Interaction of Adrenodoxin with P4501A1 and Its Truncated Form P450MT2 through Different Domains: Differential Modulation of Enzyme Activities†

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ABSTRACT: Recently we showed that the β-naphthoflavone-inducible liver mitochondrial P450MT2 consists of two N-terminal truncated forms of the microsomal P4501A1, termed P450MT2a (+5/1A1) and MT2b (+33/1A1) [Addya et al. (1997) *J. Cell Biol. 139*, 589−599]. In the present study, we demonstrate that intact P4501A1 and the major mitochondrial form, P450MT2b (routinely referred to as P450MT2), show distinct substrate specificities and preference for different electron transport proteins. Enzyme reconstitution and spectral studies show that the wild-type adrenodoxin (Adx), but not the mutant Adx, binds to P450MT2 in a functionally productive manner (K_d = 0.6 μM) and induces a characteristic high-spin state. Adx binding to intact P4501A1 or +5/1A1 is less efficient as seen from spectral shift patterns (K_d = 1.8−2.0 μM) and reconstitution of enzyme activity. Use of Adx−Sepharose affinity matrix yielded <90% pure P450MT2 (specific activity: 13.5 nmol/mg of protein) starting from a partially purified fraction of 10−15% purity, further demonstrating the specificity of P450MT2 and Adx interaction. Chemical cross-linking studies show that the bovine Adx forms heteroduplexes with both P450MT2 and intact P4501A1, though at different efficiencies. Our results show that Adx interacts with P450MT2 through its C-terminal acidic domain 2, while interaction with intact P4501A1 likely involves the N-terminal acidic domain 1. These results point to an interesting possibility that different electron transfer proteins may differently modulate the enzyme activity. Our results also demonstrate for the first time as to how a different mode of Adx interaction differently modulates the substrate specificities of the two P450 forms.

Cytochromes P450 belong to a multigene family of heme proteins which catalyze the NADPH-dependent oxidation of different exobiotic and endobiotic substrates (*1*, *2*). Constitutively expressed P450[1] forms involved in various physiological pathways are localized in both the ER and mitochondria of different tissues (*3*). A majority of the xenobiotic-inducible P450s are localized in the ER, although mitochondria from liver and brain are also known to contain some of the xenobiotic-inducible forms (*4*−*7*). It is generally believed that (*2*, *3*) the microsomal P450s require P450 reductase for the transfer of electrons from NADPH, while the mitochondrial P450s require mitochondrial-specific ferridoxin (Fdx or Adx) and ferridoxin reductase (Fdr or Adr) for activity. Recently exceptions to this generalization have been noted in studies on the reconstitution of bacterially expressed microsomal P450c17 and P4501A2 with bacterial flavodoxin and flavodoxin reductase (*8*, *9*), and reconstitution of N-terminal-modified rat mitochondrial P450c27 targeted to the ER with the microsomal P450 reductase (*10*).

A recent study from our laboratory showed that the β-naphthoflavone (BNF)-inducible rat liver mitochondrial P450MT2 consists of two N-terminal truncated forms of the similarly induced microsomal P4501A1 termed P450MT2a and P450MT2b (*11*). P450MT2a lacks the first four residues (+5/1A1) and represents only 15−25% of the total mitochondrial 1A1 antibody reactive protein pool. P450MT2b is devoid of the entire transmembrane domain (+33/1A1) and constitutes the major fraction (>75%) of the mitochondrial 1A1 antibody reactive form. We also demonstrated that the N-terminus of P4501A1 contains a chimeric signal for targeting the protein to both the ER and mitochondrial compartments. The biogenesis of mitochondrial P450MT2 involves a novel targeting mechanism in which the N-terminus of P4501A1 is cleaved past the 4th and 32nd residues by an endoprotease, thus exposing the cryptic mitochondrial targeting signal.

Previous studies from our laboratory showed that under both *in vivo* and *ex vivo* conditions structurally diverse carcinogens are activated within the mitochondrial membrane compartment and bind to mitochondrial DNA at frequencies higher than the nuclear DNA (*12*−*15*). It was also shown that the mitochondrial monooxygenase activities for different xenobiotic substrates with total mitochondrial extracts (*4*, *6*, *16*−*18*) as well as purified enzyme preparations were highly dependent on the presence of Adx and Adr electron

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[1] Abbreviations: P450, cytochrome P450; P450 reductase, NADPH cytochrome P450 reductase; Adx, adrenodoxin; Adr, adrenodoxin reductase; EROD, ethoxyresorufin *O*-deethylase; ERND, erythromycin *N*-demethylase; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BNF, β-naphthoflavone; Adx-N, N-terminal acidic domain 1 of adrenodoxin; Adx-C, C-terminal acidic domain 2 of adrenodoxin; ER, endoplasmic reticulum.

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transfer proteins. The BNF- and 3-methylcholanthrene-inducible P450MT2 was an exception in that it also showed partial arylhydrocarbon hydroxylase activity in a P450 reductase supported system (*17*). These results in conjunction with the results on the precursor−product relationship between the microsomal P4501A1 and mitochondrial P450MT2 appear to be in variance with the general belief that the microsomal P450s, including P4501A1, have selective requirement for P450 reductase electron transfer protein. In this paper, we have reinvestigated this problem and found that both intact P4501A1 and the major mitochondrial form, P450MT2b (routinely referred to as P450MT2 in this paper), interact with different domains of Adx at different efficiencies, and exhibit different substrate specificity and catalytic activity.

MATERIALS AND METHODS

Purification of P450 and Electron Transfer Proteins. Mitochondrial P450MT2 and microsomal P4501A1 from BNF-treated rat livers were purified using a combination of PEG fractionation and chromatography on ω-octylaminoagarose, DEAE-Sephacel, and hydroxylapatite columns essentially as described before (*4*, *17*). Digitonin-fractionated mitoplasts used for the purification of mitochondrial P450MT2 contained less than 1% microsomal contamination as tested by the rotenone-insensitive NADPH cytochrome *c* reductase activity and the NADPH cytochrome P450 reductase level assayed by the Western blot analysis as described recently (*4*). Adx and Adr were purified from the bovine adrenal mitochondria essentially as described by Foster and Wilson (*19*). NADPH cytochrome P450 reductase was purified from PB-treated rat liver microsomes by AMP−Sepharose chromatography according to Yasukochi and Masters (*20*). Purified proteins were over 85−90% homogeneous as tested by SDS−polyacrylamide gel electrophoresis. The specific activities of P450 were >14 nmol/mg of protein, and the NADPH P450 reductase exhibited 45 μmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$.

Spectral Measurements. Spectroscopic measurements of Adx binding to P450MT2 were carried out as described by Kido and Kimura (*21*) using 2 μM P450 in 35 mM phosphate buffer (pH 7.4), 0.1% Emulgen 911, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol, and the indicated amounts of wild-type or mutant forms of Adx at room temperature in a Varian DMS 90 or a Cary-1 dual beam spectrophotometer, as indicated in the figure legends. Spectral changes in the presence of various concentrations of wild-type and mutant Adx were recorded between 450 and 350 nm. The free Adx was calculated using the equation $Adx_{Free} = Adx_{Total} - (\Delta A/\Delta Max) \times [P450]$ (*22*). To ensure that the absorbancy changes were not due to denaturation of the heme protein during spectral analysis, each sample was analyzed for the CO-bound spectral pattern.

