

## An Additional Electrostatic Interaction between Adrenodoxin and P450c27 (CYP27A1) Results in Tighter Binding Than between Adrenodoxin and P450scc (CYP11A1)\*

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Mitochondrial cytochrome P450c27 (product of the CYP27A1 gene) is found to have significantly higher affinity for the common redox partner adrenodoxin than another mitochondrial P450, P450scc (product of the CYP11A1 gene). To investigate the basis of the ~30-fold difference in adrenodoxin binding, two sets of P450c27 mutants were generated, expressed in *Escherichia coli*, and purified. Mutations of one set were within the putative adrenodoxin-binding site containing conserved lysine residues also crucial in P450scc for binding adrenodoxin. The second set included mutations within a sequence aligning with the “meander region” of P450BM-3 proposed to be a site of redox-partner interactions in P450s (Hasemann, C. A., Kurumbail, R. G., Boddupalli, S. S., Peterson, J. A., and Deisenhofer, J. (1995) *Structure* 3, 41–62). Mutation of the P450c27 conserved lysines (K354A and K358A) led to a ~20-fold increase in apparent  $K_s$  for adrenodoxin, confirming that these two positively charged residues conserved in mitochondrial P450s are important for adrenodoxin binding. Mutation of Arg-418, conserved in the CYP27A1 family, to serine also decreased the affinity for adrenodoxin ~20-fold. This residue is predicted to be located in the meander region. A triple K354A/K358A/R418S mutation profoundly reduced adrenodoxin binding. Thus, in contrast to P450scc, where mutation of the two conserved positively charged residues results in virtually complete inhibition of adrenodoxin binding, in P450c27 there are three of such residues (Lys-354, Lys-358, and Arg-418) important for adrenodoxin interaction.

Cytochromes P450 (P450)<sup>1</sup> are a large superfamily of hemo-

proteins found in bacteria, yeast, fungi, nematodes, plants, insects, fish, and mammals (1). These monooxygenases metabolize different endogenous and exogenous substrates including steroids, fatty acids, drugs, and xenobiotics. Eukaryotic P450s are membrane-bound enzymes residing either in the endoplasmic reticulum or the inner mitochondrial membrane. Microsomal P450s accept electrons from NADPH through NADPH-cytochrome P450 reductase (P450 reductase) while mitochondrial P450s receive electrons from NADPH via a two-protein redox chain consisting of ferredoxin reductase and ferredoxin. Whereas different forms of P450s are necessary to metabolize different substrates, a single form of P450 reductase for microsomal P450s and ferredoxin for mitochondrial P450s function as P450 redox partners.

Mammalian mitochondrial P450s are involved in the biosynthesis of steroid hormones, the production of bile acids, and the formation of active and inactive vitamin D<sub>3</sub> metabolites. Among mitochondrial P450 systems that involved the conversion of cholesterol to pregnenolone (P450scc) in bovine adrenal cortex and other steroidogenic tissues have been most extensively characterized because of its relative abundance. Development of efficient *Escherichia coli* expression systems leading to availability of large quantities of adrenodoxin reductase (Adx) (2), adrenodoxin (Adx) (3) (the names of ferredoxin reductase and ferredoxin in adrenal glands) and P450scc (CYP11A1) (4), and the opportunity for site-directed mutagenesis, has greatly facilitated structure-function studies of mitochondrial P450s. By site-directed mutagenesis, lysine residues at positions 338 and 342<sup>2</sup> (according to numbering of the mature protein) in P450scc were found to play crucial roles in binding bovine Adx (4). The importance of these residues for Adx binding was implicated initially by chemical modification studies (5, 6). The fact that the key basic residues identified in P450scc are conserved (as lysine or arginine) among all mitochondrial but not in microsomal P450s (Fig. 1) suggests that a similar ferredoxin-binding domain is present in all mitochondrial P450s.

Recently, we have overexpressed P450c27 (CYP27A1), another mitochondrial P450, in *E. coli* (7). This ubiquitous enzyme participates in bile acid biosynthetic pathways catalyzing multiple oxidation reactions at the C-27 atom of steroids in the classical (hepatic) pathway (8) and cholesterol in the alternative (peripheral) pathway (9). The present study was initiated by the observation that the two mitochondrial P450s, P450scc and P450c27, show significantly different elution times from Adx-Sepharose, an affinity resin used for purification of both. Quantitation of the binding affinities using a spectral binding assay revealed an almost 30-fold difference in apparent  $K_s$

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<sup>1</sup> The abbreviations used are: P450, cytochrome P450; P450scc, cholesterol side chain cleavage cytochrome P450, product of the CYP11A1 gene; P450c27, cytochrome P450 catalyzing the multiple oxidation reactions at C-27 of steroids, product of the CYP27A1 gene; P450cam, cytochrome catalyzing the stereospecific hydroxylation of the camphor, product of the CYP101 gene; P450terp, cytochrome catalyzing the oxidation of the  $\alpha$ -terpinol, product of the CYP108 gene; P450BM-3, cytochrome catalyzing the oxidation of a variety of fatty acids, product of the CYP102 gene; P45011 $\beta$ , cytochrome P450 catalyzing the 11 $\beta$ -hydroxylation of 11-deoxycortisol to cortisol and the production of aldosterone, product of the CYP11B1 gene (1); Adx, adrenodoxin; Adr, adrenodoxin reductase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry.

<sup>2</sup> Lys-377 and Lys-381 are according to numbering of the precursor form of P450scc which is used in Ref. 4. In the present paper, numbering of the residues is based on their positions in the primary sequence of the mature forms of P450scc and P450c27.

