Charge-Dependent Sidedness of Cytochrome P450 Forms
Studied by Quartz Crystal Microbalance and Atomic Force Microscopy

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Quartz crystal microbalance (QCM) resonance measurements were used to examine the surface charge characteristics of cytochrome P450 forms and the influence of charge on the docking of redox partners like cytochrome b5. The distal surface of cytochrome P450 (CYP)101 (pI 4.5), relative to the heme, is fairly anionic, as is the proximal surface. The latter, however, also has two cationic clusters. A considerably greater extent of CYP101 binding was seen to the cationic, polyethylene-surfaced resonators. CYP2B4 (pI 8.5) preferentially bound to the polyanionic, polystyrene sulfonate-surfaced resonators. Cytochrome b5 is an acidic protein that had a preferential binding to the poly(ethyleneimine (PEI)-surfaced resonators. When binding to CYP2B4-surfaced films, cytochrome b5 preferentially bound to those cytochrome P450 molecules that were adsorbed to cationic (PEI) films. It is suggested that adsorption of CYP2B4 to an anionic polystyrenesulfonate (PSS) surface is with cationic clusters that include the cytochrome b5 docking domain. This diminishes the extent of docking of the cytochrome b5. In contrast, when CYP2B4 is adsorbed to a cationic film the proximal surface with the cytochrome b5-docking site is available for cytochrome b5 binding. A film of the polycation PEI was adsorbed to the silver QCM surface. It formed polymer islands when viewed with atomic force microscopy. Polyanionic PSS was adsorbed intermittently with the PEI. By the third and fourth layer of polyions the polymer islands were essentially merged and protein adsorption as a fourth or fifth layer formed a nearly continuous film. CYP101 was seen to adsorb as globules with a molecular diameter of about 10 nm. CYP2B4 adsorbed to the polyanionic films had a slightly elliptical globular shape, also with a molecular diameter of about 10 nm.

Key Words: P450 topology; P450 sidedness; P450 surface charges; QCM adsorption of P450; AFM of P450.

Most early attempts to rationalize the structure of the mammalian forms of cytochrome P450 (CYP)2 were based on recognized homologies in the amino acid sequences in alignments of the mammalian forms of cytochrome P450 with CYP101 (cytochrome P450CAM) (1), the first form of cytochrome P450 to be crystallized and studied (2). Alignments of the about 40 forms of cytochrome P450 sequenced in the late 1980s confirmed the similarities of primary structures of the mammalian forms of cytochrome P450 with CYP101 (3, 4) and made possible the identification of a substrate recognition domain for the mammalian forms (5). In the absence of any crystalline form of the mammalian cytochrome P450, a number of attempts were made to model mammalian forms of cytochrome P450. These were based upon different amino acid alignments with sequences of other crystallized bacterial species such as CYP101 (2), CYP108 (cytochrome P450TERP) (6), CYP107 (7), and CYP102 (cytochrome P450BM3) (8). Graham-Lorence and Peterson have suggested an alignment by secondary and tertiary structure

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2 Abbreviations used: AFM, atomic force microscopy; P450cam, CYP101; CYP, cytochrome P450; E. coli, Escherichia coli; PEI, poly(ethyleneimine); PSS, poly(styrenesulfonate); QCM, quartz crystal microbalance; Pdx, putidaredoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
predictions, based upon analyses of four bacterial cytochrome P450 crystal structures (9), that differs from those using primary sequence alignments. In other models homologies of primary sequence with CYP101 have been used (e.g., see (10)), or structural alignments have been made in which the α-carbons of mammalian forms of cytochrome P450 have been superimposed on a number of crystallized forms of cytochrome P450 (9). However, because of the greater size of the mammalian forms of cytochrome P450 than the bacterial forms and the fact that the mammalian forms are membrane-bound, it is difficult to use the bacterial hemoprotein structure to predict the surface topology or domains of interaction between the mammalian cytochromes P450 and their redox partners. A paper has appeared very recently in which a modified form of rabbit cytochrome P450, CYP2C5, has been crystallized and shown to have structural parameters very similar to the bacterial forms of cytochrome P450 (11). This should aid attempts to model the structures of the mammalian form of cytochrome P450.