In Vitro Reconstitution of Monooxygenase Activity. Reconstitution of monooxygenase activities for EROD (*4*) and ERND (*23*) was carried out in the presence of dilauroylphosphatidylcholine liposome (20 μg/mL mixed vesicles) using 60 pmol of P450, and either 0.2 nmol of Adx plus 0.02 nmol of Adr or 0.1 nmol of P450 reductase, in 200 μL reaction volumes (*4*). The BROD activity was assayed by measuring the formation of resorufin as described before (*4*) in a reaction mixture containing 20 mM Tris-HCl buffer, pH 7.8, 20 mM $MgCl_2$, 10 μM dicoumarol, 3.2 mg/mL BSA, and 20 μM ethoxyresorufin as substrate. The reaction was initiated by adding 3 mM NADPH at 37 °C in a shaking water bath. The reaction was terminated after 30 min of incubation by adding 2 mL of ice-cold methanol. The insolubles were sedimented at 10000*g* for 10 min, and the resorufin content of the supernatant was measured fluorometrically at the extinction and emission wavelengths of 528 and 590 nm, respectively. The reaction mixture for ERND activity contained 50 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, and 0.4 mM [*N-methyl*-^{14}C]erythromycin (1 mCi/mmol). After a 3 min preincubation, the reaction was initiated by adding 3 mM NADPH and was carried out for 30 min at 37 °C in a shaking water bath. The reaction was terminated by adding 60 μL of 1 M $NaCH_3COO$ (pH 4.5). The reaction product, $H^{14}CHO$, was measured according to Yang et al. (*24*).

Protein Cross-Linking Studies. Covalent complexes between P450 and Adx were generated by cross-linking with EDC, essentially as described before (*25*). Cross-linking was carried out in a 100 μL reaction in 50 mM phosphate buffer (pH 7.4) containing 10% (v/v) glycerol, 0.05% Emulgen 911, 0.1 mM DTT, 0.1 mM EDTA, 0.5 μM P450s, and ^{35}S-labeled or bacterially expressed Adx as stated in the figure legends. The cross-linking reaction was initiated by adding 9 mM EDC, was carried out for 60 min at room temperature, and was terminated by adding 10 volumes of phosphate-buffered saline. The cross-linked complexes were subjected to immunoprecipitation or Western blot analysis as indicated.

Adx−Sepharose Chromatography. Adx−Sepharose was prepared as described by Honukoglu et al. (*26*); 1 μmol of recombinant human Adx (generously provided by Dr. Larry Vickery, University of California at Irvine) was conjugated to 15 mL of CNBr-activated Sepharose 4B (7 mL bed volume) by incubation at 4 °C for 24 h as described (*26*). Partially purified P450MT2 of about 10−15% purity (10 mg) was applied to the Adx−Sepharose column (1.5 mL bed volume), which was preequilibrated with 10 mM K_2HPO_4 buffer (pH 7.4) containing 10% glycerol, 0.1% sodium cholate, 100 μM EDTA, 100 μM DTT, and 100 μM PMSF. The column was washed with 10 column volumes of the same buffer, and the proteins were eluted sequentially with a step gradient of 5 mL each, containing 50, 100, 150, and 200 mM KCl. The absorbance at 418 nm was monitored, and 1 mL fractions were collected.

Bacterial Expression of Adx and P450 Proteins. The protein coding region of the bovine Adx cDNA (*27*) was amplified by PCR and cloned in the pET-28a(+) vector (Novagen) and expressed as an N-terminal His-tagged protein in *E. coli* cells. Adx carrying D76N and D79N mutations (Mut Adx) were generated by overlap PCR and expressed in *E. coli* cells. Reconstitution of 2Fe-2S clusters of wild-type and Mut Adx was carried out according to Coghlan and Vickery (*22*). The induction of protein expression and purification of His-tagged proteins were carried out according to the Novagen protocol.

cDNAs for N-terminal truncated forms of P4501A1, +5/1A1 and +33/1A1, were generated so as to contain a C-terminal His tag as described before (*11*). The cDNA constructs were cloned in the bacterial expression vector PCW (*28*) and expressed in *E. coli* strain JM-109 as

Table 1: List of Synthetic Peptides Used for Competition

Adx-N (residues 26−48 of human Adx)‘	GDSLLDVVVENNLDIDGFGACEG
Adx-C (residues 70−85 of human Adx)	ITDEENDMLDLAYGLT
C27 peptide (residues 387−399 of rat P450c27)	LLKAVIKETLRLY
NS1 (nonspecific)	DGSGPYSTLSSPQC
NS2 (nonspecific)	STYMETYVYFYRSC

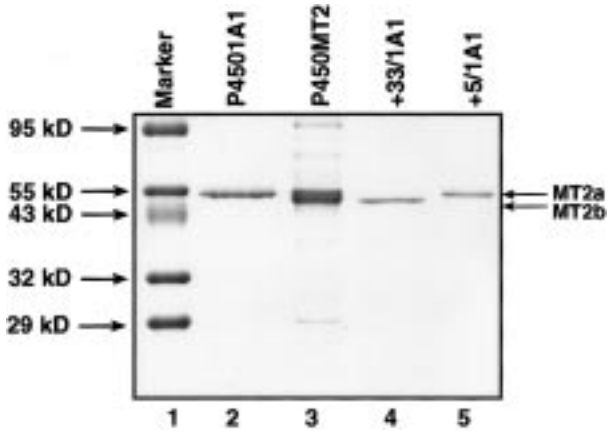


FIGURE 1: Electrophoretic patterns of purified microsomal P4501A1 and mitochondrial P450MT2. P4501A1 and P450MT2 were purified from BNF-induced rat liver microsomes and mitochondria, respectively. Recombinant +33/1A1 and +5/1A1, cloned in PCW vectors, were expressed in *E. coli* (JM109) by induction with IPTG. Bacterially expressed P450s were purified by chromatography on Ni^{2+}−agarose, DEAE-Sephacel, and hydroxylapatite columns as described (*29*). About 1−2 μg of purified proteins was subjected to gel electrophoresis on a 14−16% gradient polyacrylamide gel as recently described (*11*), and stained with Coomassie blue.

described by Imai et al. (*29*). The proteins were purified to about 65−80% purity (9−12 nmol of P450/mg of protein) by using a combination of Ni^{2+}−agarose, DEAE-cellulose, and hydroxylapatite column chromatography (*29*).

In Vitro Translation of Adx. Wild-type and mutant Adx constructs were generated as described above, cloned in pCRTM II vector (Invitrogen), and used as templates for generating ^{35}S-labeled translation products as described before (*11*).

Immunoprecipitation and Western Blot Analysis. Monospecific antibodies to P4501A1 or purified human Adx (*4*) were used for immunoprecipitation using conditions described before (*30*). The immunoprecipitated proteins were dissociated at 98 °C for 5 min in Laemmli’s sample buffer and subjected to electrophoresis on 12% polyacrylamide gels. The Western blot analysis was carried out as described before (*4*).

Synthetic Peptides. Peptides were synthesized using an ABI Model 431A synthesizer and purified by HPLC on a reverse phase column. A list of the various synthetic peptides used in this study has been presented in Table 1.

RESULTS

Reconstitution of P450s with Adx+Adr and P450 Reductase Electron Donor Proteins. The electrophoretic patterns of P450s purified from BNF-induced livers and also +5/1A1 and +33/1A1, expressed in *E. coli* cells, are presented in Figure 1. As expected, P4501A1 purified from BNF-induced microsomes resolved as a single major component of about 54 kDa (lane 2), while P450MT2, purified from the similarly induced mitochondrial fraction, resolved as two closely migrating components, designated as P450MT2a and MT2b (lane 3). In a recent study (*11*), N-terminal and internal protein sequence analysis showed that both of the mitochondrial forms represent N-terminal truncated versions of the microsomal P4501A1; P450MT2a is devoid of the first 4 residues, and MT2b is devoid of the N-terminal 32 residues. The results also show that bacterially expressed purified +33/1A1 (lane 4) comigrated with the mitochondrial MT2b protein, while +5/1A1 protein (lane 5) migrated similar to MT2a in lane 3 and also intact P4501A1 in lane 2. The SDS gel electrophoresis system used in this study is unable to resolve proteins differing by four amino acid residues.

