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Studies of membrane topology of mitochondrial cholesterol hydroxylases CYPs 27A1 and 11A1

Irina A. Pikuleva,
Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77555-1031, USA irpikule@utmb.edu

Natalia Mast,
Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77555-1031, USA

Wei-Li Liao, and
Center for Advanced Research in Biotechnology, National Institute of Standards and Technology and University of Maryland Biotechnology Institute, Rockville, MD 20850, USA

Illarion V. Turko
Center for Advanced Research in Biotechnology, National Institute of Standards and Technology and University of Maryland Biotechnology Institute, Rockville, MD 20850, USA

Abstract

Mitochondrial cytochrome P450 enzymes (CYP or P450, EC 1.14.13.15) play an important role in metabolism of cholesterol. CYP27A1 hydroxylates cholesterol at position 27 and, thus, initiates cholesterol removal from many extrahepatic tissues. CYP11A1 is a steroidogenic P450 that converts cholesterol to pregnenolone, the first step in the biosynthesis of all steroid hormones. We utilized a new approach to study membrane topology of CYPs 27A1 and 11A1. This approach involves heterologous expression of membrane-bound P450 in *E. coli*, isolation of the P450-containing *E. coli* membranes, treatment of the membranes with protease, removal of the digested soluble portion and extraction of the membrane-associated peptides, which are then identified by mass spectrometry. By using this approach, we found four membrane-interacting peptides in CYP27A1, and two peptides in CYP11A1. Peptides in CYP27A1 represent a contiguous portion of the polypeptide chain (residues 210-251) corresponding to the putative F-G loop and adjacent portions of the F and G helices. Peptides in CYP11A1 are from the putative F-G loop (residues 218-225) and the C-terminal portion of the G helix (residues 238-250). This data is consistent with those obtained previously by us and others and provide new information about membrane topology of CYPs 27A1 and 11A1.

Keywords

CYP27A1; CYP11A1; membrane topology; mass spectrometry analysis

Introduction

Cytochromes P450 (CYP or P450, EC 1.14.13.15) are obligatory enzymes in metabolism of cholesterol, steroid hormones and vitamin D_3, in mitochondria (1). We are interested in

Correspondence to: Irina A. Pikuleva.

Current address: Department of Ophthalmology and Visual Sciences, Case Western Reserve University and University Hospitals Case Medical Center, Cleveland, OH 44106, USA irina.pikuleva@case.edu

delineation of structure/function relationships in P450s that initiate cholesterol transformations to bile acids and steroid hormones. Cholesterol serves as the primary substrate for only four mammalian enzymes (CYPs 7A1, 46A1, 27A1, and 11A1), and two of them (CYPs 27A1 and 11A1) reside in mitochodria (2). CYP27A1 is a ubiquitously expressed multifunctional sterol 27-hydroxylase that catalyzes cholesterol 27-hydroxylation in many extrahepatic tissues. In addition, CYP27A1 also metabolizes bile acid intermediates in the liver and vitamin D_3 in the kidney (3). Partial or complete lack of CYP27A1 activity results in a disease cerebrotendinous xanthomatosis manifested by a variety of phenotypes including tendon xanthomas, bilateral cataracts, premature atherosclerosis, osteoporosis, neurological and neuropsychiatric abnormalities (4). Unlike CYP27A1, expression of CYP11A1 is limited to steroidogenic tissues and the brain, where it catalyzes the conversion of cholesterol to pregnenolone, the first step in overall steroid hormone biosynthesis (5). Partial or complete lack of CYP11A1 activity results in a disorder of steroidogenesis in which cholesterol accumulates within the steroidogenic tissues, and the synthesis of all adrenal and gonadal steroids is impaired (2). Several different approaches have been used previously to study membrane topology of mitochondrial P450s (6-10). Ou *et al*. treated mitochondria from the bovine adrenal cortex with trypsin and digested CYP11A1 present in these mitochondria into two fragments. Mitochondria were then washed with sodium carbonate followed by evaluation of the association of the two fragments with the membrane (6). Usanov *et al*. studied the interaction of the mitochondrion-associated CYP11A1 with antibodies to the whole enzyme and its fragments (7). We investigated CYPs 27A1 and 11A1 by combining heterologous expression in *E. coli* and site-directed mutagenesis (8,11). Residues in the F-G loop, a putative site of the interaction with the membrane, were mutated, the mutant P450s expressed in *E. coli* and their subcellular distribution and catalytic activities compared with those of the wild type enzyme. Simultaneously with our studies, Headlam *et al*. investigated the F-G loop as well as several other regions in CYP11A1 by utilizing cysteine mutagenesis followed by fluorescent labeling of the mutated residues and measurements of the changes in the fluorescence upon association of the mutant P450 with phospholipid vesicles. The N- and C-terminal truncated variants of CYP11A1 were also generated (10). Both groups suggested that the F-G loop is the site of interaction of mitochondrial P450s with the membrane. In the present investigation we continued elucidation of the membrane topology of mitochondrial P450s. We tested whether we can identify membrane-interacting areas in mitochondrial P450 other than the F-G loop by combining the methodologies of heterologous expression and proteolytic digestion with advances in mass spectrometry. CYPs 11A1 and 27A1 were expressed in *E .coli*, the *E. coli* membranes isolated and treated with trypsin. Peptides remaining after extensive washes of the trypsin-treated membranes with sodium carbonate were extracted and identified by MALDI MS. The data obtained suggest that not only the F-G loop but also a part of the G helix is embedded in the membrane in mitochondrial P450s.

