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Dissection of NADPH–cytochrome P450 oxidoreductase into distinct functional domains

(drug metabolism/electron transport/flavoproteins/nitric oxide synthase)

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ABSTRACT **NADPH–cytochrome P450 oxidoreductase transfers electrons from NADPH to cytochrome P450 and catalyzes the one-electron reduction of many drugs and foreign compounds. This enzyme is a flavoprotein containing the cofactors FMN and FAD, which are essential for its function. We have expressed the putative FMN and FAD/NADPH binding domains of P450 reductase and show that these distinct peptides fold correctly to bind their respective cofactors. The FAD/NADPH domain catalyzed the one-electron reduction of a variety of substrates but did not efficiently reduce cytochrome *c* or cytochrome P450 (as judged by the oxidation of the CYP1A1 substrate 7-ethoxyresorufin). However, the domains could be combined to provide a functional enzyme active in the reduction of cytochrome *c* and in transferring electrons to cytochrome P450. Both the reconstitution of the domains and the direct binding of cytochrome *c* to the FMN domain were ionic-strength dependent. The FMN domain containing the hydrophobic membrane anchor sequence was a potent inhibitor of reconstituted monooxygenase activity. These data strongly support the hypothesis that FMN/FAD-containing proteins have evolved as a fusion of two ancestral genes and provide fundamental insights into how this and structurally related proteins, such as nitric oxide synthase and sulfite reductase, have evolved and function.**

The microsomal flavoprotein NADPH–cytochrome P450 oxidoreductase (EC 1.6.2.4) shuttles electrons from NADPH via its FMN and FAD prosthetic groups to cytochrome P450 (1). The reductase also has the ability to reduce a host of exogenous electron acceptors, including cytochrome *c*, potassium ferricyanide, and 2,6-dichloroindophenol (DCIP) (2), as well as therapeutically important compounds such as mitomycin c (3) and the benzotriazine SR4233 (4). In addition, P450 reductase is also an electron donor to heme oxygenase (5), the fatty acid elongation system (6), and cytochrome b_5 (7) and initiates lipid peroxidation by the one-electron reduction of molecular oxygen (8). P450 reductase was the first protein found to contain FMN and FAD in an equimolar ratio of 1:1 (9). Since its isolation, two other groups of proteins have been identified which contain these flavins as prosthetic groups: (*i*) the nitric oxide synthases, involved in the production of the neurotransmitter, vasodilator, and cytotoxic agent nitric oxide from L-arginine (10, 11), and (*ii*) the α subunit of the bacterial sulfite reductases, which participate in the six-electron reduction of sulfite to sulfide (12). In addition to this similarity, these proteins have been shown to share significant sequence identity, particularly within the suggested functional domains (12, 13).

Initial sequence comparisons of P450 reductase with other proteins reveal a striking homology with two distinct flavoproteins (14, 15). The N-terminal region of the reductase shows homology with the FMN-containing bacterial flavodoxins. The C-terminal portion of the reductase is homologous with the FAD-containing ferredoxin $NADP^+$ reductases. These observations led to the proposal that P450 reductase has evolved as a fusion of two ancestral proteins (15).

We therefore set out to separate the proposed domains of P450 reductase into discrete functional units by expressing specific peptides in *Escherichia coli*. The peptides were designed on the basis that exons or groups of exons within the P450 reductase gene defined specific functional domains, as has been observed for the steroid hormone receptors (16) and the glyceraldehyde phosphate dehydrogenases (17). The ability to dissect P450 reductase into functionally viable domains would provide new avenues for studying the structure and function of this and related proteins.

MATERIALS AND METHODS

Constructs. The cDNA for human NADPH–cytochrome P450 oxidoreductase was obtained from a human skin fibroblast cDNA library (kindly provided by S. M. Keyse, Imperial Cancer Research Fund, Dundee, UK) by PCR. Oligonucleotides for the 5′ (incorporating an *Eco*RI site) and 3′ ends of the reductase cDNA were derived from the sequence reported previously (18). After treatment with Klenow DNA polymerase and digestion with *Eco*RI, the cDNA was ligated into the *Eco*RI/*Sma* I site of pTZ19R (St. Albans, Hertfordshire, UK) and the sequence was confirmed. The human cDNA was the template for further PCR amplifications. Oligonucleotides were generated for the 5′ and 3′ ends of the proposed exons shown in Fig. 1 in order to obtain cDNAs encoding domains of P450 reductase. The exon/intron boundaries were derived from the rat P450 reductase gene (19). The 5′ oligonucleotides were synthesized with an overhanging *Nde* I restriction site whereas the 3′ oligonucleotides contained an *Xho* I site. After amplification and digestion, the cDNAs were ligated into the unique *Nde* I/*Xho* I sites of the expression plasmid pET15b (Novagen). The authenticity of regions generated by PCR was confirmed by sequencing. The plasmids were used to transform *E. coli* BL21(pLysS).

