Mitochondrial Targeted Cytochrome P450 2E1 (P450 MT5) Contains an Intact N Terminus and Requires Mitochondrial Specific Electron Transfer Proteins for Activity*

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Hepatic mitochondria contain an inducible cytochrome P450, referred to as P450 MT5, which cross-reacts with antibodies to microsomal cytochrome P450 2E1. In the present study, we purified, partially sequenced, and determined enzymatic properties of the rat liver mitochondrial form. The mitochondrial cytochrome P450 2E1 was purified from pyrazole-induced rat livers using a combination of hydrophobic and ion-exchange chromatography. Mass spectrometry analysis of tryptic fragments of the purified protein further ascertained its identity. N-terminal sequencing of the purified protein showed that its N terminus is identical to that of the microsomal cytochrome P450 2E1. In reconstitution experiments, the mitochondrial cytochrome P450 2E1 displayed the same catalytic activity as the microsomal counterpart, although the activity of the mitochondrial enzyme was supported exclusively by adrenodoxin and adrenodoxin reductase. Mass spectrometry analysis of tryptic fragments and also immunoblot analysis of proteins with anti-serine phosphate antibody demonstrated that the mitochondrial cytochrome P450 2E1 is phosphorylated at a higher level compared with the microsomal counterpart. A different conformational state of the mitochondrial targeted cytochrome P450 2E1 (P450 MT5) is likely to be responsible for its observed preference for adrenodoxin and adrenodoxin reductase electron transfer proteins.

Cytochromes P450 (P450s) belong to a superfamily of heme proteins, which catalyze the oxidation of exogenous as well as endogenous compounds. P450 proteins are located in different compartments of the cell. In addition to their localization in the endoplasmic reticulum, also referred as microsomes, a number of different forms have been detected in mitochondria (1, 2), Golgi apparatus (3), and plasma membrane (4, 5). Targeting of P450 proteins to ER and mitochondria require different types of signals. Microsomal P450 is co-translationally inserted in the ER membrane through a non-cleavable N-terminal hydrophobic signal sequence (6). Mitochondrial P450 forms, such as P450scc (P450 11A1), P450 11β (P450 11B1), and P450c27 (P450 27A1) contain cleavable N-terminal amphipathic sequences that are important for the post-translational targeting of the precursor protein to mitochondria (7).

Recently we demonstrated that, in rat liver and brain, β-naphthoflavone-inducible P450 1A1 and phenobarbital-inducible P450 2B1, both known to be bona fide microsomal forms, are also targeted to mitochondria (8–10). The mitochondrial targeted P450 forms were referred to as P450 MT2 and P450 MT4, respectively. These studies described a new concept of chimeric N-terminal signal that was responsible for targeting both of these proteins to two distinct cytoplasmic organelles. In the case of P450 1A1, processing past the 4th or the 32nd amino acid residue by a cytosolic endoprotease resulted in the activation of a cryptic mitochondrial targeting signal at amino acid sequence 33–44, which directed the truncated proteins (P450 MT2) to mitochondria (8, 10, 11). In the case of P450 2B1 (P450 MT4), protein with an intact N-terminal end was targeted to mitochondria, although PKA-dependent phosphorylation at Ser128 was essential for mitochondrial targeting (9). We postulated that a conformational shift induced by Ser128 phosphorylation helped expose a cryptic mitochondrial targeting signal at amino acid sequence 21–36 of the protein. Thus, the two mechanisms have a common theme in that both require the activation of chimeric N-terminal signals for mitochondrial targeting (8, 9, 11), although mechanisms of signal activation were different.

P450 2E1 has been implicated to have important roles in human health, as its activity is affected by pathophysiological conditions such as diabetes, starvation, and obesity (12). Furthermore, it is readily induced by acute and chronic alcohol ingestion, and the enzyme is known to actively metabolize alcohol and acetaldehyde. Additionally, the enzyme efficiently catalyzes the metabolism of a wide spectrum of low molecular weight hydrophobic compounds, including carbon tetrachloride, chloroform, and vinylidene chloride in addition to chemical additives of toxicological and carcinogenic significance (13). Thus, P450 2E1 induction may underlie the increased risks of exposure to such industrial and environmental chemicals. It is well established that P450 2E1 causes oxidative stress through the production of reactive oxygen species in vivo (14) and in vitro (15). However, the role of mitochondrial targeted P450 2E1 in oxidative stress remains unknown.