Experimental Procedure

Materials

Sequencing grade modified trypsin was from Promega Corp. (Madison, WI, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Membrane fractionation

Recombinant human CYP27A1 and bovine CYP11A1 were expressed in *E. coli* as described (12,13). Cells were harvested by centrifugation at 10,000 *g* for 10 min, resuspended in 10 mM HEPES (pH 7.8) containing 10% sucrose, and treated with 0.2 mg/ml lysozyme on ice for 30 min. Spheroplasts were pelleted at 10,000 *g* for 10 min, resuspended in 10 mM HEPES (pH 7.8) containing 10% sucrose and sonicated using six 10 seconds pulses at 40% duty cycle (Sonifier 450, Branson Ultrasonics Corp., Dandury, CT). The suspension was then layered

over a cushion of 55% sucrose topped with 10% sucrose in 10 mM HEPES (pH 7.8) and subjected to centrifugation in a Ti70 rotor at 35,000 rpm for 60 min. The total membrane fraction was recovered from the 55% sucrose interface, washed twice with 25 mM NH_4HCO_3 (pH 7.9), and placed in pre-weighted tubes.

Trypsin treatment

Wet membrane pellet (5 mg) was resuspended in 1 ml of 25 mM NH_4HCO_3 (pH 7.9) followed by addition of 10 μg of sequencing grade modified trypsin. The suspension was sonicated as described above and left for proteolysis for 15 hrs at 37°C. The sample was then subjected to 106,000 *g* centrifugation for 20 min in Optima TLX ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA). Pellet was collected and washed sequentially with 100 mM Na_2CO_3 (pH 11.5) containing 0.3 M NaCl (5 times) and with water (1 time). Traces of water from the wet pellet were removed by adding 0.2 ml of methanol/chloroform mixture (4/1, v/v) and drying the sample in Vacufuge (Eppendorf AG, Hamburg, Germany). Dried pellet was then vortexed with 0.2 ml of chloroform, organic phase discarded, and the membranes dried again. Extraction of peptides from the dried membranes was carried out with 100 μl of 50% acetonitrile/0.1% trifluoroacetic acid (TFA). The extracted sample was further purified using ZipTip C_{18} (Millipore Corporation, Bedford, MA, USA) according to the manufacture protocol.

