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Enzymatic Study on Acetanilide *p*-Hydroxylase in *Streptomyces fradiae*

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Abstract □ *S. fradiae* exhibited the highest acetanilide *p*-hydroxylation activity among the *Streptomyces* spp. screened. Studies with inhibitors (metyrapone, 2,6-dichloroindophenol, α,α′-dipyridyl, *o*-phenanthroline) and an absorption peak after CO treatment suggested that *S. fradiae* hydroxylase activity was due to cytochrome p-450. This hydroxylase activity was increased to ten times in the cell extract containing 0.5 mM sodium azide. Furthermore, the sedimentary activity in 105,000×g centrifugal forces and solubilization of the activity with Triton-X 100 implied that this enzyme was membrane bound monooxygenase. pH Optimum of the enzyme was 6.5 in membrane bound state.

Keywords □ cytochrome P-450, membrane bound enzyme, sodium azide for enzyme activation, optimum pH for acetailide *p*-hydroxylase

Monooxygenases are capable of carrying out a myriad of chemical reactions. Among these reactions, stereospecific and regiospecific hydroxylation by microorganisms is a well used tool in research and industry for the introduction of oxygen at defined positions of the molecules. And this monooxygenase catalyzed microbial hydroxylations are of great interest as a model system for similar reactions in mammalian cells. In *Streptomyces*, there are a few examples of monooxygenases characterized. The cytochrome p-450 in *S. griseus* is induced by soybean flour and catalyzes the various oxidative reactions including aromatic and aliphatic hydroxylation[1]. Another p-450 monooxygenase from *S. erythreus* catalyzes the conversion of 6-deoxyerythronolide B to the 14-membered macrolactone erythronolide B[2]. Vancurova *et al.* also purified the monooxygenase responsible for conversion of anhydrotetracycline to dehydrotetracycline from *S. aureofaciens*[3]. The catalytic activity of p-450 dependent monooxygenase system is located in the endoplasmic reticulum of higher animal cells as well as in most other eukaryotic cells[4]. However, the bacterial proteins, including the p-450 components, are generally but not always soluble[4,5]. In these regards, we investigated several aspects of acetanilide *p*-hydroxylase in *Streptomyces fradiae*.

We reported previously "Microbial Transformation of Aniline to Acetaminophen[6]", "Genetic Analysis on Bioconversion of aniline to Acetaminophen in *Streptomyces fradiae*[7]", and "Bioconversion of Aniline to Acetaminophen and Overproduction of Acetaminophen by *Streptomyces* spp.[8]"

EXPERIMENTAL METHODS

Microoganisms and cultivation conditions

The microorganism used was *Streptomyces fradiae* NRRL 2702. The culture was inoculated in modified Theriault's screening medium (7% sucrose, 0.5% soybean flour, 0.5% yeast extract, 0.5% NaCl, 0.295% K_2HPO_4, and 0.162% K_3PO_4) and grown on a rotary shaker (180 rpm, 1″ stroke) at 29℃ until mycelia mass developed (usually more than 48 hrs). The culture was transferred (10% v/v) again to fresh modified Theriault's screening medium and cultivated for 80-90 hrs. The resulting mycelia were washed with distilled water three times and stored at −70℃.

Inhibition study

Mycelia stored at −70℃ were thawed and suspended in 4 volumes of 0.05 M potassium phosphate buffer, pH 7.0. Each inhibitor (metyrapone, 2,6-dichloroindophenol, sodium azide, sodium cyanide, $CuSO_4$, $CdCl_2$, *o*-phenanthroline and α,α′-dipyridyl) was prepared at 0.1 M concentration and added to the cell suspension at the final concentration of 5 mM. The mixture was incubated with acetanilide (250 μg/m*l*) on a rotary shaker (180 rpm, 1″ stroke) at 29℃ for 12 hrs. The formation of acetaminophen was estimated by a colorimetric method which is given in "Analysis of enzyme activity".