Purification of the Domains. Cloning into the *Nde* I/*Xho* I sites of pET15b engineers a His_6 linker and a thrombin cleavage site onto the N terminus of the expressed protein. Purification of the His-tagged domains on nickel-agarose columns was carried out according to Novagen's recommendations. BL21 strains harboring the domain expression plasmids were grown at 37°C overnight in LB broth containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). Five hundred milliliters of fresh LB broth was inoculated with 5 ml of the overnight culture and the bacteria were grown at 37°C

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Abbreviations: DCIP, 2,6-dichloroindophenol; EROD, 7-ethoxyresorufin O-deethylation.

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to an OD of 0.5–0.6. Isopropyl β-D-thiogalactopyranoside was then added (0.5 mM) and the culture was allowed to grow for 2.5 hr before harvest at 5000 × *g* for 10 min. The pellet from a 500-ml culture was suspended in 20 ml of binding buffer (5 mM imidazole/500 mM NaCl/20 mM Tris·HCl, pH 7.9) and frozen at −70°C for 1 hr. Upon thawing the bacteria were lysed, since the expression strain BL21 contains a plasmid bearing T7 lysozyme. Sonication (MSE probe at full power, 2 × 35 sec with 1 min of incubation on ice between steps) was carried out to shear genomic DNA present in the lysate. The extract was then centrifuged (39,000 × *g* for 30 min) to remove cellular debris and insoluble protein. Bacteria were analyzed for domain expression by SDS/PAGE of soluble and insoluble fractions (data not shown). The FMN/anchor, FMN (no anchor), and FAD/NADPH domains (Fig. 1) produced soluble protein extracts. However, the FAD and FAD/NADPH (small) domains were insoluble, as was the FMN/FAD domain, with only a very small percentage present in the soluble fraction. The supernatant containing the soluble domains was applied onto 2-ml nickel-agarose columns previously equilibrated with binding buffer. Columns were washed with 10 volumes of binding buffer and then 10 volumes of wash buffer (binding buffer containing 20 mM imidazole). The FMN (no anchor) and the FAD/NADPH domains could be eluted from the nickel-agarose column by increasing the imidazole concentration to 60 mM, while the FMN/anchor region was eluted by increasing the imidazole to 300 mM. Insoluble proteins were purified in a manner similar to the soluble domains but in the presence of 6 M urea. Attempts to renature the domains were unsuccessful. The purity of the domains was determined by SDS/PAGE. The insoluble domains were not used in any further experiments. Purified soluble domains were extensively dialyzed against 10 mM potassium phosphate buffer (pH 7.7).

Protein was determined by the method of Lowry *et al.* (20). Thrombin cleavage was performed in 20 mM Tris·HCl, pH 8.4/150 mM NaCl/2.5 mM $CaCl_2$ with a domain/thrombin weight ratio of 5000:1. Cleavage took place at 4°C and was monitored by SDS/PAGE. One hundred percent cleavage occurred after 10 min for the FMN (no anchor) domain and after 4 hr for the FAD/NADPH domain. The His_6 tag could not be removed from the FMN/anchor domain. The cut domains were separated from the His_6 tag by passing the sample through a nickel-agarose column.

Spectral Analysis. Absorption spectra were obtained with a Shimadzu UV 2000 spectrophotometer. FMN and FAD content was calculated after releasing the flavin from the domains by boiling for 3 min in the dark, followed by centrifugation at 20,000 × *g* for 10 min to remove denatured protein. The flavin concentration was determined at 450 nm by using extinction coefficients of 12.2 $mM^{-1} \cdot cm^{-1}$ for FMN (21) and 11.3 $mM^{-1} \cdot cm^{-1}$ for FAD (22).

