Electron Transport Pathway for a Streptomyces Cytochrome P450

Cytochrome P450 105D5-CATALYZED FATTY ACID HYDROXYLATION IN STREPTOMYCES COELICOLOR A3(2)*[1]

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Streptomyces and other bacterial actinomycete species produce many important natural products, including the majority of known antibiotics, and cytochrome P450 (P450) enzymes catalyze important biosynthetic steps. Relatively few electron transport pathways to P450s have been characterized in bacteria, particularly streptomycete species. One of the 18 P450s in Streptomyces coelicolor A3(2), P450 105D5, was found to bind fatty acids tightly and form hydroxylated products when electrons were delivered from heterologous systems. The six ferredoxin (FdX) and four flavoprotein Fdx reductase (FDR) proteins coded by genes in S. coelicolor were expressed in Escherichia coli, purified, and used to characterize the electron transfer pathway. Of the many possibilities, the primary pathway was NADH → FDR1 → Fdx4 → P450 105D5. The genes coding for FDR1, Fdx4, and P450 105D5 are located close together in the S. coelicolor genome. Several fatty acids examined were substrates, including those found in S. coelicolor extracts, and all yielded several products. Mass spectra of the products of lauric acid oxygen binding were relatively fast in the catalytic cycle; high kinetic deuterium isotope effects for all four lauric acid hydroxylations indicated that the rate of C–H bond breaking is rate-limiting in every case. Thus, an electron transfer pathway to a functional Streptomyces P450 has been established.

Cytochrome P450 (P450) enzymes catalyze a variety of oxidations and related reactions (2, 3). The P450s are found throughout the biological kingdoms, from eubacteria and archaebacteria to mammals. Many of the bacterial P450s catalyze reactions of environmental or medical significance. The actinomycete species produce a wealth of natural products, including about two-thirds of the antibiotics used in medical practice today. These antibiotics are presumably produced by these bacteria as a defense mechanism against neighboring microorganisms in the environment; various other roles have been postulated for the other natural products.

The genomes of three streptomycete species have now been determined, the first being Streptomyces coelicolor A3(2), followed by Streptomyces avermectilis and Streptomyces peuceticus. These three genomes contain 18, 33, and 19 P450 genes, respectively (4–6). Knowledge about the catalytic activities of these proteins is limited, but some are known or at least suspected to be involved in a number of important reactions (7).

P450s cannot receive electrons directly from reduced pyridine nucleotides and therefore require auxiliary “transformer” proteins that can function in both 1- and 2-electron transfers (with one notable exception, the P450 55A subfamily) (8). Bacterial P450s are known to use a variety of electron transfer protein mechanisms. Electrons always move from NAD(P)H to flavoproteins in these pathways. Various bacterial P450s accept electrons from either flavoproteins or Fdx proteins (which receive them from FDRs) (9). In one seemingly unusual case in Sulfolobus solfataricus, a pyruvate oxidase (containing thiamine pyrophosphate) transfers electrons to a Fdx, which in turn reduces P450 119A1 (10). The streptomycete species examined thus far contain a plethora of Fdx and FDR proteins, and attempts to discern the relevant electron transfer pathways

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S11.

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[5] The abbreviations used are: P450, cytochrome P450 (also termed “heme thiolate P450” (1)); EI, electron impact; Fdx, ferredoxin; FDR, ferredoxin reductase; Fdx, flavodoxin; GC, gas (liquid) chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; NBT, nitro blue tetrazolium; NTA, nitrilotriacetic acid; PBS, 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl; Pdx, putidaredoxin; PDR, putidaredoxin reductase; TMS, trimethylsilyl.
have not been carried out in sufficient detail. Nearly all of the conclusions about the catalytic activities of *Streptomyces* P450s have come from studies in which electrons are shuttled from heterologous electron transfer components (11), most commonly commercially available spinach FDR and Fdx.

We expressed and purified an *S. coelicolor* P450, P450 105D5, and 10 potential electron transfer proteins, including four FDR and six Fdx proteins. With these, we established the primary electron transfer pathway used by this P450 in the hydroxylation of fatty acids, including several present in the organism that may be considered as potential physiological substrates.

**EXPERIMENTAL PROCEDURES**

*Chemicals*—Streptavidin-peroxidase, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, spinach Fdx and FDR, lauric acid, myristic acid, [1-14C]myristic acid (specific activity 24 mCi mmol⁻¹), palmitic acid, [1-14C]palmitic acid (specific activity 56 mCi mmol⁻¹), oleic acid, [1-14C]oleic acid (specific activity 54 mCi mmol⁻¹), linoleic acid, arachidonic acid, [1-14C]arachidonic acid (specific activity 50 mCi mmol⁻¹), 12-hydroxy-lauric acid, N,N-diisopropyl-ethylamine, and pentafluorobenzyl bromide were purchased from Sigma. [1-14C]Lauric acid (specific activity 50 mCi mmol⁻¹) was from GE Healthcare. [1-14C]Linoleic acid (specific activity 51 mCi mmol⁻¹) was purchased from PerkinElmer Life Sciences. d$_{13}$-Lauric acid was obtained from Cambridge Isotope Laboratories (Andover, MA). N,O-bis-(Trimethylsilyl)-trifluoroacetamide (containing 1% trimethylchlorosilane) and succinimidyl-6’-(biotinamido)-6-hexanamidohexanoate were from Pierce. Oligonucleotides for cDNA synthesis were purchased from Operon (Huntsville, AL). All oligonucleotides were of salt-free quality and were used directly without further purification. *Pseudomonas putida* PDR and Pdx were kindly donated by Dr. T. Poulos (University of California). *Escherichia coli* flavodoxin reductase was prepared as described elsewhere (12). *E. coli* Flx was purified as described earlier (12). Other chemicals and reagents were obtained from sources described previously or were of the highest quality commercially available.

*Synthesis of 11-Hydroxylauric Acid*—The material was prepared by the treatment of 11-decenoic acid (Indofine, Hillsborough, NJ) with mercuric acetate followed by NaBH$_4$ (13, 14).

