

# Characterization of the FAD Binding Domain of Cytochrome P450 Reductase<sup>1</sup>

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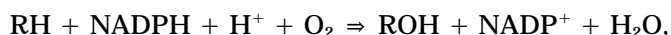
Received July 14, 1995, and in revised form October 12, 1995

**The redox potentials of FAD and FMN of Cytochrome P450 reductase (reductase) are equivalent in solution but differ by 138 mV when bound to reductase. The interaction of each flavin with its flavin binding domain confers the unique electron transferring abilities to each flavin. In order to determine flavin binding properties and activity of the FAD binding domain, we have expressed in pTrcHis three fragments (1161, 1244, and 1556 bp) of rat liver reductase cDNA encompassing the proposed FAD and NADPH binding domain. The FAD binding fragments from cells harboring the 1161- and 1556-bp-containing vectors were stable and bound 0.66 and 0.71 mol FAD/mol enzyme, respectively. Both fragments reduce ferricyanide (54 and 104% of FMN-less reductase/mol bound flavin, respectively) and participate in the transhydrogenation reaction of 3-AcPy-ADP (41 and 65% of FMN-less reductase/mol bound flavin, respectively). FAD-less fragments were purified and reconstituted with 8-amino-FAD and 8-chloro-FAD to determine binding efficiencies.** © 1996 Academic Press, Inc.

**Key Words:** Cytochrome P450 reductase; flavin binding; expression, PCR.

P450 reductase<sup>4</sup> is a flavoprotein that provides the MFO system with reducing equivalents. The MFO has

been implicated in the processes of carcinogenesis and detoxification and is minimally composed of P450, reductase, and lipid, the active component being phosphatidylcholine (1). The general mechanism for this system is the hydroxylation reaction



where RH represents a large variety of compounds including drugs, polycyclic aryl hydrocarbons, fatty acids, pesticides, and chemical carcinogens (2–4). Rat liver reductase is composed of a 78,225-Da single polypeptide chain which binds one molecule each of FMN and FAD (5–7) and is attached to the endoplasmic reticulum by an N-terminal hydrophobic membrane binding domain. The details of reductase structure and function are of particular importance because there is only one form of reductase identified per species which may interact with all of the different forms of P450. Reductase may also provide an interesting model for the structure of flavoproteins and the evolution of proteins since it has been proposed to have arisen from the fusion of two separate genes, each encoding proteins that specifically bind either FMN or FAD (8).

Reductase was first purified without the hydrophobic domain by steapsin solubilization, found to be catalytically competent with many electron acceptors (with the notable exception of P450) and thus was termed the catalytic domain. Detergent-solubilized reductase, or holoreductase, was later purified by our laboratory (9) and others (6, 10–14) in the native (full-length) form. Holoreductase is capable of reducing P450 as well as other electron acceptors (ferricyanide, cyt c, menadi-one, DCIP, 3-AcPyADP) in a reconstituted system.

The rat reductase cDNA has been cloned, sequenced, and expressed in a prokaryotic system (15). Three contiguous function domains have been elucidated: a hydrophobic domain, and two flavin binding domains. Identification of the prosthetic group binding sites were

<sup>1</sup> This research was partly supported by Grant CA53191 from the National Cancer Institute, DHHW.

<sup>2</sup> Some of the data in this paper are taken from the doctoral thesis project of A.V.H.

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<sup>4</sup> Abbreviations used: 3-AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate; CG, circlegrow; cyt c, cytochrome c; P450, cytochrome P450; DCIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; kb, kilobase pairs; PAGE, polyacrylamide gel electrophoresis; MFO, mixed function oxidase; reductase, NADPH cytochrome P450; SDS, sodium dodecyl sulfate.

based on homology studies of a family of flavoenzymes which includes ferredoxin-NADP<sup>+</sup>, NADPH-sulfite reductase, NADH-cytochrome b<sub>5</sub> reductase, glutathione reductase, and NADPH cytochrome P450 reductase (8, 16).

In solution FMN and FAD maintain the same redox potential (17); however, the unique interaction of each flavin within its own domain produces a 138-mV difference between the reductase-bound flavins (18). Understanding the interaction of each flavin with the reductase is central to discerning the enzymatic mechanism of reductase. Chemical modification studies suggest that cysteine and lysine (19–24) residues are involved in reductase catalysis and binding of prosthetic groups to reductase. Two residues in the FMN binding domain were confirmed by site-directed mutagenesis (25). Reductase purified without the hydrophobic domain has recently been crystallized and the unit cell dimensions were determined, but as of this writing, the crystal structure has not yet been defined (26).

