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

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ABSTRACT: Purified adrenocortical microsomal P-450C21 was incorporated into vesicle membranes composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at a molar ratio of 5:3:1. Trypsinolysis of the incorporated P-450C21 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-450C21 IgG. In the detergent-solubilized state, trypsin cleaved P-450C21 into very small peptides. Washing of the trypsin-treated vesicles with 500 mM Na2CO3 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-450C21. 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-450C21 differed from those of undigested P-450C21. Heat treatment at 50 °C for 20 min did not cause any decrease in activity of digested P-450C21, when the substrate progesterone was present. This high stability toward heat treatment was not observed in the solubilized state. Rotational diffusion experiments on P-450C21 showed that the size of the molecule holding the heme was not changed significantly after digestion. On the basis of these results, P-450C21 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 Δ5,Δ3-isomerase (Takemori & Kominami, 1984). P-450C21 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-450C21 have been studied using a system in which purified P-450C21 is incorporated in phospholipid vesicle membranes. Some kinetic studies indicate that P-450C21 in vesicle membranes metabolizes substrates concentrated in membranes (Kominami et al., 1986). In a reconstituted electron-transfer system in vesicle membranes composed of P-450C21 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-450C21 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 hydrophobic 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-450cam. On the basis of the crystallographic data of the P-450cam 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 b5 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-450C21 and P-45017α,12α-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-450c17 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-450C21 was examined in detail by analysis of tryptic fragments. Trypsin cleaved membrane-bound P-450C21 at two sites, but this did not affect significantly the enzymatic and physicochemical
properties as long as the cleaved protein continued to remain in membranes.

EXPERIMENTAL PROCEDURES

Preparation of Proteoliposomes. P-450C21 and NADPH-cytochrome P-450 reductase were purified from bovine adrenal and hepatic microsomes, respectively, as previously described (Kominami et al., 1986; Takemori & Kominami, 1982). Unilamellar vesicles containing P-450C21 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-450C21 was estimated from the dichoronte-reduced CO difference spectrum using \( \Delta A(450-490 \text{ nm}) = 91 \text{ mM}^{-1} \text{ cm}^{-1} \) (Omura & Sato, 1964). The vesicles used in this experiment contained P-450C21 and phospholipid 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-450C21 vesicles at a molar ratio of 1:2 to P-450C21 (Kominami et al., 1987). Bovine adrenocortical microsomes were prepared by differential centrifugations (Kominami et al., 1980).

Proteolysis and SDS-PAGE. Proteolysis of P-450C21 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-450C21 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% glycerol, 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-450C21 and adrenal microsomes was carried out as previously described (Shinazawa et al., 1988). The fragments separated by SDS-PAGE were transferred to a nitrocellulose sheet (BA-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-450C21 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., Tokyo). The fragments were precipitated in 90% (v/v) acetone and collected by centrifugation (24000g, 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-450C21. 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:

\[
r(t) = \frac{A_0(t) - A_H(t)}{A_0(t) + 2A_H(t)} \]

where \( A_0(t) \) 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, 16384 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:

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r(t) = r_1 \exp(-\phi t) + r_2 \exp(-4\phi t) + r_3 \]

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

Other Methods. The 21-hydroxylase activity of P-450C21 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-450C21 Proteoliposomes. The incorporation of P-450C21 in vesicle membranes was confirmed by density gradient centrifugation using 0–10% (w/v) Ficol (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-450C21 was reduced by the external addition of NADPH-cytochrome P-450 reductase and NADPH, showing most of the P-450C21 molecules to possess an electron-accepting site on the external side of the vesicles. The stability of P-450C21 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-450C21 in vesicle membranes was 14 ± 2 nmol of product formed (nmol of P-450C21)⁻¹ min⁻¹ and showed no decrease by heat treatment.
Digestion and the circles activity after trypsin digestion. The open symbols show activity after heat treatment in the presence of 5 mM substrate progesterone and the closed symbols activity without substrate. Proteolysis of P-450(21) 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(21). To reconstitute the electron-transfer system, 100% activity indicates activity before trypsin digestion in vesicle membranes. The triangles show activity without trypsin at 50 °C. Lane 4 shows fragments from undigested P-450(21) in vesicle and microsome membranes. Lanes 1 and 3 are digested P-450(21) in vesicle and microsome membranes, respectively. The details of trypsin digestion are the same as in Figure 1. The details of assay of 21-hydroxylase activity are presented under Experimental Procedures. The open circles, open triangles, and closed triangles represent relative amounts of uncleaved molecule and fragments from P-450(21) whose molecular masses were estimated as 30, 25, and 20 kDa, respectively, from mobility in SDS-PAGE. SDS-PAGE of the digested P-450(21) showed four fragments, P-450(21) in the solubilized form was not unique to the detergent-solubilized form. In both the substrate-bound and substrate-free forms, instability in the detergent-solubilized form was not unique to the detergent-solubilized form. Emulgen 913.

