NADPH−Flavodoxin Reductase and Flavodoxin from *Escherichia coli*: Characteristics as a Soluble Microsomal P450 Reductase†

Christopher M. Jenkins and Michael R. Waterman*

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received December 15, 1997; Revised Manuscript Received February 26, 1998

ABSTRACT: In addition to their endogenous roles as an activation system for various *Escherichia coli* metabolic pathways, the soluble flavoproteins flavodoxin (Fld) and NADPH−flavodoxin (ferredoxin) reductase (Fpr) can serve as an electron-transfer system for microsomal cytochrome P450s. Furthermore, since Fld and Fpr are structurally similar to the functional domains (FMN binding and NADPH/FAD binding domains, respectively) of NADPH−cytochrome P450 reductases (P450 reductases), these bacterial proteins represent a potentially useful model system for eukaryotic P450 reductases. Here we delineate similarities and differences between the *E. coli* Fpr−Fld system and rat P450 reductase as electron donors to bovine 17α-hydroxylase/17,20-lyase P450 (P450c17). Importantly, recombinant Fpr, in combination with recombinant Fld, supports both the hydroxylase and lyase activities of P450c17 to the same proportional extent (hydroxylase-to-lyase ratio) as does P450 reductase. Maximum P450c17 turnover [5−6 mol of 17α-OH-progesterone (mol of P450c17)$^{-1}$ min^{-1}] was achieved using a large molar excess (50−100-fold over P450c17) of a 1:1 ratio of Fpr−Fld, although this rate was an order of magnitude less than the maximal P450 reductase-supported activity. Using these conditions, we have examined the effects of increasing ionic strength and the presence of cytochrome b_5 (b_5) on these two systems. Critical Fld−P450c17 electrostatic interactions are disrupted at moderate ionic strength (>100 mM NaCl) as evidenced by significant inhibition (>50%) of Fpr−Fld-supported P450c17 activity while much higher ionic strength (300 mM NaCl) is required to disrupt P450 reductase−P450c17 interactions to the same extent. Interestingly, cytochrome b_5 was found to dramatically inhibit both P450 reductase- and Fpr−Fld-supported P450c17 progesterone 17α-hydroxylase activity while in contrast 17α-OH-pregnenolone lyase activity was stimulated by b_5. Investigation of the fate of reducing equivalents from NADPH added to Fpr under aerobic conditions revealed that the majority of the protein-bound FAD of Fpr is converted to the hydroquinone form. In constrast, the FMN of Fld is reduced by Fpr to a stable blue, neutral semiquinone which serves as the predominant electron donor to P450c17 in reconstitution assays. Thus, while the Fpr−Fld system and P450 reductase are fundamentally different with respect to their electrostatic interactions with P450c17, their ability to support maximal P450c17 turnover, and the FMN redox states (one-electron-reduced for Fld and two-electron-reduced for P450 reductase) capable of transferring electrons to microsomal cytochrome P450s, these differences do not appear to influence the relative catalytic efficiency of the P450c17 hydroxylase and lyase reactions.

Ferredoxin−NADP$^+$ oxidoreductases (EC 1.18.1.2, FNRs)[1] reversibly transfer reducing equivalents between NADP(H) and the iron−sulfur clusters of various ferredoxins (Fds) and in some cases between NADP(H) and the FMN cofactors of flavodoxins (Flds). All FNRs contain noncovalently bound FAD (1 mol/mol of protein) which remains tightly associated with the enzyme during catalysis and is the conduit through which electron transfer occurs. A number of different FNRs have been extensively characterized, most notably from spinach chloroplasts (*1*−*3*), bovine mitochondria (*4*, *5*), and the cyanobacterium *Anabaena* (*6*−*8*). These FNRs are essential for such diverse metabolic pathways as the generation of NADPH from photosynthetically reduced ferredoxin (*9*) or flavodoxin (*10*) and the biosynthesis of steroid hormones (*11*) and activation of vitamin D_3 (*12*). The FNR adrenodoxin reductase reduces the 2Fe-2S protein adrenodoxin which supports reactions catalyzed by mitochondrial P450s in the latter two pathways.

In addition to ferredoxins, some FNRs, such as those from *Anabaena* and *Escherichia coli*, transfer electrons to flavodoxins, an important class of microbial FMN-containing redox proteins. Due to their low molecular weight, solubility, and stability, flavodoxins have proven to be ideal models for studies of FMN binding (*13*, *14*), redox potential modulation (*15*−*17*), and electron transfer to other redox proteins (*18*−*20*). Unlike ferredoxins, which are one-

† This work was supported in part by National Institutes of Health Grants T32-ES07028 (to C.M.J.), P30 ES00267, and GM37942 (to M.R.W.).

* To whom correspondence should be addressed at the Department of Biochemistry, 607 Light Hall, 21st at Garland, Vanderbilt University School of Medicine, Nashville, TN 37232-0146. Telephone: (615) 322-3318. FAX: (615) 322-4349. E-mail: water@lhmrba.hh.vanderbilt.edu.

[1] Abbreviations: FNR, ferredoxin−NADP$^+$ oxidoreductase; Fpr, *Escherichia coli* NADPH−flavodoxin (ferredoxin) reductase; Fld, *Escherichia coli* flavodoxin; P450c17, cytochrome P450 17α-hydroxylase/17,20-lyase; b_5, cytochrome b_5; cyt *c*, cytochrome *c*; KP_i, potassium phosphate buffer.

S0006-2960(97)03076-6 CCC: $15.00
Published on Web 04/04/1998

electron acceptors, flavodoxins can exist in either oxidized, one-electron reduced/semiquinone ($FMNH^{\bullet}$) or two-electron reduced/hydroquinone ($FMNH^-$ or $FMNH_2$) states.

NADPH−flavodoxin (ferredoxin) reductase (Fpr) from *E. coli* participates in the flavodoxin-dependent activation of at least four enzymes: cobalamin-dependent methionine synthase (*21*), pyruvate-fomate lyase (*22*), anaerobic ribonucleotide reductase (*23*), and biotin synthase (*24*). Although Fpr will reduce *E. coli* ferredoxin (*22*), the function of which is still unknown (*25*), only reduced flavodoxin (semiquinone or hydroquinone) has been demonstrated to be an electron donor for these four enzyme systems. Protection against paraquat-generated oxygen radicals in vivo has been correlated with Fpr expression (*26*), although its exact role in this mechanism has not yet been determined.

The FNR catalytic unit, as defined by contiguous FAD and NADP(H) binding domains, has been incorporated into several more complex enzymes during evolution. *E. coli*, for example, possess at least two enzymes, NADPH−sulfite reductase (*27*) and a hemoglobin-like ferrisiderophore reductase (*28*), which contain regions which are conserved among FNR proteins. NADPH−cytochrome P450 reductase (P450 reductase) has been proposed to have arisen from the fusion of a flavodoxin and an FNR protein (*29*), as have the reductase domains of cytochrome P450BM-3 from *Bacillus megaterium* (*30*) and the mammalian nitric oxide synthases (*31*).