CYP101 uses putidaredoxin (Pdx), a small (ca. 12 kDa), acidic nonheme iron sulfur protein, as an electron donor. Studies indicated that the interaction of CYP101 with putidaredoxin could be competitively inhibited by another acidic redox protein, mammalian cytochrome b5 (12). The interaction was shown to be by complementary charge-pairing (13). Modeling and site-directed mutagenesis of cationic residues on the putative proximal docking surface of CYP101 indicated the proximal surface of CYP101 as the docking domain of Pdx (14). In contrast to CYP101, mammalian forms of cytochrome P450 have the larger (78 kDa) NADPH-cytochrome P450 reductase as a redox donor, and some forms also interact catalytically with cytochrome b5 as well. The manner and site of interaction of forms of mammalian cytochrome P450 with the small (17 kDa) redox hemoprotein cytochrome b5 has been of some interest. Our studies indicated that at least two forms of cytochrome P450, CYP2B4 and CYP2C11, make use of complementary charge pairing in their interaction with cytochrome b5 (14–18). The site of docking of cytochrome b5 on the cytochrome P450 was suggested as being a region corresponding in alignments to the putative docking site of Pdx on CYP101 (19, 20).

Recently considerable attention has been paid to the development of biosensors, in which biological reactions are induced on the surface of transducers and result in output of a signal that can be quantified electrically. AT-cut quartz crystal resonators are readily available and serve as very sensitive mass detectors (21). These have been used as a biosensor for immunosorbent assay of biochemical processes (22), for binding of concanavalin A to glycolipid monolayers (23), and protein–DNA interactions (24), as well as for electrochemical oxidation of environmental chemicals by hemoproteins adsorbed to charged films (25–28). In the present studies we have made use of the nanogram sensitivity of the QCM and layer-by-layer methodologies (28, 29) to examine the interaction between these components of the cytochrome P450 monoxygenase system. The results of our studies indicate a charge-dependent sidedness of interactions for both CYP101 and CYP2B4 that is different for the two forms of cytochrome P450. The data, further, support the concept that the docking sites of cytochrome b5 on CYP2B4 is on a more cationic surface of this latter hemoprotein, in agreement with our prior suggestion (19, 20).

MATERIALS AND METHODS

Materials. CYP2B4 (49 kDa) and rabbit cytochrome b5 (17 kDa) were prepared from rabbit liver as described earlier (30) and NADPH–cytochrome P450 reductase (78 kDa) was purified from the same tissues, or from an Escherichia coli (E. coli) expression system with a construct containing rabbit NADPH–cytochrome P450 reductase cDNA in the vector pCWoI; essentially by the method of Yasukochi and Masters (31). The proteins were kept at –86°C in 25 mM sodium phosphate, pH 7.4, 20% glycerol, until use. CYP101 was expressed in E. coli DH5α cells using pBl24 expression vector, clone H9, generously provided by Dr. Julian Peterson. The soluble protein was released upon lysing the cells in a French pressure cell and purified on DEAE Sepharose followed by Sephadex G-100. Putidaredoxin, from the same source, was similarly prepared. All of the purified enzyme preparations migrated as single bands on SDS–PAGE. Protein concentrations were determined using the Pierce BCA protein assay, reductase was also quantified enzymatically, and hemoprotein integrity was determined spectrophotometrically (32). Polysaccharides used were sodium poly(styrenesulfonate) (PSS, 70 kDa, Aldrich, St. Louis) and branched poly(ethyleneimine) (PEI, 25 kDa, Wako, Japan). Benzphetamine·HCl was a gift from Upjohn, Co.

Methods. Film assembly proceeded as follows: Polysaccharide films were assembled on the sensitive QCM resonator basically as described earlier (28, 29, 33). The films had a definite positive or negative charge depending on which polyion layer was the final step in the multilayer to which the protein was adsorbed. Every adsorption step was monitored by the frequency shift of the resonator. This permitted the calculation of the adsorbed mass of protein. Films were assembled on silver-coated 9 MHz AT-cut quartz QCM resonators (USI Systems, Fukuoka, Japan) and International Crystal Mfg. Co., Inc., Oklahoma City, OK). The silver surface was cleaned and made receptive to the polyelectrolyte binding by sonication for 30 s in a bath sonicator with 1% KOH in 60% ethanol. After rinsing and drying in a stream of N2, the resonant frequency of the resonators was determined. In subsequent adsorption cycles the resonators were immersed in a solution of PEI, 1.5 mg/ml, for 20 min, giving it a cationic charge. The resonators were then rinsed in water and dried in a gentle stream of dry N2, and the resonant frequency shift determined. This was followed by immersion in PSS, 3 mg/ml in 0.1 M NaCl, for 20 min and, after rinsing, by drying in the gentle stream of dry N2, followed by the frequency shift determination. A subsequent treatment with the PEI solution was carried out for terminally positively charged resonators. For negatively charged resonators this was followed by an additional treatment with the PSS solution. Thus, resonators with a final positive charge have received three layers and anionic resonators have received four polyionic layers. Proteins were adsorbed to the resonators in the same manner, but the resonators were immersed in the protein solutions for 30 min to assure complete adsorption. Prior studies indicated that protein adsorption was complete within 15 min (33). Binding of cytochrome b5 was carried out in solutions containing 1 mM benzphetamine as
this CYP2B4 substrate increases the affinity between the two hemoproteins (15). Changes in resonant frequency were determined on dried films after adsorption of each layer. In the absence of variations in viscoelasticity the change in resonant frequency of the shear mode QCM resonator is related to the mass as determined from the Sauerbray equation (21),

