Published in final edited form as:

Biochem J. ; 474(20): 3523–3542. doi:10.1042/BCJ20170543.

Towards a systems approach to the human cytochrome P450 ensemble: Interactions between CYP2D6 and CYP2E1 and their functional consequences

Dmitri R. Davydov^{1,*}, Nadezhda Y. Davydova¹, John T. Rodgers¹, Thomas H. Rushmore², and Jeffrey P. Jones¹

¹Department of Chemistry, Washington State University, Pullman, WA, 99164

²Department of Pharmaceutical Sciences, University of the Sciences, Philadelphia, PA 19104

Abstract

Functional cross-talk among human drug-metabolizing cytochromes P450 through their association is a topic of emerging importance. Here we studied the interactions of human CYP2D6, a major metabolizer of psychoactive drugs, with one of the most prevalent human P450 enzymes, ethanol-inducible CYP2E1. Detection of P450-P450 interactions was accomplished through luminescence resonance energy transfer (LRET) between labeled proteins incorporated into human liver microsomes and the microsomes of insect cells containing NADPH-cytochrome P450 reductase. The potential of CYP2D6 to form oligomers in the microsomal membrane is among the highest observed with human cytochromes P450 studied up to date. We also observed the formation of heteromeric complexes of CYP2D6 with CYP2E1 and CYP3A4, and found a significant modulation of these interactions by MDMA, a widespread drug of abuse metabolized by CYP2D6. Our results demonstrate an ample alteration of the catalytic properties of CYP2D6 and CYP2E1 caused by their association. In particular, we demonstrated that pre-incubation of microsomes containing co-incorporated CYP2D6 and CYP2E1 with CYP2D6-specific substrates resulted in considerable time-dependent activation of CYP2D6, which presumably occurs via a slow substrate-induced reorganization of CYP2E1-CYP2D6 heterooligomers. Furthermore, we demonstrated that the formation of heteromeric complexes between CYP2E1 and CYP2D6 affects the stoichiometry of futile cycling and substrate oxidation by CYP2D6 by means of decreasing the electron leakage through the peroxide-generating pathways. Our results further emphasize the role of P450-P450 interactions in regulatory cross-talk in human drug-metabolizing ensemble and suggest a role of interactions of CYP2E1 with CYP2D6 in pharmacologically important instances of alcohol-drug interactions.

*Corresponding author: Dmitri R. Davydov, Department of Chemistry, Washington State University, 100 Dairy Rd, 99164-0202; Tel. +1 509 335-5983; dmrdavyd@gmail.com.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: DRD designed the study and wrote the paper, DRD, ND, JPJ and JR conducted the experiments; THR designed the insect cell microsomes used in the study; DRD and JPJ analyzed the results.

Keywords

cytochrome P450; protein-protein interaction; allosteric regulation; membrane protein; drug metabolism

The system-wide parameters of the drug-metabolizing cascade of human liver are largely determined by the properties of the ensemble of cytochromes P450, which is responsible for metabolism of over 75% of all marketed drugs and new drug candidates (1–3). It has been long recognized that the inter-individual, age-dependent and temporal variations in drug metabolism are largely dictated by the respective changes in the composition of the ensemble of multiple drug-metabolizing cytochromes P450. However, in spite of the initial premise that the cumulative properties of this ensemble represent a simple aggregate of the properties of the constituting enzymes, there is a growing body of evidence that suggests important non-additive contribution of mutual functional effects of multiple P450 species. Thus, it becomes increasingly evident that the catalytic properties of the ensemble of drug-metabolizing cytochromes P450 cannot be ascertained from a simple summation of the properties of the contributing P450 species (4–6).

According to the modern concept, the properties of this ensemble are largely affected by physical interactions of multiple P450 species and formation of their heteromeric complexes (7–12). Growing evidence of functionally-consequential high-affinity interactions between various cytochrome P450 species (9–13) points out the association of dissimilar P450s as an important determinant of the catalytic properties of the P450 ensemble. Thus, further elucidation of the mechanistic basis and functional consequences of the interactions between different P450 species is of vital importance for elaborating the systems biology approaches to predicting the fate of foreign chemicals in human body and understanding the changes in drug metabolizing profile under influence of developmental, temporal and environmental factors.

Despite quite a few studies of the effects of interactions in several pairs of drug-metabolizing cytochromes P450 (14–22), our general comprehension of the mechanisms of P450-P450 interactions, their functional effects and physiological significance remains sparse. Further systematic studies of the ability of various combinations of human drug-metabolizing P450 to form heteromeric complexes and detailed exploration of functional consequences of their interactions are of critical importance for establishing integrative approaches to analyzing human drug metabolism.

An important prerequisite for these studies is an availability of an appropriate *in vitro* model system that reproduces the native membrane environment where proteins interact via dynamic encounters. An ultimate model of that kind would allow easy variation in the composition of the multienzyme P450 ensemble and its concentration in the membrane. In a search for such a system we introduced a method based on incorporation of purified P450 proteins into insect cell microsomes (ICM) containing recombinant CPR, but lacking any P450 enzymes. Incubation of these microsomes designated here as ICM(CPR) with purified cytochromes P450 results in efficient incorporation of these proteins and yields catalytically competent microsomal systems. Varying the composition of the mixture of purified P450s

and their amount added to ICM(CPR) gives membranes with the desired P450 concentration and composition (15,23).

In a recent study we combined this new approach with our LRET-based method for detecting P450-P450 interactions to explore oligomerization of cytochromes 3A4, 3A5 and 2E1 and demonstrated that all three proteins are prone to oligomerization in the microsomal membrane (15). The same kind of experiments applied to pairwise P450 combinations demonstrated high affinity interactions in CYP2E1/CYP3A4, CYP2E1/CYP3A5 and CYP3A4/CYP3A5 pairs and revealed their significant functional effects (15).

Here we extended the use of this methodology to human CYP2D6 and its pairs with CYP2E1 and CYP3A4. Functional effects of interactions of CYP2D6 with ethanol-inducible CYP2E1, the most abundant P450 enzyme in human liver (6), are of special interest due to the role played by CYP2D6 in the metabolism of psychoactive agents including those known of clinically important interactions with alcohol (24). In particular, the role played by CYP2D6 in the metabolism of MDMA (ecstasy) (25–27) suggests a potential involvement of CYP2D6-CYP2E1 interactions in exacerbation of hepato- and neurotoxicity of this widespread drug of abuse by alcohol consumption (28).

In addition to exploring P450-P450 interactions in model systems based on ICM, we further developed our methodology by probing a possibility to vary the composition of the P450 ensemble in human liver microsomes (HLM) by co-incorporating additional amounts of selected cytochromes P450 into their membrane. These experiments demonstrated efficient incorporation of purified human CYP2E1 and CYP2D6 into HLM and permitted the detection of the formation of their homo- and heterooligomers by LRET in HLM. Taken together our results exhibit a high degree of homo- and heterooligomerization of CYP2D6 in human microsomes, reveal profound effects of interactions between CYP2D6 and CYP2E1 on their functional properties and demonstrate a high potential of our methods for further studies of drug metabolism by the multienzyme P450 ensembles.

Experimental

Materials

7-Hydroxy-4-(trifluoromethyl) coumarin, glucose-6-phosphate, protocatechuate 3,4-dioxygenase from *Pseudomonas sp.*, protocatechuic acid, DL-dithiothreitol (DTT) and L- α -phosphatidylcholine from egg yolk were the products of Sigma-Aldrich (St. Louis, MO). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and NADPH tetrasodium salt were from EMD Millipore (Billerica, MA). L- α -phosphatidylethanolamine from bovine liver and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (phosphatidic acid) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 7-methoxy-4-(trifluoromethyl) coumarin (7-MFC), and 3-[2-(N, N-diethyl-N-methyl ammonium)ethyl]-7-hydroxy-4-methylcoumarin (AHMC) were the products of Corning Life Sciences (Corning, NY). 3-[2-(N, N-diethyl-N-methyl ammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC) was the product of BioVision Inc. (Milpitas, CA). Erythrosine 5'-iodoacetamide (ERIA) was from AnaSpec (San Jose, CA, USA). DY-731 maleimide (DYM) was obtained from Dyomics (MoBiTec; Göttingen, Germany). Octyl- β -D-glucopyranoside

(octylglucoside) was the product of Fluka (Switzerland). Bufuralol hydrochloride and 3,4-methylenedioxymethylamphetamine (MDMA) hydrochloride were the products of Cayman Chemical (Ann Arbor, MI). MDMA was obtained as a certified reference material (CRM) solution in methanol (1 mg/ml), which was evaporated and the chemical was re-dissolved in acetone to the concentration of 25 mM. All other chemicals were of ACS grade and were used without further purification.

Protein expression and purification

Recombinant NADPH cytochrome P450 reductase (CPR) from rat liver was expressed in *E. coli* Topp3 cells and purified as described (29). N-terminally truncated (3–20) and C-terminally His-tagged CYP2E1 (30) was expressed in *E. coli* and purified following the procedure similar to that described for CYP2B6 (31). The plasmid expressing N-terminally modified construct of CYP2D6 identical to that described by Wang and co-authors (32) was a generous gift of Dr. Thomas Pochapsky. The protein was expressed in *E. coli* DH5 α transformed with pGro7 chaperone plasmid encoding groES-groEL chaperones (Takara Bio Inc., Japan). The procedure of expression and cell growth was similar to that described by Wang and co-authors (32). After treatment with lysozyme and precipitation (31) the cell pellets were resuspended in 100 mM Na-Hepes buffer, pH 7.4, containing 3 mM TCEP, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ M leupeptin, 700 mM KCl and 20% v/v glycerol (Buffer A) and sonicated on ice. After addition of 10% solution of CHAPS to the final concentration of 14 mM (0.86%) the suspension was incubated at continuous stirring for 3 h at 4°C and centrifuged for 1 h at 105,000g. After addition of 1 mM PMSF, solid ATP to the concentration of 5mM and MgCl₂ (as 2 M stock) to the concentration of 20 mM, the supernatant was loaded onto a column of His-Pur Ni-NTA resin (Thermo Scientific, Rockford, IL) taken in the amount of 1 ml per 60 nmols of P450 and pre-equilibrated with Buffer A containing 0.86% CHAPS. The column was successively washed with 10 volumes of buffer A containing 0.5% CHAPS, 5 mM ATP and 20 mM MgCl₂ and 5 volumes of Buffer A with the concentration of KCl decreased to 150 mM and containing 0.5% CHAPS and 7.5 mM imidazole. The P450 was eluted with 100 mM Na-Hepes, pH 7.4, containing 10% glycerol, 3 mM TCEP, 150 mM KCl, pH 7.4 (Buffer B) containing 0.5% CHAPS and 150 mM histidine. The fractions containing P450 were combined, diluted 5 times by water containing 10% glycerol (v/v), 3mM TCEP and 0.5% CHAPS and applied onto a column of Macro-Prep® CM resin (Bio-Rad, Hercules, CA) taken in the amount of 1 ml per 100 nmols of P450. The column was washed with 10 volumes of buffer B containing 0.5% CHAPS and eluted with 0 – 300 mM linear gradient of KCl in the same buffer (25–30 volumes of gradient per volume of column). The protein starts to elute at 70 mM KCl and the peak of the elution band is observed around KCl concentration of 110 mM. The P450-containing fractions were combined, concentrated to the concentration of 100 – 150 μ M and dialyzed against Buffer B. CHAPS was removed using Detergent-Out Medi columns (EMD Millipore).

