Membrane Topology of Bovine Adrenocortical Cytochrome P-450$_{C21}$: Structural Studies by Trypsin Digestion in Vesicle Membranes

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ABSTRACT: Purified adrenocortical microsomal P-450$_{C21}$ was incorporated into vesicle membranes composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at a molar ratio of 5:3:1. Trypsinolysis of the incorporated P-450$_{C21}$ resulted in the formation of 30-, 25-, and 20-kDa fragments. Similar fragment formation was observed by trypsinolysis of bovine adrenocortical microsomes with Western blotting using anti-P-450$_{C21}$ IgG. In the detergent-solubilized state, trypsin cleaved P-450$_{C21}$ into very small peptides. Washing of the trypsin-treated vesicles with 500 mM Na_2CO_3 failed to cause these fragments to separate from membranes. N-Terminal amino acid sequencing of these fragments showed that trypsin cleaved the 267Arg–268Val and 332Arg–333Val bonds of P-450$_{C21}$. The time course of fragment formation indicated that trypsin cleaved the 267Arg–268Val bond first to produce 30- and 25-kDa fragments and subsequently the 332Arg–333Val bond in the 25-kDa fragment to produce the 20-kDa fragment. Neither 21-hydroxylase activity, the reduced CO difference spectrum, nor the EPR spectrum of digested P-450$_{C21}$ differed from those of undigested P-450$_{C21}$. Heat treatment at 50 °C for 20 min did not cause any decrease in activity of digested P-450$_{C21}$, when the substrate progesterone was present. This high stability toward heat treatment was not observed in the solubilized state. Rotational diffusion experiments on P-450$_{C21}$ showed that the size of the molecule holding the heme was not changed significantly after digestion. On the basis of these results, P-450$_{C21}$ is concluded to be deeply embedded in the vesicle membranes.

Adrenocortical steroid hormones are synthesized from cholesterol by the actions of several cytochrome P-450s and 3β-hydroxysteroid dehydrogenase Δ^4,Δ^5-isomerase (Takemori & Kominami, 1984). P-450$_{C21}$[1] in the endoplasmic reticulum hydroxylates progesterone and 17α-hydroxyprogesterone at the 21-position (Kominami et al., 1980). These steroids are very hydrophobic and concentrated in biological membranes and may possibly be metabolized in the membrane (Flynn, 1971; Ahmad & Mellors, 1978; Arowsmith & Hadgraft, 1983; Tomida et al., 1978). The characteristics of membrane-bound P-450$_{C21}$ have been studied using a system in which purified P-450$_{C21}$ is incorporated in phospholipid vesicle membranes. Some kinetic studies indicate that P-450$_{C21}$ in vesicle membranes metabolizes substrates concentrated in membranes (Kominami et al., 1986). In a reconstituted electron-transfer system in vesicle membranes composed of P-450$_{C21}$ and NADPH–cytochrome P-450 reductase, electron transfer has been shown to occur through their random collision in membranes (Kominami et al., 1989). A very recent study with rotational diffusion measurements suggested that P-450$_{C21}$ was deeply embedded in vesicle membranes (Ohta et al., 1992).

Two models have been proposed for the topology of cytochrome P-450 in membranes. Tarr et al. (1983) and Ozols et al. (1985) proposed that the major part of a hepatic microsomal cytochrome P-450 was embedded in membranes, based on the hydropathy profile of the primary structure. Nelson and Strobel (1988) showed significant homology in the amino acid sequences of various vertebrate cytochrome P-450s and P-450$_{cam}$. On the basis of the crystallographic data of the P-450$_{cam}$ structure, they proposed that membrane-bound cytochrome P-450 was attached to membranes only by the N-terminal region as in the case of NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase (Gum & Strobel, 1979; Ozols et al., 1984). The N-terminal anchor model has been supported for cytochrome P-450 by trypsin digestion experiments (Vergeres et al., 1989; Brown & Black, 1989), as well as the location of epitopes for reactive antibodies (De-Lemos-Chiarandini et al., 1987), deletions of DNA sequences around the N-terminal signal sequence (Sakaguchi et al., 1987), and fluorescence energy transfer (Centeno & Gutierrez-Merino, 1992). Several contradicting experimental results have been presented. The importance of hydrophobic amino acids other than the N-terminal region has been reported for P-450$_{C21}$ and P-450$_{17\alpha,lyase}$ (Chiou et al., 1990; Yanase et al., 1989). A genetically expressed cytochrome P-450 not possessing the N-terminal hydrophobic peptide has been found to bind to membranes (Yabusaki et al., 1988; Clark & Waterman, 1991). A shortened P-450 2E1 lacking amino acids 3–21 was reported to bind to *Escherichia coli* membranes and to retain the catalytic activity (Larson et al., 1991; Pernecky et al., 1993). Mitochondrial P-450$_{SCC}$ does not have an N-terminal hydrophobic segment but is an integral membrane protein, for which several regions have been proposed to be responsible for the membrane attachment (Vijayakumar & Salerno, 1992).

