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# Purification of the NADH Reductase Component of the Steroid 9α-Hydroxylase from *Mycobacterium fortuitum*

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The NADH reductase component of the steroid 9α-hydroxylase from *Mycobacterium fortuitum* was purified to homogeneity. Recovery of the enzyme from the 50~60% ammonium sulfate saturated fraction was 49%, with a purification factor of 100-fold. The NADH reductase has a relative molecular mass of 60 KDa as determined by SDS-PAGE. The absorption maxima at 410 and 450 nm indicate the presence of iron-sulfur group and flavin. These prosthetic groups seemed to function as redox groups that transfer electrons from NADH to the following protein. The $K_M$ value for NADH as substrate was 68 μM. The $NH_2$-terminal amino acid sequence of the reductase was determined as Met-Asp-Ala-Ile-Thr-Asn-Val-Pro-Leu-Pro-Ala-Asn-Glu-Pro-Val-His-Asp-Tyr-Ala-Thr. This sequence does not show a homology with the $NH_2$-terminal sequences reported for the reductase component of other monooxygenases, suggesting that the NADH reductase component of the steroid 9α-hydroxylase system is novel.

**Key words :** Steroid 9α-hydroxylase, NADH reductase component, Iron-sulfur group and flavin

## INTRODUCTION

Steroid 9α-hydroxylase is an enzyme found in nocardioform bacteria which can utilize steroids as a sole carbon source. Microorganisms containing the steroid 9α-hydroxylase may be of interest in the production of 9α-hydroxy-androstenedione, an important intermediate for the semi-synthesis of potent anti-inflammatory drugs, such as 9α-fluorocorticoids.

When the steroid 9α-hydroxylase activity in cytosol fraction of *Mycobacterium fortuitum* is plotted against the protein concentration in the assay, a sigmoidal relationship is observed, indicating that 9α-hydroxylase is a multicomponent enzyme (Kang, in press). The soluble methane monooxygenase (MMO) from the type I methanotroph *Methylococcus capsulatus* (Bath) (Colby & Dalton, 1978) has been resolved into three components: an oxygenase, an NADH reductase, and a regulatory protein. Very similar enzymes have been reported for the type II methanotrophs *Methylosinus trichosporium* OB3b (Fox *et al.*, 1989) and *Methylosinus sporium* 5 (Pilkington & Dalton, 1991). The facultative methanotroph *Methylobacterium* strain CRL26 possesses a two-component enzyme which has been purified and does not require the regulatory component (Patel, 1987). Most bacterial P450 monooxygenases have been reported for the camphor 5-exo-monooxygenase from *Pseudomonas putida* (Katagiri *et al.*, 1968) and 15β-steroid-hydroxylase from *Bacillus megaterium* ATCC 13368 (Berg *et al.*, 1979), and resolved into three components: a cytochrome P450, a redoxin, and an NAD(P)H reductase. It has been known that microsomal P450 monooxygenases consist of two components, a cytochrome P450 and a NAD(P)H reductase (Fulco, 1991). And, $P450_{BM-3}$ monooxygenase from *Bacillus megaterium* is one-protein system, a reductase-cytochrome P450 protein (Narhi & Fulco, 1987). Regardless of the number of components, most of monooxygenases may need NAD(P)H reductase activity.

The steroid 9α-hydroxylase from *Nocardia* sp. has been reported to represent an electron-transport chain consisting of an NADH-dependent flavoprotein reductase and two iron-sulfur proteins (Strijewski, 1982). But, none of the steroid 9α-hydroxylase has been purified. This report deals with the purification and characterization of the NADH reductase of the steroid 9α-hydroxylase from *Mycobacterium fortuitum.*

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## MATERIALS AND METHODS

### Strain and cultivation

*Mycobacterium fortuitum* KCTC 1122 (*Mycobacterium fortuitum* ATCC 6842) was obtained from Korean Collection for Type Cultures. This microorganism was grown in a medium contained per l 8 g nutrient broth

(Difco), 5 g glycerol, 1 g yeast extract (Difco), and 1 g tween 80, at 28°C on a rotary shaker. Steroid 9α-hydroxylase activity was induced by the addition of 0.2 g/l progesterone (Sigma) dissolved in *N,N'*-dimethylformamide (DMF) 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 J 2-21M/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

The frozen cells 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 mixture was 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 (Beckman Model L-80 ultracentrifuge) to give a clear cytosolic fraction.

