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Comparison of Reactivity of Alkane Hydroxylation with Intact Cells and Cell-Free Extracts of *Methylosinus trichosporium* OB3b

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ABSTRACT

Kinetic studies for hydroxylation of a series of alkanes (methane, ethane and propane) with intact cells and cell-free extracts of *Methylosinus trichosporium* OB3b were carried out. K_m values for alkane hydroxylation with cell-free extracts were lower than those with intact cells, suggesting that cytoplasm plays an important role in the solubility of alkanes to increase their concentration.

Index Entries: Alkane hydroxylation; methane monooxygenase; *Methylosinus trichosporium* OB3b.

INTRODUCTION

Methylosinus trichosporium OB3b is a methanotrophic bacterium having methane monooxygenase in the cells, which catalyzes the hydroxylation of methane to methanol. Methane monooxygenase also catalyzes the hydroxylation of lower alkanes, such as ethane, propane, and *n*-butane, and the corresponding alcohols are produced as follows (*1,2*):

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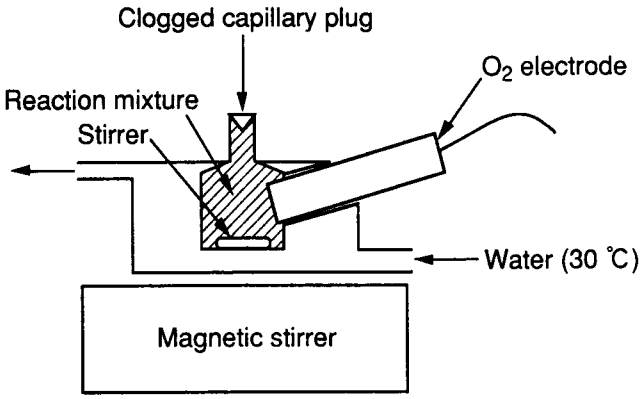

Fig. 1. Oxygen consumption measurement apparatus. The apparatus consists of a Galvanic cell-type electrode with a reaction chamber of 1.74-mL capacity, and was maintained at 30°C by a water jacket and circulator.

$$C_nH_{2n+2} + O_2 + NADH + H^+ \rightarrow C_nH_{2n+1}OH + NAD^+ + H_2O \quad (1)$$

To compare the reactivity of alkanes, alkane hydroxylation should be carried out in the same reaction conditions. Since the solubility of alkane to aqueous solution depends on the type of alkanes, a cell-type chamber was used as a reactor in this study in order to keep the alkane concentration in the reaction mixture constant.

When intact cells and cell-free extracts are used for alkane hydroxylation, the subsequent oxidation, alcohol oxidation, proceeds with alcohol dehydrogenase contained in the same bacterium (*1,3*). Since cyclopropanol inhibits alcohol dehydrogenase selectivity and irreversibly (*4*), the kinetic study was carried out with the intact cells and the cell-free extracts treated with cyclopropanol. Polarographic assay was used with a Galvanic cell-type oxygen electrode as shown in Fig. 1. Hydroxylation reactivity of various alkanes was compared and the role of cell membrane, and cytoplasm on hydroxylation reactivity was discussed.

MATERIAL AND PROCEDURE

Materials

All the chemicals used were of the highest grade available. Methane and nitrogen were purchased from Fujiibussan Co. Ethane was obtained from GL Sciences Co. Propane was obtained from Takachiho Chemical Industry Co. MOPS, DNase I (from bovine pancreas), and bovine serum albumin (RIA grade, Fraction V Powder) were obtained from Sigma Chemical Co. (St. Louis, MO). β-NADH (Grade I) was obtained from Boehringer Mannheim Yamanouchi K. K. Ethanol in NADH (20–40 mol/100 mol) was removed by evaporation at room temperature before use. 8-Hydroxyquino-

line sulfate was purchased from Tokyo Kasei Kogyo Co., Ltd. Cyclopropanol was prepared by the method reported previously (3). The other chemicals were purchased from Kanto Chemical Co., Inc.