Modification of the cytochrome P450 proteins with thiol-reactive probes ERIA and DYM was performed as described earlier (15).

Preparation of proteoliposomes

Proteoliposomes containing NADPH cytochrome P450 reductase were obtained by the octylglucoside dialysis/sorption technique as described earlier (15). Concentrated stock of purified NADPH cytochrome P450 reductase (100–200 μM) was added to the lipid solution to attain the protein:lipid ratio of 1:1000 prior to starting dialysis. Incorporation of cytochromes P450 into pre-formed CPR-containing protoliposomes was performed by incubation of a concentrated proteoliposomal suspension (8–10 mM by phospholipid content) in 100 mM Na-Hepes buffer, pH 7.4, containing 150 mM KCl, 3 mM TCEP and 20% glycerol (v/v) (LS buffer) with purified cytochromes P450 added as a concentrated stock solutions (100–200 μM) to a desired P450:lipid ratio ($R_{L/P}$). Incubation of these mixtures for 6 h at 4 °C at continuous stirring under argon atmosphere resulted in complete incorporation of the added protein into the membrane. The proteoliposomes were sedimented by centrifugation at 105,000g for 3 h at 4 °C and resuspended in a small volume of the LS buffer.

The content of phospholipids in membranous preparations (proteoliposomes and microsomes) was quantified based on the determination of total phosphorus in a chloroform/methanol extract according to Bartlett (33).

The concentration of NADPH-cytochrome P450 reductase in microsomal and proteoliposomal membranes was determined based on the rate of NADPH-dependent reduction of cytochrome *c* at 25°C (34) monitored by an increase in the difference in absorbencies at 550 and 541 nm and calculated using the extinction coefficient of 18 mM⁻¹cm⁻¹ (35). Effective molar concentration of CPR was estimated using the turnover number of 3500 min⁻¹, which we determined with our preparations of purified CPR quantified from its absorbance at 456 nm using the extinction coefficient of 21.4 mM⁻¹ cm⁻¹ (36).

Preparation of the model microsomes

The microsomes of Sf21 insect cells co-infected with baculovirus containing the cDNA for human NADPH-cytochrome P450 and CYP2D6 or CYP2E1 were produced by the Department of Drug Metabolism, Merck Research Labs (West Point, PA) (37). These CYP2D6 and CYP2E1-containing insect cell microsomes are designated here as ICM(2D6) and ICM(2E1), respectively. The insect cell microsomes containing rat recombinant NADPH-cytochrome P450 reductase (“control” SupersomesTM, cat. number 456514) were obtained from Corning Life Sciences (Corning, NY). These microsomes we designate in this paper as ICM(CPR). Incorporation of purified cytochromes P450 into the above microsomal preparations was performed by incubation of an undiluted microsomal suspension (5 mg/ml protein, 2–3.5 mM phospholipid) with purified CYP2D6, CYP2E1 or their mixture added to desired lipid:P450 ratio. After incubation for 16 h at 4°C at continuous stirring under argon atmosphere, the suspension was diluted 8 times with 100 mM K-Phosphate buffer, pH 7.4 and centrifuged at 105,000 rpm for 90 min at 4 °C. The pellet was resuspended in LS buffer (400 μl per 500 μl of initial microsomal suspension).

Activity measurements with fluorogenic substrates

The rate of *O*-demethylation of 7-MFC and AMMC were measured with a real-time continuous fluorometric assay. A suspension of microsomes or proteoliposomes was added to 300 μ l of 0.1 M Na-HEPES buffer, pH 7.4, to a final P450 concentration of 0.02 – 0.1 μ M. The mixture was placed into a 5 $\times$ 5 mm quartz cell with continuous stirring and thermostated at 30 $^{\circ}$ C. An aliquot of a 12–20 mM stock solution of substrate (AMMC or 7-MFC) in acetone was added to attain the desired concentration in the range of 1 – 200 μ M. The reaction was initiated by addition of 10 μ l of a cocktail of NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, which resulted in final concentrations of these ingredients of 200 μ M, 10 mM, and 2 units/ml respectively. An increase in the concentration of the products (AHMC or 7-HFC) was monitored with a Cary Eclipse fluorometer (Agilent Technologies, Santa Clara, CA, USA) or a custom-modified PTI QM-1 fluorometer (Photon Technology International, New Brunswick, NJ). The modified PTI instrument was equipped with a Hamamatsu H9059 photon-counting photomultiplier module (Hamamatsu Photonics, Japan) and a custom data acquisition software. With both instruments the emission wavelength was set to 500 or 470 nm (for 7-MFC and AMMC, respectively) with 20 nm slit. The excitation was performed with a monochromatic light centered at 405 nm with 5 nm bandwidth (Cary Eclipse) or with a light emitted by a 405 nm CPS405 laser diode module (Thorlabs Inc, Newton, NJ) combined with a 400 nm long-pass filter (PTI QM-1). The rate of formation of the fluorescent products was estimated by determining the slope of the linear part of the kinetic curve recorded over a period of 3 – 5 min. Calibration of the assay was performed at the end of each day by measuring the intensity of fluorescence in a series of 4–5 samples of the same reaction mixture containing HFC or AHMC at concentrations from 0.1 to 5 μ M.

Activity measurements with bufuralol were performed in 0.1 M Na-HEPES buffer, pH 7.4. The reaction was started by addition of NADPH to the final concentration of 1 mM and carried on for 10 min at 30 $^{\circ}$ C. Bufuralol was added as a 20mM solution in water. Reaction was quenched with an addition of 25% of the incubation volume of 1 M solution of formic acid in acetonitrile containing a known concentration phenacetin as internal standard, followed by centrifugation at 9,300 g for 10 min.

The amount of formed 1'-hydroxybufuralol was quantified by liquid chromatography-mass spectrometry. An LC-20AD series high-performance liquid chromatography system (Shimadzu, Columbia, MD) fitted with a HTC PAL autosampler (LEAP Technologies, Carrboro, NC) was used to perform chromatography on a Luna $^{\circ}$ reverse-phase column (50 $\times$ 2.0 mm, 5 μ m; Phenomenex, Torrance, CA), using a flow rate of 400 μ L/min. Chromatographic separation was performed using a linear gradient over the next 2.2 min to 25% mobile phase A. Mobile phase A was then held constant at 25% over 0.1 min, followed by a linear gradient back to 90% A over 0.7 min. Finally, the column was re-equilibrated to the initial conditions over the last 0.9 min. The quantification of the metabolite was conducted using an API 4000 Q-Trap tandem mass spectrometry system manufactured by Applied Biosystems/MDS Sciex (Foster City, CA) using turbospray ESI operating in positive ion mode. The analyte (1'-hydroxybufuralol) and the internal standard (phenacetin) were detected using multiple reaction monitoring mode by monitoring the *m/z* transition

from 278.31 to 186.2 and 180.2 to 110.1, respectively. Quantitation of product was achieved with a calibration curve ranging from 0 to 200 nM concentration of 1'-hydroxybuturalol.

Phosphorescence spectroscopy measurements were performed with the use of a Cary Eclipse spectrofluorometer equipped with a Peltier 4-position cell holder. The excitation of donor phosphorescence was performed with monochromatic light centered at 540 nm with 20 nm bandwidth. The spectra in the 620 – 850 nm wavelength region were recorded with 20 nm emission bandwidth and 250 μs delay time.

Interactions of DYM- and ERIA-labeled cytochromes P450 were judged from the kinetics of increase in the amplitude of LRET assessed from a series of spectra of delayed emission taken in 2 min time intervals during co-incubation of DY-labeled proteins with membranes containing an erythrosine-labeled enzyme. The incubation was performed at 4 °C under anaerobiosis, which was achieved by addition of the oxygen-scavenging enzyme protocatechuate 4,5-dioxygenase (1 unit/ml) and protocatechuic acid (20 mM). The experiments were performed in 0.1 M Na-Hepes buffer, pH 7.4, containing 150 mM KCl and 20% glycerol. The total P450 concentration in the incubation media was in the range of 0.2 – 1.5 μM.

Data analysis

Analysis of series of spectra obtained in fluorescence spectroscopy experiments was done by principal component analysis (PCA) (38), which was used as a method of global analysis that minimizes the contribution of incidental variation in the shape of the spectra and makes the results more robust to a minor displacement of the DY-731 emission band (a blue shift of approx. 3–5 nm) that takes place upon the interactions of DY-731-labeled proteins with the membrane and/or with the erythrosine-labeled protein partner. As a rule, the LRET-attributable changes were represented solely by the first principal component whose contribution to the total spectral changes was in the range of 98–99.5%. To resolve the changes in the fluorescence of the donor and acceptor we used a linear least-squares fitting of the spectra of the first principal component by a combination of the standard spectra of emission of ERIA and DYM.

The equation for the equilibrium of binary association (dimerization) used in the fitting of oligomerization isotherms (dependencies of LRET efficiency on the concentration of P450 in membranes) had the following form:

$$[X] = \frac{1}{2} \cdot K_D + [E]_0 - \sqrt{\frac{1}{4} \cdot K_D^2 + [E]_0 \cdot K_D} \quad (1)$$

where $[E]_0$, $[X]$, and K_D are the total concentration of the associating compound (enzyme), the concentration of its dimers, and the dissociation constant, respectively.

