Microbial Demethylation of Immunosuppressant FK-506: Isolation of 31-O-FK-506-Specific Demethylase Showing Cytochrome P-450 Characteristics from *Streptomyces rimosus* MA187

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As a result of an extensive screening program for the microbial modification of the immunosuppressant FK-506, one culture, *Streptomyces rimosus* MA187, which specifically catalyzed the C-31 demethylation of FK-506 was identified. Treatment of the biotransforming culture with FK-506 increased demethylase activity 2.4-fold and stabilized the cytochrome P-450 protein. The enzyme responsible for this demethylation (31-O-FK-506 demethylase) was isolated and shown to be a soluble cytoplasmic protein which is constitutively expressed in the cells, which requires NADPH, ferredoxin-NADP⁺-reductase, and ferredoxin for activity, and which shows a cytochrome P-450 light absorption characteristic. Carbon monoxide saturation of the enzyme preparation and known mammalian cytochrome P-450 inhibitors such as quinidine HCl, ketoconazole, troleandomycin, and sulfaphenazole abolish the demethylating activity extensively. The purified enzyme is a monomeric protein with a molecular mass of 42 kDa and shows its maximal activity at a pH of 7.4 and an incubation temperature of 34°C. The first 19 N-terminal amino acids in the sequence of the purified protein have been determined, with no cytochrome P-450 match found in the OWL and Swiss-Prot 23 databases. The isolated demethylase is therefore a cytochrome P-450 protein that can be used as a catalyst for the synthesis of 31-O-desmethylFK-506, an important immunosuppressant and a known metabolite of FK-506 metabolism by human liver microsomes.

Enzymes and microorganisms are increasingly being used as catalysts in organic synthetic reactions and in detoxification of hazardous chemicals (10, 19). In our laboratories, enzymatic and microbiological systems are used for the structural modification of known chemicals and for the generation of metabolites from therapeutically promising agents. Recently, we have reported the microbial (1) and enzymatic (15) modifications of two immunosuppressant compounds, FK-520 (immunosuppressant) and FK-506. These compounds are microbial products which are being developed as therapeutic agents in human organ transplants (18) and are being utilized as tools in basic research (9). Furthermore, the analogs of these compounds are in demand for both their pharmacological effects and use as starting materials for the synthesis of other derivatives with useful properties. One desired analog is 31-O-desmethylFK-506. Because of the presence of several methoxys and other functional groups in the structure of FK-506, the chemical methods for the specific demethylation at the C-31 methoxy are complex. These complexities prompted us to contemplate biological approaches for the preparation of this compound. Toward this end, a microbial screening program which resulted in the discovery of one culture, *Streptomyces rimosus* MA187, was initiated (1). This culture was found to have remarkable specificity for the C-31 demethylation of FK-506 (Fig. 1). Because of the unique substrate specificity and also potential use of this culture in catalyzing the reaction for the preparation of 31-O-desmethylFK-506 from FK-506, we deemed it necessary to investigate the enzymatic system that was responsible for the demethylating reaction in this organism. We now report the isolation and characterization of this demethylase from *S. rimosus* MA187 and present evidence for

the cytochrome P-450 properties that are associated with this enzyme.

MATERIALS AND METHODS

Materials. Columns for fast-performance liquid chromatography (FPLC) purification were purchased from Pharmacia (Piscataway, N.J.). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, NADP⁺, spinach NADP⁺-reductase, spinach ferredoxin, 4-methylpyrazole, quinidine HCl, troleandomycin, and sulfaphenazole were obtained from Sigma (St. Louis, Mo.). Ketoconazole was purchased from Research Diagnostic Inc. (Flanders, N.J.). Polyvinylidene fluoride membrane was from Millipore (Bedford, Mass.). All other chemicals were of the highest quality available.

Culture development and biotransformation of FK-506. The seed and fermentation cultures were developed according to established procedures in our laboratories (1). In the present work, a frozen sample of a mycelial preparation of *S. rimosus* MA187 from the Merck culture collection was used for both the establishment of the biotransforming culture and the preparation of mycelial cells for enzyme isolation. The product of the biotransformation reaction was identified according to previously published procedures (1).

