Complex Formation in Vesicle-Reconstituted Mitochondrial Cytochrome P450 Systems (CYP11A1 and CYP11B1) As Evidenced by Rotational Diffusion Experiments Using EPR and ST-EPR†

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ABSTRACT: Rotational diffusion measurements using EPR and saturation transfer EPR were applied to analyze complex formation between the electron-transfer components of the mitochondrial steroid-hydroxylating cytochrome P450 systems (CYP11A1 and CYP11B1) in phosphatidylcholine/phosphatidylethanolamine/cardiolipin vesicles prepared by octyl glucoside dialysis/adsorption. Octyl glucoside reconstitution of P450SCC results in large vesicles, which have an advantage over small vesicles in that vesicle tumbling does not contribute to measured rotational diffusion rates. Immobilization of spin-labeled adrenodoxin by both P450SCC and adrenodoxin reductase indicates equimolar complexation between P450SCC and adrenodoxin as well as between adrenodoxin reductase and adrenodoxin. Combination of rotational diffusion and antibody cross-linking confirmed the complexation of adrenodoxin with P450SCC and for the first time provided direct evidence of a complex between P450SCC and P45011β in the membrane. In contrast, no evidence was found for the existence of adrenodoxin reductase−P450SCC complexes or a ternary complex of all three proteins. Thus, these experiments confirm the shuttle mechanism of electron transfer to vesicle-reconstituted P450SCC and P45011β.

The first, as well as the final, step in biosynthesis of steroids occurs in mitochondria of the adrenal cortex and includes at least two cytochrome P450 enzymes, P450SCC and P45011β. Both are localized in the inner mitochondrial membrane and obtain the reducing equivalents via a common short electron-transfer chain consisting of adrenodoxin (an Fe_2-S_2 protein, AD)[1] and NADPH-dependent adrenodoxin reductase (an FAD-containing flavoprotein, AR) (*1*, and references therein). With regard to the mechanism of electron transfer, three different models have been proposed. The so-called "shuttle mechanism" with AD as the mobile electron shuttle between P450 and AR (*2*, *3*) and mutually exclusive binding of AD to P450 and subsequently to AR is supported by a wide range of observations, among them cross-linking studies (*4*), chemical modification studies (*4*, *5*), and site-directed mutagenesis (*6*). In an alternative model, the three proteins function as a ternary complex (*7*). Support for this model comes from chemical modification experiments (*8*) and protein rotation studies of mitochondria and submitochondrial particles (*9*). Finally, a quaternary cluster model involving an AD dimer has been proposed (*10*) and subsequently supported by activity titration studies (*11*).

P450SCC catalyzes the side chain cleavage of cholesterol to produce pregnenolone, the common precursor of all steroid hormones. P45011β purified from bovine adrenal mitochondria exhibits different catalytic activities, including 11β- and 18-hydroxylations and, under certain circumstances, aldosterone synthase activity (*12*). To explain the zone-specific aldosterone synthesis in bovine adrenal, recently it has been hypothesized that P450SCC might play an important role in regulating the various catalytic activities of P45011β, perhaps via protein−protein interaction in the membrane. An interaction between both mitochondrial P450s has been proposed on the basis of catalytical experiments, but until now never shown directly by structural methods (*13*).

Many of the experiments to date concerning electron transfer to P450 have been performed with P450 in solution rather than in membrane systems. Moreover, few studies have been performed to directly analyze protein−protein interactions between the components of the mitochondrial P450 monooxygenase systems. In this paper we report, for the first time, the direct analysis of complex formation between the electron transfer partners in PC/PE/CL vesicle systems by rotational diffusion. The lipids used for vesicle preparation were selected to approximate the composition of the inner mitochondrial membrane. The inclusion of CL seems particularly important (i) for physiological relevance (it is a specific lipid of the inner mitochondrial membrane), (ii) because it is essential for complete membrane incorporation of P450SCC into certain vesicle systems, and (iii) because

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[1] Abbreviations: P450SCC, cytochrome P450SCC (CYP11A1); P45011β, cytochrome P45011β (CYP11B1); AD, adrenodoxin; AR, adrenodoxin reductase; OG, octyl glucoside; PC, egg phosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; CL, bovine heart cardiolipin; PE, egg phosphatidylethanolamine; DOC, deoxycorticosterone; MSL(6)**, [^{15}N]perdeuterio-*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmaleimide).

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of its extraordinary stimulating role in side chain activity and other functions of P450SCC (*1*, *14*). The level of rotational diffusion was measured by EPR and saturation transfer EPR (ST-EPR) using ^{15}N- and ^{2}H-substituted spin-labels that improve sensitivity and resolution compared to conventional spin-labels (*15*, *16*). Octyl glucoside (OG) reconstitution of P450SCC vesicles was used because it yields relatively large vesicles. With these vesicles, there is no significant contribution of vesicle tumbling to the measured mobility of P450SCC in the membrane. We found evidence for complexation of AD and P450SCC, of AD and AR, and between the two P450s, P450SCC and P45011β, but no indication for the existence of AR−P450SCC complexes or ternary complexes of all three proteins.

MATERIALS AND METHODS

Materials. The double-isotope-substituted spin-label [^{15}N]-perdeuterio-*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmaleimide) [MSL(6)**] was from the the Novosibirsk Institute of Organic Chemistry (Novosibirsk, Russia).

Egg PC and egg PE were from Lipid Products, and bovine heart CL was from Sigma and OG from Calbiochem. [^{14}C]-Cholesterol, [^{14}C]DPPC, and [^{3}H]DOC were from Amersham; Sephacryl S-1000 was from Pharmacia, and BioBeads SM-2 were from Biorad.

P450SCC, AD, and AR were purified from bovine adrenocortical mitochondria to electrophoretic purity using specific affinity adsorbents (*17*, *18*). They were stored until they were used at −80 °C at high concentrations in 50 mM phosphate buffer (pH 7.4), 1 M NaCl, 0.3% sodium cholate, 1 mM EDTA, and 20% (v/v) glycerol (storage buffer). P45011β was prepared as described in ref *19*. Antibodies to P450SCC, AD, and AR were raised in rabbits and purified according to the procedure described previously (*20*).