Alternatively, we fitted the oligomerization isotherms with the equation for equilibrium of the formation of enzyme trimers (trimerization)(15):

$$[X] = \frac{[E]_0}{3} - \frac{C_{50}}{\sqrt{27}} \cdot \sinh(\phi);$$

$$\phi = \frac{1}{3} \cdot \operatorname{arsinh}\left(\frac{\sqrt{27} \cdot [E]_0}{C_{50}}\right) \quad (2)$$

Here $[X]$ is the steady-state concentration of trimers and C_{50} designates the concentration of E at which the degree of oligomerization is equal to 50%. The relationship between C_{50} and K_D is defined as

$$C_{50} = \sqrt{\frac{4}{3} \cdot K_D}$$

To fit the dependencies of the relative decrease in donor fluorescence (A) on P450 concentration to Eq. 1 and Eq. 2 we supplemented these equations with the parameter $A_{\max}$, which represents the amplitude of the curve extrapolated to infinite concentration of the enzyme and corresponds to LRET efficiency in the oligomer:

$$A = A_{\max} \cdot \frac{n \cdot [X]}{[E]_0} \quad (3)$$

where n is the number of the enzyme molecules in the oligomer (i.e., 2 and 3 for $[X]$ calculated according to equations (1) and (2), respectively).

Fitting of the titration isotherms to the above equations was performed with non-linear regression using a combination of Nelder-Mead and Marquardt algorithms as implemented in our SpectraLab software (38).

Determination of the partial stoichiometry of futile NADPH consumption and substrate oxidation was performed with a setup combining a MC2000-2 two-channel CCD spectrometer (Ocean Optics, Inc., Dunedin, FL) with an OXR430 retractable optical oxygen minisensor (PyroScience, Aachen, Germany). Utilization of NADPH and consumption of oxygen were monitored simultaneously using two channels of the MC2000-2 spectrometer for simultaneous detection of the spectra of UV-Vis absorbance of the sample and the oxygen-sensitive luminescence of the OXR430 sensor. We used a PX-2 pulsed xenon light source (Ocean Optics) and a 617 nm M617L3 LED (Thorlabs) as the light sources for the absorbance and luminescence channels, respectively.

The experiments were performed at 30 °C in 100 mM Na-Hepes buffer at the initial concentration of NADPH of 100 μM. The sample with a volume of 1 ml was placed into a cylindrical optical cell (1 cm path length) inserted into a CUV-ALL-UV thermostated cell holder (Ocean Optics) equipped with a motor-less magnetic stirrer (Variomag-USA, Daytona Beach, FL). The absorbance-beam optical windows of the holder were connected via optical fibers to a PX-2 light source and a master channel of the MC2000-2 spectrometer

for absorbance measurements. The cell was closed with a Teflon stopper with a 1.5 mm bore for insertion of the OXR430 sensor. The stopper was designed in the way that ensures a direct contact of its surface with the sample and eliminates air bubbles. After inserting the sensor connected via a bifurcated fiber bundle to the slave channel of MC2000-2 and a M617L3 LED light source, the reaction was initiated by adding 5–10 μ l of microsomal suspension with a gas-tight syringe through a bore in the stopper. A series of spectra of absorbance (320–500 nm) and luminescence (700–900 nm) were collected simultaneously in 2 s time intervals for 5–10 min with a custom-designed software. The rates of utilization of NADPH and consumption of oxygen were determined from the changes in absorbance at 340 nm and the integral intensity of luminescence, respectively. Calibration of the oxygen sensor was performed using an oxygen scavenging system consisting of glucose (60 mM), glucose oxidase (30 u/ml) and catalase (2000 u/ml).

Ratio of the rates of NADPH and oxygen consumption (R_{NADPH}) determined in these experiments was used to estimate the fraction of reducing equivalents used through the “oxidase” shunt (uncoupled production of water) that requires two NADPH molecules per one oxygen molecule as opposed to the fraction used for substrate monooxygenation and generation of superoxide and H_2O_2 that require one molecule of NADPH per one molecule of oxygen. The percent of reducing equivalents utilized through the “oxidase” shunt was calculated as $F_{\text{H}_2\text{O}} = 100 * (R_{\text{NADPH}} - 1)$.

The experiments performed in the presence of CYP2D6 substrate (bufuralol) were accompanied with measuring the rate of bufuralol hydroxylation in separate experiments performed in the same experimental conditions. The degree of coupling was determined as the ratio of the rate of bufuralol hydroxylation to the rate of NADPH consumption.

Results

Oligomerization of CYP2D6 and its interactions with CYP2E1 and CYP3A4 in model microsomes

To probe oligomerization of CYP2D6 and its heteroassociation with CYP2E1 and CYP3A4 we used incorporation of the proteins into the membrane of ICM(CPR) combined with LRET-based detection of their complexes (15). In the experiments with CYP2D6 and CYP2E1 we used N-terminally truncated wild type proteins, while the studies with CYP3A4 employed our cysteine-depleted CYP3A4(C58, C64) construct (15,23). The procedure of modification of the proteins with ERIA and DYM resulted in the preparations with a label-to-P450 ratio of 0.9 – 1.05. The modification did not cause any heme bleaching, P450-to-P420 conversion or any considerable displacement of the spin equilibrium in both CYP2D6 and CYP2E1.

Incubation of CYP2D6-ERIA with ICM(CPR) added at the lipid-to-P450 molar ratio ($R_{\text{L/P}}$) in the range of 150:1 – 4800:1 resulted in efficient incorporation of the protein into the membranes. The recovery of the labeled heme protein in microsomes after their sedimentation and re-suspension was in the range of 75–85%.

Incubation of ICM(CPR) microsomes containing CYP2D6-ERIA with an equimolar amount of either CYP2D6-DYM, CYP2E1-DYM or CYP3A4(C64, C58)-DYM resulted in appearance of LRET due to the formation of mixed oligomers of labeled proteins. A series of spectra obtained with the CYP2D6-ERIA/CYP2D6-DYM pair is shown in Fig. 1. The maximal amplitude of LRET that characterizes the steady state concentration of protein-protein complexes were determined from the fitting of the kinetic curves of the decrease in the intensity of donor fluorescence by a bi-exponential equation (Fig. 1a, inset).

In order to probe the degree of P450 oligomerization in the membrane we performed these experiments with a series of microsomes with different lipid-to-P450 (L/P) ratios. Increase in the L/P ratio may be thought equivalent to diluting the membrane with lipids. According to the law of mass action the concentration of dissociating complexes of membrane-bound proteins decreases progressively as the lipid content increases. A series of experiments carried out at varying L/P ratios was used therefore to determine the actual concentration of P450 oligomers in the membrane and obtain an estimate of LRET efficiency in the formed complex. In order to probe possible effect of interactions of CYP2D6 with its substrates the experiments were performed either in the absence of substrate or in the presence of 50 μ M MDMA ("ecstasy") known to be a high affinity substrate of CYP2D6 (26,39).

The dependencies of the LRET amplitude on P450 concentration obtained with CYP2D6-ERIA/CYP2D6-DYM, CYP2D6-ERIA/CYP2E1-DYM and CYP2D6-ERIA/CYP3A4(C64, C58)-DYM pairs obtained in the absence of added substrate and in the presence of MDMA are shown in Fig. 2. The X-axes of these plots represent the surface density of P450 in the membrane calculated based on $R_{L/P}$ using the value of 0.95 nm² for the area of microsomal membrane corresponding to one molecule of phospholipid in a monolayer (40), similar to the approach used in our earlier reports (15,23).

Even a visual comparison of the three panels shown in Fig. 2 reveals that the homooligomerization of CYP2D6 (Fig. 2a) is considerably more favorable as compared to the formation of its heteromeric complexes with CYP2E1 (Fig. 2b) and CYP3A4 (Fig. 2c). As we will discuss in more details below, the propensity of CYP2D6 to oligomerize was among the highest observed with any homo- and heterotropic pairs of human cytochromes P450 studied up to date.

In most cases the results of fitting of the experimental data shown in Fig. 2 with the steady-state equations for dimerization (eq. 1) and trimerization (eq. 2) were of equally satisfactory quality (ρ^2 0.94). The only exception is the case of CYP2D6-ERIA/CYP3A4(C64, C58) pair in the presence of MDMA, where more adequate approximation was obtained with the trimerization equation due to noticeably sigmoidal character of the titration curve.

It should be also noted than in the case of CYP3A4-CYP2D6 with no substrate added, the points obtained at the lowest $R_{L/P}$ ratios probed (100:1 and 75:1) deviate far below the general dependence (data not shown). A most likely reason for this irregular behavior might be a limited capacity of the membrane to bind CYP3A4 when no substrate is added. As a result, at very high CYP2D6 concentrations the requirement for the concentration of acceptor-labeled protein to be large or equal to the concentration of the donor-labeled

partner becomes unreachable, so that the necessary condition for the intensity of donor fluorescence to reflect the LRET amplitude remains unsatisfied.

The parameters derived from the approximations of the experimental data with the trimerization equation are shown in Table 1. As seen from this table, the propensity of CYP2D6 to form homooligomers in the microsomal membrane is very high and characterized with a $[P450]_{50\%}$ value of 0.066 ± 0.026 pmol/cm², which is the lowest of the values reported for all P450-P450 pairs characterized to date (15,23). In contrast, the tendency of CYP2D6 to form heterooligomers with CYP3A4 is very low and it further decreases in the presence of MDMA, which is known to be a substrate of both enzymes (41).

Of notable importance is a pronounced effect of MDMA on interactions between CYP2E1 and CYP2D6. CYP2D6 is known as the major MDMA-metabolizing P450 enzyme in human liver, whereas no metabolism of this substrate by CYP2E1 has been detected (26). While the addition of MDMA results in a decrease in the degree of association in CYP2D6/CYP2D6 and CYP2D6/CYP3A4 pairs, it considerably promotes the formation of the heterooligomers between CYP2D6 and CYP2E1. Another important effect of MDMA is a profound decrease in LRET efficiency observed in the presence of this substrate in CYP2D6-ERIA/CYP2E1-DYM pair. This effect presumably reveals a substrate-induced rearrangement in the architecture of the CYP2D6 complex with CYP2E1, which results in an increase in distance between fluorescent probes attached to the proteins (Table 1).

Oligomerization of CYP2D6 and its interactions with CYP2E1 in human liver microsomes

In order to better reproduce the environment of the ER membranes of human liver cells we applied our LRET-based method to probe the interactions of ERIA- and DYM-labeled cytochromes P450 incorporated into human liver microsomes. In these studies we used pooled human microsomes of 50 male donors (preparation H1000) obtained from Sekisui Xenotech LLC (Kansas City, KS). According to our determination, the P450 and the phospholipid contents in this preparation were equal to 400 pmol and 504 nmol per mg of protein, respectively. These values correspond to $R_{L/P}$ ratio of 1260:1 and the P450 surface density in the membrane of 0.27 pmol/cm².