Mass spectrometry analysis

Peptide samples after ZipTip purification were dried, dissolved in 5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA, and manually spotted onto the ABI 01-192-6-AB target plate. Mass spectrometry (MS) analyses were performed using an AB4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). MS-mode acquisitions consisted of 1,000 laser shots averaged from 20 sample positions. In the MS/MS-mode acquisitions, 6,000 laser shots were averaged from 60 sample positions for PSD fragments. Automated acquisition of MS and MS/MS data was controlled by 4000 Series Explorer software 3.0.

Computational analysis

Free energy of transfer from water to bilayer interface (ΔG_{WW}) was computed using Totalizer module of MPEx (http://blanco.biomol.uci.edu/mpex) based on the Wimley-White hydrophobicity scale. A function of “no end groups” as a subsequence of a longer sequence was used for this calculation. Hydrophobic moment (μ_H) was also computed using the Totalizer module of MPEx.

Results

Identification of the membrane-interacting peptides in CYP27A1 and CYP11A1

Representative MS spectrum of the extract derived from trypsin-treated CYP27A1-containing *E. coli* membranes is shown in Fig. 1a. Manual analysis revealed 4 peptides with *m/z* $[M+H]^+$ values expected for the CYP27A1 hydrolysis with trypsin. Two of these peptides with *m/z* $[M+H]^+$ values 1329.72 and 1485.81 are labeled in Figure 1a. Signals for two other peptides with *m/z* $[M+H]^+$ values 1645.78 and 1929.95 were very small and could be seen only after zooming (not shown). In MALDI experiment, signal intensity does not linearly correlate with the concentration of analyte, therefore very small peaks were annotated as well. A summary of all the peptides identified in CYP27A1 is given in Table 1. The expected *m/z* $[M+H]^+$ values were calculated using MASCOT software and rounded to two decimals. The accuracy of mass measurement in *ppm* was calculated as a difference between the expected and observed value divided by observed value. Without the internal calibration, the observed *m/z* $[M+H]^+$ values

for these peptides deviate from the expected *m/z* $[M+H]^+$ values in a range of 40 ppm. Fig. 1b shows the MS/MS spectrum for the peptide ion with *m/z* $[M+H]^+$ value 1329.72. This spectrum has immonium ion for Trp and various *y*- and *b*-type fragment ions that collectively confirm the correct assignment of amino acid sequence. The MS analysis of the CYP11A1 sample revealed two CYP11A1 peptides. One of them with *m/z* $[M+H]^+$ value 1675.80 is labeled in Fig. 1c. The zoomed spectrum for the second peptide with *m/z* $[M+H]^+$ value 1107.59 is shown in Fig. 1d. The intensity for this peptide was very low.

Analysis of the identified peptides

Membrane-interacting protein sequences usually possess a unique combination of hydrophobicity and amphiphilicity. Therefore, the identified peptides were evaluated for two parameters that render peptides "membrane-seeking": free energy of transfer from water to bilayer interface and hydrophobic moment. Free energy of transfer (ΔG_{WW}) is a measure of solubility in non-polar phase with the positive value of ΔG_{WW} being unfavorable for partitioning the peptide sequence into the membrane interface. Hydrophobic moment (μ_H) detects periodicities in the hydrophobicity of amino acid sequences and is a measure of the amphiphilicity of a protein fragment (14,15). The ΔG_{WW} and μ_H values of the CYP27A1 and CYP11A1 peptides are shown in Table 1. All of the identified peptides except one, ^{238}AEKYTEIFYQDLR^{250}, have negative values of ΔG_{WW} and values of μ_H from 1.98 to 6.12. This is consistent with the fact that they were extracted from the membrane. The ^{238}A-R^{250} peptide has a positive ΔG_{WW} value but a high μ_H value which strongly increases the chances for this sequence to interact with the membrane. Trypsin can not cleave amino acid residues embedded in the membrane. Therefore, the identified peptides contain both membrane-bound and soluble regions. If the soluble region is too long, this may affect the calculated ΔG_{WW}, and could be the case with the ^{238}A-R^{250} peptide.