Spin-Labeling. P450SCC was spin-labeled with MSL(6)**. Labeling conditions (molar label/P450SCC ratio, incubation time, temperature, and presence of cholate) were systematically varied to achieve optimal conditions. Finally, standard labeling was performed by incubation of P450SCC with a 10-fold excess of label for 1 h at room temparature (or equivalent, overnight at 4 °C) and resulted in about 1.2 molecules of label per P450SCC molecule. Typically, 0.4 mL of 50 μM P450SCC in storage buffer (20 nmol) was diluted with the same volume of standard buffer [50 mM phosphate (pH 7.4), 1 mM EDTA, and 20% (v/v) glycerol] and incubated for 1 h at room temperature with 2 μL of a MSL(6)** stock (0.1 M in ethanol) (200 nmol). Buffer with cholate between 0 and 0.15% could be used, but needed to be free of DTT. Excess free, unbound label was removed by gel filtration using a PD-10 column (Pharmacia). Alternatively, P450SCC was spin-labeled after incorporation into the vesicle membrane. Labeling of AD and P45011β was performed under similar conditions; however, P45011β labeling required that it be in the membrane-bound state, i.e., after it had been incorporated into the vesicles.

Incorporation of P450 into Vesicles. The labeled enzyme was incorporated into PC/PE/CL (2/2/1, w/w/w) vesicles by octyl glucoside dialysis/adsorption of detergent/lipid/P450 mixed micelles according to a protocol described for nonlabeled P450SCC (*21*). P450SCC could not be reconstituted successfully by octyl glucoside dialysis under conditions which resulted in proper incorporation of microsomal P450 enzymes. In the presence of higher OG concentrations that were used for microsomal P450s, P450SCC showed a strong tendency to precipitate and to degrade to cytochrome P420. We found it optimal to use OG concentrations of ≤0.43% at a molar detergent/protein ratio of about 3300 and a detergent/lipid ratio of about 10. These conditions guaranteed both monomerization of the P450 and no precipitation and/or degradation. P450SCC was incorporated into vesicles by combining OG dialysis and simultaneous adsorption of OG by BioBeads SM-2 for effective detergent removal. In brief, 10 mg of lipid [standard mixture being PC/PE/CL (2/2/1, w/w/w)] was dried and subsequently solubilized by an OG standard buffer solution. This suspension was incubated with P450 for 45 min at room temperature in a final volume of 9 mL [final concentrations of 0.43% OG, 1.1 mg/mL lipid, and 4 μM P450, with a lipid/protein ratio of 5 (w/w)] followed by dialysis for 48 h at 4 °C in the presence of BioBeads. After dialysis, the suspension was gel filtrated using Sepacryl S-1000 (i) to reduce the residual OG further, (ii) to separate possible nonincorporated P450, and (iii) to fractionate the vesicles according to size. Fractions containing the larger P450SCC vesicles were collected and sedimented by ultracentrifugation at 100000*g* for 90 min. Finally, the pellet was resuspended in standard buffer and transferred to a standard flat cell for the EPR measurements. The exclusive use of larger vesicles prevented vesicle tumbling contributing to the measured mobility of P450SCC in the membrane.

Co-reconstitution of both P450 enzymes into vesicles was performed with a two-step protocol, similar to a procedure described previously (*13*). First, P45011β was incorporated into vesicles by OG dialysis/adsorption as described for P450SCC. In a second step, the P45011β-containing vesicles were incubated with P450SCC for 5 min at 25 °C. The latter method has been used previously by other workers (*22*−*24*) and recently by us for incorporation of P450SCC into different types of vesicles (*14*).

EPR and ST-EPR Measurements. EPR and ST-EPR spectra were recorded using a computer-controlled Bruker ESP 300E EPR spectrometer. Usually, 100 kHz modulation with a 1−2 G modulation amplitude, depending on the line widths, and a 5−20 mW microwave power were applied to record (conventional) EPR spectra. ST-EPR spectra were monitored as the second harmonic of absorption out-of-phase (v_2' spectra) (*25*). Phase setting was performed according to the "self null method" (*25*); the modulation amplitude was 2.8 G, and the microwave setting was adjusted to give an effective H_1 value of 0.2 G, depending on the dielectric properties of the sample. The recommendations given elsewhere (*26*, *27*) were closely followed. Sometimes, we recorded the spectra with digital phase sensitive detection using the Bruker DPSD preamplifier with 62.5 kHz modulation and 125 kHz signal detection. The sample was placed in a standard flat cell.

Following the general classification of spin-label spectra on the basis of typical line shapes reflecting certain motional states of the label, we could distinguish the spectra as being "fast motional" ($\tau_R = 10^{-11}-10^{-9}$ s), "intermediate motional" (10^{-9} to 3×10^{-9} s), "slow motional" (3×10^{-9} to 2×10^{-7} s), and "very slow motional" ($10^{-7}-10^{-3}$ s). Because of the dramatic changes in the line shapes observed in this work, it was generally unnecessary to extract exact rotational

correlation times from the EPR line shape. When τ_R values are reported, they were determined by simulation of the EPR spectra using the program of Schneider and Freed (*28*).

In the case of ST-EPR, τ_R values were determined from v_2' spectra by comparison of the spectra with reference spectra obtained from MSL(6)**-labeled hemoglobin in aqueous glycerol and sucrose solutions. For quantitation, the intensity ratio parameters L'/L, L''/L, and H''/H (*25*) and defined for ^{15}N spin-label spectra (*15*) were used. Our reference system is closely related to that described previously (*27*) apart from the use of MSL(6)**-labeled hemoglobin. This is a reliable model for isotropic rotational diffusion, and consequently, the τ_R values determined this way for membrane systems can only be used as effective correlation times and have to be interpreted carefully as discussed in detail elsewhere (*29*). However, the main conclusions drawn in this paper do not depend on the exact determination of the effective τ_R reported here.

Analytical Methods and Enzymatic Activity Assays. Determination of the concentration of spin-labels was achieved by double integration of EPR spectra and comparison with spectra of a standard spin-labeled sample at a known concentration, recorded under the same experimental conditions and instrumental settings.