11A1hum	PLIKASIKETLRLHP
11A1rat	PLLKASIKETLRLHP
11A1pig	PLLKASIKETLRLHP
11A1trout	PLVKGALKETLRLHP
11A1bov	335 PLLKASIKETLRLHP 349
27A1hum	351 PLLKAVLKETLRLYP 365
27A1rab	PLLKASIKETLRLYP
27A1rat	PLLKASIKETLRLYP
27B1hum	PLLKAVVKEVLRYP
27B1rat	PLLKAVVKEVLRYP
27B1mou	PLLKAVVKEVLRYP
24Amou	PYLKACIKESMELTP
24Arat	PYLKACIKESMELTP
24Ahum	PYLKACIKESMELTP
24Achic	PYLKACIKESMELTP
11B1hum	PLLRAALKETLRLYP
11B1rat	PLLRAALKETLRLYP
11B1bov	PLLRAALKETLRLYP
11B1mou	PLLRAALKETLRLYP
11B2hum	PLLRAALKETLRLYP

FIG. 1. The alignment of amino acid sequences of mitochondrial P450s in the region of the putative adrenodoxin-binding site (4). Original references for these sequences can be found in the P450 superfamily summary (1). Residues mutated in the previous (4) and present studies are *underlined*. Two positively charged lysines important for the interaction with Adx in mitochondrial P450s are boxed in black. The outlined glutamate and arginine are the part of the ERR triad (19).

values between these two P450s for ferredoxin, with P450c27 binding more tightly. Utilizing site-directed mutagenesis we have established the primary basis of this difference. Three positively charged residues of P450c27 (Lys-354, Lys-358, and Arg-418) play essential roles in binding Adx. Two of them (Lys-354 and Lys-358) are aligned with residues previously identified as the 2 residues essential for ferredoxin binding to P450scc (4), while Arg-418 is a newly identified residue important for the interaction of mitochondrial P450c27 with Adx. This residue aligns with Ser-402 in P450scc. The much tighter binding of P450c27 to Adx due to the presence of the additional positively charged residue may be important because of the low levels of ubiquitously expressed P450c27 and Adx relative to much higher levels of P450scc and Adx in steroidogenic mitochondria.

#### EXPERIMENTAL PROCEDURES

**Materials**—Restriction endonucleases and other modifying enzymes were purchased from Boehringer-Mannheim. Reagents for bacterial growth were from Difco. [4-<sup>14</sup>C]Cholesterol and [7-<sup>3</sup>H]pregnenolone were from DuPont, while 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol, [3H]5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol, 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ ,27 $\alpha$ -tetrol, and [3H]5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ ,27 $\alpha$ -tetrol were kindly provided by Dr. I. Bjorkhem (Karolinska Institute, Sweden). Other chemicals were purchased from Sigma. Recombinant bovine Adx and ADR were expressed and purified as described previously (2, 3).

**Site-directed Mutagenesis**—This was carried out using *in vitro* QuikChange™ Site-directed Mutagenesis Kit (Stratagene) according to the instruction manual. Templates and complementary mutagenic oligonucleotides used to introduce mutations by polymerase chain reaction are shown in Table I. Correct generation of desired mutations was confirmed by DNA sequencing. The *StuI/XbaI* fragments of P450c27 and the *BsmI/KpnI* fragment of P450scc containing the mutations were ligated into *StuI/XbaI*- and *BsmI/KpnI*-digested expression constructs containing wild-type P450c27 and P450scc, respectively. For each P450c27 mutant the region from upstream of the *StuI* site to beyond the *XbaI* site (*BsmI* and *KpnI* sites for P450scc) was sequenced to make sure that there are no undesired mutations as well as to confirm correct ligation.

**Preparation of Mutant P450s**—Expression in *E. coli* was as described previously for wild-type P450s (4, 7), except that *E. coli* strain DH5 $\alpha$ F'IQ (Life Technologies, Inc.) was used, and chloramphenicol at a concentration of 1  $\mu$ g/ml culture was added at the same time as isopropyl-1-thio- $\beta$ -D-galactopyranoside and  $\delta$ -aminolevulinic acid. These

changes were found to increase the level of expression.<sup>3</sup> The purification procedure for all mitochondrial P450s was the same as described for human recombinant P450c27 (7). After the last purification step, chromatography on Adx-Sepharose, both wild types and mutated forms of P450 were concentrated to 30  $\mu$ M using ultrafiltration membrane cones (Amicon), dialyzed overnight against 100 volumes of 40 mM phosphate buffer (pH 7.4) containing 20% glycerol to remove 1 M NaCl and 0.5% sodium cholate present in the elution buffer which interfere with spectral binding and enzymatic assays, and then frozen at -70 °C.

**Spectral Studies**—Adrenodoxin-induced spectral changes of P450s (spectral binding assay) were measured at room temperature as described (10) using 0.6–4  $\mu$ M P450 in 20 mM potassium phosphate buffer (pH 7.4), 20% glycerol, 0.1% Tween 20, and 50  $\mu$ M substrate (5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol for P450c27 or cholesterol for P450scc). The concentration of free ligand was calculated as described previously (11). After titration, P450 content was measured by reduced CO-difference spectrum (12) to confirm that there was no denaturation during titration.

**Enzyme Assays**—Enzymatic activities of wild-type and mutated forms of P450c27 were determined using 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol as a substrate as described previously (7), except that a much higher molar excess of Adx (140-fold, final concentration 5.6  $\mu$ M) over P450 was used. The reaction time was 10 min. To measure the activity of the triple K354A/K358A/R418S P450c27 mutant, the reaction time was increased to 20 min, and the final concentrations of the P450, Adx, and ADR were 0.6, 22, and 1  $\mu$ M, respectively. Enzymatic activities of the wild-type and mutated forms of P450scc were determined as described previously (13) using 50  $\mu$ M cholesterol as substrate in 40 mM phosphate buffer (pH 7.2) containing 0.3% Tween 20. The reaction mixture (1 ml) contained 0.2  $\mu$ M P450, 8  $\mu$ M Adx, and 1  $\mu$ M ADR, and the reaction time was 10 min. Steroids were separated as described previously after extraction with CH<sub>2</sub>Cl<sub>2</sub> and evaporation (7).

