Membrane Topology of Guinea Pig Cytochrome P450 17α Revealed by a Combination of Chemical Modifications and Mass Spectrometry

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ABSTRACT: Cytochrome P450s in endoplasmic reticulum membranes function in the hydroxylation of exogenous and endogenous hydrophobic substrates concentrated in the membranes. The reactions require electron supplies from NADPH−cytochrome P450 reductase in the same membranes. The membranes play important roles in the reaction of cytochrome P450. The membrane topology of guinea pig P450 17α was investigated on the basis of the differences in reactivity to hydrophilic chemical modification reagents between those in the detergent-solubilized state and proteoliposomes. Recombinant guinea pig cytochrome P450 17α was purified from *Escherichia coli* and incorporated into liposome membranes. Lysine residues in the detergent-solubilized P450 17α and in the proteoliposomes were acetylated with acetic anhydride at pH 9.0, and the acidic amino acid residues were conjugated with glycinamide at pH 5.0 by the aid of a coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The modifications were performed under conditions where the denatured form, P420, was not induced. The modified P450 17α's were digested by trypsin, and the molecular weights of the peptide fragments were determined by MALDI-TOF mass spectrometry. From the increase in the molecular weights of the peptides, the positions of modifications could be deduced. In the detergent-solubilized state, 11 lysine residues and 7 acidic amino acid residues were modified, among which lysine residues at positions 29, 59, 490, and 492 and acidic residues at 211, 212, and/or 216 were not modified in the proteoliposomes. Both the N- and C-terminal domains and the putative F−G loop were concluded to be in or near the membrane-binding domains of P450 17α.

17α-Hydroxylase cytochrome P450 (P450 17α;[1] EC 1.14.99.9) catalyzes both 17α-hydroxylation and C17−20 bond cleavage reactions of C21-steroids, and is located in the membranes of the endoplasmic reticulum of adrenal and gonadal glands (*1*−*3*). The substrate steroids progesterone and pregnenolone are hydrophobic and favor partitioning into the membrane lipid phase rather than into the aqueous phase (*4*, *5*). It is a reasonable proposal that steroidogenic P450 metabolizes the substrates concentrated in the membranes and the substrates enter the substrate-binding site directly from the membranes (*6*, *7*). The possibility that the F−G loop of the protein might be located close to the substrate access channel has been shown in P450 cam, P450 BM3, and P450 14M, water-soluble P450s (*8*−*10*). It is crucial for understanding the reactions of steroidogenic P450s whether the substrate access channel of the P450s is facing the membrane or aqueous phase.

The cytochrome P450-catalyzed hydroxylation reaction requires two electron equivalents that are transferred from NADPH−cytochrome P450 reductase through the interaction between the two proteins in the membranes (*11*, *12*). P450 associates with the reductase via the electrostatic interaction between the cationic residues of P450 and the anionic residues of the reductase (*13*−*15*). NADPH−cytochrome P450 reductase loses the N-terminal membrane-spanning domain through limited proteolysis and becomes soluble in aqueous solution (*16*). The soluble reductase hardly delivers electrons to P450 and cannot support the P450 reactions (*17*). These experimental results show that the proper orientation of P450 with respect to the reductase on the membranes is necessary for the electron transfer from the reductase to P450.

The membrane topology of microsomal P450s has been studied by various methods, limited proteolysis (*18*−*20*), rotational diffusion experiments (*20*, *21*), deletion of the N-terminal peptide, site-directed mutagenesis (*22*−*27*), immunochemical methods using antibodies against specific epitopes of P450s (*28*, *29*), atomic force microscopy (*30*), and molecular modeling (*31*, *32*). These studies show that

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[1] Abbreviations: P450 17α, cytochrome P450 having steroid 17α-hydroxylase activity in adrenal and gonadal microsomes; P450 c17, same species as P450 17α; P450 C21, cytochrome P450 having steroid 21-hydroxylase activity in adrenal microsomes; P450 2C5, cytochrome P450 having progesterone 21-hydroxylase activity in liver microsomes; P450 cam, cytochrome P450 having camphor hydroxylation activity; P450 14M, cytochrome P450 having 14α-sterol demethylase activity; MALDI-TOF mass spectrometry, a method of mass spectrometry using a matrix-assisted laser desorption ionization and time-of-flight techniques; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; P450 BM3, a soluble cytochrome P450 from *Bacillus megaterium*; HPLC, high-performance liquid chromatography; DPPC, dipalmitoylphosphatidylcholine.