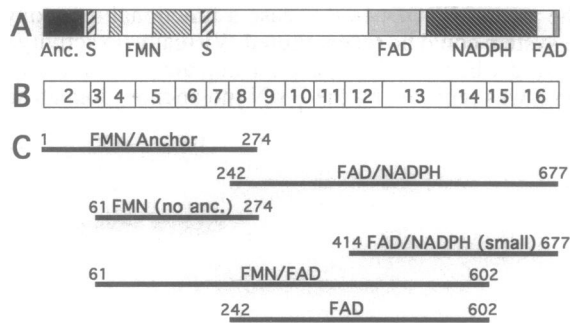

FIG. 1. Organization of P450 reductase domains (*A*), exon organization (*B*), and domains made for this study (*C*). Amino acids at the ends of the domains are shown. Anc., membrane-anchoring domain; S, substrate recognition sites. FMN-, FAD-, and NADPH-binding domains are shown. Calculated molecular masses of the domains: FMN/anchor, 31.1 kDa; FAD/NADPH, 49.7 kDa; FMN (no anchor), 24.2 kDa; FAD/NADPH (small), 30.2 kDa; FMN/FAD, 61.3 kDa; FAD, 41.0 kDa.

Enzyme Assays. The one-electron reduction of cytochrome *c*, potassium ferricyanide, DCIP, menadione, and 3-acetylpyridine adenine dinucleotide phosphate was carried out in 50 mM potassium phosphate (pH 7.7) at 37°C (23). For reconstitution of reductase activity, the FMN (no anchor) and the FAD/NADPH domains (0.1 nmol of each) were mixed in 10 mM potassium phosphate (pH 7.7) for 2 hr at 4°C before assay. The FMN/anchor domain was used for the reconstitution of P450 reductase activity. 7-Ethoxyresorufin O-deethylation (EROD) was determined (24) with a Perkin–Elmer LS-3 fluorescence spectrophotometer. Details of the reconstitution assays are in the figure legends. Rat CYP1A1 and P450 reductase were purified as described (25, 26).

RESULTS

Expression and Purification of P450 Reductase Domains. *E. coli* cells were transfected with expression plasmids encoding the P450 reductase domains (Fig. 1). All the peptides tested were expressed at high level (10–15% of total cell protein) but not all were soluble (data not shown). The FAD and the FAD/NADPH (small) domains were totally insoluble and could be purified only under denaturing conditions. The FMN/FAD domain was partially soluble but upon isolation was very susceptible to proteolysis. The FMN (no anchor) domain was predominantly soluble (up to 35 mg of purified protein could be obtained from a 1-liter culture), with the FMN/anchor and the FAD/NADPH domain less so (up to 5 mg of purified protein from a 1-liter culture).

The recombinant His-tagged proteins were purified to homogeneity by nickel-agarose chromatography (Fig. 2). The His_6 tag was completely removed from the N terminus of the FMN (no anchor) and FAD/NADPH domains. However, the tag could not be removed from the N terminus of the FMN/anchor domain (data not shown).

Absorption Spectra of the Oxidized FMN and FAD/NADPH Domains. The FMN/anchor, FMN (no anchor), and FAD/NADPH domains purified from the bacterial supernatant (under nondenaturing conditions) were yellow, indicating the presence of flavin. To confirm this, UV/visible spectra were recorded. The spectrum of the FMN (no anchor) domain had peaks at 370 and 453 nm and a broad absorption band between 570 and 630 nm (probably due to some reduced FMN being present in this sample) (Fig. 3*A*). This spectrum was virtually identical to that reported by Kurzban *et al.* (27) for the FAD-depleted P450 reductase and similar to that predicted from the computer model of Oprian and Coon (28). The spectrum of the FAD/NADPH domain had absorption maxima at 382 and 454 nm (Fig. 3*B*), similar to the FMN-depleted preparations of P450 reductase (29, 30). However, this spectrum had more definition than the FMN-depleted reductase produced by site-directed mutagenesis (31). Analysis of flavin content showed that the FMN/anchor domain contained 0.67 mol of FMN per mol of protein, and the FMN domain, 0.63:1. The FAD/NADPH domain contained 0.70 mol of FAD per mol of protein. When the FMN and FAD/NADPH domains were combined in a 1:1 ratio, a spectrum identical to that of native P450 reductase was obtained (Fig. 3 *C* and *D*).