**Matrix-assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)**—Two microliters of wild-type P450c27 (250  $\mu$ M in 30 mM potassium phosphate (pH 7.2), containing 15% glycerol) was mixed with 8  $\mu$ l of ferulic acid (10 mg/ml) in tetrahydrofuran, spotted on a MALDI-TOF sample plate, and washed with 1% aqueous trifluoroacetic acid to remove glycerol. Mass spectrometry was performed in a linear positive ion mode with a 350-ns delayed pulse (linear delayed extraction mode) using a laser desorption ionization mass spectrometer with a N<sub>2</sub> pulse laser at 337 nm (PerSeptive Biosystems Voyager Elite). Signals from 255 excitation pulses were accumulated using accelerating voltage, 25,000 V; grid voltage, 94.0%; and guidewire voltage, 0.2%. Mass calibration was performed using bovine serum albumin (average molecular weight is 66,431).

#### RESULTS

**Comparison of Binding of Wild-type P450c27 and P450scc to Adx**—To date, the mode of interaction of mitochondrial P450s with the redox partner Adx has only been investigated for bovine P450scc (4–6). When we overexpressed a second mitochondrial P450, human P450c27 in *E. coli*, affinity chromatography on the Adx-Sepharose column was used as a final purification step (7). P450c27 was found to elute from this column at a much higher salt concentration than P450scc. Since different detergents were used for solubilization of the mitochondrial P450s from the *E. coli* membranes, and different chromatographic steps preceded chromatography on Adx-Sepharose P450scc (4) and P450c27 (7), it was possible that these differences affected chromatographic behavior on Adx-Sepharose. To test this possibility, recombinant P450scc was purified exactly in the same way as P450c27 and applied to Adx-Sepharose. Changing the purification protocol did not affect the elution time of P450scc from Adx-Sepharose. It is eluted as one peak at 0.54 M NaCl, 0.34% sodium cholate, while P450c27 is eluted as two overlapping peaks, one at 0.9 M NaCl, 0.47% sodium cholate and the other at 1 M NaCl, 0.5% sodium cholate (Fig. 2). P450c27 in the two peaks has identical spectral parameters in the region between 260 and 715 nm, and identical electrophoretic mobility and purity as judged by the silver-stained SDS-polyacrylamide gel electrophoresis (data not shown). The

<sup>3</sup> K. Kusano, unpublished data.

TABLE I  
Oligonucleotides and templates used to generate P450c27 and P450scc mutants

Template	Mutation	Oligonucleotides
P450c27	K354A	5'-GCCCACATGCCGTTGCTCGCAGCTGTGCTTAAGGAG-3' <sup>a</sup> 5'-CTCCTTAAGCACAGCTCGAGCAACGCATGTGGGC-3'
P450c27	V356S	5'-CGTTGCTCAAAGCTAGCCTTAAGGAGACTCTGC-3' 5'-GCAGAGTCTCCTTAAGGCTAGCTTTGAGCAACG-3'
P450c27	L357I	5'-GCTCAAAGCTGTGATTAAGGAGACTCTGCG-3' 5'-CGCAGAGTCTCCTTAATCACAGCTTTGAGC-3'
P450c27	K358A	5'-CTCAAAGCTGTGCTTGGGAGACTCTGCGTCTC-3' 5'-GAGACGCAGAGTCTCCGCAAGCACAGCTTTGAG-3'
P450c27	Y364H	5'-GAGACTCTGCGTCTCCACCCTGTGGTCCCC-3' 5'-GGGGACCACAGGGTGGAGACGCAGAGTCTC-3'
P450c27	H414T	5'-GAAAGCTTCCAGCCACGCGTTGGCTGAGAAACAGC-3' 5'-GCTGTTTCTCAGCCAACGCGTGGGCTGGAAGCTTTC-3'
P450c27	R418S	5'-CCCCACCGCTGGCTGAGTAACAGCCAGCCTGC-3' 5'-GCAGGCTGGCTGTTACTCAGCCAGCGTGGGG-3'
P450c27	V356S/L357I/Y364H	5'-CTCAAAGCTAGCATTAAGGAGACTCTGCGTCTCCACCCTGTGGTC-3' 5'-GACCACAGGGTGGAGACGCAGAGTCTCCTTAATGCTAGCTTTG0AG-3'
R418S P450c27	K354A/K358A/R418S	5'-GCCGTTGCTCGCAGCTGTGCTTGGGAGACTCTGCGTCTCTACCC-3' 5'-GGGTAGAGACGCAGAGTCTCCGCAAGCACAGCTCGCAGCAACGGC-3'
P450scc	S402R	5'-CCAACCAGGTGGCTGCGCAAAGACAAAGACCTC-3' 5'-GAGGTCTTTGTCTTTGCGCAGCCACCTGGTTGG-3'

<sup>a</sup> The mutated nucleotides are underlined.

two peaks were pooled and subjected to MALDI-TOF MS to check if there is any heterogeneity in the fraction. Three peaks are seen in the MALDI-TOF mass spectrum of P450c27 (Fig. 3). The first and the second peaks correspond to doubly and singly charged monomers of P450c27, respectively, and the third peak is a singly charged dimer of the protein which is a laser induced artifact of MALDI-TOF MS. The molecular weight of the singly charged monomer of P450c27 was 56,909 while the theoretical value of 56,910 is calculated from the primary sequence. The results of MALDI-TOF MS rule out heterogeneity of the sample due to post-translational modifications of P450c27 or its proteolytic digestion during purification. One possible explanation for the two-peak elution profile of P450c27 from Adx-Sepharose is based on our observation that in solution in the absence of detergent, P450c27 forms aggregates with an average molecular mass of 1,146 kDa (data of light scattering),<sup>4</sup> approximately 20 molecules per aggregate. As a result of the aggregation, not all the residues important for binding Adx in P450c27 molecule may be available for interaction with immobilized Adx because of steric hindrance. The peak eluted first from Adx-Sepharose could result from nonspecific aggregation of P450c27 having only some of these residues available for Adx interaction. The second peak could be aggregates in which all residues in P450c27 important for the binding to Adx are available for interaction.