The concentration of P450SCC was determined from difference spectra of the reduced P450SCC−CO complex versus reduced P450SCC using a difference extinction coefficient of 91 mM^{-1} for A_{450} minus A_{490} according to ref *30*. The concentrations of AR and AD were determined using extinction coefficients of 10.9 mM^{-1} at 450 nm (*31*) and 11 mM^{-1} at 415 nm (*32*), respectively. Absorption spectra were recorded at room temperature on a double-beam model UV2401PC spectrophotometer (Shimadzu).

Cholesterol and lipid were quantitated by liquid scintillation counting using ^{14}C-labeled cholesterol and ^{14}C-labeled DPPC, respectively.

Reconstitution of the P450SCC vesicles with NADPH, AD, and AR resulted in a catalytically active monooxygenase system as shown by side chain cleavage of cholesterol which produced pregnenolone according to the following assay. Vesicle-reconstituted P450SCC (0.25 nmol of P450, 6 mol % cholesterol, and a trace of [^{14}C]cholesterol in the membrane), 5 nmol of AD, and 0.25 nmol of AR were incubated in a volume of 0.5 mL. The reaction was started by addition of NADPH (final concentration of 0.25 mM). Tubes were incubated for 5 min at 37 °C, and then the reaction was stopped by addition of 0.1 mL of 0.5 N HCl. The residual substrate (cholesterol) and the product (pregnenolone) were extracted with 2 mL methylene chloride, and pregnenolone was separated from cholesterol by thin-layer chromatography on silica gel (0.25 mm, 20 cm × 20 cm; Merck) followed by counting the ^{14}C radioactivity of cholesterol and pregnenolone using an LB284 Linear Analyzer (Berthold).

Characterization of the activities of vesicle-reconstituted P45011β was carried out with DOC as the substrate as described in ref *33*. Briefly, vesicle-reconstituted P45011β (0.04 μM) was incubated with 4 μM AD, 0.4 μM AR, and 12 μM DOC (including a small aliquot of [^3H]DOC as a tracer) and the reaction was started by addition of NADPH for 20 min at 33 °C. Then the reaction was stopped, and the residual substrate (DOC) and the different metabolites were extracted and separated by HPLC followed by counting the

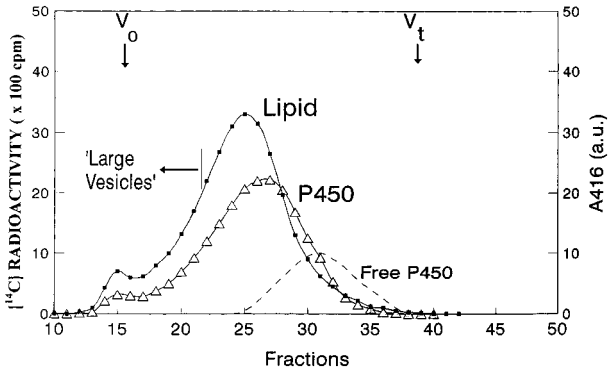

FIGURE 1: Elution profile after Sephacryl S-1000 gel chromatography of P450SCC vesicles. P450SCC-containing PC/PE/CL (2/2/1, w/w/w) vesicles [lipid/protein ratio of 10 (w/w)] were applied to the column. In a separate experiment (free), P450SCC in aqueous solution (without lipid) was applied to the same column. To monitor the lipid, a small aliquot of [^{14}C]DPPC was added to the lipid mixture; P450SCC was monitored by its heme absorption at 416 nm. The column was calibrated with phospholipid vesicles of different sizes. V_o was determined with very large aggregated vesicles and V_t with [^{14}C]glucose.

^3H radioactivity and the UV absorption at 240 nm of the substrate and metabolites. Metabolites were identified by coelution with reference steroids on the basis of their UV bands. Apart from the vesicle preparation described above, vesicles for catalytical experiments with P45011β were prepared by cholate dialysis.

RESULTS

Characterization of P450SCC-Containing Vesicles. Figure 1 shows a typical elution profile of P450SCC and vesicles after Sephacryl S-1000 gel filtration. Almost all lipid (≥90%) and 55−70% of the P450SCC elute together in two relatively broad peaks between the void volume V_o and the total volume V_t of the column. The coincidence of the fractions containing P450SCC (A416) and lipid ([^{14}C]DPPC) and the enzymatic activity measured in these fractions indicates that P450SCC is associated with the vesicles. Almost no P450SCC eluted at the position where free P450SCC in aqueous solution (without lipid) elutes. Without CL, some P450SCC did elute in this position, indicating incomplete incorporation of P450SCC.

We used Sephacryl S-1000 because it is well-suited for fractionating vesicles according to size (*34*). Thus, large vesicles (average diameter of ≥150 nm) were separated and collected for the rotational diffusion measurements. To do this, the column was calibrated in relation to particle size using phospholipid vesicles of known sizes which were determined by light scattering. In agreement with electron microscopy analyses of the size distribution, these studies showed that P450SCC vesicles consist of mainly two populations: larger ones with an average diameter of about 150 nm (about 27%) and smaller ones with a diameter of ∼60 nm (about 73%). Freeze-fracture electron microscopy of P450SCC-containing PC/PE/CL (2/2/1) vesicles showed that they were mainly spherical with very little aggregation.