Incubation of HLM with CYP2D6-ERIA taken in the amount of 0.1 nmol per mg of microsomal protein resulted in complete incorporation of the labeled protein into the membrane. Similar to that observed with ICM(CPR) microsomes, incubation of HLM-CYP2D6-ERIA with either of CYP2D6-DYM or CYP2E1-DYM resulted in the appearance of LRET indicative of the formation of the complexes between ERIA- and DYM-labeled proteins. A series of spectra obtained upon incubation of CYP2D6-ERIA-containing HLM with the equimolar amount of CYP2D6-DYM and kinetic curves of the changes in donor fluorescence in the course of interactions of CYP2D6-ERIA with DYM-labeled CYP2D6 (circles) and CYP2E1 (triangles) in the absence (open symbols) and in the presence of MDMA (closed symbols) are shown in Fig. 3.

In contrast to that observed with ICM(CPR) microsomes, where the kinetic curves of LRET appearance obey a bi-exponential equation, the kinetic curves obtained in HLM could be approximated with a single exponential (Fig. 3, inset). As derived from exponential

approximations of the kinetic curves obtained in these experiments, the limiting amplitudes of LRET observed in interactions of CYP2D6-ERIA with CYP2D6-DYM in HLM in the absence and in the presence of MDMA were equal to 5.5 and 11.8% respectively, while in the case of interactions of CYP2D6-ERIA with CYP2E1-DYM the respective amplitudes were found to be of 4.3 and 2.2% respectively. Congruence of most these values with those reported for LRET efficiencies in CYP2D6-CYP2D6 and CYP2D6-CYP2E1 complexes (Table 1) suggests a high degree of oligomerization of CYP2D6 and its heterotropic association with CYP2E1 at probed protein concentrations in HLM.

A notable difference between HLM and ICM(CPR) revealed in these experiments is represented by the case of CYP2D6 homooligomerization in the presence of MDMA. An ample increase in LRET amplitude caused by addition of MDMA (from 5.5 and 11.8%) is a distinctive feature of HLM, which is not observed in ICM(CPR) (Fig. 2a, Table 1). This peculiarity may reveal an effect of other P450 species present in HLM on CYP2D6 oligomerization and its modulation by MDMA.

Effects of interactions between CYP2D6 and CYP2E1 on their catalytic activity. Time-dependent activation of CYP2D6 by its substrates in the presence of CYP2E1

Probing the functional consequences of CYP2D6-CYP2E1 interactions we studied the effect of co-incorporation of the two enzymes into ICM(CPR) microsomes on the parameters of oxidation of CYP2E1- and CYP2D6-specific substrates. As a probe for the activity of CYP2E1 we used 7-MFC, a fluorogenic substrate which is not metabolized by CYP2D6. To investigate the effect of CYP2E1 on CYP2D6 we studied CYP2D6-dependent *O*-demethylation of fluorogenic substrate AMMC (42) and hydroxylation of bufuralol, a beta-adrenoceptor antagonist known as a highly selective substrate of CYP2D6 (43). The experiments with ICM(CPR) system were performed at $R_{L/P}$ ratio of 600:1 and the molar ratio of P450 to CPR of 2.8:1. In all three systems used in this study, namely in ICM(CPR), ICM(2D6) or proteoliposomes, the cytochromes P450 were present in molar excess over the CPR (P450:CPR ratio varied from 2.8:1 to 13:1, depending on the system). In our previous publication (15) we demonstrated a linear relationship between the rate of CYP2E1-dependent metabolism of 7-MFC and the CPR:P450 ratio in ICM(CPR) system containing P450 in excess over the reductase. Therefore, the CPR was attributed to be the rate limiting component in all systems used in this study and the turnover numbers were calculated relative to its concentration.

As illustrated in Fig. 4a, co-incorporation of CYP2D6 into CYP2E1-containing microsomes results in a considerable decrease in the rate of CYP2E1-dependent *O*-demethylation of 7-MFC. Interestingly, substrate dependence of the reaction rate both in the absence and in the presence of CYP2D6 reveals a pronounced homotropic cooperativity. As seen from Table 2, interactions with CYP2D6 result in over 3-fold decrease in k_{cat} , while having no substantial effects on S_{50} and the Hill coefficient (n_H) values.

In contrast to a prominent inhibition of CYP2E1 by CYP2D6, no significant effect of CYP2E1-CYP2D6 interactions on CYP2D6-dependent oxidation of AMMC in ICM(CPR) microsomes was detected in the experiments where the addition of NADPH immediately followed the addition of substrate. However, an important activation of CYP2D6 by

CYP2E1 was observed when the microsomes were preincubated with the substrate (AMMC) for 15–30 min prior to initiating the reaction by the addition of NADPH (Fig. 4b). As seen from the data presented in Table 2, preincubation with AMMC resulted in 2.5-fold increase in k_{cat} in ICM(CPR) microsomes, while having no effects on S_{50} and n_{H} values. In contrast, no effect of preincubation with substrate was observed with microsomes containing CYP2D6 only. Preincubation with the CYP2E1 substrate 7-MFC also had no effect on the activity of CYP2E1 either in the absence of or in the presence of CYP2D6 (data not shown).

A similar effect for preincubation with AMMC was observed in ICM(2D6) containing c-DNA expressed recombinant full-length CYP2D6, where CYP2E1 was introduced by incorporation of the purified enzyme (Table 2). The preparation of ICM(2D6) used in our studies was characterized with an $R_{\text{L/P}}$ ratio of 1220:1 and the P450-to-CPR ratio of 6.5. Incorporation of equimolar amount of CYP2E1 into these CYP2D6-containing microsomes resulted in a noticeable decrease in the rate of catalysis, while preincubation with substrate restored the value of k_{cat} back to the level characteristic of CYP2D6 in the absence of co-incorporated CYP2E1 (Table 2). A similar effect of CYP2E1 co-incorporation into ICM(2D6) and preincubation was also observed with another CYP2D6 substrate, bufuralol (Table 2).

Investigating the kinetics of substrate-induced activation of CYP2D6 in the presence of CYP2E1 in microsomal membranes we found that the increase in the rate of *O*-demethylation of AMMC in the process of incubation of ICM(2D6) with this substrate is more rapid than that observed in ICM(CPR) system (Fig. 5a). In both cases the process obeyed monoexponential kinetics and the kinetic constant was equal to $0.22 \pm 0.01 \text{ min}^{-1}$ and $0.08 \pm 0.04 \text{ min}^{-1}$ for the ICM(2D6) and the ICM(CPR) system respectively. However, this difference between ICM(CPR) and ICM(2D6) may be misleading, as it may be caused by faster inactivation of ICM(2D6) at incubation in the reaction buffer at 30 °C. Indeed, the incubation of the latter preparations in the presence of AMMC for over 20 min resulted in a gradual decrease in their enzymatic activity (data not shown), in contrast to ICM(CPR) preparations, where no loss of activity was observed with incubation time up to 1 h. Due to rapid inactivation of ICM(2D6) microsomes, the data set for the dependence of their enzymatic activity on preincubation time used for exponential approximation was limited to the first 16 min of incubation (Fig. 5a, triangles).

We also probed the effect of CYP2D6 interactions with CYP2E1 in CPR-containing proteoliposomes where the P450 enzymes were incorporated at $R_{\text{L/P}}$ ratio of 150:1. While the proteoliposomes, where the only incorporated P450 enzyme was CYP2D6, exhibited the parameters of *O*-demethylation of AMMC analogous to those observed in ICM(CPR) system (Table 2), co-incorporation of CYP2E1 resulted in deep inhibition of the CYP2D6 activity measured without preincubation with substrate. In preincubation experiments in this system we probed the effect of MDMA, another CYP2D6-substrate. After preincubation with 50 μM MDMA a concentrated proteoliposomal suspension was diluted 50 times and the reaction was started with simultaneous addition of NADPH and AMMC. Similar to that observed in other CYP2D6/CYP2E1 systems, preincubation with CYP2D6 substrate resulted in an ample activation of the enzyme. Interestingly, in this case the activating effect was also associated with an important decrease in the affinity of CYP2D6 to AMMC (Table

2). In these experiments we also compared the kinetics of the activating action of MDMA and AMMC. As seen from Fig 5b, the amplitude and the rate of time-dependent activation of CYP2D6/CYP2E1 proteoliposomes by MDMA were similar to those observed with AMMC.

Effect of CYP2D6-CYP2E1 interactions on futile cycling of the system and the stoichiometry of monooxygenation

A very important characteristics of the drug-metabolizing microsomal cytochrome P450 system (microsomal monooxygenase, MMO) is its susceptibility to futile cycling and low degree of coupling of NADPH consumption to substrate oxidation (44–47). This peculiarity makes the MMO energetically wasteful and potentially hazardous due to continuous generation of reactive oxygen species (ROS) (45,48–49). However, this striking imperfection observed *in vitro* is likely to be eliminated in the living cells through several compensatory mechanisms including the functional effect of formation of heteromeric complexes of multiple P450 species (46). In order to probe if the interactions between CYP2D6 and CYP2E1 are involved in these regulatory mechanisms we studied the effect of co-incorporation of these enzymes into the microsomal membrane on futile cycling of the system and on the coupling of bufuralol oxidation to NADPH and oxygen consumption.

In these studies we used the molar ratio between the consumed NADPH and oxygen as a criterion of partitioning between the “oxidase” shunt resulting in production of water and the substrate monooxygenation together with peroxide-generating pathways that produce H_2O_2 either directly or through dismutation of generated superoxide anion-radical. The premise is that the four-electron reduction of oxygen resulting in the production of water (“oxidase” shunt) requires two moles of NADPH per one mol of consumed oxygen molecule, while both the substrate monooxygenation and the pathways resulting in generation of superoxide and H_2O_2 require one molecule of NADPH per one molecule of oxygen (50). Hence, the fraction of reduction equivalents utilized through the “oxidase” shunt was calculated from the ratio of the rates of NADPH and oxygen consumption (R_{NADPH}), as described in *Experimental* section.