Preparations of reductase with a selectively removed flavin, or aporeductases, have been used to identify electron acceptors specific for each reductase-bound flavin. FAD-less reductase (27) contains one molecule of FMN per one molecule of reductase and FMN-less reductase (28) contains one molecule of FAD per molecule of reductase. The FAD binding domain has been more difficult to study using flavin removal techniques than the FMN binding domain because FAD binds more tightly to reductase than FMN. FAD-less reductase has very little ability to donate electrons to one-electron acceptors (such as cyt c or P450) or to accept electrons from two-electron donors such as the physiologically intended donor NADPH (27). FMN-less reductase is able to utilize NADPH to reduce ferricyanide and participate in the transhydrogenation of 3-AcPyADP. Aporeductase studies have demonstrated that electrons flow two at a time (28), from NADPH to FAD, and are then shuttled one at a time to FMN and subsequently to P450. These data are consistent with FAD serving as the direct acceptor of electrons from NADPH, interflavin electron transfer one at a time, and FMN as the sole electron donor to substrates (cyt c, NBT, and DCIP). Thus, each flavin serves as an independent redox center in reductase. The conclusions of single flavin aporeductase studies may be limited due to incomplete removal of one flavin and the ability of each flavin to dissociate from its flavin binding site and bind to the other site, presumably generating some background electron transport activities, spectral peaks, and flavin contents. The flavin background problems were eliminated by expression and purification of enzymatically active reductase fragments encompassing the putative FAD and NADPH binding domains. We have previously reported the cloning and expression of the FAD and NADPH domain of rat re-

ductase (29). In the course of our work on rat reductase, Smith *et al.* (30) reported the expression of the FAD and NADPH domain of human reductase. Enzymatic activities of both rat and human fragments and the spectra of the human reductase fragment were consistent with the fragments binding FAD.

In order to extend these FAD domain-focused studies, quantify FAD binding, and more specifically determine residues which confer FAD and NADPH binding, three differently sized cDNA fragments of the rat reductase putative FAD and NADPH binding domain were ligated into pTrcHis. The purified protein products from two of these plasmids were compared to FMN-less rat reductase with respect to FAD and FAD analog binding properties and enzymatic competence.

## MATERIALS AND METHODS

**Chemicals and biochemicals.** Renex 690 was supplied by ICI America (Atlanta, GA). Molecular weight markers were from Bio-Rad (Richmond, CA). Restriction endonucleases and ligase were obtained from Promega (Madison, WI), New England Biolabs (Beverly, MA), or Boehringer Mannheim (Indianapolis, IN). GeneClean II and CG media were from Bio 101 (La Jolla, CA). Biozyme Laboratories Intl. Ltd. (San Diego, CA) supplied Enterokinase. Cloning vector pKK233-3 and its upstream primer were purchased from Pharmacia (Madison, WI).

**Protein analytical methods.** Protein concentration was determined by bicinchoninic acid, supplied by and performed according to Pierce Chemical Co. (Rockford, IL). SDS-PAGE (31) and protein immunoblot (32) were conducted as previously described. Cyt c, DCIP, ferricyanide, menadione, and 3-AcPyADP reduction assays were determined spectrophotometrically and performed in triplicate as previously described (27).

**Cloning and sequencing of reductase.** Recombinant DNA techniques were performed as described (33). A 2.1-kb-pair cDNA clone that spans from nucleotides 193–2293 of reductase was isolated from a rat liver library by Dr. James W. White in our laboratory by hybridization to a polyclonal rabbit anti-rat reductase antibody and restriction digestion analysis. The reductase cDNA was subcloned into pKK233-2 (Pharmacia) and confirmed by protein immunoblot and nucleotide sequencing, performed according to the U.S. Biochemicals Sequenase (Cleveland, OH) protocol. The sequence and nucleotide location of oligonucleotides used for DNA sequencing and PCR are shown in Table I. The nucleotide sequences of the clones were identical to the published sequence of the corresponding region of the rat liver sequence (15). *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). The primer sets OR25/18 and OR23/18 were amplified in 20 mM Tris-Cl, pH 8.2, 10 mM KCl, 6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1% Triton X-100, 2 mM MgCl<sub>2</sub> and annealed at 65°C. Set OR21/18 was amplified in 20 mM Tris-Cl, pH 8.2, 10 mM KCl, 6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1% Triton X-100, 5.5 mM MgCl<sub>2</sub> and annealed at 40°C. PCR was performed as previously described (34).