Table 2: Enzyme Activities of Intact and Truncated P450s with Different Electron Transfer Proteins

		activities[a]	
P450	electron transfer proteins	EROD	ERND
P4501A1[b]	P450 reductase	5.0 ± 0.1	0.05 ± 0.01
P4501A1	Adx + Adr	0.07 ± 0.02	0.36 ± 0.02
P450MT2[b]	P450 reductase	0.03 ± 0.01	0.72 ± 0.04
450MT2	Adx + Adr	0.05 ± 0.02	2.89 ± 0.2
+5/1A1[c]	P450 reductase	3.65 ± 0.2	0.21 ± 0.02
+5/1A1	Adx + Adr	0.10 ± 0.02	0.58 ± 0.03
+33/1A1[c]	450 reductase	0.33 ± 0.06	0.68 ± 0.07
+33/1A1	Adx + Adr	0.03 ± 0.01	2.30 ± 0.2

[a] EROD = nmol of resorufin formed min^{-1} (nmol of P450)$^{-1}$, ERND = nmol of $H^{14}CHO$ formed min^{-1} (nmol of P450)$^{-1}$. [b] P4501A1 and P450MT2 were purified from BNF-induced rat liver microsomes and mitochondria, respectively. [c] +5/1A1 and +33/1A1 were purified forms expressed in *E. coli* cells.

In a previous study, we showed that the mitochondrial P450MT2 exhibits high ERND and low EROD activities in an Adx+Adr-supported system (*4*). Because of our recent findings that P450MT2 consists of two forms of N-terminal truncated P4501A1 (*11*), we compared the enzyme activities of the purified P450 forms and also the two recombinant bacterially expressed forms in assay systems reconstituted with different electron transfer proteins. Results in Table 2 show that in a P450 reductase-supported system, intact P4501A1 exhibited high EROD activity while the mitochondrial P450MT2 exhibited negligible activity. Additionally, bacterially expressed +5/1A1 showed a moderately high P450 reductase-supported EROD activity, while +33/1A1 showed very low activity. For yet unknown reasons, the bacterially expressed +33/1A1 showed a significantly higher EROD activity in a P450 reductase-supported system than P450MT2 purified from BNF-induced liver mitochondria. All these forms of enzymes, however, showed consistently reduced EROD activity in an Adx+Adr-supported system. Both liver mitochondrial P450MT2 and bacterially expressed +33/1A1 showed the highest ERND activity [2.9 and 2.3 nmol of $H^{14}CHO$ formed min^{-1} (nmol of P450)$^{-1}$, respectively] in an Adx+Adr-supported system, and significant but vastly reduced activity in a P450 reductase-supported system. The intact P4501A1, on the other hand, showed negligible ERND activity in a P450 reductase-supported system, while it showed a low, but significant activity with the Adx+Adr electron transfer system. These results suggest that the intact P4501A1 has high preference for ethoxyresorufin in a P450 reductase-supported system, while the mitochondrial P450MT2

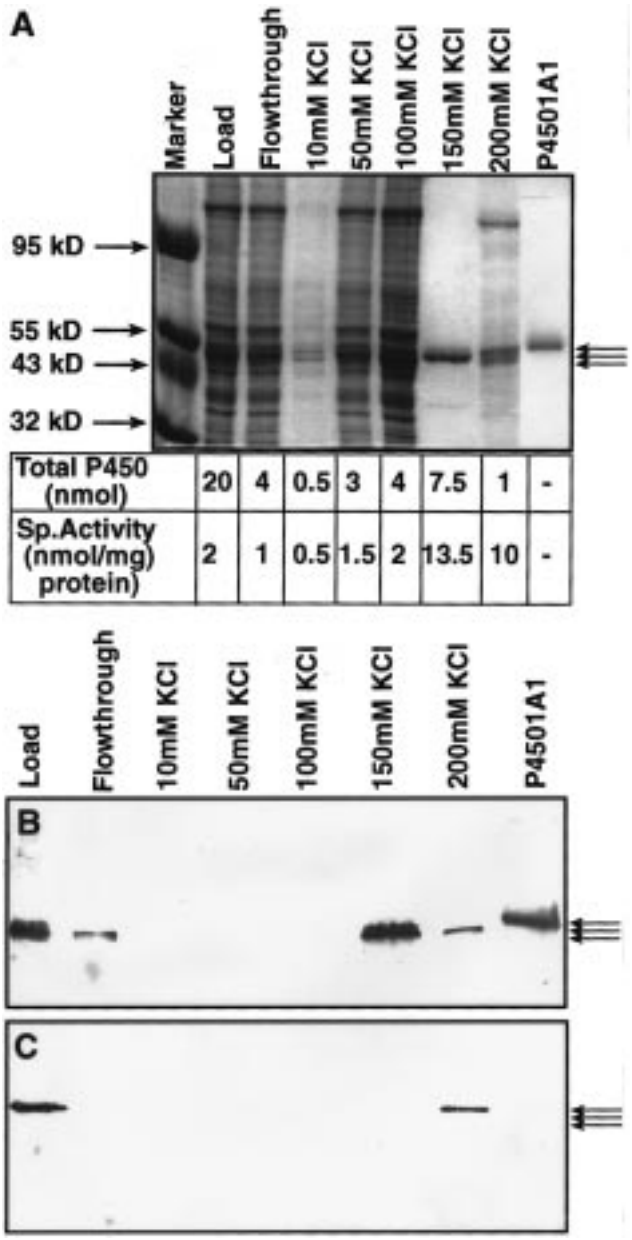


FIGURE 2: Ionic strength dependent elution of Adx−Sepharose-bound P450MT2 and P450c27. About 10 mg of partially purified protein from BNF-induced rat liver mitochondria was subjected to chromatography on an Adx−Sepharose column (100 nmol of Adx, 1.5 mL bed volume) as described under Materials and Methods. About 15−25 μL of each fraction and 1 μg of purified P4501A1 were subjected to electrophoresis on a 14−16% gradient acrylamide gel as in Figure 1. Panel A represents the Coomassie-stained gel pattern. The total P450 contents (nmol) and specific activities (nmol of P450/mg of protein) of each fraction are indicated underneath. Panels B and C represent duplicate gels subjected to Western blot analysis using antibodies to P4501A1 and P450c27, respectively. In each case, an antibody dilution of 1:1000 was used as described before (*4*). The three arrows on the right-hand side of each pattern indicate the positions of the 54 kDa, 52.5 kDa and 51 kDa bands in fractions eluted with the 150 mM and 200 mM KCl containing buffers.

exhibits a higher preference for erythromycin in an Adx+Adr-supported system. It should be pointed out that the +5/1A1 with nearly intact transmembrane domain (P450MT2a in the purified mitochondrial fraction) resembles the intact P4501A1 more closely in terms of enzyme activity. The purified P450MT2 used in this study (see Figure 2A) consists mostly (>75% mass) of the faster migrating MT2b (+33/1A1) form as tested by the gradient gel electrophoresis. Thus, the enzymatic and physical properties of purified P450MT2 mostly reflect the properties of the +33/1A1 form.