The above data showed that the FMN and FAD domains could be expressed separately and could fold independently to bind their respective cofactors. To investigate whether the FAD/NADPH protein could transfer electrons to the FMN (no anchor) domain to produce an air-stable semiquinone, similar to the native P450 reductase (Fig. 3*C*), the two domains were mixed and an excess of NADPH was added. A spectrum characteristic of the air-stable semiquinone form of

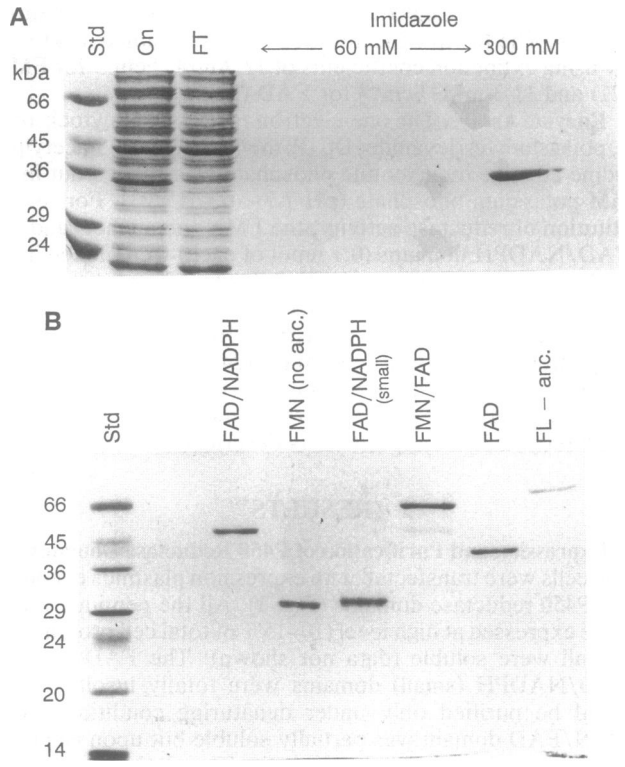

FIG. 2. Expression of P450 reductase domains in *E. coli*. The cDNA sequences encoding the domains shown in Fig. 1 were expressed in *E. coli* BL21(pLysS) using the pET15b vector. Domains were purified to apparent homogeneity on nickel-agarose columns. (*A*) Purification of the FMN/anchor domain. Std, molecular size standards; On, soluble fraction applied to the column (15 μg); FT, flowthrough (15 μg); 300 mM, domain eluted with 300 mM imidazole after washing of the column with 60 mM imidazole (2 μg). (*B*) SDS/12% PAGE analysis of the other purified domains (1 μg) illustrated in Fig 1. FL − anc., full-length P450 reductase minus the anchor domain.

native P450 reductase was obtained (Fig. 3 *C* and *D*). This slowly reoxidized over a period of 4 hr, which is unlike intact P450 reductase, where this intermediate is stable for up to 48 hr. This reduction occurs via electron transfer from NADPH through FAD (30). The similarity of the spectra (Fig. 3 *C* vs. *D*), which are characteristic of FMN semiquinone, indicates that electrons are transferred from FAD to the FMN domain. This is supported by the finding that the FAD semiquinone rapidly reoxidized (25 min in a similar experiment using the FAD/NADPH domain; data not shown) and that NADPH did not directly reduce the FMN (no anchor) domain (data not shown).

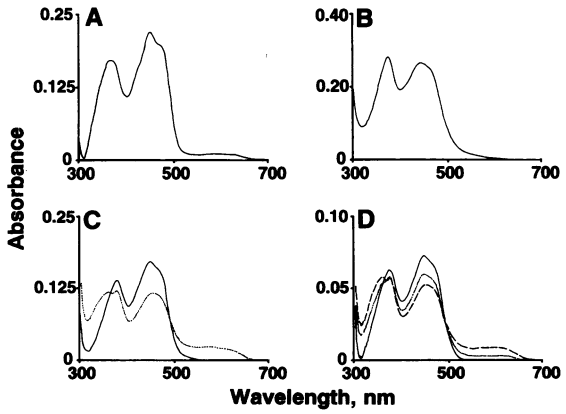

FIG. 3. Absorption spectra of purified P450 reductase domains. (*A*) FMN (no anchor) domain, 55 μM. (*B*) FAD/NADPH domain, 50 μM. (*C*) Native P450 reductase, 10 μM: ——, oxidized; - - - -, air-stable semiquinone 24 hr after addition of 200 μM NADPH. (*D*) Spectrum obtained on mixing the FMN (no anchor) (7.5 μM) and FAD/NADPH (7.5 μM) domains: ——, oxidized; — —, reduced spectrum 30 min after the addition of 200 μM NADPH; - - - -, reduced spectrum 4 hr after the addition of 200 μM NADPH.