In addition to the computational analysis, peptides were examined for the presence of the missed cleavage sites. One may assume that the missed cleavage sites are located either in the membrane or close to the membrane surface, and therefore resistant to proteolysis. Two peptides in CYP27A1, ^{227}W-R^{237} and ^{238}Y-K^{251}, and one peptide in CYP11A1, ^{238}A-R^{250}, contain one missed cleavage site, and one peptide in CYP11A1, ^{218}L-R^{225}, has two missed cleavage sites. Also, the CYP27A1 peptides with *m/z* $[M+H]^+$ values 1329.72 and 1485.81 represent the same sequence without and with the trypsin missed cleavage site, respectively.

The position of the membrane-interacting peptides in the primary and secondary sequences of CYPs 27A1 and 11A1 is shown in Fig. 2. Peptides in CYP27A1 represent a contiguous portion of the polypeptide chain comprising the putative F-G loop and flanking portions of the F and G helixes. In CYP11A1, the ^{218}L-R^{225} peptide is located in the F-G loop and the ^{238}A-R^{250} peptide in the C-terminal portion of the G helix.

Discussion

In the current study, we used CYP27A1- and CYP11A1-containing *E. coli* membranes as a model system to study membrane topology of these mitochondrial enzymes. Previously, we developed *E. coli* expression for both proteins, showed that they stay associated with the membrane fraction when expressed in *E. coli*, and are catalytically active in the *E. coli* membranes (8,9,12,13). The membrane topology of CYPs 27A1 and 11A1 was then studied by selecting a hydrophobic region in their primary sequence, the F-G loop, and evaluating how mutations within this region affect binding to the membrane and activity of the mutant P450s (8,9). Weakened binding of some the mutants indicated that the F-G loop indeed interacts with the membrane. Mitochondrial P450s are suggested to have a peripheral association with the membrane (16,17). However, it is not currently clear whether one or several segments of the primary sequence are associated with the membrane. Headlam and colleagues began to address

this question by investigating three regions in CYP11A1, the F-G loop, A' helix, and the 14 amino acid residue-segment at the C-terminus (10). These regions were selected based on hydrophobicity profiling and secondary and tertiary structure predictions. The data obtained indicate the involvement of the F-G loop and possibly A' helix, but not the C-terminus of CYP11A1, in the interactions with the membrane. Thus, although the computational analysis provided a reasonable prediction, not all of the putative membrane-interacting regions were then validated by other methods. In the present study a principally different approach was undertaken. It includes the protease degradation of the solution-exposed portion of the membrane-associated CYPs 27A1 and 11A1 and subsequent MS analysis of the recovered membrane-bound peptides. Consistent with previous studies, peptide(s) from the F-G loop were extracted from the membrane in CYPs 27A1 and 11A1. Identification of these peptides lends support to the validity of our approach and serves as additional evidence for the role of the F-G loop. We also identified one peptide in CYP27A1 (^{238}Y-K^{251}) and one peptide in CYP11A1 (^{238}A-R^{250}) that are located outside the F-G loop, in the N- and C-terminal parts of the G helix, respectively. The N-terminal part of the G helix represents a continuation of the F-G loop. Therefore identification of the ^{238}Y-K^{251} peptide in CYP27A1 is not an unexpected finding. In fact, it provides an explanation to our previous observation that CYP27A1 is associated tighter with the *E. coli* membrane than CYP11A1 (8). A longer stretch of amino acid residues in the F-G loop region seem to interact with the membrane in CYP27A1 than in CYP11A1. Identification of the ^{238}A-R^{250} peptide in CYP11A1 is a more interesting finding because the C-terminal part of the G helix has never been considered as a potential membrane-binding site in CYP11A1. Further studies are required to confirm this unexpected finding. As any method, the MS-based approach has a limitation. This limitation is hidden in the procedure of analysis. Certain classes of long peptides with high hydrophobicity can escape MS analysis (18), therefore a set of identified peptides may never reach a full completeness. For example, when purified CYPs 27A1 and 11A1 are digested with trypsin in solution, we usually obtain peptides covering about 54% of the primary sequence. If each of the P450s is subjected to SDS PAGE and digested in-gel, the coverage of the primary sequence by the identified peptides is ~46%. When combined, tryptic peptides from solution and in-gel digestion cover ~65% of the primary sequence. Thus, MS studies with purified CYPs 27A1 and 11A1 raise a possibility that not all of the membrane-interacting peptides in CYPs 27A1 and 11A1 were identified in the present study. Nevertheless, our approach provided further insight into membrane topology of CYPs 27A1 and 11A1 and is a valuable addition to the other methods in the field.