Trypsinolysis of P-450(21) in Vesicle and Microsomal Membranes. P-450(21) in vesicle membranes was digested at 37 °C for 2 h with trypsin at a weight ratio of 1:25 to P-450(21). SDS-PAGE of the digested P-450(21) 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(21) 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(21) in vesicle and microsomal membranes using anti-P-450(21), 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(21) from trypsin digestion. P-450(21) may possibly be buried deeply in membranes. For confirmation of this possibility, the membrane fraction of digested P-450(21) was suspended in 500 mM Na2CO3. 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 60 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 peptide.
corresponds to the 333-496 fragment, apparently formed by cleavage of the F2 fragment.

Termination of heat treatment at 50°C for 20 min caused no observable trypsinolysis (Figure 1). In the presence of substrate progesterone, heat treatment of membranes protected the activity to 50% of the original. The activity of P-450~21, 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.

**Stability of Digested P-450~21 in Vesicle Membranes.**

It was striking that vesicle-bound P-450~21 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-450~21, although digested P-450~21 showed not the original band but the F1, F2, and F3 bands on SDS-PAGE. The stability of vesicle-bound P-450~21 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-450~21 in membranes. Without progesterone, heat treatment for 2 min decreased the activity to 50% of the original. The activity of P-450~21, 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-450~21 and its conformation is quite stable against thermal perturbation even after digestion. Vesicle membranes protected P-450~21 from thermal denaturation even after digestion, and substrate progesterone also exerted a protective effect.

**Spectroscopic Properties of Digested Liposomal P-450~21.**

The dithionite-reduced CO difference spectra of vesicle-bound P-450~21 is shown in Figure 6(a) before and after trypsinolysis. The optical difference spectra of digested and undigested P-450~21 were the same. The EPR spectrum of vesicle-bound P-450~21 was measured at 77 K [Figure 6(b)]. Typical low-spin signals of cytochrome P-450 have been 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 bond of P-450~21 causes little detectable change about the heme.

**Rotational Mobility of Digested P-450~21 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-450~21 about the axis perpendicular to the membrane plane. The rotational relaxation time of digested P-450~21 [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-450~21 in membranes is basically the same as undigested P-450~21.

The effect of high ionic concentration on the rotational anisotropy decay curves was examined. If the fragments in digested P-450~21 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-450~21 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-450~21 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-450~21, however, rapidly lost activity during heat treatment. Detergent-solubilized P-450~17α,21-lyase and P-450~11b 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-450~21/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 b5 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-450C21 with P-4502B1/2B2 shows a homologous sequence about 21 Gly-22 Arg-23 Trp-24 Lys-25 Leu-26 Arg of P-450C21 (Nelson & Strobel, 1988). No cleaved sites in vesicle-bound P-450C21 have an N-terminal sequence corresponding to that around 21 Gly-26 Arg. The F1 fragment (30 kDa) contains the original N-terminal sequence of undigested P-450C21. These data indicate that trypsin cannot attack the N-terminal region of P-450C21. Large fragments, 30, 25, and 20 kDa were observed after trypsinoysis of P-450C21 in vesicle membranes. On the basis of the fact that the detergent-solubilized P-450C21 was readily 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 Na2CO3 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-45010cV7 and P-450 1A1 (Clark & Waterman, 1991; Yabusaki et al., 1988). The sequence around 172 Ile in P-450C21 has been demonstrated to be necessary for binding of P-450C21 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; Pernecke et al., 1993).

N-Terminal sequencing of the fragments from vesicle-bound P-450C21 showed bond cleavages to occur at 267 Arg-268 Val and 332 Arg-333 Val. The time dependence of fragment formation indicated that trypsin cleaved the 267 Arg-268 Val bond first to produce the Met-267 Arg (30 kDa) and 286 Val-496 Gln (25 kDa) fragments and subsequently the 332 Arg-333 Val bond to produce the 268 Val-332 Arg and 333 Val-496 Gln (20 kDa) fragments. According to the alignments reported by Nelson and Strobel, 267 Arg and 332 Arg in P-450C21 might locate near both sides of the I-helix, assuming the conformation of the active site of P-450C21 to be similar to that of P-450 am (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-450C21 caused no decrease in activity at all. Neither the reduced CO difference spectrum nor the EPR spectrum was different before and after digestion (Figure 7). 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 P-450C21. It might be possible that the F3 fragment (C-terminal fragment) might hold the native conformation around the active site of P-450C21 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-450C21 (Raag et al., 1991). In the molecular structure of P-450C21, the I-helix is surrounded by β-sheets and α-helices. 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-450C21, at least around the active site of P-450C21. 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 (Tsukos et al., 1992).

The rotational relaxation time of digested liposomal P-450C21 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-450C21 by trypsinoysis. This result completely rules out the possibility that F3 is an active fragment that maintains the native conformation around the active site of P-450C21 without other fragments. The rotational relaxation time of P-450C21 in vesicle membranes is about 180 μs. A much shorter relaxation time is expected if the heme-containing compartment of P-450C21 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-450C21.

Concerning the membrane topology of P-450C21, a considerable portion of P-450C21 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-450C21 in membranes. Four membrane binding segments have been proposed in the molecular structure of P-450ccc by the sequence alignment study of P-450ccc with P-450 am (Vijayakumar & Salerno, 1992). A similar membrane topology can be assumed for P-450C21 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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