\[ \Delta F = \frac{-2f_0^2 \Delta m}{(\rho \mu A)^{1/2}} \]

where the characteristics of the quartz, \(2f_0^2/(\rho \mu A)^{1/2}\), can be summarized by the constant \(k\), which in our system has been experimentally determined to be \(1.83 \times 10^8\). The increase in mass (\(\Delta m\)), in grams, can be determined from the frequency shift (\(\Delta F\)) of the dry films or the proteins adsorbed to them after taking into account the resonator characteristics, according to the equation

\[ \Delta F = 1.832 \times 10^8 \Delta m/A. \]

The measured area (A) of the microbalance resonators used is \(0.16 \pm 0.01\) cm\(^2\), and the change in mass can be determined from the change in frequency after adding adsorbing material:

\[ \Delta F/1.145 \times 10^9 = \Delta m. \]

The thickness of the adsorbed layers can be calculated from the density of the polyionic material (1.2 g/cm\(^3\)) and the mass of protein embedded in and on it (1.3 g/cm\(^3\)) and assumes that the film layer is uniform.

Space-filling models of CYP101 were constructed using a Silicon Graphics workstation with Sybyl, and coordinates of the molecule were obtained from Entrez at the NCBI web site.

Analyses of the binding of films and proteins to the resonators using AFM was carried out on a Mac-mode AFM (Molecular Imaging). Commercial supersharp Si\(_3\)N\(_4\) tips attached to triangular cantilevers were used, as described earlier (34). The probing force was minimized in each experiment to reduce tip perturbation of the samples. Samples dried on QCM resonators were inserted, positioned, and a drop of 10 mM sodium phosphate, pH 7.5, was put on the surface to wet the samples before analysis.

RESULTS

Figure 1 shows a sketch of the silver-coated QCM resonator used, cartooning the adsorption of polyions. The adsorption of multilayer films of charged surfactants on silver-coated QCM resonators is exemplified in the side view, with an initial layer of PEI (mass about 25 kDa) shown as thin lines adsorbed to the silver layer. Subsequent binding of the polyion, PSS (mass about 70 kDa), was essentially as described earlier (33), and is depicted by the thicker line attached in places to the PEI, with which it makes numerous salt bridges with cationic charges on the latter. Binding of the initial layer of PEI resulted in a positively charged resonator and decreased the oscillation frequency of the resonator (Fig. 2). A greater decrease in frequency was accomplished by subsequent incubation of the resonator in PSS, due to a greater mass adsorption. Sequential incubations of PEI and PSS resulted in similar adsorption of polyions with decreases in resonant frequencies. The right-hand scale shows the adsorbed masses of polyions resulting in the decreases in resonant frequency.