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Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology (NIST) nor does it imply that the materials, instruments, or equipment identified is necessarily the best available for the purpose.

Abbreviations

CYP27A1	Cytochrome P450 27A1
CYP11A1	Cytochrome P450 11A1
MS	mass spectrometry
TFA	trifluoroacetic acid
MALDI MS	matrix assisted laser desorption ionization mass spectrometry

TOF	time-of-flight

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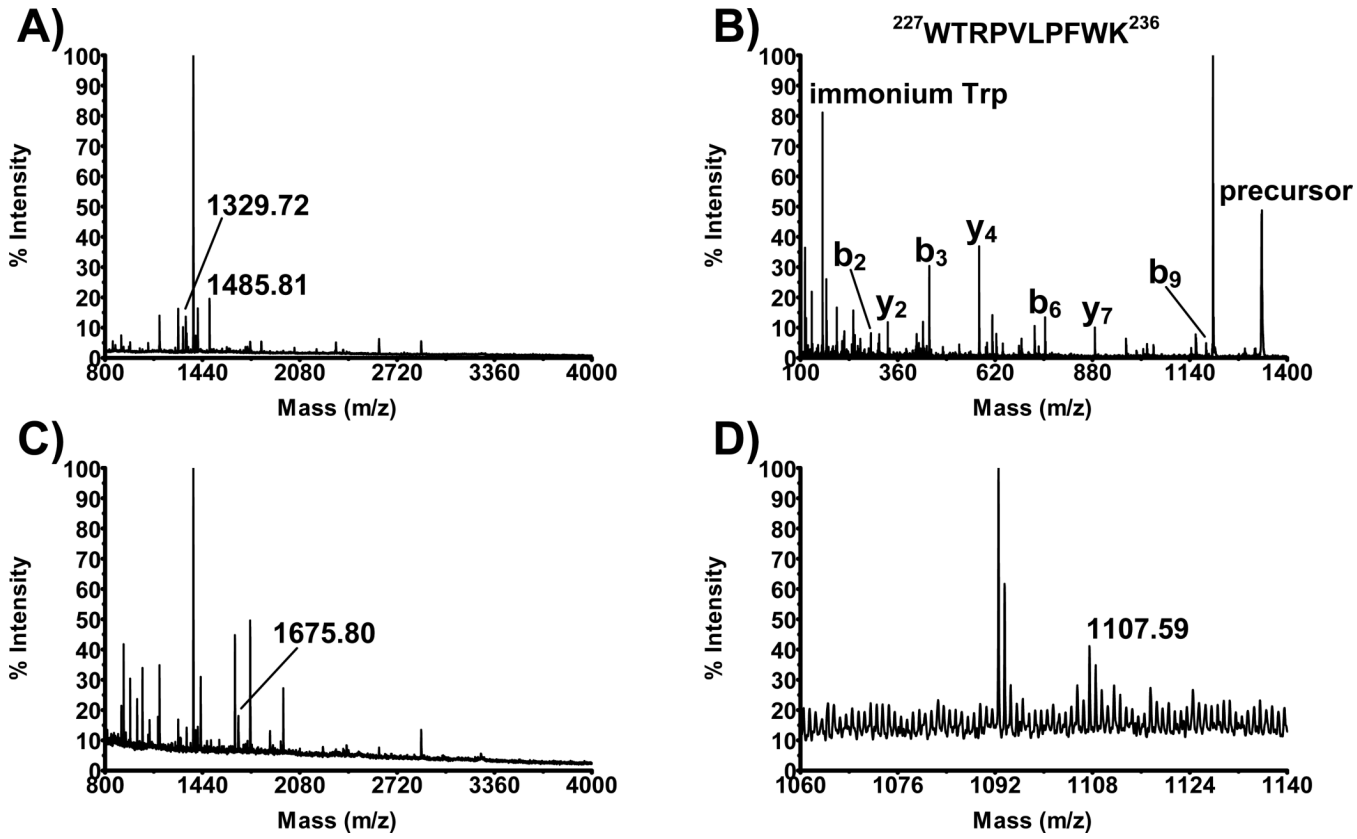

Fig. 1.