**Construction of reductase expression vectors.** PCR products were ligated directly into pCRII (Invitrogen, San Diego, CA) using the TA Cloning Kit according to the manufacturer's instruction. The *EcoRI* fragment from each subcloned pCRII vector was ligated into the *EcoRI* site of pTrcHis (Invitrogen), an oligo-His sequence containing expression vector, to create the reductase expression vectors pAH21/18, pAH23/18, and pAH25/18. A schematic diagram showing reductase protein products from these plasmids is presented in Fig. 1. Holoreductase and its putative flavin and NADPH binding domains (35) are illustrated for comparison. *EcoRI* fragments were isolated on 1% agarose gels and purified according to the instructions of Gene-

**TABLE I**  
Sequencing and PCR Primers

Nucleotide location	Sequence names	Sequence (5'–3')
466–483	OR19	CTTCTATGACTGGCTGCA
664–681	OR21	CACGTGGAGGGGAGCAGTT
976–993	OR23	TGTGTACCCAGCCAATGA
1051–1068	OR25	GTCTCTAAACAATCTCGA
1230–1247	OR15	CCTCGCCTGAGGATGACG
1310–1327	OR8	GACTACCCATACCTGGCGG
1474–1493	OR9	GGGGGTGGCCACTAGCTGGC
1707–1727	OR10	CCTCATCCGAGCGCCGGCAGC
1707–1724	OR13	GCTGCCGGCGCTCGGATG
1816–1831	OR11	CTGGACATAGACCTTG
1874–1891	OR14	CACGAGGGCGGTGCCAC
2154–2171	OR20	CAGGCCAGGAGGAGTCAG
2202–2219	OR18	CTGATACAATGGACCGGG
2231–2249	OR16	CTCTTGCCATGTGCCCTGG

Clean II. Top 10F Electrocompetent Cells (Invitrogen) were transformed with a BTX Electro Cell Manipulator 600 in a 2-mm-gap BTX disposable cuvette at 2.5 kV/resistance, 129 ohm, and 2.45 kV.

Cells harboring expression vectors pAH21/18, pAH23/18, and pAH25/18 were screened by protein immunoblotting. Vectors pAH25/18 and pAH21/18 each directed the expression of an IPTG inducible, immunoreactive protein fragment of the appropriate apparent molecular weight (52 or 55 kDa, respectively) protein (data not shown). No bands were present in the lanes for vector only or after zero time of induction. The protein immunoblot of pAH 23/18 indicated that the protein was partially degraded and therefore, the protein was not used in any further experiments.

**Large-scale cell growth.** Typically six 1-liter flasks of CG medium supplemented with ampicillin (100  $\mu$ g/ml) and riboflavin (1  $\mu$ g/ml) were each inoculated with 10 ml of an overnight culture and shaken at 275 rpm at 37°C. When the optical density at 600 nm reached 0.6 units, the cultures were treated with IPTG (240  $\mu$ g/ml). Cultures were allowed to continue shaking for 4 h. Cells were pelleted at 2500g for 20 min and stored at –70°C until further purification.

**Cell lysis.** Pelleted cells from 6 liters of cell culture were resuspended on ice in 120 ml of 100 mM Tris–Cl, pH 7.7, 1 mM EDTA, 0.1 mM DTT, 250  $\mu$ M PMSF. Resuspended cells were lysed with a large-sized probe secured to a Model W185 Cell Sonicator (Branson Sonic Power Co., Plainview, NY) and treated with RNase and DNase as stated in the EXPRESS system instructions. Cellular debris was removed by centrifugation at 3000g for 15 min and passage through a 0.45- $\mu$ m filter.

**DEAE–Sephadex A-25 chromatography.** The protein purification was a modification of the holoreductase purification method (11). The cellular lysate was applied to a 33.7-ml bed volume DEAE A-25 column preequilibrated with A-25 buffer (100 mM Tris–Cl, pH 7.2 (at 4°C), 1 mM EDTA, 0.15% Renex, 0.1 mM DTT). When the leading red band reached the bottom of the column, the column was eluted with a 100-ml gradient of 0–0.4 M KCl in A-25 buffer. Fractions were sequentially analyzed for absorption at 280 and 454 nm, ability to reduce ferricyanide, and ultimately for protein immunoreactivity. Fractions reacting positively with reductase antibody were pooled (220 ml).