P450 2E1, alternately designated as P450 MT5, has been...
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Aliquots of fractions were analyzed by CO-binding difference spectrum and subjected to SDS-polyacrylamide gel electrophoresis. Fractions containing relatively pure P450 were pooled, concentrated by ultrafiltration through Amicon filters, and dialyzed against buffer A.

The microsomal P450 2E1 was purified from the microsomal membrane of pyrazole-treated rat livers according to a previously described procedure, except that 0.6% sodium cholate was used for solubilization of microsomal P450 proteins.

Immunoprecipitation—Liver mitochondria and microsomes (200 μg of each) from control and pyrazole-treated rats were solubilized in 500 μl of phosphate-buffered saline (10 mM potassium phosphate, pH 7.4, 0.2% sodium cholate and phosphatase inhibitors (0.5 mM sodium orthovanadate, 0.05 mM sodium molybdate, and 2 mM sodium fluoride). The 15,000 g supernatant fractions were incubated with anti-P450 2E1 antibody (5 μg of IgG) (Oxford Biochemical Research, Oxford, MI) overnight at 4 °C. 30 μl of protein A-agarose (Life Technologies, Inc.) was added to each sample and shaken for 3 h at room temperature. Beads were pelleted by centrifugation and washed four times with phosphate-buffered saline containing 0.05% Tween 20 and phosphatase inhibitors. Immunoprecipitated proteins were eluted from protein A-agarose beads by heating at 95 °C for 5 min in 30 μl of 2× Laemmli buffer (23) without added 2-mercaptoethanol.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—Proteins were resolved by electrophoresis on a 12% SDS-polyacrylamide gel and visualized by Coomassie Blue staining or transferred to nitrocellulose membrane for immunoblot analysis as described (24). Polyclonal antibodies against P450 2E1, mtTFA, and CPR, and monoclonal antibody against Ser-phosphate (Sigma) were used. Blots were developed by chemiluminescence with the Pierce Super Signal Ultra kit. Imaging and quantitation were performed using a Fluor-S imaging system (Bio-Rad).

N-terminal Amino Acid Sequencing—Purified proteins were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and transferred to Seviquel polyvinylidene difluoride membrane (Bio-Rad). Protein bands were visualized by reversible staining with xylene cyanol, and the amino acid sequencing was performed by phenylthiohydantoin-derivatization procedure in a Beckman LH 2600 gas phase sequencer.

Peptide Analysis by MALDI-TOF Spectrometry—Purified P450 2E1 proteins were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and visualized by staining with Coomassie Blue. Purified P450 2E1 bands were excised, and gel slices were washed three times with 50% acetonitrile in 25 mM NH4HCO3 (pH 8), soaked in 100% acetonitrile, and dehydrated in a vacuum centrifuge. Gel fragments were rehydrated with a minimal volume of trypsin (10 μg/μl) on ice for 3 min. Equal volume of 10 N NaOH was added to the supernatant, and the reaction product, p-nitrophenol, and 200 μg of proteins as enzyme source. The reaction mixture was pre-incubated with or without added antibodies or inhibitors at 4 °C for 20 min. Following a 3-min incubation at 37 °C in a shaking water bath, the reaction was initiated by adding 1 mM NADPH and the incubation was continued for 20 min. These incubation conditions cause mitochondrial swelling making the membrane permeable to antibodies. The reaction was terminated by adding 0.6 N perchloric acid. Insolubles were removed by centrifuging at 10,000 g for 3 min. Equal volume of 10 N NaOH was added to the supernatant, and the reaction product, p-nitrophenolate, was measured at 540 nm. Reconstitution with purified mitochondrial or microsomal P450 2E1 was carried out essentially as described for intact organelles, except that the reactions were carried out in the presence of 0.2% sodium cholate and phenylmethylsulfonyl fluoride (26).

