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Heterogeneous Natures of the Microbial Steroid 9α-Hydroxylase in Nocardioforms

Hee Kyoung Kang and Sang Sup Lee

College of Pharmacy, Seoul National University, Seoul 151-742, Korea

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Steroid 9α-hydroxylase is an enzyme found in nocardioform microorganisms which can utilize steroids as a sole carbon source. After fractional centrifugation of the cell homogenates, the enzyme activity in *Nocardia* and *Rhodococcus* was found in cytoplasmic membrane fraction. On the contrary, *Mycobacterium* had its 9α-hydroxylation activity in cytosolic fraction. To characterize the enzyme in these microorganisms, several potential inhibitors of 9α-hydroxylase were tested and the cofactor requirement for the same enzyme was also examined. The inhibitory effect of ferrous ion chelators indicated involvement of iron containing proteins in the 9α-hydroxylase system. On the other hand, metyrapone, an inhibitor known to be specific for cytochrome P450 interfered with the enzyme in *Mycobacterium,* but didn't inhibit the enzyme activity in *Nocardia* and *Rhodococcus.* While the 9α-hydroxylase system in *Nocardia* and *Rhodococcus* required NADPH, NADH was required as an election donor in *Mycobacterium.*

Key words : Steroid 9α-hydroxylase, *Nocardioforms*, Iron-containing protein, Cytochrome P450

INTRODUCTION

Stereospecific hydroxylation by microorganisms is a well-used tool in research and industry (Sebek & Perlman, 1979) for the introduction of oxygen at defined non-activated positions of the molecules. Some hydroxylase systems which have been resolved into their components contain cytochrome P450. Among the steroid-hydroxylating systems, the 15β-hydroxylase from *Bacillus megaterium* was shown to contain cytochrome P450. One of the most thoroughly investigated hydroxylase systems is the camphor 5-exo-monooxygenase from *Pseudomonas putida,* which utilizes cytochrome P450 as terminal oxidase (Katagiri *et al.*, 1968). In recent years, many different cytochrome P450-containing hydroxylases have been discovered (Asperger *et al.*, 1984: Berg *et al.*, 1979: De Frank & Ribbons, 1977: Ho & Fulco, 1976: O'keefe *et al.*, 1988: Trower *et al.*, 1989: Ullah *et al.*, 1990). However, there are other hydroxylase systems that contain non-heme iron proteins as terminal oxidases (Bernhardt *et al.*, 1975: Ericson *et al.*, 1988: Peterson *et al.*, 1966). One of them is methane monooxygenase (MMO) found in methanotrophic bacteria. Two distinct forms of the enzyme differing in cellular location are known to exist: a cytoplasmic ("soluble") MMO and a membrane-bound ("particulate") MMO (Prior & Dalton, 1985: Stanley *et al.*, 1983).

Correspondence to: Sang Sup Lee, College of Pharmacy, Seoul National University, Seoul, 151-742, Korea

The steroid 9α-hydroxylase occurs in many bacterial genera used in industrial process (Dodson & Muir, 1961). These include *Nocardia, Mycobacterium, Arthrobacter* and *Corynebacterium.* In previous study, Chang and Sih found that the enzyme in *Nocardia restrictus* is located in the cytoplasmic membrane fraction (Chang & Sih, 1964). However, their attempts to isolate the enzyme from *N. restrictus* did not lead to a soluble enzyme preparation which was suitable for chromatographic purification. Thereafter, Strijewski reported the enzyme from *Nocardia* species M117 is located in the cytosolic fraction, and represents an electron-transport chain consisting of NADH-dependent flavoprotein reductase and two iron-sulfur proteins (Strijewski, 1982).

In the present study, heterogeneous natures of the steroid 9α-hydroxylase in nocardioform microorganisms were examined : the location of the enzyme, influence of pyridine nucleotides as electron donors, and effects of several potential inhibitors of 9α-hydroxylation activity.