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the N-terminal hydrophobic sequence in microsomal P450 contains a signal for localization to the endoplasmic reticulum membranes and is the primary site for binding to the membranes. The experiments using N-terminal-truncated (about 20 amino acids) P450s showed, however, that there are membrane-binding domains in microsomal P450s other than the N-terminal domain and the deletion of the N-terminal region from P450s does not destroy the enzyme activities. The results of site-directed mutagenesis and crystallographic studies on P450 2C5 were consistent with the hypothesis that the F−G loop interacts with the membranes and the entrance of the putative substrate access channel orients toward the surface of the membrane.

Chemical modification is a powerful method in the identification of amino acid residues important for the function of proteins. The combination of HPLC and amino acid sequencing requires milligrams of protein for the identification of modified sites. The development of mass spectrometry in protein science provides a sensitive methodology for the measurement of molecular weights of peptide fragments, which allows one to identify the modified sites in the protein using micrograms of protein. In this study, we chemically modified the ionic amino acid residues lysine, glutamate, and aspartate of recombinant guinea pig adrenal cytochrome P450 17α both in the detergent-solubilized state and in proteoliposomes. The positions modified in the detergent-solubilized state were identified by the mass spectrometry of the tryptic peptide fragments. It is found that some of them could not be modified in the proteoliposomes, and we concluded that the amino acid residues not modified in the proteoliposomes are in positions at or near the membrane-binding domains in P450 17α.

EXPERIMENTAL PROCEDURES

Purification of Enzymes and Preparation of Proteoliposomes. Recombinant guinea pig P450 17α $(His)_4$ was purified by sequential chromatographies with DEAE-Sephacel (Pharmacia-Amerciam) and Ni-NTA (Quiagene) as described previously (*33*, *34*). NADPH−cytochrome P450 reductase was purified from bovine liver microsomes as described elsewhere (*35*). Proteoliposomes containing P450 17α in a ratio of 1:2000 (mol/mol) to phospholipids were prepared by the cholate dialysis method using egg yolk phosphatidylcholine (Sigma-Aldrich) (*36*). The concentration of P450 17α in the sample was determined using a difference absorption coefficient of $\Delta\epsilon(448-490\ \text{nm}) = 91\ \text{mM}^{-1}\ \text{cm}^{-1}$ for the CO−dithionite-reduced spectrum (*37*). The concentration of lipids was estimated from the radioactivity of [^{14}C]-DPPC (Dupont-NEN) that had been mixed with the phospholipids before dialysis (*38*). More than 80% of the incorporated P450 17α is located on the outside surface of the liposome membranes.

Chemical Modifications and Trypsin Digestion. Acetylation of the lysine residues of P450 17α was carried out with acetic anhydride (Nakalai Tesque, Kyoto, Japan). P450 17α's in 0.1% β-dodecylmaltoside (Dojinn, Kumamoto, Japan) solution and in prepared proteoliposomes were diluted to 4 μM with about 25 volumes of 100 mM H_3BO_3 (pH 9.0). To each 20 μL of sample solution, 2 μL of freshly prepared 10 mM acetic anhydride was added, in which the molar ratio of P450 17α to acetic anhydride was 1:250, and the mixture was incubated for 15 min at 4 °C. The reaction was terminated by the addition of 40 μL of 100 mM NH_4HCO_3. A 40 μL sample of chilled acetone (−20 °C) was mixed with the solution to precipitate acetylated P450 17α, and the mixture was kept at −20 °C overnight. The precipitates were collected and dissolved again in 40 μL of 100 mM NH_4HCO_3, and then the precipitation was repeated again with 40 μL of chilled acetone. The precipitates were dried by evacuation and dissolved in 40 μL of 100 mM NH_4HCO_3. The acetylated P450s were digested at 25 °C for 2 h with trypsin (Sigma) at a molar ratio of trypsin to P450 17α of 1:100 (mol/mol).

