Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 468 (2007) 226–233
ABB
www.elsevier.com/locate/yabbi

Lateral segregation of anionic phospholipids in model membranes induced by cytochrome P450 2B1: Bi-directional coupling between CYP2B1 and anionic phospholipid

Keon-Hee Kim [a], Dong-Hyun Kim [a], Hyun-Hee Jang [a], Mihee Kim [a,b], Dae-Hwan Kim [a], Joon-Sik Kim [c], Jeong-Il Kim [d], Ho Zoon Chae [a], Taeho Ahn [b,*], Chul-Ho Yun [a,*]

[a] *School of Biological Sciences and Technology and Hormone Research Center, Chonnam National University, Gwangju 500-757, Republic of Korea*
[b] *Department of Biochemistry, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea*
[c] *Peptron, Inc., Daejeon 305-340, Republic of Korea*
[d] *Department of Biotechnology and Kumho Life Science Laboratory, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Republic of Korea*

Received 6 August 2007, and in revised form 2 October 2007
Available online 12 October 2007

Abstract

The lateral segregation of anionic phospholipids phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylserine (PS) was detected after addition of cytochrome P450 2B1 (CYP2B1). The tendency of lipid clustering was highly dependent on the type of anionic phospholipids examined. PA was the most highly clustered while PI and PS clustered to a lesser degree. Moreover, liposomes containing anionic phospholipids form anionic phospholipid-rich microdomains in the presence of CYP2B1. Anionic phospholipids (mostly notably PA) also increased the ability of CYP2B1 to bind to lipid monolayers. In addition to the ability of CYP2B1 to modulate the physical properties of the membrane, the membrane itself can have reciprocal effects on the activity and conformation of CYP2B1. The catalytic activity of CYP2B1 increased as a function of anionic phospholipid concentration and in the presence of 10 mol% PA, the activity increased by 85%. These results suggest a bi-directional coupling between the CYP2B1 and anionic phospholipids.

Keywords: Rat cytochrome P450 2B1; Anionic phospholipids; Phase separation; Phosphatidic acid; Catalytic activity; Protein conformation

The cytochrome P450 (P450 or CYP)[1] enzymes are a superfamily of proteins that play an important role in the oxidation of many foreign chemicals and endogenous compounds [1,2]. The majority of P450s are membrane-anchored proteins that must be incorporated into the lipid bilayer of the ER. Studies on microsomes have shown that P450s are located on the cytosolic face and their N-terminal hydrophobic domains are capable of insertion into the membrane. The microsomal monooxygenase system includes P450, CPR, phospholipids, and at times, cytochrome b_5 and NADH-cytochrome b_5 reductase [3]. P450-dependent activities can be reconstituted in vitro by

* Corresponding authors. Fax: +82 62 530 1919 (C.H. Yun).
E-mail addresses: thahn@jnu.ac.kr (T. Ahn), chyun@jnu.ac.kr (C.-H. Yun).

[1] *Abbreviations used:* P450 or CYP, cytochrome P450; CPR, NADPH-P450 reductase; PC, phosphatidylcholine; PE, phosphatidylethanolamime; PS, phosphatidylserine; PA, phosphatidic acid; PI, bovine heart phosphatidylinositol; NBD-labeled phospholipids, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzodiazol-4-yl)-amino]dodecanoyl]-*sn*-glycero-3-phosphoglycerides; pyrene-labeled phospholipids, 1-palmitoyl-2-(pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerides; BODIPY-labeled phospholipids, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoglycerides; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; LUVs, large unilamellar vesicles; SUVs, small unilamellar vesicles; ER, endoplasmic reticulum; EFC, 7-ethoxy-4-(trifluoromethyl)coumarin.

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.10.003

mixing purified P450, CPR, and phospholipids [4,5]. P450 and CPR are distributed randomly on the plane of membranes, and they interact through lateral diffusion [6,7].

The functional properties of many proteins in biological membranes, including ER membranes, seem to be closely related to the microenvironment provided by membrane lipids [8,9]. Phospholipids in the vicinity of P450 in rabbit liver microsomes have been suggested to be highly organized as compared with those in bulk membranes [10]. Moreover, it has been proposed that the specific interaction of phospholipids with P450 may be necessary for maintaining its active protein conformation, for interactions with CPR, and for efficient electron transfer [11]. Although the significance of P450's interaction with phospholipids for its function has been suggested, a specific mechanism is not yet fully understood. Previous work has shown that anionic phospholipids are required for the optimal activities of several microsomal P450s including 1A2 [12], 2B1 [13], and 3A4 [14].

Membranes composed of mixed phospholipids (and other lipids) can form membrane microdomains with locally unique surface properties. A classic example of the microdomains is the formation of cholesterol- and sphingolipid-rich lipid rafts and caveolae on cell surfaces [15,16]. Liposomes containing simple binary mixtures of neutral and anionic phospholipids spontaneously form anionic phospholipid-rich membrane microdomains only in the presence of calcium ions, which can become even more pronounced in the presence of membrane-binding proteins [17,18]. Thus, anionic phospholipids can coalesce via lateral diffusion over relatively large distances into subdomains several microns in size.