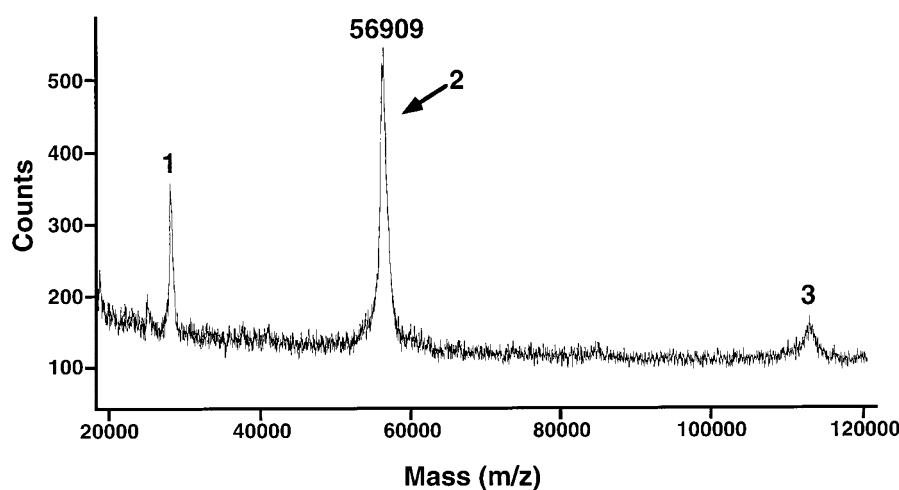
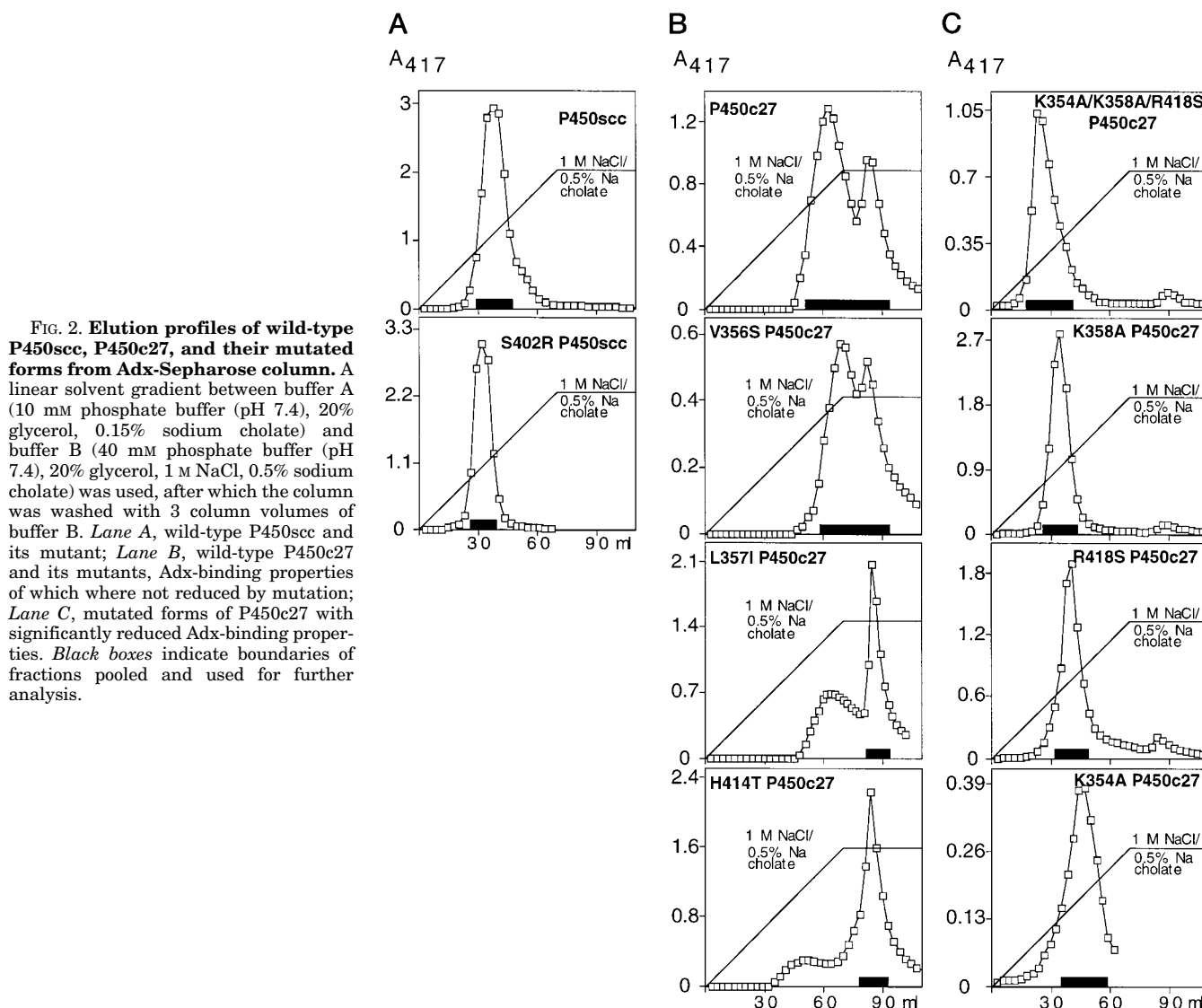
Chromatography on Adx-Sepharose clearly shows that there are significant differences in affinities between the substrate-free forms of two mitochondrial P450s. To quantitate these differences, Adx binding constants were estimated using a spectral binding assay. As shown earlier (14), binding of adrenodoxin to P450scc in the presence of substrate (cholesterol) and the nonionic detergent Tween 20 induces a change in spin state of the heme iron and a shift in the absorbance from 418 to 389 nm. When cholesterol is absent, the Adx-induced spin state change is much smaller (14). The value of the apparent binding