Interaction Between the FMN and FAD/NADPH Domains. In view of the above observation, we determined whether the two domains could bind directly to each other. The His-tagged FMN (no anchor) domain was bound to a nickel-agarose column and the FAD/NADPH domain (without the His_6 tag) applied. The FAD/NADPH domain became tightly bound to the column, but only when the FMN domain was bound. The FAD/NADPH domain could be dissociated from the FMN (no anchor) domain and eluted by increasing the ionic strength of the column buffer (Fig. 4), indicating that ionic interactions were important for binding to occur.

In addition, the ability of the domains to bind cytochrome *c* was studied. When cytochrome *c* was applied to the column containing the immobilized FMN (no anchor) domain it was retained on the column. This could also be eluted by increasing the ionic strength of the buffer (data not shown). In a similar experiment with the FAD/NADPH protein immobilized on a nickel-agarose column, cytochrome *c* did not bind (data not shown).

Functional Analysis of Domains and Reconstitution of Activity. The above data indicated that the FAD/NADPH and FMN domains folded correctly and could interact to form a complex with each other. We then established whether the domains could couple to form a catalytically active unit. The catalytic activity of the FAD/NADPH domain alone was also assessed.

The FAD/NADPH domain could reduce a range of one-electron acceptors and showed transhydrogenase activity in the absence of the FMN domain but could not efficiently reduce cytochrome *c* (Table 1). The activities measured were to a varying degree decreased relative to native P450 reductase. Activity toward these one-electron acceptors was not significantly increased by the presence of the FMN (no anchor) domain. However, cytochrome *c* reductase activity was reconstituted when a combination of the FMN (no anchor) and FAD/NADPH domains was used. The activity of the reconstituted domains was found to be around 2% that of the native enzyme.

To test for the reconstitution of cytochrome P450 monooxygenase activity, the rat cytochrome P450 CYP1A1 was incorporated into incubation mixtures containing the FMN/anchor and FAD/NADPH domains. Activity was monitored as the rate of EROD. In this case a functional monooxygenase system could be reconstituted. Virtually no activity was measured when the FMN/anchor domain was omitted, but when it was replaced by the FMN (no anchor) domain some

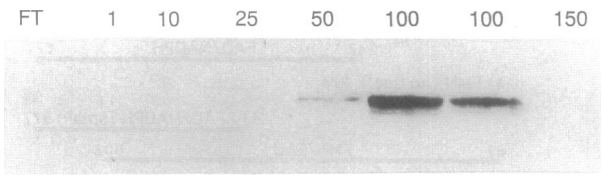

FIG. 4. Interaction of the FMN (no anchor) domain with the FAD/NADPH domain. His-tagged FMN (no anchor) domain (2 mg) was bound to a 1-ml nickel-agarose column and the FAD/NADPH domain (0.1 mg/ml) was applied in 1 mM potassium phosphate (pH 7.7). This resulted in the binding of the latter domain. The concentration of the buffer passed through the column was then increased from 1 to 150 mM in a stepwise fashion and the resulting eluates were analyzed by Western blotting using an antibody to full-length P450 reductase. FT, flowthrough.

Table 1. Specific activities of P450 reductase and domains

Domain(s)	Specific activity (%)					
	$Fe(CN)_6^{3-}$	DCIP	Menadione	3-AcPyADP*	CYP1A1	Cytochrome *c*
P450 reductase	33.1	11.2	7.67	1.14	6.34	23.5
FAD/NADPH	17.9 (54.1)	0.456 (4.1)	0.145 (1.9)	0.228 (20)	0.001 (0.016)	0.025 (0.11)
FAD/NADPH plus FMN	16.1 (48.6)	0.705 (6.3)	0.134 (1.7)	0.207 (18)	0.121 (1.9)	0.410 (1.7)
FMN	–	–	–	–	–	–

Specific activities are expressed as μmol of substrate reduced per min per mg of protein, except for menadione, where the oxidation of NADPH was followed, and CYP1A1 activity, which was determined as EROD activity and is expressed as nmol of resorufin produced per min per nmol of P450. All assays were carried out in triplicate with SEM < 5%.
*3-Acetylpyridine adenine dinucleotide phosphate.

deethylation was measured (around 20% of that seen with the FMN/anchor; data not shown). Also, when P450 was not present no activity was measured. The reconstitution was time dependent (Fig. 5*A*). Reconstitution of cytochrome *c* reductase activity was also time dependent (data not shown). The ability to reconstitute P450 reductase activity was ionic-strength dependent (Fig. 5*B*).