Vesicles prepared this way were stable for at least several days at 4 °C under an athmosphere of N_2. During this time, no lipid degradation and little P450 denaturation could be detected; the P450 concentration only decreased about 22%. The vesicles were stable in relation to size and size

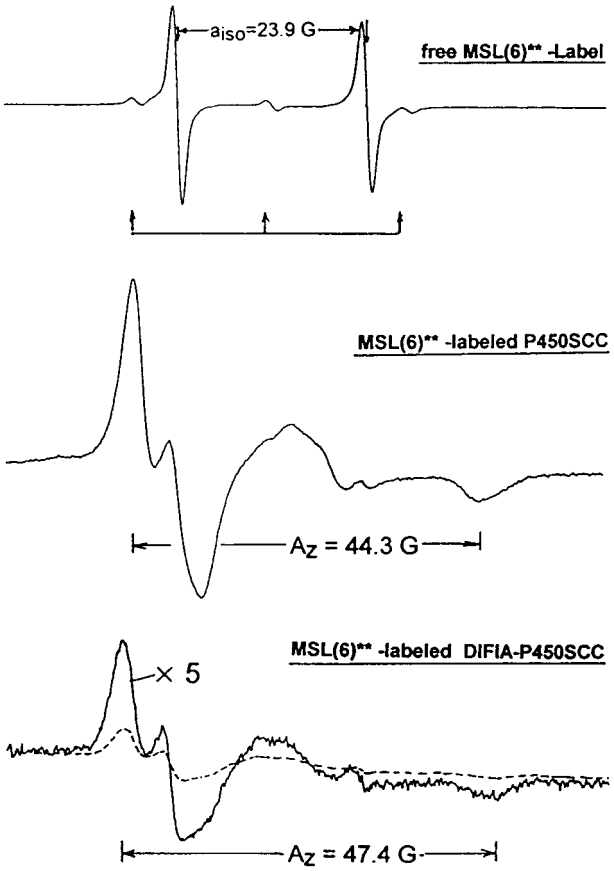

FIGURE 2: Spin-labeling of P450SCC. EPR spectra of free (unreacted) label after its separation using gel filtration (top spectrum), the bound label (spin-labeled P450SCC) (middle spectrum), and the inhibiton of labeling after modification of P450SCC with DIFIA (bottom spectrum) (dashed line, spectrum recorded under normal experimental conditions; and solid line, spectrum recorded with a 5-fold higher magnification).

homogeneity as checked by dynamic light scattering; no aggregation and/or fusion took place.

Spin-Labeling of P450SCC, AD, and P45011β. P450SCC was covalently labeled with MSL(6)** with high specificity for its single free accessible SH group, Cys264. The top spectrum of Figure 2, consisting of two sharp lines of equal height and separated by the isotropic hyperfine splitting constant a_{iso}, is the typical line shape of "very fast rotating" free, unbound labels. The three small lines represent residual ^{14}N labels in our preparation. The spectrum of MSL(6)**-labeled P450SCC is a typical one for "slow motion", characterized by differential broadening and the outward shift of the lines. The *z* component of the hyperfine tensor $\mathbf{A}_z$ is indicated (Figure 2, middle spectrum).

Prior labeling of P450SCC with diiodofluorescin iodoacetamide (DIFIA), a fluorophore known to bind selectively to Cys264 (*35*), effectively prevented labeling with MSL(6)** (Figure 2, bottom spectrum). By double integration of the spectra, the level of residual labeling after blocking Cys264 was determined to about 15%. Thus, MSL(6)** has a high specificity for labeling Cys264. The nonspecific labeling probably represents labels bound to other groups, probably lysines, and/or unspecific noncovalently bound spin-labels. The nonspecific labeling does not cause any problems in the analysis or in the interpretation of the experiments. Labeling of P450 at the observed molar ratio of 1.2 has little effect on the native structure of the enzyme as checked by heme iron EPR, CO difference spectroscopy, and cholesterol binding. However, it influences the interaction of P450SCC with AD as indicated by a 30−50% reduction in activity and a weakening in AD binding compared to those of the native enzyme. It is interesting that Cys264 is located in the so-called interdomain "hinge region" which lies along a surface-exposed loop. It is only three residues away from Lys245 which might, according to a recent model of P450SCC, participate in one of the electrostatic contacts with AD (*36*). Interestingly, labeling of P450SCC associated with vesicles inhibited catalytic activity less than labeling P450SCC prior to incorporation into vesicles for activity measurements.

The MSL(6)** label covalently links to the single free and accessible Cys95 of AD, as evidenced by the absence of any significant labeling of the AD mutant Cys95His (not shown). The AD mutant was kindly provided by R. Bernhardt (University of Saarland, Saarbruecken, Germany).

Because of the known instability of purified P45011β, it was not possible to prepare (at least not with a sufficient yield) spin-labeled P45011β in solution. After reconstitution of P45011β into vesicles, it was remarkably more stable and could be successfully labeled. The degree of labeling was determined to be about 1.1 mol of label per mole of P45011β. CO difference spectroscopy indicated that there was no change in the native structure of the enzyme. However, unlike the case for P450SCC, we do not know the position of labeling. According to an alignment of various CYP11 family sequences, bovine P45011β has no Cys corresponding to Cys264 of P450SCC, but has two others (Cys20 and Cys103) (*36*). One of these two has probably been modified and consequently must be relatively accessible to the label in the membrane-bound state.

Effect of AD and AR on the Mobility of Spin-Labeled P450SCC. Rotational diffusion of vesicle-reconstituted P450SCC was studied by ST-EPR because conventional EPR is not sensitive to the very slow motion occurring for proteins in membranes (*25*). The υ_2' spectrum of P450SCC in PC/PE/CL vesicles exhibits the typical line shape of microsecond rotational motion (Figure 3, bottom trace). From the parameters L'/L and L''/L, an average effective τ_R value of about 8.5 μs for the rotation of P450SCC in the vesicle membrane was estimated. Addition of AD to P450SCC vesicles or coreconstitution of P450SCC with AR did not lead to any significant changes in the υ_2' spectra. However, a specific effect was observed after addition of anti-AD IgG to AD-containing P450SCC vesicles.