### NADH reductase assay

Activity of NADH reductase component of steroid 9α-hydroxylase was assayed by NADH-2,6-dichlorophenolindophenol (DCPIP:Sigma) reductase activity by measuring the rate of decrease in absorbance at 600 nm resulting from the reduction of DCPIP, using an extinction coefficient of 13 $mM^{-1}cm^{-1}$. The reaction was carried out at 30°C in 1 ml of 25 mM MOPS buffer, pH 8.0, containing 1 μmol DCPIP, 1 μmol NADH, and 5~50 μl of reductase fraction. One unit of DCPIP reductase activity is defined as the amount required for reduction of 1 μmol of DCPIP/min at 30°C (Hultquist, 1978).

### Purification of NADH reductase

All procedures were performed at 4°C unless stated otherwise.

**Step 1: Ammonium sulfate precipitation:** The cytosolic fraction was brought to 50% ammonium sulfate saturation by addition of solid ammonium sulfate (Sigma). After stirring at 2°C precipitated proteins were collected by centrifugation (15,000×g, 30 min). The supernatant was brought to 60% saturation with solid ammonium sulfate. After an additional 10 min, the precipitated proteins were collected by centrifugation and redissolved in 50 ml of buffer B (25 mM MOPS, pH 8.0, containing 10% glycerol and 100 M PMSF). This was ulfrafiltrated against 1 l of buffer B with using YM 10 membrane (Amicon) to remove most of ammonium sulfate.

**Step 2: DEAE-Cellulose chromatography:** The redissolved and desalted protein solution was loaded on a DEAE-Cellulose (Sigma) column (35 by 2.5 cm) equilibrated with buffer B. After application, a 0 to 0.6 M KCl gradient in 1.5 l of the same buffer was started at a flow rate of 2 ml/min. Fractions of 13.5 ml were collected, and those containing NADH reductase activity were pooled.

**Step 3: Superose 6 chromatography:** The pooled NADH reductase fraction from the DEAE-Cellulose chromatography was concentrated with using YM 10 membrane and applied to a Superose 6 column (HR 10/30:Pharmacia) equilibrated with buffer B. This column was linked to the FPLC system and eluted with buffer B at a 0.25 ml/min flow rate with collection of 0.5 ml fractions. The NADH-DCPIP reductase fractions were combined and concentrated.

**Step 4: 5'-AMP Sepharose 4B column chromatography:** Final purification step of NADH reductase was achieved by 5'-AMP Sepharose 4B chromatography. Concentrated protein solution from Superose 6 was applied to a 5'-AMP Sepharose 4B (Sigma) column (1.0 ×9.0 cm) equilibrated with buffer B. This column was eluted with 8 ml of buffer B containing 0.1 M NaCl and washed with buffer B, then the enzyme was eluted with 15 ml of buffer B containing 1 mM NADH with collection of 2 ml fractions.

### Protein determination

Protein was determined by using the micro-assay of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

### SDS-PAGE

SDS-polyacylamide gel electrophoresis (PAGE) was used to determine the subunit molecular weight and purity of the NADH reductase. A 12% (wt/vol) separation slab gel was used with the discontinuous buffer system of Laemmli (Laemmli, 1970). Proteins were stained with silver nitrate solution using a kit from Sigma. Myosin, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin and lysozyme were used as standards.