*Expression and Purification of Recombinant S. coelicolor FDR1 and FDR4*—The plasmids coding for FDR1 and FDR4 (designated as SCO2106/SCF15.02 and SCO2106/SC2C1A.02 in gene annotation tables, respectively) were transformed into *E. coli* BL21(DE3)pLysS (Novagen). A single ampicillin-resistant colony of the transformed cells was selected and grown in overnight culture to saturation at 37 °C in Luria-Bertani medium containing 100 μg ml⁻¹ ampicillin. A 10-ml aliquot was used to inoculate each liter of Terrific Broth medium, which contained 0.2% bactopeptone (w/v), trace elements, and 100 μg ml⁻¹ ampicillin. Upon reaching an A$_{600}$ of 1.0, the cells were induced with 1.0 mm isopropyl β-D-thiogalactopyranoside and 1 μg ml⁻¹ chloramphenicol. The temperature was cooled to 25 °C, and the cells were further cultured for 24 h with shaking at 180 rpm in a New Brunswick Inova shaker (New Brunswick, Edison, NJ). After incubation, cells were harvested by centrifugation at 6.5 × 10⁵ × g for 20 min. Spheroplasts were prepared using lysozyme and disrupted by sonication. The cellular sonicates were centrifuged at 10⁴ × g for 20 min, and the cytosolic fractions were obtained by centrifugation at 10⁵ × g for 90 min (to remove insoluble material). The cytosolic fraction was applied to an Ni$_2$+-NTA column (Qiagen) that had been pre-equilibrated with 50 mm potassium phosphate (pH 7.4) buffer containing 20% glycerol (v/v), 0.5 M NaCl, 1.5% Tween 20 (v/v), and 0.10 mm phenylmethylsulfonyl fluoride. The column was washed with 50 column volumes of the same buffer followed by a second wash with the same buffer containing 20 mm imidazole. The FDR proteins were eluted using a stepwise gradient increasing from 40 to 200 mm imidazole in the same buffer. The eluate was dialyzed against 50 mm Tris-HCl (pH 7.4) buffer containing 20% glycerol (v/v) and 0.1 mm dithiothreitol and loaded onto a 1-ml MonoQ column (Amersham Biosciences). The FDR proteins were eluted using a linear gradient increasing from 0 to 0.4 M NaCl in the same buffer. Fractions were pooled, concentrated, and further resolved.
on a Superdex 200 gel filtration column (Amersham Biosciences) with the same buffer. Eluted fractions were analyzed by SDS-PAGE with Coomassie Brilliant Blue R-250 staining. FDR1 and FDR4, as well as FDR2 and FDR3, were electrophoretically homogeneous and showed the expected flavin spectra (Fig. 1). Pooled fractions were concentrated using an Amicon Ultra centrifugal filter (Millipore) and stored in 50 mM Tris-HCl (pH 7.4) buffer containing 20% glycerol (v/v), 1.0 mM EDTA, and 0.10 mM dithiothreitol at −80 °C.

Expression and Purification of Other Recombinant Proteins—S. coelicolor P450s, FDRs, and Fdx proteins were cloned into the E. coli expression vector pET17b (Novagen, Madison, WI) (4). Four histidine residues were engineered into the C terminus of the recombinant proteins for ease of purification. In the cases of expression of Fdx1, Fdx3, and Fdx6, an automatic codon optimization to suit the codon preference bias of E. coli and oligonucleotide design for PCR-based gene synthesis was performed commercially by DNA 2.0, Inc. (Menlo Park, CA). The P450 105D5 (3SCF60.06c) (supplemental Fig. S1) and FDR2 (SCO7117/SC4B10.18c), and FDR3 (SCO2469/SC7A8.08c) (Fig. 1); and Fdx1 (SCO7110/SC4B10.11), Fdx2 (SCO5135/SC9E12.20), Fdx3 (SCO7676/SC4C2.11), Fdx4 (SCO0773/SCF60.05c), Fdx5 (SCO3867/SCI41.32) (supplemental Fig. S2) proteins (S. coelicolor annotated gene names in parentheses) were expressed essentially by the methods described above, except that the chaperone groEL/ES was also co-expressed in these systems, and the expression was conducted at 22 °C for 30–40 h after treatment with 1 mM isopropyl β-D-thiogalactopyranoside and 4 mg ml−1 L-(-)-arabinose. The cytosolic fraction of the cell lysates obtained after centrifugation at 8,000 g (60 min) was applied to a Ni2+-NTA column equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 500 mM NaCl and 20% glycerol (v/v). The column was washed with 50 mM potassium phosphate buffer (pH 7.4) containing 500 mM NaCl, 20% glycerol (v/v), 0.5% sodium cholate (w/v), and 8 mM imidazole, and the His-tagged proteins were then eluted with the same buffer containing 80 mM imidazole. The eluates were directly applied to a DEAE-Sepharose (Fastflow) column that had been equilibrated with 5 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.10 mM EDTA, and 0.10 mM dithiothreitol. After the column was
washed with the same buffer containing 100 mM NaCl, the proteins were eluted with this buffer containing 250 mM NaCl. The purified P450 and FDR enzymes were dialyzed three times against 100- fold volumes of 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.10 mM EDTA, and 0.10 mM dithiothreitol. Fdx proteins were stored at −80 °C without dialysis because of the low Mr.

Assays of Rates of Electron Transfer to Artificial Acceptors—Rates of FDR electron transfer were determined by measuring cytochrome c reduction or NBT reduction. Cytochrome c reduction assays were done in 0.30 M potassium phosphate buffer (pH 7.7) containing 100 mM FDR and 40 mM horse heart cytochrome c. Reactions were initiated by the addition of 200 μM NADH and followed by the increase in absorbance at 550 nm using an Amino DW-2a/OLIS spectrophotometer. For measurement of rates of NBT reduction, the reactions were done in 50 mM Tris buffer (pH 7.5) containing 100 mM FDR and 200 μM NBT. Reactions were initiated with the addition of 200 μM NADH (or NADPH) and followed by the increase in absorbance at 535 nm. Concentration changes were calculated using an extinction coefficient of 18.3 mM−1 cm−1 for NBT.

Protein-Protein Interactions—Interactions between P450, FDR, and Fdx proteins were examined using a streptavidin-biotin method as described elsewhere (16). Briefly, varying concentrations of purified enzymes, in 50 μl of PBS, were applied to the wells of a Nunc 384-well polystyrene plate (Fisher) and incubated in a humid atmosphere for 2 h at room temperature. After binding of enzymes to the wells, unreacted material was removed by extensively washing the wells with PBS containing 0.1% Tween 20 (w/v). Biotinylation of purified enzymes was done by incubating proteins with a 2-fold molar excess of succinimidyl-6-(biotinamido)-6-hexanamidohexanoate in PBS at room temperature for 2 h in the dark. Samples were diluted (166-fold) with 100 mM Tris-HCl buffer (pH 7.2) containing 100 mM glycine (volume 50 μl) to quench the residual succinimide reagent, and the biotinylated proteins (in a 50-μl volume) were applied to the wells in which the free enzymes had been attached. After a 1-h incubation at room temperature, the unbound biotinylated proteins were removed by washing the wells six times with PBS containing 0.1% Tween 20 (w/v). A streptavidin-peroxidase solution (50 μl) was added to the wells, followed by incubation at room temperature for 1 h. The wells were washed again with PBS. Peroxidase substrate (50 μl of a solution of 50 mM sodium citrate buffer (pH 4.2) containing 90 mM 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 0.05 mM H2O2) was added to each well, and substrate color intensities were measured at 405 nm using an ELX800NB plate reader (Bio-Tek Instruments, Winooski, VT).