Preparation of the cell free extracts

The frozen mycelia (5g cell paste) were thawed, suspended in 10 m*l* of buffer A (0.05 M potassium phosphate, pH 6.5, 2 mM dithiothreitol and 1 mM EDTA) cooled below 4℃ in an ice bath and sonicated for eight to nine 15 sec periods at 30 μ. A 40 to 50 sec time interval was allowed between each sonication period to maintain the temperature. All subsequent steps were carried out below 4℃. The sonically disrupted cells were centrifuged for 30 min at 20,000×g to remove the cell debris. Further centrifugation of the supernatant was performed for 90 min at 105,000×g to give supernatant and pellet. For membrane fraction, the pellet was resuspended in half of the volume of same buffer. To investigate the effect of pH on hydroxylase activity, pH of buffer A was changed from 6.0 to 9.0 and incubated in these buffers.

Measurement of reduced CO difference spectrum

Reduced CO difference spectrum was measured according to Omura and Sato[9]. The enzyme preparation was reduced with dithionite and then saturated with carbon monooxide. The difference spectrum between dithionite-reduced CO complex and the reduced form was measured in the range of 400-600 nm.

Analysis of enzyme activity

The enzyme activity was determined by the amounts of acetaminophen formed. The reaction mixture contained 250 μg/m*l* of acetanilide, 0.1 mM of NADPH, NADPH-regenerating system (glucose-6-phosphate, 1.3 mM; nicotinamide, 20 mM; $MgCl_2$, 1.2 mM; glucose-6-phosphate dehydrogenase, 100 unit/*l*), 0.5 mM NaN_3 and 2 m*l* of enzyme preparation. This reaction mixture was incubated on a rotary shaker (180 rpm, 1″ stroke) at 29℃ for 12 hrs. Acetaminophen formed in the incubation mixture was analyzed by TLC, colorimetry and (or) HPLC. For TLC, the mixture incubated with acetanilide was adjusted to pH 1 with d-HCl, extracted with equal volume of ethylacetate three times. The pooled ethylacetate extracts were evaporated to dryness in nitrogen atmosphere. The residues were solubilized with 50 μ*l* methanol. About 10 μ*l* solutions were taken up for thin layer chromatography. Composition of developing solvent is $CHCl_3 : CH_3OH : NH_4OH = 85 : 15 : 1.5$. For HPLC, methanol extracts were centrifuged (15,000×g, 15 min) to remove the particulate impurities. HPLC was carried out with acetonitrile/1% acetic acid in water (15 : 85 v/v), on a μ Bondapak C 18 column (30 cm×4 mm) at a flow rate of 1.1 m*l*/min. Injection volume was 10 μ*l* and 280 nm UV absorbance detector was used. For the colorimetric assay, 1 m*l* of reaction mixture was removed and mixed with 2-nitroso-1-naphthol-4-sulfonic acid (NNS) and trichloroacetic acid solution (final concentration, 10 g/*l* and 200 g/*l*, respectively) vigorously. The mixture was centrifuged (4,500×g, 20 min) to remove the protein precipitate. For full color development, 0.2 m*l* of $NaNO_2$ solution (1 g/*l*) was added to 1.8 m*l* supernatant, allowed to stand for 10 min and then optical density was determined at 530 nm against a blank (reaction mixture in the absence of substrate) in the reference beam.

RESULTS AND DISCUSSION

By use of the appearance of acetaminophen in the incubation broth as an indicator of the enzyme titer *in vivo* and examination of the growth of *S. fradiae* for the production of acetaminophen as a function of time, 80-90-hr-old mycelia from cultures grown in modified Theriault's screening medium were found to have the highest hydroxylation activity[8]. This condition was used for preparation of cell free extracts. *S. fradiae* cells were disrupted by sonication and the cell debris was removed by centrifugation at 20,000×g. Acetanilide *p*-hydrxylation activity was estimated by HPLC and colorimetric method. The reaction time of 12 hrs was chosen because the hydroxylase activity did not increase significantly after 12 hrs.