The Nature of Adx Binding to P450MT2. It is well established that constitutive mitochondrial P450s like P450scc and P450c27 bind to Adx with high affinity through charge interactions, such that solid matrix-conjugated Adx could be used for the affinity purification of the P450 forms (*26*, *31*, *32*). The affinity binding and the salt requirements for elution also help resolve specific binding from nonspecific binding of charged proteins to Adx (*32*−*34*). We therefore tested the affinity of P450MT2 to bind to an Adx−Sepharose column and verified the KCl concentration required for disrupting this interaction. As shown in Figure 2A, of the 20 nmol of P450 (specific activity of 2 nmol/mg of protein) loaded on the column, about 16 nmol bound to the column and only about 4 nmol was recovered in the flow-through fraction. Similarly, the P450 contents of fractions eluted with the wash fraction and also buffers containing 50 and 100 mM KCl were low, with specific activities in the range of 0.5−2 nmol/mg of protein. Nearly 85% of the bound proteins eluted in these three fractions. Fractions eluted with 150 and 200 mM KCl containing buffers had the highest specific content of 13.5 and 7.5 nmol/mg of protein, respectively. The gel pattern in Figure 2A shows that the input as well as the flow-through fractions contained multiple bands. Similar was the case with fractions eluted in the wash and the 50 and 100 mM KCl containing buffers. The fraction eluted with 150 mM KCl containing buffer, however, contained nearly 90% homogeneous protein with a major ~52.5 kDa band (putative MT2b) and a minor ~54 kDa band (putative MT2a), the latter comigrating with P4501A1. Finally, the fraction eluted with the 200 mM KCl containing buffer consisted of a major 150−160 kDa protein of unknown nature and three closely migrating proteins with apparent molecular mass of ~51 to ~54 kDa. Western immunoblots in Figure 2B,C show that the major 52.5 kDa (and also the minor ~54 kDa) proteins eluted with 150 mM KCl containing buffer and also the ~52.5 kDa protein from the 200 mM KCl eluted fraction cross-reacts with antibody to P4501A1 but not with antibody to P450c27. Additionally, only the 54 kDa protein eluted with the 200 mM KCl containing buffer cross-reacted with antibody to P450c27, suggesting that this P450 may form a more stable complex with Adx. Finally, the nature of the 51 kDa protein eluted with the 200 mM KCl containing buffer remains unknown as it failed to interact with any of the antibodies tested. These results indeed show that the interaction of P450MT2 with Adx is specific and resembles the binding properties of the constitutive mitochondrial P450s.

Adx- and P450 Reductase-Induced Spin State Changes in P450MT2. Binding of electron transfer proteins or added substrates is known to induce a high-spin state in both the mitochondrial and the microsomal P450 heme, which is characterized by increased absorbancy at 390 nm and a reduced peak at 420 nm (*21*, *26*, *35*). It is also known that the two conserved acidic amino acid residues, D76 and D79, from the Adx-C (residues 70−85) are important for binding to P450scc and for the reconstitution of cholesterol side chain cleavage activity (*22*). We have determined the binding efficiencies of the wild-type and mutant Adx with P450MT2

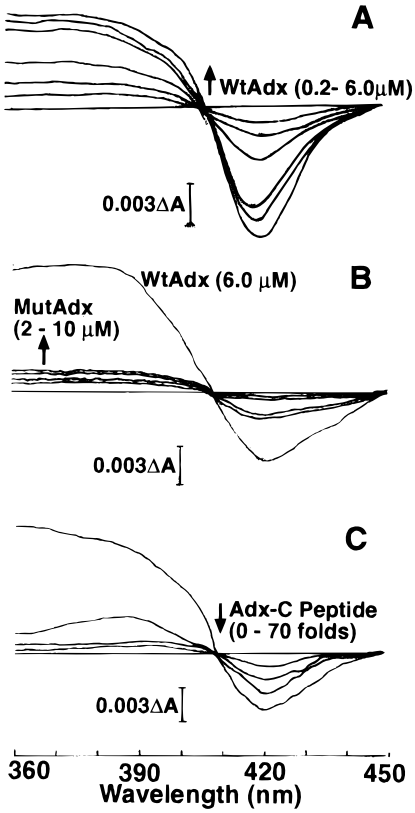


FIGURE 3: Spectral analysis of Adx binding to P450MT2 and induction of high-spin state. Spectral measurements were carried out as described under Materials and Methods using 2 μM purified P450MT2 in a Varian DMS 90 dual beam spectrophotometer. In (A) the measurements were carried out using varying concentrations of human recombinant Adx ranging from 0.2 μM (lowest absorbance) to 6 μM (highest absorbance). In (B) measurements were carried out either in the presence of 6 μM wild type Adx (highest absorbance) or in the presence of 2−10 μM MutAdx carrying D76N and D79N mutations. In (C), measurements were carried out in the presence of 6 μM wild-type Adx, with added 0−70-fold molar excess of Adx-C peptide.

and verified the effects of added peptides from the two different acidic domains, Adx-C and Adx-N listed in Table 1. As shown in Figure 3A, bacterially expressed human Adx was able to induce a shift in the spin state of the P450MT2 heme iron as indicated by Soret bands at 420 and 385−390 nm. The shift in absorbancy at these two wavelengths was dependent on the concentration of wild-type Adx added. As shown in Figure 3B, Mut Adx carrying D76N and D79N substitutions induced diminished spectral shifts in the P450MT2 heme. Similar effects of mutant Adx protein on the P450scc were shown before (*22*). Figure 3C shows that 20−70 molar excess of Adx-C peptide effectively competed for binding. The hyperbolic type of curves in the 385−390 nm region indeed represents the high-spin state of the heme, rather than denatured heme protein, as these samples exhibited characteristic CO-bound absorption spectra. It should be noted that some studies have reported symmetrical peaks in the 380−90 nm region in response to added substrate or electron donor proteins (*8*, *22*, *26*), while others (*36*, *37*) have observed hyperbolic patterns similar to those obtained in the present study. Currently the reason for these differences remains unclear, although it may be due to inherent differences in the P450 types used. These spectral results provide evidence that the bovine Adx indeed binds

Table 3: Effects of Wild-Type and Mutated Adx on the P450MT2-Catalyzed ERND Activity

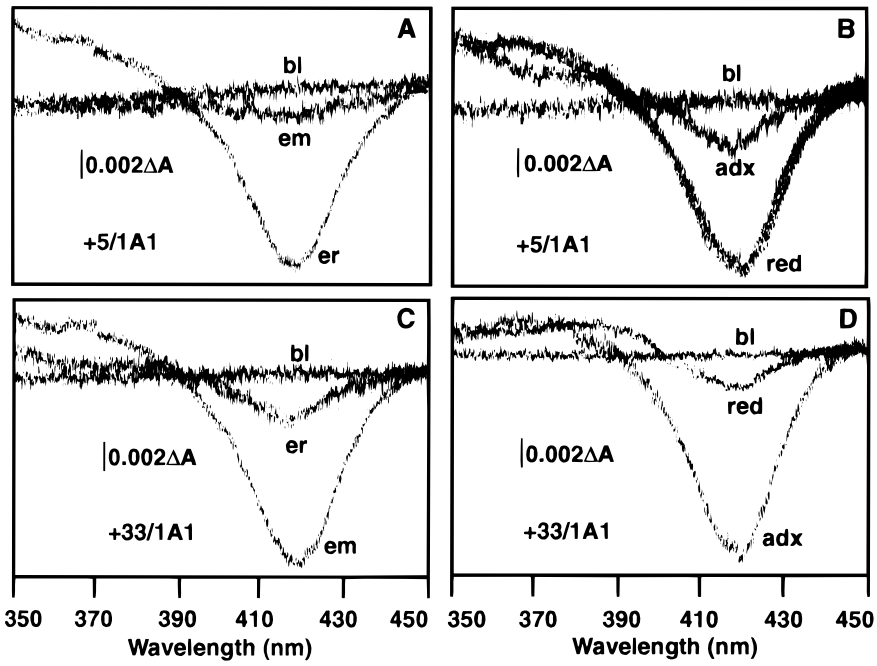
reconstituted with	competitor	activity[a]	K_d (μM)[d]
Adx + Adr	none	2.57 ± 0.06	0.6
	C27 peptide[b]	0.51 ± 0.01	ND[f]
	NS1[c]	2.06 ± 0.07	ND
	NS2[b]	2.07 ± 0.1	ND
	P450 reductase[c]	3.10 ± 0.2	ND
Mut Adx + Adr	none	0.35 ± 0.04	6.1
P450 reductase	none	0.82 ± 0.08	∼1.8[e]
	C27 peptide	0.76 ± 0.1	ND
	NS1[b]	0.78 ± 0.07	ND
	NS2[b]	0.72 ± 0.05	ND
	Adx[c]	0.95 ± 0.09	ND

[a] ERND activity [nmol of $H^{14}CHO$ formed min^{-1} (nmol of P450)$^{-1}$]. [b] 600 pmol (10-fold excess) of the various peptides shown in the table was added. [c] 10 μM P450 reductase and Adx were added as competitors. [d] Binding constants (K_d) were calculated based on spectral measurements presented in Figure 3. [e] The corresponding K_d for P4501A1 in the presence of added ethoxyresorufin is 0.4 μM. [f] ND = not done.

to P450MT2 and the binding may involve the acidic domain 2 of the Adx, referred to as Adx-C. Although binding studies were carried out in the presence of 0.1% Emulgen, measurements in the presence of added phospholipids yield essentially similar patterns.