Subsequent to the adsorption of the polyion films the binding of CYP101 was examined. Using a three-layered film with the cationic PEI as the surface layer, the adsorption of CYP101 was determined as a function of the concentration of cytochrome P450 (Fig. 3). As the concentration of cytochrome P450 was increased the amount of...
the protein adsorbed increased. The solid line is the fit of the equation to a rectangular hyperbola, and has a half-maximal binding value of 0.617 μM and a ΔF_{max} of -810.4. Differences were observed for the binding of the CYP101 to differently charged polyion films. Figure 4 shows the adsorption of CYP101 and Pdx to the cationic surface (PEI, a three-layer alternating polyion film) when the concentrations of the proteins were 8 μM in the medium. Approximately 15 pmol of CYP101 was adsorbed, as seen by a frequency decrease of almost 800 Hz. Immersion of the PEI-surfaced resonator in buffer containing Pdx resulted in adsorption of 16.6 pmol of protein, as seen by a decrease in resonant frequency of 228 Hz. Immersion of resonators with an anionic surface charge (PSS, a four-layer alternating polyion film) in buffer containing these proteins resulted in considerably lower molar adsorption (Fig. 4, PSS). A 6-fold lower amount of CYP101 was adsorbed to the PSS film than to the PEI film (2.6 pmol vs 15 pmol). Similarly, Pdx adsorption to the anionic surface was about 3-fold lower (4.7 pmol vs 16.6 pmol). These experiments indicate a differential distribution of charged residues on the surface of both protein and, at least for CYP101, is in agreement with the crystal structure of the protein. Immersing the CYP101-coated resonators in solutions containing 8 μM Pdx resulted in similar low levels of binding to the CYP101, despite the greater amount of the hemoprotein adsorbed to the PEI-surfaced resonator (data not shown). The charge distribution on the surface of a space filling model of CYP101, showing the acidic and basic residues on the proximal surface relative to the heme, is seen in Fig. 5. The cluster of blue (basic) residues in the center of this surface represents a functionally active cationic domain above the heme (cyan) on the proximal surface of CYP101, believed to be the docking site for Pdx. Another basic cluster is seen on the lower right side of this surface. A number of accessible acidic residues (red) is scattered over this surface of the hemoprotein. The distal surface of CYP01 (Fig. 6) has an even greater number of accessible acidic residues without the local concentrations of cationic residues of the proximal surface. This asymmetry of charge creates a sidedness to the molecule, and explains the greater degree of adsorption of the hemoprotein to the cationic PEI film than to the PSS film on the silver QCM resonator (Fig. 4). The charge-dependent sidedness of CYP101 serves to increase the efficiency of electron transfer by orienting the acidic Pdx molecule toward the proximal surface of the hemoprotein where the heme prosthetic group is closest to the surface (Fig. 5).

The pattern of adsorption of CYP2B4, a mammalian form of cytochrome P450, to charged polyions films was distinctly different from the adsorption of CYP101. Its binding to the anionic PSS film was twice as high as its extent of adsorption to the cationic PEI film (Fig. 7, cycle 1), indicating that in contrast to the acidic CYP101, CYP2B4 has a highly cationic molecular surface. The pI of CYP2B4, determined from isoelectric focusing, was 8.5 (data not shown). A sidedness with respect to charge distribution also exists on this protein, and influences the docking of its redox partner, cytochrome b₅, on the CYP2B4. As shown in Fig. 7, cytochrome b₅ preferentially bound to CYP2B4 that was adsorbed to the cation-surfaced resonator. The molar ratio of cytochrome b₅ bound to CYP2B4 was almost 4:1, when the latter hemoprotein was adsorbed to the cationic film, and was 0:5:1 when the CYP2B4 was adsorbed to the anionic PSS.

In agreement with earlier studies indicating the docking of cytochrome b₅ on CYP2B4 is influenced by electrostatic interactions (18), the binding as measured by QCM also showed interference by increased ionic strength. The influence of ionic strength on the binding of cytochrome b₅ to CYP2B4 is shown in Fig. 8. The immersion of the QCM resonator with a cationic surface in a solution of 8 μM CYP2B4 in 10 mM buffer (Fig. 8) resulted in 2 pmol of cytochrome P450 adsorption (cycle 1). The subsequent immersion in 8 μM cytochrome b₅ in 10 mM buffer (cycle 2) resulted in a molar cytochrome b₅:cytochrome P450 ratio of 4:1, as indicated above. However, if the cytochrome b₅ was in 200 mM buffer the amount bound was reduced by more than half (cycle 2, solid bar).

