nmol of purified hydroxylase, 0.12 mg protein from the reductase fraction, 0.15 mg protein from the component B fraction, 500 nmol NADH, and 140 μmol propene. Specific activity is reported relative to the hydroxylase present. Assays were performed in 25 mM MOPS, pH 7.5 at 30°C as reported in the Methods.**

bound form of MMO since detergent solubilization was required. The tendency of the cells to elaborate only the membrane bound form of the enzyme has been a recurring problem in the purification of soluble MMO in quantity. In contrast to the other methanotrophs which have been utilized, *M. trichosporium* OB3b can readily be grown to high cell densities (20 to 28 g / liter) and reproducibly elaborates the soluble form of the enzyme.

Analysis of the metal content of the hydroxylase by ICPES indicates that iron is the only metal present in stoichiometric amounts or greater. Copper, manganese, cobalt, molybdenum, and nickel are specifically absent. Colorimetric and ICPES iron analysis show that the ratio of iron to hydroxylase is variable. Using the purification procedures described above, preparations containing 4 to 8 mol Fe / mol hydroxylase have been observed. The high field Mössbauer spectrum of the $^{57}$Fe- enriched hydroxylase as isolated is shown in Fig. 2. At least two distinct iron species contribute to the observed spectrum. A fraction (ca. 70%) of the iron exhibits broad and poorly defined hyperfine interactions typical of paramagnetic (or possibly ferromagnetic) ferric species. In weak applied fields (~50 Tesla) a portion of this magnetic material appears as a broadened quadrupole doublet, suggesting intermediate to fast electronic spin relaxation. Such a spectrum is typical of adventitious ferric aggregates which are frequently observed in preparations of enzymes purified in iron containing buffers (11). Further studies to be reported elsewhere indicate that a substantial fraction of this iron species can be removed without affecting the activity of the hydroxylase[2]. The sharp central feature shown in Fig. 2 results from the presence of a diamagnetic iron center. The isomer shift observed for the spectrum shows that all iron present is high-spin ferric (inherently paramagnetic). Thus the diamagnetic center must consist of an even number of strongly coupled iron atoms.

The EPR spectrum of the hydroxylase is shown in Fig. 3. As isolated (dashed line), the hydroxylase exhibits weak resonances at g=4.3 (not shown) and g=2.0. The resonance at g=4.3 probably arises from the EPR active portion of adventitiously bound $Fe^{3+}$. Upon partial reduction with sodium dithionite (solid line), an EPR spectrum with all resonances below g=2 ($g_{ave}$=1.85) appears. Similar spectra have been reported for the hydroxylase



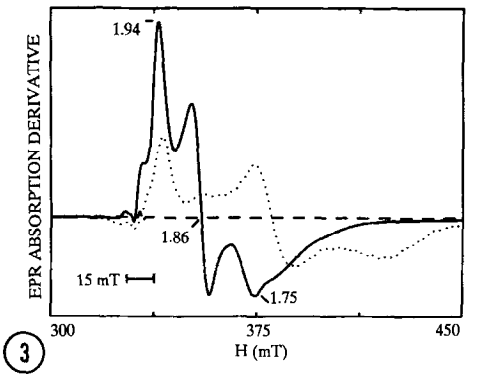




**Figure 2.** Mössbauer spectrum of purified, $^{57}$Fe- enriched hydroxylase. The spectrum was recorded at 4.2 K in a 6.0 Tesla field applied parallel to the observed γ-radiation. The solid line is the theoretical curve for a diamagnetic ferric center with a quadropole splitting of 1.06 mm / s and an isomer shift of 0.51 mm / s with an asymmetry parameter of 1.0. The broad magnetic spectrum cannot be fit with any single set of parameters and appears to result from high spin $Fe^{3+}$ in a variety of environments. (Protein data: 300 mg / ml protein; specific activity 525; 7.5 Fe / protein).

**Figure 3.** X-band EPR spectra of the purified hydroxylase. Dashed line: hydroxylase as isolated; solid line: hydroxylase partially reduced by the anaerobic addition of sodium dithionite. The dotted line shows the EPR spectrum of partially reduced uteroferrin. Instrument settings: gain 250; modulation amplitude 1 mT; modulation frequency 100 kHz; microwave power 10 mW; microwave frequency 9.22 GHz; temperature 8.8 K. (Protein data: 85 mg / ml protein, specific activity 764, 5.2 Fe / protein).

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components isolated from both Type I and Type II methanotrophs (17,18). This EPR spectrum as well as the Mössbauer spectrum of Fig. 2 resemble those observed for proteins containing oxo-bridged iron dimers such as hemerythrin, uteroferrin, and purple acid phosphatase (19-23). The EPR spectrum of reduced uteroferrin is shown in Fig. 3 (dotted line) for comparison. The hydroxylase EPR spectrum differs from those of uteroferrin-like proteins in that it has a significantly larger $g_{ave}$ value (1.85 vs 1.74) and the spectrum is more axial. Nevertheless, the assignment of the center as μ-oxo or μ-hydroxo binuclear iron is probably correct. Recent EXAFS studies of the hydroxylases from both *M. species* CRL-26 and *M. capsulatus (Bath)* showing Fe-Fe distances on the order of 3.05-3.4 Å are consistent with this assignment (18,24). Such centers in proteins have not previously been reported to catalyze monooxygenase reactions, thus it is likely that a new oxygenase mechanism is employed. The availability of large quantities of highly purified hydroxylase component will facilitate more detailed studies of the structure and function of this novel enzyme.

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