Double reciprocal plots ($1/\Delta A$ versus 1/[Adx]) of titration values with Adx were used to calculate the apparent K_d values essentially as described (*21*, *22*, *26*) and compared to the ERND activities in a reconstituted system. As shown in Table 3, in reconstitution assays with P450MT2, the wild-type Adx yielded high ERND activity and also high binding efficiency (as determined by spectral measurements) in the range of 0.6 μM K_d, while MutAdx carrying D76N and D79N substitutions yielded low activity and a 10-fold higher apparent K_d value of 6.1 μM. Reconstitutions with the wild-type Adx are efficiently competed by a 10-fold excess of the C27 peptide from the Adx binding domain of rat P450c27, but not by two different nonspecific peptides, NS1 and NS2. Similarly, the C27 peptide drastically reduced the binding efficiency, while the nonspecific peptides had no significant effect. Reconstitution with P450 reductase yielded 3-fold lower ERND activity and also 3-fold lower binding efficiency than that observed with Adx. Interestingly, neither the C27 peptide nor added Adx protein affected the activity of the enzyme in a P450 reductase-supported system. These results suggest that P4501A1 and P450MT2 have different affinities for Adx and different substrate specificities. The results of competition with different proteins and peptides also suggest a nonoverlapping type of P450MT2 interaction with the two electron transfer proteins.

The possible differences in the physical and catalytic properties of the full-length and truncated P4501A1 were further investigated using bacterially expressed and purified +5/1A1 with nearly intact transmembrane domain, and the truncated +33/1A1 species. The spectral patterns in Figure 4A,B show that ethoxyresorufin (er) and P450 reductase (red) induced maximum shifts in spin state of +5/1A1 while erythromycin (em) and Adx induced minimal changes. In contrast, the spectral patterns with +33/1A1 show maximal shifts with erythromycin (em) and Adx and minimal changes with ethoxyresorufin (er) and P450 reductase (see Figure 4C,D). The binding constants calculated from the spectral changes at 418 and 390 nm are presented in Figure 4E. The



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	K_d Values(μM)			
Enzyme	EM	ER	Adx	Red
+5/1A1	N.S	2	2	0.5
+33/1A1	65	25	0.6	1.5

FIGURE 4: Spectral analysis of bacterially expressed +5/1A1 and +33/1A1 for binding to different substrates and electron donor proteins. Spectral measurements using bacterially expressed and purified +5/1A1 (A and B) and +33/1A1 (C and D), 2 μM in each case, were carried out as described in Figure 3 in a Cary Model 1E spectrophotometer. The term bl indicates the base line. The spectral analyses were carried out using 6 μM purified human Adx, 4 μM purified rat P450 reductase (red), 40 μM ethoxyresorufin (er), or 500 μM erythromycin (em). K_d values were calculated based on measurements carried out in the presence of varying levels of substrates or electron donor proteins as in Table 3. The term N.S (not significant) represents binding below the level of detection.

results show that the nearly intact +5/1A1 binds ethoxyresorufin and P450 reductase at higher efficiency while the more truncated +33/1A1 binds erythromycin and Adx at higher efficiency. The overall binding affinity of +33/1A1 for erythromycin (65 μM) is about 3−4-fold lower than the values reported for P4503A1/2 that are considered as markers for this activity (37). Finally all of these P450 samples yielded a characteristic CO absorbance spectra suggesting that they were not in a denatured state.

Chemical Cross-Linking of Adx with Intact and Truncated P4501A1. The water-soluble carbodiimide EDC has been used to cross-link various redox partner proteins, including Adx, Adr, cytchrome b_5, cytochrome *c*, and cytochrome P450 (*25*, *38*−*40*). EDC cross-linking of wild-type Adx and P450scc (*39*) has shown that the Adx-C domain of the human Adx interacts with the basic region of P450scc, through charge pairing (41). In the present study, we used EDC cross-linking to investigate the mode of interaction of Adx with P4501A1 and P450MT2 (containing more than 80% of the +33/1A1form) by two parallel approaches. In the first approach, ^{35}S-labeled Adx was used for cross-linking with unlabeled P4501A1 and P450MT2, and the cross-linked products were identified by immunoprecipitation with polyclonal antibody to Adx or P4501A1. In the second approach, unlabeled bacterially expressed Adx and P4501A1 or P450MT2, purified from BNF-induced livers, were subjected to EDC conjugation, the unmodified and also the cross-linked P450s were immunoprecipitated with P4501A1 antibody, and the products were visualized by Western blot analysis using the same antibody.

The autoradiogram in Figure 5A shows that in reactions without added EDC, the immunoprecipitated protein with Adx antibody consisted exclusively of labeled Adx, while the P4501A1 antibody failed to immunoprecipitate any labeled protein. In reaction mixtures with added EDC, however, both Adx antibody and P4501A1 antibody immunoprecipitated a 68 kDa cross-linked product with P4501A1 and a major 66 kDa cross-linked product with P450MT2. The apparent sizes of these cross-linked products are consistent with cross-linked heteroduplexes of Adx with P4501A1 or P450MT2, respectively. Figure 5C,D shows the extent of cross-linking as a function of increasing concentrations of the two P450s and increasing Adx, respectively. The results show that the extent of heteroduplex formation follows a linear relationship with the concentrations of P450MT2 and Adx, respectively, and reached a saturation when one of the components became limiting. The results also show that cross-linking with intact P4501A1