MATERIALS AND METHODS

Strains

Mycobacterium fortuitum KCTC 1122 (*Mycobacterium fortuitum* ATCC 6842), *Rhdococcus rhodochorus* KCTC 1061 (*Nocardia erythropolis* ATCC 17895), *Rhdococcus erythropolis* KCTC 1062 (*Nocardia erythrop-*

olis ATCC 25544), and *Nocardia restrictus* ATCC 14887 were used throughout the experiments. These microorganisms are able to utilize steroids as carbon and energy sources. *N. restrictus* was obtained from American Type Culture Collection and the others were purchased from Korean Collection for Type Cultures.

Cultivation and media

N. restrictus was cultivated in a medium contained per l 8 g nutrient broth (Difco), 10 g yeast extract (Difco), and 10 g glucose. *M. fortuitum* was grown in a medium contained per l 8 g nutrient broth, 5 g glycerol, 1 g yeast extract, and 1 g tween 80. And in the cultivation of *R. rhodochorus* and *R. erythropolis*, a medium containing nutrient broth 8 g was used. These microorganisms were grown in 2 l Erlenmeyer flask with a working volume of 500 ml at 28°C on a rotary shaker. Hydroxylase activity was induced by the addition of 0.2 g/l progesterone (Sigma) dissolved in N,N'-dimethylformamide during the late logarithmic growth phase. 10 h after steroid addition, cells were harvested at 10,000×g (4°C, 7 min) using Beckman Model J2-21M/E centrifuge. Each pellet of the microorganisms was washed with 25 mM 3-[morpholino]-propane-sulfonic acid (MOPS) buffer and the cell pastes were stored at -70°C.

Preparation of cell-free extract

Frozen cell pastes were thawed and suspended in the same volume of buffer A (25 mM MOPS, pH 8.0, containing 10% glycerol, 2 mM 1,4-dithiothreitol (DTT: Sigma), and 100 μM phenylmethyl sulfonyl fluoride (PMSF: Sigma)). Cells were disrupted by grinding with acid-washed glass bead (150~212 microns: Sigma) of a fourth of cell weight for 5 min with a mortar and pestle. The mixtures were diluted with the same volume of buffer A, and sonicated with a Branson 450 Sonifier at 30 μ output for 6 min (50% duty cycle). All subsequent steps were carried out at 4°C. The cell debris was removed by centrifugation at 20,000×g for 60 min, yielding cell-free extract. The supernatant was again centrifuged at 105,000×g for 90 min using Beckman Model L-80 ultracentrifuge. The resulting clear supernatant, cytosolic fraction was collected and the pellet was resuspended in buffer A, yielding cytoplasmic membrane fraction.

Protein determination

The concentration of protein (soluble or insoluble) was determined by the method of Bradford with bovine serum albumin as a standard (Bradford, 1976: Bradford, 1987). In case of insoluble proteins, hexyl-β-D-glucopyranoside was used to solubilize membrane-bound proteins.

9α-Hydroxylase assays

The steroid 9α-hydroxylase activity was measured by the transformation of 9(11)-dehydro-17α-methyl-testosterone (DHMT: Sigma) to the corresponding 9(11)-epoxides, 9α,11α-oxido-17β-hydroxy-17α-methyl-4-androstene-3-one (9α,11α-oxido-MT) and 9α,11α-oxido-17β-hydroxy-17α-methyl-1,4-androstadiene-3-one (9α,11α-oxido-Δ^1-MT). The enzyme assay was carried out in 5 ml assay mixture: cytosol fraction and/or cytoplasmic membrane fraction, containing variable amount of protein. The reaction was started by the addition of 50 μg DHMT dissolved in 10 μl ethanol and the mixture was incubated for 20 min at 30°C. The assay was stopped by the addition of 10 ml ethyl acetate and through mixing. After extraction two times, the organic phase was evaporated to dryness. The analysis was carried out with TLC or HPLC. Firstly, in the analysis with TLC, the residue was dissolved in 100 μl of ethyl acetate and 3 μl sample was chromatographed on precoated thin-layer silica gel plates with 254 nm fluorescent indicator (Sigma) using the following solvent system: chloroform/methanol (96/4, v/v). The developed plates were photographed under short wave ultraviolet illumination. The HPLC analysis was performed as follows. The residue was dissolved in 1.5 ml of methanol-chloroform-water (1:1:1). The steroids-containing chloroform phase was separated and 25 μl of chloroform extract was diluted with 475 μl of acetonitrile. The steroids in 100 μl sample were analyzed by HPLC (pump: LC-9A, UV detector: SPD-6AV, system controller: SCL-6B, integrator: C-R6A, Shimadzu) with a solvent system of 50% acetonitrile-water on an ODS column (2.5×150 mm, particle size 4 μm: Cosmosil) at a flow rate of 0.5 ml/min. The chromatogram was monitored at 238 nm. Under these conditions, the retention times of 9α,11α-oxido-MT and 9α,11α-oxido-Δ^1-MT are 8.3 and 6.7 min, respectively.