constant,  $K_s$ , is obtained from double reciprocal plots of the concentration of free Adx and the differences in absorbance change upon titration with Adx (Fig. 4).  $K_s$  and hence the interaction between P450scc and Adx is found to be affected by several factors. Presence of cholesterol increases the affinity of P450scc for Adx (13–20-fold) (14), while binding of Adx to both substrate-free P450scc and the cholesterol complex is significantly reduced as ionic strength is increased (10, 14). Binding is unaffected by the nonionic detergent Tween 20 (14). The fact that the  $K_s$  value for Adx is substrate-dependent means that apparent binding constants of different mitochondrial P450s determined in the presence of different substrates may not accurately reflect physiological differences in the affinity of these P450s for Adx. P450c27 has a wide substrate specificity, the best physiological substrate being 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol (15). Cholesterol is also an important physiological substrate, although the rates of metabolism by P450c27 are much slower than those for the triol substrates. Titrations of recombinant P450c27 with up to 10  $\mu$ M Adx in the presence of 0.1% Tween 20 and either 50  $\mu$ M 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol, or 50  $\mu$ M cholesterol, or without any substrate showed spectral changes only in the presence of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol. Thus the titration buffer (20 mM potassium phosphate, 20% glycerol, 0.1% Tween 20) for P450scc contained 50  $\mu$ M cholesterol while that for P450c27 contained 50  $\mu$ M 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol. The apparent binding constants of Adx for recombinant P450c27 and recombinant P450scc were determined to be 0.049 and 1.42  $\mu$ M, respectively (Table II), confirming the original chromatographic observation that P450c27 binds much more tightly to Adx than P450scc. We believe that the almost 30-fold difference in  $K_s$  values between two P450s rather accurately depicts the difference in affinities to Adx-Sepharose (Fig. 2), and two independent methods (chromatography on Adx-Sepharose and spectral binding assay) can be used for evaluation of the effect of amino acid substitutions in P450s on their binding properties to Adx.

*Selection of Amino Acid Residues for Mutation*—Calculation

<sup>4</sup> I. A. Pikuleva, C. Cao, and M. R. Waterman, unpublished data.





of the difference in free energy of binding,  $\Delta(\Delta G)$ , between P450scc-Adx, and P450c27-Adx using spectral binding constants permits prediction of the type of additional protein-protein interactions which might be involved in formation of the P450c27-Adx complex. While dependent on the environment and solvent exposure, the value of 1.97 kcal/mol indicates that it could be either one additional salt bridge (while envi-

ronmental dependent, the disruption of electrostatic interaction between charged groups is reported to weaken binding energy by about 2.0 kcal/mol (16)), or more than one hydrogen bond, involving non-polar groups (the weakening of binding energy in this case is 0.5–1.5 kcal/mol (17)). Previous site-directed mutagenesis studies of human Adx identified two negatively charged residues (Asp-76 and Asp-79) as being impor-

FIG. 4. Double reciprocal plots of titration of wild-type and selected mutant forms of P450c27 (A) and P450scc (B) with Adx. Titrations have been carried out as described under "Experimental Procedures." Panel A:  $\square$ , P450c27;  $\diamond$ , K354A P450c27;  $\circ$ , K358A P450c27;  $\triangle$ , R418S P450c27. Panel B:  $\square$ , P450scc;  $\circ$ , S402R P450scc.

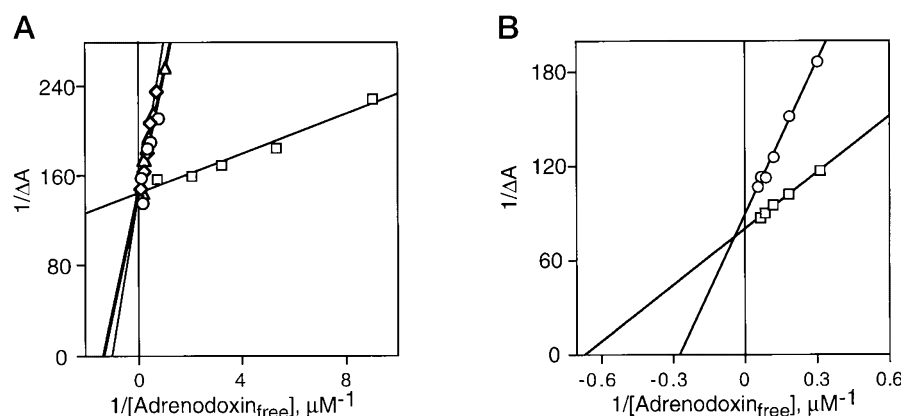


TABLE II

Apparent binding constants, differences in free energies of binding ( $\Delta(\Delta G)$ ) for the interaction of P450 with adrenodoxin and enzymatic activities of the wild-type and mutant forms of P450c27 and P450scc

Form	P450 spectral binding assay		Turnover
	$K_s$ $\mu\text{M}$	$\Delta(\Delta G)$ $\text{kcal/mol}$	
Wild-type P450c27	$0.049 \pm .006^a$		$40.0 \pm 2.26^b$
K354A	$1.06 \pm 0.17$	$1.8^c$	$20.3 \pm 3.61$
V356S	$0.014 \pm 0.004$	$-0.7$	$29.9 \pm 2.82$
L357I	$0.023 \pm 0.006$	$-0.4$	$27.4 \pm 1.99$
K358A	$0.88 \pm 0.15$	$1.7$	$15.4 \pm 0.83$
H414T	$0.025 \pm 0.006$	$-0.4$	$25.8 \pm 1.40$
R418S	$0.83 \pm 0.02$	$1.7$	$23.6 \pm 3.00$
K354A/K358A/R418S	ND <sup>d</sup>		$0.30 \pm 0.01^e$
Wild-type P450scc	$1.42 \pm 0.22$		$1.43 \pm 0.06$
S441R	$3.65 \pm 0.07$	$0.6$	$1.28 \pm 0.03$

<sup>a</sup> Titrations has been carried out as described under "Experimental Procedures," the results are means of three to four experiments.

<sup>b</sup> Enzymatic activities were measured as described under "Experimental Procedures," the results are means of triplicate experiments.

<sup>c</sup> Calculated from the following equation:  $\Delta(\Delta G) = -RT \ln (K_{\text{wild type}}/K_{\text{mutant}})$ .

<sup>d</sup> ND, not detectable.

