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STRUCTURES OF MITOCHONDRIAL P450 SYSTEM PROTEINS

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ABSTRACT

Mitochondrial P450 type enzymes are generally involved in the metabolism of cholesterol derived steroidal compounds. The reactions catalyzed by these enzymes include cholesterol conversion to pregnenolone, 11-beta and 18 hydroxylation reactions in adrenal steroid biosynthesis, C-27 hydroxylation of cholic acid in bile acid metabolism, and 1alpha and 24 hydroxylations of vitamin D. These P450 mediated reactions require molecular oxygen and two electrons donated by NADPH. The electrons of NADPH are transferred to P450 by an electron transfer system that includes a specific flavoprotein, adrenodoxin reductase, and an iron-sulfur protein, adrenodoxin. These proteins are not specific for individual P450s and serve as electron donors for different P450 in different tissues. This review presents an overview of the major sequence and structural characteristics of the mitochondrial P450 system proteins.

1. INTRODUCTION

Mitochondrial P450 type enzymes are generally involved in the metabolism of cholesterol derived steroidal compounds. The reactions catalyzed by these enzymes include cholesterol conversion to pregnenolone, 11β and 18 hydroxylation reactions in steroid biosynthesis, C-27 hydroxylation of cholic acid in bile acid metabolism, and 1α and 24 hydroxylations of 25-OH-vitamin D (Table 1).[1–6] These reactions are catalyzed by specific P450s and follow the usual monooxygenation stoichiometry.[7,8]

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Table 1. Reactions catalyzed by the mitochondrial P450 systems

P450	Gene	Major reaction	Highest levels in
P450scc	CYP11A1	Cholesterol side chain cleavage	Steroidogenic cells in adrenal cortex and gonads
P450c11	CYP11B1	Steroid 11β-hydroxylation	Zona fasciculata of adrenal cortex
P450c18	CYP11B2	Steroid C-18 hydroxylation	Zona glomerulosa of adrenal cortex
P450cc24	CYP24	25-OH-vitamin D3-24 hydroxylation	Kidney tubules
P450c27	CYP27A	Sterol C-27 hydroxylation Vitamin D3-25 hydroxylation	Liver
P450l?	CYP27B	25-OH-vitamin D3-1α hydroxylation	Kidney

$$R\text{–}H + NADPH + H^+ + O_2 \longrightarrow R\text{–}OH + NADP^+ + H_2O$$

In these reactions NADPH donates two electrons which are transferred to P450 via two electron transfer proteins, adrenodoxin reductase, which is an FAD containing flavoenzyme, and adrenodoxin, which is a [2Fe-2S] ferredoxin type iron-sulfur protein.[1,2,9,10] FAD of adrenodoxin reductase accepts two electrons from NADPH, and these are transferred one at a time to adrenodoxin which is a one electron carrier. There is evidence that the availability of NADPH to the mitochondrial P450 systems is regulated in coordination with steroid biosynthesis.[11] The mitochondrial P450 electron transfer system is similar to that of some bacterial P450's such as P450cam from Pseudomonas putida that includes a ferredoxin reductase and a ferredoxin (named putidaredoxin) as the electron transfer proteins.[9,12]

All three proteins of mitochondrial P450 systems are located on the matrix side of the inner mitochondrial membrane.[1,13] Whereas the mitochondrial P450s are tightly bound to the membrane, the electron transfer proteins are soluble in the matrix. All three proteins are encoded as larger precursors, and their signal peptides are cleaved during transfer into mitochondria.[14,15]

In contrast to the multiplicity of P450 forms,[12,16] there is generally only one form of adrenodoxin reductase, and adrenodoxin, encoded by one or two similar nuclear genes in all animal species.1 Thus, the electron transfer proteins are not specific to individual P450s and serve as electron donors for different cytochromes P450 in different tissues. Adrenodoxin reductase and adrenodoxin are expressed in all human tissues examined.[1,16] Their highest levels of expression are observed in steroidogenic cells especially in adrenal cortex and ovarian corpus luteum.[1] The levels of these proteins show no significant sex, or interindividual variation in bovine adrenal cortex.[17]