Glycinamidation of the acidic residues of P450 17α was performed as follows (*39*). The solutions of detergent-solubilized P450 17α and the proteoliposomes were diluted to 1−2 μM with about 25 volumes of 50 mM glycinamide−HCl solution (pH 5.0). The modification reaction was started by the addition of 4 μL of 4 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; Sigma) to the sample solution, and the mixture was incubated for 2 h at room temperature. The reaction was terminated by the addition of 10 μL of 500 mM sodium acetate (pH 5.0). The precipitations and trypsin digestion were preformed in the same way as those after acetylation.

Mass Spectrometry and Identification of Modified Positions. The positions of modified amino acid residues of P450 17α were deduced from the increase in molecular weights of the tryptic fragments determined by MALDI-TOF mass spectrometry. Zip Tip C18 (Millipore) was used to remove metal ions from the sample. The peptide fragments adsorbed on the tip were eluted with tetrafluoroacetic acid (TFA; Sigma) to plastic tubes and dried. A 3 μL aliquot of matrix solution (α-cyano-4-hydroxy-*trans*-cinnamic acid (Sigma)) was added to the tube, and 1 μL of the sample-matrix mixture was spotted on a stainless steel probe tip of the instrument and allowed to air-dry for 10 min at room temperature. Mass spectra for most of the samples from acetylated P450 17α were measured with a Bruker Bioflex II MALDI-TOF mass spectrometer equipped with a nitrogen laser (337 nm). Spectra for other samples, mainly from glycinamidated P450 17α, were measured on a Bruker Ultraflex MALDI-TOF mass spectrometer. Mass spectra were obtained by accumulating data for 200 laser shots under the threshold irradiance in the reflector positive mode at a 28.5 kV acceleration voltage and 1.5 kV in the detector. Fragments in the range from *m*/*z* 800 to *m*/*z* 3000 were mainly observed. Well-resolved spectra arising from three to five selected target spots were analyzed. All MALDI-TOF mass spectra were calibrated externally using a standard peptide mixture (angiotensin II (*m*/*z* 1047.2), adrenocorticotropic hormone fragment 18−39 (*m*/*z* 2466.7), and insulin (*m*/*z* 5734.6); Sigma). The identification of the peptide fragments was carried out with a software MASCOT (http://www.matrixscience.com) or Protein Prospector (http://prospector.ucsf.edu). The acetylated fragments were identified by an increase in the mass number of 42 in the fragments containing lysine residues, and a mass number of 56 was used for the identification of glycinamidation of acidic amino acid residues. An error in mass number of ±1.0 was tolerated in the identification of the modified peptides, if there were no unmodified fragments in this error range.

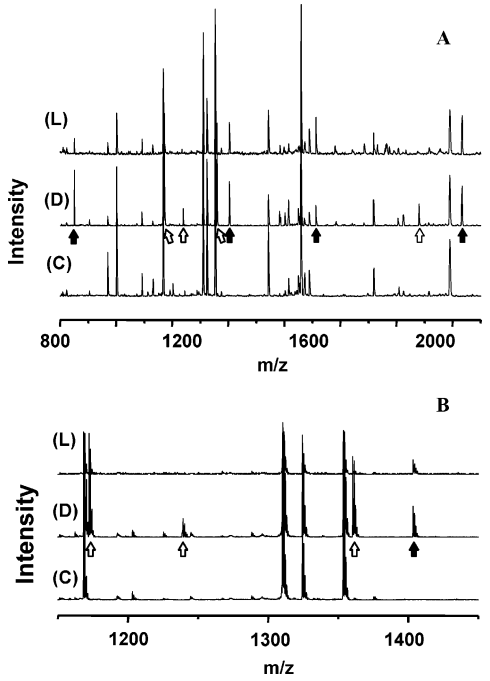

FIGURE 1: MALDI-TOF mass spectra of acetylated guinea pig cytochrome P450 17α: (C) unmodified, (D) acetylated in the detergent-solubilized state, (L) acetylated in the proteoliposomes. Panel A shows the spectra in the region from *m*/*z* 800 to *m*/*z* 2300. Those from *m*/*z* 1150 to *m*/*z* 1450 are expanded in panel B. Arrows show the positions of peaks that are not detected in unmodified P450 17α. Open arrows show the positions of peaks that cannot be detected in the proteoliposomes but can be in the detergent-solubilized state. The details of the reaction conditions and the identifications are described in the Experimental Procedures.