In this study, the topology of membrane-bound P-450$_{C21}$ was examined in detail by analysis of tryptic fragments. Trypsin cleaved membrane-bound P-450$_{C21}$ at two sites, but this did not affect significantly the enzymatic and physicochemical

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[1] Abbreviations: P-450$_{C21}$, cytochrome P-450 having steroid 21-hydroxylase activity (P-450 21A1); P-450$_{17\alpha,lyase}$, cytochrome P-450 having steroid 17α-hydroxylase and C17,C20-lyase activities (P-450 17A1); P-450$_{SCC}$, cytochrome P-450 having cholesterol desmorase activity (P-450 11A1); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EPR, electron paramagnetic resonance.

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properties as long as the cleaved protein continued to remain in membranes.

EXPERIMENTAL PROCEDURES

Preparation of Proteoliposomes. P-450$_{C21}$ and NADPH–cytochrome P-450 reductase were purified from bovine adrenal and hepatic microsomes, respectively, as previously described (Kominani et al., 1986; Takemori & Kominami, 1982). Unilamellar vesicles containing P-450$_{C21}$ were prepared by cholate dialysis using phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at a molar ratio of 5:3:1 (Kominami et al., 1988). The concentration of incorporated P-450$_{C21}$ was estimated from the dithionite-reduced CO difference spectrum using $\Delta\epsilon$(450–490 nm) = 91 mM^{-1} cm^{-1} (Omura & Sato, 1964). The vesicles used in this experiment contained P-450$_{C21}$ and phospholipids at a molar ratio of about 1:2000. For measurements of 21-hydroxylase activity following trypsin digestion or heat treatment, the reductase was incorporated by incubation at 0 °C for 1 h with the treated P-450$_{C21}$ vesicles at a molar ratio of 1:2 to P-450$_{C21}$ (Kominami et al., 1987). Bovine adrenocortical microsomes were prepared by differential centrifugations (Kominami et al., 1980).

Proteolysis and SDS–PAGE. Proteolysis of P-450$_{C21}$ in vesicle membranes or in its solubilized form was carried out at 37 °C with trypsin at a weight ratio of 1:25 to P-450$_{C21}$ in 50 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl. The solubilization of vesicles was conducted using 1% (w/v) sodium cholate. Tryptic digestion was also performed on the microsomes under the same conditions. Digestion was terminated by adding phenylmethanesulfonyl fluoride to a final concentration of 5 mM. For samples of SDS–PAGE, digestion was terminated by heating at 90 °C for 5 min in the presence of 5% SDS, 5% glyerol, and 2.5% mercaptoethanol.

SDS–PAGE was performed using Tricine buffer according to the method of Schaegger and Von Jagow (1987). The concentration of polyacrylamide in the separating (6 cm), spacer (5 cm), and stacking (3 cm) gels was 16.4%, 10%, and 4%, respectively. Electrophoresis was performed at 30 V for 2 h and subsequently at 80 V for 7 h with a Biocraft BE110 device. Western blotting of fragments from incorporated P-450$_{C21}$ and adrenal microsomes was carried out as previously described (Shinzawa et al., 1988). The fragments separated by SDS–PAGE were transferred to a nitrocellulose sheet (BA5-85, Schleicher & Schuell, Inc., Dassel, Germany) at 10 V for 30 min with a semidry blotting device (Biocraft Co., Tokyo). After the reactive sites were blocked with 3% (w/v) bovine serum albumin (Nacalai Tesque, Inc., Kyoto), the sheet was soaked in a solution of anti-P-450$_{C21}$ IgG prepared from rabbit sera (Kominami et al., 1983). The fragments bound to the IgG were stained using horseradish peroxidase-labeled anti-rabbit sera IgG, 4-chloro-1-naphthol (Nacalai Tesque, Inc.), and H_2O_2.