N-Demethylation of DMNA was assayed as described in detail in a recent study (11). Briefly, reactions were carried out in 50 mM Tris-HCl
RESULTS

Extent of Induction of Mitochondrial P450 2E1 by Pyrazole—The rat liver mitochondrial preparations were routinely checked for purity by marker enzyme assays as described previously (11, 26, 28). The digitonin-stripped mitochondria used in this study contained approximately 95% of mitochondrial-specific marker enzyme activity, cytochrome c oxidase, but less than 1% microsome-specific, retinone-insensitive NADPH cytochrome c reductase activity. The rate of induction of mitochondrial P450 2E1 by pyrazole treatment was studied by immunoblot analysis of mitoplast preparations from control untreated and pyrazole-treated rat livers. As shown in Fig. 1, pyrazole treatment induced the level of antibody-reactive 52-kDa putative P450 2E1 protein by 3-fold, whereas the level of the protein in the microsomes was induced by 2-fold. Based on the recovery of 7.5 mg of mitochondrial proteins and 9 mg of microsomal proteins/g of liver, the mitochondrial content in the pyrazole-treated liver corresponds to 30–40% of the total tissue pool of P450 2E1. In order to ascertain the purity of membrane isolates, the immunoblot was co-developed with antibodies to the mitochondria-specific protein mtTFA, and the microsome-specific marker protein CPR. Results show that only the 28-kDa mtTFA is detected in the mitochondrial fractions with no detectable 78-kDa CPR protein, indicating the purity of mitochondrial preparation. The microsomal fractions, on the other hand, contained only the 78-kDa CPR protein with no detectable 28-kDa mitochondria-specific mtTFA protein.

The effects of pyrazole treatment on P450 2E1-dependent PNPH and DMNA N-demethylation (25, 29) were measured to gain further insight on the extent of induction (Fig. 2, A and B, respectively). Results show that, in both mitochondrial and microsomal fractions, pyrazole treatment resulted in 2–3-fold increased N-demethylation of DMNA and PNPH activities. Furthermore, both the activities were inhibited 83–97% by P450-specific inhibitor SKF-525A and CO (latter results not shown). Thus, the extent of increase in enzyme activity with both substrates is consistent with the increased antibody reactive protein in pyrazole-treated fractions. Results also show that the activities with both pyrazole-treated and untreated livers were effectively inhibited by Adx antibody, but minimally with antibody to CPR, further demonstrating the purity of mitochondrial preparations. As expected, the activity of the microsomal fraction was specifically inhibited by antibody to CPR.

Purification of Mitochondrial P450 2E1—In order to determine if the mitochondrial targeted P450 2E1 is N-terminally truncated or not, we purified the protein from both mitochondria and microsomes from pyrazole-treated rat livers using the conventional purification procedures (20). As shown in Table II, the cholate extract of mitoplasts from pyrazole-treated livers contained 0.6 nmol of P450/mg of protein. The 20% PEG precipitation contained 83% of the input P450. The protein fraction eluted with 0.06% Emulgen 911 from the o-octylamine-agarose column showed a very low P450 content of 0.3 nmol/mg of protein. On the other hand, the fraction eluted with 0.2% Emulgen 911 contained approximately 45% of input P450 with a specific activity of 4.8 nmol of P450/mg of protein and was therefore used for further purification by DEAE-Septacel column chromatography. The eluates from DEAE-Septacel column were collected as three fractions. The first fraction contained only 5% of the input P450, although it exhibited the highest specific activity of 10.2 nmol/mg protein. The second and third fractions showed a lower specific activity of 4 and 7 nmol/mg protein, respectively (Table II). Although not shown, the microsomal P450 2E1 exhibited a similar pattern of purification and recovery.

Fig. 3A shows the electrophoretic pattern of protein fractions...
at various stages of purification of mitochondrial P450 2E1. Proteins in the size range of 50 and 55 kDa appeared to be enriched in the PEG fraction. One protein of approximately 52 kDa was further enriched in the o-octylamine-agarose step (Fig. 3A, lane 4). As indicated by different specific activities (Table II), the three DEAE-Sephacel column fractions showed different levels of purity. Proteins from fraction 1 resolved as a major component of 52 kDa, and exhibited approximately 70–80% purity (Fig. 3A, lanes 5–7). Fractions 2 and 3 contained additional bands suggesting a lower level of purity (Fig. 3A, lanes 8 and 9, respectively). The P450 from DEAE-Sephacel column fraction 1 was therefore used for further characterization.