Bacterial Growth and Preparation of Cell Suspensions

M. trichosporium OB3b was kindly provided by John D. Lipscomb (Department of Biochemistry, University of Minnesota) and cultivated in a 500-mL baffle-walled shaking flask containing ca. 130 mL of mineral salts medium with methane and air (1:4 v/v) as described by Fox et al. (5). The cells were grown at 30°C in batch culture. Methane was used as the sole carbon source. After cultivation for 3 d, the cells were harvested by centrifugation at 6800*g* for 10 min and then washed with a 10-mmol/dm^3 potassium phosphate-NaOH buffer (pH 7.0). Cell suspensions (0.4 g cell wet wt mL^{-1}) were frozen in liquid nitrogen and stored at −80°C. The activity of the cell suspensions was maintained for 4 d.

Cyclopropanol treatment of cell suspensions was carried out as follows. The equivalent volume of 44 μmol/dm^3 cyclopropanol was added to the cell suspensions and incubated for 30 min at 30°C.

Preparation of Cell-Free Extracts of *M. trichosporium* OB3b

The cell paste (24 g) was resuspended with 1.5 mL of 1.76 mmol/dm^3 cyclopropanol and 10 mL of the 10 mmol/dm^3 potassium phosphate-NaOH buffer (pH 7.0), and incubated for 10 min at room temperature. The cell suspensions were centrifuged at 28,000*g* for 10 min. The supernatant was removed and resuspended in 24 mL of 25 mmol/dm^3 MOPS (pH 7.0), containing 200 μmol/dm^3 $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 2 mmol dm^3 L-cysteine (buffer A) as described by Fox et al. (6), 10 μg/mL DNase I and 4 mmol/dm^3 $MgCl_2 \cdot 6H_2O$ were added. The cells were sonicated for 24 min at 70 W with sufficient cooling to maintain the temperature below 4°C. The sonicated suspension was centrifuged at 28,000*g* for 20 min at 4°C. The supernatant was further centrifuged at 187,000*g* for 1 h at 4°C. The supernatant (cell-free extracts) was frozen in liquid nitrogen and stored at −80°C. The activity of cell-free extracts was maintained for more than 1 mo. Protein concentrations were determined by the Lowry method with bovine serum albumin as a standard.

Assays of Intact Cells and Cell-Free Extracts

Assays of bacterial cells were carried out in the reactor shown in Fig. 1 with the Galvanic cell-type oxygen electrode (Iijima Electric Industry Co.). The assay temperature was maintained at 30°C by a water jacket. The oxygen electrode was calibrated by 0.5 mol/dm^3 sodium sulfite (oxygen-free) and air-saturated distilled water (O_2 conc. = 235 μmol/dm^3). Two milliliters of 0.1 mol/dm^3 potassium phosphate-NaOH buffer (pH 7.0)

and 0.5 mL of 0.1 mol/dm^3 sodium formate were mixed in a 10-mL pear-shaped flask and sealed using septa in advance; alkane and nitrogen were injected into the flasks with a gas-tight syringe. The flasks containing the assay mixture were then incubated for 30 min at 30°C. A portion of the assay mixture was transferred into the reaction chamber by using a syringe. The concentration of substrate in the assay mixture was immediately determined by Hitachi 263-30 gas chromatograph with a column of Sorbitol 25%-Gasport B (2 m × 3 mm, temp. 100°C, carrier gas; N_2, flow rate 40 mL/min). The reaction was initiated by injection of cell suspensions (30 μL) treated with cyclopropanol and monitored by the dissolved oxygen meter.

Initial rates were determined from the initial O_2 consumption rates. During the reaction, the reaction mixture was analyzed by gas chromatograph. Good agreement between O_2 consumption and alcohol formation was obtained.

For the cell-free extracts, the assay mixture containing 1.5 mL of 0.1 mol/dm^3 potassium phosphate-NaOH buffer (pH 7.0) and 0.5 mL of 25 mmol/dm^3 NADH (ethanol-free) in the 10-mL pear-shaped flask was prepared and sealed using septa in advance. The successive procedure is the same as the method of intact cells.