Isolation of 31-O-FK-506 demethylase. (All operations were carried out at 4°C or on ice). After 2 days of incubation, the contents of 75 flasks containing actively growing biotransformation cultures of *S. rimosus* MA187 were pooled and centrifuged at 20,000 × g for 30 min. The mycelial cell pellet was washed three times in 0.1 M phosphate buffer, pH 7.4, by resuspension and recentrifugation and was finally suspended (at 137 g [wet weight]) in buffer A (0.1 M phosphate buffer, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, pepstatin [1 mg/liter], leupeptin [1 mg/liter], and 10% glycerol [pH 7.4]). The suspension thus prepared (300 ml) was subjected to the French press method of cell disruption (20,000 lb/in²), and the cell lysate was centrifuged at 200,000 × g for 45 min. The supernatant (crude extract, 260 ml, 40 mg/ml) was subjected to sequential ammonium sulfate precipitation, and the pellet from the 40 to 60% saturation was recovered after centrifugation at 20,000 × g for 45 min. The pellet was dissolved in buffer A containing 0.1 M KCl (buffer S) and dialyzed overnight against the same buffer with a membrane casing (Spectrum, Los Angeles, Calif.) with a molecular weight cutoff of 15,000. The dialyzed sample (45 ml, 140 mg/ml) was applied to a Q-Sepharose column (2.5 by 75 cm) previously equilibrated with buffer S, and the column was eluted with buffer S containing a linear gradient of KCl from 0.1 to 0.5 M. A total of 100 fractions (7 ml per fraction) at a flow rate of 60 ml/h were collected. Enzymatically active fractions (fractions 41 to 69) from the Q-Sepharose column were concentrated with an ultrafiltration cell equipped with YM-3 membrane (Amicon, Beverly, Mass.) and dialyzed against buffer A by passing the sample through Sephadex G-25 columns (PD-10; Pharmacia). The

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FIG. 1. The 31-O-FK-506 demethylation reaction catalyzed by *S. rimosus* MA187.

dialyzed sample (11 ml, 60 mg/ml) was further fractionated with a MonoQ HR10/10 column. This column was eluted at a flow rate of 60 ml/h with buffer A containing a gradient of 0 to 0.5 M KCl which was formed during a running time of 95 min. Fractions eluting from the MonoQ column were examined for both enzyme activity and cytochrome P-450 content. Most of the enzyme activity and cytochrome P-450-containing material were eluted between fractions 55 to 60. Minor amounts of cytochrome P-450 and some enzyme activity also eluted in earlier fractions. On the basis of the enzyme specific activity, cytochrome P-450-specific content, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) quality, fractions 56 and 57 from the MonoQ column were pooled, concentrated, and applied to a calibrated Superose-12 column. This column was eluted with buffer A containing 0.10 M NaCl with a flow rate of 18 ml/h, and 0.3-ml fractions were collected. Each fraction was examined for the enzyme activity and cytochrome P-450 content. Fraction 48 was found to have the highest specific enzyme activity and specific cytochrome P-450 content.

Molecular weight determination. Fraction 48 from the Superose-12 column was used to estimate the molecular weight of both native and denatured 31-O-FK-506 demethylase by gel filtration chromatography and SDS-PAGE, respectively (17).

31-O-FK-506 demethylase assay and enzymatic product identification. The enzyme assay supporting the 31-O-demethylation of FK-506 was run in a total volume of 1 ml containing 0.1 M phosphate buffer (pH 7.4), 1 mM NADPH, 1 mM NADP⁺, 17 mM glucose-6-phosphate, 0.1 U of glucose-6-phosphate dehydrogenase, and appropriate quantities of the fractions under analysis. The assay mixture was supplemented with 44 µg of ferredoxin and 0.05 U of ferredoxin-NADP⁺-reductase. The reaction was started by the addition of 0.25 nmol of ³H-labelled FK-506 (2.63 µCi/nmol), and the reaction mixture was incubated at 34°C for 20 min. Under these conditions, the rate of the demethylation of FK-506 was directly proportional to the amount of protein (ammonium sulfate fraction) added up to 4.5 mg. With this amount of protein in the assay mixture, the rate was first order for 2 h. At the end of the incubation time, the reaction mixture was extracted with 2 ml of ethyl acetate, 1 ml of which was used for the thin-layer chromatography (TLC) and radio high-pressure liquid chromatography (HPLC) analysis (15). For the TLC analysis, the extract was spiked with standard 31-O-desmethylFK-506 and applied to the plastic support TLC sheet (silica gel; Merck), which was then developed in a chloroform-methanol (9:1) solvent system. The developed TLC sheet was cut in the area of the standard 31-O-desmethylFK-506, which had an *R_f* value of 0.19 in this solvent system (for purposes of comparison, the *R_f* value of FK-506 is 0.44), and the radioactivity in the cut plastic strip was measured with a Beckman liquid scintillation spectrometer (LS 5801) after the addition of 7 ml of scintillation fluid (ScintiVerse I; Fisher Scientific, Pittsburgh, Pa.). Radio HPLC analysis of the extract of the enzymatic reaction mixture was carried out under conditions previously described (1, 15) for the identification of the biotransformation product, and 1-ml fractions were collected. A portion from each of the collected fractions was mixed with 7 ml of the scintillation fluid, and radioactivity was determined.