Spectral Binding Titrations—Purified P450 105D5 was diluted to 1 μM in 0.10 M potassium phosphate buffer (pH 7.4) and divided between two cuvettes. Spectra (350–500 nm) were recorded with subsequent additions of fatty acids, using an Aminco DW-2a/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA). The difference in absorbance between the wavelength maximum (390 nm) and minimum (418 nm) (∆A390 − ∆A418) was plotted versus the substrate concentration to estimate Ks with analysis using hyperbolic or quadratic equations as appropriate (17, 18). Spectral dissociation constants (Ks) were estimated using GraphPad Prism software (GraphPad Software, San Diego, CA) with fits to a quadratic equation using the following form,

$$K_s = \frac{([E]_{total} - [EL])([L]_{total} - [EL])}{[EL]}$$

(Eq. 1)

with E denoting enzyme and L denoting ligand, and Equation 2,

$$[EL] = 0.5(K_0 + E_0 + L_0) - 0.5((K_0 + E_0 + L_0)^2 - 4E_0L_0)^{1/2}$$

(Eq. 2)

with the following expression,

$$Y = B + \left(\frac{A}{2}\right) \times \left(\frac{1}{E}\right) \times \left( (K_r + E + X) - \sqrt{((K_r + E + X)^2 - (4 \times E \times X))} \right)$$

(Eq. 3)

utilized in Prism whenever a preliminary hyperbolic fit yielded an apparent Ks value less than 3-fold greater than the total enzyme concentration.

Enzymatic Assays for Fatty Acid Hydroxylation—The hydroxylation of fatty acids was generally measured by incubating purified P450 105D5 (1 μM) with either Fdx, Pdx, or Flx (1 μM) and either FDR, PDR, or flavodoxin reductase (1 μM), respectively, in a standard reaction mixture containing substrates (e.g. [1-14C]oleic acid (8 μM, 0.2 μCi) or [1-14C]lauric acid (7.3 μM, 0.2 μCi) in 0.10 M potassium phosphate buffer (pH 7.4). The reactions were initiated by the addition of 2 mM NADH or NADPH. After 30 min at 37 °C, reactions were terminated by the addition of 40 μl of a 1 M HCl, 2 M NaCl solution. The products and residual substrate were extracted twice with 0.7 ml of ethyl acetate. The organic phase was reduced to dryness under an N2 stream, and the residue was dissolved in CH2CN for HPLC analysis. The products and residual substrate were separated using a 3-μm Prodigy octadeclsilane (C18) HPLC column (4.6 × 150 mm; Phenomenex, Torrance, CA). The mobile phase (0.2% CH2CO2H in H2O/CH3CN mixtures, v/v) program began with a 20:80 ratio of the two solvents (v/v) at a flow rate of 1.0 ml min−1, followed by a linear gradient to 10:90 (v/v) H2O/CH3CN for 10 min, and then followed by a linear gradient to 100% CH3CN for 2 min and held at 100% CH3CN for 20 min. The chromatography apparatus was attached to a β-RAM Flow-Through System model 2 (IN/US Systems, Tampa, FL) flow counter.

Characterization of Hydroxylated Fatty Acids by GC-MS—In vitro incubations were carried out under the same conditions as described above except using 100 μM concentrations of fatty acid substrates. After drying the extracts under a stream of N2, each residue was mixed with 100 μl of N1O-bis(3trimethylsilyl)- trifluoroacetamide containing 1% trimethylchlorosilane (w/v) at 60 °C for 1 h. After derivatization, the samples were directly analyzed on a ThermoFinnigan TRACE GC 2000 gas chromatograph coupled to a Finnigan Trace GC DSQ mass-selective detector (ThermoFinnigan, Austin, TX). The capillary column Omegawax 320 (30 m × 0.32 mm × 0.25 μm) was obtained from Supelco (Bellefont, PA). The temperature program for this analysis had an initial temperature of 80 °C, held for 1 min, and then was increased at 15 °C per min to 140 °C, at 5 °C per min to 180 °C, at 25 °C per min to 280 °C, and then held
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at 280 °C for 3 min. Mass spectra were recorded in the scanning EI mode.

For the *in vivo* analyses, *S. coelicolor* was grown on yeast extract malt extract medium for 3 days. The cells were separated from the media by centrifugation, washed with H2O twice, suspended in 0.5 ml of H2O, and sonicated for 3 min using the probe tip of a VWR sonicator. The cell pellet suspension was adjusted to pH 1–2 using concentrated HCl. A Folch solvent (1.0 ml of a CHCl3/CH3OH mixture, 2:1, v/v) was used to extract the fatty acids from the cell pellet. The combined Folch extract, containing the fatty acids, was evaporated to dryness and dissolved in 0.5 ml of CH2Cl2, to which was added 40 μl of disisopropylethylamine and 10 μl of pentafluorobenzyl bromide. The derivatization reaction temperature was 60 °C, and the reaction time was 60 min. The organic phase was collected and evaporated under a N2 stream. The sample was reconstituted in 200 μl of hexane for GC-MS analysis (EI negative ion mode) using a column described for TMS esters (see above), with a temperature program beginning at 150 °C and increasing to 280 °C over 10 min (and then held at 280 °C for 6 min).

**Stopped-flow Kinetics**—All reactions were measured at 23 °C using an OLIS RSM-1000 system (OLIS, Bogart, GA) in the rapid scanning mode (10^3 scans s⁻¹) or occasionally at a single wavelength, with a fixed slit. In the anaerobic studies, samples were deaerated in glass 90-ml tonometers using intermittent cycles of vacuum and equilibration with argon gas that had been scrubbed through copper catalytic traps (and a bubbler containing photoreduced safranin T dye) in a manifold system described elsewhere (19–21). Except in the case of samples that containing photoreduced safranin T dye) in a manifold system described elsewhere (19–21). Except in the case of samples that were mixed with O2 in reactions, the tonometers contained an O2-scrubbing system composed of 0.3 M glucose oxidase, 0.02 M catalase, and 0.13 M glucose. The deaerated samples were transferred to the syringes of the stopped-flow spectrophotometer after previous treatment (of the stopped-flow syringes) sequentially with 0.1 M Na2S2O4 (overnight), safranin T, and anaerobic buffer solutions (20, 21). In most cases, the results are expressed as means ± S.D. calculated from three or more measurements, and representative traces are presented. The results were processed with the OLIS software, usually extracting kinetic traces from the collection of traces obtained using rapid scanning at the wavelengths showing maximum amplitude changes. In some cases, global fitting/singular value decomposition was used in the analysis.