The electrophoretic patterns of protein fractions at various stages of microsomal P450 2E1 purification are presented in Fig. 3B. As shown above for the purification of the mitochondrial protein, fraction 1 from DEAE-Sephacel column, showing a P450 content of approximately 10 nmol/mg protein (data not shown) exhibited the highest purity (75–80%) and was used for further characterization and comparison with the mitochondrial P450 2E1.

The immunoblots of mitochondrial and microsomal proteins at different stages of purification are presented at the bottom of Fig. 3 (A and B), respectively. The results show that DEAE fractions representing different levels of purity indeed cross-react with monoclonal antibody to P450 2E1.

As seen in Fig. 3C, N-terminal amino acid sequencing of the rat liver mitochondrial P450 2E1 yielded a sequence identical to that of the rat liver microsomal P450 E1.

**Phosphorylation of Mitochondrial P450 2E1**—In a recent study we showed that PKA-mediated Ser128 phosphorylation of P450 2B1 is a critical factor, which modulates the rate of protein targeting to ER and mitochondrial membranes (9). We also showed that P450 2B1 purified from phenobarbital-induced mitochondria (termed as P450 MT4) was phosphorylated at a higher level compared with the microsomal counterpart (9). Since members of family 2 P450 are known to be phosphorylated, it was decided to see if mitochondrial targeted P450 2E1 (designated as P450 MT5) is also phosphorylated using two different approaches. In the first approach, proteins solubilized from mitochondria and microsomes of livers from control and pyrazole-treated rats or P450 2E1 purified from these organelles were immunoprecipitated with P450 2E1 antibody, and the immunoprecipitated proteins were probed with antibody to Ser-phosphate by immunoblot analysis. As shown in Fig. 4, only P450 2E1 immunoprecipitated from mitochondria or purified from mitochondrial membrane cross-reacted with anti-Ser-phosphate antibody. The similarly treated microsomal protein fractions showed very low to negligible cross-reactivity. It is also seen that the antibody cross-reacted with a single protein band with apparent molecular mass of 52 kDa with purified mitochondrial P450 2E1, whereas the antibody cross-reacted with two closely migrating bands of 52 and 51 kDa with immunoprecipitates from total mitochondrial proteins. Although the nature of the faster migrating (~51 kDa) band remains unclear, it may represent a contaminant or degradation product of P450 2E1 generated during immunoprecipitation.

In the second approach, MALDI-TOF analysis was carried out to evaluate the phosphorylation status of purified microsomal and mitochondrial P450 2E1. P450 2E1 sequence (Swiss-Prot no. P05182) was loaded into the GPMAW software (PerSeptive Biosystems) for theoretical digestion analysis. The PKA target site on P450 2E1 is part of the tryptic fragment FS(129)/LSILR. With Ser128-phosphorylated P450 2E1 (30), one expects a mass of 914.50 for this fragment and with unphosphorylated protein, a mass of 835.50. Fig. 5A shows the spectra of the digested peptide mass fingerprint of P450 purified from rat liver mitochondria and microsomes. The data base search using MSFit identified both of these proteins as P450 2E1 with 48–58% matched masses. Chromatograms with both mitochondrial and microsomal P450 2E1 also showed some fragments (marked with arrows) that did not match either with P450 2E1 or with any of the known proteins from the data base. These fragments may therefore be derived from unknown contaminating proteins. It is also seen that the mass spectra with both mitochondrial and microsomal P450 2E1 showed the presence of peaks with molecular mass of 835.50, suggesting the presence of unphosphorylated species. The mass spectrum with mitochondrial P4502E1 also showed a small, yet detectable peak with a mass of 914.55. Based on the relative heights of peaks with masses 835.50 and 914.55, approximately 20–25% of the mitochondrial P450 2E1 is phosphorylated. An enlargement of the area of the chromatogram corresponding to masses 914 to 918, presented in Fig. 5B, further demonstrates the presence of the peptide with molecular mass of 914.55 in the
mitochondrial P450 2E1, but negligible amount in the microsomal protein digest. Thus, the results of MALDI-TOF analysis together with the immunoblots in Fig. 4 show that, under steady state conditions, the mitochondrial imported protein is phosphorylated at higher level than the microsomal P450 2E1.