The molecular weight of the native protein was estimated by means of gel filtration on a Superose 6 fast protein liquid chromtography column and electrophoresis on 10% SDS-polyacrylamide gel.

### 9α-Hydroxylase assay

To identify that purified NADH-reductase is a com-

**Table I.** Summary of the purification of NADH reductase component of the steroid 9α-hydroxylase from *M. fortuitum*

| Step | Total protein (mg) | Total activity (mU) | Specific activity (mU/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| 50~60% $(NH_4)_2SO_4$ saturated fraction | 67.5 | 41,900 | 620 | 100 | 1.0 |
| DEAE-Cellulose chromatography | 44.1 | 38,900 | 880 | 92.8 | 1.4 |
| Superose 6 chromatography | 2.81 | 33,500 | 1190 | 80.0 | 19.2 |
| 5'-AMP Sepharose 4B chromatography | 0.33 | 20,400 | 6180 | 48.6 | 99.7 |

ponent of the steroid 9α-hydroxylase, the reaction mixture contained 0.2 umol NADH, 1.34 mg of protein solution containing steroid 9α-hydroxylase (fraction from testosterone affinity gel chromatography of 50~60% ammonium sulfate saturated fraction), and 0.09 unit (1.5 μg) of NADH-reductase in a final volume of 1 ml of buffer A (Kang, in press) . The reaction was started by the addition of 50 μg 9(11)-dehydro-17α-methyl-testosterone (DHMT: Sigma) dissolved in 10 μl ethanol and the mixture was incubated for 20 min at 30°C. The assay was stopped by the addition of 2 ml ethyl acetate and through mixing. The amount of 9(11)-epoxides formed was determined by HPLC analysis as described (Kang & Lee, in press).

### $NH_2$-terminal amino acid sequence analysis

NADH reductase on SDS-polyacrylamide gel was electrotransferred to the polyvinylidene fluoride (PVDF) membrane (Millipore) with Towbin buffer (192 mM glycine, 25 mM Tris, pH 8.3, 1.3 mM SDS, 20% methanol) by using the semi-dry blotter. Then, electrotransferred protein was stained with 0.2% ponceau S (in 1% acetic acid) for 1 min and washed with Milli Q water. The $NH_2$-terminal amino acid sequence of the band containing NADH reductase was determined with Milligen 6600B protein sequencer.

## RESULTS

The purification steps for NADH reductase of the steroid 9α-hydroxylase from *M. fortuitum* are summarized Table I. Briefly, DEAE-Cellulose (Fig. 1), Superose 6 (Fig. 2) and 5'-AMP Sepharose 4B (Fig. 3) chromatographies were used. The final yield was as much as about 49%, starting from the 50~60% ammonium sulfate saturated fraction of cytosol from *M. fortuirum*. The purified sample gave a single protein-staining band on analysis by SDS-PAGE (Fig. 4) and its molecular weight was estimated to be about 60 KDa (Fig. 5). When assayed as described in materials and methods, the addition of purified NADH-reductase enhanc-