Optical absorption spectra were measured with a Beckman DU-650 spectrophotometer. 17α-Hydroxylase activities before and after modifications were measured only for P450 17α in liposome membranes using [^{3}H]progestrone (Dupont-NEN) as described previously, since P450 17α does not show any significant activity in the detergent solution (*33*, *40*).

RESULTS

More than 25 tryptic peptide fragments from unmodified guinea pig P450 17α were detected in the mass spectrum in the region of *m*/*z* 800−3500 (Figure 1A (C)). It is reasonable that almost the same spectra were observed for detergent-solubilized P450 17α and for the proteoliposomes, because the detergents or membrane lipids were removed before the trypsin treatments and the conditions for the proteolysis were the same for both samples. With mass spectrometry under the conditions used in this study, fragments containing less than 7 or more than 40 amino acid residues are difficult to detect. Identified peptides in the mass spectra from unmodified P450 17α covered about 70% of the sequence of the parent protein. This sequence coverage showed that the peptides from most parts of P45017α were detected in the mass spectra in the present study. Almost similar sequence coverage was found for P450 17α's modified by the reagents.

We examined several conditions for acetylation of P450 17α, pH, temperature, reaction time, and the molar ratio of acetic anhydride to P450 17α. Acetic anhydride is reactive to the $-NH_2$ group rather than the $-NH_3^+$ group, where the pK_a for the amino group in the lysine side chain is about 10 (*41*). A pH higher than 10 induced denaturation of P450 17α,

Table 1: Identification of the Positions of Acetylated Lysine Residues in Detergent-Solubilized Guinea Pig P450 17α and in the Proteoliposomes[a]

Observed Mass (m/z)	No. of Modifications	Deduced Peptide	Modified Position	Detergent Solubilized	Proteoliposome
851.2	1	234 239 KYTEIR	234	+++	+++
1172.2	1	59 67 KYGPIYSFR	59	+++	
1239.4	1	491 500 VKITVRPAWK	492	+++	
1360.4	1	482 492 IVFLIDPFKVK	490	+++	
1403.3	1	223 233 IFPNKTLEMIR	227	+++	+++
1713.3	1	327 340 KIQEEIDHNVGFSR	327	+++	+++
2080.6	1	27 45 YPKSLPSLPVVGSLPFLPK	29	++	
2232.6	1	90 110 GKEFSGRPLTTTVALLSDNGK	91	+++	+++
2843.8	1	363 388 IRPVAPILIPHKANTDSSIGEFAIDK	374	++	++
3169.2	1	201 227 FTTGFVNSLSDDNLVDIFPWLKIFPNK	222	+	+
4979.6	1	375 ANTDSSIGEFAIDKDTNVLVNL 416 WALHHNEQEWDRPDQFMPER	388	+	+

[a] The details of the reaction conditions and the identifications are described in the Experimental Procedures. The symbols +++, ++, and + represent the appearance of the peak in most, more than half, and less than half of the samples, respectively.

and 100 mM H_3BO_3 (pH 9.0) was used for acetylation. A pH lower than 4, reaction at room temperature for 2 h, and/or too high a concentration of acetic anhydride induced the denatured form of the enzyme, P420. We confirmed that acetic anhydride at a molar ratio of 250:1 to P450 17α could not induce P420 when reacted at 4 °C for 2 h. Trypsin digestion was performed after acetylation and subsequent removal of detergents or phospholipids by repeated acetone precipitations. Two times more concentrated trypsin (a molar ratio of 1:50 to P450 17α) did not make any significant changes in the mass spectra even after incubation of 4 h at room temperature.