<sup>e</sup> Enzymatic activity of the fraction bound to Adx-Sepharose.

tant for high affinity binding to bovine P450scc (18). These 2 residues are located in a region (residues 68–86) within mitochondrial ferredoxins containing 5 additional acidic residues that could also potentially be involved in the interaction with P450c27. Based on the fact that much higher ionic strength is needed to elute P450c27 from Adx-Sepharose, we surmised that stronger binding between P450c27 and Adx compared with the P450scc-Adx pair is due to an additional electrostatic interaction between the two proteins involving a positive charge on P450c27 and a negative one on Adx. Alignment of amino acid sequences in the region which is highly conserved among mitochondrial P450s containing the two lysine residues crucial for the interaction of P450scc with Adx (Lys-338 and Lys-342) (Fig. 1) shows that only three amino acid residues at positions 356, 357, and 364 (P450c27 numbering) are different between human P450c27 and bovine P450scc, and none of these are positively charged. The conserved Lys-354 and Lys-358 were converted to Ala to determine whether they are also important for the binding to Adx in P450c27. We also mutated each of 3 residues at positions 356, 357, and 364 in P450c27 to the corresponding residues in P450scc in addition to generating a triple V356S/L357I/Y364H P450c27 mutant making the complete stretch (residues 351–365) in P450c27 identical to that in P450scc.

The putative Adx-binding site in mitochondrial P450s also contains the glutamate and arginine residues (Glu-359 and Arg-362 in P450c27, Fig. 1) which are invariant in all P450

sequences. In three soluble bacterial P450s with known tertiary structure, these invariant glutamate and arginine are part of the so called ERR triad in which the glutamate forms salt bridges with both arginines located 2 and ~50 residues away from it in the P450 primary sequences (19). The second arginine in the ERR triad is located in the meander region (Arg-415 in P450c27 aligns with the conserved ERR arginine in the meander region, Fig. 5) and in different P450s it is arginine, histidine, or asparagine (19). The meander region (~20 amino acids) has been proposed to be a site of interaction with redox partners in P450s (19). Despite more than 50 amino acids separating the first 2 residues in the ERR triad from the last one, they are spatially very close in the three bacterial P450s forming a set of salt bridge interactions between each other and carbonyl oxygens in the meander (19). This implies that in mitochondrial P450s the regions containing residues of the ERR triad (the conserved Adx-binding site (Fig. 1) containing the first 2 residues of the ERR triad and the meander region containing the last residue of the ERR triad, Fig. 5) may be spatially close, and that the meander region could contain residues which interact with Adx. Alignment of mitochondrial P450s in the meander region (Fig. 5) shows that Arg-418 is conserved within the CYP27A1 family but is not conserved among all mitochondrial P450s. This arginine was mutated to serine in P450c27. Later, after obtaining data on properties of this serine mutant, a reciprocal mutant of P450scc, S402R, and additional mutants of P450c27, H414T, and K354A/K358A/R418S were generated.

**Expression and Purification of Mutant P450s**—All the mutants except two were overexpressed at levels comparable with the wild-type enzymes. Immunoblotting analysis using anti-serum against human recombinant P450c27 showed no detectable protein after the expression of the Y364H P450c27 and V356S/L357I/Y364H P450c27 mutants in *E. coli* (data not shown). Thus of the 10 mutants only eight were studied. The last purification step, chromatography on Adx-Sepharose, was also used to monitor the affinity of the substrate-free forms for Adx in the absence of nonionic detergent. Three single P450c27 mutants, K354A, K358A, and R418S, were eluted much earlier from Adx-Sepharose than wild type (Fig. 2C), indicating alteration of Adx binding properties. The elution of these mutants was close to that of wild type P450scc (Fig. 2A). When the triple K354A/K358A/R418S P450c27 mutant was subjected to chromatography on Adx-Sepharose, ~50% of the amount applied was found in the flow-through fraction, while the rest, bound to the resin, was eluted much earlier than any of the single mutants (Fig. 2C). The fact that a portion of K354A/K358A/R418S P450c27 mutant bound to Adx-Sepharose may indicate that besides Lys-354, Lys-358, and Arg-418 there are some other residues in P450c27 involved in the binding to Adx. However, because very low ionic strength was needed to elute

11A1hum	FFDPENFDPT	WLSKDKNITYF	
11A1rat	FFPNPKFDPT	WLEKSQNTTHF	
11A1pig	FSNPGQFDPT	WLGKERDLIHF	
11A1trout	FPRPEKYLPS	WLRTEQYFRS	
11A1bov	389 FSSPKFDPT	WLSKDKDLIHF	410
27A1hum	405 FSEPSFQPH	WLENSQPATPR	426
27A1rab	FSEPDTFWPY	WLRKQPETSK	
27A1rat	FPEPSFQPH	WLRKREDDNSG	
27B1hum	FPEPNSFRPA	WLGEGPTPHPF	
27B1rat	FREPNSFNPA	WLGEGPAPHPF	
27B1mou	FDPNSFNPA	WLGEGPTPHPF	
24Amou	FEDADKFRPE	WLEKEKKINPF	
24A1rat	FEDSHKFRPE	WLQKEKKINPF	
24A1hum	FEDSSQFRPE	WLQKEKKINPF	
24A1chic	FNGWTQFKPE	RWLQKHSINPF	
11B1hum	FPRPERYNPQ	WLDIRGSGRNF	
11B1rat	FPRPERYMPQ	WLERKRSFQHL	
11B1bov	FARPEYHPQ	WLDKQSGSRF	
11B1mou	FPRPERYMPQ	WLERKRSFQHL	
11B2hum	FPRPERYNPQ	WLDIRGSGRNL	

FIG. 5. The alignment of amino acid sequences of mitochondrial P450s in the meander region (19). Original references for these sequences can be found in the P450 superfamily summary (1). Residues mutated are *underlined*. A conserved arginine within the CYP27A1 family important for the interaction with Adx is *boxed in black*. The *outlined* arginine is a part of the ERR triad (19).

a triple mutant from Adx-Sepharose, the total contribution of such residues appears to be much smaller than those of Lys-354, Lys-358, or Arg-418. The flow-through portion of the K354A/K358A/R418S P450c27 mutant may represent nonspecific aggregates in which these additional residues are not available for interaction with Adx-Sepharose. The elution profiles of the other single P450c27 mutants, V356S, L357I, and H414T, showed modest changes in binding to the column compared with the wild type (Fig. 2B). The reciprocal S402R P450scc mutant was generated to determine if this substitution will improve the interaction of P450scc with Adx. Chromatographic behavior of this mutant on Adx-Sepharose, however, indicated it actually binds more weakly to Adx-Sepharose than wild type (Fig. 2A).