In this report, we have studied the influence of CYP2B1 on the organization of membranes and conversely, the effect of membrane composition on the CYP2B1 activity.

Materials and methods

Materials

All phospholipids and NBD-labeled phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Pyrene- and BODIPY-labeled phospholipids were purchased from Molecular Probes (Eugene, OR). The lipids were stored as chloroform solutions under argon at −20 °C. All other chemicals were of the highest grade commercially available.

Protein purification

Rat CYP2B1 was purified from liver microsomes of phenobarbital-treated male rats as described previously [19]. Purified CYP2B1 was electrophoretically homogeneous and had a specific P450 content of 12 nmol/mg of protein. Protein was assayed using a bicinchoninic acid procedure (Sigma). The concentration of P450 was determined by Fe^{2+}-CO *versus* Fe^{2+} difference spectroscopy [20]. Recombinant rat CPR was expressed in *Escherichia coli* and purified as described [21].

CYP2B1 was made metal-free by extensive dialysis against a 100 mM potassium phosphate (pH 7.4) buffer containing 0.1 mM EDTA and 20% glycerol. Inductively coupled plasma massspectroscopy analysis was performed on a VQ3 ICP-MS (Thermo Elemental, Cambridge, UK) in the Korea Basic Science Institute facility. Purified enzymes contained no detectable metals. The measured values for all enzymes were less than 0.01 of Cu^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, or Mn^{2+} per enzyme subunit (beside heme iron). The buffers used for all experiments were metal-ion free by passage through a Chelex column and storage in EDTA-treated glassware.

Liposome preparation

In all experiments, POPC liposomes were used as a standard vesicle. For fluorescent liposomes, 1 mol% pyrene-labeled anionic phospholipids, 1 mol% BODIPY-labeled anionic phospholipids, or 5 mol% NBD-labeled anionic phospholipids were incorporated instead of normal PC. To study the penetration and binding of CYP2B1 to membranes by resonance energy transfer, 2 mol% of pyrene-PA (or pyrene-PS and PI) were incorporated into membranes. In all experiments using model membranes, PA, PS, and PI were incorporated into the membrane at a final concentration of 5 mol%.

After mixing the appropriate amount of lipids in chloroform, the solvent was evaporated under a stream of argon gas. The dry lipids were hydrated in a buffer solution (25 mM Tris–HCl, pH 7.4, containing 100 mM NaCl) by vortexing and subsequently sonicating for 30 s. To obtain homogeneous LUVs, the dispersion was frozen and thawed five times and extruded 25 times through two polycarbonate membranes (100 nm pore size). All LUVs used for this work were stable for at least 3 days as determined by <10% deviation in light scattering values. Using this same method, the possibility for protein-induced aggregation of vesicles was also tested.

The concentration of liposome stock solution was 0.2–1.0 mM, and each solution was diluted to study the interaction of CYP2B1 with membranes. The concentrations of non-fluorescent phospholipids were determined by a phosphorus assay [22]. The concentrations of the fluorescent probes were determined spectrophotometrically at 342 nm for pyrene-lipids, at 465 nm for NBD-lipids, and at 500 nm for BODIPY-lipids with the molar extinction coefficients of 38,000, 22,000, and 80,000 cm^{-1}, respectively.

Monolayer experiments

Monolayer surface pressures were measured as described previously [23]. The Teflon dishes had a volume of 12 ml and a surface area of 20.3 cm^2. The sub-phase buffer used was 50 mM Tris–HCl (pH 7.4) containing 100 mM NaCl. The lipid layers were spread from a chloroform solution to give an initial surface pressure of 18 mN/m^2 at 30 ± 0.2 °C. The sub-phase (total 5 ml) was injected with CYP2B1 in the presence or absence of lipid monolayers. Any excess CYP2B1 present in the sub-phase was washed away with 10 times the sub-phase volume after 30 min. Pressure changes were measured until the surface pressure reached a maximal value.

Fluorescence measurements

All fluorescence experiments were performed at 30 °C and samples (500 μl of sample volume) were maintained in a circulating water bath for 5 min before measurements were taken. Fluorescence emission spectra were recorded with a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostated cuvette compartment using a quartz cuvette. The emission fluorescence of the Trp and Tyr residues in CYP2B1 were measured at 341 nm after exitation at 280 nm. Excimer (E) and monomer (M) fluorescence of pyrene-containing liposomes were measured over the range of 360–500 nm after excitation at 342 nm. The E/M ratio of pyrene-labeled phospholipids was calculated from the fluorescence intensity measurements at 375 nm (for the monomer) and 480 nm (for the excimer). The emission fluorescence of NBD-labeled phospholipids was measured at 534 nm with an excitation wavelength of 465 nm using a 500 nm cutoff filter. In all fluorescence experiments, each measurement under the experimental condition was corrected for inner filter effect due to light scattering and absorption, as described elsewhere [24].