In the spectra of tryptic peptides from P450 17α acetylated in the detergent-solubilized state (Figure 1A (D)), we can see several new peaks (shown by closed and open arrows), which are not detected in the unmodified P450 17α. In Figure 1B, the spectra in the range from *m*/*z* 1150 to *m*/*z* 1450 in Figure 1A were expanded. New peaks at *m*/*z* 1172.2, 1239.4, 1360.4, and 1403.3 are clearly detected after acetylation in comparison of spectrum D with spectrum C in Figure 1B. We also noticed ^{13}C satellite peaks on the right side of each peak, which provide confirmation of the high resolution of the measurements. From the molecular weights of the peptides that appeared after the acetylation, we can deduce the sequences of the peptides in which one of the lysine residues was acetylated. The sequences and the positions of acetylated lysine residues are listed in Table 1. Several more peaks could be candidates for acetylated peptides, but they had molecular weights similar to those of unacetylated tryptic peptides (within $\Delta(m/z) = \pm 1.0$), which are neglected in this table. Some of the peaks in the unacetylated P450 17α decreased in the acetylated sample, which can be attributed to an increase in the molecular weight due to acetylation and also to inhibition of the trypsin digestion by the acetylated lysine at the C-terminal of the peptide fragments, the cleavage position of trypsin. The percentage of individual lysine residues acetylated was estimated to be 10−50% using the decrease in the original peak heights and the increase in

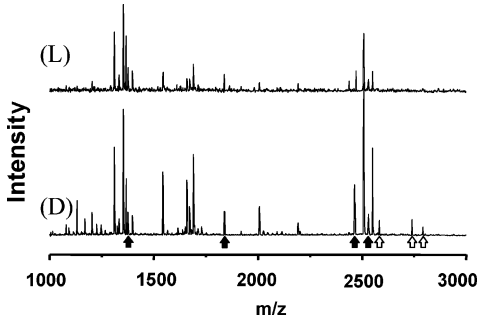

FIGURE 2: MALDI-TOF mass spectra of glycinamidated guinea pig cytochrome P450 17α: (D) glycinamidated in the detergent-solubilized state, (L) glycinamidated in the proteoliposomes. Arrows show the positions of peaks that are not detected in unmodified P450 17α. Open arrows show the positions of peaks which cannot be detected in the proteoliposomes but can be in the detergent-solubilized state. The details of the reaction conditions and the identifications are described in the Experimental Procedures.

those of the corresponding acetylated fragments. The acetylated P450 17α in the proteoliposomes showed less than 10% of the 17α-hydroxylase activity for progesterone of the unacetylated P450 17α. More than 20 repetitions of preparation of acetylated samples confirmed that 11 lysine residues were acetylated in the detergent-solubilized state, as shown in Table 1. Parts A (L) and B (L) of Figure 1 show typical spectra of P450 17α acetylated in the liposome membranes. It is clear that some of the peaks (shown by open arrows) in parts A (D) and B (D) of Figure 1 cannot be detected in the sample from the acetylated proteoliposomes. In other words, the acetylation of some positions of P450 17α was inhibited by the incorporation of the enzyme into liposome membranes. The reproducibility of the appearances of a particular acetylated peptide signal in the mass spectra was shown by +++, ++, or + for the peptide observed in most, more than half, or less than half of the spectra, respectively, in Table 1. Differences in the intensities of the signals of various peptides cannot be attributed, however, to differences in the yields of the peptides, since those are highly dependent on the ionizations by the MALDI method (*42*). The reproducibility in Table 1 does not reflect correctly the reactivity of the peptide to the reagent. The conditions for proteolysis and ionization are the same for samples from P450 17α's both in the detergent-solubilized state and in the liposomes, because the detergent and the phospholipids were removed before the proteolysis. The difference in the reproducibility of a particular modified peptide in Table 1 between those from the detergent-solubilized P450 17α and the proteoliposomes showed the difference in reactivity of the peptide to the modification reagent in the two states.