**Nickel affinity chromatography.** Chromatography was performed using nickel affinity columns, which took advantage of the histidine-rich nature of the leader sequence of the fusion protein, as described by Invitrogen's EXPRESS System using Native Binding Buffer

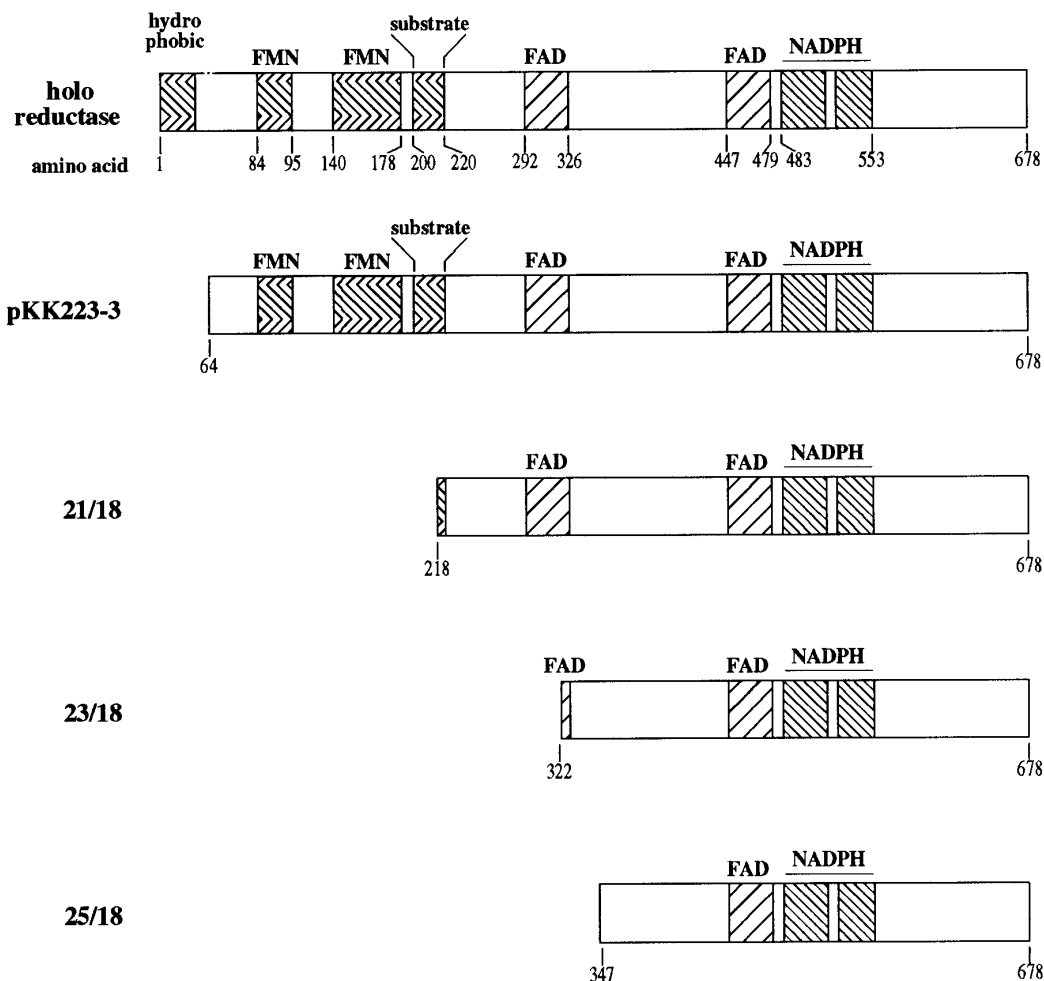
(NBB). Four 1.5-ml fractions were collected and analyzed by protein immunoblotting. Typically the first two fractions contained almost all of the eluted protein and were pooled. The eluate was rechromatographed on the column in the same manner in an effort to separate a 79-kDa band that copurified with the flavin binding fragments. The leader sequences were cleaved with 1  $\mu$ g of enterokinase/mg fragment in 50 mM sodium acetate, pH 5.0, 10 mM CaCl<sub>2</sub>, 0.1% Triton X-100 overnight at 37°C in the dark.

**Flavin determination and FAD analog reconstitution assays.** Concentrations of flavin were determined as previously described (36) on a Perkin Elmer LS-5 Fluorescence Spectrophotometer with emission slit 10, excitation slit 15, and fixed absorbance scale 10 at pH 2.6 and 7.7. Concentrations of flavin standard solutions were determined using  $\epsilon_{450} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$  for FAD and  $\epsilon_{450} = 12.2 \text{ mM}^{-1} \text{ cm}^{-1}$  for FMN. FAD analogs were generous gifts of Dr. Vincent Massey (Dept. of Biol. Chem., Univ. of Michigan). Concentrations for stock solutions of flavins were calculated from the molar extinction coefficients: 8-amino-FAD, 46.2  $\text{mM}^{-1} \text{ cm}^{-1}$  at 482 nm; and 8-chloro-FAD, 10.6  $\text{mM}^{-1} \text{ cm}^{-1}$  at 448 nm. FAD-depleted FAD binding fragments were prepared by a modification of the procedure described for the FAD-bound fragments. Riboflavin was not added to the medium and cells were grown for only 2 h after IPTG induction. Aliquots of flavin analog were added to a solution of 2.5  $\mu$ M FAD-depleted FAD binding fragments at 25°C in 100 mM KPi, pH 7.7, 10% (v/v) glycerol, 0.1 mM EDTA. With the excitation at 450 nm, fluorescence emission was monitored at 525 nm after each aliquot of flavin analog was added to FAD-depleted enzyme until a stable value was reached.  $K_d$  values for FAD and 8-Cl-FAD were determined by Lineweaver–Burk plots. The  $K_d$  values for 8-NH-FAD were determined spectrophotometrically.