**Deuterium Isotope Effects**—Incubations were done with the substrate lauric acid (d₆ or d₂₃) at a concentration of 50 μM with the reconstituted FDR/Fdx/P450 105D5 enzyme system. The products were extracted, derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (w/v) (see above), and analyzed by GC-MS as described (see below).

**Other Assays**—Protein concentrations were estimated using a bicinchoninic acid (BCA) assay (Pierce). P450 concentrations were estimated using previously reported methods (22). FDR concentrations were estimated from the flavin absorption spectra, using a general ε₄₅₀ value of 11.3 mm⁻¹ cm⁻¹. For measurement of NADH oxidation, P450 105D5 was preincubated with Fdx and FDR for 5 min at 37 °C in the presence of absence of lauric acid (20 μM). Reactions were initiated with the addition of 200 μM NADH, and the decrease in A₃₄₀ was monitored using a Cary14/OLIS spectrophotometer (On-Line Instrument Systems). Rates were calculated using the extinction coefficient Δε₃₄₀ = 6.22 mm⁻¹ cm⁻¹.

**RESULTS**

**Interaction of Fatty Acids with P450 105D5**—The initial studies on the separation and reconstitution of the mammalian P450 system were done using fatty acid hydroxylation (26), and many mammalian P450s are capable of catalyzing these reactions (27, 28). Some bacterial P450s are also known to be highly active in fatty acid hydroxylation (29), and we considered fatty acids as potential substrates for *S. coelicolor* P450s. Preliminary studies showed interaction of P450 105D5 with lauric acid, as judged by the conversion of the iron from the low to high spin state (“Type I” ligand binding interaction) (30) (supplemental Fig. S3A). The estimated Kᵣ was 0.12 μM (supplemental Fig. S3B).

**TABLE 1**

| Fatty acid | P450 105D5 | P450 158A2 |
|-----------|------------|------------|
| Lauric     | 0.12 ± 0.04 | 0.025 ± 0.001 | – | – |
| Myristic   | 0.02 ± 0.03 | 0.046 ± 0.003 | – | – |
| Palmitic   | 0.01 ± 0.02 | 0.032 ± 0.001 | – | – |
| Stearic    | 1.3 ± 0.04  | 0.24 ± 0.011 | – | – |
| Oleic      | 0.40 ± 0.10 | 0.054 ± 0.002 | 6.4 ± 1.2 | 0.062 ± 0.001 |
| Linoleic   | 0.47 ± 0.05 | 0.050 ± 0.001 | 15 ± 1 | 0.062 ± 0.001 |
| Arachidonic| 0.37 ± 0.04 | 0.073 ± 0.007 | 7.6 ± 0.4 | 0.068 ± 0.001 |

ΔA₃₄₀: extrapolated maximum ΔA₃₄₀ = A₃₄₀ for a 10 μM P450 105D5 concentration (measured in 0.10 M potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v)).

For measurement of NADH oxidation, P450 105D5 was preincubated with Fdx and FDR for 5 min at 37 °C in the presence or absence of lauric acid (20 μM). Reactions were initiated with the addition of 200 μM NADH, and the decrease in A₃₄₀ was monitored using a Cary14/OLIS spectrophotometer (On-Line Instrument Systems). Rates were calculated using the extinction coefficient Δε₃₄₀ = 6.22 mm⁻¹ cm⁻¹.

7-Ethoxyresorufin O-deethylation (23), 7-ethoxycoumarin O-deethylation, benzo[a]pyrene 3-hydroxylation, and coumarin 7-hydroxylation (24, 25) activities were determined as described.
activity was completely dependent on all of the components (FDR, Fdx, P450, and NADPH) (data not presented).

This catalytic activity of P450 105D5 could also be supported by a combination of *P. putida* PDR and Pdx, which had been used successfully to support oxidative reaction by *Streptomyces griseolus* P450 by O’Keefe et al. (11) or a combination of two flavoproteins, *E. coli* flavodoxin reductase and Flx (Fig. 2A). The PDR/Pdx system has been shown to be relatively selective for *P. putida* P450 101A1 (P450cam), at least with regard to high catalytic activity, but the *E. coli* flavodoxin reductase/Flx system is known to support slow electron transfer to mammalian P450s (12, 31). As in the case of the spinach FDR/Fdx system, all components were required in the cases of the PDR/Pdx- and flavodoxin reductase/Flx-supported systems. The PDR/Pdx system was more active with NADH than NADPH, in line with the known selectivity of PDR (32, 33).

Further experiments showed that one of the *S. coelicolor* Fdx proteins, Fdx4, could couple with PDR to support oleic acid oxidation (Fig. 2B). The activity was roughly equivalent to that seen in the spinach FDR/Fdx- and PDR/Pdx-supported systems. None of the other *S. coelicolor* Fdx proteins had substantial activity when tested with PdR (Fig. 2B).

Searches for a Homologous Electron Transfer System with *S. coelicolor* Components—Initial studies to reconstitute P450 105D5 oleic acid hydroxylation activity with purified *S. coelicolor* FDR2 or FDR3 and any of the *S. coelicolor* Fdx proteins were unsuccessful (and positive control experiments (e.g. see Fig. 2) were done at the same time). We utilized a heterologous binding assay, based on the use of biotin tagging (16), to demonstrate that both biotinylated FDR2 and FDR3 could interact directly with P450 105D5 (results not shown); FDR2 and FDR3 showed very weak interactions with Fdx4. *S. coelicolor* Fdx1, Fdx2, Fdx4, and Fdx6 could all bind tightly to P450 105D5, but Fdx3 and Fdx5 did not (Table 2). However, none of these data provided direct information about the productive interactions of P450 105D5 with the FDR and Fdx proteins of *S. coelicolor*.

Early efforts with *S. coelicolor* P450 systems had been focused on FDR2 and FDR3 (34). We re-examined the available genome of *S. coelicolor* A3 (2) (using the *S. coelicolor* annotation server ScoDB (available on the World Wide Web at streptomyces.org.uk) and considered two other candidate FDRs (Fig. 3), FDR1 and FDR4. FDR1 had been postulated to be a secreted or membrane protein, and its role had not been pursued. The four FDR sequences have 24–60% identity with each other (with the highest, 60%, for FDR2 and FDR3) (supplemental Fig. S4). Upon further inspection (Fig. 3B), FDR1 is located nearest the genes coding for Fdx4 and P450 105D5 but clearly not close enough to be considered part of an operon (Fig. 3B).