Previous studies established several general principles of function for P450 system electron transport chains:[7–10] 1) The reductases are generally expressed at much lower levels than P450s, there being only one molecule of reductase per about 10 or more molecules of P450. 2) The protein components are independently mobile and do not form static multicomponent complexes. 3) Proteins that are redox partners form transient high affinity 1:1 complexes during their random diffusions, in accordance with the principles of mass action. Dissociation constants of these protein-protein complexes are strongly influenced by the redox states of the proteins and other molecules in the environment, such as P450 substrate, ions, and phospholipids. 4) The transfer of an electron between two redox partners depends on the formation of a specific high affinity 1:1 complex between the two proteins. In P450 systems electron transfer is not always coupled to substrate monooxy-

genation. P450s and their electron transfer proteins may transfer electrons to other acceptors, such as O_2. This type of "uncoupling" or "leaky electron transport" is observed in both mitochondrial and microsomal systems.[7,9,11] The regulation of protein-protein complex formation generally enhances productive associations for monooxygenase activities and helps to minimize uncoupled reactions that produce harmful free radicals.

The sections below present a general overview of the major sequence and structural characteristics of adrenodoxin reductase, adrenodoxin and mitochondrial P450s. As the extensive number of references are already listed in the sequence databases, the codes of the proteins are provided in the tables below for retrieval of individual references.

2. STRUCTURE OF ADRENODOXIN REDUCTASE

In both human and bovine genomes there is only one gene that encodes for adrenodoxin reductase.[1] The sequences of human, bovine, rat, and mouse adrenodoxin reductase have been deduced from cloned DNAs (Table 1). Whereas the signal peptides of these proteins share 55–82% identity, the mature peptide sequences show 88–95% identity among the enzymes from four different species (Figure 1). In addition to these, a yeast gene homologous to the mammalian adrenodoxin reductase gene has been identified, yet its function remains uncharacterized.[18] Despite functional similarities, adrenodoxin reductase shows no sequence homology with the bacterial P450cam system putidaredoxin reductase or other types of oxidoreductases.[19]

In immunoelectron microscopy of adrenal cells, adrenodoxin reductase appears as membrane associated.[20] However, its sequence does not have a hydrophobic membrane spanning segment (Fig. 1) and, it probably functions as a peripheral membrane protein.

The FAD and NAD(P) binding sites of adrenodoxin reductase were identified using an ADP dinucleotide binding site consensus motif.[19] FAD and NAD(P) both have ADP as a common part of their structures. In most FAD or NAD(P) binding enzymes, the sites that bind this ADP portion also share a similar conformation of a βαβ-fold. The most highly conserved sequence in this fold is Gly-X-Gly-X-X-Gly/Ala which forms a tight turn between the first β-strand and the α-helix.[19,21,22] Analysis of adrenodoxin reductase sequence led to the discovery that in NADP binding sites of this type, there is an Ala instead of the third Gly residue, and it was proposed that this is a major determinant of NADP vs. NAD specificity of enzymes.[19] This hypothesis was verified for glutathione reductase.[23,24]

The full-length sequence of adrenodoxin reductase shows no similarity to that of the bacterial putidaredoxin reductase, yet, the FAD and NAD(P) motifs appear in both enzymes at nearly identical positions:[9] the FAD site at the amino terminus, and the NAD(P) site at 146–151 residues from the amino terminus (Fig. 1). Similar spacing of the FAD and NADP sites is also observed in many other flavoenzymes.[19] The sequence of the FAD binding amino terminus is highly conserved across species.[25] A bovine adrenodoxin reductase cDNA expressed in yeast with an extra segment encoding four additional residues at the N-terminus did not yield an active enzyme, suggesting that the four residues disrupted the incorporation of FAD into the apoprotein.[26] One alternative splicing product of adrenodoxin reductase gene encodes six extra residues in both bovine and human genes.[27,28] However, this form represents ~1% of the total reductase mRNA population,[28] and expression of its cDNA in E. coli did not yield active enzyme, suggesting that the structure of the enzyme.[29]

Whereas the bovine adrenodoxin reductase was reported to be glycosylated,[30,31] a recent study could not confirm previous evidence for functional glycosylation of the bovine