## RESULTS AND DISCUSSION

Cytochrome P450 reductase is an unusual mammalian enzyme in that it binds two flavin moieties, FMN and FAD, which are each proposed to bind a specific domain within the reductase protein and confer electron transport activity. Identification of the electron acceptors for the two aporeductases, FMN-less (37) and FAD-less (38), has contributed greatly to the understanding of the role of flavins bound to reductase; however, both studies are limited in their conclusions due to flavin contamination of the presumed flavin-free redox centers. Further complicating these studies is the possibility that the flavin observed bound to reductase was not exclusively bound to the original flavin binding site, but instead partially or totally dissociated from one redox center and bound to the other site. If either of these possibilities were the case, the assignment of the proposed electron flavin binding sites and the electron entry and egress points from reductase may have been compromised. To address this issue in the study of the FAD binding domain, we have designed reductase expression vectors that contain only the FAD and NADPH binding domains of reductase and therefore exclude the possibility of contamination from the FMN binding site.

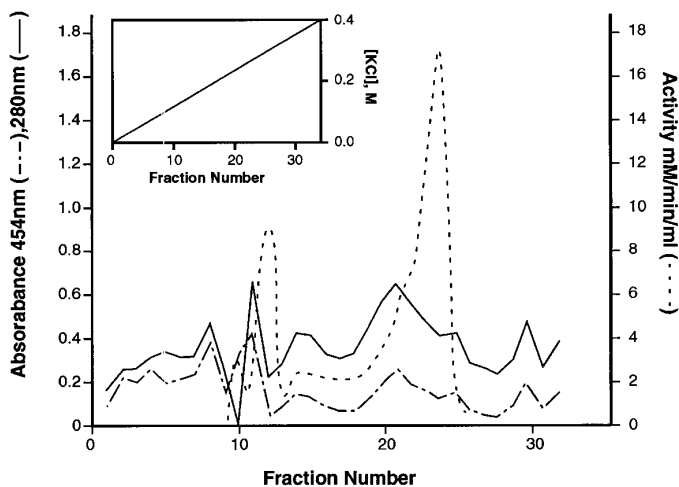
**Purification of FAD binding fragments.** Cell lines harboring pAH25/18 and pAH21/18 plasmids were grown and harvested as described under Materials and Methods. The FAD binding fragments 21/18 and 25/18



**FIG. 1.** FAD and NADPH binding domain fragments. A diagram of the holoreductase is shown with a labeled hydrophobic domain, putative prosthetic binding sites, and their corresponding amino acid residues as reported by Porter and Kasper (35). The putative binding site for FAD includes residues 292–326 and 447–476 and for NADPH includes residues 483–519 and 524–553. Residues 84–95 and 140–178 were predicted as the FMN/substrate-binding site. The line labeled pKK223-3 represents a reductase fragment expressed by a rat liver reductase cDNA subcloned into the expression vector pKK223-3. The fragments labeled 21/18, 23/18, and 25/18 were generated by PCR from the reductase cDNA in pKK223-3. All of the fragments contain the 447- to 479-residue FAD and all of the NADPH binding sites. The fragment labeled pKK223-3 contains all of the prosthetic group binding sites and differs from the full-length reductase in that it lacks the hydrophobic domain. Fragment 21/18 contains all of the putative FAD and NADPH sites and all of the region connecting the FMN/substrate and FAD/NADPH binding domains. Fragment 23/18 contains only four residues of the 292–326 FAD binding site. Fragment 25/18 contains no residues of the 292–326 FAD binding domain.

from these cultures were purified by a modification of the holoreductase purification method (9) as described under Materials and Methods. The A-25 elution profiles and purification efficiency data are very similar for both fragments; therefore, for simplicity only the FAD binding fragment 21/18 is shown. Two protein peaks eluting from the A-25 column were identified by absorbance at 280 nm as shown in Fig. 2. The fractions containing the highest absorbance at 450 and 280 nm were analyzed for ferricyanide reduction activity as shown and for immunoreactivity (not shown). Fractions of the two major activity containing peaks were pooled separately.