**Fig. 3. Electrophoretic patterns of proteins at different stages of purification.** Figure shows mitochondrial P450 2E1 (A) and microsomal P450 2E1 (B). In upper panel of A, protein fractions at various steps of purification of mitochondrial P450 2E1 (as indicated in Table II) were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and visualized by staining with Coomassie Blue. Lane 1, molecular weight markers; lane 2, 25 μg of cholate extract; lane 3, 50 μg of PEG fraction; lane 4, 15 μg of α-octylamine-agarose column fraction (OAA); lanes 5–7, 0.5, 1, and 2 μg of DEAE-Sephacel column fraction 1; lane 8, 2 μg of DEAE-Sephacel column fraction 2; lane 9, 2 μg of DEAE-Sephacel column fraction 3. In the upper panel of B, microsomal protein fractions at different steps of purification were processed as in A. Lane 1, molecular weight markers; lane 2, 20 μg of microsomal protein; lane 3, 40 μg of cholate extract; lane 4, 10 μg of α-octylamine-agarose column fraction (OAA); lane 5, 1 μg of DEAE-Sephacel column fraction 1; lane 6, 1 μg of DEAE-Sephacel column fraction 2; lane 7, 2 μg of DEAE-Sephacel column fraction 3. The lower panels in A and B represent duplicate gels subjected to immunoblot analysis using anti-P450 2E1 antibody as described in Fig. 1. C shows the N-terminal amino acid sequence of the mitochondrial P450 2E1, compared with the reported sequence of microsomal P450 2E1. About 8 pmol of purified mitochondrial P4502E1 (DEAE-Sephacel fraction 1 from Fig. 3A) were analyzed by N-terminal sequencing. Numbers in parentheses show percentage of recovery of residues at each cycle.

Peptides was less efficient and corresponded to approximately 36% of the protein. The N-terminal as well as middle regions of the protein poorly represented in the mass spectra in Fig. 5A yield short peptides of less than 8 amino acid residues, which are not efficiently recovered by the present extraction procedure. The differential recovery of peptides as indicated in Fig. 5C may also be responsible for the difference in the mass spectrometry patterns between the mitochondrial and microsomal P450 2E1 (see Fig. 5A). Nevertheless, results of MALDI-TOF analysis, along with the N-terminal sequence data and also the 52-kDa apparent mass of the purified protein esti-

| Fraction | Protein content (mg) | P450 (nmol) | Specific activity (nmol/mg) | Purification (fold) | Yield (% input) |
|----------|----------------------|------------|-----------------------------|--------------------|----------------|
| Cholate-solubilized fraction | 1500 | 960 | 0.6 | 100 | |
| 20% PEG fraction | 810 | 801 | 1 | 1.7 | 83 |
| OAA 0.06% Emulgen eluate | 7.6 | 2.2 | 0.3 | 0.5 | 0.2 |
| OAA 0.2% Emulgen eluate | 90 | 431 | 4.8 | 8 | 45 |
| DEAE-Sephacel Fraction 1 | 5 | 51 | 10.2 | 17 | 5 |
| Fraction 2 | 4.2 | 17 | 4 | 6.7 | 2 |
| Fraction 3 | 29.4 | 206 | 7 | 11.7 | 21 |

*a* OAA, α-octylamine-agarose.
mated by SDS-polyacrylamide gel analysis in Fig. 3A, provide evidence that the mitochondrial P450 2E1 contains an unprocessed intact N terminus.

Reconstitution of Enzyme Activity with Purified P450 2E1—The PNPH and N-demethylase of DMNA activities of the purified mitochondrial P450 2E1 were reconstituted with CPR or Adx + Adr systems, and compared with those of enzyme purified from the microsomal fraction. Mitochondrial P450 2E1 in the presence of Adx + Adr yielded the same range of activity as the microsomal P450 2E1 in the presence of CPR: 1.23 and 1.61 nmol/min/nmol of P450, respectively, for PNPH and 15.4 and 13.5 nmol/min/nmol of P450, respectively, for N-demethylation of DMNA (Figs. 6 and 7). Notably, the mitochondrial enzyme yielded very low to negligible activity with the CPR system, whereas the microsomal enzyme was nearly completely inactive with the Adx + Adr system. The activities with both enzymes were inhibited by P450 inhibitors SKF 525-A and CO and also by the P450 2E1-specific inhibitor, 4-methylpyrazole. Furthermore, the activities were highly dependent on the addition of electron transport proteins and also NADPH (results not shown). Finally, a polyclonal antibody specific for P4502E1 inhibited the PNPH activity of both the Adx + Adr-supported