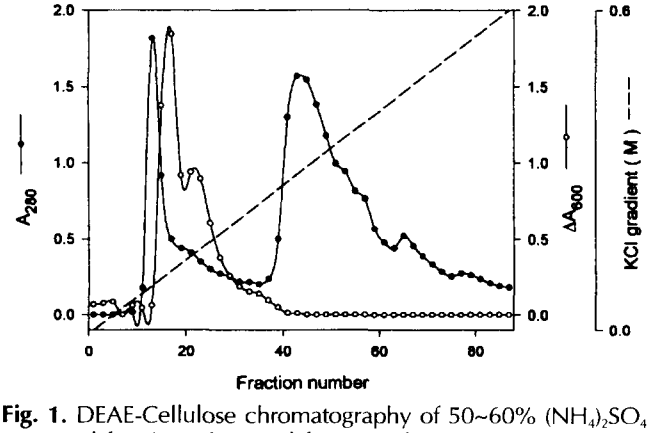


**Fig. 1.** DEAE-Cellulose chromatography of 50~60% $(NH_4)_2SO_4$ saturated fraction of cytosol from *M. fortuitum*.

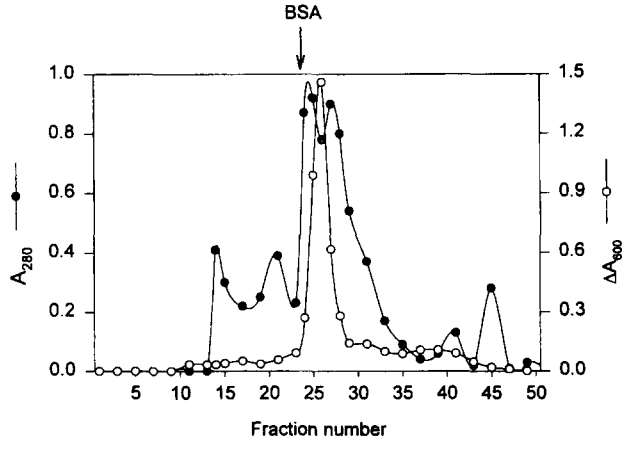


**Fig. 2.** Superose 6 chromatography of NADH reductase containing fractions after DEAE-Cellulose chromatography. * BSA: Bovine serum albumin (68,000).

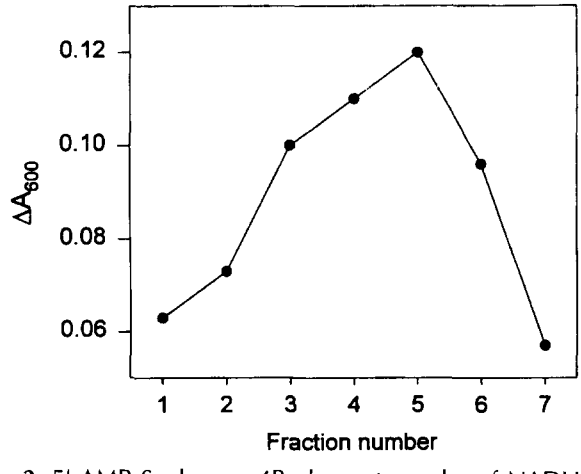


**Fig. 3.** 5'-AMP Sepharose 4B chromatography of NADH reductase containing fractions from Superose 6 chromatography.

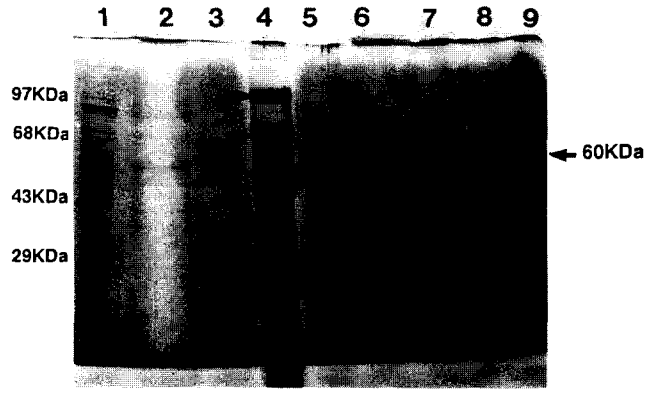


**Fig. 4.** SDS-polyacrylamide gel electrophoresis of NADH reductase containing fractions after 5'-AMP Sepharose 4B chromatography. Lane 4 showed SDS-PAGE patterns of molecular weight standards (myosin, 200,000; phosphorylase B, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 29,000; β-lactoglobulin, 18,400; lysozyme, 14,300). Lane 1 exhibited electrophoresis pattern of NADH reductase containing fraction after Superose 6 chromatography. Lane 2, 3, 5, 6, 7, 8, and 9 exhibited electrophoresis of fraction 1, 2, 3, 4, 5, 6, and 7 eluent from 5'-AMP Sepharose 4B chromatography with 1 mM NADH, respectively.

