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Interactions of mammalian cytochrome P450, NADPH-cytochrome P450 reductase, and cytochrome b_5 enzymes

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Abstract

An immobilized system was developed to detect interactions of human cytochromes P450 (P450) with the accessory proteins NADPH-P450 reductase and cytochrome b_5 (b_5) using an enzyme-linked affinity approach. Purified enzymes were first bound to wells of a polystyrene plate, and biotinylated partner enzymes were added and bound. A streptavidin–peroxidase complex was added, and protein–protein binding was monitored by measuring peroxidase activity of the bound biotinylated proteins. In a model study, we examined protein–protein interactions of *Pseudomonas putida* putidaredoxin (Pdx) and putidaredoxin reductase (PdR). A linear relationship ($r^2 = 0.96$) was observed for binding of PdR-biotin to immobilized Pdx compared with binding of Pdx-biotin to immobilized PdR (the estimated K_d value for the Pdx · PdR complex was 0.054 μM). Human P450 2A6 interacted strongly with NADPH-P450 reductase; the K_d values (with the reductase) ranged between 0.005 and 0.1 μM for P450s 2C19, 2D6, and 3A4. Relatively weak interaction was found between holo-b_5 or apo-b_5 (devoid of heme) with NADPH-P450 reductase. Among the rat, rabbit, and human P450 1A2 enzymes, the rat enzyme showed the tightest interaction with b_5, although no increases in 7-ethoxyresorufin O-deethylation activities were observed with any of the P450 1A2 enzymes. Human P450s 2A6, 2D6, 2E1, and 3A4 interacted well with b_5, with P450 3A4 yielding the lowest K_d values followed by P450s 2A6 and 2D6. No appreciable increases in interaction between human P450s with b_5 or NADPH-P450 reductase were observed when typical substrates for the P450s were included. We also found that NADPH-P450 reductase did not cause changes in the P450 · substrate K_d values estimated from substrate-induced UV–visible spectral changes with rabbit P450 1A2 or human P450 2A6, 2D6, or 3A4. Collectively, the results show direct and tight interactions between P450 enzymes and the accessory proteins NADPH-P450 reductase and b_5, with different affinities, and that ligand binding to mammalian P450s did not lead to increased interaction between P450s and the reductase.
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The cytochrome P450 (P450)[1] superfamily of enzymes catalyzes the oxidative metabolism of a number of endobiotic and xenobiotic chemicals [1,2]. Liver microsomes contain multiple forms of P450 and individual P450 enzymes have different, but overlapping, substrate specificities for diverse chemicals [3–6]. Recent studies have established that the P450 (*CYP*) 1, 2, and 3 gene families code for the major enzymes involved in

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[1] *Abbreviations used:* P450, cytochrome P450; b_5, cytochrome b_5; NPR, NADPH-P450 reductase; DLPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine, ELISA, enzyme-linked immune absorbent assay; Pdx, putidaredoxin; PdR, putidaredoxin reductase; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; PBS, phosphate-buffered saline solution (10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl); αNF, α-naphthoflavone (5,6-benzoflavone).

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the metabolism of drugs; polymorphisms of these P450 genes have been shown to be important factors controlling individual differences in responses to the pharmacologic and toxic actions of drugs in humans [7–11].

Human P450 enzymes have been purified from liver samples and from heterologous expression systems [12–14]. Reconstitution of P450s with the accessory proteins NADPH-P450 reductase (NPR) and cytochrome b_5 (b_5) in phospholipid vesicles has suggested that some of the P450 enzymes (e.g., P450s 2A6, 2B6, 2C9, 2C19, 2E1, 3A4, and 3A5, plus some involved in steroidogenesis) require b_5 for full catalytic activities, although mechanisms underlying stimulation by b_5 have been shown to differ depending on the P450 enzyme used [14–20]. For example, both holo-b_5 and apo-b_5 have been shown to be able to enhance oxidation activities catalyzed by P450s 2A6, 2B6, 2C8, 2C9, 2C19, 3A4, and 3A5 in reconstituted monooxygenase systems, although P450 2E1-dependent chlorzoxazone 6-hydroxylation is enhanced only when holo-b_5 is included [14,17]. It has also been shown that P450 3A4-catalyzed testosterone 6β-hydroxylation is enhanced by P450s 1A1 and 1A2, which are nearly devoid of this hydroxylation activity, as well as by holo- and apo-b_5 [14]. Other work with rabbit P450s has also demonstrated enhancement of the activity of one P450 by another [21], suggesting that protein–protein interactions may be one of the mechanisms causing enhancement of P450-catalytic activities by b_5 [19,22].