**Fig. 4. Levels of phosphorylation of mitochondrial and microsomal P450 2E1.** Proteins solubilized from 200 μg each of mitochondria (MT) and microsomes (MIC) or proteins purified from these membranes (5 μg each) were subjected to immunoprecipitation with anti-P450 2E1 antibody. The immunoprecipitates were divided into two equal fractions, and each fraction was subjected to electrophoresis on a 12% SDS-polyacrylamide gel. One gel was subjected to immunoblot analysis with anti P450 2E1 antibody (top panel), and the companion gel was subjected to immunoblot analysis with mouse monoclonal anti-phosphoserine antibody (1:1000) as described under “Materials and Methods” and in Fig. 1.

**Fig. 5. MALDI-TOF analysis of tryptic digests of purified P450 2E1.** Purified mitochondrial and microsomal proteins (10 μg each) were subjected to electrophoresis on a 12% SDS-polyacrylamide gel, stained with Coomassie Blue. Single stained bands were excised from the gel and subjected to trypsin digestion, and the peptides were extracted and subjected to MALDI-TOF analysis as described under “Materials and Methods.” A, mass fingerprints of peptides from mitochondrial P4052E1 (upper panel) and microsomal P450 2E1 (bottom panel). Ion 835.5 in both panels represents the non-phosphorylated peptide fragment from the Ser129 region. Ion 914.5 in the panel for mitochondrial P405 2E1 represents phosphorylated peptide fragment. B, selective enlargement of chromatograms of mitochondrial (upper panel) and microsomal (lower panel) proteins corresponding to masses 914–918 to visualize ion 914.5, representing the phosphorylated fragment. C, alignment of 2E1-specific fragments identified in the mitochondrial and microsomal P4502E1 digests along the protein sequence. The N-terminal 10-amino acid sequence region was identified by direct sequencing as shown in Fig. 3C.
mitochondrial and CPR-supported microsomal P450 2E1 enzymes by over 90%. These results further confirm that the PNPH activity is catalyzed by mitochondrial and microsomal P450 2E1, rather than any possible contaminating proteins. We have previously shown that Adx interacts with P450 MT2 (N-terminal truncated P4501A1), through its conserved C-terminal acidic domain 2, which spans amino acid sequence 70–85 of human Adx (see Table I) (28). This is the same domain that was shown to interact with the constitutively expressed mitochondrial P450 forms, such as P450scc, P450c27, etc. (31, 32). As shown for P450 MT2, Adx+Adr-supported PNPH activity of the mitochondrial P450 2E1 was inhibited by nearly 100% by a 30 μM excess of Adx-C peptide (Fig. 7A). However, the Mut Adx-C peptide in which negatively charged residues are substituted by neutral residues (see Table I) failed to inhibit significantly the Adx+Adr-supported PNPH activity. On the other hand, both peptides had no effect on CPR-supported PNPH activity of the microsomal P450 2E1, showing their specificity. These results suggest that a functionally productive interaction of Adx with mitochondrial P450 2E1 occurs through the same C-terminal acidic domain as that involved in interaction with other mitochondrial P450 forms and P450 MT2.

Recently, we mapped the region of P450 MT2 interfacing...
with both Adx and CPR to amino acid sequence 266–279 (putative helix G, sequence of P450 MT2 peptide in Table I) of the protein, which contains 5 positively charged residues (33). We also showed that Lys267 and Lys271 were critical for binding to Adx, while Lys268 and Arg275 were important for binding to CPR (33). Results of reconstitution in Fig. 7 (A and B) show that P450 MT2 peptide also inhibited both the Adx- and CPR-supported PNPH activity of mitochondrial P450 2E1 by over 95%. Although not shown, P450 MT2 peptide also inhibited both the Adx- and CPR-supported DMNA N-demethylase activities of mitochondrial and microsomal enzymes to >90%. These results suggest that the same structural domain of P450 2E1, similar to that of P450 MT2, may be involved in interaction with both Adx and CPR. An analysis of P450 2E1 sequence indeed suggests the occurrence of a domain similar to the P450 MT2 peptide at sequence 231–244 (see Fig. 7C) (34), which exhibits positional identities with Lys267, Lys268, and Lys271 of the MT2 sequence. The 4th positively charged residue (Lys243) of the putative G helix of P450 2E1 is positionally removed by two residues as compared with Arg275 of MT2 sequence.