Measurement of Dissolution Rate of Alkane to Intact Cells and Cell-Free Extracts

To measure the dissolution rate of alkane, MMO was inactivated with 8-hydroxyquinoline sulfate aqueous solution (8-HQS). The cell paste (0.8 wet g) or cell-free extracts treated with 8-HQS were suspended by 0.8 mL of 10 mmol/dm^3 potassium phosphate-NaOH buffer (pH 7.0). The assay mixture containing 2.0 mL of 0.1 mol/dm^3 potassium phosphate-NaOH buffer (pH 7.0), 0.5 mL of 0.1 mol/dm^3 sodium formate, 0.5 mL of distilled water, and 0.5 mL of the cell suspension in the 10-mL pear-shaped flask was prepared and sealed using septa in advance and incubated for 5 min at 30°C. Alkane (2.5 mL) was then injected into the flasks with the gas-tight syringe. Dissolution rate was determined by gas chromatography.

RESULTS AND DISCUSSION

Alkane Hydroxylation

Figures 2 and 3 show the relationship between the initial alkane hydroxylation rate and alkane concentration. In every alkane hydroxylation, the initial rate increased with alkane concentration and reached a constant value. Since the relationship obeyed the Michaelis-Menten equation, V_{max} and K_m values for methane, ethane, and propane were obtained as shown in Tables 1 and 2. As shown in these tables, V_{max} values for these alkanes were different, and the order of V_{max} values was methane > ethane > propane for intact cells and ethane > propane > methane for cell-free

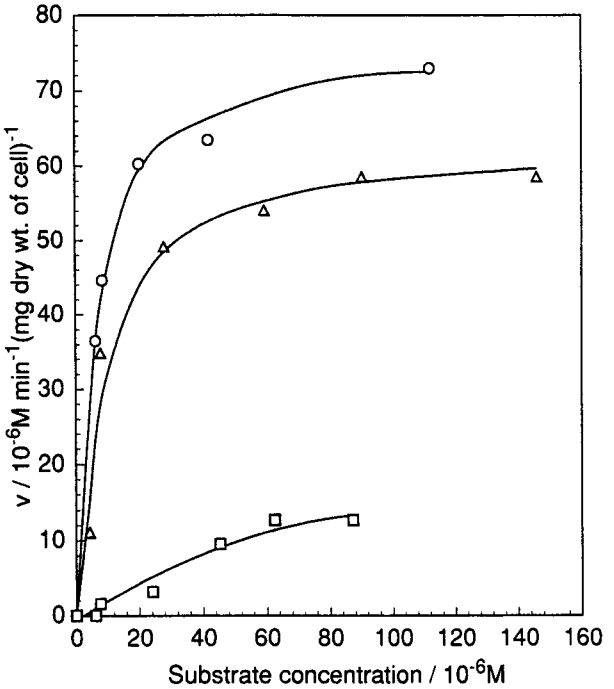

Fig. 2. Initial velocity vs [S] plot for alkane hydroxylation with intact cells of *M. trichosporium* OB3b. O_2 concentration, 200 μmol/dm³; cell dry wt, 0.37 mg. ○, Methane; △, ethane; □, propane.