Analysis of Tryptic Fragments. Separated fragments in the SDS–PAGE gel were electroeluted. The area of the gel containing a fragment was carefully scraped out with a razor, cut into small pieces, and suspended in 0.125 M Tris-HCl buffer (pH 6.8) containing 0.1% SDS. A glass tube (0.5 × 5 cm) was connected to a visking tube (0.5 × 5 cm) and loosely sealed with glass wool just above the point of connection. The visking tube was knotted on the lower side and dipped in 0.1 M Tris-HCl buffer (pH 8.3) containing 0.1% SDS. A suspension of the gel was placed on glass wool, and 150 V was applied for 3 h. The glass wool prevented the falling of small pieces of suspended gel into the visking tube. The solution in the visking tube was dialyzed extensively against pure water. The fragments were precipitated in 90% (v/v) acetone and collected by centrifugation (24000*g*, 20 min). N-Terminal amino acid sequence of fragments was determined by a gas-phase protein sequencer (ABI 477/120A; Applied Biosystem, Inc., Forster City, CA).

Rotational Diffusion Measurements of Liposomal P-450$_{C21}$. Rotational diffusion measurements were performed at 20 °C. 58% (w/w) sucrose was dissolved so as to reduce light scattering and vesicle tumbling. The absorbance change due to photolysis of the cytochrome P-450–CO complex by a vertically polarized flash was measured at 450 nm. Signals were analyzed by calculating absorption anisotropy, $r(t)$, in the equation:

$$r(t) = [A_V(t) - A_H(t)]/[A_V(t) + 2A_H(t)] \quad (1)$$

where $A_V(t)$ and $A_H(t)$ are absorption changes for vertical and horizontal polarization, respectively, at time t after a laser flash at 532 nm. In each experiment, 16 384 signals were averaged using a Toyo Technica 2805 transient memory. $r(t)$ was analyzed on the basis of a model of rotation about the membrane normal with the equation:

$$r(t) = r_1 \exp(-t/\phi) + r_2 \exp(-4t/\phi) + r_3 \quad (2)$$

where ϕ is the average rotational relaxation time and r_1, r_2, and r_3 are constants (Kawato et al., 1981; Kawato & Kinoshita, 1981; Ohta et al., 1992). No denaturation of P-450$_{C21}$ during the measurement was observed.

Other Methods. The 21-hydroxylase activity of P-450$_{C21}$ vesicles was measured 37 °C in 100 mM potassium phosphate buffer (pH 7.3) containing 0.1 mM EDTA and 40 μM progesterone as substrate (Kominami et al., 1989). Optical absorption spectra were measured at 25 °C with a Beckmann UV-7 spectrophotometer. EPR spectra were measured at 77 K with 1 mW of microwave power and 100-kHz field modulation frequency using an X-band ESR spectrometer (JES-FE2, JEOL Inc., Tokyo). The relative amount of each fragment on the SDS–PAGE gel was determined using a densitometer with 565-nm light (DMU-33C, Advantec Toyo Inc., Tokyo) after staining with Coomassie brilliant blue (R250; Nacalai Tesque). The protein concentration was determined using a BCA protein assay kit from Pierce Chemical Co. (Rockford, IL).

Materials. L-α-Phosphatidylserine from bovine spinal cord and L-α-phosphatidylethanolamine from egg yolk were purchased from Lipid Products (Surrey, U.K.), and L-α-phosphatidylcholine from egg yolk, trypsin from bovine pancreas (type III), and sodium cholate were from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of the highest grade commercially available.