All purified mutants exhibited UV and visible absorption spectra indistinguishable from wild-type P450s. They all were isolated as substrate-free, low spin forms with specific heme contents of 16.5–17.5 nmol/mg of protein, except the triple mutant, K354A/K358A/R418S, which had lower heme content (9.2 nmol/mg of protein in the flow-through and 11.4 nmol/mg of protein for the fraction bound to Adx-Sepharose). After chromatography on hydroxylapatite, which precedes chromatography on Adx-Sepharose, the specific heme content of the K354A/K358A/R418S P450c27 mutant was 9.8 nmol/mg of protein, a value close to that obtained for the wild-type P450c27 at this step (7). The lack of significant enrichment of specific heme content of the fraction eluted from Adx-Sepharose accompanied by no significant change of specific heme content of the flow-through fraction indicates that simultaneous replacement of 3 residues did not cause major structural perturbations resulting in the formation of apoprotein or denatured protein. No P420 form was detected in any of the purified mutants.

**Spectral Assay of Adx Binding**—Addition of adrenodoxin to purified P450 mutants produced difference spectra qualitatively similar to those elicited by the wild-type enzymes allowing determination of apparent binding constants summarized in Table II. The double reciprocal plots for titration of wild-type and mutated forms of P450scc and P450c27 are shown in Fig. 4. Approximately the same increase in  $K_s$  values (20-fold) was obtained for each of the three single P450c27 mutants (K354A, K358A and R418S) corresponding to an increase in apparent free energy of binding  $\Delta(\Delta G)$  of  $\sim 1.7$  kcal/mol (Table II). These data are consistent with results from chromatography on Adx-

Sepharose and suggest that all three positively charged residues at positions 354, 358, and 418 are important for interaction with Adx. The other three P450c27 mutants (V356S, L357I and H414T) showed slight decreases (2–3.5-fold) in apparent binding constants with only small decreases of apparent free energy of binding  $\Delta(\Delta G)$  of 0.4–0.7 kcal/mol (Table II). Only the triple K354A/K358A/R418S P450c27 mutant (both bound and unbound to Adx-Sepharose fractions) did not show any spectral shift even upon addition of a very high concentrations of Adx (up to 36  $\mu$ M), or when the concentration of the P450 used in the spectral binding assay was increased 7-fold (data not shown). Mutation of serine to arginine in P450scc at position 402 which was predicted to increase the affinity for Adx based on studies with P450c27, in contrast led to a 2.6-fold increase in  $K_s$  value (Table II).

**Enzyme Assays**—The mutant forms of P450c27 and P450scc were also tested for 27-hydroxylation of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol and side chain cleavage activity of cholesterol, respectively, by reconstitution with Adx and Adr. Activity in this assay requires binding of Adx to Adr and P450 and productive electron transfer. These experiments were carried out using a 140-fold molar excess of Adx over P450 to compensate for the differences in affinity of Adx for different mutant P450s. To measure the specific activity of the triple K354A/K358A/R418S P450c27 mutant (both unbound and bound to Adx-Sepharose fractions) a 15-fold higher concentration of P450 was used and the reaction time was 2 times longer. All mutants except the unbound fraction of the triple K354A/K358A/R418S P450c27 mutant showed enzymatic activities but had reduced turnover numbers (Table II).

## DISCUSSION

Details of the interactions of different mitochondrial P450s with their common redox partner ferredoxin are derived from chemical modification and site-directed mutagenesis studies using bovine P450scc. Finding that the key basic residues for interaction with Adx in P450scc are conserved among all mitochondrial P450s suggests that this interaction involves a similar conserved binding domain. In the present study it is confirmed with a second mitochondrial P450, human P450c27, that these conserved lysine residues are important for Adx binding. However, it is also shown that an additional positively charged residue (Arg-418) participates in Adx binding, leading to tighter binding of Adx to P450c27 than to P450scc. This residue is located outside the immediate environment of the conserved lysine residues.

Binding of Adx to P450c27 exhibits several differences compared with that to P450scc. First, it is 30-fold tighter. Despite this, the contribution of each of the conserved lysines (Lys-354 and Lys-358) is approximately an order of magnitude weaker than in P450scc. For example, in P450scc mutation of K338A resulted in about a 250-fold increase in apparent  $K_s$  value, while the replacement of the corresponding lysine residue in P450c27 (K354A) led to only about a 20-fold change of  $K_s$ . A smaller 150-fold increase in  $K_s$  was observed upon the mutation of the second conserved lysine in P450scc (K342Q) with  $\sim 20$ -fold increase in  $K_s$  arising from mutation of the corresponding K358A in P450c27. The finding that P450c27 binds more tightly to Adx than P450scc and that the conserved lysine residues in P450c27 play a smaller role in Adx binding than in P450scc suggests that additional residue(s) in P450c27 participate in the interaction with Adx. At first we checked the effect of differences in the primary sequence between the two P450s in the 15-amino acid stretch containing the conserved lysine residues important for Adx binding. Between bovine P450scc and human P450c27 there are three differences in this stretch (Val-356, Leu-357, and Tyr-364) and replacement of Val-356



and Leu-357 in P450c27 with the corresponding residue in P450scc (Fig. 1) showed no significant effect on Adx binding. Neither the Y364H nor V356S/L357I/Y364H mutants were detected using immunoblotting analysis during expression in *E. coli*. Apparently Tyr-364 in P450c27 is important for maintaining structural integrity of the protein, and P450c27 cannot be properly folded when this residue is replaced with histidine, the residue present in P450scc. Mutation of V356S and L357I actually slightly improved binding of Adx to P450c27 indicating that these 2 residues do not contribute to tighter complex formation between Adx and P450c27. Despite the fact that affinity to Adx was increased, the V356S and L357I mutants showed moderately decreased enzymatic activity implying that these mutations directly or indirectly affect some other steps in the P450 catalytic cycle. Because Val-356 and Leu-357 are located within the Adx-binding site, their mutation could affect the rate of electron transfer and hence the turnover number. Thus, while the conserved lysine residues are important for Adx binding to P450c27, additional residues essential for this are located outside this region.