FDR1 and FDR4 were both expressed in *E. coli* and purified (Fig. 1). Both proteins showed catalytic activity in reducing the artificial electron acceptors cytochrome c and NBT. Selectivity for NADH > NADPH was seen for all FDRs in the NBT reduc-
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tion assays (Fig. 1C), and some stimulation of the cytochrome c reduction activity (∼25%) was observed with Fdx4 (results not shown). In other experiments (not shown), none of the S. coelicolor Fdx proteins could stimulate the cytochrome c reduction activity of FDR2 or FDR3. Thus, the dependence of cytochrome c reduction by S. coelicolor FDRs on Fdx proteins appears to be much weaker than with spinach FDR (35). Of relevance later for the P450 105D5 assays, the $K_m$ value for NADH was found to be 58 ± 8 μM for FDR1 in the NBT assay.

**Reconstitution of Oleic Acid Hydroxylation System with S. coelicolor Proteins**—As indicated previously, neither FDR2 nor FDR3 could support fatty acid hydroxylation with any of the six purified S. coelicolor Fdx proteins. We found that Fdx4 was the Fdx that enhanced cytochrome c reduction most with PDR or spinach FDR (supplemental Fig. S5). The oleic acid hydroxylation results obtained with the PDR-supported systems indicated that only Fdx4 appears to be able to productively interact with P450 105D5 (Fig. 2B). A mixture of Fdx4 and P450 105D5 was examined with each of the four purified S. coelicolor FDRs, and only FDR1 supported the oxidation (Fig. 4) (results with FDR4 not shown).

When FDR1 was used with individual Fdx proteins, Fdx4 was clearly the most efficient in supporting oleic acid hydroxylation (Fig. 5). The system was further optimized and a 1:3:1 molar ratio of FDR1/Fdx4/P450 105D5 gave the highest activity (supplemental Fig. S6).

**Interaction of FAD with Fdx4**—We considered the possibility that one or more of the FDR proteins we purified might not

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**TABLE 2**

| Fdx | $K_m$ | $\Delta A_{max, max}^c$ |
|-----|-------|------------------|
| 1   | 0.24 ± 0.09 | 1.2 ± 0.2 |
| 2   | 0.32 ± 0.18 | 0.53 ± 0.10 |
| 3   | ND | ND |
| 4   | 0.11 ± 0.05 | 0.07 ± 0.01 |
| 5   | ND | ND |
| 6   | 0.45 ± 0.20 | 0.24 ± 0.04 |

*The concentration of each Fdx was set at 0.10 μM, and the P450 105D5 concentration varied from 0.025 to 1.6 μM.*

*Estimated from a fit to a quadratic expression.*

*Data from Ref. 16.*

*No detectable binding ($K_a > 5 \mu M$).*

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**FIGURE 3.** Locations of FDR, Fdx (FDX), and P450 (CYP) genes in the S. coelicolor chromosome. A, total map from the S. coelicolor annotation server ScoDB (available on the World Wide Web at streptomyces.org.uk). B, expansion of the region containing FDR1, Fdx4, and P450 105D5, with positional bases numbered.
have a full complement of FAD and therefore be unable to reconstitute the catalytic activity. Preliminary experiments with NBT reduction indicated some apparent effect of added FAD with FDR2 and FDR3, and this effect was maximal with $\sim 100 \mu M$ FAD (preincubated for 1 h at 4 °C). The initial results with oleic acid oxidation also suggested a stimulation of the Fdx4/P450 105D5-catalyzed reaction (supplemental Fig. S7). However, further analysis showed that FAD could support the oxidation of oleic acid by P450 105D5/Fdx4 in the absence of any FDR (supplemental Fig. S7B), and this effect was specific for Fdx4 among the six Fdx proteins (results not shown).

The result may be explained by a slow transfer of a hydride ion from NADH to FAD at these concentrations, which are not considered to be physiologically relevant (i.e. 100 $\mu M$ FAD, 2 mM NADH). Attempts to demonstrate binding of FAD to Fdx4 or P450 105D5 were unsuccessful (using UV or fluorescence measurements). The results could be explained, in principle, by interaction of FAD with P450 105D5 or Fdx4. We conclude that the interaction of FAD is with Fdx4, because Fdx4 could also stimulate the slow reduction of cytochrome c by NADH.

Electron Transfer between Protein Components—The rate of reduction of FDR1 was estimated by measuring the decrease in the absorbance ($A_{450}$) after mixing with excess NADH ($500 \mu M$). The estimated rate was 1400 ± 30 min$^{-1}$ at 25 °C (results not shown), indicating rapid transfer of the hydride ion to the FDR flavin (results not shown).

The rate of electron transfer from FDR1 to Fdx4 was difficult to measure because of the low extinction coefficient of Fdx4 ($\epsilon_{406} \sim 1 \text{ mm}^{-1} \text{ cm}^{-1}$; supplemental Fig. S2C) relative to the flavin of FDR1 (and particularly the heme of P450 105D5). When a low concentration of FDR1 (0.067 $\mu M$) and excess Fdx4 (9.6 $\mu M$) were mixed with NADH (500 $\mu M$), the decrease in $A_{406}$ associated with oxidized Fdx4 (supplemental Fig. S2C) could be fit to a single exponential plot with an initial rate of $80 \pm 5$ min$^{-1}$ at 25 °C, accounting for the reduction of most of the Fdx (Fig. 6).

The rate of reduction of P450 105D5 was estimated in a complete system containing 0.5 $\mu M$ FDR1, 1.5 $\mu M$ Fdx4, 0.5 $\mu M$ P450 105D5, 50 $\mu M$ lauric acid, and 500 $\mu M$ NADH, utilizing the difference in the spectra of the ferric and ferrous heme spectra of P450 105D5 (see below). A rate of $30 \pm 4$ min$^{-1}$ was estimated at 25 °C (using singular value decomposition global analysis software). This rate is considered to reflect the individual transfers from NADH to FDR1 (1400 min$^{-1}$), from FDR1 to Fdx4 (80 min$^{-1}$; Fig. 6), and from Fdx4 to P450 105D5, so the actual rate of the last step (Fdx4 to P450) must be somewhat
In principle, this ion could be derived from a cleavage at the arid 12-hydroxylauric acid, the esters (Fig. 7). None of these co-chromatographed with stand-products, which could be further resolved by GC of the TMS below) and also showed higher rates of hydroxylation.