The EPR spectra of labeled P450SCC recorded in the presence of AD and anti-AD apparently indicate immobilization of P450SCC. However, on the basis of EPR alone, it is not possible to discriminate between two possible interpretations of these observations. It might be that either (i) the observed spectra arise from one single motional P450SCC species which successively becomes more and more immobilized or (ii) the spectra are indicative of the presence of two motional species. In the latter case, the observed spectra may originate from overlapping of a component arising from mobile P450SCC ($\tau_R \sim 8.5$ μs) and another one arising from P450SCC that is almost immobilized ($\tau_R > 100$ μs). Such a coexisting equilibrium of mobile and immobile protein populations has also been observed for

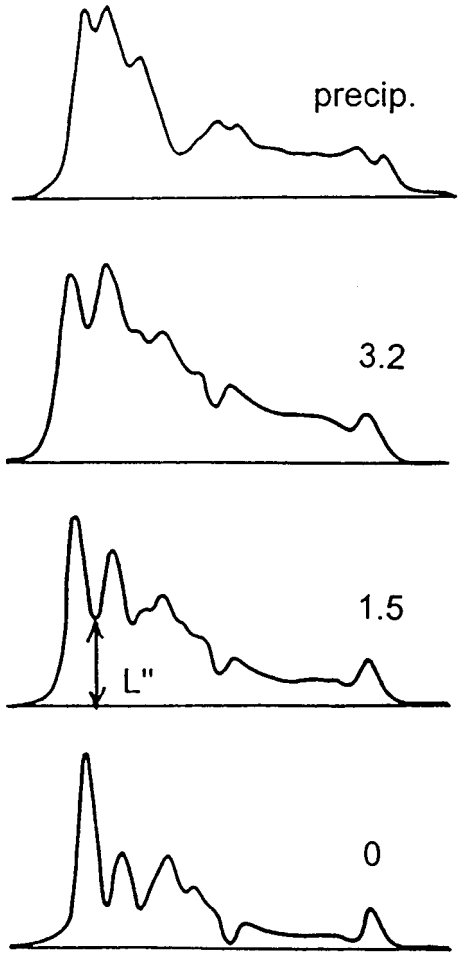

FIGURE 3: Effect of anti-adrenodoxin IgG on the rotation of spin-labeled P450SCC in PC/PE/CL (2/2/1, w/w/w) vesicles in the presence of adrenodoxin. The figure shows the υ_2' spectra of spin-labeled P450SCC; the numbers at the curves on the right indicate the molar anti-AD/AD ratio. The molar AD/P450SCC ratio was 7.5. Prior to rotational diffusion measurements, P450SCC vesicles were incubated with anti-AD IgG at the indicated excess over P450SCC for 15 min at room temperature. The top spectrum is a υ_2' spectrum taken from a precipitated sample corresponding to total immobilization of the spin-labeled P450SCC.

other membrane-reconstituted P450 systems, for instance, microsomal steroid-hydroxylating P450 enzymes (*37*). Additional justification of the following analysis of the spectra as weighted sums of mobile and immobile P450SCC can be gained from rotational diffusion experiments with DIFIA-labeled P450SCC by delayed fluorescence depolarization. These studies unequivocally prove the existence of at least two populations of P450SCC in the membrane, a mobile and an immobile one (*38*).

Initially, the spectrum of labeled P450SCC reconstituted with AD was characteristic of the mobile spin-label (f_m), but a component representative of immobilized P450SCC increased with increasing amounts of added anti-AD IgG. The spectra were analyzed as weighted sums of mobile and immobile P450:

$$L'' = (1 - f_{im})L''(\mathrm{m}) + f_{im}L''(\mathrm{im})$$

where L''(m) and L''(im) are the amplitudes of the fraction of mobile and immobile P450, respectively, each referring to the same concentration. The spectra of the mobile and immobile component were approximated by the spectrum of vesicle-reconstituted P450SCC (Figure 3, bottom spectrum) and by the spectrum of precipitated P450SCC (Figure 3, top spectrum), respectively. With this assumption, the fraction of immobilized P450SCC was estimated as a function of the anti-AD to AD molar ratio. Without antibodies, 27% of the P450SCC molecules were immobilized. The addition of a 1.5-, 3.2-, and 7-fold excess (moles per mole) of anti-AD over AD successively increased the proportion of immobile P450SCC molecules to 36, 48, and 57%, respectively. Thus, in the presence of a 7-fold excess of antibodies, only 43% of the P450SCC molecules were still rotating. These results imply that cross-linking of AD by anti-AD IgG immobilizes the P450SCC as a result of complex formation. A schematic model illustrating the effect of antibodies on P450 rotation is shown in Figure 6 for the reconstituted system involving P45011β, P450SCC, and anti-P450SCC IgG. It illustrates how anti-AD induced cross-linking of AD causes immobilization of P450SCC. Thus, the data directly demonstrate the existence of binary AD−P450SCC complexes for vesicle-associated P450SCC.

Similar experiments were performed with nonimmune IgG to rule out nonspecific effects on P450SCC rotation. The presence of an 8-fold molar excess of nonimmune IgG over AD did not affect the spectrum within experimental error, indicating no immobilization of P450SCC by nonimmune IgG, in contrast to what is observed with anti-AD IgG.

In contrast to the results with anti-AD IgG, co-reconstitution of AR with P450SCC and addition of anti-AR IgG in the presence or absence of AD did not influence the mobility of P450SCC in vesicles (not shown). These findings indicate the absence of both P450SCC−AR complexes and ternary complexes of all three proteins.

Effect of P450SCC and AR on the Mobility of Spin-Labeled AD. In another series of EPR experiments, spin-labeled AD was used as a macromolecular probe to analyze both the stoichiometry of the complex formation between P450SCC and AD and between AR and AD. Because AD is a relatively small molecule, it rotates with correlation times corresponding to "intermediate immobilization" ($\tau_R \sim 10^{-9}$ to 3×10^{-9} s) and consequently, its mobility can be monitored by conventional spin-label EPR. The corresponding spectra in the "intermediate motional region" typically consist of two lines which differentially broaden while the line positions remain constant as the motion of the label becomes progressively slower.

An EPR titration of labeled AD was performed using P450SCC vesicles. In the absence of P450SCC, the spectrum of spin-labeled AD exhibits the typical line shape for labels of "intermediate immobilization" characterized by a τ_R of ~3 ns (Figure 4, bottom spectrum, mobile AD). Successive addition of P450SCC vesicles causes the appearance of a component in the spectrum representative of "strong immobilized" spin-labels, indicating immobilization of AD by complexation with P450SCC (Figure 4, top spectrum, immobilized AD).