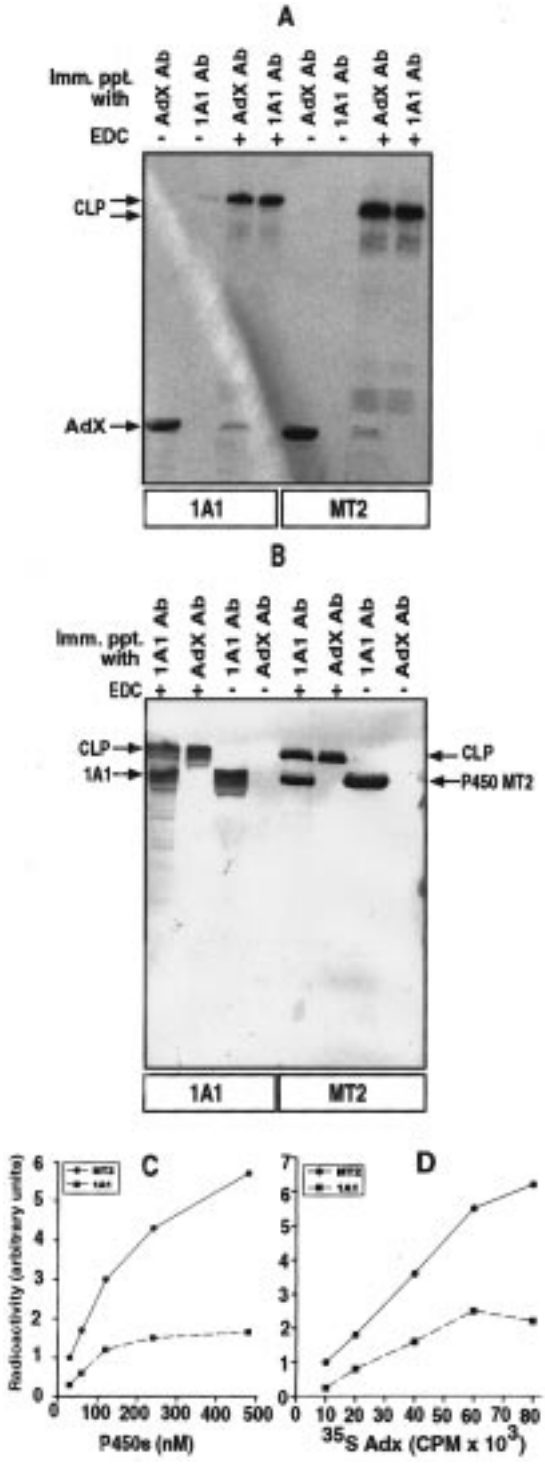

FIGURE 5: Chemical cross-linking of Adx with P450MT2 and intact P4501A1. In (A), ^{35}S-labeled Adx was conjugated to P4501A1 or P450MT2 as described under Materials and Methods; the products were immunoprecipitated with either Adx IgG or P4501A1 IgG and subjected to acrylamide gel electrophoresis and fluorography. In (B), cross-linking was carried out using unlabeled bovine Adx and P450s, and the immunoprecipitated products were subjected to Western immunoblot analysis using P4501A1 antibody. In panels C and D, the extent of cross-linking in the presence of increasing concentrations of the two P450s and ^{35}S-labeled Adx, respectively, was examined. Constant amounts of Adx (80×10^3 cpm) in (C) and constant amounts of P450MT2 or P4501A1 (500 nM) in (D) were used. The cross-linked products (CLP) were subjected to immunoprecipitation and electrophoresis as in (A), and the extent of cross-linking was quantitated by excising and counting the gel pieces.

was less efficient and reached a saturation point at about one-third to one-fourth the level of that with P450MT2. Although not shown, bacterially expressed +5/1A1 yielded efficiency comparable to P4501A1. Because of the low abundance of +5/1A1, coupled with its low binding efficiency with Adx, the cross-linked product with P450MT2 contained a very low level of 67.5 kDa species that was visible only in overexposed gels.

The Western blot in Figure 5B developed with P4501A1 antibody shows that incubation of P4501A1 and Adx with added EDC yielded two immunoprecipitable products with P4501A1 antibody that are consistent with 54 kDa P450 and 68 kDa P450−Adx cross-linked product. The Adx antibody, on the other hand, immunoprecipitated only the 68 kDa putative cross-linked product. Reaction mixtures without added EDC yielded only the 54 kDa protein band with the P4501A1 antibody and no band with Adx antibody. Essentially similar patterns were obtained with P450MT2, except that the unmodified P450MT2 and the putative cross-linked product (66 kDa) migrated faster than the corresponding P4501A1 reaction products. These results demonstrate that Adx indeed interacts with both P4501A1 and P450MT2, and the interacting complexes can be cross-linked as respective heteroduplexes.

Different Domains of Adx Interacting with P450MT2 and P4501A1. In Figure 6, cross-linking reactions were carried out with saturating levels of ^{35}S-labeled Adx (80 000 cpm) and P450MT2 (500 nM), and equal portions of reaction mixtures were subjected to immunoprecipitation with excess Adx antibody (Figure 6B) or Western blot analysis using P4501A1 antibody (see Figure 6B). The immunoprecipitation was used for the quantitative analysis, and the Western blot was carried out to ensure that the P450MT2 concentrations were not limiting in the reaction mixtures. Quantitation of representative gels from Figure 6A shows that the wild-type Adx cross-linked with P450MT2 efficiently (100%) while the MutAdx carrying D76N and D79N mutations yielded only 3−4% cross-linking (see Figure 6C). Similarly, addition of a 30-fold molar excess of a moderately hydrophobic nonspecific peptide, NS1, or a hydrophilic nonspecific peptide, NS2, and also addition of a 5 molar excess of BSA did not cause any inhibition. Addition of a 30 molar excess of C27 peptide, on the other hand, yielded only 4% cross-linking. Furthermore, addition of 10 and 30 molar excess of Adx-C peptide, corresponding to sequence 70−85 of the human Adx (*42*), inhibited cross-linking by 30 and 95%, respectively. These results demonstrate that Adx interacts with P450MT2 through its conserved C-terminal acidic domain 2, which is also believed to be the domain for interaction with the constitutively expressed mitochondrial P450s (22).

Figure 7 represents a companion cross-linking experiment as in Figure 6, except that intact P4501A1 was used. Equal portions of the reaction mixture were subjected to immunoprecipitation with Adx antibody (Figure 7A) and Western blot analysis with 1A1 antibody (Figure 7B) to ensure that the levels of P4501A1 in various reactions were not limiting. As seen from Figure 7C, Adx cross-linking with intact P4501A1 shows a highly contrasting pattern of competition as compared with P450MT2. First, the MutAdx that showed a markedly reduced binding to P450MT2 exhibited nearly 80% cross-linking as compared to the wild type. Second, a

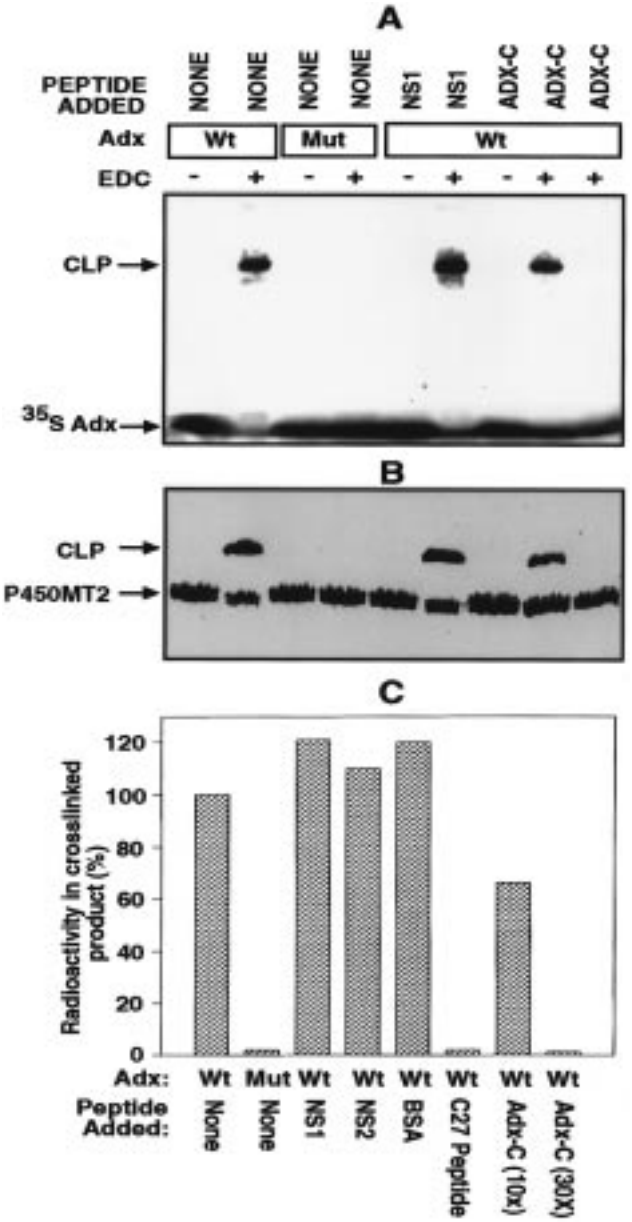

FIGURE 6: Specificity of Adx cross-linking with P450MT2. Incubations with or without added EDC were carried out using 80 $\times$ 10^3 cpm of ^{35}S-labeled wild-type Adx or MutAdx and 500 nM P450MT2 as described under Materials and Methods. Half of the reaction products were subjected to immunoprecipitation with excess Adx antibody followed by electrophoresis and radioautography (A), and the other half was subjected to Western blot analysis using P4501A1 antibody (B). In (C), representative gels as in (A) containing reactions with additional indicated competitors were quantitated as described in Figure 4C,D. A 30-fold molar excess of NS1 and NS2 peptides, 5 molar excess of BSA, 30 molar excess of Adx binding domain C27 peptide, 10- and 30-fold molar excess of Adx-C peptides, and 10-, 30-, and 70-fold molar excess of Adx-N peptide were used for competition. Results in (C) are the average of two separate cross-linking reactions.