Membrane-interacting peptides in CYP27A1 and CYP11A1. A) MS spectrum of the extract from the trypsin-treated CYP27A1-containig *E. coli* membranes. Peptides with m/z $[M+H]^+$ values 1329.72 and 1485.81 correspond to CYP27A1, all the other peptides are of the *E. coli* origin. B) MS/MS spectrum of the peptide ion with m/z $[M+H]^+$ value 1329.72 that corresponds to the ^{227}WTRPVLPFWK^{236} peptide in CYP27A1. Multiple fragment ions matching to this sequence are labeled as follows: b_2, WT; b_3, WTR; b_6, WTRPVL; b_9, WTRPVLPFW; y_2, WK; y_4, PFWK; and y_7, PVLOFWK. C) and D) Regular and zoomed MS spectrum of the extract from the trypsin-treated CYP11A1-containig *E. coli* membrane. Peptides with m/z $[M+H]^+$ value 1675.80 and 1107.59 correspond to CYP11A1.

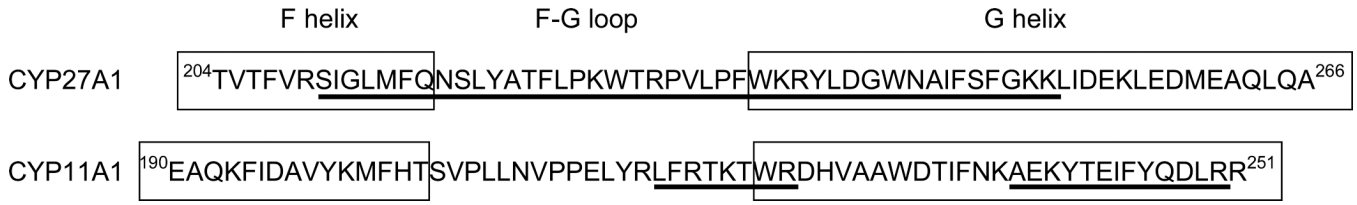

Fig. 2.

Location of the membrane-interacting peptides (underlined) in the primary and secondary structures of CYPs 27A1 and 11A1. Boxes indicate putative F and G helices. The secondary structure consensus prediction program (http://npsa-pbil.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_seccons.html) was used to identify these structural elements.

Table 1

Membrane-interacting peptides in CYPs 27A1 and 11A1. Missed cleavage sites are underlined.

Peptide	$[M+H]^+$ expected	$[M+H]^+$ observed	Delta (*ppm*)	ΔG_{WW} (kcal mol^{-1})[a]	Hydrophobic moment (μ_H)[a]
CYP27A1					
^{210}SIGLMFQNALYATFLPK^{226}	1930.01	1929.95	31	-2.36	1.98
^{227}WTRPVLPFWK^{236}	1329.75	1329.72	23	-2.48	5.25
^{227}WTRPVLPFWKR^{237}	1485.85	1485.81	27	-1.67	5.59
^{238}YLDGWNAIFSFGKK^{251}	1645.84	1645.78	36	-1.97	6.12
CYP11A1					
^{218}LFRTKTWR^{225}	1107.64	1107.59	45	-0.65	1.39
^{238}AEKYTEIFYQDLR^{250}	1675.83	1675.80	18	4.08	6.11

[a] Free energy of transfer from water to bilayer interface based on the Wimley-White hydrophobicity scale (ΔG_{WW}) and hydrophobic moment (μ_H) were computed using Totalizer module of MPEx (http://blanco.biomol.uci.edu/mpex).