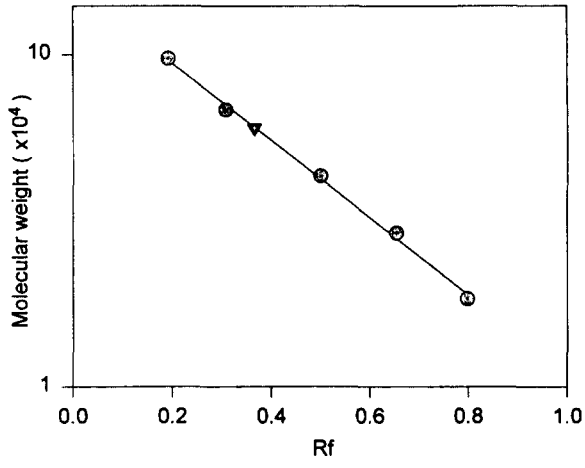


**Fig. 5.** Estimation of molecular weight of NADH reductase on 10% SDS-polyacrylamide gel. ⊙designated molecular weight markers (phosphorylase B, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 29,000; β-lactoglobulin, 18,400). ▼expected to be NADH reductase. Rf value was derived from distance of protein migration/distance of tracking dye migration.

ed the hydroxylation of DHMT to corresponding 9(11)-epoxides (Table II).

The absorption spectrum of NADH reductase showed absorption maxima at 408 nm and 455 nm (Fig. 6). Fig. 7 shows optical absorption spectrum of reduced NADH reductase with absorption peak at 408 nm. The absorption maximum at 455 nm indicates the presence of a flavin as a prosthetic group. This was confirmed by the disappearance of absorption peak

**Table II.** Enhancement of the steroid 9α-hydroxylation of DHMT to corresponding 9(11)-epoxides[a] by the addition of purified NADH-reductase

| Reaction mixture | Produced 9α,11α-oxido compds. of DHMT (nmol) | Hydroxylase activity (%) |
|---|---|---|
| control[b] | 3.25 | 100 |
| +Purified NADH-reductase | 4.85 | 150 |

[a]DHMT: 9(11)-dehydro-17α-methyl-testosterone. Corresponding 9(11)-epoxides (9α,11α-compds. of DHMT): 9α,11α-oxido-17β-hydroxy-17α-methyl-4-androstene-3-one and 9α,11α-oxido-17β-hydroxy-17α-methyl-1,4-androstadiene-3-one
[b]Reaction mixture of control contained NADH, DHMT, and fraction exhibited steroid 9α-hydroxylase activity from testosterone affinity chromatography.