Spectral analysis and cytochrome P-450 assay. Visible and UV absorption spectra of various fractions were obtained with a Hewlett-Packard 84151A diode array spectrometer. All samples were analyzed in buffer A for spectral analysis and determination of cytochrome P-450 contents. The cytochrome P-450 content was determined from the difference spectrum after the reduction of the carbon monoxide-treated (1 min) sample with sodium dithionite according to procedures reported by Omura and Sato (12). An extinction coefficient of 100 cm⁻¹ M⁻¹ was used for the quantitation of the cytochrome P-450 content in various fractions.

Demethylase inhibition studies. All of the inhibition experiments were designed on the basis of the assumption that the demethylase was a cytochrome P-450 and were carried out under the conditions described above for the 31-O-FK-506 demethylase assay.

(i) Carbon monoxide saturation. The enzyme preparation, while on ice, was saturated with carbon monoxide (CO) by passing a stream of this gas through the

solution for 20 min. An appropriate volume of this CO-saturated preparation was then used as the source of the enzyme in the demethylase assay.

(ii) Mammalian cytochrome P-450 inhibitors. Five known mammalian cytochrome P-450 inhibitors consisting of 4-methylpyrazole, quinidine HCl, ketoconazole, troleandomycin, and sulfaphenazole were chosen and used for the demethylase inhibition studies; all were used at a concentration of 1 mM. The stock solutions of 4-methylpyrazole and quinidine HCl were made by dissolution of these compounds directly in the assay buffer, and appropriate aliquots were used. Because of the lack of solubility, ketoconazole, troleandomycin, and sulfaphenazole were first dissolved in acetone. An aliquot of each acetone solution was then added to the assay buffer. The resulting solution was then placed under a stream of nitrogen gas in order to free the buffer from acetone before the addition of other components of the enzymatic reaction mixtures.

(iii) Metal ions. Stock solutions of chloride salts of the metal ions Mg²⁺, Co²⁺, Ca²⁺, Mn²⁺, Cu²⁺, and Fe²⁺ were prepared, with assay buffer being used as the solvent. An appropriate volume of each stock solution was added to the reaction mixture to give a concentration of the metal ion in the final volume of 1 mM.

N-terminal amino acid sequence analysis. A sample of a highly purified preparation was subjected to SDS-PAGE, and the resulting Coomassie blue-stained gel was blotted on a polyvinylidene difluoride membrane. The area of the membrane showing the major band with a molecular mass of 42 kDa was cut out and used for N-terminal amino acid sequence analysis with the ABI gas phase sequencer (Tazana, Calif.) under standard conditions.

Other analytical procedures. Protein concentrations were determined with the Pierce (Rockford, Ill.) Coomassie blue protein assay kit in which bovine serum albumin was used as a standard. SDS-PAGE was performed according to the method of Laemmli (8), in which 3 and 15% acrylamides containing 0.1% SDS were used for the formation of the stacking and separating gels, respectively.

RESULTS AND DISCUSSION

Isolation of 31-O-desmethylFK-506 demethylase. The radioactivity-based assay system using ³H-labelled FK-506 as a substrate was used to fractionate and monitor the purification of the demethylase from a crude extract that had been prepared from the mycelial cells of *S. rimosus* MA187 grown in the absence of FK-506. Various fractions thus prepared were also examined for the presence of cytochrome P-450 protein, and as a result, a highly purified preparation was obtained. A summary showing the results of the purification procedures is presented in Table 1. As can be seen from Table 1, a 488-fold purification of demethylase activity is achieved, despite the loss of some activity after ammonium sulfate fractionation. Similarly, if cytochrome P-450-specific contents are used for the estimation of purification, a value of 307 is obtained, suggesting that the demethylase activity and P-450 protein are copurified.