To establish whether the FMN/anchor domain could interact with P450 to affect the coupling of P450 reductase to P450, the effect of adding this domain to a reconstituted system containing native P450 reductase and CYP1A1 was investigated. Preincubation of CYP1A1 with the FMN/anchor domain resulted in almost complete inhibition of EROD activity (Fig. 6). Fifty percent inhibition was observed at a domain/native reductase ratio of ≈4:1. The FAD/NADPH domain had little effect on EROD activity, although a reproducible slight decrease in activity was seen at the lowest domain concentration tested. An interesting result was observed when titrating the FMN (no anchor) domain into the reconstituted system. This domain was found to activate EROD activity up to 3-fold. This appeared to be specific, as the denatured FMN domain or free FMN did not cause this effect (data not shown).

DISCUSSION

We describe the dissection of an FMN/FAD-containing flavoprotein, NADPH–cytochrome P450 oxidoreductase, into structurally and functionally independent domains. This provides strong experimental evidence to substantiate the hypothesis that this protein has evolved from two distinct ancestral genes (15). Indeed, many of the properties of the ancestral proteins appear to have been retained and there is no need for direct interaction between the two domains for FMN and FAD binding. However, for stabilization of the incorporated flavin in the protein (i.e., to maintain a 1:1 molar ratio FAD or FMN to protein) and for efficient electron transfer, interactions may be needed between the domains and/or flavins.

The expressed FAD/NADPH domain could catalyze the reduction of certain compounds, indicating correct folding of both the FAD and NADPH binding regions. Certain reaction rates were comparable to those of the native enzyme, indicating that the FAD/NADPH domain is at least in part responsible for the one-electron reduction of many compounds, including redox cycling drugs. The much slower rate of reduction of substrates such as menadione by the FAD/NADPH domain indicates that for certain compounds this region is not the major site of reduction. Previous work on the FMN-depleted native P450 reductase also indicated that this was the case (29, 30). The FAD/NADPH and FMN domains could be reconstituted to form a complex active in the reduction of cytochrome *c* and in donating electrons for cytochrome P450-dependent monooxygenase reactions. This fascinating finding demonstrated that electron transfer between the domains, although not optimal, could be achieved. Studies into why these interactions are so sensitive to ionic strength, and studies into optimal domain size should identify some of the structural and functional requirements needed for efficient transfer between the peptides.

Comparison of the FMN-binding region of P450 reductase with the flavodoxin from *Desulfovibrio vulgaris* shows that highest homology lies within exons 4–6 (19). However, we chose to express exons 2–8 and exons 3–8 as the FMN-binding domain. In recent experiments we have expressed only exons 4–6 but found that this protein did not bind FMN. Similarly, sequence alignments based on the crystal structure

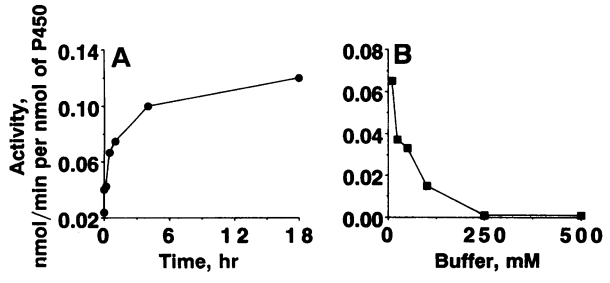

FIG. 5. (*A*) Reconstitution of cytochrome P450 monooxygenase activity. The FMN/anchor and FAD/NADPH domains (0.1 nmol) were mixed in 10 mM potassium phosphate (pH 7.7) in a volume of 20 μl at 4°C. After various times the domains were incubated with dilauroyl phosphatidylcholine (25 μg) and CYP1A1 (37.5 pmol in 5 μl) at 37°C for 5 min before assay EROD activity (24). (*B*) Effect of ionic strength on the reconstitution of EROD. Domains were preincubated, as described for *A*, for 2 hr in various concentrations of potassium phosphate buffer at pH 7.7. Dilauroyl phosphatidylcholine (25 μg) and CYP1A1 (37.5 pmol in 5 μl) were then added and incubated at 37°C for 5 min before assay for EROD.