Development of a high-level expression system for recombinant eukaryotic microsomal P450s in *E. coli* lead to the surprising observation that such enzymes are active in the intact microbe (*32*, *33*; E. F. Johnson, personal communication). This was surprising since *E. coli* do not contain endogenous P450s and were reported not to contain an immunologically detectable P450 reductase (*34*). Preliminary experiments revealed this *E. coli* P450 reductase to be cytosolic (*32*), and subsequent purification identified Fpr in combination with Fld as the components of this system (*35*). These experiments and those of others (*33*, *36*) have shown that the soluble *E. coli* Fpr−Fld system is able to replace, although less efficiently, P450 reductase in supporting microsomal cytochrome P450 activity. A more detailed characterization of the *E. coli* system, however, has been hampered by the low endogenous levels of these proteins. In this report, we have overexpressed and purified Fpr for further characterization, in combination with recombinant Fld, as a P450 reductase for bovine cytochrome P450 17α-hydroxylase/17,20-lyase (P450c17). This microsomal enzyme is of particular interest since it catalyzes two distinct reactions: 17α-hydroxylation of progesterone or pregnenolone and cleavage of the 17,20 carbon−carbon bond of 17α-OH-pregnenolone, which are essential for glucocorticoid (cortisol) and androgen (testosterone and estrogen) biosynthesis, respectively. Evidence is presented which indicates that the *E. coli* system represents a viable (although different?) model for P450 reductase for examining protein−protein interactions (e.g., flavodoxin−P450c17 vs P450 reductase−P450c17 interactions) and electron transfer (flavodoxin to P450c17).

EXPERIMENTAL PROCEDURES

Cloning and Overexpression of Fpr. Genomic DNA was purified from *E. coli* strain JM109 and digested with *Pst*I. Forward (5′-GCAGCCATATGGCTGAT-TGGGTAACAG-GCAAAGTCACTAAAGTG-3′ which introduced an *Nde*I site at the start codon) and reverse (5′-GCTGCGAATTCT-TACCAGTAATGCTCCGCTGTCATGTGGCCCGG-TCG-3′ which introduced an *Eco*RI site at the 3′ end) primers were used for PCR amplification (cycle: 92 °C, 1 min; 56 °C, 1 min; 70 °C, 1 min) of *fpr* using *Pfu* DNA polymerase (Stratagene). Klenow fragment (10 units) was added to the reaction mixture following PCR and incubated for 15 min at 37 °C. The single major PCR product (approximately 700 bp) was gel-purified and blunt-end-ligated into pBluescript (Stratagene) at the *Eco*RV site. Competent DH5α cells were electroporated with the pBluescript−*fpr* ligation mixture, and white colonies were selected. Of the clones containing inserts, three were sequenced, one of which contained the entire *fpr* coding sequence and the introduced restriction sites. Orientation of the *fpr* coding sequence in pBluescript allowed convenient excision using *Nde*I and *Bam*HI for insertion into a pET11a vector (Novagen). *E. coli* strain HMS174 (DE3) (Novagen) was transformed with the pET11a−*fpr* ligation mixture, and clones were selected for overexpression.

HMS174 (DE3) cells containing pET11a−*fpr* were grown overnight in LB media containing 100 mg of ampicillin/mL which was used to inoculate (1:100 dilution) TB media containing 1 mM $MgCl_2$, 5 mM NaCl, and 50 μg of ampicillin/mL. Cells were grown at 33 °C and 250 rpm in a shaker−incubator (Innova) for 4 h after which isopropyl thiogalactopyranoside (Calbiochem) was added to 1 mM final concentration, followed by 4 h of growth/induction.

Purification of Fpr. Cells overexpressing Fpr were pelleted (2000*g* for 10 min) and resuspended in ice-cold 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). Lysozyme was added to 0.5 mg/mL and the suspension stirred at 4 °C for 30 min. Following sonication (3 × 20 s bursts), the lysed cells were centrifuged at 100000*g* for 30 min. The supernatant was diluted 1:10 with 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 0.1 mM DTT (DE52 equilibration buffer) and applied to a DE52 column equilibrated with the same buffer. This column was washed with equilibration buffer containing 40 mM NaCl, and Fpr was eluted with equilibration buffer containing 150 mM NaCl. Fractions containing Fpr ($A_{456} > 0.07$) were diluted 1:5 with DE52 equilibration buffer and applied to a red Sepharose (Pharmacia) column equilibrated with the same buffer. Following a wash step using equilibration buffer containing 50 mM NaCl, Fpr was eluted with equilibration buffer containing 1 M NaCl. Fractions containing Fpr were pooled, dialyzed against 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol, and frozen at −70 °C. Fpr concentration was determined using $\epsilon_{456} = 7100\ cm^{-1}\ M^{-1}$ (*21*).

Assay for P450c17 Catalytic Activities. P450c17 progesterone hydroxylase and 17α-OH-pregnenolone 17,20-lyase activities were assayed with progesterone (50 μM unlabeled and 1×10^5 cpm [^{3}H]progesterone) and 17α-OH-pregnenolone (10 μM unlabeled and 1×10^5 cpm [^{3}H]-17α-OH-pregnenolone), respectively, as substrates in the presence of P450c17 (0.2 μM), in reconstitution buffer (10 mM KP_i buffer, pH 7.4, 0.1 mg of L-α-dilauroylphosphatidylcholine/mL, 20% glycerol), 0.6 unit/mL glucose-6-phosphate dehy-

drogenase, and the indicated reductase system (P450 reductase or *E. coli* Fpr−Fld). Reaction mixtures were incubated at 37 °C for 2 min before initiation with NADPH (final concentration = 0.3 mM) and glucose 6-phosphate (final concentration = 3 mM). Chloroform was used to terminate and extract the steroids which were then separated by thin-layer chromatography (*37*). Areas corresponding to the expected products were excised and quantitated by scintillation counting.

Expression and Purification of Recombinant Rat Cytochrome P450 Reductase and E. coli Fld. The plasmid encoding recombinant rat P450 reductase, pOR263 (*38*), was kindly provided by Prof. Charles B. Kasper (University of Wisconsin, Madison). The recombinant reductase was expressed in *E. coli* and purified as described (*39*). *E. coli* flavodoxin was expressed and purified as described (*40*).