Properties of the 31-O-FK-506 demethylase. The purified demethylase is a soluble cytoplasmic protein which could be isolated from mycelial cells grown in the absence or presence of the FK-506 substrate, indicating the constitutive nature of the enzyme. Addition of the substrate (FK-506) to the culture at the onset of the biotransformation increased the demethylase activity 2.4-fold in the cell extract. In light of the fact that the isolated demethylase had properties characteristic of cytochrome P-450 protein (see below), the induction effect of FK-506 was examined with samples of the cell extracts prepared from both FK-506-treated and untreated mycelial cells. The

TABLE 1. Purification of the 31-O-FK-506 demethylase from *S. rimosus* MA187

Fraction ^a	Amt of protein (mg)	Sp act (pmol/mg/h)	Specific content of P-450 (µg/mg)
Crude extract	10,400	0.14	0.13
(NH ₄) ₂ SO ₄	6,300	0.04	0.28
Q-Sepharose	660	0.56	0.63
MonoQ-FPLC	4.0	8.27	8.00
Superose-12	0.15	63.04	40.00

^a From 137 g (wet weight) of mycelia.

FIG. 2. Dithionite-reduced CO complex and dithionite-reduced difference spectra for the fractions isolated from an uninduced (A) and induced (B) culture of *S. rimosus* MA187. Both preparations contained equal amounts of protein.

results of these studies are presented in Fig. 2. As can be seen from Fig. 2A, the dithionite-reduced CO complex of the FK-506 untreated sample showed the presence of a small amount of cytochrome P-450 protein with a large amount of what appears to be a cytochrome P-420 protein. In contrast, a relatively large amount of cytochrome P-450 protein, compared with that of the cytochrome P-420 protein, was detected in the FK-506-treated sample (Fig. 2B). The simplest explanation for this difference in spectral profile is the possible stabilization of the cytochrome P-450 protein by either FK-506 or its metabolite, 31-O-desmethylFK-506, at the expense of the formation of cytochrome P-420, which is a known phenomenon for this class of proteins (13). The other possible explanation would be the induction of the constitutive P-450 and demethylase activity by FK-506. The 2.4-fold increase in the demethylase specific activity support both explanations.

The purified enzyme had an apparent molecular mass of 42 kDa on the basis of an examination of it in its native state by Superose-12 gel filtration chromatography. The SDS-PAGE gel of this fraction showed one major and several minor bands

FIG. 3. SDS-PAGE of the isolated (Superose-12) 31-O-FK-506 demethylase (lane 3) and Q-Sepharose (lane 2) fractions. Lane 1 shows standard molecular weight markers. The numbers at the left indicate molecular weights.

TABLE 2. Effect of various components of the assay mixture on the activity of the partially purified (MonoQ fraction) 31-O-FK-506 demethylase

Expt	Assay component	Activity (%)
1	Complete ^a	100
2	Minus NADPH	3
3	Minus NADPH and NADH	67
4	Minus demethylase	4
5	Minus ferredoxin	4
6	Minus NADP ⁺ -reductase	4

^a Defined in Materials and Methods.

(Fig. 3). The molecular mass of the major band was estimated to be 42 kDa, which together with the native molecular weight establishes a monomeric nature for the demethylase protein. The N-terminal amino acid sequence of this protein has been determined to be PEIIDLGA YGPDFVADPHP, with no cytochrome P-450 match found in the OWL and Swiss-Prot 23 databases. This is not unusual, as most sequence homologies seen with streptomycete cytochrome P-450s occur in the heme- and substrate-binding areas of the protein (14).

The purified fraction showed its maximal demethylase activity at pH 7.4 and a temperature of 34°C. Under optimal assay conditions, the isolated fraction required ferredoxin, ferredoxin-NAD(P)⁺-reductase, and NADPH as cofactors for the demethylase activity. The results of the investigation to determine these required components are shown in Table 2. According to these data, over 96% of the demethylase activity was lost when any one of the components was eliminated from the reaction mixture. In a different set of experiments, we have also shown that a NADPH regenerating system consisting of NADP⁺, glucose-6-phosphate, and NADP⁺-glucose-6-phosphate dehydrogenase could completely substitute for NADPH. However, only 67% of the demethylase activity was observed when NADH was used as the sole source of the reducing power. In the same study, addition of the NADH to the complete reaction mixture in which partially purified demethylase had been reconstituted increased the activity by about 30%, indicating the possible facilitation of the transfer of the second electron to the demethylase (cytochrome P-450), as has been suggested previously for other cytochrome P-450-dependent enzymes (4, 5). In this regard, similar properties have previously been reported (3, 16) for other monooxygenase systems of *Streptomyces* spp., supporting our assumption that the demethylase is a cytochrome P-450-type protein.