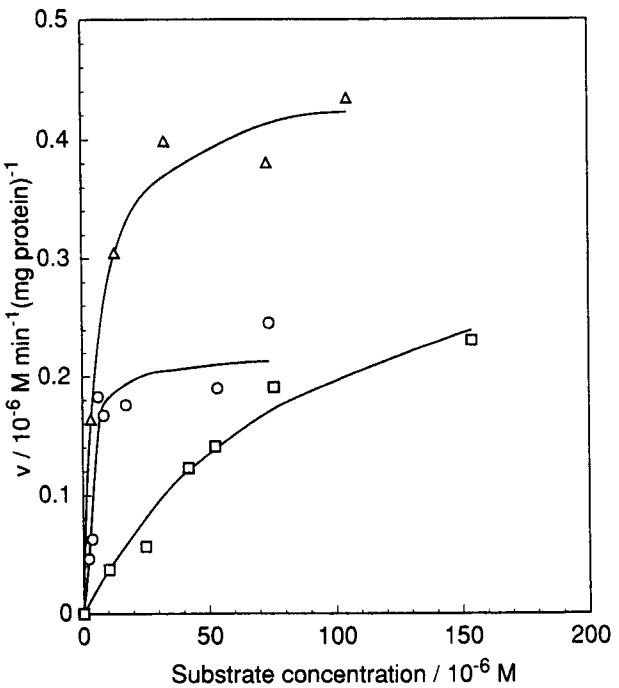

Fig. 3. Initial velocity vs [S] plot for alkane hydroxylation with cell-free extracts of *M. trichosporium* OB3b. O_2 concentration, 200 μmol/dm³; NADH concentration, 5 mmol/dm³. Protein concentration: Methane, 1.91 mg protein/mL; ethane, 0.956 mg protein/mL; propane, 4.78 mg protein/mL. ○, Methane; △, ethane; □, propane.

Table 1
Kinetic Constants for Intact Cells
of *M. trichosporium* OB3b for Alkane Hydroxylation

Substrate	V_{max}[a]	$K_m/\mu M$	k[b]
Methane	76.2	6.32	12.1
Ethane	64.3	9.96	6.46
Propane	44.7	193	0.232

[a] V_{max}: μM/min/(mg dry wt of cell).
[b] $k = V_{max}/K_m$: min^{-1}/(mg dry wt of cell).

Table 2
Kinetic Constants for Cell-Free Extracts
of *M. trichosporium* OB3b for Alkane Hydroxylation

Substrate	V_{max}[a]	$K_m/\mu M$	k[b]
Methane	0.218	2.01	1.08×10^{-1}
Ethane	0.457	7.77	5.88×10^{-2}
Propane	0.387	94.4	4.10×10^{-3}

[a] V_{max}: μM/min/(mg protein).
[b] $k = V_{max}/K_m$: min^{-1}/(mg protein).

extracts. For both intact cells and cell-free extracts, K_m values for these alkanes were remarkably different. The order of K_m value was methane < ethane < propane, and K_m value for propane was remarkably high. These results indicate that the alkane with the shorter carbon chain length has the lower K_m values, and these alkanes have the higher affinity to methane monooxygenase in the cells.

For both intact cells and cell-free extracts, V_{max}/K_m ratio (= k, first-order rate constant) is methane > ethane > propane. The substrate with shorter carbon chain length has lower K_m values and higher k values, showing that the alkane with shorter carbon chain length has higher affinity to methane monooxygenase in the cells. From the above results, we concluded that the reactivity depends on the affinity of substrate and methane monooxygenase in the cells. Similar findings were obtained in alkene epoxidation (*7*). On comparison of K_m values, K_m values for every alkane with cell-free extracts were lower than those with intact cells, indicating that cytoplasm plays an important role in the solubility of alkanes. In alkene epoxidation, K_m values for alkenes, except for ethylene, with cell-free extracts were lower than those with intact cells, suggesting that the cell membrane acts to avoid higher alkenes.

Thus, the cell membrane may act to avoid higher alkane and alkene, but cytoplasm may help to dissolve alkane and alkene.

Table 3
Relative Dissolution Rate of Alkanes
to Intact Cells and Cell-Free Extracts of *M. trichosporium* OB3b

	Relative dissolution rate/%	
Substrate	Intact cells	Cell-free extracts
Methane	2.56	9.27
Ethane	4.30	9.43
Propane	6.34	19.0

Relative Dissolution Rate of Alkane to Intact Cells and Cell-Free Extracts

Relative dissolution rate of alkane to intact cells and cell-free extracts was measured and compared. The results are summarized in Table 3. The relative dissolution rate for cell-free extracts was higher than that for intact cells. These results strongly indicate that cytoplasm helps to increase the concentration of alkane. On comparison of K_m value and relative dissolution rate, we concluded that cytoplasm may help to dissolve alkane, and thus, alkane is easily incorporated into methane monooxygenase.

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