RESULTS

Characterization of P-450$_{C21}$ Proteoliposomes. The incorporation of P-450$_{C21}$ in vesicle membranes was confirmed by density gradient centrifugation using 0–10% (w/v) Ficoll (Kominami et al., 1989). Electron microscopic observations indicated that the prepared vesicles were unilamellar of about 50 nm in average diameter. More than 90% of the incorporated P-450$_{C21}$ was reduced by the external addition of NADPH–cytochrome P-450 reductase and NADPH, showing most of the P-450$_{C21}$ molecules to possess an electron-accepting site on the external side of the vesicles. The stability of P-450$_{C21}$ in vesicle membranes and in the solubilized state was assessed by measuring residual hydroxylase activity after heat treatment at 50 °C (Figure 1). The hydroxylase activity of P-450$_{C21}$ in vesicle membranes was 14 ± 2 nmol of product formed (nmol of P-450$_{C21}$)$^{-1}$ min^{-1} and showed no decrease by heat treatment

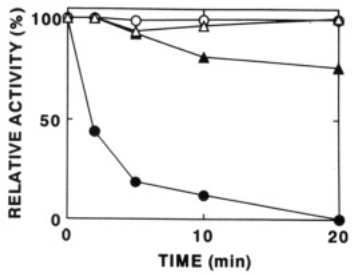

FIGURE 1: Stability of P-450$_{C21}$ in vesicle membranes during heat treatment at 50 °C. The residual 21-hydroxylase activity of P-450$_{C21}$ in vesicle membranes was plotted against the time of heat treatment at 50 °C. 100% activity indicates activity before trypsin digestion and heat treatment. The triangles show activity without trypsin digestion and the circles activity after trypsin digestion. The open symbols show activity after heat treatment in the presence of 5 μM substrate progesterone and the closed symbols activity without substrate. Proteolysis of P-450$_{C21}$ in the vesicle membranes was performed at 37 °C for 2 h in the presence of trypsin at a weight ratio of 1:25 to P-450$_{C21}$. To reconstitute the electron-transfer system, NADPH–cytochrome P-450 reductase was incorporated into P-450$_{C21}$ vesicles after heat treatment. The details of assay of 21-hydroxylase activity are presented under Experimental Procedures.

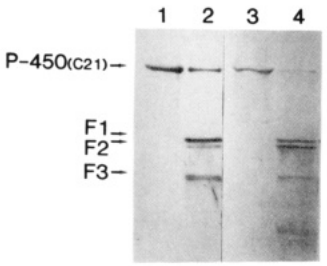

FIGURE 2: Western blotting of P-450$_{C21}$ and fragments before and after trypsin digestion. Lane 2 shows fragments produced by trypsin digestion on P-450$_{C21}$ in vesicle membranes. Lane 4 shows fragments from P-450$_{C21}$ in microsome membranes. Lanes 1 and 3 are undigested P-450$_{C21}$ in vesicle and microsome membranes, respectively. The conditions for trypsin digestion are the same as in Figure 1. The details of SDS–PAGE and blotting are given under Experimental Procedures. F1, F2, and F3 show the fragments from P-450$_{C21}$ whose molecular masses were estimated as 30, 25, and 20 kDa, respectively, from mobility in SDS–PAGE.

at 50 °C for 20 min, provided substrate progesterone was present. Vesicle-bound P-450$_{C21}$ in the substrate-free form showed a slight decrase (about 20%) in activity by the same treatment. P-450$_{C21}$ in the detergent-solubilized state lost activity completely during heat treatment for less than 2 min, in both the substrate-bound and substrate-free forms. Instability in the detergent-solubilized form was not unique to cholate and was also observed with a nonionic detergent, Emulgen 913.

Trypsinolysis of P-450$_{C21}$ in Vesicle and Microsomal Membranes. P-450$_{C21}$ in vesicle membranes was digested at 37 °C for 2 h with trypsin at a weight ratio of 1:25 to P-450$_{C21}$. SDS–PAGE of the digested P-450$_{C21}$ showed four fragments, with molecular masses of about 30, 25, 20, and 6 kDa. The solubilized sample showed no bands in SDS–PAGE after trypsinolysis, indicating that P-450$_{C21}$ in the solubilized form was cleaved into very small peptides (Ohta et al., 1992). Trypsin digestion was also conducted on bovine adrenal microsomes. Figure 2 shows the results of Western blotting of digested P-450$_{C21}$ in vesicle and microsomal membranes using anti-P-450$_{C21}$ IgG. The same pattern was detected on the blotting, showing that trypsin cleaved the same sites of protein in both membranes.