Several compounds known to inhibit hepatic cy-

Table I. Effect of inhibitors on acetanilide *p*-hydroxylase activity of *S. fradiae*

Inhibitors	Production of acetaminophen (μg/ml)	Rate of inhibition (%)
Sodium cyanide	10.8	0
Sodium azide	0	100
$CuSO_4$	0.75	93
$CdCl_2$	7.55	26
o-phenanthroline	0.50	95
2,6-dichloroindophenol	1.30	87
α,α′-dipyridyl	1.25	88
Metyrapone	3.00	70
Control	10.2	

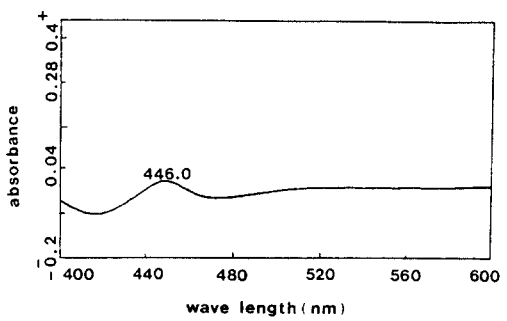

Fig. 1. Reduced CO difference spectrum of *S. fradiae*.

tochrome p-450 or electron transport system were examined for its effect on the *S. fradiae* monooxygenase (Table 1). Metyrapone, a competitive inhibitor of hepatic cytochrome p-450 and 2,6-dichloroindophenol, inhibitor of hepatic cytochrome p-450[10] inhibited the *p*-phydroxylase activity considerably. Iron chelators, such as α,α′-dipyridyl and *o*-phenanthroline also showed strong inhibition (about 90%) but cyanide showed no inhibitory effect. These results suggested that *S. fradiae p*-hydroxylase activity was due to cytochrome p-450. Furthermore, characteristic cytochrome p-450 peak of reduced CO difference spectrum convinced that *S. fradiae* monooxygenase was related to cytochrome p-450 (Fig. 1). In contrast, hydroxylase found in *S. aureofaciens* ATCC 10762 was reported not to be inhibited by hepatic cytochrome p-450 inhibitors but inhibited by iron chelators and electron transport chain blockers[11]. In addition, absorption peak of cytochrome p-450 was not observed and this hydroxylase was categorized as non-heme iron sulfur protein as steroid 9α-hydroxylase from *Norcardia* species M117[12].

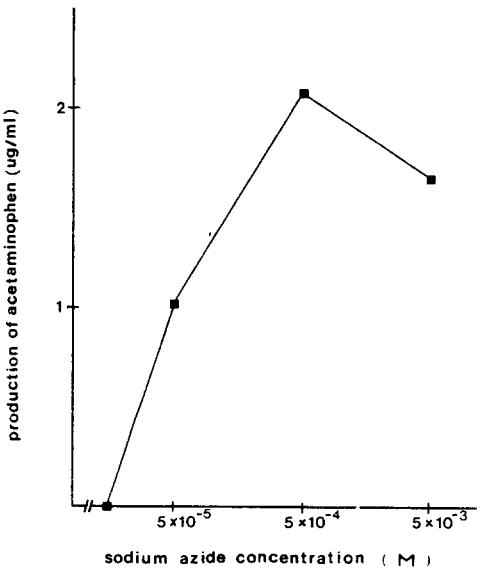

Fig. 2. Effect of sodium azide on acetanilide *p*-hydroxylation activity in cell free extract of *S. fradiae*. Sodium azide was added to cell free extracts to the final concentration of 1.5×10^{-6}, 5×10^{-5}, 5×10^{-4} and 5×10^{-3} M.