3). Of the fatty acids, lauric and oleic acids were examined in what higher than the Arachidonic 11.3

from the reconstituted FDR1-Fdx4-P450 105D5 system (supplemental Fig. S8). The assignment existing literature relevant to fragmentation of hydroxylated fatty acid TMS esters (36–38). Further, the other products (Fig. 7B) showed fragmentation patterns in which the base peak m/z was successively shifted +14 atomic mass units in each case, which we attribute to the scission shown in Fig. 7A. Thus, the assignments of the products are for ω-1, ω-2, ω-3, and ω-4 hydroxylation based on the MS fragmentation analysis, although we have not isolated the products and done NMR analysis for confirmation.

An M-15 peak (m/z 345) was also observed with 11-hydroxylauric acid. In principle, this ion could result from the loss of a TMS methy1, but a more probable explanation is the loss of the ω-methyl group. With the ω-2, ω-3, and ω-4 hydroxyauric acid products, strong ions were seen at m/z 331, 317, and 303, respectively (Fig. 7B). Taken together, the structural assignments we present are consistent with the mass spectra, which appear to be dominated by the scissions in the carbon chain at either site of the hydroxyl introduced by the P450.

Oleic acid was oxidized to multiple products. The most prominent was assigned as 17-hydroxyoleic acid, the ω-1 hydroxylauric acid product (supplemental Fig. S8). The assignment is based on a strong m/z 117 peak (75% relative abundance) and the analysis of the hydroxylated lauric acid products (Fig. 7).

The steady-state kinetic parameters for oleic acid hydroxylation (total) were measured with the optimized reconstituted system, yielding \( k_{cat} = 5.0 \pm 0.7 \text{ min}^{-1} \) and \( K_m = 2.4 \pm 1.0 \text{ \mu M} \left( k_{cat}/K_m = 2 \times 10^6 \text{ M}^{-1} \text{ min}^{-1} \right) \). The latter value (\( K_m \)) is somewhat higher than the \( K_e \) estimated for binding (Table 1).

The relative composition of some of the fatty acids present in S. coelicolor A3 (2), grown under laboratory conditions (and pelleted and washed), was determined by GC-MS (Table 3 and Fig. 8). Only traces of linolenic and arachidonate acid (~1% of palmitic acid; data not shown) were found, and the origin of these fatty acids may be the media. Analysis of the pentafluorobenzyl esters provides a relatively direct comparison based upon the ionization response, with little variation expected among the compounds. The traces (Fig. 8) at the m/z values 16 atomic mass units higher than the parent fatty acids show a number of peaks eluted later than the fatty acids, as would be expected for hydroxylated fatty acids. The detector responses indicate that in all cases, the putative hydroxylated fatty acids are present at levels an order of magnitude less than the corresponding parent fatty acids, as indicated by the response scales (Fig. 8).
Rate-limiting Steps in P450 105D5 Fatty Acid Hydroxylation—

On the basis of the results obtained with the individual components, we conclude that the pathways of electron transfer relevant to P450 105D5 are those shown in Fig. 9, based on the generally accepted P450 mechanism (39). We considered rates of some of the individual steps and their relevance to overall rates of fatty acid hydroxylation.

Rates of the electron transfer steps have already been discussed and are included in Fig. 9. Steps 1 and 7 (Fig. 9) were analyzed directly, with observation of the rates of changes in the heme Soret spectrum upon binding of fatty acids (supplemental Fig. S3). Single exponential kinetics were observed for the binding of the substrate lauric acid and also the major product 11-hydroxylauric acid (supplemental Fig. S9). The complex, as expected, was unstable and decayed in a biexponential manner ($k_1 = 30 \pm 1$ min$^{-1}$, $k_2 = 4 \pm 1$ min$^{-1}$ at 23 °C) (supplemental Fig. S10, B and C). The rate of the reaction of ferrous P450 with O$_2$ is clearly not rate-limiting; the rate of decomposition of the FeO$_2^{2+}$ complex may compete with productive substrate hydroxylation.

Kinetic Deuterium Isotope Effects—One method of probing the contribution of a C–H bond breaking event in a hydroxylation reaction is with measurement of noncompetitive kinetic deuterium isotope effects (20, 40). Parallel incubations of the lauric acid hydroxylation reactions were done with 50 μM $d_{22}$- and $d_{22}$-lauric acid (with NADH, FDR1, Fdx4, and P450 105D5; Fig. 5), and equal volumes of the reaction were mixed and analyzed together (as TMS esters) (supplemental Fig. S11). The ratios of $d_0$ to $d_{22}$ products (areas of integrals) were used as estimates of the kinetic isotope effects. For the 8-, 9-, 10-, and 11-hydroxy products, the ratios were 17, 30, 21, and 30, respectively. These values are high for P450 reactions but not unprecedented (e.g. intrinsic $k$ values of 15 estimated for human P450 3A4-catalyzed testosterone 6β-hydroxylation (41) and 14–15 for p-nitroanisole O-demethylation by rabbit P450 1A2 (20)). These values (for P450 105D5) are not corrected for any secondary isotope effects, and the multiplicative nature of secondary geminal and vicinal effects could be considerable (42). How-

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**FIGURE 7. Determination of positions of hydroxylation of lauric acid by MS analysis.** TMS esters were analyzed by GC-MS. A, mass spectrum and fragmentation pattern of 11-hydroxylauric acid-TMS ester (prepared from synthetic 11-hydroxylauric acid). In principle, the m/z 117 fragment could be derived from either end. B, mass spectra of four hydroxylauric acid-TMS ester products (peaks I, II, III, and IV) derived from P450 105D5 oxidation of lauric acid. The MS panel for the GC peak IV matched that of the standard 11-hydroxylauric acid TMS ester (A), with the m/z 117 base peak. The base peak is shifted by $\pm$ 14 atomic mass units in each of the other peaks, consistent with an additional –CH$_2$– unit at the ω-end and leading to the assignments shown. None of the peaks were observed in control experiments devoid of NADPH (or critical protein components).
ever, even a substantial secondary isotope effect of 1.2 (42) for each of the five neighboring deuteriums (four vicinal and one geminal) would yield a contribution of \(\frac{1}{2.5}\); the observed isotope effects of 17–30 would still correspond to primary isotope effects of 7–12, which are generally accepted to be evidence for a rate-limiting contribution in a multistep reaction (20, 40).