Expression and Purification of Recombinant Bovine P450c17(His)$_4$. Bovine P450c17 containing four histidine residues at the C-terminus was expressed in *E. coli* as previously described (*32*) and purified with the following modifications. Cells containing P450c17(His)$_4$ were resuspended in 0.1 M Tris−acetate, pH 7.6, 0.5 M sucrose, and 1 mM EDTA (5× w/v of cells) to which lysozyme was added (final concentration = 0.5 mg/mL) at 4 °C. After slowly adding an equal volume of ice-cold 0.1 mM EDTA, pH 8.0, the suspension was stirred for 30 min. Spheroplasts were pelleted and resuspended in 50 mM KP$_i$ buffer, pH 7.4, 33% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 5 μg of DNase/mL, and 40 μM progesterone using a Teflon homogenizer. Triton X-114, precondensed as previously described (*41*), was added dropwise to 0.7% while stirring. The dark reddish detergent-rich phase, formed after centrifugation (100000*g* for 30 min), was slowly diluted 10-fold with 50 mM KP$_i$ buffer, pH 7.4, 20% glycerol, 0.1 mM PMSF, 40 μM progesterone, 0.1% Emulgen 913, and 0.1% sodium cholate, which was also used to equilibrate a Ni−NTA (Qiagen) column. The diluted detergent-rich phase was applied to Ni−NTA, washed with equilibration buffer including additional Emulgen (final concentration = 0.2%) and cholate (final concentration = 0.2%), 0.1 M NaCl, and 0.05 M glycine, and slowly eluted with the wash buffer containing 30 mM histidine. Fractions containing P450c17-(His)$_4$ were diluted 10-fold in 1 mM KP$_i$, pH 7.4, 20% glycerol, 0.1% Emulgen 913, 0.1% sodium cholate, 0.1 mM EDTA, 0.1 mM DTT, and 40 μM progesterone and applied to a hydroxylapatite column equilibrated with the same buffer. After washing the column extensively with 20 mM KP$_i$, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.3% sodium cholate, P450c17(His)$_4$ was eluted with 400 mM KP$_i$, pH 7.4, 20% glycerol, 0.1 mM DTT, and 0.6% sodium cholate. Fractions containing P450c17(His)$_4$ were dialyzed against 50 mM KP$_i$, pH 7.4, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.05% sodium cholate and stored at −70 °C.

Spectrophotometric Assays. Spectra of Fpr, a 1:1 complex of Fpr−Fld, and aerobic reduction in the presence of NADPH were collected using an Aminco DW-2 scanning spectrophotometer. The amount of Fld semiquinone was calculated using $\epsilon_{579} = 4.57$ mM^{-1} cm^{-1} (*42*). Oxidation of NADPH by either P450 reductase or Fpr−Fld in reconstitution buffer (without the NADPH-regenerating system) was measured by the decrease in absorbance ($\epsilon_{340} = 6.22$ cm^{-1} M^{-1}) using a Beckman DU 640 spectrophotometer with a kinetics program.

Table 1: Purification of Overexpressed Fpr from *E. coli*

fraction	protein (mg)	Fpr (nmol)	sp content (nmol of Fpr/ mg of protein)	reductase act. (μmol of cyt *c* min^{-1} mg^{-1})
cytosol[a]	508	3370	6.6	0.12
DE52	142	2230	15.7	0.30
Red Sepharose	58	1920	33.1	0.53[b]

[a] From 4 × 500 mL cultures. [b] 15.6 μmol of cyt *c* reduced (μmol of Fpr)$^{-1}$ min^{-1} at 25 °C. 25.5 μmol of cyt *c* reduced (μmol of Fpr)$^{-1}$ min^{-1} at 35 °C.

Other Methods. Identification of the Fpr flavin was determined by boiling the protein in the dark for 5 min, removing the precipitated protein by centrifugation, and injecting the filtered (0.2 mm) supernatant into a Waters HPLC system equipped with a C18 column equilibrated as described (*43*). The flavin was confirmed as FAD based on the elution time of an FAD standard. FMN was found to elute 1.4 min later than FAD. The assay for cytochrome *c* reduction was performed as previously described (*42*).

RESULTS

Because native *E. coli* NADPH−ferredoxin (flavodoxin) reductase (Fpr) is a minor component of the total cellular protein [approximately 0.1% (*21*)], a method was developed for obtaining large quantities of this flavoprotein for further characterization of the Fpr−Fld system as a microsomal P450 reductase. To be certain that the gene encoding Fdr from *E. coli* JM109, the strain from which the soluble P450 reductase system was originally purified (*35*), was the same as that reported previously from strain K-12 C-600 (*23*), primers were designed and, following PCR, a single 700 bp product was obtained using partially digested JM109 genomic DNA (data not shown). DNA sequencing revealed that the JM109 *fpr* clone was identical to that isolated from K-12 strain C-600 (*23*) (data not shown). A pET vector (11a) was used to overexpress Fpr as has been used for *E. coli* flavodoxin (*40*). After growth and IPTG induction, harvested cells containing pET11a−*fpr* were bright yellow in color, indicating the presence of Fpr. The results from a typical two-step purification are summarized in Table 1 showing that approximately 1 μmol or about 30 mg of purified Fpr is obtained per liter of cultured cells. This corresponds to greater than 10% of the total cytosolic protein or a greater than 100-fold increase over endogenous levels of Fpr.

The spectral properties of recombinant oxidized Fpr were identical to those reported for the native enzyme (*21*), exibiting characteristic peaks at 400 and 456 nm with a shoulder at around 480 nm (Figure 1A). This unusual absorbance profile has been attributed to a bent conformation of the FAD in which the adenine moiety is hydrogen bonded to the isoalloxazine ring as seen in the crystal structure of this flavoprotein (*44*). On the basis of specific content, greater than 95% of the purified preparation contained flavin, which was subsequently confirmed as FAD by HPLC (data not shown). Purified recombinant Fpr can reduce cytochrome *c* (cyt *c*) (Table 1) at the rate of 25.5 nmol of cyt *c* (nmol of enzyme)$^{-1}$ min^{-1} at 37 °C which is the same as the rate measured for the native enzyme [25.6 nmol of cyt

c (nmol of Fpr)$^{-1}$ min^{-1}] (*42*). If a 10-fold excess of *E. coli* Fld (relative to 0.5 μM Fpr) is included in the assay, this rate increases approximately 3-fold, indicating that Fld is also able to transfer electrons to cyt *c* as has been well established for other flavodoxins (*45*, *46*). By comparison, recombinant rat cytochrome P450 reductase will catalyze the reduction of 1650 nmol of cyt *c* (nmol of enzyme)$^{-1}$ min^{-1} under these conditions, a value comparable to that reported for native rat P450 reductase (*47*).

Addition of excess NADPH to *E. coli* NADPH−flavodoxin (ferredoxin) reductase under aerobic conditions results in a biphasic bleaching of the flavin absorbance, consistent with reduction of the FAD cofactor (Figure 1A). A small shoulder at 526 nm and a broad shoulder around 610 nm may be indicative of a small amount of the Fpr semiquinone species. The presence of an imperfect isosbestic point at 506 nm and incomplete reduction of oxidized Fpr would suggest that all redox states of the protein are present, with the hydroquinone being predominant. Another possibility is that the increase in long-wavelength absorbance (>500 nm) is indicative of a charge-transfer complex between NADP(H) and $FADH_2$, although this was not observed upon anaerobic reduction of the native enzyme with sodium dithionite or NADPH (*42*).