RESULTS

We initially determined whether the 9α-hydroxylases in nocardioform microorganisms are located in cytoplasmic membrane fraction or cytosolic fraction. As illustrated in Figs, 1-4, *Mycobacterium* contained the 9α-hydroxylation activity in cytosolic fraction, whereas in *Nocardia* and *Rhodococcus*, the 9α-hydroxylase was found in the cytoplasmic membrane fraction.

The steroid 9α-hydroxylase system requires reduced pyridine nucleotides as electron donors. Requirement of pyridine nucleotide for the 9α-hydroxylase activity was investigated by the HPLC analysis. As shown in Table I, NADH was prerequiste for the 9α-hydroxylation in *Mycobacterium.* NADPH substituted for NADH to a lesser extent. In contrast, NADPH was found to be most effective for the 9α-hydroxylase

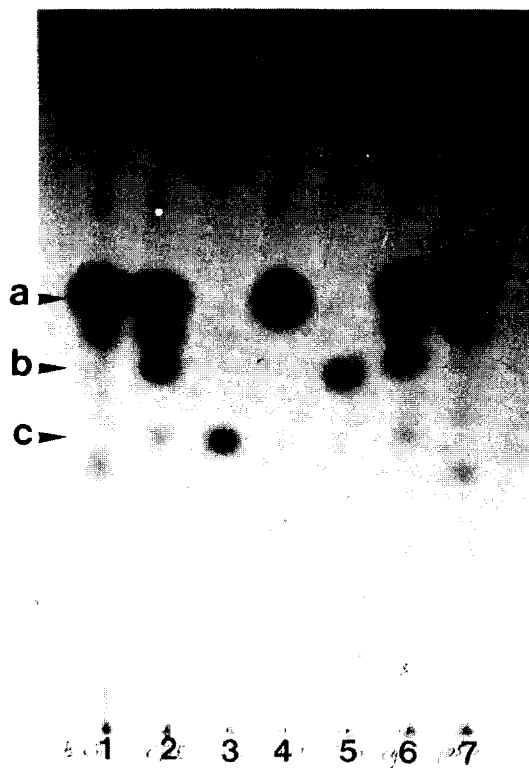

Fig. 1. The TLC profile of conversion of 9(11)-dehydro-17α-methyl-testosterone by different cellular fractions of *M. fortuitum.* Lane 1, 2, 6, and 7 exhibited biotransformation patterns of DHMT by denatured cell free extract, cell free extract, cytosol fraction, and cytoplasmic membrane fraction, respectively. Lane 3, 4, and 5 designated [c]9α,11α-oxido-Δ^1-MT, [a]DHMT, and [b]9α,11α-oxido-MT as standards.