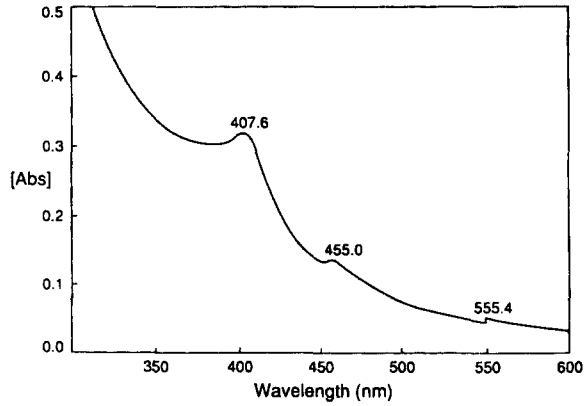


**Fig. 6.** Absorption spectrum of NADH reductase containing fraction after DEAE-Cellulose chromatography.

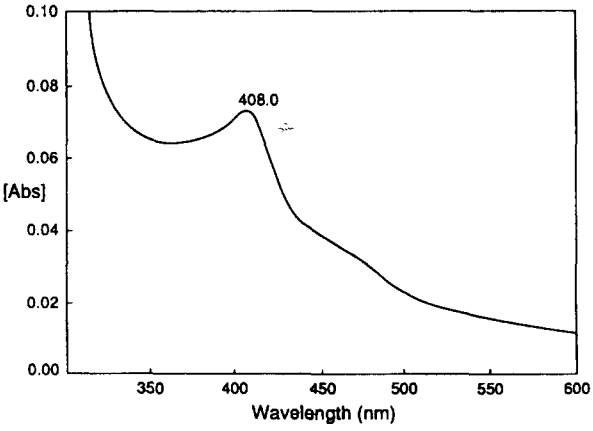


**Fig. 7.** Absorption spectrum of NADH reductase containing fraction after 5'-AMP Sepharose 4B chromatography.

at 455 nm in the reduction of NADH reductase with the addition of NADH. The absorption maximum at 408 nm indicates the presence of a second prosthetic group, an [Fe-S] cluster.

The $NH_2$-terminal amino acid sequence has been determined as Met-Asp-Ala-Ile-Thr-Asn-Val-Pro-Leu-Pro-Ala-Asn-Glu-Pro-Val-His-Asp-Tyr-Ala-Thr. This sequence does not show homology with the $NH_2$-terminal se-

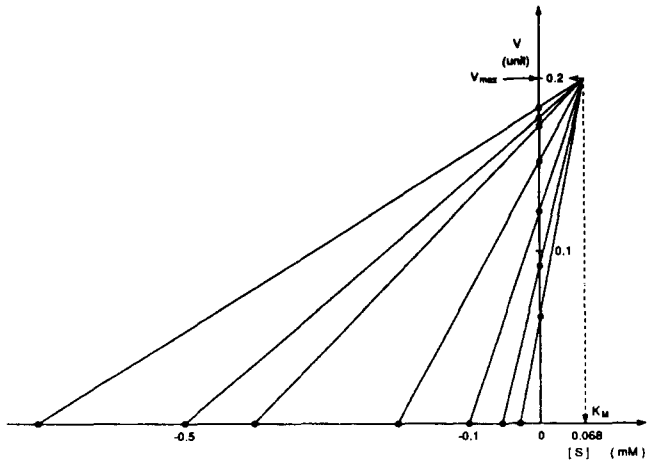


**Fig. 8.** Direct linear plot of NADH reductase for NADH. A unit is defined as the reduction of 1 umol/min of DCPIP. Variable concentration of NADH (25-700 uM), 250 uM DCPIP, an appropriate amount of enzyme and 25 mM MOPS buffer, pH 8.0 in a total volume of 1 ml were used.

quences reported for the reductase component of the alkene monooxygenase from *Mycobacterium* strain E3 (Frans *et al.*, 1992), the soluble MMO from *Methylosinus trichosporium* OB3b (Fox *et al.*, 1991), naphthalene dioxygenase from *Pseudomonas* strain NCIB 9816 (Haigler & Gibson, 1990) or xylene monooxygenase from *Pseudomonas putida* mt-2 (Shaw & Harayama, 1992). The direct linear plot, shown in Fig. 8, gave the kinetic constant as apparent $K_M$ value 68 uM for NADH. The NADH reductase of xylene monooxygenase was reported to have a $K_M$ value for NADH of 22 uM (Shaw & Harayama, 1992).