**Difference in the Secondary Structure of Microsomal and Mitochondrial P450 2E1**—To understand reasons for the vast differences in the affinity of P450 2E1 from the two cellular compartments for Adx and CPR, we compared the purified proteins for secondary structure contents by CD spectroscopy. Despite identical primary sequence, the mitochondrial and microsomal enzymes exhibited a marked difference in the CD spectra (Fig. 8). It is seen that the microsomal P450 2E1 preparation exhibited negative bands at 208 and 223 nm at equal intensity, characteristic for proteins in the α-helical conformation (35). Although α-helices are characterized by an additional positive band near 190 nm, this band was apparently masked by the noise from the inorganic salts and other additives of the solvent (buffer C). The solvent conditions may also explain the somewhat higher than 100% α-helix content, as determined by the manual algorithm established by Greenfield and Fasman (36). The intensity ratio of the 208 nm/223 nm bands was greatly reduced for the mitochondrial variant, and in fact the CD spectrum resembled more of those representing β-pleated sheets (36). A computer-based analysis of the CD spectra for the composition of different secondary structural elements (35) indicated that the microsomal P450 2E1 contains approximately 36% α-helix, and relatively low β-sheet structure, while the mitochondrial P450 contains 13% α-helix, and markedly increased β-sheet structure. As reported for P4502B1 previously (9), the mitochondrial targeted P450 2E1 contains lower helical content, suggesting a less compact structure.

**DISCUSSION**

In this study, we have used a combination of protein purification, enzyme reconstitution, and protein biochemistry for characterizing the molecular and enzymatic properties of rat liver mitochondrial P450 2E1. Our results demonstrate that N-terminally intact P450 2E1 is targeted to mitochondria. The immunoblot analysis of P450 from crude membrane fractions, as well as purified proteins, suggest that the mitochondrial P450 2E1 is phosphorylated at a significantly higher level as compared with the microsomal counterpart. These results are further supported by MALDI-TOF analysis of gel-purified pro-
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teins (Fig. 5). The latter results show the occurrence of a protease fingerprint product of 914.55 mass, representing the Ser\textsuperscript{129} phosphorylated fragment only with the mitochondrial purified enzyme but not the microsomal purified enzyme. These results suggest that similar to that shown for P450B1 (P450 MT4), intact Ser\textsuperscript{129} phosphorylated P450 2E1 (P450 MT5) is targeted to mitochondria. Despite an extensive analysis, we failed to detect the putative 40-kDa N-terminal cleaved product (16) either in intact mitoplasts (Fig. 1) or as part of purified P450 2E1 (Fig. 3A). Recent results\textsuperscript{2} on the in vivo protein targeting by cell transfection and in vitro mitochondrial import showed that the full length P450 2E1 with an intact N terminus is targeted to mitochondria. Furthermore, as suggested previously, the mitochondrial imported P450 2E1 is a membrane extrinsic protein based on its solubility in alkaline Na\textsubscript{2}CO\textsubscript{3} (2, 37).