In a further evaluation of the possible cytochrome P-450 nature of the preparation showing the demethylase activity, studies to determine the effect of several known inhibitors of mammalian cytochrome P-450s were carried out. These inhibitors were carbon monoxide, 4-methylpyrazole, quinidine HCl, ketoconazole, troleandomycin, sulfaphenazole, and various metal ions. As can be seen from Table 3, carbon monoxide saturation of the enzyme preparation caused a more than 96% reduction of the demethylase activity. Similarly, quinidine HCl, ketoconazole, troleandomycin, and sulfaphenazole gave rise to 76, 96, 95, and 89% reductions in the demethylase activity, respectively. The only inhibitor which did not show any inhibitory effect was 4-methylpyrazole. The inhibitory activity of this compound is reported to be specific for the cytochrome P-450 2E isozyme whereas quinidine HCl, ketoconazole, troleandomycin, and sulfaphenazole are known to be specific inhibitors for the cytochrome P-450 2D6, 3A4, 3A4, and 2C isozymes, respectively (7, 20). These results suggest that not only is the microbial demethylase isolated in our study a cytochrome

TABLE 3. Inhibitory effect of known mammalian cytochrome P-450 inhibitors on 31-O-FK-506 demethylase isolated from *S. rimosus* MA187

Inhibitor	Inhibition (%)
Carbon monoxide	96
4-Methylpyrazole.....	0.0
Quinidine HCl.....	76
Ketoconazole.....	96
Troleandomycin	95
Sulfaphenazole	89

P-450, but also because of its enzyme activity and its behavior toward inhibitors, it has structural and conformational properties which are similar to properties shown by such isozymes of mammalian cytochrome P-450 as 2D6, 3A4, and 2C (7). In this regard, there is a recent published report (6, 7) which indicates that FK-506 is mainly metabolized by human cytochrome P-450 isozymes 3A4 and 2D6 and that one of the metabolites is characterized as 31-O-desmethylFK-506. This observation, while reinforcing our other evidence regarding the cytochrome P-450 nature of the 31-O-desmethylFK-506 demethylase, gives credence to the opinion which was presented by Clark and Hufford (2) and which concerns the use of microorganisms as models for the study of human drug metabolism.

The effect of various metal ions at 1 mM on the enzyme activity was also studied, and the results are presented in Table 4. As can be seen from Table 4, the most extensive inhibition was observed with Cu^{2+} , Mn^{2+} , Co^{2+} , and Fe^{2+} , with copper inhibiting over 97% of the FK-506 demethylase activity. Similar metal effects have previously been reported for the two cytochrome P-450s isolated from *Streptomyces carbophilus* SANK 62585 (11), thereby strengthening the evidence for the identity of the 31-O-FK-506 demethylase as a cytochrome P-450-type protein. In further analysis, the absorption and difference spectra for the oxidized and dithionite-reduced CO complex and dithionite-reduced types of the isolated 31-O-FK-506 demethylase were determined. The oxidized preparation had absorption maxima at 420 (Soret), 536 (β), and 568 (α) nm, indicating the low-state form of cytochrome P-450 in the demethylase preparation (Fig. 4A). This preparation gave a typical cytochrome P-450 difference spectral profile, with the maximum absorption at 450 nm (Fig. 4B).

In conclusion, the results presented above collectively show that we have identified a *Streptomyces* sp., *S. rimosus* MA187, which can specifically demethylase FK-506 at the C-31 position. The enzyme responsible for this reaction is isolated and purified and can be used for the synthesis of 31-O-desmethylFK-506. We have also shown that this enzyme is a cytochrome P-450-

TABLE 4. Inhibitory effect of metal ions (1 mM) on the activity of the 31-O-FK-506 demethylase

Inhibitor	Inhibition (%)
None	0.0
MgCl_2	8
CoCl_2	59
CaCl_2	3
MnCl_2	71
CuCl_2	98
FeCl_2	51

FIG. 4. Absorption spectra of the oxidized (A) and dithionite-reduced CO complex (B) isolated fractions, showing 31-O-FK-506 demethylating activity. The x and y axes show wavelength (nanometers) and absorption intensity, respectively.

type protein which mimics mammalian cytochrome P-450-dependent metabolism of FK-506 and, therefore, can be used as a model for the study of drug metabolism in humans and animals. The physiological role of this constitutive cytochrome P-450 in organisms which produce it is not known. However, several cytochrome P-450s which have been shown to function as enzymes in the biosynthesis of the secondary metabolites (16) or as catalysts in the metabolism of xenobiotics (14) have previously been isolated from different species of the genus *Streptomyces*.

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