Results of these studies are presented in Table 3. As seen from this table, although the futile cycling of CYP2D6 taken alone was twice as high as that observed with CYP2E1, the fates of the electrons transferred through these enzymes were different. In the case of CYP2E1 75% of the futile NADPH consumption went through the peroxide-generating pathways. In contrast, the CYP2D6-containing system in the absence of substrate used all reducing equivalents for water production. Addition of substrate does not eliminate futile cycling by CYP2D6, and the coupling of bufuralol monooxygenation to NADPH consumption is low (<7%). However, interactions of CYP2D6 with bufuralol alter the fate of the transferred electrons by redirecting them through the peroxide-generating pathways (Table 3).

Co-incorporation of CYP2E1 together with CYP2D6 changes the situation dramatically. Although the rate of oxygen consumption by the mixed system represents an intermediate between the values specific to CYP2D6 and CYP2E1, the fate of the transferred electrons in the absence of substrate is similar to that specific to CYP2D6. In the mixed system saturated with bufuralol most of the NADPH consumption results in the production of water, in

contrast to what is observed with CYP2D6 taken alone. Therefore, although the interactions between CYP2D6 and CYP2E1 do not eliminate wasteful consumption of NADPH, they considerably decrease the NADPH-dependent generation of H₂O₂ and other toxic oxygen intermediates.

Discussion

This report presents the first investigation of P450-P450 interactions of human CYP2D6 in microsomal membrane, including its homooligomerization and its interactions with CYP2E1, the major ethanol-inducible human cytochrome P450. Capitalizing on our advances in developing model microsomal systems and methods for detecting P450-P450 interactions, we demonstrated that the propensity of CYP2D6 to form oligomers in microsomal membrane is among the highest observed with human cytochromes P450 enzymes studied to date (15). We also demonstrated the formation of heteromeric complexes in the CYP2D6/CYP2E1 and CYP2D6/CYP3A4 pairs, found an ample effect of CYP2D6 interactions with MDMA, its substrate, on enzyme homo- and heterooligomerization and discovered a noticeable alteration of the catalytic properties of CYP2D6 and CYP2E1 caused by their interactions.

Similarly to what we observed earlier with CYP3A4, CYP3A5 and CYP2E1 (15), the use of N-terminally modified CYP2D6 enzyme does not preclude its incorporation into the microsomal and proteoliposomal membrane. Furthermore, the results obtained in the present study with ICM(CPR) containing N-terminally modified CYP2D6 were completely consistent with the results obtained with ICM(2D6), where CYP2D6 is represented by a full-length wild type protein. In particular, the time-dependent activation of CYP2D6 by its interactions with CYP2E1 in the presence of CYP2D6-specific substrates, which is, perhaps, the most remarkable effect of CYP2D6-CYP2E1 interactions observed in this study, was similar to the full-length protein in ICM(2D6) and the N-terminally modified enzyme in ICM(CPR) (Table 2).

A similar agreement between the full-length and the N-terminally modified enzymes we also reported for CYP3A4, CYP3A5 and CYP2E1 in our earlier study (15). As it has been already articulated in the above publication, these findings suggest that the N-terminal modifications implemented in the constructs of microsomal enzymes used in our studies do not change the mode of their interactions with the membrane considerably and do not affect the functional properties of the membrane-incorporated enzymes in any critical way. This conclusion is in agreement with the results of Baylon and co-authors, who concluded that the membrane binding of P450 is largely independent of the presence of the N-terminal hydrophobic anchor (51). According to the current concepts, the interactions of the microsomal P450 enzymes with the membrane are in large part determined by the regions between the N-terminal anchor and α -helix A (52–54), as well as by the hydrophobic surface in the region of α -helices F' and G' (51,55–56). Furthermore, according to the X-ray structure of the full-length CYP51, the constrained orientation of the P450 catalytic domain relative to the membrane is dictated by a network of interactions of polar residues in the C-terminal part of the transmembrane helix and the following proline-rich region (52), which are retained in our constructs.

Besides extending the use of our model system with purified recombinant P450 proteins incorporated into ICM(CPR) microsomes (15,23) to new P450 pairs, we further developed our methodology by applying it to the studies in human liver microsomes with co-incorporated labeled proteins. This innovative approach allows studying P450-P450 interactions in microsomal membranes with the protein composition, P450-to-CPR ratio and lipid environment closely approaching those characteristic of the ER membranes of human liver cells. In the present study the amount of the incorporated labeled P450 proteins does not exceed 50% of the native P450 content in HLM. Although this treatment resulted in an increase in the total P450 content from 0.4 to 0.6 nmol/mg protein, this content still remains within the limits routinely observable in HLM (0.2–0.7 nmol/mg protein (57–59)).

As described above, the congruence of the amplitudes of LRET observed upon formation of CYP2D6-CYP2D6 and CYP2D6-CYP2E1 complexes in HLM (Fig. 3) with the respective LRET efficiencies determined in the ICM(CPR) system (Table 1) suggests high steady state concentrations of homo- and heterooligomeric states of CYP2D6 in HLM. More specifically, based on the values of LRET efficiencies reported in Table 1 and the limiting (steady state) amplitudes of LRET derived from the exponential approximation of the kinetic curves shown in Fig. 3 (inset), we can calculate that the amplitudes observed for CYP2D6-CYP2D6 interactions in HLM correspond to 78% and 100% degree of homo-oligomerization of the enzyme in the absence and in the presence of MDMA respectively. This is significantly higher than the levels of 67% and 42% which could be expected at the probed concentration of labeled CYP2D6 proteins (0.14 pmol/cm²) according to [P450]_{50%} values determined in ICM(CPR) microsomes (Figure 2a, Table 1). In the case of CYP2D6-CYP2E1 complexes our calculation based on the maximal amplitudes of the respective kinetic curves (Fig. 3, inset) and LRET efficiencies reported in Table 1 resulted in apparent degrees of heterooligomerization as high as 40% and 39%, as compared to 7.2 and 13% calculated for the probed concentrations of the labeled P450 proteins using the [P450]_{50%} values determined in ICM(CPR) (Table 1). These results suggest that the propensities of CYP2D6 to oligomerize and form complexes with CYP2E1 in HLM are higher than those measured in the insect cell microsomes.

A conceptually important finding made in this study is a discovery of time-dependent activation of CYP2D6 in its complexes with CYP2E1 caused by CYP2D6-specific substrates. This effect apparently reveals a slow substrate-dependent reorganization or dissociation of heteromeric complexes of the two enzymes which is triggered by interactions of CYP2D6 with a specific substrate of the enzyme – either AMMC, bufuralol or MDMA.

A very slow rate of developing the activating effect of substrates observed in our experiments is consistent with a concept of “persistent heterogeneity” which we introduced to explain a persistent (quasi-irreversible) distribution of P450 pool in oligomers in solution and in the membrane between the fractions with different functional properties (9–10,15). This concept, which may also be called positional heterogeneity (13), is based on an assumption that the P450 subunits forming homo- and heterooligomers are not identical in their conformation and orientation, but are characterized by different position of spin equilibrium and different abilities to be reduced, bind substrates and interact with redox partners. The transition between dissimilar subunit positions is extremely slow and requires

dissociation and re-association of oligomers. A detailed examination of our hypothesis, an overview of a large body of supporting observations and a discussion of possible structural basis for positional heterogeneity in P450 oligomers has been recently presented by Reed and Backes in their comprehensive review (13).

According to our hypothesis (10,15) the minimal building block of P450 oligomers in the membrane is a trimer that comprises of two different types of subunits. The subunits occupying two different positions in the oligomer, which may be designated as “active” and “hindered”, differ in their spin state, propensity to form productive electron-transfer complexes with CPR and the ability to interact with substrates. “Hindered” subunits are hypothesized to have limited ability (or be completely unable) to bind substrates and interact with CPR. The oligomers, where the conformational state of the enzyme is “frozen” by subunit interactions are in slow equilibrium with the P450 monomers, where the conformational state is dynamic and substrate-dependent. As illustrated in the schematic representation of our hypothesis shown in Fig. 6, in the system comprising of two different P450 species, the distribution of the two interacting P450s between the two subunit positions in the absence of P450 substrates is random (Fig. 6, top right group of trimers). However, addition of a substrate specific to one of the interacting P450 species (“target P450”) will result in its binding to the molecules of the target P450 occupying active positions. The substrate will also interact with the enzyme monomers and cause a displacement of their conformational equilibrium towards a state matching the conformation of the “active” subunit in the oligomer. This conformational shift will make the “active” position in the oligomer preferable for the target enzyme. Therefore, after a slow dissociation and reassembly of the oligomers, the substrate-bound P450 enzyme will by preference occupy the “active” positions, while the enzyme lacking substrate will be displaced to the “hindered” locations (Fig. 6, bottom right group of trimers). The transitions between the oligomeric and monomeric states in crowded environment of the microsomal membrane (60) are presumed to be slow and rate limiting for this process. An important feature of the proposed mechanism is that the degree of activation caused by hetero-association is determined by the disparity between the interacting enzymes in their affinities for each particular P450 substrate. This mechanism of activation may be triggered only by a substrate highly specific to one of the interacting species.

The proposed model of substrate-induced reorganization of heterooligomers is corroborated by our observation of a profound decrease in LRET efficiency in CYP2D6-ERIA complexes with CYP2E1-DYM caused by the addition of MDMA. This effect presumably reveals an increase in distance between fluorescent probes caused by MDMA-induced relocation of CYP2D6 to “active” positions caused by substrate induced displacement of conformational equilibrium and subsequent change in the preferential mode of CYP2D6-CYP2E1 interactions.

Another important finding reported here regards the effect of CYP2D6 interactions with CYP2E1 on futile cycling of the monooxygenase system and the stoichiometry of substrate oxidation by CYP2D6. Surprisingly, we found that the futile cycling of CYP2D6 is considerably more extensive than that observed with CYP2E1, which has a reputation of one of the most uncoupled P450 enzymes (61–62). However, in contrast to CYP2E1, where

uncoupled NADPH consumption results in massive production of H_2O_2 (61), the CYP2D6-containing system in the absence of substrate uses all reducing equivalents for water production. Importantly, when CYP2D6 is the only P450 enzyme present in the microsomal membrane, addition of its substrate (bufuralol) does not eliminate the futile cycling but rather redirects it through the peroxide-generating pathways (Table 3). Co-incorporation of CYP2E1 together with CYP2D6 changes the situation dramatically. In this case most of the NADPH consumption, either in the presence of bufuralol or in the absence of added substrate, results in the production of water. This effect suggests a CYP2E1-induced change in the degree of hydration of the CYP2D6 heme pocket and/or the structure of the network of sequestered water molecules that serves for proton delivery (63–65). This change in the conformational landscape decreases the probability of proximal protonation of the peroxycomplex of CYP2D6 that results in unwanted production of hydrogen peroxide. Therefore, although the interactions between CYP2D6 and CYP2E1 do not eliminate wasteful consumption of NADPH, they considerably decrease unwanted NADPH-dependent generation of H_2O_2 and other toxic oxygen intermediates.