Figure 2 shows spectra of tryptic peptides from P450 17α glycinamidated in the detergent-solubilized state (Figure 2(D)) and in the liposome membranes (Figure 2(L)). As in the case of acetylation, one can recognize the appearance of several new peaks (shown by arrows) in the spectrum from glycinamidated P450 17α in the detergent-solubilized state (Figure 2 (D)). We can identify seven glycinamidated acidic residues in P450 17α in the detergent-solubilized state, and the sequences and the modified positions are shown in Table 2. The percentage of individual acidic residues glycinamidated was about 5−10% of that in the original P450, estimated from the peak heights of the new peaks. We could detect almost no changes in the catalytic activity and no

Table 2: Identification of the Positions of Glycinamidated Acidic Amino Acid Residues in Detergent-Solubilized Guinea Pig P450 17α and in the Proteoliposomes[a]

Observed Mass (m/z)	No. of Modifications	Deduced Peptide	Modified Position	Detergent Solubilized	Proteoliposome
1366.4	1	348 358 NHLLMLEATIR	354	++	++
1838.0	2	127 141 LVLSSFSLFRDGEQK	137 and 139	+	+
2469.5	1	68 89 LGSTTTVVIGHHQLARELLIKK	84	++	+
2550.1	1	228 248 TLEMIRKYTEIRGAMLSKILK	230 or 237	++	+
2582.2	1	201 222 FTTGFVNSLSDDNLVDIFPWLK	211, 212 or 216	+	
2740.4	1	200 222 RFTTGFVNSLSDDNLVDIFPWLK	211, 212 or 216	+	
2796.0	2	200 222 RFTTGFVNSLSDDNLVDIFPWLK	211, 212 or/and 216	+	

[a] The details of the reaction conditions and the identifications are described in the Experimental Procedures. The symbols +++, ++, and + represent the appearance of the peak in most, more than half, and less than half of the samples, respectively.

denatured form of P420 after the glycinamidation. A few of the glycinamidated peptide peaks (shown by open arrows) in Figure 2 (D) cannot be observed in the spectrum of glycinamidated P450 17α in proteoliposomes (Figure 2 (L)). The disappearances of the peaks at *m*/*z* 2582.2, 2740.4, and 2796.0 must be due to the inhibition of glycinamidation by liposome membranes, which corresponds to the inhibition of the glycinamidation at positions D211, D212, and/or D216 as shown in Table 2. We could not determine the precise positions of the modifications, because these three acidic amino acid residues are located in the same peptide fragments (peptides 201−222 and 200−222). Since increases in the mass number of 56 and 56 × 2 were observed in these fragments, there is no doubt about the modifications at D211, D212, and/or D216.

DISCUSSION

It is a reasonable assumption that the reactivity of water-soluble chemical modification reagents to lysine and acidic amino acid residues of guinea pig cytochrome P450 17α incorporated into the liposome membranes might be different from that of the protein in the detergent-solubilized state. The main differences could be expected in the residues located in the membrane-binding domains. Previously, we reported that P450 17α and P450 C21 in the detergent-solubilized state were easily digested to small peptide fragments by trypsin treatments but not those incorporated into liposome membranes (*21*). Tables 1 and 2 show differences in reactivities of lysine and acidic amino acid residues in P450 17α between those in the detergent-solubilized state and in the liposome membranes. It is possible that the difference in the reactivity is due to the conformational change of the membrane protein caused by the solubilization in detergent solution. P450 17α showed, however, similar activity in the presence of a very low concentration of a nonionic detergent, and thus, it can be assumed that not much change in the conformation occurred in the detergent-solubilized state compared to the native form in the membranes (*2*). The difference in the reactivity must be due to the accessibility of the small and water-soluble modification reagents to those amino acid residues, and the positions that are not modified in the proteoliposomes must

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MWELVTLLGLILAYLFWPRQGSSGTKYP[K]SLPSLPVVGSLPFLPKSGHMH          50
 N-terminal Helix
VNFFKLQK[K]YGPIYSFRLGSTTTVVIGHHQLAR[E]LLIKKG[K]EFSGRPLTT      100
 A-Helix                    B-Helix
TVALLSDNGKGIAFADSSATWQLHRRLVLSSFSLFR[D]G[E]QKLENIICQEL        150
B'-Helix          C-Helix              D-Helix
SALCDFLATCDGQVKDLSSSIFMTVVNIICMICFSVSYKEGDMELVTIRR            200
                    E-Helix
FTTGFVNSLS[DD]NLV[D]IFPWL[K]IFPN[K]TL[E]MIR[K]YT[E]IRGAMLSKILKEC 250
  F-Helix                   G-Helix
KEKFRSDSVSNLIDLLIQAKVNENNNNSSLDQDSNLFSDKHILTTLGDIF            300
         H-Helix                    I-Helix
GAGVETSSSVVLWVIAFLLHNPQVKK[K]IQEEIDHNVGFSRTPTFSDRNHL          350
   I-Helix          J-Helix          J'-Helix
LML[E]ATIREVLRIRPVAPILIPH[K]ANTDSSIGEFAID[K]DTNVLVNLWALH       400
    K-Helix                          K'-Helix
HNEQEWDRPDQFMPERFLDPTGSQIIVPSSSYLPFGAGPRSCVGEALARQ            450
                              L-Helix
EIFLITAWLLQKFDLEVPEGGQLPSLEGIPKIVFLIDPF[K]V[K]ITVRPAWK        500