Recombinant *E. coli* flavodoxin will bind Fpr under conditions of low ionic strength as evidenced by an increase in absorbance from approximately 350 to 500 nm (Figure 1B). A similar spectral change has been observed upon binding of *Anabaena* FNR to *Anabaena* Fld and was used to calculate a binding constant of 6.4 μM at pH 7.0 (*48*). The binding constant of *E. coli* Fld to Fpr in a 1:1 complex has recently been determined to be 1.0 μM in 10 mM potassium phosphate buffer (pH 7.0) (*49*). While the flavin peaks of Fpr and Fld overlap at approximately 450 nm, they are distinct at 400 nm (Fpr, Figure 1A) and 369 nm (Fld, Figure 1B). The increase in absorbance of both of these peaks upon interaction may indicate that the isoalloxazine rings of each flavin cofactor are more exposed to the surrounding solvent during binding of Fld to Fpr and less affected by the quenching effects of the individual polypeptides.

Addition of an excess of NADPH to the 1:1 Fpr−Fld complex under aerobic conditions results in the formation of a stable flavodoxin semiquinone (Figure 1C) with a maximum at 580 nm, a shoulder around 620 nm, and an isosbestic point at 518 nm. A similar one-electron reduction of native *E. coli* flavodoxin by native Fpr has been observed under anaerobic conditions using either NADPH or sodium dithionite (*42*). Approximately 80% of Fld is present in the semiquinone form after 3 min (Figure 1C), and no significant change was observed over 30 min, despite the accumulation of NADPH (absorbance increase at 340 nm). Thus, under conditions similar to those of the P450c17 assay, the semiquinone of flavodoxin is predominant and therefore is the most probable electron donor to P450c17. Doubling the amount of flavodoxin increases the amount of semiquinone formed (Figure 1D).

Like their native counterparts, recombinant Fpr and Fld will support P450c17-catalyzed 17α-hydroxylation of progesterone (Figure 2). Previous experiments with the native enzymes at a 1:10 Fpr−Fld ratio (10 μM) achieved a turnover number of approximately 1. Here, a large molar excess (50-fold relative to P450c17) of a 1:1 Fpr−Fld ratio (10 μM) has been found to approach a maximal rate, producing a P450c17 turnover number of 5. Lower concentrations of the 1:1 ratio result in a linear decrease in P450c17 turnover (Figure 2, inset) while doubling the concentration of Fpr and Fld to 20 μM caused only a 20% increase in P450c17 turnover. In the presence of a large excess (50-fold) of flavodoxin, Fpr is able to reduce Fld under aerobic conditions at 1.6 nmol of Fld semiquinone (nmol of Fpr)$^{-1}$ min^{-1}. Since this value is much less than the rate of P450c17 turnover, a

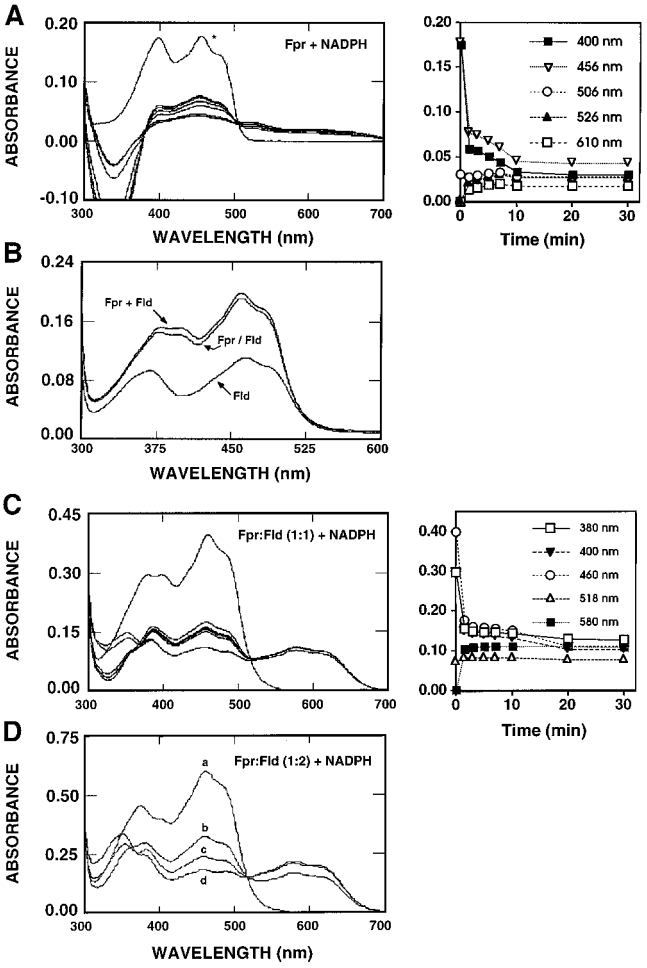

FIGURE 1: (A) Absorbance changes upon aerobic reduction of Fpr by NADPH. NADPH (0.15 mM final concentration) and glucose 6-phosphate (1.5 mM final concentration) were simultaneously added to 1 mL of 25 μM Fpr in 10 mM KP_i, pH 7.4, 20% glycerol, and 1.2 units of glucose-6-phosphate dehydrogenase [sample cuvette spectrum indicated by an asterisk (*)] and to an identical (reference) cuvette containing the same solution minus Fpr. Absorbance changes were recorded after NADPH addition at the time points indicated (right). (B) Absorbance changes upon mixing Fpr with Fld (1:1 molar ratio). Fld, absorbance spectrum of oxidized recombinant *E. coli* flavodoxin (25 μM, path length = 0.5 cm). Fpr (25 μM) and Fld (25 μM) were placed in separate chambers (path length = 0.5 cm) of a tandem cuvette, and absorbance spectra before (Fpr/Fld) and after (Fpr+Fld) mixing were recorded using buffer as a reference. (C) Aerobic reduction of a 1:1 Fpr−Fld (25 μM) mixture by NADPH (0.15 mM) in the presence of an NADPH-regenerating system as described in (A) above. Absorbance changes were recorded after NADPH addition at the time points indicated (right). (D) Aerobic reduction of a 1:2 Fpr−Fld (25:50 μM) mixture by NADPH (0.15 mM) in the presence of an NADPH-regenerating system as described in (A) above. Absorbance changes were recorded at 0 (a), 1.5 (b), 9 (c), and 18 (d) min after addition of NADPH.

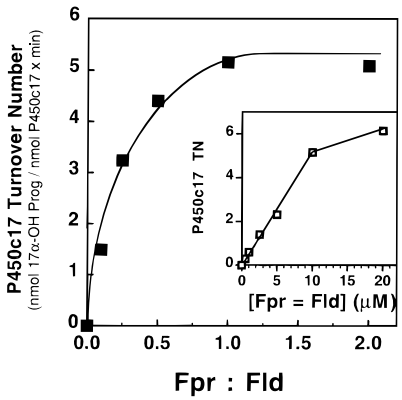

FIGURE 2: P450c17 progesterone 17α-hydroxylase activity reconstituted with recombinant Fpr and Fld. P450c17 (0.2 μM) samples were preincubated for 2 min at 37 °C in the presence of 10 mM KP_i, pH 7.4, 20% glycerol, DLPC (0.1 mg/mL), progesterone (50 μM), 0.6 unit of glucose-6-phosphate dehydrogenase, 10 μM Fld, and increasing amounts of Fpr before initiation with NADPH (0.3 μM final concentration) and glucose 6-phosphate (3 mM final concentration). Inset: P450c17 activity using equimolar ratios of Fpr to Fld.