To identify these additional residues we utilized the results of a comparative analysis of crystal structures of three bacterial P450s (19). This analysis argues that the general structure of eukaryotic P450s resembles that of the bacterial enzymes, and most relevant to this study identifies the ERR triad and the meander region as typical features of P450 structure (19). The meander region was proposed to be a likely site of redox-partner interactions (19). Mutation of the positively charged Arg-418, predicted to be located in the P450c27 meander region and conserved in the CYP27A1 family decreased the affinity of P450c27 to Adx ~20-fold, a comparable effect to that caused by the mutation of conserved Lys-354 and Lys-358. This mutant was expressed at the same level as the wild-type enzyme, did not exhibit any abnormalities during purification, showed a peak at 448 nm without any trace of a peak at 420 nm in the reduced CO-difference spectrum and was able to support the activity in the reconstituted system, although full activity was not achieved because of the lower affinity to Adx. This mutation did not cause any major structural perturbations in the protein and the substantial decrease of affinity for Adx is most likely the result of the neutralization of a positive charge at position 418. The 1.7 kcal/mol increase in apparent free energy of binding caused by this mutation is not significantly different from the value of 1.97 kcal/mol, characterizing  $\Delta(\Delta G)$  between P450scc-Adx and P450c27-Adx. Such close correspondence of these two values implies that Arg-418 is the most important among the residue(s) in P450c27 which confer(s) the additional strength for Adx binding. Mutation of His-414 which is also located in the meander region, only 3 residues away from Arg-418, to Thr the corresponding residue in P450scc, did not decrease but slightly improved binding to Adx, indicating that His-414 is not the additional residue in P450c27 important for the interaction with Adx.

The present studies raise new questions regarding the interaction of mitochondrial P450s with the common ferredoxin redox partner. The most important one is: what is the physiological significance of greatly different affinities of mitochondrial P450s for ferredoxin? Besides the present work demonstrating that two mitochondrial P450s have significantly different Adx-binding properties, there are several studies showing that mitochondrial P45011 $\beta$  (CYP11B1) has lower affinity for Adx than P450scc (11, 20, 21). Thus, differences in binding affinity for ferredoxin may be a general phenomenon among mammalian mitochondrial P450s. Perhaps the different levels of expression of ferredoxin in different tissues and the tissue distribution pattern of mitochondrial P450s provide an

explanation. The lower the level of expression of ferredoxin and P450 in a given tissue (liver or kidney, for example) the higher the affinity between two proteins and vice versa. In tissues where several mitochondrial P450s are present simultaneously at relatively high levels (P450scc and P45011 $\beta$  in adrenal cortex), different affinities may serve to regulate the preferential partitioning of electron transfer toward one P450. If the level of expression of two P450s is significantly different, higher binding affinity of the more poorly expressed P450 is a way to compete efficiently for ferredoxin.

Another unanswered question is how many binding sites for Adx are in mitochondrial P450s, and if there are several sites, are they formed by the same stretch of primary sequence in different mitochondrial P450s? In the absence of the tertiary structure of P450c27 we do not know if Lys-354, Lys-358, and Arg-418 are part of one site. If the overall tertiary structure of P450c27 is not too different from that of bacterial P450s, P450cam, P450terp, and P450BM-3, then these 3 residues should be spatially close to each other forming a single binding site. Our data also indicates that there may be other residues in P450c27 interacting with Adx which form a separate binding site, which contributes much less to Adx binding than Lys-354, Lys-358, or Arg-418. Thus, in mitochondrial P450s with high affinity for Adx there may be two sites for interaction with Adx, one being common and the other one being specific for each P450.

Site-directed mutagenesis studies have shown that four conserved negatively charged residues (Asp-72, Glu-73, Asp-76, and Asp-79) located within the highly acidic region (positions 68–86) in mitochondrial ferredoxins are important for binding of human ferredoxin to bovine P450scc (18). The contribution of these residues to the affinity for P450scc is different being the greatest for Asp-76 and Asp-79 ( $K_s$  for D76N and D79A mutants was increased ~6- and 8-fold, respectively) followed by Asp-72 and Glu-73 (3- and 4-fold increase of  $K_s$  for D72N and E73Q mutants) (18). Recently the tertiary structure of a truncated form of bovine Adx where 3 residues from the N terminus and 20 residues from the C terminus were removed by genetic engineering has been determined (22). This truncated form exhibits 3.2- and 6-fold decreased  $K_m$  values for P450scc and P45011 $\beta$ , respectively, compared with that of the wild type, and 4.5-fold enhanced efficiency of substrate conversion catalyzed by P45011 $\beta$  (23). Subsequently we determined the tertiary structure of full-length bovine Adx.<sup>5</sup> Residues Asp-72, Glu-73, Asp-76, and Asp-79 are located on the surface of the molecule in both structures with their side chains directed toward the solvent. The carbon atoms of the carboxylate groups of these 4 residues form the corners of a quadrilateral with length of the sides between 6.6 and 9.1 Å. Thus, 4 acidic residues in adrenodoxin are potentially available for the pairwise electrostatic interactions with mitochondrial P450s. Determination of which of the residues are paired in electrostatic interactions with P450c27 and P450scc by analysis of the interactions of mutant forms of both partners could establish whether differences in paired interactions exist between mitochondrial P450s which contribute to both binding and catalytic activity.

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