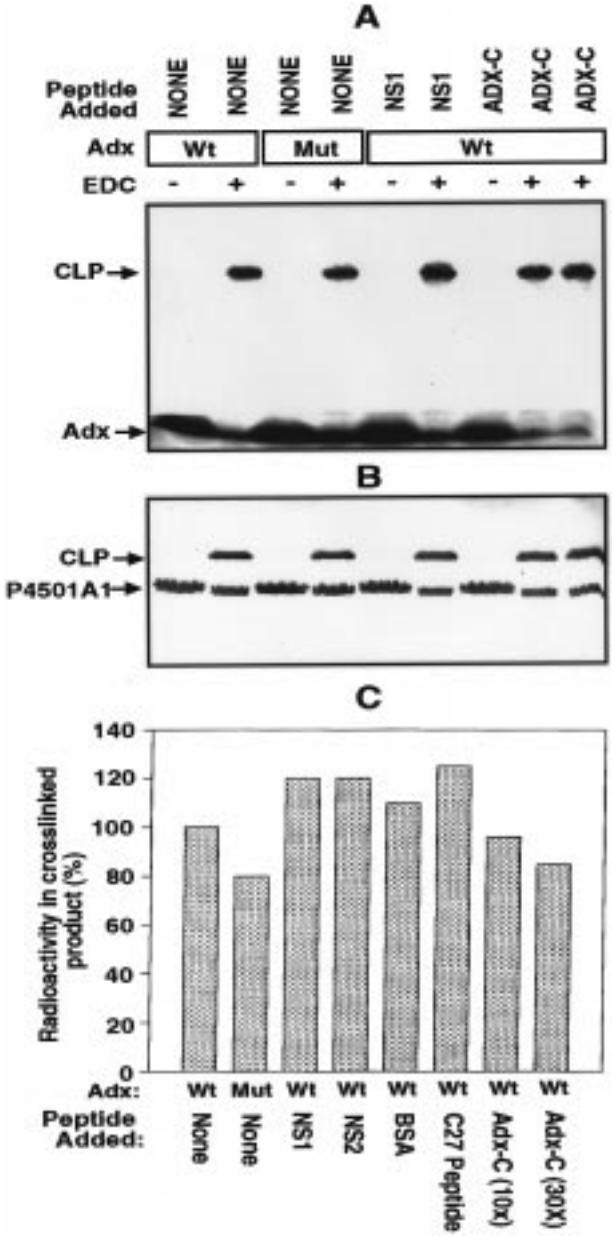

FIGURE 7: Interaction of wild-type and mutant Adx with intact P4501A1. Reactions were carried out using ^{35}S-labeled wild-type Adx or MutAdx with microsomal P4501A1 as described in Figure 5. (A) Half of the reaction mixture was immunoprecipitated with Adx antibody and electrophoresed. (B) The second half was subjected to Western blot analysis using 1A1 antibody. (C) Quantitation of representative gels as in (A) containing reactions with the additional indicated competitors. The concentrations of competitors used were as in Figure 5. The values in (C) are the average of two separate cross-linking reactions.

30-fold excess of C27 peptide did not inhibit the cross-linking. Additionally, 5-fold molar excess of either BSA, the nonspecific peptides (NS1 and NS2), or the Adx-C peptides failed to compete for Adx cross-linking to P4501A1. These results suggest that Adx interaction with intact P4501A1 may involve a domain other than the Adx-C acidic domain 2.

Since Adx also contains an N-terminal acidic domain (sequence 26−48 of human Adx), which was initially implicated in binding to some P450 forms (*43*), we tested its ability to compete for Adx cross-linking with P450MT2 and also P4501A1. The cross-linking reactions were carried out in the presence of saturating levels of ^{35}S-labeled Adx (80 000 cpm) and unlabeled P450s (500 nM) as described in Figure 6, and the cross-linked products were assayed by radiometric analysis. As seen from Figure 8A, the Adx cross-linking to P450MT2 was observed only in the presence of added EDC, and in contrast to the effect of Adx-C peptide (Figure 6), 10-, 30-, and 70-fold excess of Adx-N peptide only marginally inhibited (10−15%) the cross-linking. In

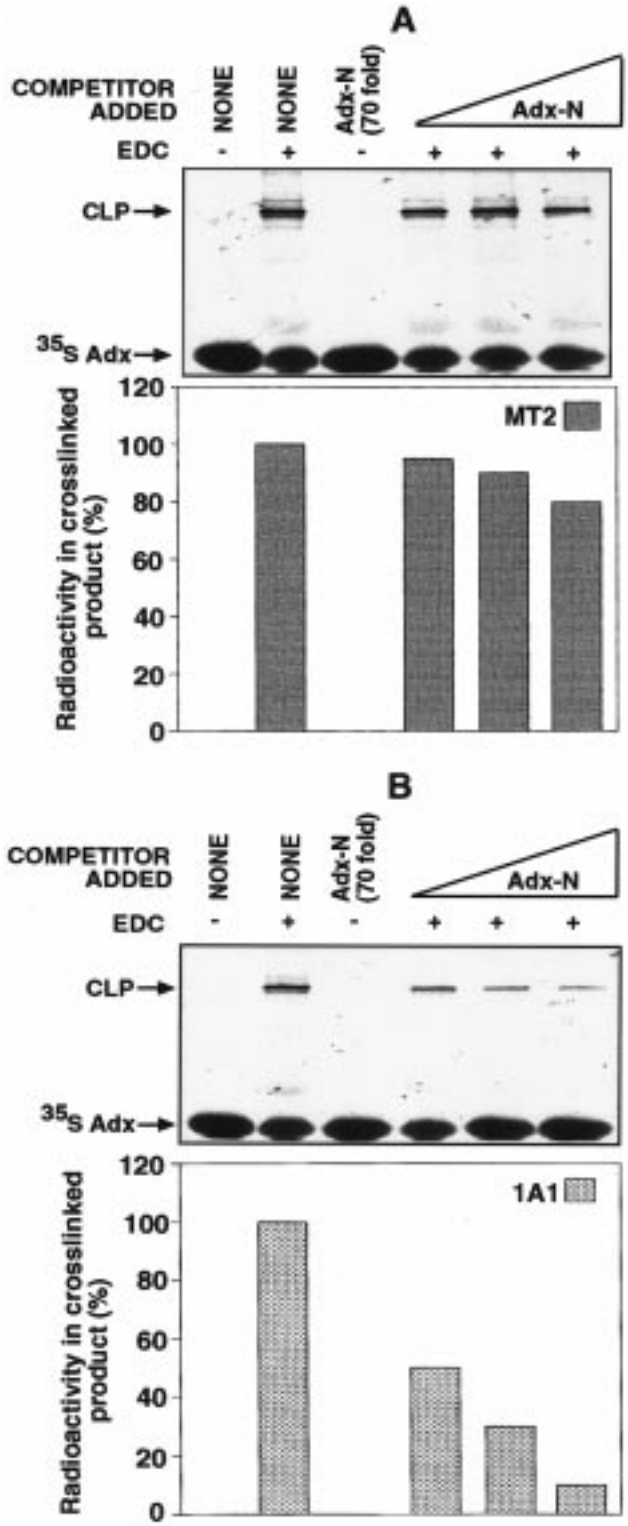

FIGURE 8: Different mode of Adx interaction with intact P4501A1 and P450MT2. Cross-linking reactions were carried out with or without added EDC, 80 × 10^3 cpm of ^{35}S-labeled Adx, and 500 nM P450s as described under Materials and Methods. The cross-linked products with P450MT2 (presented in panel A) and P4501A1 (presented in panel B) were resolved on polyacrylamide gels and subjected to autoradiography. The radiometric quantitation of CLP from representative gels is presented underneath each autoradiogram. Values represent the average of 2−3 separate cross-linking reactions. The Adx-N (from N-terminal acidic domain 1) peptide was added at 10-fold, 30-fold, and 70-fold molar excess in the last three lanes of both (A) and (B).

contrast, results in Figure 8B show that Adx cross-linking to P4501A1 was inhibited by 50%, 70%, and >95% by addition of 10, 30, and 70 molar excess, respectively, of the Adx-N peptide. These results suggest that P4501A1 may interact with Adx through its N-terminal acidic domain 1.