A closely related observation has been made by Tan and co-authors for the pair of CYP2E1 with another human enzyme, CYP2A6, co-expressed in insect cell microsomes (66). These authors demonstrated that, in the system where CYP2A6 is the only P450 present in the membrane, the addition of coumarin, a CYP2A6 substrate, results in a drastic increase in the rate of NADPH-dependent generation of H_2O_2 . Co-expression of CYP2E1 together with CYP2A6 prevents this effect, so that the mixed system exhibit low rate of H_2O_2 production either in the absence or in the presence of substrates of CYP2A6 and CYP2E1 (66).

A striking peculiarity of eukaryotic cytochromes P450, which is in a stark contrast to their bacterial progenitors, is that they are catalytically inefficient (turnover numbers $<10 \text{ min}^{-1}$) and energetically wasteful (coupling of NADPH consumption to substrate oxidation $<50\%$), at least in the *in vitro* studies. Leakiness of the MMO system makes it potentially deleterious to cells due to continuous generation of reactive oxygen species (ROS) (44–47), which contributes significantly to overall ROS formation in the cell (45,48–49). The effect of interactions between dissimilar P450 species on the routes of uncoupling of the monooxygenase system, demonstrated in this study and also observed by Tan and co-authors, may be involved in the mechanisms that regulate production of ROS by the P450 ensemble and minimize the unwanted effects of its uncoupling on living cells (46).

In summary, this study represents a next step towards characterization of interactions in heterologous pairs of human cytochromes P450 in microsomal membranes, their functional effects and modulation by specific substrates of interacting species. Application of our LRET-based techniques to the studies of P450-P450 interactions in human liver microsomes offers exciting possibilities for untangling the routes of P450-P450 crosstalk functioning in living cells. Furthermore, profound functional effects of CYP2D6-CYP2E1 interactions and their modulation by MDMA may play an important role in the mechanisms of hepatotoxic interactions of alcohol with this CYP2D6-metabolized drug of abuse.

Acknowledgments

This research was supported by NIH grant R01-GM114369 to JPI. The authors thank Dr. Thomas C. Pochapsky and Dr. F. Peter Guengerich for providing us with the CYP2D6-expressing plasmids and sharing with us their experience in CYP2D6 expression and purifications.

Abbreviations

ICM	insect cell microsomes
ICM(CPR)	insect cell microsomes containing recombinant CPR

References

1. Rendic S. Summary of information on human CYP enzymes: Human P450 metabolism data. *Drug Metab Rev.* 2002; 34:83–448. [PubMed: 11996015]
2. Rendic S, Guengerich FP. Update Information on Drug Metabolism Systems-2009, Part II. Summary of Information on the Effects of Diseases and Environmental Factors on Human Cytochrome P450 (CYP) Enzymes and Transporters. *Curr Drug Metab.* 2010; 11:4–84. [PubMed: 20302566]
3. Wienkers LC, Heath TG. Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Disc.* 2005; 4:825–833.
4. Gao N, Tian X, Fang Y, Zhou J, Zhang HF, Wen Q, Jia LJ, Gao J, Sun B, Wei JY, Zhang YF, Cui MZ, Qiao HL. Gene polymorphisms and contents of cytochrome P450s have only limited effects on metabolic activities in human liver microsomes. *Eur J Pharm Sci.* 2016; 92:86–97. [PubMed: 27339126]
5. Zhang HF, Li ZH, Liu JY, Liu TT, Wang P, Fang Y, Zhou J, Cui MZ, Gao N, Tian X, Gao J, Wen Q, Jia LJ, Qiao HL. Correlation of Cytochrome P450 Oxidoreductase Expression with the Expression of 10 Isoforms of Cytochrome P450 in Human Liver. *Drug Metab Disp.* 2016; 44:1193–1200.
6. Zhang HF, Wang HH, Gao N, Wei JY, Tian X, Zhao Y, Fang Y, Zhou J, Wen Q, Gao J, Zhang YJ, Qian XH, Qiao HL. Physiological Content and Intrinsic Activities of 10 Cytochrome P450 Isoforms in Human Normal Liver Microsomes. *J Pharm Exp Ther.* 2016; 358:83–93.
7. Backes WLK, RW. Organization of multiple cytochrome P450s with NADPH-cytochrome P450 reductase in membranes. *Pharm Ther.* 2003; 98:221–233.
8. Kandel SE, Lampe JN. Role of Protein-Protein Interactions in Cytochrome P450-Mediated Drug Metabolism and Toxicity. *Chem Res Toxicol.* 2014; 27:1474–1486. [PubMed: 25133307]
9. Davydov DR. Microsomal monooxygenase as a multienzyme system: the role of P450-P450 interactions. *Expert Opin Drug Metab Toxicol.* 2011; 7:543–558. [PubMed: 21395496]
10. Davydov R. Molecular Organization of the Microsomal Oxidative System: a New Connotation for an Old Term. *Biochemistry (Moscow) Suppl B- Biomed Chem.* 2016; 10:10–21.
11. Reed JR, Backes WL. Formation of P450-P450 complexes and their effect on P450 function. *Pharm Ther.* 2012; 133:299–310.
12. Reed JR, Backes WL. The functional effects of physical interactions involving cytochromes P450: putative mechanisms of action and the extent of these effects in biological membranes. *Drug Metab Rev.* 2016; 48:453–469. [PubMed: 27500687]
13. Reed JR, Backes WL. Physical Studies of P450-P450 Interactions: Predicting Quaternary Structures of P450 Complexes in Membranes from Their X-ray Crystal Structures. *Frontiers Pharmacol.* 2017:8.
14. Cawley GF, Zhang SX, Kelley RW, Backes WL. Evidence supporting the interaction of CYP2B4 and CYP1A2 in microsomal preparations. *Drug Metab Disp.* 2001; 29:1529–1534.
15. Davydov DR, Davydova NY, Sineva EV, Halpert JR. Interactions among Cytochromes P450 in Microsomal Membranes: Oligomerization of cytochromes P450 3A4, 3A5, and 2E1 and its functional consequences. *J Biol Chem.* 2015; 290:3850–3864. [PubMed: 25533469]

16. Davydov DR, Petushkova NA, Bobrovnikova EV, Knyushko TV, Dansette P. Association of cytochromes P450 1A2 and 2B4: Are the interactions between different P450 species involved in the control of the monooxygenase activity and coupling? *Adv Exp Med Biol.* 2001; 500:335–338. [PubMed: 11764964]
17. Hazai E, Kupfer D. Interactions between CYP2C9 and CYP2C19 in reconstituted binary systems influence their catalytic activity: Possible rationale for the inability of CYP2C19 to catalyze methoxychlor demethylation in human liver microsomes. *Drug Metab Disp.* 2005; 33:157–164.
18. Kelley RW, Cheng DM, Backes WL. Heteromeric complex formation between CYP2E1 and CYP1A2: Evidence for the involvement of electrostatic interactions. *Biochemistry.* 2006; 45:15807–15816. [PubMed: 17176103]
19. Kelley WK, Reed JR, Backes WL. Effect of ionic strength on the functional interactions between CYP2B4 and CYP1A2. *Biochemistry.* 2005; 44:2632–2641. [PubMed: 15709776]
20. Reed JR, Eyer M, Backes WL. Functional interactions between cytochromes P450 1A2 and 2B4 require both enzymes to reside in the same phospholipid vesicle. Evidence for physical complex formation. *J Biol Chem.* 2010; 285:8942–8952. [PubMed: 20071338]
21. Subramanian M, Low M, Locuson CW, Tracy TS. CYP2D6-CYP2C9 protein-protein interactions and isoform-selective effects on substrate binding and catalysis. *Drug Metab Disp.* 2009; 37:1682–1689.
22. Subramanian M, Tam H, Zheng H, Tracy TS. CYP2C9-CYP3A4 protein-protein interactions: Role of the hydrophobic N-terminus. *Drug Metab Disp.* 2010; 38:1003–1009.
23. Davydov DR, Davydova NY, Sineva EV, Kufareva I, Halpert JR. Pivotal role of P450-P450 interactions in CYP3A4 allostery: the case of alpha-naphthoflavone. *Biochem J.* 2013; 453:219–230. [PubMed: 23651100]
24. Tanaka E. Toxicological interactions involving psychiatric drugs and alcohol: an update. *J Clin Pharm Ther.* 2003; 28:81–95. [PubMed: 12713604]
25. Antolino-Lobo I, Meulenbelt J, van den Berg M, van Duursen MBM. A mechanistic insight into 3,4-methylenedioxymethamphetamine (“ecstasy”)-mediated hepatotoxicity. *Veterinary Quart.* 2011; 31:193–205.
26. Kreth KP, Kovar KA, Schwab M, Zanger UM. Identification of the human cytochromes P450 involved in the oxidative metabolism of “Ecstasy”-related designer drugs. *Biochem Pharm.* 2000; 59:1563–1571. [PubMed: 10799653]
27. Meyer MR, Peters FT, Maurer HH. The Role of Human Hepatic Cytochrome P450 Isozymes in the Metabolism of Racemic 3,4-Methylenedioxyethylamphetamine and Its Single Enantiomers. *Drug Metab Disp.* 2009; 37:1152–1156.
28. Pontes H, de Pinho PG, Fernandes E, Branco PS, Ferreira LM, Carmo H, Remiao F, Carvalho F, Bastos ML. Metabolic interactions between ethanol and MDMA in primary cultured rat hepatocytes. *Toxicology.* 2010; 270:150–157. [PubMed: 20170704]
29. Harlow GR, Halpert JR. Analysis of human cytochrome P450 3A4 cooperativity: Construction and characterization of a site-directed mutant that displays hyperbolic steroid hydroxylation kinetics. *Proc Natl Acad Sci USA.* 1998; 95:6636–6641. [PubMed: 9618464]
30. Spatzenegger M, Liu H, Wang QM, Debarber A, Koop DR, Halpert JR. Analysis of differential substrate selectivities of CYP2B6 and CYP2E1 by site-directed mutagenesis and molecular modeling. *J Pharm Exp Ther.* 2003; 304:477–487.
31. Jang HH, Davydov DR, Lee GY, Yun CH, Halpert JR. The role of cytochrome P450 2B6 and 2B4 substrate access channel residues predicted based on crystal structures of the amlodipine complexes. *Arch Biochem Biophys.* 2014; 545:100–107. [PubMed: 24445070]
32. Wang A, Savas U, Hsu MH, Stout CD, Johnson EF. Crystal Structure of Human Cytochrome P450 2D6 with Prinomastat Bound. *J Biol Chem.* 2012; 287:10834–10843. [PubMed: 22308038]
33. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem.* 1959; 234:466–468. [PubMed: 13641241]
34. Shen AL, Porter TD, Wilson TE, Kasper CB. Structural analysis of the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase by site-directed mutagenesis. *J Biol Chem.* 1989; 264:7584–7589. [PubMed: 2708380]