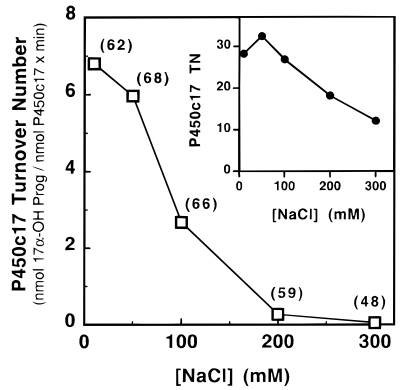

FIGURE 3: Effect of [NaCl] on Fpr−Fld (10 μM 1:1 ratio) and P450 reductase (0.2 μM, inset) supported P450c17 hydroxylase activity. Reaction conditions were similar to those described in Figure 2. Numbers in parentheses represent the percentage of Fld semiquinone (measured at 579 nm) after 4 min incubation at 37 °C.

two-electron process, electron transfer from flavodoxin to P450c17, must be faster than that from Fpr to Fld. Indeed, anaerobic stopped-flow experiments examining the transfer of the first electron to P450c17 (by formation of the reduced CO complex) using *E. coli* Fpr−Fld indicate that it is direct and occurs at an apparent rate of 2.4 min^{-1} when 20 μM Fpr, 20 μM Fld, and 2 μM P450c17 are used. Presumably, by using a large excess of Fpr and Fld relative to P450c17, the apparent rate-limiting step of flavodoxin reduction is overcome.

The ability of *E. coli* flavodoxin, both native and recombinant, to bind P450c17 and other microsomal P450s in an ionic-strength-dependent manner strongly suggests that electrostatic forces play an important role in this interaction (*35*, *40*). As shown in Figure 3, P450c17 activity, as reconstituted by either Fpr−Fld or P450 reductase (inset), is inhibited by high ionic strength (200−300 mM NaCl). This effect is more pronounced with the *E. coli* reductase system which is essentially unable to provide electrons to P450c17 above 200 mM NaCl. The ionic-strength-dependent activity profile observed with P450 reductase and P450c17 (inset) appears to be typical relative to other P450s which have been studied

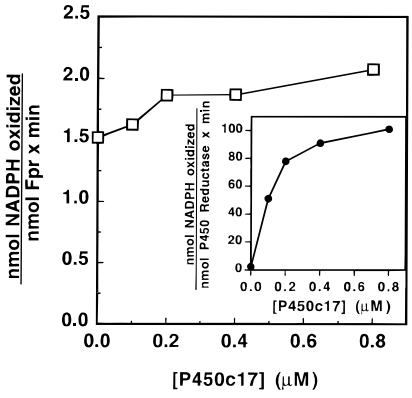

FIGURE 4: Rate of NADPH oxidation by Fpr−Fld (10 μM 1:1 ratio) and rat P450 reductase (0.2 μM, inset) in the presence of increasing amounts of P450c17. Samples in the presence of unlabeled progesterone (50 μM) and buffer minus glucose-6-phosphate dehydrogenase were preincubated at 37 °C for 2 min prior to addition of NADPH (0.15 mM final concentrated) and recording of the absorbance change at 340 nm.

(*50*, *51*). Specifically, these profiles are generally characterized by an increase in activity from low to moderate ionic strength, followed by a decline at higher buffer or salt concentrations. The loss of activity observed with the Fpr−Fld system under these conditions could be explained by disruption of either the Fpr−Fld and/or the Fld−P450c17 interaction. However, even at the highest salt concentration tested (300 mM), almost 50% of the available flavodoxin is in the semiquinone form, while at lower ionic strengths this value is not greater than 70%. Collectively, these data indicate that the Fld−P450c17 interaction, in terms of steady-state activity, is governed by electrostatic forces more strongly than that of Fpr−Fld.

In the absence of a suitable electron acceptor, either artificial or physiological, recombinant rat P450 reductase will oxidize NADPH at a rate of approximately 2 mol (mol of reductase)$^{-1}$ min^{-1} (Figure 4, inset). Upon coupling to an acceptor, such as P450c17 in the presence of substrate, diaphorase activity increases dramatically (50-fold) and hyperbolically as a function of P450c17 concentration. NADPH oxidation by a 1:1 complex of Fpr−Fld, by comparison, increases only 30% (Figure 4) in the presence of P450c17 and substrate. This modest P450-dependent increase in NADPH oxidation by Fpr−Fld [equivalent to 10 nmol of NADPH (nmol of P450c17)$^{-1}$ min^{-1}] is approximately 50% coupled relative to the rate of progesterone 17α-hydroxylation [5 nmol of product (nmol of P450c17)$^{-1}$ min^{-1}], which is similar to the coupling observed for P450 reductase [a 1:1 ratio of P450 reductase to P450c17 will hydroxylate approximately 40 nmol of progesterone (nmol of P450)$^{-1}$ min^{-1}]. Thus, the relatively low P450c17 reductase activity of the Fpr−Fld system is not due to a deficiency in the coupling of NADPH oxidation to substrate turnover, but rather probably due to differences in protein−protein interactions which ultimately determine the rates of electron transfer.

In addition to its 17α-hydroxylase activity, bovine cytochrome P450c17 cleaves the 17,20 carbon−carbon bond of 17α-OH-pregnenolone to form the C19 androgen dehydroepiandosterone (*52*). This reaction is catalyzed at a much slower rate than that of progesterone 17α-hydroxylation (Table 2). Interestingly, the hydroxylase to lyase ratio

Table 2: Comparison of P450 Reductase- and Fpr−Fld-Supported P450c17 Hydroxylase and Lyase Activities

	hydroxylase[a]	lyase[b]	hydroxylase/ lyase
P450 reductase	42 ± 2	1.6 ± 0.2	26
P450 reductase + b_5	22 ± 1	12 ± 1	1.8
Fpr−Fld	5.9 ± 0.1	0.19 ± 0.1	31
Fpr−Fld + b_5	0.2 ± 0.04	0.49 ± 0.08	0.4
P450 reductase + SOD[c] + catalase	39 ± 2	1.6 ± 0.2	24
Fpr−Fld + SOD[c] + catalase	5.7 ± 0.2	0.16 ± 0.02	36

[a] Progesterone 17α-hydroxylase activity [nmol of 17α-OH-progesterone (nmol of P450c17)$^{-1}$ min^{-1}]. [b] 17α-OH-Pregnenolone 17,20-lyase activity [nmol of DHEA (nmol of P450c17)$^{-1}$ min^{-1}]. [c] Superoxide dismutase.