To quantitate the immobilization of spin-labeled AD, the amplitude of the $I(+1/2)$ EPR line in the spectrum of mobile AD was used. This amplitude successively decreased with increasing P450SCC content until finally, at equimolar stoichiometry, a spectrum typical of immobilized label was obtained, reflecting slow rotation of AD now bound to

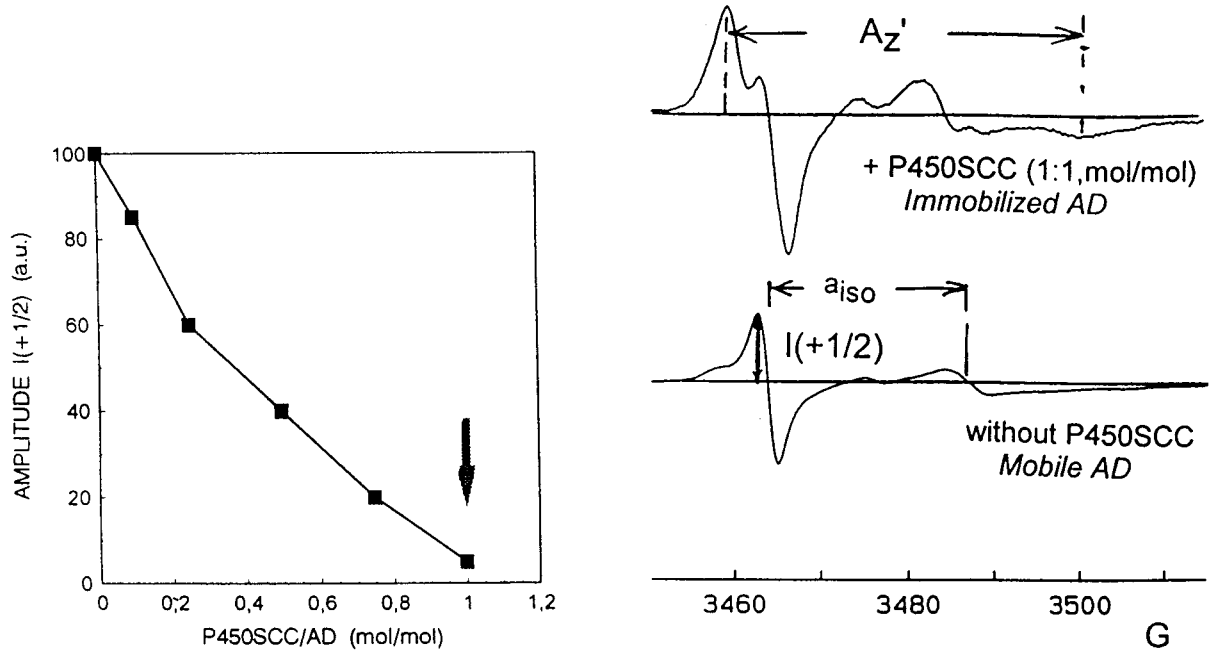

FIGURE 4: Effect of vesicle-reconstituted P450SCC on the rotation of spin-labeled adrenodoxin. (Right) EPR spectra of spin-labeled AD without P450SCC (bottom spectrum) and with an equimolar amount (moles per mole) of liposomal P450SCC (top spectrum). (Left) Decrease of the fraction of mobile AD caused by addition of P450SCC. At approximately equimolar concentrations of AD and liposomal P450SCC, nearly all the AD was immobilized.

P450SCC. Therefore, these EPR titrations demonstrated the existence of equimolar complexes between AD and membrane-bound P450SCC.

The effect of AR on the mobility of spin-labeled AD was also studied. AR was successively added to a solution of spin-labeled AD. Again, with increasing amounts of AR, the spectrum of AD changed from one typical of mobile molecules (Figure 5, upper part, mobile AD) to one of molecules with only slow rotation (Figure 5, upper part, immobilized AD). The transition was almost complete when the concentrations of AD and AR were equal. The bottom spectrum of Figure 5 was taken after addition 0.8 M KCl (final concentration) and shows that the AD−AR complex dissociates at high salt and therefore that the binding is reversible. The total concentration of spin-label was essentially constant throughout the titration experiment, indicating that the reversible interconversion between the different states occurred without any protein degradation.

Effect of P450SCC on the Mobility of Spin-Labeled P45011β. To study possible interactions of P450SCC with P45011β, the influence of P450SCC on the rotation of spin-labeled P45011β was investigated in vesicles containing both P450 enzymes, P450SCC and P45011β. To monitor the mobility of P45011β, ST-EPR was used because conventional EPR is not sensitive to the very slow motion of membrane-reconstituted P45011β. A specific complexation could be evidenced by a combination of rotational diffusion and antibody cross-linking. The strategy was the same as in the experiments described above which showed complexation of AD and P450SCC. Spin-labeling of P45011β was successful only after incorporation of the enzyme into the membrane (see Materials and Methods). In the vesicle-reconstituted state, the P45011β stability was comparable to that of P450SCC.

The υ_2' spectrum of spin-labeled P45011β in PC/PE/CL vesicles exhibits the typical line shape of microsecond

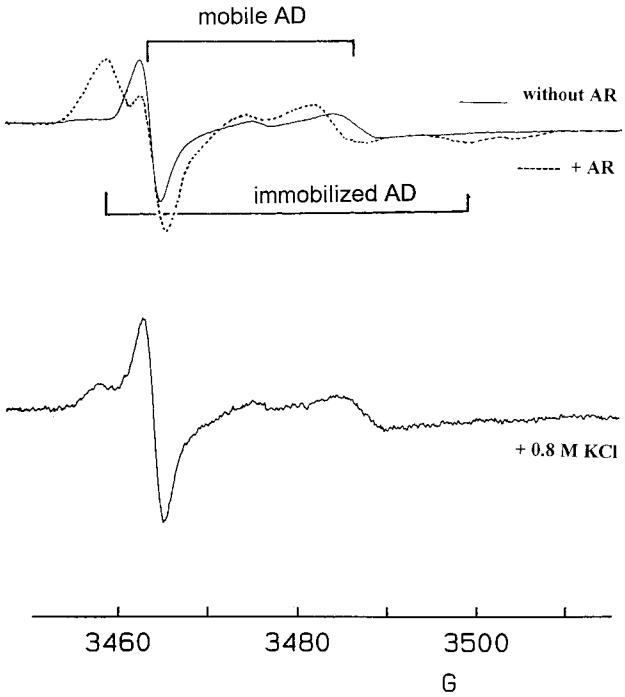

FIGURE 5: Effect of adrenodoxin reductase on the rotation of spin-labeled adrenodoxin. (Top) EPR spectra of spin-labeled AD without AR (solid line) and in the presence of AR (AR/AD ratio of 1.1, moles per mole) (dashed line) in buffer solution. (Bottom) Spectrum after addition of 0.8 M KCl (final concentration). The total concentration of spin-label in all three samples, measured by double integration of the spectra taking into account the different amplification used in recording the spectra, was constant.