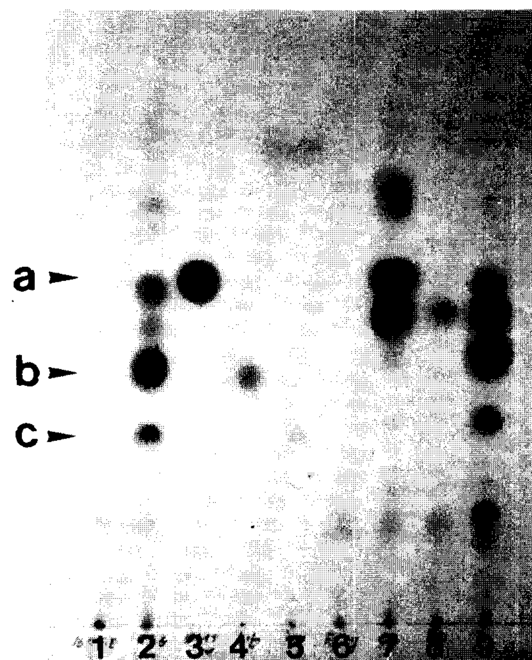

Fig. 2. The TLC profile of conversion of 9(11)-dehydro-17α-methyl-testosterone by different cellular fractions of *N. restrictus.* Lane 1, 6, and 8 exhibited patterns of cell free extract, cytosol fraction, and cytoplasmic membrane fraction as blanks. Lane 2, 7, and 9 showed biotransformation patterns of DHMT by cell free extract, cytosol fraction, and cytoplasmic membrane fraction, respectively. Lane 3, 4; and 5 designated [a]DHMT, [b]9α,11α-oxido-MT, and [c]9α,11α-oxido-Δ^1-MT as standards.

from *Nocardia and Rhodococcus.*

Table II shows the influences of different inhibitors on 9α-hydroxylase from nocardioform bacteria. The inhibition by iron chelators, 2,2'-dipyridyl, *o*-phenanthroline, and 8-hydroxyquinoline, indicates the participation of iron-containing proteins in the 9α-hydroxylase system. In addition, sodium cyanide, respiratory inhibitor, inhibited 9α-hydroxylase activity. D-Penicillamine which formed complex with copper ion exhibited no inhibitory effect on the 9α-hydroxylase system. Mercuric acetate, an inhibitor of sulfhydryl group, did not interfere with 9α-hydroxylase. On the other hand, metyrapone, an inhibitor known to be specific for cytochrome P450, did not interfere with the 9α-hydroxylase system in *Nocardia* and *Rhodococcus.* But, in *Mycobacterium,* metyrapone inhibited the enzyme.

DISCUSSION

The chemical and enzymatic characters of the steroid 9α-hydroxylase have remained unclear for many years. Conflicting reports about 9α-hydroxylase have created confusion concerning the nature of the enzyme (Chang & Sih, 1964: Strijewski, 1982). Since Strijewski's report (1982), prior to purification and characterization of the enzyme, natures of steroid 9α-hydroxylase in nocardioforms which are capable of steroid-ring-B-splitting were compared.

Nocardia and *Rhodococcus* showed 9α-hydroxylation activity in the cytoplasmic membrane fraction. In contrast to these results, in *Mycobacterium,* the cytosolic fraction exhibited the enzyme activity. These results are helpful to understand the difference in the location of its activity in *Nocardia restrictus* (Chang & Sih, 1964) and *Nocardia* sp. M117 (Strijewski, 1982) despite the same genera. Furthermore, in the case of methane monooxygenase (MMO) in the methanotrophic bacteria, the form expressed (soluble or particulate) depends on the availability of copper during growth (Prior & Dalton, 1985).