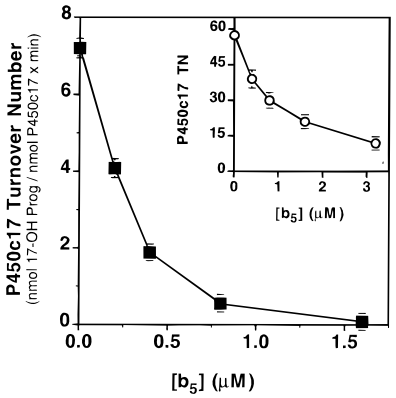

FIGURE 5: Effect of cytochrome b_5 on P450c17 progesterone 17α-hydroxylase activity as supported by Fpr−Fld (10 μM 1:1 ratio) and rat P450 reductase (1 μM, inset). Reaction conditions were similar to those described in Figure 2.

obtained using P450 reductase is very similar to that obtained with *E. coli* Fpr−Fld (26 vs 31). Inclusion of cytochrome b_5 (b_5) with either system decreases this ratio by increasing lyase activity (8-fold for P450 reductase and 2-fold for Fpr−Fld) and by inhibiting hydroxylase activity. The inhibition of hydroxylase activity is b_5-dependent (Figure 5) with the apparent K_i of b_5 equal to 0.8 μM in the presence of P450 reductase (1 μM) and 0.2 μM in the presence of a large excess of Fpr−Fld (1:1, 10 μM). Fpr alone or in combination with flavodoxin, like rat P450 reductase (*53*), can reduce recombinant human cytochrome b_5 [>50 nmol of b_5 reduced (nmol of Fpr)$^{-1}$ min^{-1}]. From this, it would appear that it is reduced b_5 which is inhibitory. Addition of a combination of superoxide dismutase and catalase has virtually no effect on either activity catalyzed by either reductase system, indicating that P450c17 activity is not mediated by superoxide or hydrogen peroxide (Table 2).

DISCUSSION

Escherichia coli, as probably all living organisms, possess an NADP(H)−ferredoxin (flavodoxin) reductase which, in this bacterium, fulfills several roles. Specifically, Fpr primarily functions to provide reduced flavodoxin which activates, directly or indirectly, cobalamin-dependent methionine synthase, pyruvate-formate lyase, anaerobic ribonucleotide reductase, and biotin synthase. A secondary, less well-defined role for Fpr could be in the defense against reactive oxygen. As a consequence of observing P450c17 activities in *E. coli* expressing P450c17 (*32*), we identified this enzyme, in combination with flavodoxin, as a soluble P450c17 reductase system (*35*). Subsequently, the catalytic activities of other microsomal cytochrome P450s have been observed to be supported by this system (*33*, *36*). In this report, we describe the overexpression of *E. coli* Fpr and further characterization of the Fpr−Fld system in comparison to rat NADPH−cytochrome P450 reductase.

Reduction of Fpr by NADPH under aerobic conditions similar to those used for the P450c17 reconstitution assay occurs primarily as a two electron reduction of the FAD cofactor. Although some semiquinone Fpr may be present, the rapid formation of the hydroquinone is in agreement with previous anaerobic experiments with the native enzyme (*42*). In this respect, *E. coli* Fpr is similar to other FNRs such as spinach NADP$^+$−ferredoxin reductase (*3*) and adrenodoxin reductase (*5*) for which the semiquinone states of each enzyme are relatively unstable. Not surprisingly, the redox potential for the two-electron reduction of Fpr is essentially the same or perhaps more electropositive [−300 mV (*42*)] than that for NADP$^+$/NADPH [−317 mV (*54*)].

Recombinant Fpr, like its native counterpart, binds Fld and reduces it to the semiquinone in the presence of NADPH under aerobic conditions. The 2e$^-$ reduced form of Fld has been reported to form under anaerobic conditions in the presence of Fpr and an NADPH-regenerating system, although only half the available Fld could be fully reduced (*22*). Certainly, under the conditions of the P450c17 assay, the majority of the flavodoxin (80%) can be accounted for as the blue, neutral semiquinone with no detectable further reduction of this species, even when using a stoichiometric amount of Fpr. The polypeptide of Fld decreases both the oxidized/semiquinone ($E_{ox/sq}$) and semiquinone/reduced ($E_{sq/red}$) midpoint potentials of FMN [$E_{ox/sq} = -238$ mV and $E_{sq/red} = -172$ mV (*55*)] to −285 and −455 mV, respectively (*56*). The extremely low Fld $E_{sq/red}$ couple provides a thermodynamic reason for the very low level of fully reduced flavodoxin in the presence of Fpr and NADPH. Structurally, this lowering of the FMN $E_{sq/red}$ redox potential by the Fld polypeptide is probably due to restriction of protonation at N(1) of the isoalloxazine ring and/or destabilization of the hydroquinone negative charge due to charge repulsion caused by neighboring acidic amino acid residues, as has been reported for other flavodoxins (*16*, *57*). The precise environment of the isoalloxazine N(1), however, cannot be discerned from the present structure of *E. coli* Fld (*49*). Interestingly, the ox/sq couple of *E. coli* Fld (−285 mV) is comparable to that of the microsomal P450 reductase FMN sq/red potential [−270 mV (*58*, *59*)]. It is the hydroquinone ($FMNH_2$) and not the stable one-electron-reduced form (FAD/FMNH$^\bullet$) of P450 reductase which has been established to be the electron donor to microsomal cytochrome P450s (*60*−*62*). In comparison, recent experiments with P450BM-3 indicate that the FMN semiquinone, either red anionic (*63*) or blue neutral (*64*), is the electron donor to the BM-3 heme, whereas the hydroquinone has been demonstrated to be a catalytic "dead end" (*63*). Thus, the redox states of the protein-bound FMNs capable of electron transfer to the appropriate P450 hemes are similar for the *E. coli* reductase (blue neutral FMNH$^\bullet$ to microsomal P450s, as well as other similar FNR−Fld systems, e.g., *Anabaena*) and the flavoprotein domain of P450BM-3 (FMN$^{-\bullet}$ or FMNH$^\bullet$ to the heme domain), but

distinct from NADPH−cytochrome P450 reductase ($FMNH_2$ to microsomal P450s).

The interaction of P450 reductase with microsomal P450s is at least partially electrostatic as determined by results from chemical modification (*65*−*68*), chemical cross-linking (*69*, *70*), ionic strength (*50*, *51*), and site-directed mutagenesis (*71*) experiments. The effect of ionic strength on P450c17 activity as supported by P450 reductase (Figure 3, inset) would appear to be typical. That is, the highest activity observed at moderate ionic strength is an optimal balance of electrostatic attraction and repulsion between the two proteins during catalytic turnover. The activity profile of the *E. coli* Fpr−Fld system is clearly different. Namely, Fpr−Fld-supported P450c17 activity is inversely proportional to ionic strength, and it is inhibited to a greater extent relative to the membrane-bound P450 reductase under the same conditions. Hydrophobic forces between the P450 reductase membrane anchor and P450c17 may help tether the complex and make it more resistant to ionic strength effects than the Fld−P450c17 interaction. Interestingly, the amount of flavodoxin semiquinone at 200−300 mM ionic strength is only 25% less than that present at low ionic strength where P450c17 activity is greatest. This would suggest that electron transfer between Fld and Fpr is less sensitive to ionic strength than the Fld−P450c17 interaction and that the observed loss of P450c17 activity is due primarily to a disruption of the Fld−P450c17 complex.