The adsorption of cytochrome P450 to polyion films on solid supports was also examined using AFM. This procedure has been employed as a valuable tool for determining the surface of various solid supports and can yield greater discrimination than scanning electron microscopy. It is useful for showing layering effects of lipids and polyions with protein. QCM experiments, while useful for quantifying protein binding to solid supports, do not give an indication of the uniformity of the surfaces formed. Thus, although one can calculate the “thickness” of the binding layers, the assumption is that the layers are uniform and complete. After multiple polyion layers, a uniform surface is seen...
by scanning electron microscopy (28, 29). An AFM scan of a single layered PEI-coated QCM resonator is shown in Fig. 9. The polyion adsorption formed islands, rather than a film surface of uniform thickness. The PEI (25 kDa) is visible as blobs of about 7 nm diameter. Subsequent binding of CYP101 to the PEI provided an indication of the appearance of the hemoprotein (Fig. 9, right). There is an increase in the island size due to a PEI-CYP101 interaction. The CYP101 appears to aggregate, forming a layer over the PEI islands (see enhanced image portion). The insert points to a molecule of the 45-kDa hemoprotein, seen as a globule of about 10 nm diameter. In contrast, Pdx adsorption resulted in a uniform dispersal of the Pdx over the surface of the polyion film (Fig. 10, right). Zooming in on a section of the Pdx-bound PEI film reveals the 12-kDa Pdx molecules as smaller flecks associated with the PEI blobs. The anionic polyion PSS, too, takes on a globular shape in the film when adsorbed to the PEI on the solid support (Fig. 11). The larger (70 kDa) PSS has a molecular diameter of almost 11 nm. The island nature of the two polyion-layered film is clearly visible. CYP101 binds more sparingly to the PSS film (Fig. 11, right), and these smaller molecules are difficult to differentiate from the similar-appearing PSS.

When more than three polyion film layers are adsorbed the surface is more uniform and the islands appear to merge into a continuous film. Binding of CYP2B4 to a four-layer anionic surface (left) and three-layer cationic surface (right) is shown in Fig. 12. The polyion island nature of the three-layered underlying film is still discernable, while with the four-layered polyion film the island-like structure is almost nondetectable under the protein. The CYP2B4-bound resonators were immersed in a cytochrome b_{5} containing solution. One can clearly see a greater extent of bind-

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**FIG. 5.** Space filling model of CYP101, proximal surface. Side chain carboxyl residue oxygens are in red and side chain nitrogens are in blue. The buried heme is visible in cyan and the cysteine 357 sulfur is in yellow. Models were constructed with a Silicon Graphics computer using Sybyl.
FIG. 6. Space filling model of CYP101, distal surface. Side chain carboxyl residue oxygens are in red, side chain nitrogens are in blue.

FIG. 9. AFM of silver coated QCM resonator to which the cationic polyion, PEI, was adsorbed and the resonator with subsequent binding of CYP101 (P450cam). For this experiment only a single polyion layer was used. The insets point to individual PEI and CYP101 molecules.
ing of CYP2B4 to the PSS-coated resonator (left) than to the PEI-coated resonator. The 49-kDa CYP2B4 appears to have a more oval shape than the CYP101 with a molecular diameter of about 10 nm (Fig. 12, right). Unfortunately, at this time it is not possible to resolve the binding of the smaller (17 kDa) cytochrome b5 on the larger CYP2B4 molecules. However, it is possible to see smaller, rectangular specks attributable to cytochrome b5 in the figure. There appears to be more cytochrome b5 on the cation-surfaced resonator, in agreement with the QCM results of Fig. 6.

DISCUSSION

CYP101 has a pI of about 4.5 (35) and an equal number of acidic and basic residues. However, there is an inequality in the surface distribution of the charged residues, such that the side distal to the heme is heavily anionic and the side proximal to the heme, where the heme is closest to the surface, has a few cationic clusters among the distribution of anionic residues. As with purple membrane (36), CYP101 has an asymmetry in surface charge distribution on the molecule that results in a sixfold greater adsorption of CYP101 to the cationic PEI surface film than to the anionic PSS surface film. This inequality in surface charge distribution produces a sidedness to the molecule that would be expected to facilitate proper orientation of its redox partner, Pdx. Pdx also is an acidic protein, with a pI below 4.5 (35). It has been shown to charge-pair with a cationic cluster on the surface proximal to the heme cysteine ligand of CYP101. Included in this cluster are amino acids R112, R72, K344, and R364 (12, 14).