It is clear that membranes protect vesicle-bound P-450$_{C21}$ from trypsin digestion. P-450$_{C21}$ may possibly be buried deeply

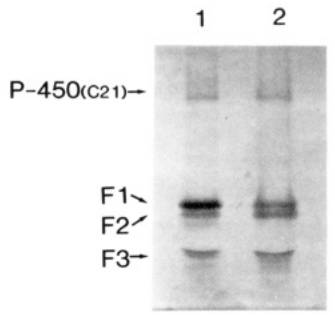

FIGURE 3: Membrane-bound fragments produced by trypsin digestion on P-450$_{C21}$. Lane 2 shows the pattern of SDS–PAGE of membrane fragments from P-450$_{C21}$ in vesicle membranes obtained by centrifugation (100000g, 3 h) after trypsin digestion and subsequent treatment with 500 mM Na_2CO_3 at 25 °C for 1 h. The conditions of trypsin digestion are the same as in Figure 1. The details of salt treatment are given in the text. The control sample in lane 1 was obtained in the same way as that of lane 2 but without Na_2CO_3.

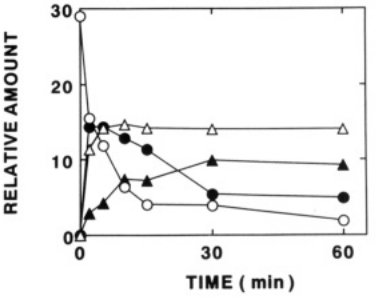

FIGURE 4: Formation of tryptic fragments from P-450$_{C21}$ in vesicle membranes. Trypsin digestion was continued until the time indicated on the vertical axis, and the digested P-450$_{C21}$ in the vesicle membranes was electrophoresed. The SDS–PAGE gel was stained with Coomassie brilliant blue. The optical density of the bands was monitored with a densitometer. Relative amounts of the uncleaved molecule and fragments from P-450$_{C21}$ were estimated from the peak areas in densitometry divided by molecular weights. The open circles, open triangles, closed circles, and closed triangles represent relative amounts of uncleaved P-450$_{C21}$, 30-kDa (F1), 25-kDa (F2), and 20-kDa (F3) fragments, respectively. The conditions for trypsin digestion are the same as in Figure 1.

in membranes. For confirmation of this possibility, the membrane fraction of digested P-450$_{C21}$ was suspended in 500 mM Na_2CO_3. After the suspension had been left at room temperature for 1 h, the membrane fraction was collected by centrifugation (10000g, 3 h). In SDS–PAGE, the membrane fraction showed the same pattern as that without salt treatment (Figure 3). Fragments obtained after digestion are not simply adsorbed on membranes but are deeply embedded in vesicle membranes.

Time Course of Fragment Formation. Figure 4 shows the time course of fragment formation during trypsinolysis. The density of the original protein band on SDS–PAGE decreased with time. Within 2 min, bands of F1 (30 kDa) and F2 (25 kDa) appeared. The density of the F2 band gradually decreased with a simultaneous increase in the F3 (20 kDa) band after 5 min. The relative amount of each fragment was estimated from the density of each band divided by its molecular weight (Figure 4). It is apparent that the increase in the F3 band is correlated with the decrease in the F2 band. The F3 fragment might be formed from the F2 fragment. The presence of the substrate progesterone during proteolysis had no significant effect on fragment formation. N-Terminal amino acid sequences of fragments, collected by electroelution from the SDS–PAGE gel, were determined with a gas-phase amino acid sequencer. N-Terminal sequences are depicted in Figure 5 with numbers corresponding to the position in the

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                1 . . . 5 . . . . 10 .
F1(30kDa)  MVLAGLLLLLT···

               268 . 270 . . . . 275 . . . . 280 . . . . 285
F2(25kDa)  VEEGPGQLLEGHVHMSVV···

               333 . 335 . . . . 340 . 342
F3(20kDa)  VTYKDRARLP···
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FIGURE 5: N-Terminal amino acid sequences of fragments produced by trypsin digestion on P-450$_{C21}$ in vesicle membranes. Fragments produced by trypsin digestion were separated by SDS–PAGE, collected by electroelution, and subjected to N-terminal analysis. The number above each amino acid shows its position in the primary structure of P-450$_{C21}$.