Inclusion of cytochrome b_5 in P450 reconstitution systems has been observed to have stimulatory, inhibitory, or negligible effects on a multitude of P450-catalyzed reactions (*72*−*74*). In several cases, the effect of b_5 is in part determined by the P450 substrate and the rate at which it is metabolized (*75*). For cytochrome P450c17, b_5 has been shown to dramatically stimulate the 17,20-lyase reaction for bovine (*76*) and human (*77*) P450c17, while the hydroxylase reaction is comparatively unaffected. While Estabrook and co-workers (*76*) have detected a slight decrease in progesterone 17α-hydroxylation using a bovine P450c17/rat P450 reductase fusion protein in combination with recombinant rat b_5, we have observed a more dramatic decrease using the separate proteins (bovine P450c17 and rat P450 reductase or Fpr−Fld) and recombinant human cytochrome b_5 (Figure 5). One possible explanation for these results is that b_5 is competing for a mutually exclusive binding site on P450c17 as that for P450 reductase or *E. coli* Fld in a manner perhaps analogous to the competitive binding of b_5/putidaredoxin for P450cam (*78*). Since cytochrome b_5 has been proposed to be capable of donating only the second, but not the first, electron in the P450 catalytic cycle (*79*, *80*), it would be expected that, at least during the relatively fast and coupled hydroxylase reaction, b_5 might have an inhibitory effect. Furthermore, the equilibrium association of cytochrome b_5 with P450c17 in the presence of a large excess of Fld (refer to Figure 5) appears to greatly favor the more physiological P450c17−b_5 interaction over P450c17−Fld. For the slower lyase reaction, the beneficial influence of b_5, of increasing the coupling efficiency of the P450c17 reaction (*53*), may outweigh its inhibitory effects. *E. coli* Fpr−Fld can, in fact, support the lyase activity of P450c17 (Table 2), in contrast to our initial observations (*35*), which is enhanced 2-fold by b_5. Further investigation will be required to address whether the *E. coli* reductase system can provide insight into the differing effects of b_5 on P450c17 hydroxylase and lyase activities.

In summary, *E. coli* flavodoxin and NADPH−flavodoxin reductase are, not unexpectedly, quite different from the physiological eukaryotic P450 reductase in many respects. Both proteins are soluble, and neither possesses domains resembling the N-terminal membrane anchor or the FMN−FAD linker domains of P450 reductase. Absence of a Fld membrane anchor may partially explain the dramatic effect of ionic strength on P450c17 activity. Furthermore, absence of a domain to connect and tightly couple individual Fpr and Fld proteins may contribute to the relatively low enhancement of NADPH oxidation observed in the presence of P450c17. Importantly, differences in the redox potentials (as determined by protein environment) of the P450 reductase and *E. coli* flavodoxin FMN cofactors determine which reduced states are effective electron donors. For *E. coli* Fld, electron transfer to P450c17 is most likely mediated by the blue, neutral FMN semiquinone and not $FMNH_2$, the electron donor of P450 reductase to microsomal P450s. Despite these differences, however, bacterial Fpr−Fld systems may have useful applications in the study of microsomal P450−electron donor interactions and electron transfer between these proteins.

REFERENCES

1. Aliverti, A., Bruns, C. M., Pandini, V. E., Karplus, P. A., Vanoni, M. A., Curti, B., and Zanetti, G. (1995) *Biochemistry 34*, 8371−8379.
2. Batie, C. J., and Kamin, H. (1984) *J. Biol. Chem. 259*, 11976−11985.
3. Batie, C. J., and Kamin, H. (1986) *J. Biol. Chem. 261*, 11214−11223.
4. Brandt, M. E., and Vickery, L. E. (1993) *J. Biol. Chem. 268*, 17126−17130.
5. Lambeth, J. D., and Kamin, H. (1976) *J. Biol. Chem. 251*, 4299−4306.
6. Medina, M., Mendez, E., and Gomez-Moreno, C. (1992b) *FEBS Lett. 298*, 25−28.
7. Pueyo, J. J., Gomez-Moreno, C., and Mayhew, S. G. (1991) *Eur. J. Biochem. 202*, 1065−1071.
8. Serre, L., Vellieux, F. M., Medina, M., Gomez-Moreno, C., Fontecilla-Camps, J. C., and Frey, M. (1996) *J. Mol. Biol. 263*, 20−39.
9. Shin, M., and Arnon, D. I. (1965) *J. Biol. Chem. 240*, 1405−1411.
10. Fillat, M. F., Sandmann, G., and Gomez-Moreno, C. (1988) *Arch. Microbiol. 150*, 160−164.
11. Hanukoglu, I. (1992) *J. Steroid Biochem. Mol. Biol. 43*, 779−804.
12. Okuda, K. I. (1994) *J. Lipid Res. 35*, 361−372.
13. Pueyo, J. J., Curley, G. P., and Mayhew, S. G. (1996) *Biochem. J. 313*, 855−861.
14. Stockman, B. J., Krezel, A. M., Markley, J. L., Leonhardt, K. G., and Straus, N. A. (1990) *Biochemistry 29*, 9600−9609.
15. Ludwig, M. L., Pattridge, K. A., Metzger, A. L., Dixon, M. M., Eren, M., Feng, Y., and Swenson, R. P. (1997) *Biochemistry 36*, 1259−1280.
16. Zhou, Z., and Swenson, R. P. (1995) *Biochemistry 34*, 3183−3192.
17. Zhou, Z., and Swenson, R. P. (1996) *Biochemistry 35*, 15980−15988.
18. Cheddar, G., Meyer, T. E., Cusanovich, M. A., Stout, C. D., and Tollin, G. (1986) *Biochemistry 25*, 6502−6507.
19. De Francesco, R., Edmondson, D. E., Moura, I., Moura, J. J., and LeGall, J. (1994) *Biochemistry 33*, 10386−10392.
20. Medina, M., Hervas, M., Navarro, J. A., De La Rosa, M. A., Gomez-Moreno, C., and Tollin, G. (1992a) *FEBS Lett. 313*, 239−242.