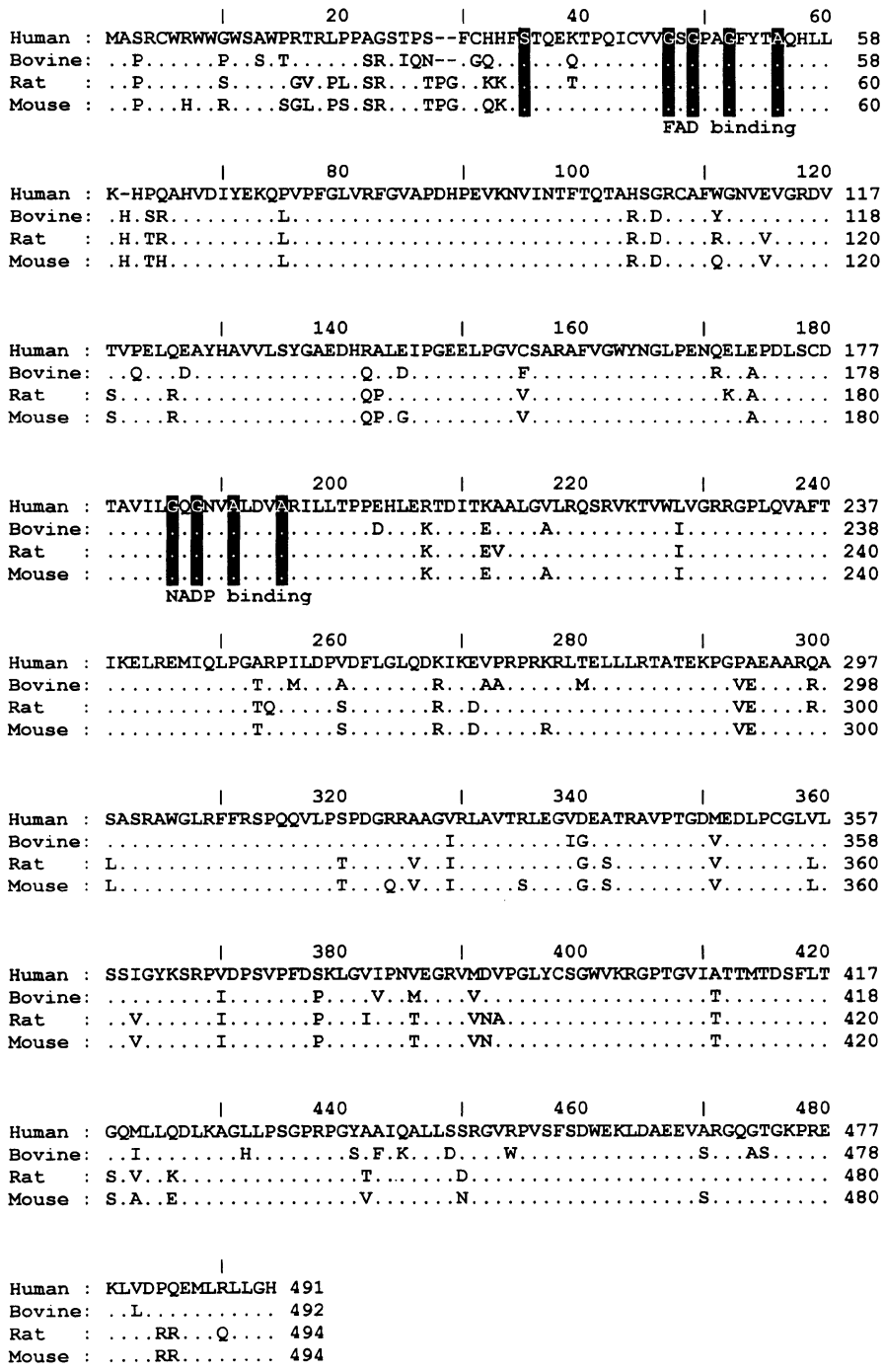

Figure 1. Alignment of adrenodoxin reductase sequences from vertebrate species. The amino terminal Serine of the mature peptides, and the conserved glycines and alanines in the FAD and NADP binding sites are marked by background shading. Dashes in sequence represent gaps inserted for alignment.