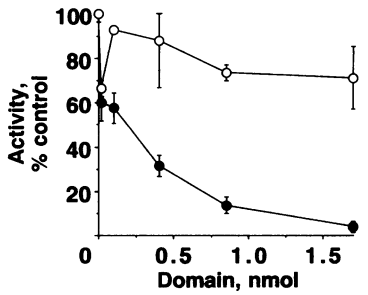

FIG. 6. Inhibition of monooxygenase activity by the FMN/anchor domain of P450 reductase. CYP1A1 (7.5 pmol) and 25 μg of dilauroyl phosphatidylcholine were incubated for 5 min with various concentrations of the FMN/anchor (●) or FAD/NADPH (○) domain (0.04–1.7 nmol) in 47.5 μl at 37°C for 5 min. Native P450 reductase (0.05 nmol in 2.5 μl) was then added and the sample was incubated for 5 min before measurement of EROD activity. Results (mean ± SEM, n = 3) are expressed as percentage of the activity in the absence of the domain; 100% = 6.34 nmol/min per nmol of P450.

of spinach ferredoxin $NADP^+$ reductase have revealed that the minimal FAD/NADPH-binding region of the protein should be contained within exons 12–16 (32). However, expression of exons 12–16 yielded an insoluble protein which, from its white appearance, did not bind FAD. In other cases, even when insoluble products were formed, the color of the protein suggested that flavin had been incorporated.

Expression of the FAD-binding region of P450 reductase, excluding the proposed NADPH-binding domain (exons 15 and 16) (Fig. 1), generated an insoluble protein that did not bind FAD. Alignment of this region with spinach ferredoxin $NADP^+$ reductase shows that exons 15 and 16, in addition to containing important amino acids for $NADP^+$ binding, contain residues involved in covering the face of the flavin without hydrogen bonding to it (32). It has also been suggested that the large contact surface between the FAD- and NADPH-binding domains would not allow them to be functionally separated (32). These observations would explain why the FAD domain did not fold in the absence of the NADPH domain.

The binding between the FMN (no anchor) and FAD/NADPH domains, the interaction of the FMN (no anchor) domain with cytochrome *c*, and the reconstitution of monooxygenase activity were all ionic-strength dependent. These findings are probably related to the highly charged nature of P450 reductase, which contains 97 Asp and Glu residues and 74 Arg and Lys residues out of a total of 677 amino acids. The direct involvement of the FMN (no anchor) domain with cytochrome *c* binding and in its reduction is in agreement with previous studies (29, 31, 33). Crosslinking studies of native P450 reductase with cytochrome *c* indicate that amino acids 207–215 of the reductase are involved in the binding between the two proteins (33). These residues are located in the FMN domain. Various amino acids within the FMN domain have also been implicated in P450 interactions (34).

The FMN/anchor domain was found to be a potent inhibitor of reconstituted monooxygenase activity. This could be explained by the domain preventing the association of the P450 to P450 reductase. This suggests a role for the hydrophobic anchor in directing P450 reductase to the P450, as suggested in earlier studies (23, 35). However, the His_6 tag and thrombin cleavage site could not be removed from this domain. It cannot be ruled out that the His_6 tag may affect the association of the domain with the bilayer and that the inhibitory effect we observe may be occurring outside the bilayer. The FMN (no anchor) domain did not inhibit but significantly increased monooxygenase activity. This could be explained if the FMN (no anchor) domain can accept electrons from the FAD of native P450 reductase and supply electrons directly to P450.

Our data support the hypothesis that P450 reductase has evolved as a fusion of two ancestral proteins (15). Indeed, we may speculate that as two independent proteins the ancestors of P450 reductase functioned as a dehydrogenase/electron-transferase in a primitive organism. By fusion of the genes encoding these ancient flavoproteins a more catalytically efficient electron-transfer system was produced.

The ability to dissociate the protein into domains will be of significant value for x-ray and NMR studies as well as in understanding how the domains interact with each other and with cytochrome P450 to form a functional electron-transfer unit. Such studies will provide insights into the function of structurally related proteins such as nitric oxide synthase and sulfite reductase.

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