Table II presents the purification of the rat liver recombinant reductase FAD binding fragment 21/18. The specific activity of 42.2  $\mu\text{mol}/\text{min}/\text{mg}$  protein is about 67% of that which we typically obtain for holoreductase. Fifteen milligrams of fragment 21/18 were purified from 3800 mg of *E. coli* cellular protein. Given a 52% yield, 0.76% of the total cellular protein was reported as the FAD binding fragment. Porter and Kasper (8) reported that the expression in *E. coli* of recombinant rat liver holoreductase was 1% of the total cellular protein content and Smith *et al.* (30) found that the expression in *E. coli* of the recombinant human FAD binding domain was 10–15% of the total cellular



**FIG. 2.** Elution profile of FAD fragment 21/18 from DEAE-Sephadex A-25 column. Absorbance at 454 and 280 nm are plotted for all fractions. The ferricyanide activity was determined as described under Materials and Methods for fractions 10–25 and is shown (---). The 0–0.4 M KCl gradient is indicated in the inset graph.

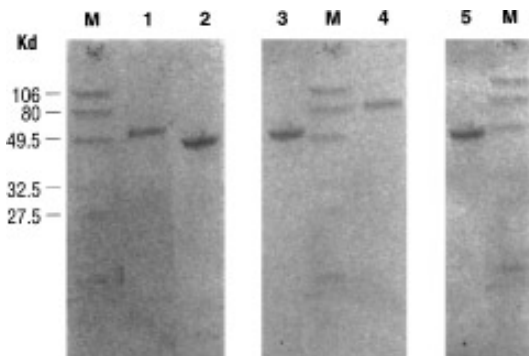
protein. The purified, uncut, and cleaved fragments are shown in Fig. 3.

**Enzymatic activity of the FAD fragments.** The enzymatic activities of the FAD fragments with four electron acceptors are shown in Table III. To provide a more valid assessment of flavoprotein enzymatic activity we have compared the activity of proteins that bind only FAD to the exclusion of FMN, specifically, FMN-less reductase, FAD binding fragment 21/18 and FAD binding fragment 25/18. All of these proteins bind approximately the same molar ratio of FAD: the FAD binding fragments because they do not contain the FMN site and the FMN-less reductase because FMN was selectively removed (28). The activities have been normalized to moles of flavin content/mole of protein to account for the lower affinity of flavin for the fragments compared to the full-length FMN-less reductase. FMN-

**TABLE II**  
Purification of FAD Binding Fragment 21/18

Sample	Vol (ml)	Total protein (mg)	Specific activity $\mu\text{mol}/\text{min}/\text{mg}$ protein	% Yield
Cell paste (resuspended)	240	3800	0.32	—
Cytosolic fraction	200	2700	0.40	86%
DEAE pool	220	270	3.6	78%
Nickel affinity column	7.5	15	42	52%

*Note.* The protein yield and enzymatic activity were calculated as described under Methods and Materials.



**FIG. 3.** SDS-PAGE (12.5%) of purified FAD binding fragments. Ten micrograms of each fragment was applied to each gel lane. Lanes M, molecular weight marker; Lane 1, fragment 21/18; Lane 2, enterokinase-digested fragment 21/18; Lane 3, 25/18; Lane 4, holoreductase; Lane 5, enterokinase-digested fragment 21/25.

less reductase studies demonstrate that the FAD redox center, in the absence of an active FMN redox center, can accept electrons from NADPH to reduce ferricyanide and that the FAD redox center but not the FMN redox center of reductase can participate in the 3-AcPyADP transhydrogenase reaction. Holoreductase is able to reduce ferricyanide, cyt c, and DICP and catalyze the 3AcPyADP transhydrogenase reaction. Aporeductase studies also included enzymatic analysis of all of the above electron acceptors and the data were in accord (Table III) with the proposed electron transfer sequence through the enzyme; NADPH  $\rightarrow$  FAD  $\rightarrow$  FMN  $\rightarrow$  acceptor. Consistent with this sequence, the FAD fragments should have activity only toward ferricyanide and 3-AcPyADP and not toward cyt c or DICP, although both aporeductases retained a small residual amount of cyt c and DICP reductase activity (37, 38). As expected, the fragments had significant activity as a ferricyanide reductase and a 3-AcPyADP *trans*-hy-

**TABLE III**  
Enzymatic Activities of Holoreductase, FMN-less Reductase, and Reductase FAD Binding Fragments 21/18 and 25/18

Electron acceptor	Activities of proteins (mol/min/mol flavin)			
	Holoreductase	FMN-less reductase <sup>a</sup>	25/18 (% of FMN-less reductase)	21/18 (% of FMN-less reductase)
Fe(CN) <sub>6</sub>	3100	4300	2400 (56%)	4500 (105%)
3AcPyADP	59	48	20 (42%)	31.0 (65%)
DICP	1200	130	0.00 (0%)	00.0 (0%)
Cyt c	2200	68	9.30 (14%)	8.00 (12%)

*Note.* Protein concentrations and enzymatic activity were determined as described under Materials and Methods.