EAQAEGSAHHHH                                                   512
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FIGURE 3: Positions of lysine and acidic amino acid residues in guinea pig P450 17α modified by acetic anhydride and glycinamide, respectively. Bold letters show the modified positions, and bold letters in squares show the positions that cannot be modified in the proteoliposomes but can be modified in the detergent-solubilized state. The positions of the helix (underlined) are deduced on the basis of the structure of P450 BM3 in refs 9 and 32. It is not clear from the results which two aspartic acid residues among D211, D212, and D216 are modified, but three of them are shown by bold letters in squares.

be at or near the membrane-binding domains of P450 17α. A similar inhibition of chemical modifications has been reported at the interaction surfaces of two proteins (*43*). Oligomer formation in the detergent-solubilized state had been reported for P450 C21, and an oligometric structure has been observed recently for P450 2C2 in living cells (*40*, *44*). These oligomer formations might occur also in guinea pig P450 17α and might affect the reactivity of the amino acid residues to the modification reagents. In this study it is assumed that the oligomer formation of P450 17α might affect the reactivity of amino acid residues less than the membranes does.

In Figure 3, the positions of modifications are shown in the sequence of guinea pig P450 17α. The positions of the helices were deduced from the alignment of guinea pig P450 17α with the oxyganase domain of P450 BM3 (*32*). The modified positions in the detergent-solubilized state are shown by bold letters, and bold letters in squares show the positions where the modifications were inhibited by liposome membranes. One notices that the lysine and acidic amino acid residues in the range 210−250 (F−G loop and G-helix) are easily modified. The unmodified positions in Figure 3 do not mean that the hydrophilic modification reagent cannot access those residues but show that the modifications of these residues cannot be detected under these experimental conditions. We cannot exclude the possibility that some other lysine and acidic residues were modified but the tryptic peptides were not observed in this range of measurements or the peptides were not easily ionized under these experimental conditions (*42*). Bold letters in the squares are near both N- and C-terminals and in the sequence between F and G helices. These amino acid residues must be near or in the membrane-binding domains, and the membranes disturb the

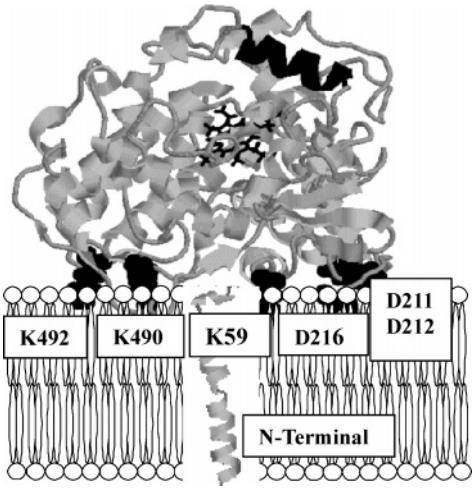

FIGURE 4: Membrane topology of guinea pig P450 17α. The upper part of the molecular model (48-C-termical) was constructed by superimposition of the amino acid sequence of guinea pig P450 17α on a molecular model of human P450 c17 simulated by Auchus and Miller (ref 32). The black residues are the positions that cannot be modified in the proteoliposomes but can be modified in the detergent-solubilized state. Lysine 29 is one of them but is not shown in the model, because no information is available about the structure of the region that connects the N-terminal membrane-spanning helix to the upper part of the molecule. The black helix shows the putative C-helix.

access of the modification reagents to these residues in the proteoliposomes.