Most of monooxygenases require the reduced pyridine nucleotides as electron donors. So far as an electron donor of the steroid 9α-hydroxylase is concerned, the enzyme in *Nocardia* and *Rhodococcus* required NADPH. However, the 9α-hydroxylase in *Mycobacterium* was dependent on NADH. Cytochrome $P450_{cam}$ in *Pseudomonas putida* (Katagiri *et al.,* 1968), cytochrome $P450_{soy}$ in *Streptomyces griseus* (Ramanchandra & Saviaslani, 1990), alkene monooxygenase in *Myco-*

bacterium (Frans *et al.*, 1992), and soluble methane monooxygenase in *Methylosinus trichosporium* OB 3b (Fox *et al.*, 1991) require NADH as the external electron donor. On the other hand, steroid monooxygenase from *Cyclindrocarpon radicicola* (Itagaki, 1986), cytochrome $P450_{meg}$ (Berg *et al.*, 1976), and cytochrome $P450_{BM-3}$ (Ho & Fulco, 1976) are dependent on NADPH for their monooxygenase activity.

Using cytosolic fraction or cytoplasmic membrane fraction of nocardioforms, several potential inhibitors of the steroid 9α-hydroxylase activity were tested. 2, 2'-Dipyridyl, 8-hydroxyquinoline, and *o*-phenanthroline all significantly inhibited the steroid 9α-hydroxylase activity. These results suggest that involvement of iron-containing proteins in the enzyme are common in nocardioform bacteria. The strong inhibitory effect observed with iron-chelators has also been reported for the enzyme in *Nocardia* sp. (Strijewski, 1982). In case of alkene monooxygenase in *Mycobacterium*, 2, 2'-dipyridyl did not interfere alkene monooxygenase activity in spite of inhibitory effect with 8-hydroxyquinoline and *o*-phenanthroline (Hartmans *et al.*, 1991). These differences in the inhibitory effects of monooxygenase activity between iron-chelators are supposed to be due to their differential ability of shielding from attacks. On the other hand, metyrapone did not in-

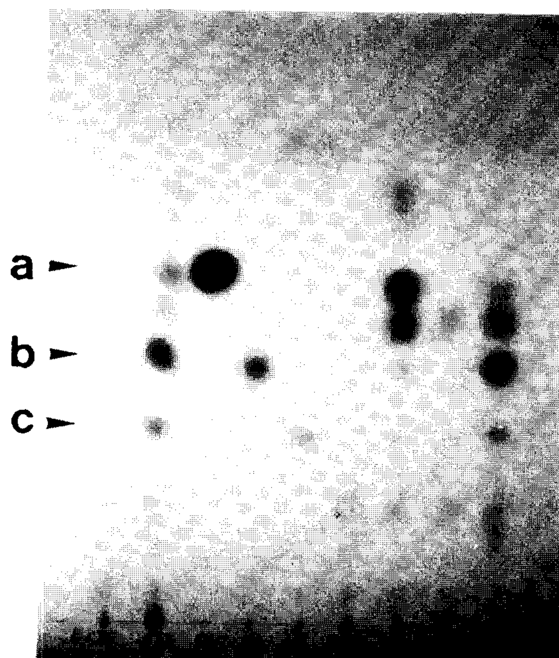

Fig. 3. The TLC profile of conversion of 9(11)-dehydro-17α-methyl-testosterone by different cellular fractions of *R. rhodochorus*. Lane 1, 6, and 8 exhibited patterns of cell free extract, cytosol fraction, and cytoplasmic membrane fraction as blanks. Lane 2, 7, and 9 showed biotransformation patterns of DHMT by cell free extract, cytosol fraction, and cytoplasmic membrane fraction, respectively. Lane 3, 4, and 5 designated [a]DHMT, [b]9α,11α-oxido-MT, and [c]9α,11α-oxido-Δ^1-MT as standards.