<sup>a</sup> As previously described (28).

**TABLE IV**  
Flavin Content of Holoreductase and Reductase FAD Binding Fragments

Enzyme	FAD content	FMN content	FAD/FMN
		(mol/mol enzyme)	
Native (27)	0.89	0.88	1.0
FMN-less (37)	0.83	0.10	8.7
FAD-less (27)	0.012	0.93	0.013
Fragment 21/18	0.71	0.030	24
No FAD	0.57	0.02	
No FAD, no Rib.	0.08	0.02	
Fragment 25/18	0.66	0.020	33
No FAD	0.54	0.01	
No FAD, no Rib	0.08	0.01	

*Note.* Flavin contents (FMN and FAD) were determined as described under Materials and Methods. FMN-less and FAD-less indicate reductase prepared without FMN and FAD, respectively. No FAD indicates reductase analyzed without prior reconstitution with FAD. No Rib indicates reductase prepared from medium that was not supplemented with riboflavin.

drogenase, but little to none as a cyt c or DCIP reductase. Since residual cyt c reductase activity was observed for both aporeductases, we are not surprised to see some small residual cyt c reductase activity in the expressed fragments. The observed activity may be due to a loosely folded enzyme leaking a small number of reducing equivalents to cyt c.

The bound FAD, therefore, accepted two electrons at a time from NADPH and was able to donate electrons to one-electron acceptors. These results are consistent with the proposal that the FAD moiety of reductase accepts electrons directly from NADPH and donates electrons to FMN. The fragment 25/18 had less activity toward both compounds than fragment 21/18. This is not surprising given the fact that the 25/18 fragment lacks the amino acid residues 292–326, which are proposed to participate in the binding of FAD, and binds FAD less tightly than the 21/18 fragment does as discussed below. The proposed cyt c binding site (39) is not present in the fragment 25/18.

*Flavin determinations.* The flavin concentrations were determined as described under Materials and Methods and are shown in Table IV. Flavin binding contents of other reductase preparations are shown for reference. This is the first report quantifying the flavin binding behavior of an isolated P450 reductase flavin binding fragment. Both of the FAD fragments 21/18 and 25/18 bind FAD after reconstitution to a catalytically significant degree (0.71 and 0.66 mol/mol protein, respectively). This represents 80% (for fragment 21/18) and 74% (for fragment 25/18) of the FAD binding observed for holoreductase (after a 15-min incubation with a FAD-containing buffer). The fact that the 25/18

fragment binds about 10% less FAD than the 21/18 FAD binding fragment may be due to the lack in the 25/18 fragment of the amino acid residues 292–326 which are proposed to bind the FAD pyrophosphate group. This deletion may cause the entire polypeptide to be more loosely folded or may simply cause the FAD binding site to be more loosely folded. After purification, the proportion of flavin-bound reductase for both fragments was about 20% lower than for holoreductase. Holoreductase has also been observed to lose some FAD and FMN upon purification and is regularly reconstituted in our laboratory prior to analysis.

These data further define and confirm the amino acid residues required to confer the proposed FAD binding site (8) and redox center enzymatic activities (26). A human reductase fragment from residues 242 to 677 maintained the ability to reduce ferricyanide at ~50% of the level of holoreductase (30). Quantification of FAD content was not determined for this human reductase fragment. By collating our results with those of Smith *et al.* (30) on human reductase fragment, we can deduce that the amino acid residues preceding 347 do not seem to be necessary to confer FAD binding or associated activity since the FAD binding fragment 25/18 (encompassing residues 347–678) does not contain these residues and yet binds FAD (0.66 mol/mol enzyme, Table IV) and is approximately equal in ferricyanide reductase activity (~50% of FMN-less reductase activity, Table III, fragment 25/18) as the 242–677 human fragment. On the other hand, with rat reductase we observe a 7% increase in FAD binding (Table IV, 21/18) and a dramatic 188% increase in ferricyanide reductase activity (Table III, 21/18 FAD binding fragment) for the longer fragment which represents the addition of the preceding 129 residues (347 to 218).