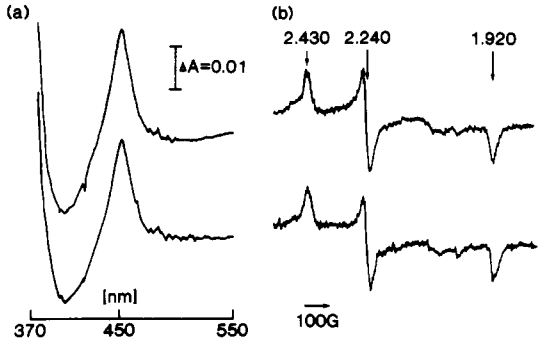

FIGURE 6: Dithionite-reduced CO difference spectra (a) and EPR spectra (b) of P-450$_{C21}$ in vesicle membranes before and after trypsin digestion. The upper spectra represent those before trypsin digestion, and the lower spectra, after digestion. Optical spectra were measaured at 25 °C, and EPR spectra were measured at 77 K. The numbers above the arrows of the EPR signals indicate *g* values. The conditions of trypsin digestion are the same as in Figure 1. The details of the measurements are indicated under Experimental Procedures.

primary structure of P-450$_{C21}$ (Yoshioka et al., 1986). If trypsin cleaves the 267Arg–268Val bond of P-450$_{C21}$, two fragments should have molecular masses of 29 and 25 kDa. From molecular weights and N-terminal sequences, the F1 and F2 fragments could be identified as the 1–267 and 268–496 fragments of original P-450$_{C21}$. The F3 fragment corresponds to the 333–496 fragment, appparently formed by cleavage of the F2 fragment.

Stability of Digested P-450$_{C21}$ in Vesicle Membranes. It was striking that vesicle-bound P-450$_{C21}$ showed no decrease in hydroxylase activity after digestion at 37 °C for 2 h with trypsin at a weight ratio of 1:25 to P-450$_{C21}$, although digested P-450$_{C21}$ showed not the original band but the F1, F2, and F3 bands on SDS–PAGE. The stability of vesicle-bound P-450$_{C21}$ toward thermal denaturation at 50 °C was assessed after trypsinolysis (Figure 1). In the presence of substrate progesterone, heat treatment at 50 °C for 20 min caused no observable change in the activity of digested P-450$_{C21}$ in membranes. Without progesterone, heat treatment for 2 min decreased the activity to 50% of the original. The activity of P-450$_{C21}$, solubilized after digestion, disappeared completely during heat treatment for 2 min even in the presence of progesterone. These results showed that trypsin digestion brings about no drastic change around the active site of vesicle-bound P-450$_{C21}$ and its conformation is quite stable against thermal perturbation even after digestion. Vesicle membranes protected P-450$_{C21}$ from thermal denaturation even after digestion, and substrate progesterone also exerted a protective effect.

Spectroscopic Properties of Digested Liposomal P-450$_{C21}$. The dithionite-reduced CO difference spectrum of vesicle-bound P-450$_{C21}$ is shown in Figure 6(a) before and after trypsinolysis. The optical difference spectra of digested and undigested P-450$_{C21}$ were the same. The EPR spectrum of vesicle-bound P-450$_{C21}$ was measured at 77 K [Figure 6(b)]. Typical low-spin signals of cytochrome P-450 have been

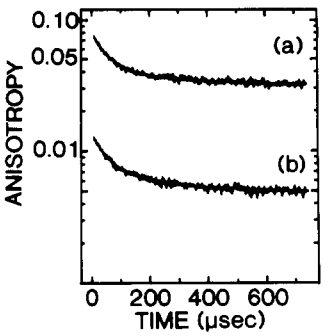

FIGURE 7: Time-dependent absorption anisotropy of P-450$_{C21}$ in vesicle membranes before and after trypsin digestion. Samples (2–3 μM in heme) were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm as described under Experimental Procedures. Measurements were performed in 58% sucrose and 11% glycerol solution at 20 °C. Curve (b), P-450$_{C21}$ in vesicle membranes after digestion with trypsin. Curve (a), control P-450$_{C21}$ obtained by the same treatment as for curve (b) but without trypsin. The initial anisotropy of curve (b) is vertically displaced for illustrative purposes; otherwise, it is almost completely superimposed on curve (a). The conditions of trypsin digestion are the same as in Figure 1.

detected (Peisack et al., 1971). No detectable difference in the EPR spectra before and after digestion could be found. These spectroscopic studies show that cleavage of the 267Arg–268Val and 332Arg–333Val bonds causes little detectable change about the heme.