35. Massey V. The microestimation of succinate and the extinction coefficient of cytochrome c. *Biochim Biophys Acta*. 1959; 34:225–256.
36. French JS, Coon MJ. Properties of NADPH-cytochrome P-450 reductase purified from rabbit liver microsomes. *Arch Biochem Biophys*. 1979; 195:565–577. [PubMed: 112928]
37. Rushmore TH, Reider PJ, Slaughter D, Assang C, Shou MG. Bioreactor Systems in Drug Metabolism: Synthesis of Cytochrome P450-Generated Metabolites. *Metab Eng*. 2000; 2:115–125. [PubMed: 10935727]
38. Davydov DR, Deprez E, Hui Bon Hoa G, Knyushko TV, Kuznetsova GP, Koen YM, Archakov AI. High-pressure-induced transitions in microsomal cytochrome P450 2B4 in solution - evidence for conformational inhomogeneity in the oligomers. *Arch Biochem Biophys*. 1995; 320:330–344. [PubMed: 7625841]
39. Segura M, Farre M, Pichini S, Peiro AM, Roset PN, Ramirez A, Ortuno J, Pacifici R, Zuccaro P, Segura J, de la Torre R. Contribution of Cytochrome P450 2D6 to 3,4-Methylenedioxymethamphetamine Disposition in Humans: Use of Paroxetine as a Metabolic Inhibitor Probe. *Clin Pharmacokinet*. 2005; 44:649–660. [PubMed: 15910012]
40. Wibo M, Amar-Costesec A, Berthet J, Beayfay H. Electron microscope examination of subcellular fractions III. Quantitative analysis of the microsomal fraction isolated from rat liver. *J Cell Biol*. 1971; 51:52–71. [PubMed: 4329524]
41. Antolino-Lobo I, Meulenbelt J, Nijmeijer SM, Maas-Bakker RF, Meijerman I, van den Berg M, van Duursen MBM. 3,4-Methylenedioxymethamphetamine (MDMA) interacts with therapeutic drugs on CYP3A by inhibition of pregnane X receptor (PXR) activation and catalytic enzyme inhibition. *Toxicol Lett*. 2011; 203:82–91. [PubMed: 21402137]
42. Chauret N, Dobbs B, Lackman RL, Bateman K, Nicoll-Griffith DA, Stresser DM, Ackermann JM, Turner SD, Miller VP, Crespi CL. The use of 3- 2-(N, N-diethyl-N-methylammonium)ethyl -7-methoxy-4-methylcoumarin (AMMC) as a specific CYP2D6 probe in human liver microsomes. *Drug Metab Disp*. 2001; 29:1196–1200.
43. Kronbach T. Bufuralol, dextromethorphan, and debrisoquine as prototype substrates for human P450 IID6. *Meth Enzymol*. 1991; 206:509–517. [PubMed: 1686064]
44. Gorsky LD, Koop DR, Coon MJ. On the stoichiometry of the oxidase and monooxygenase reactions catalyzed by liver microsomal cytochrome P-450. Products of oxygen reduction. *J Biol Chem*. 1984; 259:6812–6817. [PubMed: 6725272]
45. Kuthan H, Ullrich V. Oxidase and oxygenase function of the microsomal cytochrome P450 monooxygenase system. *Eur J Biochem*. 1982; 126:583–588. [PubMed: 7140747]
46. Zangar RC, Davydov DR, Verma S. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicol Appl Pharm*. 2004; 199:316–331.
47. Zhukov AA, Archakov AI. Stoichiometry of Microsomal Oxidation Reactions. Distribution of Redox-Equivalents in Monooxygenase and Oxidase Reactions Catalyzed by Cytochrome P-450. *Biochemistry (Moscow)*. 1985; 50:1659–1672.
48. Bondy SC, Naderi S. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem Pharmacol*. 1994; 48:155–159. [PubMed: 8043018]
49. Rashba-Step J, Cederbaum AI. Generation of reactive oxygen intermediates by human liver microsomes in the presence of NADPH or NADH. *Mol Pharmacol*. 1994; 45:150–157. [PubMed: 8302274]
50. Grinkova YV, Denisov IG, McLean MA, Sligar SG. Oxidase uncoupling in heme monooxygenases: Human cytochrome P450 CYP3A4 in Nanodiscs. *Biochem Biophys Res Commun*. 2013; 430:1223–1227. [PubMed: 23266608]
51. Baylon JL, Lenov IL, Sligar SG, Tajkhorshid E. Characterizing the Membrane-Bound State of Cytochrome P450 3A4: Structure, Depth of Insertion, and Orientation. *J Am Chem Soc*. 2013; 135:8542–8551. [PubMed: 23697766]
52. Monk BC, Tomasiak TM, Keniya MV, Huschmann FU, Tyndall JDA, O'Connell JD III, Cannon RD, McDonald JG, Rodriguez A, Finer-Moore JS, Stroud RM. Architecture of a single membrane spanning cytochrome P450 suggests constraints that orient the catalytic domain relative to a bilayer. *Proc Natl Acad Sci USA*. 2014; 111:3865–3870. [PubMed: 24613931]

53. Williams PA, Cosme J, Sridhar V, Johnson EF, McRee DE. Mammalian microsomal cytochrome P450 monooxygenase: Structural adaptations for membrane binding and functional diversity. *Molecular Cell*. 2000; 5:121–131. [PubMed: 10678174]
54. Liu J, Tawa GJ, Wallqvist A. Identifying Cytochrome P450 Functional Networks and Their Allosteric Regulatory Elements. *PLOS One*. 2013; 8:e81980. [PubMed: 24312617]
55. Berka K, Paloncyova M, Anzenbacher P, Otyepka M. Behavior of Human Cytochromes P450 on Lipid Membranes. *J Phys Chem B*. 2013; 117:11556–11564. [PubMed: 23987570]
56. Cojocaru V, Balali-Mood K, Sansom MSP, Wade RC. Structure and Dynamics of the Membrane-Bound Cytochrome P450 2C9. *Plos Computational Biology*. 2011; 7:e1002152. [PubMed: 21852944]
57. Boobis AR, McKillop D, Robinson DT, Adams DA, McCormick DJ. Interlaboratory comparison of the assessment of P450 activities in human hepatic microsomal samples. *Xenobiotica*. 1998; 28:493–506. [PubMed: 9622851]
58. Forrester LM, Henderson CJ, Glancey MJ, Back DJ, Park BK, Ball SE, Kitteringham NR, McLaren AW, Miles JS, Skett P, Wolf CR. Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Biochem J*. 1992; 281:359–368. [PubMed: 1736885]
59. Nelson AC, Huang W, Moody DE. Variables in human liver microsome preparation: Impact on the kinetics of L-alpha-acetylmethadol (LAAM) N-demethylation and dextromethorphan O-demethylation. *Drug Metab Disp*. 2001; 29:319–325.
60. Melo E, Martins J. Kinetics of bimolecular reactions in model bilayers and biological membranes. A critical review. *Biophys Chem*. 2006; 123:77–94. [PubMed: 16730881]
61. Ekstrom G, Ingelman-Sundberg M. Rat-liver microsomal NADPH-supported oxidase activity and lipid-peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). *Biochem Pharm*. 1989; 38:1313–1319. [PubMed: 2495801]
62. Cederbaum AI. CYP2E1 - Biochemical and toxicological aspects and role in alcohol-induced liver injury. *Mt Sinai J Med*. 2006; 73:657–672. [PubMed: 16878272]
63. Loida PJ, Sligar SG. Molecular recognition in cytochrome P-450: mechanism for the control of uncoupling reactions. *Biochemistry*. 1993; 32:11530–11538. [PubMed: 8218220]
64. Makris TM, Davydov R, Denisov IG, Hoffman BM, Sligar SG. Mechanistic enzymology of oxygen activation by the cytochromes P450. *Drug Metab Rev*. 2002; 34:691–708. [PubMed: 12487147]
65. Makris TM, von Koenig K, Schlichting I, Sligar SG. Alteration of P450 distal pocket solvent leads to impaired proton delivery and changes in heme geometry. *Biochemistry*. 2007; 46:14129–14140. [PubMed: 18001135]
66. Tan Y, Patten CJ, Smith T, Yang CS. Competitive interactions between cytochromes P450 2A6 and 2E1 for NADPH-cytochrome P450 oxidoreductase in the microsomal membranes produced by a baculovirus expression system. *Arch Biochem Biophys*. 1997; 342:82–91. [PubMed: 9185616]
67. Sineva EV, Rumfeldt JAO, Halpert JR, Davydov DR. A large-scale allosteric transition in cytochrome P450 3A4 revealed by luminescence resonance energy transfer (LRET). *PLOS One*. 2013; 8:e83898. [PubMed: 24376769]

Fig 1.

Interactions of CYP2D6-ERIA with CYP2D6-DYM mutants in ICM(CPR) microsomes studied with LRET. Main panel shows a series of spectra of delayed emission recorded during incubation of a 0.4 μM suspension of CYP2D6-ERIA incorporated into ICM(CPR) at $R_{L/P}=300$ with 0.4 μM CYP3A4(C166)-DYM. (The $R_{L/P}$ ratio in the membrane after incorporation of DYM-labeled enzyme is equal to 150). The inset shows the time dependencies of normalized intensity of donor fluorescence obtained in the experiments at the $R_{L/P}$ of 150, 225, 300 and 1200. Solid lines represent the approximation of the kinetic curves with a bi-exponential equation.

Fig. 2.

The dependencies of the LRET amplitude on P450 concentration in the membrane of ICM(CPR) microsomes for the interactions in CYP2D6-ERIA/CYP2D6-DYM (*a*), CYP2D6-ERIA/CYP2E1-DYM (*b*), and CYP2D6-ERIA/CYP3A4(C58, C64)-DYM (*c*) pairs. The data sets shown in circles were obtained at no substrate added, while the data sets shown in triangles were recorded in the presence of 50 μM MDMA. Solid and dashed lines show the approximations of the data sets with the equations (2) and (1), respectively.