DISCUSSION

It is well established that P450 reductase is targeted to the ER, while the Adx and Adr are targeted to the mitochondrial matrix compartment (*3*). Studies on the membrane topology and enzyme reconstitution demonstrate that the microsomal P450s, that are transmembrane proteins with an N-terminal membrane anchor domain, are heavily dependent on P450 reductase for the transfer of electrons from NADPH (*44*, *45*). In contrast, the constitutive mitochondrial P450s such as P45011A, P45011B, and P450c27, which lack the transmembrane anchor (*47*, *48*), are membrane extrinsic proteins although some of them are tightly associated with the mitochondrial inner membrane possibly through internal hydrophobic domains (*2*, *4*, *46*, *48*). Studies with a number of mitochondrial xenobiotic-inducible P450s, which cross-react with antibodies to similarly induced microsomal P450s, suggest a membrane extrinsic organization (*4*, *48*). Recent experiments in our laboratory showed that the mitochondrial P450MT2 consists of two progressive N-terminal truncated versions of the microsomal P4501A1, raising questions on the nature of P450MT2 interaction with mitochondrial resident electron transfer proteins. To address these questions, we have investigated the physical and structural interaction of human and bovine Adx with rat mitochondrial P450MT2, as well as recombinant +5/1A1 and +33/1A1 expressed in bacterial cells.

A notable observation of this study is that the intact 1A1 and truncated P450MT2 differ significantly with respect to their substrate preferences; the intact protein yields high EROD activity in a P450 reductase-supported system, while the truncated P450MT2 yields higher ERND activity in an Adx+Adr-supported system (Tables 2 and 3). Since ERND activity is a specific marker for the 3A subfamily (*49*), the observed ERND activity with +33/1A1 is intriguing. Although the reasons for the altered substrate specificity of P450MT2 remain unknown, a conformational change due to the loss of the N-terminal hydrophobic transmembrane domain is a likely possibility. Conformational shifts and associated changes in the activity due to elimination of transmembrane anchor domains or mutations targeted to the transmembrane helical domains have been reported for a number of membrane-bound enzymes and receptors (*50*−*52*). Our results also suggest that the P450 reductase is the preferred electron donor for the intact P4501A1 and also +5/1A1, while the Adx+Adr proteins are the preferred electron transfer proteins for the activity of +33/1A1, which is the predominant constituent of the P450MT2 purified from BNF-induced liver mitochondria. These results are in general conformity with the published results from our own and other laboratories (*4*−*7*) on the distinctive features of the xenobiotic-inducible mitochondrial and microsomal P450s. They also provide a valuable new insight on how the same P450 apoprotein targeted to two different membrane compartments exhibits different structural and catalytic properties.

Since the concept of Adx+Adr interaction with intact or truncated P4501A1 questions existing views on the functional specificities and the electron transport protein requirements

of P450s targeted to the two different cytoplasmic organelles, we investigated this problem using multiple approaches. First, the reconstitution of ERND activity and the spectral absorbancy shift to a high-spin state clearly demonstrate that Adx+Adr indeed interacts with P450MT2 in a functionally productive manner. The interaction is specific by the following criteria: (1) the use of mutated Adx, or addition of peptides from the Adx binding domain of P450c27 (C27 peptide) and the Adx C-terminal acidic domain (Adx-C peptide) which is known to interact with the constitutive mitochondrial P450s, yielded low enzyme activity, and suppressed the absorbancy shifts; (2) the use of an Adx− Sepharose affinity column yielded nearly homogeneous P450MT2, and the elution of the P450 from the affinity column resembles the conditions reported for other mitochondrial P450s; and (3) chemical cross-linking with EDC demonstrated that the wild-type Adx, but not the mutated form, can physically interact with P450MT2 to form a heterodimer. Our results on chemical cross-linking also demonstrate that the interaction of Adx with P450MT2 involves the same C-terminal acidic domain 2.

The results on the productive interaction of Adx with truncated P4501A1 reported in this study support and extend recent observations on the reconstitution of microsomal and mitochondrial P450 forms with heterologous, previously thought to be nonconventional, electron transfer proteins. Jenkins and Waterman (*8*) have successfully reconstituted the 17α-hydroxylase activity of the bacterially expressed P450c17 with flavodoxin and flavodoxin reductase purified from *E. coli* cells (*8*). They also showed that flavodoxin binds to P450c17 with high affinity (∼0.2 μM K_d) at low ionic strengths. Dong et al. (*9*) showed that both bacterial flavodoxin and flavodoxin reductase or spinach Fdx and Fdr could support the ethoxyresorufin *O*-deethylase activity of the N-terminal truncated bacterially expressed P4501A2, though the overall levels were significantly lower than that obtained with the P450 reductase system. Finally, Sakaki et al. (*10*) showed that 27-hydroxylation of 5β-cholestene-3α,7α,12α-triol, an N-terminal-modified rat mitochondrial P450c27 targeted to the yeast microsomal compartment, could be reconstituted with yeast microsomal P450 reductase, suggesting a possible evolutionary relationship between some of the mitochondrial and microsomal P450 forms.

An important implication of this study is that although Adx physically interacts with intact P4501A1 its mode of association appears to be quite different from that with P450MT2. In an Adx+Adr-supported system, P4501A1 yields marginal ERND and no significant EROD activities, and also Adx failed to induce a measurable shift in the spin state of P4501A1 heme. Additionally, use of mutated Adx, competition with Adx binding domain C27 peptide, and also competition with Adx-C as well as Adx-N peptides (Figures 6, 7, and 8) suggest a different mode of Adx interaction with P450MT2 and P4501A1. While the conserved acidic domain 2 of Adx is involved in a functionally productive interaction with P450MT2, the N-terminal acidic domain appears to be involved in interaction with P4501A1. Although an effective competition with Adx-N peptide and no significant competition with nonspecific peptides suggest the specificity of Adx and P4501A1 cross-linking, a nonspecific interaction between these two proteins cannot be ruled out. However, this interaction appears to be minimally productive as far as the catalytic function of the P450 heme group is concerned. Thus, our results raise two important and interrelated issues on the cytochrome P450 structure and activity: (1) A highly interesting possibility is that different electron donor proteins may differently modulate the activities of P450 enzymes. (2) Different modes of interfacing with the electron donor protein, possibly affecting the routes of electron flow, may differently modulate the catalytic properties of the mammalian P450 enzymes.

The functional and physiological significance of the minor species of +5/1A1 detected in the mitochondrial compartment is intriguing, since similar to the full-length 1A1, it is minimally active with the Adx+Adr system. It is likely that the +5/1A1 exists as an inactive precursor, that may be converted to the active +33/1A1 form by an as yet unidentified mechanism inside the mitochondrial membrane compartment. In summary, results of the physical interaction of Adx with +33/1A1 (P450MT2) provide a compelling argument in support of coevolution of some of the xenobiotic-inducible microsomal and mitochondrial P450s, a process that may involve the evolution of a chimeric N-terminal signal for targeting the protein to both the ER and mitochondria, and also coevolution of domains needed for interaction with P450 reductase and Adx in their respective membrane lipid environments. Finally, although the precise region of the P450MT2 involved in Adx binding remains unknown, results of competition with C27 peptide and also preliminary mutational data suggest that a domain similar to the conserved Adx binding domains of the constitutive mitochondrial P450s may be involved.

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