In contrast to CYP101, rabbit liver CYP2B4 is a basic protein, with a pI of 8.5 (Jansson and Schenkman unpublished data). Like CYP101, its primary sequence contains 52 acidic amino acids. In contrast to the bacterial protein, which has an equal number of basic residues, CYP2B4 contains 71 basic residues. The crystal structure of this membrane protein has, to date, not been determined. Our QCM studies on the binding of CYP2B4 protein to polycation charged films indicate that it, too, has an asymmetrical distribution of surface charges that imparts a sidedness to this molecule. Almost twice as much of the hemoprotein bound to the resonators charged with the anionic PSS than to the resonators charged with the cationic PEI. This charge-sidedness indicates, in contrast to CYP101, a greater cationic surface charge than anionic charge. As noted by He et al. (36), an asymmetry of charge on purple membranes provides for a high degree of orientation of bacteriorhodopsin in thin films. The same would be true for an asymmetry of charge on a membrane protein, with respect to its membrane orientation. Earlier studies on the docking of cytochrome b5 to different forms of cytochrome P450 indicated that the interaction between the hemoproteins is by complementary charge pairing (15, 18), making use of carboxyl residues on the cytochrome b5. Cytochrome b5 is an acidic protein with 23 of its 133 residues either aspartate or glutamate. Its docking to a functionally active site on CYP2B4 has been shown to involve complementary charge pairing of acidic amino acid residues of the cytochrome b5 with cationic amino acids on the surface of CYP2B4 (18–20). Examination of the amino acid sequence of CYP2B4 revealed the pres-

FIG. 7. Charge-dependent sidedness of CYP2B4. Comparison of adsorption of CYP2B4 to PEI- and PSS-coated QCM resonators and influence of this binding on subsequent docking of cytochrome b5 to the cytochrome P450. Resonators prepared as in Fig. 2 were immersed in 10 mM sodium phosphate buffer containing 8 μM CYP2B4 (cycle 1) for 30 min. The resonators were then rinsed in deionized water and dried under a gentle stream of nitrogen and the frequency change measured. The cytochrome P450-bound resonators were then immersed in 10 mM sodium phosphate buffer containing 8 μM cytochrome b5 and 1 mM benzphetamine for 30 min and were subsequently rinsed and dried under a gentle stream of nitrogen for measurement of frequency change (cycle 2).

FIG. 8. The influence of ionic strength on the interaction of cytochrome b5 with CYP2B4. The resonators coated with three alternating polycation film layers were immersed in 8 μM CYP2B4 for 30 min and then were rinsed, dried, and the frequency shift measured. They were then immersed in solutions of cytochrome b5 in either 10 mM sodium phosphate (cycle 2) or 200 mM sodium phosphate buffer, pH 7.5 (cycle 2, solid bar), for 30 min. Benzphetamine was present at 1 mM level during the binding of cytochrome b5 in 10 mM as well as in 200 mM buffer.
FIG. 10. AFM of Pdx on silver coated QCM resonator to which the cationic polyion, PEI, was previously adsorbed (right). For comparison, the PEI-coated resonator is shown on the left. The inset points to an individual PEI and Pdx molecule.

FIG. 11. AFM of CYP101 binding to a PSS film. The two-layer polyion film (surface layer PSS) before (left) and after (right) immersion in 8 μM CYP101 (P450cam) solution in 10 mM sodium phosphate buffer, pH 7.5.

FIG. 12. Comparison of binding of CYP2B4 and cytochrome b₅ to anionic and cationic polyion multilayers by AFM. The support used was gold and cytochrome P450 binding was to three-layer (PEI surface) and four-layer (PSS surface) films. After binding of the CYP2B4 (30 min) the resonators were rinsed and immersed in the cytochrome b₅ solution containing 1 mM benzphetamine for 30 min.
ence of a region containing a substrate recognition motif, RRFS, of cAMP-dependent protein kinase (37). Phosphorylation of the serine in this sequence in several forms of cytochrome P450, which is also found in some 62 other forms of cytochrome P450, mostly in cytochrome P450 family 2, was shown to inhibit binding of cytochrome b5 (20, 38, 39). On the basis of competitive inhibition between cytochrome b5 and phosphorylation of cytochrome P450 by cAMP-dependent protein kinase it was concluded that the docking site of cytochrome b5 on CYP2B4 overlapped the protein kinase substrate recognition motif, amino acids R125–S128 (20). This region corresponds to one of the cationic clusters on the proximal surface of CYP101 in alignments (40). That region on CYP101 was elegantly assigned to the docking site for its redox partner, Pdx (12, 14), and contains the amino acid R112, which was shown to be essential for electron transfer to the heme iron from Pdx (41). In alignment studies with CYP101 the corresponding amino acid on CYP2B4 is R125, one of the above-mentioned residues of the cAMP-dependent protein kinase substrate recognition motif. Further support for the identification of the cytochrome b5-docking domain on the mammalian cytochrome P450 came from studies on mouse cytochrome P450 (CYP2A5). It could be shown that alteration of residue R129 (a component of the protein kinase substrate recognition motif, RRFS) resulted in decreased binding of cytochrome P450 to cytochrome b5-affinity columns (42).