Table 2. Characteristics of adrenodoxin reductase and adrenodoxin amino acid sequences determined from cloned cDNA or gene sequences

Species	SWISSPROT code	Prepeptide	Signal peptide	Mature peptide	
				Length	MW
Adrenodoxin reductase					
Human	adro_human	491	32	459	49967
Bovine	adro_bovin	492	32	460	50296
Rat	adro_rat	494	34	460	50316
Mouse	adro_mouse	494	34	460	50128
Yeast	adro_yeast	493			
Adrenodoxin					
Human	adx_human	184	60	124	13561
Bovine	adx1_bovin	186	58	128	14048
Sheep	adx_sheep	ND			
Pig	adx_pig	186	58	128	14012
Rat	adx_rat	188	64	124	13588
Mouse	adx_mouse	188	64	124	13617
Chick	adx_chick	ND		124	13558

ND: Not determined.

enzyme.[32] The porcine enzyme is free of carbohydrate.[33] Adrenodoxin reductase expressed in E. coli functioned as well as the native enzyme in a reconstituted mitochondrial P450scc system. Thus, the apoprotein may be assembled to active holoenzyme without eukaryotic posttranslational modifications.[34]

Adrenodoxin reductase has been crystallized in several laboratories.[35,36,37] The elucidation of its crystal structure is necessary to increase our understanding beyond the sequence analyses, to identify in detail the cofactor pockets and the adrenodoxin binding sites, and to elucidate the routes of electron transfer.

3. STRUCTURE OF ADRENODOXIN

The sequence of adrenodoxin has been deduced from cloned DNAs in six mammalian species and in chicken (Table 2, Figure 2). Similar to adrenodoxin reductase, the mature peptide sequence of adrenodoxin is much more conserved than its signal peptide: Whereas the homology of the mature peptide ranges between 81–97% among these species, the homology of the signal peptide ranges between 31–67% (except for rat and mouse 82%). Adrenodoxin sequence shows significant homology with bacterial putidaredoxin and their sequences can be aligned over their entire lengths with only a few gaps.[38] A [2Fe-2S] ferredoxin from E. coli also shares 36% identity with adrenodoxin and putidaredoxin.[39] Waki et al.[40] reported the purification of a [2Fe-2S] protein from bovine liver mitochondria that can support C-25 and C-27 hydroxylations of steroids, but with an amino terminus sequence completely different from adrenodoxin.

Bovine adrenodoxin is translated from multiple species of mRNA encoded by a single gene.[41] The protein sequences encoded by these mRNAs differ only in the C-terminus of the signal peptide and the first two residues of the mature sequence.[42] In the human genome there are two genes, but both encode the same protein product.[43] In polyacrylamide gel electrophoresis, adrenodoxin purified from different tissues two major bands or a broad band may be observed around ~12–14 kDa.[44,45] This heterogeneity was considered