Rotational Mobility of Digested P-450$_{C21}$ in Vesicle Membranes. Rotational diffusion measurements were performed at 20 °C (Figure 7). In all samples, the absorption anisotropy $r(t)$ decayed within 1 ms to a time-independent value r_3. The $r(t)$ curves were analyzed by eq 2 based on rotation of P-450$_{C21}$ about the axis perpendicular to the membrane plane. The rotational relaxation time of digested P-450$_{C21}$ [curve (b)] was about 180 μs, which was not significantly different from that of undigested sample [curve (a)]. This indicates that the molecular size of digested P-450$_{C21}$ in membranes is basically the same as undigested P-450$_{C21}$.

The effect of high ionic concentration on the rotational anisotropy decay curves was examined. If the fragments in digested P-450$_{C21}$ are associated with each other by electrostatic force, the fragments should disaggregate in the presence of high ionic concentration. The decay curve of digested P-450$_{C21}$ in 600 mM NaCl was not much different from that without NaCl. This result suggests that fragments are not interacting with each other by electrostatic force alone.

DISCUSSION

P-450$_{C21}$ was observed to be very stable in vesicle membranes and to show no decrease in hydroxylase activity after heat treatment at 50 °C for 20 min. Detergent-solubilized P-450$_{C21}$, however, rapidly lost activity during heat treatment. Detergent-solubilized P-450$_{17\alpha,\text{lyase}}$ and P-450$_{11\beta}$ also showed much less stability toward heat treatment than those in vesicle membranes (Kominami et al., 1988; Ikushiro et al., 1989). Vesicle membranes would thus appear essential to the stabilization of membrane proteins.

Following trypsin digestion of P-450 2B1/P-450 2B2 proteoliposomes, only an N-terminal peptide, 1Met–21Arg, has been shown to remain in the membranes (Vergeres et al., 1989). This was similar to the results of trypsin digestion of NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase (Gum & Strobel, 1979; Ozols et al., 1984) which are anchored in vesicle membranes by an

N-terminal anchor peptide. Alignment of the amino acid sequence of P-450$_{C21}$ with P-450 2B1/2B2 shows a homologous sequence about 21Gly-22Arg-23Trp-24Lys-25Leu-26Arg of P-450$_{C21}$ (Nelson & Strobel, 1988). No cleaved sites in vesicle-bound P-450$_{C21}$ have an N-terminal sequence corresponding to that around 21Gly–26Arg. The F1 fragment (30 kDa) contains the original N-terminal sequence of undigested P-450$_{C21}$. These data indicate that trypsin cannot attack the N-terminal region of P-450$_{C21}$. Large fragments, 30, 25, and 20 kDa, were observed after trypsinolysis of P-450$_{C21}$ in vesicle membranes. On the basis of the fact that the detergent-solubilized P-450$_{C21}$ was rapidly cleaved into small peptides (Ohta et al., 1992), these fragments may probably be incorporated deeply in vesicle membranes. To confirm the integration of fragments in membranes, membrane fractions were washed in 500 mM Na_2CO_3 after proteolysis. Three fragments were recovered in the membrane fraction as shown in Figure 3, indicating them to be deeply integrated in membranes. Several investigations showed the existence of membrane binding segments other than the N-terminal region in cytochrome P-450. Genetic expression experiments on N-terminal fragment-lacking cytochrome P-450s have shown that other segments besides the N-terminal hydrophobic part are responsible for membrane incorporation of P-450$_{17\alpha,\text{lyase}}$ and P-450 1A1 (Clark & Watermen, 1991; Yabusaki et al., 1988). The sequence around 172Ile in P-450$_{C21}$ has been demonstrated to be necessary for binding of P-450$_{C21}$ to microsomes (Tisie-Luna et al., 1990). An artificially mutated P-450 2E1 lacking amino acids 3–21 is located in *Escherichia coli* membranes and retains the unchanged catalytic activity (Larson et al., 1991; Pernecky et al., 1993).