rotational mobility characterized by an effective average rotational correlation time of ~3 μs (Figure 6, bottom spectrum). As can be seen from Figure 6 (middle spectrum), the mobility of spin-labeled P45011β co-reconstituted with an equimolar amount of P450SCC was similar to that of the spin-labeled P45011β alone. However, after addition of a

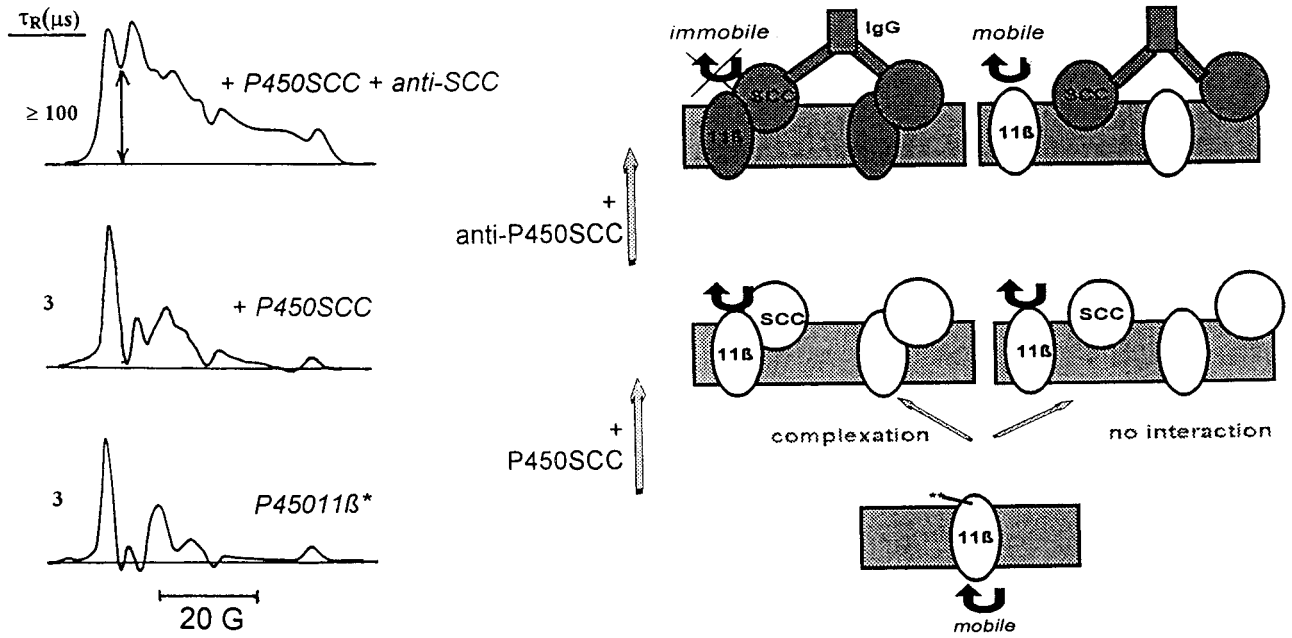

FIGURE 6: Effect of P450SCC and anti-P450SCC on the rotation of spin-labeled P45011β in PC/PE/CL vesicles. (Left) v_2' spectra of spin-labeled P45011β in PC/PE/CL vesicles (bottom trace), co-reconstituted with P450SCC (P45011β/P450SCC ratio of 1, moles per mole) (middle trace), and co-reconstituted with P450SCC in the presence of anti-P450SCC IgG (top trace). Prior to the measurements, the P45011β- and P450SCC-containing vesicles were incubated with an excess of anti-P450SCC IgG (anti-P450SCC/P450SCC ratio of 10, moles per mole) for 15 min at room temperature. The numbers on the left of the spectra represent effective average correlation times. (Right) Schematic representation of the immobilization of P45011β assuming complex formation between P45011β with antibody-cross-linked P450SCC.

10-fold excess of anti-P450SCC IgG over P450SCC, we detected a remarkable immobilization of P45011β (Figure 6, top spectrum). The line shape of the v_2' spectrum of P45011β in the presence of P450SCC and antibodies to P450SCC is characteristic of a very strongly immobilized enzyme, very similar to that of precipitated P450SCC (see Figure 3, top spectrum). These findings indicate an immobilization of P45011β caused by its binding to the P450SCC−antibody complex. The results therefore demonstrate for the first time formation of a complex between P450SCC and P45011β in the vesicle membrane.

To analyze how the complexation between P45011β and P450SCC influences the specificity of P45011β-catalyzed reactions, these structural studies were correlated with functional analysis. Catalytical characterization of vesicles containing both enzymes, P45SCC and P45011β, was carried out with DOC as the substrate. Table 1 shows the various activities of P45011β, reconstituted alone into PC/PE/CL vesicles and co-reconstituted with an equimolar amount of P450SCC. Clearly, the distribution of the different activities of P45011β is significantly influenced by the presence of P450SCC. The effect of P450SCC is a significant increase in the 11β-hydroxylating activity of P45011β paralleled by a significant reduction of both the 18-hydroxylation and aldosterone synthase activities.