Fig. 3. Oligomerization of CYP2D6 and its interactions with CYP2E1 in human liver microsomes studied with LRET. Main panel shows a series of spectra of delayed emission recorded during incubation of a 0.1 μ M suspension of CYP2E1-ERIA incorporated into HLM with 0.1 μ M CYP2E1-DYM. The inset shows the time dependencies of normalized intensity of donor fluorescence obtained in the course of interactions of CYP2D6-ERIA with DYM-labeled CYP2D6 (circles) and CYP2E1 (triangles) in the absence (open symbols) and in the presence of MDMA (closed symbols).

Fig. 4.

The effect of CYP2D6-CYP2E1 interactions in ICM(CPR) microsomes on CYP2E1-dependent *O*-demethylation of 7-MFC (*a*) and CYP2D6-dependent *O*-demethylation of AMMC (*b*). The data sets obtained with the microsomes containing individual CYP2E1 and CYP2D6 enzymes are shown in circles and solid lines, triangles and dashed lines show the data obtained with a mixture of the two enzymes. Filled circles in panel *b* designate the data obtained after 15 min of preincubation with substrate, while the open circles show the data obtained without preincubation. The lines represent the approximations of the data sets with the Hill equation.

Fig. 5.

Kinetics of activation of CYP2D6 during preincubation with substrate in mixed CYP2D6/CYP2E1 systems. Panel *a* shows the activation of AMMC *O*-demethylation during preincubation with AMMC in ICM(CPR) microsomes (circles, solid line) and CYP2D6-containing ICM(2D6) (triangles, dashed line) with co-incorporated CYP2E1. In these data sets the initial (100%) levels of activity correspond to turnover rates of 0.24 and 1.0 min⁻¹ for the data shown in circles and triangles, respectively. Panel *b* shows the result obtained with AMMC *O*-demethylation by CPR-containing proteoliposomes with co-incorporated CYP2D6 and CYP2E1 ($R_{LP}=150$) upon their preincubation with AMMC (triangles) and MDMA (circles). In both datasets shown in this figure the initial (100%) rates of turnover were equal to 0.042 min⁻¹. The lines show the approximation of the data by an exponential equation.

Fig. 6.

Schematic illustrating the hypothesis of positional heterogeneity in P450 oligomers and the proposed mechanism of time-dependent activation of CYP2D6 (green) by its substrates in the presence of CYP2E1 (magenta). The proteins are present in equilibrium between oligomers (trimers in this figure) and monomers. The architecture of the oligomer gives rise to the positional dissimilarity of the subunits, which are stabilized in "active" and "hindered" conformations. The "hindered" subunits are hypothesized to be unable to bind substrates and interact with CPR. In the absence of substrate the distribution of CYP2D6 and CYP2E1 between the two subunit locations is random. However, addition of a substrate specific to CYP2D6 (bufuralol or MDMA in this study) results in its binding to the molecules of CYP2D6 occupying active positions, as well as to the CYP2D6 monomers. These interactions displace the conformational equilibrium in monomers towards the "active" conformation, so that after a slow dissociation and reassembly of the oligomers, the CYP2D6 molecules will eventually occupy all "active" positions and CYP2E1 will be completely displaced to the "hindered" locations. The bottom part of the scheme illustrates the situation with complete saturation of CYP2D6 with its substrate, so that all CYP2D6 molecules adopting the "active" conformation are presumed to be associated with substrate.

Oligomerization of CYP2D6 and its interactions with CYP2E1 and CYP3A4 in model microsomes studied by LRET*

Table 1

.P450 pair	MDMA, μM	[P450] _{50%} , a , pmol/cm^2	(Lipid/P450) _{50%} , b	LRET efficiency, %	Interprobe distance c , $\text{\AA}$
2D6+2D6	0	0.066 $\pm$ 0.026	5242 $\pm$ 2445	6.7 $\pm$ 0.33	53.5
	50	0.20 $\pm$ 0.03	1747 $\pm$ 290	6.8 $\pm$ 0.97	53.3
2D6+2E1	0	1.1 $\pm$ 0.39	317 $\pm$ 131	10.3 $\pm$ 1.3	49.5
	50	0.64 $\pm$ 0.08	541 $\pm$ 69	4.6 $\pm$ 0.2	57.2
2D6+3A4	0	1.0 $\pm$ 0.16	346 $\pm$ 19	18.9 $\pm$ 1.2	44.0
	50	3.3 $\pm$ 0.4	105 $\pm$ 4	15.9 $\pm$ 1.0	45.5

* The values given in the Table were obtained by fitting of the titration curves with trimerization equation (Eq. 2). The “ $\pm$ ” values show the confidence interval calculated for $p = 0.05$.

a Surface density of P450 in microsomal membranes at which the amplitude of the titration curves reaches 50% of maximal.

b Lipid-to-P450 ratio at which the amplitude of the titration curves reaches 50% of maximal.

c Apparent interprobe distance based on LRET efficiency and calculated with Förster equation assuming the orientation factor (κ^2), the refractive index and the quantum yield of DY-731 fluorescence of being equal to 0.667, 1.4 and 0.35% respectively (67).

Table 2
Effect of co-incorporation of CYP2D6 and CYP2E1 on their specific activities in model membranous systems^{*}

System	Preincubation	Substrate	$k_{cat}^{a,b}$	S_{50} or K_M , μM	n_H
ICM(CPR)+2E1	-	7-MFC	0.17 $\pm$ 0.06	5.1 $\pm$ 3.1	1.8 $\pm$ 1.1
ICM(CPR)+2D6+2E1	-	7-MFC	0.05 $\pm$ 0.02 (0.02)	7.5 $\pm$ 1.3	2.3 $\pm$ 0.2
ICM(CPR)+2D6	-	AMMC	0.56 $\pm$ 0.22	7.4 $\pm$ 2.5	1.9 $\pm$ 0.5
ICM(CPR)+2D6+2E1	15 min	AMMC	0.64 $\pm$ 0.23 (0.64)	8.8 $\pm$ 2.8	2.2 $\pm$ 0.1
		AMMC	1.5 $\pm$ 0.5 (0.03)	7.0 $\pm$ 2.2	1.7 $\pm$ 0.5
ICM(2D6)	-	AMMC	2.1 $\pm$ 0.2	5.7 $\pm$ 1.4	1.7 $\pm$ 0.1
ICM(2D6)+2E1	15 min	AMMC	1.4 $\pm$ 0.2 (0.01)	6.8 $\pm$ 3.4	1.1 $\pm$ 0.1
		AMMC	2.1 $\pm$ 0.3 (0.01)	8.1 $\pm$ 2.6	1.3 $\pm$ 0.4
Liposomes(CPR)+2D6	-	AMMC	1.4 $\pm$ 0.05	4.9 $\pm$ 2.9	2.1 $\pm$ 0.5
Liposomes(CPR)+2D6+2E1	30 min	AMMC	ND ^c	ND ^c	2.7 $\pm$ 0.9
		AMMC	2.4 $\pm$ 0.1 (<0.01)	43 $\pm$ 9	
ICM(2D6)	-	Bufuralol	3.2 $\pm$ 1.0	5.7 $\pm$ 1.0	NA ^d
ICM(2D6)+2E1	30 min	Bufuralol	1.3 $\pm$ 0.5 (<0.01)	6.7 $\pm$ 2.8	
		Bufuralol	2.4 $\pm$ 0.4 (0.01)	5.8 $\pm$ 0.3	

^{*} The values given in the Table were obtained by averaging the results of 3–5 individual measurements and the “ $\pm$ ” values show the confidence interval calculated for $p = 0.05$.

^a The data were approximated with the Hill equation in all cases except for bufuralol hydroxylation by ICM(2D6)-based systems where the Michaelis-Menten equation was used..

^b Turnover rate calculated per concentration of CPR, which was the rate limiting component in all systems under study. The values in parentheses represent the p -values of Student's t -test for the hypothesis of equality of the respective values of k_{cat} with the value presented in the previous line (i.e., at no second P450 present or at no preincubation). The values of k_{cat} characterized with p -values 0.05 are shown in bold to emphasize the effects with high statistical significance.

^c ND - Not determined: the activity of CYP2D6/CYP2E1 containing proteoliposomes with AMMC at no preincubation was too low for reliable determination of its parameters.

^d NA - Not applicable: the datasets obtained with bufuralol as a substrate exhibited no cooperativity and were fitted to Michaelis-Menten equation.

Table 3
Stoichiometry of NADPH-consumption and substrate oxidation in model microsomes^{*}

System	Substrate	NADPH consumption ^a , min ⁻¹	Oxygen consumption ^a , min ⁻¹	Coupling ^b , %	H ₂ O production ^c , %
ICM(2D6)	none	48.6 ± 8.6	24.2 ± 4.9		106.0 ± 36.0
	Bufuralol, 100µM	46.9 ± 8.2	39.1 ± 8.0	6.8 ± 3.3	21.3 ± 11.7
ICM(2E1)	none	23.3 ± 3.9	18.9 ± 3.6		24.8 ± 10.6
	none	31.4 ± 2.2	17.1 ± 1.7		85.0 ± 16.9
ICM(2D6)+2E1	Bufuralol, 100µM	58.6 ± 5.4	28.2 ± 5.5	2.2 ± 1.2	110.5 ± 24.2
	Bufuralol, 100 µM after preincubation	60.0 ± 9.2	33.7 ± 2.6	4.0 ± 1.3	77.8 ± 18.3

^{*} The values given in the Table were obtained by averaging the results of 2–5 individual measurements (each with a different preparation of model microsomes) and the “±” values show the confidence interval calculated for $p = 0.05$.

^a The rates of NADPH and oxygen consumption were calculated per concentration of CPR.

^b The percent of coupling represents the fraction of NADPH-derived reducing equivalents used for oxidation of substrate (bufuralol), calculated based on the rate of the formation of the product of bufuralol hydroxylation (1'-hydroxybufuralol) determined in the same experimental conditions. The coupling is determined as the ratio of the rate of bufuralol hydroxylation by a respective system to the rate of NADPH consumption at saturating concentration of bufuralol (100 µM).

^c Fraction of the NADPH-derived reducing equivalents used for the four-electron process of formation of water estimated based on the ratio of the rate of usage of NADPH to the rate oxygen consumption.