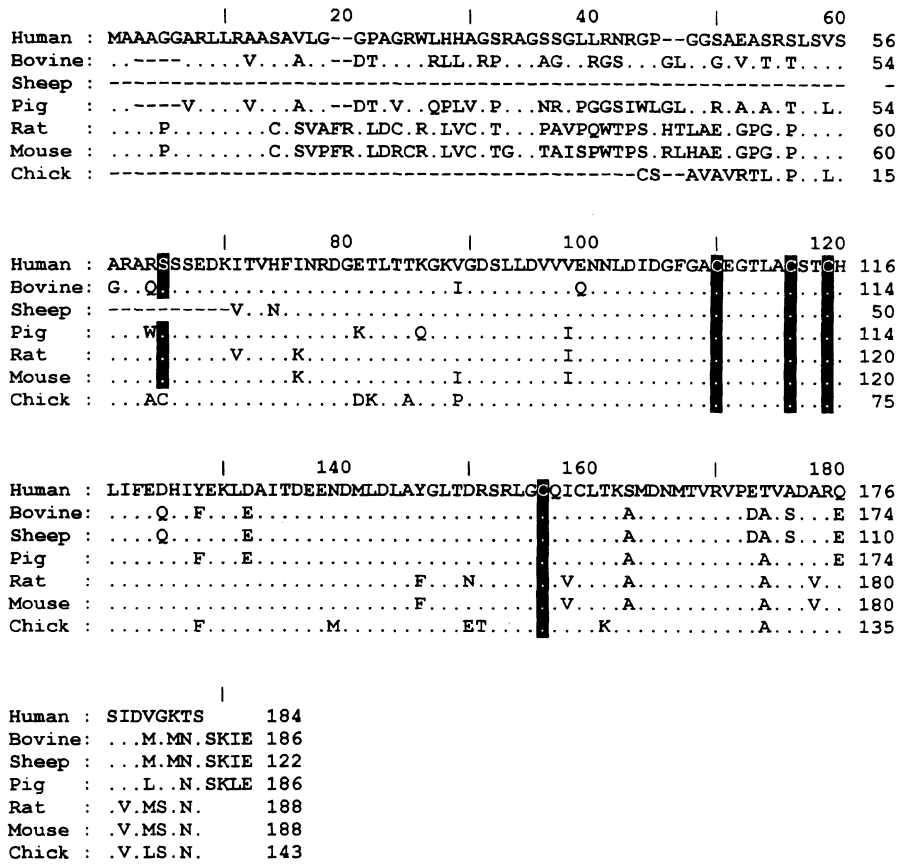

Figure 2. Alignment of the adrenodoxin sequences from six vertebrate species. The amino terminal Serine of the mature peptides, and the cysteines that are involved in the formation of [2Fe-2S] complex are marked by background shading.

to represent a multiplicity of tissue specific forms of adrenodoxin. However, it apparently results from proteolytic cleavage of up to 14 residues from the carboxy terminus of the mature adrenodoxin during the purification process.[46,47] Trypsin treatment of purified adrenodoxin produces a truncated form of adrenodoxin (des 116–128) which shows a lower Km (higher affinity) in supporting P450 activity.[48]

In the [2Fe-2S] center two iron atoms are coordinated to four cysteines and two labile sulfur atoms. Mature bovine adrenodoxin sequence includes five cysteines. Chemical modification and site-directed mutagenesis studies indicated that Cys-46, 52, 55, and 92 are involved in iron-sulfur coordination, whereas Cys-95 is free.[38,49,50]

A cluster of negatively charged residues of bovine adrenodoxin have been implicated in complex formation with both adrenodoxin reductase and mitochondrial P450s in studies employing different approaches.[51–54] Site directed mutation of Asp-76 and Asp-79 showed that these residues are essential for adrenodoxin binding to both adrenodoxin reductase and P450scc.[54] In contrast, modification of all lysine or arginine residues did not affect adrenodoxin interactions with either of its redox partners, suggesting that these are not located at

Table 3. Characteristics of mitochondrial P450 amino acid sequences determined from cloned cDNA or gene sequences

Species	SWISSPROT Code	Prepeptide	Signal peptide	Mature peptide	
				Length	MW
P450scc					
Human	cpm1_human	521	39	482	56117
Bovine	cpm1_bovin	520	39	481	56398
Sheep	cpm1_sheep	520	39	481	56399
Goat	cpm1_caphi	520	39	481	56304
Pig	cpm1_pig	520	39	481	56240
Rabbit	q28827				
Rat	cpm1_rat	526	36	490	56946
Trout	cpm1_oncmy	514	39	475	55324
P450c11					
Human	cpn1_human	503	24	479	54889
Baboon	cpn1_papha	503	24	479	55004
Bovine	cpn1_bovin	503	24	479	55087
Sheep	cpn1_sheep	503	24	479	55028
Pig	cpn1_pig	503	24	479	54647
Cavia	cpn1_cavpo	500	24	476	55146
Rat	cpn1_rat	499	24	475	54612
Mouse	cpn1_mouse	500	24	476	54468
Hamster	cpn1_mesau	499	24	475	54015
Frog	cpn1_ranca	517	45	472	54492
P450c18					
Human	cpn2_human	503	24	479	54934
Rat	cpn2_rat	500	24	476	54274
Mouse	q64661	500	24	476	54568
Hamster	cpn2_mesau	500	24	476	54526
P450c24					
Human	cp24_human	513	35	478	54935
Rat	cp24_rat	514	35	479	55537
Mouse	cp24_mouse	514	35	479	55427
P450c27A					
Human	cp27_human	531	33	498	56908
Rabbit	cp27_rabit	535	32	503	57033
Rat	cp27_rat	533	32	501	57185
P450c27B					
Human		508			
Rat		501			