N-Terminal sequencing of the fragments from vesicle-bound P-450$_{C21}$ showed bond cleavages to occur at 267Arg–268Val and 332Arg–333Val. The time dependence of fragment formation indicated that trypsin cleaved the 267Arg–268Val bond first to produce the 1Met–267Arg (30 kDa) and 286Val–496Gln (25 kDa) fragments and subsequently the 332Arg–333Val bond to produce the 268Val–332Arg and 333Val–496Gln (20 kDa) fragments. According to the alignments reported by Nelson and Strobel, 267Arg and 332Arg in P-450$_{C21}$ might locate near both sides of the I-helix, assuming the conformation of the active site of P-450$_{C21}$ to be similar to that of P-450$_{cam}$ (Poulos et al., 1987). The loop area-connected helix I of P-450 1A2 has been shown to be exposed to the water phase using a specific antibody (Edwords et al., 1990).

It was quite striking that digestion of vesicle-bound P-450$_{C21}$ caused no decrease in activity at all. Neither the reduced CO difference spectrum nor the EPR spectrum was different before and after digestion (Figure 6). The absence of a change in activity by digestion may quite likely be due to the absence of conformational change around the active site of digested P-450$_{C21}$. It might be possible that the F3 fragment (C-terminal fragment) might hold the native conformation around the active site of P-450$_{C21}$ by itself even without the F1 fragment and I-helix. The I-helix is located at the distal side of the heme and has important roles in the reaction of P-450$_{cam}$ (Raag et al., 1991). In the molecular structure of P-450$_{cam}$, the I-helix is surrounded by β-sheets and α-helixes. High stability toward heat treatment at 50 °C (Figure 1) indicates the native conformation not to unfold by cleavages close to either side of the I-helix. These fragments might be arranged as in the case of intact P-450$_{C21}$, at least around the active site of P-450$_{C21}$. The proteolytic cleavage of the interhelical turn region connected to the I-helix has been noted in cytochrome P-450 1A1, P-450 2B1, and P-450 2E1. In P-450 1A1, the cleavage did not affect benzo[*a*]pyrene binding (Tsokos et al., 1992).

The rotational relaxation time of digested liposomal P-450$_{C21}$ was basically the same as that without digestion, indicating the size of the molecule holding the heme to be essentially the same before and after digestion (Figure 7). No flexible motion was induced in membrane-bound P-450$_{C21}$ by trypsinolysis. This result completely rules out the possibility that F3 is an active fragment that maintains the native conformation around the active site of P-450$_{C21}$ without other fragments. The rotational relaxation time of P-450$_{C21}$ in vesicle membranes is about 180 μs. A much shorter relaxation time is expected if the heme-containing compartment of P-450$_{C21}$ is anchored to membranes only by an N-terminal hydrophobic segment as in the case of NADPH–cytochrome P-450 reductase (Nishimoto et al., 1983). The results of rotational diffusion experiments rule out the possibility of the N-terminal anchor model for P-450$_{C21}$.

Concerning the membrane topology of P-450$_{C21}$, a considerable portion of P-450$_{C21}$ including both the N-terminal and C-terminal regions must be deeply embedded in membranes. The sites of trypsin attack must be outside the membranes. It should be pointed out that the stability of the active site mainly derives from the deep insertion of P-450$_{C21}$ in membranes. Four membrane binding segments have been proposed in the molecular structure of P-450$_{SCC}$ by the sequence alignment study of P-450$_{SCC}$ with P-450$_{cam}$ (Vijayakumar & Salerno, 1992). A similar membrane topology can be assumed for P-450$_{C21}$ in which the proximal region of the heme is exposed to the aqueous phase and the distal region faces the membrane. NADPH–cytochrome P-450 reductase has been shown to interact with cytochrome P-450 in the direction of the proximal region (Stayton & Sliger, 1990; Simizu et al., 1991). The substrate binding site in the distal region (Poulos et al., 1985) may facilitate rapid access to the binding site of hydrophobic substrates accumulated in the membranes.

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