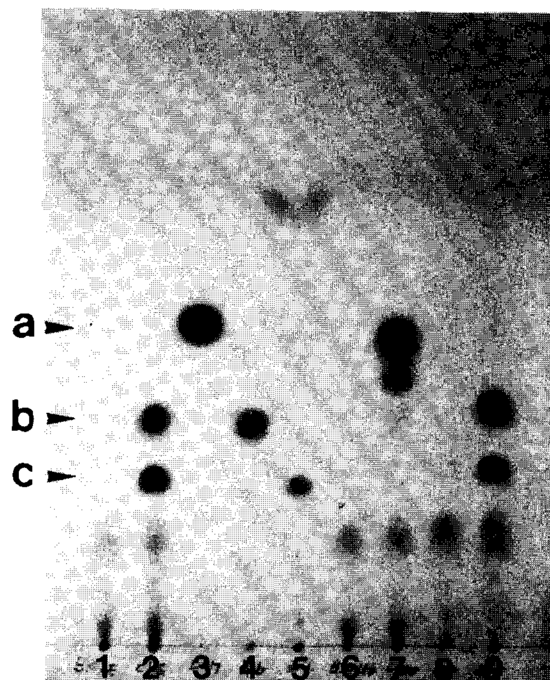

Fig. 4. The TLC profile of conversion of 9(11)-dehydro-17α-methyl-testosterone by different cellular fractions of *R. erythropolis*. Lane 1, 6, and 8 exhibited patterns of cell free extract, cytosol fraction, and cytoplasmic membrane fraction as blanks. Lane 2, 7, and 9 showed biotransformation patterns of DHMT by cell free extract, cytosol fraction, and cytoplasmic membrane fraction, respectively. Lane 3, 4, and 5 designated [a]DHMT, [b]9α,11α-oxido-MT, and [c]9α,11α-oxido-Δ^1-MT as standards.

Table I. Effects of pyridine nucleotides on the steroid 9α-hydroxylase activity

Cofactor	Hydroxylase activity (%)[a]			
	M. fortuitum	*N. restrictus*	*R. rhodochorus*	*R. erythropolis*
Not added	100	100	100	100
NADH 500 uM	182	121	105	114
NADPH 500 uM	129	145	154	202
NADH 500 uM+NADPH 500 uM	187	127	130	200

[a]Reaction was carried out in the dialyzed cytosolic fraction of *Mycobacterium*, and dialyzed cytoplasmic membrane fraction of *Nocardia* and *Rhodococcus*. The steroid 9α-hydroxylase activity in the presence of added pyridine nucleotide was compared with that in the absence of cofactor by calculation of produced 9α,11α-oxido compounds of DHMT (nmol/mg of protein). Relative to no addition of cofactor as 100%.

Table II. Effects of inhibitory compounds on the steroid 9α-hydroxylase activity

Compounds (mM)		Inhibition (%)[a]			
		M. fortuitum	*N. restrictus*	*R. rhodochorus*	*R. erythropolis*
2,2'-Dipyridyl	0.1	57	12	0	90
	1	80	68	86	100
8-Hydroxy-quinoline	0.1	25	57	0	5
	1	98	100	100	65
Mercuric acetate	0.1	0	0	0	0
	1	0	0	0	0
Metyrapone	0.1	10	0	0	15
	1	35	0	0	13
D-Penicillamine	0.1	0	0	0	0
	1	0	0	0	0
o-Phenanthroline	0.1	85	94	100	100
	1	95	100	100	100
Sodium cyanide	0.1	13	0	0	87
	1	95	100	100	100

[a]Reaction was carried out in the cytosol fraction of *Mycobacterium,* and cytoplasmic membrane fraction of *Nocardia* and *Rhodococcus.* The steroid 9α-hydroxylase activity in the presence of added inhibitory compound was compared with that in the absence of inhibitor by calculation of produced 9α,11α-oxido compounds of DHMT (nmol/mg of protein). Inhibition (%)=(1-(produced 9α,11α-oxido compds. of DHMT in the presence of inhibitor/that in the absence of inhibitor))×100.

terfere with the steroid 9α-hydroxylase activity in *Nocardia* and *Rhodococcus.* But, it resulted in significant inhibition in *Mycobacterium.* These results indicate that a P450 type of cytochrome is involved in *Mycobacterium,* but not in *Nocardia* and *Rhodococcus.*

These heterogeneous natures of the steroid 9α-hydroxylase in nocardioform bacteria, such as *Mycobacterium, Nocardia,* and *Rhodococcus* are likely to be useful in taxanomical studies of microorganisms.

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