the binding site for either protein.[52] These findings support the conclusions based on kinetic studies[9,10,55,56] that the binding sites of these two enzymes on adrenodoxin overlap. Yet, the differential effects of carboxy-terminal truncation at Arg-115 on interactions with adrenodoxin reductase and P-450 suggest that the sites are not identical.[48] Mutation of Tyr-82 did not affect reductase binding but changed Km values with P450scc and P450c11, suggesting that Tyr-82 may affect the binding of P450.[57] A mutated form of adrenodoxin missing six amino terminal residues supported only 60% of the activity of P450scc suggesting that the amino terminal residues may also play a role in P450scc binding or electron transfer.[58] Similarly deletion studies of C-terminus of bovine adrenodoxin indicate that this region, and especially Pro-108 are essential for the structural integrity of the protein.[59] Site-directed mutagenesis was also used in assigning the NMR signals from His residues.[60,61]

Table 4. Percent identity of P450c11 amino acid sequences from ten species. The bold numbers represent the number of amino acids that are identical

	Human	Baboon	Bovine	Sheep	Pig	Cavia	Rat	Mouse	Hamster	Frog
Human	**503**	96%	72%	74%	73%	63%	63%	67%	61%	44%
Baboon	485	**503**	72%	74%	73%	62%	63%	67%	61%	44%
Bovine	367	366	**503**	95%	80%	61%	59%	64%	58%	47%
Sheep	374	374	482	**503**	82%	62%	60%	65%	58%	47%
Pig	368	371	407	415	**503**	61%	61%	65%	58%	45%
Cavia	320	316	310	313	310	**500**	58%	65%	57%	42%
Rat	318	318	299	304	308	294	**499**	80%	74%	41%
Mouse	339	339	324	329	329	330	402	**500**	74%	45%
Hamster	310	307	296	294	293	289	370	372	**499**	38%
Frog	232	231	246	246	236	222	218	236	202	**517**

Bovine and chick adrenodoxins can be phosphorylated. This may affect their interaction with P450 and hence the activity of P450.[62,63] Adrenodoxin cDNAs expressed in E. coli encode proteins functionally as active as the native protein, indicating that the [2Fe-2S] centers of these proteins can be properly assembled in bacteria, and that eukaryote specific posttranslational modifications are not necessary for activity.[49,50,54,64]

The structure of adrenodoxin crystals currently available cannot be solved because of their complexity.[65] The elucidation of the complete structure of adrenodoxin awaits formation of different crystals suited for crystallographic analysis. Proton NMR studies indicate that the structure of adrenodoxin is similar to that of Spirulina platensis ferredoxin.[66]

4. STRUCTURES OF MITOCHONDRIAL P450s

The hydrophobic character of the mitochondrial P450s have hindered the crystallization of these proteins for crystallographic structure analysis. The structures of several soluble bacterial P450s have been determined.[12,67,68] Yet, the very low sequence similarity (<20%) between the mitochondrial and bacterial P450 sequences does not permit an exact alignment of these sequences along their entire lengths. A model for the 3-D structure of P450scc has been suggested based on the crystal structure of P450cam and alignment of the two sequences.[69] This model remains speculative because of the low similarity between these sequences. Thus, our present understanding of mitochondrial P450 structure is based on sequence comparisons and biochemical analyses of these proteins.

To date the sequences of six different types mitochondrial P450s have been determined from cloned DNAs from vertebrate species (Tables 1, and 3 and Figure 3). These P450s contain 24–39 residue long signal peptides. The sequences of the P450c11 and P450c18 specific for 11?- and 18-hydroxylation of steroids are most similar showing 91% amino acid sequence identity in humans. The sequences of other mitochondrial P450s in humans share between 24–37% sequence identity. The intron-exon organization of P450scc, P450c11 and P450c18 are highly similar, clearly indicating a common evolutionary origin at least for these three mitochondrial P450s.[70] Recently an insect P450 sequence has been isolated that shows strong homology to the vertebrate mitochondrial P450s, yet the function of this P450 remains to be determined.[71] There is immunological evidence that insect ecdysone 20-monooxygenase may be related to the vertebrate mitochondrial P450s.[72]