Figure 4 shows a model for guinea pig P450 17α on the membrane. The upper part of the molecular structure, corresponding to the N-terminal-deleted protein (48-C-terminal), was constructed by superimposition of the amino acid sequence of guinea pig P450 17α on a molecular model of human P450 c17 which had been simulated using the structure of P450 BM3 (*32*). The lowest energy structure for guinea pig P450 17α was determined using the coordinates of human P450c17 as the initial positions of the corresponding residues of guinea pig P450 17α with the program package TINKER (J. W. Ponder, Washington University School of Medicine, St. Louis, MO). The calculation did not change the coordinates much, and Figure 4 was made using the human coordinates for positions of the corresponding amino acid residues of guinea pig P450 17α with the software RasMol Ver. 2.7.1.1. The N-terminal region (M1−P18) is assumed to form an α-helix structure that spans the membranes as a primary membrane-binding domain (*18*). We cannot suggest anything about the structure of the region (R19−H47) connecting the N-terminal region and the upper part of the molecule (48-C-terminal). It is remarkable in this model that all the amino acid residues whose modification was inhibited by the presence of the membranes, except for K29, are almost in a plane (black residues in Figure 4). Since the ionic amino acid residues are difficult to embed into the hydrophobic region of the membranes, salt bridges might be formed between these amino acid residues and the ionic headgroups of the phospholipid molecules in the membranes. The effects of the ionic phospholipids on the P450 activities have been reported, which might be due to the interaction between ionic residues in the P450 and the phospholipids (*45*).

The inhibition of the acetylation at K29 and K59 is reasonable, because these residues are located in the region

connecting the N-terminal α-helix with the functional domain. E84 is accessible to water-soluble reagents and is the nearest amino acid residue to the N-terminal which is exposed in the aqueous solution in our results. D211, D212, and/or D216 are located near or in the putative F−G loop of P450 17α. Several studies have suggested that the F−G loop has an important role in the binding to membranes and also is localized near an entrance of the substrates (*25*, *26*). The hydrophobic substrate concentrated in the membranes by the partitioning between aqueous and membrane lipid phases might directly enter the substrate access channel through the entrance near the F−G loop. Previously, we suggested that the membrane-binding domains are located around the C-terminal side in P450 C21 on the basis of the effect on limited proteolysis of incorporation of the enzyme into liposomal membranes (*20*). The present study showed that liposome membranes inhibited chemical modification reactions at K490 and K492. The existence of the membrane-binding domains at the N- and C-terminals and the putative F−G loop can explain the high stability of P450 17α against proteolysis and heat and also the slow rotational diffusion relaxation time in the membranes (*21*).

Nishimoto showed that a cluster of acidic residues in NADPH−P450 reductase was interacting with lysine residues of cytochrome *c* in the process of electron transfer from NADPH to cytochrome *c* using a cross-linked covalent complex between the two proteins (*13*). FMN in NADPH−P450 reductase functions as storage of electrons for the acceptors cytochrome *c* and P450s and has been shown to exist near the surface of the reductase in a domain rich in acidic residues (*46*). A detailed structure analysis of P450 for the interaction with cytochrome b_5 and NADPH−P450 reductase was performed with site-directed mutagenesis in P450 2B4 by Bridges et al. (*14*). They reported that Arg and Lys residues in 122−139 and around 430 have important roles in the interaction with the reductase. We found the corresponding residues 125Arg, 126Arg, 136Arg, 141Lys, 440Arg, and 449Arg on the opposite side of P450 17α with respect to the membrane-binding sites of the molecule in the model in Figure 4. Sevriouskova et al. showed an important role for the C-helix (118−130) in a P450−redox partner electron-transfer complex in bacterial cytochrome P450 BM3 (*15*). The corresponding C-helix in P450 17α is shown by a black helix in Figure 4. Electrons might be transferred from the FNM of the reductase interacting on the opposite side of P450 17α with respect to the membranes, and the hydrophobic substrate concentrated in the membranes might enter the binding site from the membranes.

It must be pointed out that development of mass spectrometry leads to a sensitive and quick identification of modified peptides, and the method used in this experiment is powerful and convenient for the study of the topology of membrane proteins.

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