**DISCUSSION**

P450 105D5 is the product of one of the 18 P450 genes in *S. coelicolor* and catalyzes the hydroxylation of fatty acids, which are endogenous compounds in this bacterium. Evidence is also presented that the hydroxylated fatty acid products are also found to be present *in vivo* (Fig. 8). The existence of six Fdx and four FDR proteins in *S. coelicolor* A3 (2) provides 24 possible pathways for electron transfer to each P450 plus conceivable possibilities for direct transfer from the FDRs or other flavoproteins. We purified all 10 of the candidate proteins, plus P450 105D5, and were able to establish the primary pathway NADH → FDR1 → Fdx4 → P450 105D5 for fatty acid oxidation (Fig. 10). Mass spectrometry evidence suggests that the products result from \(\omega-1\), \(\omega-2\), \(\omega-3\), and \(\omega-4\) hydroxylation in the case of lauric acid; with oleic acid, the major of the several products appeared to be the \(\omega-1\) hydroxylated species, and we presume that the other fatty acids give similar mixtures.

The location of the genes coding for FDR1, Fdx4, and P450 105D5 in *S. coelicolor* may be consistent with the biological activity, at least the latter two genes (Fig. 3). The selectivity of P450 105D5 for Fdx4 is striking (Fig. 2B), and the proximity of these two genes (Fig. 4) argues that Fdx4 and P450 105D5 might be part of an operon (4). Fdx4 is selective among *S. coelicolor* Fdx proteins for interaction with P450 105D5. Fdx4 only accepted electrons (efficiently) from FDR1 of the four *S. coelicolor* FDRs, because product formation only occurred with FDR1 and Fdx4 (Fig. 4). If Fdx4 had been reduced by one of the other FDRs, then product formation should have been observed. Interestingly, Fdx4 also interacts with the heterologous *P. putida* PDR (Fig. 2B, supplemental Fig. S5). P450 105D5 apparently only accepted electrons efficiently from Fdx4 among the *S. coelicolor* Fdx proteins, as judged by the results of the experiments with PDR (Fig. 2B) and with FDR1 (Fig. 5), although another consideration is that these and the other FDRs do not reduce some of these Fdx proteins very efficiently.

![Graph showing GC-MS analysis of fatty acids of *S. coelicolor*.](image-url)
However, P450 105D5 can use electrons almost as effectively from the heterologous Fdx proteins Pdx and spinach Fdx and even the flavoprotein E. coli Flx (Fig. 2A). Thus, the system appears to be more selective among the S. coelicolor electron transfer proteins than the proteins from heterologous sources (eubacteria and plants).

Whether or not P450 105D5 and Fdx4 are truly in an operon is not clear, and the evidence should be considered carefully. Analysis of the genes surrounding P450 (CYP) 105D5 does not show the presence of fatty acid metabolism genes. Most genes in this region are predicted to encode proteins of unknown function. However, P450 105D5 and Fdx4 (Fig. 3) are part of a 10-linked gene set which forms an AUD (“amplifiable unit of DNA”) (43), related to genetic instability of the Streptomyces chromosome. Large deletions of the chromosome can occur, producing mutants that undergo DNA amplification of particular units, and P450 105D5 is a member of one such unit (AUD4) (43). Interestingly, there is a putative oxidoreductase in this set of genes that is annotated as a zinc binding oxidoreductase; the closest potential homolog is human NADPH-quinone oxidoreductase (NQO1).

We found that P450 105D5 hydroxylates a variety of fatty acids, including those found in S. coelicolor A3 (2) (Table 3, Fig. 8). With lauric acid, ω-1 hydroxylation was demonstrated unambiguously by GC-MS comparison with a synthetic standard (Fig. 7). On the basis of the 11-hydroxylauric acid fragmentation pattern and comparison with the oleic acid hydroxylation products, we conclude that the major product of the products of oleic acid hydroxylation is that generated by ω-1 hydroxylation. The sites of oxidation of the other fatty acids are not known but are presumed to be mixtures similar to those found with lauric acid. The point should be emphasized that the mass spectral assignments of the structures of the products are logical (Fig. 7) but not unambiguous in the absence of NMR data.

We postulate that the juxtaposition of fatty acids in P450 105D5 is such that several methylenes can be positioned near the FeO moiety; the lack of ω-hydroxylation is the result of either poor steric fit or the inherently more difficult thermodynamic barrier to methyl hydroxylation compared with methylene. We have tested a variety of putative P450 substrates for catalytic activity, all of which have yielded negative results (i.e. undetectable activity with a variety of assays, including benzo[a]pyrene oxidation, coumarin 3-hydroxylation, 7-ethoxycoumarin O-deethylation, and 7-ethoxyresorufin O-deethylation, all assayed together with positive controls (human liver microsomes and recombinant human P450s 1A2, 1B1, 2A6, and 2E1)). The lack of benzo[a]pyrene 3-hydroxylation activity, as judged by HPLC (25) and extraction/fluorescence arrays (24, 44), can be compared with a previous report of very low activity of P450 105D5 for benzo[a]pyrene hydroxylation (34). None of the reconstituted S. coelicolor protein or heterologous systems yielded detectable activity, in contrast to positive controls (human liver micro-

![Proposed catalytic cycle for P450 105D5](image.png)

**FIGURE 9. Proposed catalytic cycle for P450 105D5.** The scheme is based upon a generalized mechanism for P450 (39). Second-order rate constants or pseudo-first-order rates measured under experimental conditions in the work (all at 23 °C) are indicated.

![Schemes of electron flow for P450 105D5 fatty acid hydroxylation](image.png)

**FIGURE 10. Schemes of electron flow for P450 105D5 fatty acid hydroxylation.** See “Discussion.”
Streptomyces P450 Electron Transport Pathway

Somes and recombinant human P450 1B1 (25, 45). Thus, S. coelicolor P450 105D5 can be considered a fatty acid hydroxylase in light of the present results, including the finding of the hydroxylated products in vivo (as judged by the MS data) (Fig. 8), although no physiological relevance of these transformations has been established. S. coelicolor P450 105D5 cannot be considered a broad specificity oxygenase unless other catalytic activities can be rigorously established. It is of interest to note that Streptomyces griseus P450 105D1 (46), which is 70% identical to S. coelicolor P450 105D5 (4), has been reported to oxidize a wide variety of compounds, including benzo[a]pyrene, erythromycin, warfarin, testosterone, and 7-ethoxycoumarin, and the results have been corroborated in separate laboratories (47–49).