Protein–protein interactions involving P450s have been probed using plasmon resonance systems, utilizing a carboxymethylated dextran matrix as protein-trapping surface [23]. Direct protein–protein interactions were observed using this approach in several P450 monooxygenase enzyme systems, including bacterial putidaredoxin reductase (PdR)/putidaredoxin (Pdx)/P450 101A1 and mitochondrial adrenodoxin reductase/adrenodoxin/P450 11A1 [24,25]. This methodology has also been applied to the interaction of rabbit liver microsomal P450 2B4 with NPR and b_5 [26,27].

In this study, we determined the interactions of various human P450s with NPR and holo- and apo-b_5 using a system in which one protein was added to a plastic surface and the other protein of interest was tagged with biotin, i.e., an enzyme-linked immune absorbent assay (ELISA). Binding was monitored with the use of a streptavidin–peroxidase complex, using peroxidase catalytic activity. We first examined protein–protein interactions with bacterial PdR and Pdx as a model, because this system has been characterized previously. Subsequently, interactions were measured with NPR and b_5 with rat, rabbit, and human P450s 1A2 and human P450s 2A6, 2C19, 2D6, 2E1, and 3A4. The effects of substrates and inhibitors of these P450s on interactions were also examined.

Materials and methods

Chemicals

Succinimidyl-6′-(biotinamido)-6-hexanamido hexanoate (Catalog No. 21343) was purchased from Pierce (Rockford, IL). Streptavidin–peroxidase (Catalog No. S-5512), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Catalog No. A-1888), and Tween 20 (Catalog No. P-7946) were obtained from Sigma Chemical (St. Louis, MO). Other chemicals and reagents were obtained from sources described previously or were of the highest qualities commercially available.

Enzymes

Human P450s 1A2, 2A6, 2C19, 2D6, 2E1, and 3A4 [28–33] and rat NPR [34] were expressed in *Escherichia coli* and purified to apparent homogeneity by methods described previously. Recombinant human b_5 was expressed in *E. coli* JM109 cells from a plasmid (pSE420 (Amp)) kindly provided by Satoru Asahi (Takeda Pharmaceutical, Osaka, Japan). The protein was solubilized and purified to electrophoretic homogeneity using modifications of the DEAE-cellulose and hydroxylapatite chromatography methods described elsewhere [35].

Pseudomonas putida Pdx and PdR were generously provided by T. Poulos and I. Sevrioukova (University of California, Irvine, CA). *Bacillus megaterium* P450 102A1 was expressed in *E. coli* and purified using modifications of procedures described elsewhere [36], using a plasmid originally provided by T. Poulos and A.J. Fulco (University of California, Los Angeles). Rabbit b_5, rabbit NPR, and rabbit and rat P450 1A2 were purified from liver microsomes of rabbits and rats, respectively, as described previously [28,35,37,38]. Apo-b_5, devoid of heme, was prepared from rabbit liver holo-b_5 as described previously [15].

Protein–protein interactions

Varying concentrations of purified enzymes, in 50 μL of 10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS), were applied to the wells of a Nunc 384-well polystyrene plate (Fisher Scientific, Pittsburgh, PA, Catalog No. 242757) and incubated in a humid atmosphere for 2 h at room temperature. After binding of enzymes to the wells, unreacted material was removed by extensively washing the wells with PBS containing 0.1% Tween 20 (w/v) six times. Preliminary experiments were done with the biotinylated enzymes to establish the amount needed to saturate the wells (Fig. 1). A single enzyme incubation at the optimal concentration (vide infra) was sufficient to saturate the wells in all cases, as demonstrated in subsequent tests.

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