Insofar as the human and rat amino acid sequences are structurally similar, two major observations may be made regarding the structure of the FAD binding domain. First, it appears that the majority of increase in enzymatic activity observed for fragments 21/18 and 25/18 arises from the residues 218–242, since the activity of the fragment 21/18 (residues 218–677) is approximately double that observed for the 242–677 human fragment (30). Second, since a 414 to 677-residue human reductase fragment (30) remained insoluble, it seems that at least some of the residues between 347 and 414 may be required to allow proper folding of a reductase FAD binding fragment.

Without FAD incubation before assay, about 55% of the FAD binding sites are occupied on the FAD binding fragments. The fragments produced in media enriched with riboflavin show a marked increase in FAD binding over the fragments grown in media without riboflavin enrichment (For 21/18, binding increases from 0.08 to 0.71 mol FAD bound/mol FAD binding fragment; an 89% increase.). Thus, enriching the media with ribo-

TABLE V  
FAD Analog Binding

Flavin	FAD binding fragment	
	21/18	25/18
FAD (-210 mV)		
$V_{\max}$	13.3	10.6
$K_d$	1.05	1.35
8-Cl-FAD (-150 mV)		
$V_{\max}$	19.6	19.2
$K_d$	3.1	4.7
8-NH-FAD (-310 mV)		
$V_{\max}$	1.1	0.5
$K_d$	5.8	6.3

Note. The  $V_{\max}$  and  $K_m$  values were determined as described under Materials and Methods. The free redox values of each flavin is indicated in parentheses.  $V_{\max}$  values are expressed in  $\mu\text{mol}$  ferricyanide reduced/min/mol enzyme and  $K_d$  values are expressed in  $\mu\text{M}$ .

flavin *in vivo* has the most significant effect on the amount of flavin bound while preincubation of enzyme *in vitro* has a less significant effect.

The fact that FAD binds both fragments as purified from *E. coli* indicates that prior FMN binding to some portion of a recombinant reductase fragment is not required for FAD to bind the FAD binding domain. Whether prior FMN binding is required for FAD binding to holoreductase synthesized in the rat liver remains to be demonstrated. There is some indication of FMN binding in all of the various types of reductases, but it is at almost undetectable levels (0.03–0.01 mol/mol protein) in the fragments.

Riboflavin, a precursor of FAD, was added to the reductase cell cultures used by Porter and Kasper (8). They did not analyze whether this supplementation promoted FAD binding to the recombinant holoreductase; however, we have observed that an addition of riboflavin during rapid bacterial growth is critical to obtain FAD-bound reductase fragments. Perhaps this is necessary because the fragments are produced at such a higher rate in the cells after induction that the rate-limiting factor of FAD-bound production in the cells is the synthesis of FAD.

**Flavin analog reconstitution.** FAD, 8-chloro-FAD, and 8-amino-FAD were reconstituted into the flavin binding fragments to determine the binding efficiencies as described under Materials and Methods and are shown in Table V. Both of the flavin analogs used in this study were more difficult to reconstitute into the reductase fragments 21/18 and 25/18 (they have a higher  $K_d$ ) than was FAD. Since the redox potentials for FMN and FAD change drastically as they are bound to the protein, so may the FAD analogs. Further analysis to determine the flavin-bound redox potentials of

the FAD binding fragments may demonstrate that the rapid equilibrium hypothesis (40) is applicable to the FAD binding fragments.

The free redox value for FAD lies between the values for the two FAD analogs, 8-chloro-FAD and 8-amino-FAD, as shown in Table V. The  $V_{\max}$  values for the reconstituted fragments were directly proportional to the free redox potentials, increasing from 8-amino FAD to FAD to 8-chloro-FAD. Binding efficiencies to the cloned fragments did not appear to be related to the enzymatic activity since the  $K_d$  values for the FAD analogs were higher than the values for FAD and the  $V_{\max}$  value for FAD was between the  $V_{\max}$  values for the FAD analogs.

This is the first report that quantitates FAD binding to the proposed FAD binding domain and confirms the proposed FAD/NADPH binding domain elucidated by Porter and Kasper (15). Further, this study narrows the required amino acid segments necessary for FAD binding and enzymatic activity. The amino acids residues preceding 347 do not appear to be absolutely required to bind FAD and to support FAD associated electron transfer activities, but the residues from 218 to 347 do increase the degree of FAD binding 7% and double ferricyanide reduction activity. This study has provided two new and active FAD/NADPH binding fragments for studying the structure and function of reductase. Further structural analysis of these polypeptides could shed more light on the factors important in binding FAD and NADPH.

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