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# Comparison of methane hydroxylation activity with intact cells and cell-free extracts of *Methylosinus trichosporium* OB3b

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**Abstract**

The number of active sites for methane hydroxylation of *Methylosinus trichosporium* OB3b was determined by using the inhibition method. The activity per active site of methane hydroxylation with the intact cells and the cell-free extracts were compared. From the turnover numbers of both systems, the reaction mechanism is discussed.

*Keywords*: Active site number; Hydroxylation; Methane hydroxylation; Methane monooxygenase; *Methylosinus trichosporium* OB3b; Turnover number

Methane monooxygenase (MMO) from *Methylosinus trichosporium* OB3b catalyzes hydroxylation of lower alkanes and epoxidation of alkenes [1,2]. In particular, the activity of methane hydroxylation is pretty high, though methane is a low reactivity reagent. To determine the factors taking the methane hydroxylation process, such as active methane transport by cell membrane and methane condensation by cytoplasm, the turnover numbers of methane hydroxylation with the intact cells and with the cell-free extracts were compared. To calculate the turnover number, the number of active sites were determined by the inhibition method.

*M. trichosporium* OB3b was cultivated as described by Fox et al. [3]. Viable cell numbers and the turnover number for methane hydroxylation with the intact cells were determined as follows. After cultivation for 3 days, the cells were aseptically harvested by centrifugation at $6{,}800 \times g$ for 10 min at 4°C and then suspended with a sterilized 10 mmol $dm^{-3}$ potassium phosphate–NaOH buffer (pH 7.0) to give a 0.1 g wet weight of cells per ml. Viable cell numbers of the cell suspensions were determined by plate counts. When methane is oxidized with the intact cells or the cell-free extracts, the methanol produced is subsequently oxidized with the methanol dehydrogenase contained in the same bacterium [2,4]. To prevent further oxidation, the cell suspensions and the cell-free extracts were treated with cyclopropanol which is a selective and irreversible inhibitor for methanol dehydrogenase [5]. The number of active sites per cell was determined as follows. The reaction mixture (3.5 ml) containing 0.2 mmol of potassium phosphate–NaOH buffer (pH 7.0), 0.05 mmol of sodium formate, various amounts of 8-hydroxyquinoline sulfate, a selective inhibitor of MMO [6], 4.5 μmol of the same buffer, and 50 μl of the cell suspensions, was

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Table 1
The number of active sites and the turnover number per active site

| | Active sites | Turnover no./$s^{-1}$ |
|---|---|---|
| Intact cell | $8.49\times10^{4}$ [a] | 16 |
| Cell-free extract | $2.13\times10^{13}$ [b] | $3.3\times10^{-2}$ |

[a] Active sites in $cell^{-1}$.
[b] Active sites in $(mg\text{-}protein)^{-1}$.

introduced into a pear-shaped flask sealed with septa, and incubated for 5 min at 30°C. The reaction was initiated by injecting 2.5 ml of methane into the flask with a gas-tight syringe. The reaction mixture was analyzed by Hitachi 263-30 gas chromatograph with a column of Sorbitol 25%-Gasport B (4 m × 3 mm, temp. 100°C, carrier gas; $N_2$, flow rate 20 ml $min^{-1}$).

The cell-free extracts was prepared as follows. The cell paste (24 g) was resuspended in 10 ml of the 10 mmol $dm^{-3}$ potassium phosphate–NaOH buffer (pH 7.0) containing 1.5 ml of 1.76 mmol $dm^{-3}$ cyclopropanol and was incubated for 10 min at room temperature. The cell suspensions were centrifuged at 28,000 × $g$ for 10 min at 4°C. The supernatant was removed and resuspended in 24 ml of 25 mmol $dm^{-3}$ MOPS (pH 7.0) containing 200 μmol $dm^{-3}$ $Fe(NH_4)_2(SO_4)_2\cdot6H_2O$ and 2 mmol $dm^{-3}$ L-cysteine (buffer A) [7], and 10 μg $ml^{-1}$ DNase I and 4 mmol $dm^{-3}$ $MgCl_2\cdot6H_2O$ added. The ensuing procedure was the same as the method reported previously [8]. Protein concentration was determined by Lowry method with bovine serum albumin as a standard. The assay mixture (2.5 ml) contained 0.15 mmol of potassium phosphate buffer (pH 7.0), various amounts of 8-hydroxyquinoline sulfate, 12.5 μmol of purified NADH (ethanol-free), and 0.5 ml of the cell free-extracts.

At the low concentration of 8-hydroxyquinoline sulfate, the initial methane hydroxylation rate decreased linearly with increase of the concentration of 8-hydroxyquinoline sulfate. The number of active sites was estimated by the intercept obtained by extrapolation of the straight line and by viable cell numbers. These results are summarized in Table 1. As shown in the table a there is a remarkable difference between the turnover number of methane hydroxylation with the intact cells and that of the cell-free extracts was observed. As the cell-free extract is prepared by the destruction of the cell membrane by sonication, the surroundings of the MMO enzyme in the cell-free extract may be different from those in the intact cell.

Table 2
Kinetic constants for methane hydroxylation with the intact cells and the cell-free extracts of OB3b

| | $V_{max}$ | $K_m$/μM |
|---|---|---|
| Intact cells | 76.2 [a] | 6.32 |
| Cell-free extracts | 0.218 [b] | 2.01 |

[a] $V_{max}$ in $\mu M\cdot(min\ mg\ dry\ weight\ of\ cells)^{-1}$.
[b] $V_{max}$ in $\mu M\cdot(min\ mg\text{-}protein)^{-1}$.

To elucidate the factors influencing the turnover number, kinetic studies for methane hydroxylation with the intact cells and the cell-free extracts were carried out as follows. The equivalent volume of 44 μmol $dm^{-3}$ cyclopropanol was added to the cell suspensions (0.4 g wet weight of cells per ml) and incubated for 30 min at 30°C. Methane hydroxylation rate of the intact cells and the cell-free extracts obeyed Michaelis–Menten equation, and $V_{max}$ and $K_m$ were determined. The results are summarized in Table 2. $K_m$ values of intact cells is at most three times larger than that of cell-free extracts, that is, the reactivity of methane hydroxylation with intact cells and cell-free extracts does not strongly depends on $K_m$ values.

From the above results the dissociation process of the product, methanol, from the active site of MMO in intact cells is much easier than cell-free extract. In the intact cells, product dissociation may be much faster by the surroundings of the enzyme compared with the cell-free extract.

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