While both CYP101 and Pdx preferentially adsorbed to the cationic PEI surface, little difference was noted in the extent of binding of Pdx to CYP101 adsorbed to either the PEI- or PSS-surfaced resonators, despite the much greater degree of binding of the monooxygenase to the PEI-surfaced resonators. Perhaps the difference between the binding of Pdx and cytochrome b5 reflects differences in their catalytic roles. The former works with the soluble bacterial monooxygenase, CYP101, serving as an electron shuttle component in the monooxygenase reaction. The oxidized Pdx binds to and accepts reducing equivalents from the Pdx reductase enzyme. It is the reduced Pdx that interacts with ferric CYP101. In contrast, ferric cytochrome b5 works with membrane-bound monooxygenases, binding to ferric cytochrome P450 and serving as an electron buffer in the monooxygenase reaction; it takes an electron from the reduced cytochrome P450 and returns it to the oxyferrous cytochrome P450 (for details see (43)). In the studies reported in this paper we used equal concentrations of Pdx and CYP101 (8 μM) for electrostatic adsorption to the polyion films. Future studies will require higher levels of the oxidized Pdx, as a dissociation constant for the Pdx–CYP101 complex could be calculated as about 10–30 μM from equilibrium dialysis studies (44), and studies on the metabolism and CYP101 reduction both employ concentrations of Pdx about 10-fold higher than CYP101 hemoprotein concentration. In addition, future studies on docking of Pdx to the adsorbed CYP101 will utilize reduced Pdx. The affinity between the reduced Pdx and CYP101 has been shown to be about two orders of magnitude greater than with oxidized Pdx (44).

Prior studies on the charge-pairing interaction between cytochrome b5 and different forms of cytochrome P450 indicated that the proteins had high affinity for each other, with dissociation constants of less than 0.5 μM (15). Spectrophotometric titration of several forms of cytochrome P450 with cytochrome b5 indicated saturation of binding, with 1:1 stoichiometry being achieved at equimolar concentrations of the two proteins (15, 45). In contrast, the present QCM experiments indicated a cytochrome b5:CYP2B4 stoichiometry of about 4:1 (Fig. 7). This would suggest that if all of the binding was to cytochrome P450 more than one cytochrome b5-binding sites must exist on the CYP2B4. Since the earlier measurements of stoichiometry were carried out by following spin state shifts in the cytochrome P450, the additional binding sites probably do not affect the spin equilibrium of the CYP2B4. It is also not yet clear whether the additional binding is by salt bridging, since preliminary experiments with elevated ionic strength reduced the extent of cytochrome b5 binding to CYP2B4-bound resonators only by half (Fig. 8).

Geometric modeling of CYP2B4, based upon generation of a template from structures of the four crystallized forms of cytochrome P450, has been used to examine the substrate binding region of the CYP2B4 and potential docking sites of the redox proteins, cytochrome b5 and NADPH–cytochrome P450 reductase. The studies suggested three different potential cytochrome b5 docking sites on CYP2B4 (46). The models were further refined using site-directed mutagenesis and predictions were made that the proximal surface of CYP2B4 is the binding surface for both cytochrome b5 and NADPH cytochrome P450 reductase (47). This suggestion is similar to an earlier one that was based upon structural alignments with CYP101 and with several other bacterial forms of cytochrome P450 (48). Future studies will be aimed at examining the interaction between the reductase and cytochrome P450 and formation of complexes involving these plus cytochrome b5.

In more general terms, this study points to the utility of combining QCM and the layer-by-layer film growth method for studying protein–protein interactions. We have found that it is possible to orient an unsymmetrically charged protein on a polyion underlayer so that its site of binding to a second protein in solution is either available or unavailable, depending on the charge of the underlying polyion layer. QCM measurements allow quantitation of the binding events. The AFM results point to the globular nature of
the individual polyon layers and also visualize the proteins. However, AFM resolution for these proteins is limited by the island-like structure of the polyon underlayers. Using regularly ordered, smooth, charged underlayers that do not form islands, it may be possible to improve binding specificity and AFM resolution for the proteins. Such techniques may provide powerful tools for investigating biomolecular interactions and are currently under investigation.

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