## DISCUSSION

It was reported that the steroid 9α-hydroxylase from *Nocardia* sp. represents an electron-transport chain consisting of an NADH-dependent flavoprotein reductase and two iron-sulfur proteins (Strijewski, 1982). It is known that most of monooxygenases use oxygen molecules and require electron-transport chain consisting of NAD(P)H-dependent reductase. The steroid 9α-hydroxylase of *Mycobacterium fortuitum* use NADH as an electron donor (Kang & Lee, in press). The enzyme activity was not given linearly with protein concentration in the assay (Kang, in press). This feature has also been reported for benzene (Axcell & Geary, 1975), toluene (Yeh *et al.*, 1977), and naphthalene (Ensley *et al.*, 1982) dioxygenase which are multicomponent systems. The oxygenase component of the steroid 9α-hydroxylase is the most interesting protein to study in detail. However, as a simple and specific assay for the oxygenase component was not available, purification of the reductase component was chosen firstly. The NADH reductase can be specifically assayed from 50~60% ammonium sulfate saturated fraction of cytosol that exhibited steroid 9α-hydroxylation activity (Kang, in press). The NADH reductase was purified to homogeneity using affinity chromatography on 5'-AMP Sepharose 4B. Recovery of the enzyme was 49%, with a purification factor of 100-fold. This purified protein had its activity for a month at -70°C and seems to be more stable than another component of the steroid 9α-hydroxylase. The NADH reductase is a monomer with a molecular mass of 60 KDa as determined by gel filtration and SDS-PAGE. The absorption spectrum of the isolated NADH reductase revealed the presence of prosthetic groups. The absorption maximum at 455 nm indicates the presence of a flavin. The absorption maximum at 408 nm indicates the presence of a second prosthetic group, an [Fe-S] cluster. Related reductase components with the same prosthetic groups have a molecular weight lower than that of reductase of the steroid 9α-hydroxylase. For the reductase components of the MMOs (Patel, 1987; Pilkington & Dalton, 1991), phthalate oxygenase from *P. cepacia* (Batie *et al.*, 1987), 4-methoxybenzoate *o*-demethylase from *P. putida* (Bernhardt *et al.*, 1975), benzoate 1,2-dioxygenase from *P. arvilla* (Yamaguchi & Fujisawa, 1978), xylene monooxygenase (Shaw & Harayama, 1992), and alkene monooxygenase (AMO) (Weber *et al.*, 1992), molecular masses of 34 to 56 KDa have been reported. Two redox groups, a flavin and a [Fe-S] cluster, are found in most multicomponent oxygenases and form a short electron transport chain to the oxygenase. The flavin, the first redox group, accepts two electrons from NAD(P)H: these electrons are then transferred to the [Fe-S] cluster. The electrons are finally transferred to the oxygenase. These two redox groups can be located on the same protein (the reductase component), as with AMO (Weber *et al.*, 1992), the MMOs (Fox *et al.*, 1989), phthalate oxygenase (Batie *et al.*, 1987), 4-methoxybenzoate o-demethylase (Bernhardt *et al.*, 1975), and benzoate 1,2-dioxygenase (Yamaguchi & Fujisawa, 1978). The two redox centers can also be located on two different proteins (flavin on the reductase and the [Fe-S] cluster on a ferredoxin or rubredoxin type of protein) as for with alkane hydroxylase of *P. oleovorans* (Ueda *et al.*, 1972) and toluene dioxygenase and benzene dioxygenase from *P. putida* (Geary *et al.*, 1984; Subramanian *et al.*, 1981). Furthermore, a system requiring three redox groups has been described for the naphthalene dioxygenase from a *Pseudomonas* species (Haigler & Gibson, 1990). The reductase contained both a FAD and [Fe-S] cluster, and a third redox group was located on the ferredoxin, which transfers its electron to the oxygenase component. For the soluble MMOs from *M. trichosporium* OB3b (Fox *et al.*, 1989), *M. sporium* 5 (Pilkington & Dalton, 1991), and *M. capsulatus* (Bath) (Colby & Dalton, 1978), which contain a reductase with two redox group, an ad-

ditional protein has been reported. This protein contains no redox group and is thought to function as a regulatory protein. Whether a third protein (regulatory or with a third redox group) is required for steroid 9α-hydroxylase activity is still unclear, and it will be the subject of further investigations.

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