21. Fujii, K., and Huennekens, F. M. (1974) *J. Biol. Chem. 249*, 6745−6753.
22. Blaschkowski, H. P., Neuer, G., Ludwig-Festl, M., and Knappe, J. (1982) *Eur. J. Biochem. 123*, 563−569.
23. Bianchi, V., Reichard, P., Eliasson, R., Pontis, E., Krook, M., Jornvall, H., and Haggard-Ljungquist, E. (1993) *J. Bacteriol. 175*, 1590−1595.
24. Birch, O. M., Fuhrmann, M., and Shaw, N. M. (1995) *J. Biol. Chem. 257*, 19158−19165.
25. Ta, D. T., and Vickery, L. E. (1992) *J. Biol. Chem. 267*, 11120−11125.
26. Liochev, S. I., Hausladen, A., Beyer, W. F., Jr., and Fridovich, I. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1328−1331.
27. Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. M., and Kredich, N. M. (1989) *J. Biol. Chem. 264*, 15796−15808.
28. Andrews, S. C., Shipley, D., Keen, J. N., Findlay, J. B. C., Harrison, P. M., and Guest, J. R. (1992) *FEBS Lett. 302*, 247−252.
29. Porter, T. D., and Kasper, C. B. (1986) *Biochemistry 25*, 1682−1687.
30. Oster, T., Boddupalli, S. S., and Peterson, J. A. (1991) *J. Biol. Chem. 266*, 22718−22725.
31. Marletta, M. A. (1993) *J. Biol. Chem. 268*, 12231−12234.
32. Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5597−5601.
33. Dong, M. S., Yamazaki, H., Guo, Z., and Guengerich, F. P. (1996) *Arch. Biochem. Biophys. 327*, 11−19.
34. Porter, T. D., Wilson, T. E., and Kasper, C. B. (1987) *Arch. Biochem. Biophys. 254*, 353−367.
35. Jenkins, C. M., and Waterman, M. R. (1994) *J. Biol. Chem. 267*, 27401−27408.
36. Yamazaki, H., Ueng, Y. F., Shimada, T., and Guengerich, F. P. (1995) *Biochemistry 34*, 8380−8389.
37. McCarthy, J. L., and Waterman, M. R. (1988) *J. Steroid Biochem. 29*, 307−312.
38. Shen, A. L., Porter, T. D., Wilson, T. E., and Kasper, C. B. (1989) *J. Biol. Chem. 264*, 7584−7589.
39. Hanna, I. H., Teiber, J. F., Kokones, K. L., and Hollenberg, P. F. (1998) *Arch. Biochem. Biophys. 350*, 324−332.
40. Jenkins, C. M., Pikuleva, I., Kagawa, N., and Waterman, M. R. (1997) *Arch. Biochem. Biophys. 347*, 93−102.
41. Bordier, C. (1981) *J. Biol. Chem. 256*, 1604−1607.
42. Fujii, K., Galivan, J. H., and Huennekens, F. M. (1977) *Arch. Biochem. Biophys. 178*, 662−670.
43. Brock, B. J., Rieble, S., and Gold, M. H. (1995) *Appl. Environ. Microbiol. 61*, 3076−3081.
44. Ingelman, M., Bianchi, V., and Eklund, H. (1997) *J. Mol. Biol. 268*, 147−157.
45. Cusanovich, M. A., Hazzard, J. T., Meyer, T. E., and Tollin, G. (1988) *Prog. Clin. Biol. Res. 274*, 401−418.
46. Mayhew, S. G., and Tollin, G. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) pp 389−426, CRC Press, Boca Raton.
47. Yasukochi, Y., and Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337−5344.
48. Fillat, M. F., Edmondson, D. E., and Gomez-Moreno, C. (1990) *Biochim. Biophys. Acta. 1040*, 301−307.
49. Hoover, D. M., Jarrett, J. T., Sands, R. H., Dunham, W. R., Ludwig, M. L., and Matthews, R. G. (1997) *Biochemistry 36*, 127−138.
50. Schenkman, J. B., Voznesensky, A. I., and Jansson, I. (1994) *Arch. Biochem. Biophys. 314*, 234−241.
51. Yun, C., Song, M., Ahn, T., and Kim, H. (1996) *J. Biol. Chem. 271*, 31312−31316.
52. Zuber, M. X., Simpson, E. R., and Waterman, M. R. (1986) *Science 234*, 1258−1261.
53. Holmans, P. L., Shet, M. S., Martin-Wixtrom, C. A., Fisher, C. W., and Estabrook, R. W. (1994) *Arch. Biochem. Biophys. 312*, 554−565.
54. Rodkey, F. L., and Donovan, J. A. (1959) *J. Biol. Chem. 234*, 677−680.
55. Draper, R. D., and Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802−808.
56. Vetter, H., Jr., and Knappe, J. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 433−446.
57. Ludwig, M. L., and Luschinsky, C. L. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) pp 427−466, CRC Press, Boca Raton.
58. Iyanagi, T., Makino, N., and Mason, H. S. (1974) *Biochemistry 13*, 1701−1710.
59. Vermilion, J. L., and Coon, M. J. (1978) *J. Biol. Chem. 253*, 8812−8819.
60. Iyanagi, T., Makino, R., and Anan, F. K. (1981) *Biochemistry 20*, 1722−1730.
61. Oprian, D. D., Vatsis, K. P., and Coon, M. J. (1979) *J. Biol. Chem. 254*, 8895−8902.
62. Vermilion, J. L., Ballou, D. P., Massey, V., and Coon, M. J. (1981) *J. Biol. Chem. 256*, 266−277.
63. Sevrioukova, I., Shaffer, C., Ballou, D. P., and Peterson, J. A. (1996) *Biochemistry 35*, 7058−7068.
64. Murataliev, M. B., Klein, M., Fulco, A., and Feyereisen, R. (1997) *Biochemistry 36*, 8401−8412.
65. Bernhardt, R., Kraft, R., Otto, A., and Ruckpaul, K. (1988) *Biomed. Biochim. Acta 47*, 581−592.
66. Nadler, S. G., and Strobel, H. W. (1991) *Arch. Biochem. Biophys. 290*, 277−284.
67. Shen, S., and Strobel, H. W. (1992) *Arch. Biochem. Biophys. 294*, 83−90.
68. Shen, S., and Strobel, H. W. (1993) *Arch. Biochem. Biophys. 304*, 257−265.
69. Nadler, S. G., and Strobel, H. W. (1988) *Arch. Biochem. Biophys. 261*, 418−429.
70. Nisimoto, Y., and Otsuka-Murakami, H. (1988) *Biochemistry 27*, 5869−5876.
71. Shimizu, T., Tateishi, T., Hatano, M., and Fujii-Kuriyama, Y. (1991) *J. Biol. Chem. 266*, 3372−3375.
72. Gorsky, L. D., and Coon, M. J. (1986) *Drug Metab. Dispos. 14*, 89−96.
73. Peterson, J. A., and Prough, R. A. (1986) in *Cytochrome P-450: Structure, Mechanisms, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 89−118, Plenum Press, New York.
74. Gruenke, L. D., Konopka, K., Cadieu, M., and Waskell, L. (1995) *J. Biol. Chem. 270*, 24707−24718.
75. Morgan, E. T., and Coon, M. J. (1984) *Drug Metab. Dispos. 12*, 358−364.
76. Shet, M. S., Fisher, C. W., Arlotto, M. P., Shackleton, C. H., Holmans, P. L., Martin-Wixtrom, C. A., Saeki, Y., and Estabrook, R. W. (1994) *Arch. Biochem. Biophys. 311*, 402−417.
77. Katagiri, M., Kagawa, N., and Waterman, M. R. (1995) *Arch. Biochem. Biophys. 317*, 343−347.
78. Stayton, P. S., Poulos, T. L., and Sligar, S. G. (1989) *Biochemistry 28*, 8201−8205.
79. Hildebrandt, A., and Estabrook, R. W. (1971) *Arch. Biochem. Biophys. 143*, 66−79.
80. Pompon, D., and Coon, M. J. (1984) *J. Biol. Chem. 259*, 15377−15385.

BI973076P