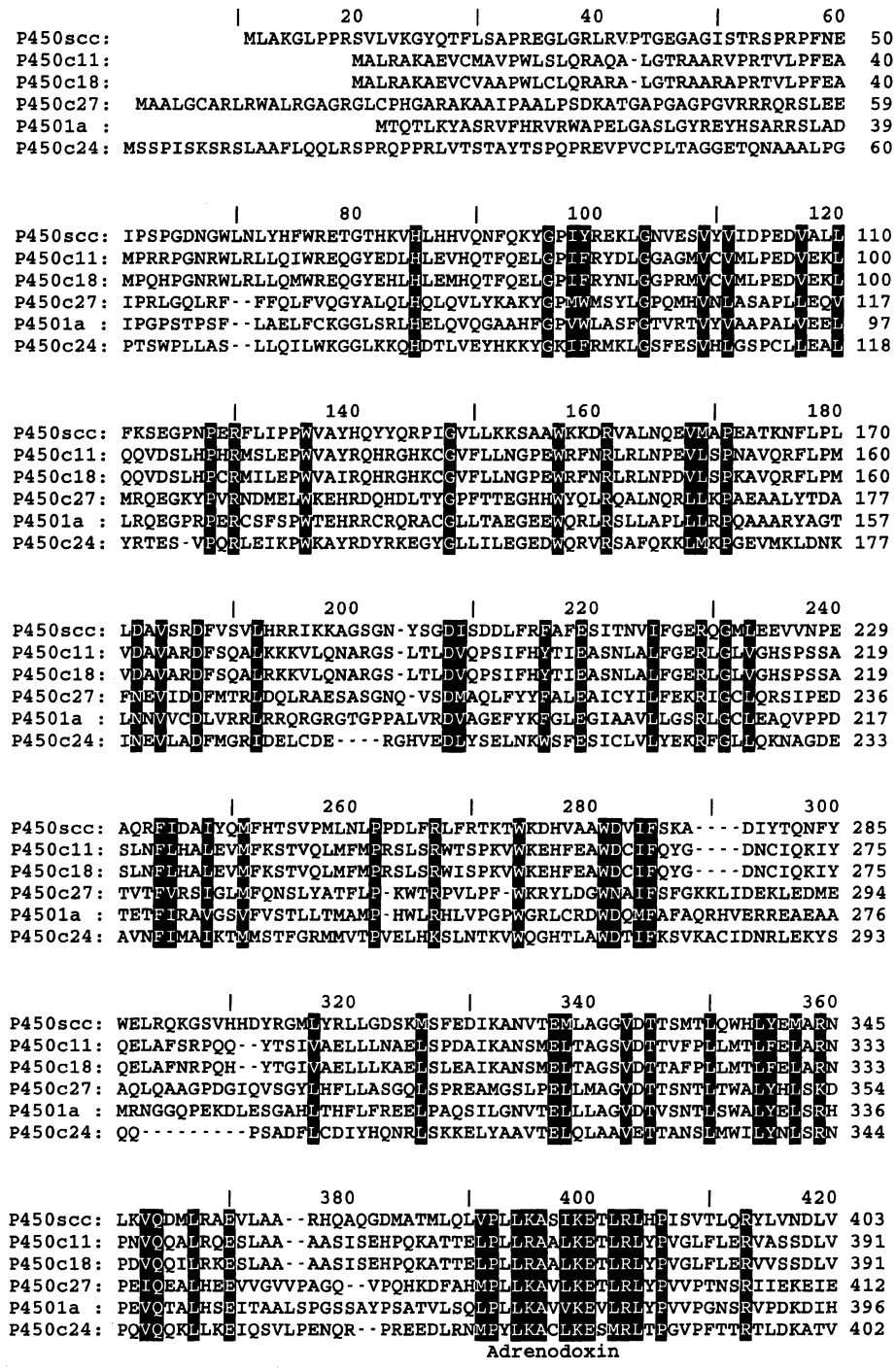

Figure 3. Alignment of six different human mitochondrial P450 sequences. Shaded regions mark identical residues. The positions of putative adrenodoxin binding and heme binding regions are marked below the sequences.