Recent studies from our laboratory demonstrated that mitochondrial targeted P450 MT2 (+33/1A1) and P450 MT4 (phosphorylated P450 2B1) interact in a functionally productive manner with mitochondrial specific electron donor proteins Adx+Adr (9, 28, 33), while retaining their ability to interact with the microsomal electron donor protein, CPR. Since the microsome-associated P450 1A1 and 2B1 showed preference for CPR as an electron donor, we hypothesized that N-terminal truncation, in the case of mitochondrial imported P450 MT2, and Ser\textsuperscript{128} phosphorylation in the case of mitochondrial P450 2B1, may induce conformational changes that allow interaction with soluble electron donor proteins. In many respects, the mitochondrial imported P450 2E1 (P450 MT5) described in this study resembles the similarly located P450 2B1 with a subtle difference. Reconstitution experiments show the exclusive requirement of mitochondrial imported P450 2E1 for Adx+Adr. These results suggest a notable structural or conformational difference between P450 molecules associated with the microsomal and mitochondrial membrane compartments. As reported before for P450 2B1 (9), immunoblot analysis with anti-Ser-phosphate antibody shows higher level of phosphorylation with mitochondrial P450 2E1 as compared with the microsomal form. Preliminary results also show that, similar to that reported for P450 2B1 (9), Ser\textsuperscript{129} phosphorylation is critical for mitochondrial targeting of protein, suggesting that it may serve to activate the cryptic mitochondrial targeting signal of P450 2E1 apoprotein. MALDI-TOF analysis, however, suggests that only approximately 20–25% of the mitochondrial P450 2E1 pool may be phosphorylated at Ser\textsuperscript{129}. These latter results suggest that mitochondrial P450 forms are subject to regulation by phosphorylation and dephosphorylation. This possibility is consistent with increasing evidence that other mitochondrial proteins, such as those associated with electron transport complexes, are subject to modulation by cAMP-dependent PKA (9, 38) and protein phosphatases (39).

It is noteworthy that P450 2E1 proteins purified from the two membrane compartments show markedly different α-helical contents (Fig. 8). In our previous study with mitochondrial P450 2B1, we rationalized that phosphorylation might be an important contributing factor in the manifestation of structural differences, as revealed by CD spectral studies (9), and binding to Adx (results not shown). Since the phosphorylated species comprise only approximately 20–25% of the mitochondrial P450 2E1 pool, additional possibilities are likely to account for this difference. The mode of mitochondrial entry of the apoprotein through the N-terminal end, and its first encounter with mitochondrial import proteins, such as HSP70 (40) may some how direct the protein to assemble in a membrane extrinsic orientation, differently from the signal recognition particle-directed membrane targeting and membrane insertion processes in the ER compartment. It is also likely that the P450 protein is folded differently in the mitochondrial compartment because of possible differences in the protein folding machinery or chaperones. It is known that a single amino acid substitution in the chaperone component of the subtilisin E causes subtle changes in the folding of its protease domain, suggesting that the same polypeptide can be folded in different manner (41).

Phosphorylation of Ser\textsuperscript{129} may be an important contributing factor in this process. Additional experiments are needed to understand the molecular and cellular processes leading to the formation of P450 2E1 with subtly different structure or conformation.

A recent study using physical and genetic mapping of electron donor protein binding sites on P450 MT2 and structural modeling of this P450 showed that both Adx and CPR bind to the putative G helix, but subtly through different contact points. Notably, both Adx and CPR bound to the same helix at 90° orientation to each other in a non-overlapping fashion (33). In the present study, the MT2 peptide representing the P450 site for binding to two different electron donor proteins inhibited both the Adx supported and CPR supported enzyme activities. These results suggest that a homologous P450 2E1 region is involved in binding to these two evolutionarily divergent electron donor proteins. Although not shown, a polar amino acid-rich, aqueous accessible helix G, spanning sequence 231–244 and containing 4 positively charged amino acids, is also conserved in P450 2E1 (see Fig. 7C). We postulate that the conserved G-helix of P450 2E1 is involved in binding to the electron donor proteins, possibly involving Arg\textsuperscript{233}, Lys\textsuperscript{234}, and Lys\textsuperscript{237} as the direct contact points. In subtle variation from the P450 MT2 model, in vitro reconstitution of purified enzymes suggests that the binding to the two electron donor proteins, Adx and CPR, is exclusive of each other. Currently, reasons for the observed preference of the microsomal P450 2E1 for binding to CPR and the mitochondrial form for binding to Adx remain unclear, although different conformations of the putative G helix in enzymes from two different membrane sources is a likely possibility.

In summary, here we demonstrate that the P450 2E1 associated with the mitochondrial membrane compartment is identical to the microsomal P450 2E1, except that the former is phosphorylated at a higher level. The results of this study also support our previous observations with P450 MT2 and P450 MT4, and provide additional evidence for the occurrence of multiple mechanisms for the activation of chimeric N-terminal signal for the mitochondrial targeting of xenobiocinducible P450 forms.

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