Interestingly, *S. fradiae* hydroxylase activity was sharply increased more than 12 times when sodium azide was added to the enzyme preparation at the final concentration of 0.5 mM (Fig. 2). However sodium azide showed inhibitory effect on bioconversion with intact whole cells (100% inhibition with 5 mM, 63% inhibition with 0.5 mM, Fig. 3). This controversal effect of sodium azide remains to be studied, but alcohol oxidase from a methanol yeast, *Candida biodinii* S2, was stabilized in the presence of 0.25 mM and 2.5 mM sodium azide through forming an enzyme-azide complex non-covalently. This phenomenon was explained with decreased inactivation of the enzyme by H_2O_2 and aldehyde in the complex[13].

S. fradiae hydroxylase was suggested to be membrane-bound monooxygenase on the basis of the following observations. The activities sedimented with the membrane fraction (105,000×g pellet) in an ultracentrifuge (Fig. 4). And on solubilization with Triton X-100, it was not attained efficiently but activities were increased gradually in proportion to

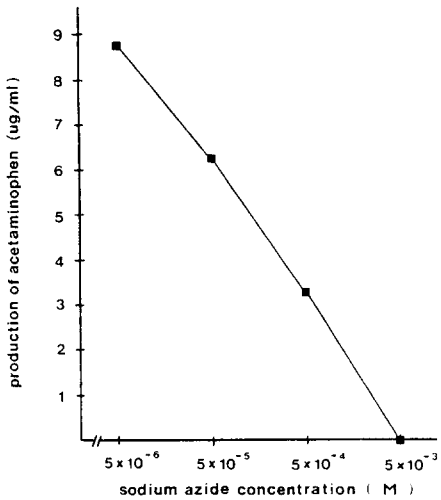

Fig. 3. Effect of sodium azide on acetanilide *p*-hydroxylation activity in intact whole cell of *S. fradiae*.

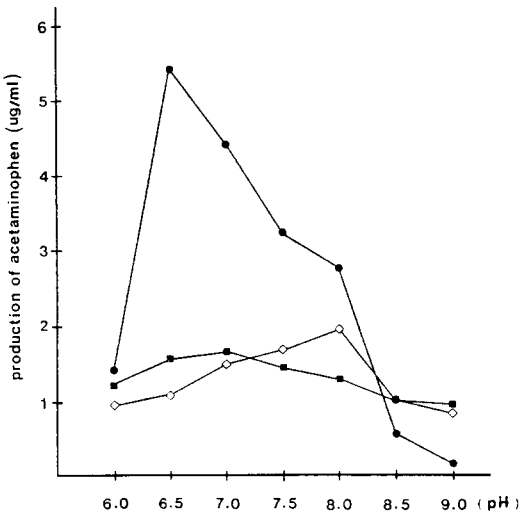

Fig. 4. Influence of pH on acetanilide *p*-hydroxylation activity in cell free extract, cytosolic fraction and membrane fraction of *S. fradiae*.
●, membrane fraction; ■, cytosolic frction; ◇, cell free extracts.

the concentration of Triton X-100 used (data not shown). Residual activites (1/3-1/4 of membrane fraction) could be detected in cytosolic fraction. These activities were thought to be caused by very small fragments generated during sonication and sedimented inefficiently by ultracentrifugation. On the other hand, hydroxylase from *S. aureofaciens* was observed to be solubilized in cytosolic fraction[11]. Bacterial p450-dependent monooxygenases are generally solubilized in cytosol[4,5]. But $p450_{oct}$ system from *Rhodococcus rhodochrous* ATCC 19067, which catalyzes the conversion of n-octane to 1-octanol, appears to resemble the microsomal P-450 monooxygenase[14,15]. P450 component (M_r=52,000), one of the $p450_{oct}$ three proteins, was not soluble protein but solubilized in the presence of Triton X-100 and denatured when the detergent was removed[16].

S. fradiae hydroxylase exhibited a pH optimum near 6.5 when the membrane fraction was resuspended in potassium phosphate buffer (Fig. 4). Small pH deviation from pH 6.5 caused considerable decrease in enzyme activity.

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