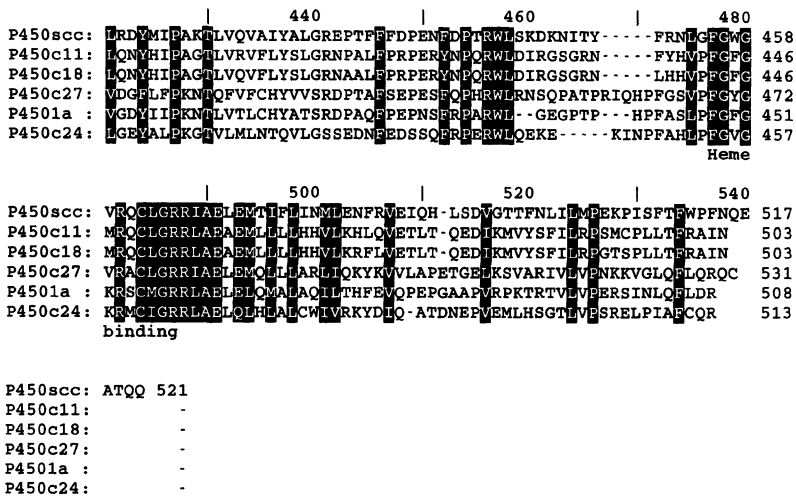

Figure 3. (*Continued*)

For each form of P450, interspecies homology varies in general according to the evolutionary relatedness of the species. The complete sequence of P450c11 has been determined in ten species (Table 3). Human and baboon P450c11 sequences share 96% identity. Whereas, frog P450c11 shares 38–47% sequence identity with other species (Table 4). If conservative substitutions are considered, (such as Thr for Ser, or Glu for Asp) then the percent similarity increases by up to 20% over the figures noted in Table 4.

Mitochondrial P450s behave as integral membrane proteins, as they are strongly hydrophobic and their isolation requires treatment with detergents. In contrast to the microsomal P450s, mitochondrial P450s do not have a hydrophobic membrane spanning segment. Thus, their mode of association with the inner mitochondrial membrane is still not understood. A mitochondrial P450c27 that was engineered to contain the microsomal targeting signal of bovine P450c17 front the mature form of rat P450c27 was localized in the microsomes and could utilize the microsomal NADPH-P450 reductase as an electron donor.[73] Yet, purified human P450c27 does not have the capability to utilize microsomal P450 reductase as an electron donor.[74] Thus, mitochondrial P450s may bind to the microsomal membranes and that there is no intrinsic requirement for the mitochondrial membrane environment for the function of the mitochondrial P450s. Mitochondrial P450s can also function in purified form in the absence of a membrane environment as well as in phospholipid vesicles.[75] The signal sequences of P450s are apparently not completely species specific as, in vitro synthesized bovine P450scc precursor can be imported into isolated soybean cotyledon mitochondria and processed therein to the mature size product.[76]

Since all the mitochondrial P450s bind heme and interact with adrenodoxin, the binding sites for these molecules should be conserved. All forms of P450, including bacterial and microsomal P450s show strong homology in the heme binding segment before the L-helix close to the carboxy termini of the enzymes.[68,77] All mitochondrial P450 sequences noted in Table 3 carry the consensus signature sequence FGxGxRxCxG in this region (Fig. 3).

The adrenodoxin binding site of P450scc was identified by chemical modification studies.[78] The sequence of this site is also conserved in P450scc from all species[79] as well as other mitochondrial P450s (Fig. 3). The labeling of Cys264 of P450scc affected the interaction of P450scc with adrenodoxin and significantly inhibited its enzymic activity.[80] Labeled and unlabelled enzymes were cleaved by trypsin and split into two fragments. It has been suggested that the hinge connecting the two domains in the region Arg250-Asn257 is exposed to the surface of the membrane and involved in the interaction of P450scc with adrenodoxin.[80]

In contrast to the conservation of binding sites for heme and adrenodoxin, the substrate binding sites of P450s would be expected to show divergence in accordance with the diversity of the substrates. Indeed among bacterial and microsomal P450s the sequences of structurally known or predicted sites of substrate binding show great diversity.[67,77] A study utilizing a suicide substrate for P450scc suggested that the substrate binding site may be located at its amino terminus.[81] Among the mitochondrial P450s the amino terminus is the least conserved segment (Fig. 3). However, a recent study showed that P450c11 could be converted into an enzyme with the activity of P450c18 by two residue substitutions of Ser288Gly and Val320Ala.[82] Thus, even if the amino terminus is involved in the formation of a substrate channel or binding site, critical residues are located in the middle of the polypeptide chain.

As noted for the electron transfer proteins, the elucidation of the structure of the mitochondrial P450s awaits isolation of enzyme crystals suitable for X-ray diffraction analysis.

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