The identified S. coelicolor NADH → FDR → Fdx4 → P450 105D5 pathway (for fatty acid hydroxylation) may have a counterpart in a study of S. griseus P450 105D1 reported by Ueno et al. (49). Streptomyces lividans was used as an expression system, and a fusion construct of 7-ethoxycoumarin to coumarine was used as an end point. A plasmid expressing S. griseus P450 105D1, an Fdx gene adjacent to P450 105D1 in the S. griseus genome, and S. coelicolor FDR1 was used to produce 7-ethoxycoumarin O-deethylating activity in S. lividans but only with the addition of the Fdx and S. coelicolor FDR1 (49). The authors concluded that S. lividans does not produce the auxiliary proteins necessary for electron transport to (S. griseus) P450 105D1. We suggest that the Fdx (the gene that is adjacent to that for S. griseus P450 105D1 (50)) is similar to S. coelicolor Fdx4.

Some limited information is available about the interaction of P450s with Fdx proteins in other systems. Mammalian adrenodoxin interacts with several P450s in mitochondria (51), including some that generally reside in the endoplasmic reticulum (52). In P. putida, the interaction of Pdx with PDR and P450 101A1 was shown to be quite specific (53). With S. griseus P450 105A1 and 105B1, either of two Fdx proteins could reconstitute activity, although the flavoprotein donor used was spinach FDR in that system (11) (and spinach Fdx was more effective). A P450 system consisting of a P450, a [2Fe-2S] cluster Fdx, and a flavoprotein FDR has been purified from a Sphingomonas sp. (strain AO1) and demonstrated to be active in hydroxylation of the pollutant bisphenol A (54); the spinach FDR/Fdx system could also support catalysis but was not as effective. Some of the Fdx proteins involved in bacterial P450 electron transport are the [2Fe-2S] cluster type (e.g., Pdx and Sphingomonas) (54); all of the S. coelicolor Fdx proteins are of the [3Fe-4S] type. The nature of the prosthetic group is apparently not a strict determinant of the ability of a Fdx to function with a P450.

Some predictions can be made about possible related pathways in related organisms. S. griseus P450 105D1 has already been mentioned; the genome is not available, but an Fdx is known to be close to the P450 gene (55) and appears to be functional (49). 7-Ethoxycoumarin O-deethylating activity could be reconstituted with an FDR, Fdx, and P450 105D1 from S. griseus (50). S. coelicolor FDR1 can (at least qualitatively) complement the system; thus, no similar FDR1 protein appears to be available in S. lividans (49). At least two other Streptomyces genomes are now available, S. avermitilis (33 P450s) and S. peucetius (19 P450s). S. avermitilis has six putative FDRs and nine Fdxs (5). Five P450 105 family genes are present, and the one with the 105D7 nomenclature is located close to genes coding for an FDR and particularly an Fdx. We can speculate that these accessory proteins will couple with P450 105D5, although we have no experimental evidence. In S. peucetius, 19 P450 genes are present, two in the 105 family (6). Interestingly, four putative FDRs and only two Fdx proteins have been located.

The results of our study clearly implicate the electron transfer pathway NADH → FDR1 → Fdx4 → P450 105D5 in fatty acid hydroxylation in S. coelicolor A3 (2) (Fig. 10). However, a number of questions remain to be addressed. One is why the hydroxylation rate is relatively slow. Others (56) have reported relatively slow rates of electron transfer in some other reactions catalyzed by bacterial P450s, and the high rates of some reactions catalyzed by classic bacterial P450 enzymes, such as P. putida P450 101A1 (53) and Bacillus megaterium P450 102A1 (57), may be misleading in terms of what should be generally expected with the bacterial P450s. At this point, we know which fatty acids are present in S. coelicolor A3 (2) (Table 3, Fig. 8) (at least grown under these conditions) and that these are substrates for the enzyme system we have characterized, but the biological significance of fatty acid hydroxylation in S. coelicolor A3 (2) is unclear (as in the case for B. megaterium P450 102A1 (58)).

The question can be raised as to which step(s) is rate-limiting in fatty acid hydroxylation within the system identified in this study (Fig. 9). Substrate binding, oxygen binding (supplemental Figs. S9 and S10, B and C), and product release (supplemental Fig. S9C) are all fast. The kinetics of several of the electron transfer steps were measured (e.g., see Fig. 6) and are consistent with the steady-state rate of NADH oxidation, which is ~70 min⁻¹ at 23 °C and 140 min⁻¹ at 37 °C. The rate was only slightly higher when lauric acid was present compared with absent (not statistically significant). The rate of NADH oxidation (at 37 °C) is much faster than lauric acid hydroxylation measured under the same conditions (~5 min⁻¹), indicating that (i) overall, reduction is not rate-limiting and (ii) the bulk of the electrons is not being used productively in the reaction. These results may explain why heterologous electron donors (e.g., Pdx, spinach Fdx, and Flx-based systems) yielded hydroxylation rates similar to the FDR1/Fdx4 system (Fig. 2). The difficulty in hydroxylating the fatty acids is also reflected in the high kinetic deuterium isotope effects (supplemental Fig. S11). The apparent isotope effects of 17–30, even corrected for the contribution of potential secondary isotope effect contributions, are still estimated to be in the range of 7–12 (see above) and consistent, in a noncompetitive intermolecular isotope effect experiment, for rate-limiting C–H bond breaking (59).

Another major question is which proteins will be involved in electron transfer to other P450s in S. coelicolor. The possibility exists that Fdx4, possibly being in an operon with P450 105D5, could work selectivity with P450 105D5 but not other P450s. We have not determined if FDR1 will reduce other S. coelicolor Fdx proteins efficiently at this point (Figs. 4 and 5 and supplemental Fig. S5), due to the difficulty of the assays (Fig. 6). However, an interesting point to consider is that Fdx4 was the most...
efficient Fdx in coupling spinach FDR and PDR to cytochrome c (in reduction) (supplemental Fig. S5). These results may suggest that the preferential efficiency of Fdx4 may be a function of inherent catalytic activity as opposed to (productive) affinity with specific proteins (Table 2), but further characterization is necessary to address such a hypothesis. The assignment of electron transfer pathways with individual proteins for *S. coelicolor* P450s will require approaches of the sort used here. At this point, we are assuming that the electron transfer protein pathway to each P450 will be largely independent of the substrate (and reaction), although we do not have specific evidence for this hypothesis beyond the fatty acids examined here. Ultimately, other approaches, such as analysis of in vivo systems with transgenic strains, will be needed to establish the biological significance of each P450-catalyzed reaction.

In summary, we have identified the primary pathway for electron transfer to an *S. coelicolor* P450 as being NADH → FDR1 → Fdx4 → P450 105D5 (Fig. 10). The enzyme is involved in fatty acid ω-1 and other hydroxylations (but apparently not ω0), and the range of substrates beyond these fatty acids is not known. Other species of *Streptomyces* have elaborate collections of P450, Fdx, and FDR genes, and these studies with P450 105D5 provide an example of the approaches needed for elucidation of electron transfer pathways.

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