Table 1: Effect of P450SCC on the Various Activities of Vesicle-Reconstituted P45011β in the Deoxycorticosterone (DOC) Metabolism[a]

metabolite	P45011β vesicles (nmol min^{-1} nmol^{-1})[c]	(P450SCC + P45011β) vesicles (nmol min^{-1} nmol^{-1})[c]
corticosterone (11β-OH-DOC)	25.4	37.1
A1[b]	2.0	nd
aldosterone	4.0	2.3
18-OH-B1[b]	8.3	10.1
18-OH-corticosterone	16.2	6.5

[a] The DOC metabolism was measured in P45011β vesicles and in (P450SCC + P45011β) vesicles as described in Materials and Methods. P450SCC/P45011β ratio = 1 mol/mol; lipid/P45011β ratio = 1000 mol/mol. [b] The metabolite 18-OH-B1 and the minor product A1 could not be identified; A1 was not detected (nd) in P45011β vesicles containing P450SCC. [c] Data represent an average of three chromatographic runs.

DISCUSSION

The successive immobilization of vesicle-reconstituted P450SCC by the addition of increasing amounts of antibodies to AD observed in the presence of AD unequivocally demonstrates the existence of binary AD−P450SCC complexes for vesicle-associated P450SCC. The combination of rotational diffusion and antibody cross-linking has been already previously demonstrated to be an efficient method for analysis of protein complex formation in membrane-bound P450 systems (*39*). Our result, that not all P450SCC molecules in the membrane were immobilized by anti-AD, is probably due to a nonhomogeneous distribution of P450SCC and AD in the vesicles and/or an insufficient concentration of antibody. Using antibodies to AR, by the same strategy we could directly prove the absence of both P450SCC−AR complexes and ternary complexes of all three proteins. However, the stoichiometry of the P450SCC−AD complex cannot be determined via experiments based on ST-EPR and antibody cross-linking. Therefore, we used spin-labeled AD as a macromolecular probe and conventional EPR to reveal the existence of both (i) equimolar complexes between AD and P450SCC and (ii) equimolar complexes between AD and AR. It should be noted that these complexes might not consist of just an AD−P450SCC dimer or an AD−

AR dimer, respectively. There could be more than one AD and an equal number of P450SCCs in the complex. On the other hand, a 1/1 complex between AD and P450SCC has been proposed previously on the basis of kinetic measurements (*1*). However, complexes in which two or more AD molecules exist as proposed previously on the basis of activity titration studies (*10*, *11*) cannot explain our findings. The EPR titration experiment clearly shows that the complexes consist of an equimolar amount of P450SCC and AD. Otherwise, all AD molecules should be already immobilized at a molar P450SCC/AD ratio of remarkably less than 1, in contrast to what is observed in the actual titration experiment.

With respect to the complexation between AD and AR, the observed dissociation of the complex in the presence of high salt also demonstrates that the interaction between AD and AR is mainly electrostatic in nature, as shown previously for the interaction between human AD and bovine AR using site-directed mutagenesis (*6*).

Taken together, the results derived from our studies therefore support the "shuttle mechanism" of electron transfer from AR to P450SCC, with AD acting as the mobile electron shuttle between the two. The result is in contrast to reports from Kawato's group who found evidence for transient ternary associates of cholesterol-bearing P450SCC with its two redox partners based on mobility studies of P450 in mitochondria and submitochondrial particles (*9*). Naturally, it is not possible to directly compare the complex situation in the mitochondrial membrane with the defined structure and composition of the vesicle system used by us. Nevertheless, the following points should be considered with respect to the results reported in ref *9*. (i) On the basis of the type of measurements (decay of absorption anisotropy after photolysis of the heme−CO complex), the method is limited to analysis of the reduced states of the heme protein. (ii) The use of natural membranes prohibited unequivocal discrimination between the contribution of P450SCC and P45011β to the decay. (iii) The protein−protein interaction may be different in the presence of cholesterol which might be indicative for a substrate-controlled interaction between the electron-transfer components (as discussed by the authors). (iv) It must be considered that the high sucrose and/or glycerol content of the samples used (up to 80%) to suppress tumbling of the mitochondrial particles may influence the rotational mobility of P450, as was demonstrated previously by us (*40*).

The results obtained from studying the mobility of spin-labeled P45011β co-reconstituted with P450SCC into PC/PE/CL vesicles demonstrate for the first time formation of a complex between P450SCC and P45011β in the vesicle membrane. Moreover, by correlating the motional analysis with enzymatic analysis, we showed that the distribution of the different activities of P45011β is significantly influenced by the presence of P450SCC. The main effect of P450SCC is an increase in the 11β-hydroxylating activity of P45011β paralleled by a significant reduction of both the 18-hydroxylation and aldosterone synthase activities. Therefore, the results demonstrate that complex formation with P450SCC leads to a system capable of modulating the various catalytical activities of P45011β in vesicle membranes. It is straightford to speculate about the importance of this finding of association between P45011β and P450SCC with regard to the regulation of the different activities of P45011β in the mitochondrial membrane. In this respect, we refer to the previously published papers from Takemori's group. They first reported that addition of P450SCC to P45011β vesicles enhanced 11β-hydroxylation activity but suppressed aldosterone synthesizing activity (*13*). On the basis of kinetic analysis, an equimolar complex between P450SCC and P45011β in the membrane was suggested. According to their hypothesis, the various activities of bovine P45011β in the different zones of adrenal cortex may result from differences in the interaction between the two P450 types in the membrane (*13*). The aldosterone synthase activity of P45011β in the zona fasciculata-reticularis of bovine adrenals is suppressed by interaction with P450SCC which might be due to a weaker binding of corticosterone to P45011β in the presence of P450SCC (*41*). Our studies directly demonstrate the existence of such complexes by direct detection of the complexes in the vesicle membrane. It should be mentioned that the interaction between both P450s may be considerably more extensive, and probably more complex, in intact mitochondria. In this regard, the studies highlight the potential usefulness of our vesicle system for future physicochemical and biochemical studies of the P450SCC−P45011β interaction, including the competition of both P450 enzymes for electron transfer and the role of P450SCC, lipid, and substrate in the regulation of the different activities of P45011β in the mitochondrial membrane.

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