Association of Cytochromes P450 with Their Reductases: Opposite Sign of the Electrostatic Interactions in P450BM-3 As Compared with the Microsomal 2B4 System

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ABSTRACT: The role of electrostatic interactions in the association of P450s with their nicotinamide adenine dinucleotide phosphate- (NADPH) dependent flavoprotein reductases was studied by fluorescence resonance energy transfer. The fluorescent probe 7-(ethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin maleimide (coumarylphenylmaleimide, CPM) was introduced into the flavoprotein molecule at a 1:1 molar ratio. The interaction of P450 2B4 and NADPH−P450 reductase (CPR) from rabbit liver microsomes was compared with that of the isolated heme domain (BMP) and the flavoprotein domain (BMR) of P450BM-3. The cross-pairs of the components were also studied. Increasing ionic strength (0.05−0.5 M) was shown to result in the dissociation of the CPR−P450 2B4 complex with the dissociation constant increasing from 0.01 to 0.09 μM. This behavior is consistent with the assumption that charge pairing between CPR and P450 2B4 is involved in their association. In contrast, the electrostatic component of the interaction of the partners in P450BM-3 was shown to have an opposite sign. The isolated BMP and BMR domains have very low affinity for each other and the dissociation constant of their complex decreases from 8 to 3 μM with increasing ionic strength (0.05−0.5 M). Importantly, the BMP−CPR and P450 2B4−BMR "mixed", heterogeneous pairs behave similarly to the pairs of BMP and P450 2B4 with their native electron donors. Therefore, the observed difference in the interaction mechanisms between these two systems is determined mainly by the different structure of the heme proteins rather than their flavoprotein counterparts. P450BM-3 is extremely efficient and highly coupled, with the reductase and the P450 domains tethered to one another. Therefore, in contrast to P450 2B4−CPR binding, very tight binding between the P450BM-3 redox partners would be of no value in the synchronization of complex formation during catalytic turnover.

P450s are heme-thiolate enzymes acting as terminal monooxygenases in multienzyme systems found in animals, plants and bacteria (*1*). These enzymes catalyze the oxidation of various hydrophobic compounds by the two-electron activation of molecular oxygen, whereby one atom of dioxygen is inserted into the organic substrate and the other is reduced to water. The membrane-bound liver microsomal monooxygenase system (MMO) of higher animals catalyzes the oxygenation of both endogenous substrates (steroids, fatty acids, etc.) and exogenous compounds (drugs, carcinogens, and other xenobiotics). A wide variety of substrates can be oxidized because of the existence of multiple P450 isozymes—some have broad substrate selectivity while others have a very narrow range of substrates. In the case of microsomal monooxygenases, electrons are transferred to P450 by the flavoprotein NADPH−P450 reductase (CPR)[1] or by cytochrome b_5. These microsomal electron carriers were shown to form dynamic complexes in the membrane (*2*−*6*). Since CPR, the most important donor of electrons to P450, is the limiting component in microsomes (*4*, *6*), the interaction of P450s with CPR may be a major determinant of the functional activity. Recently, interactions between different P450 species and their competition for CPR have been recognized to be very important in the functional and regulatory mechanisms of microsomal monooxygenases (*7*−*9*).

Besides the fact that the formation of electron-transfer complexes between P450 and its electron donors is essential for catalysis, the interactions with these proteins also affects the spin state of the microsomal P450s, their affinity for

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[1] P450BMP or BMP, the heme protein domain of P450BM-3; P450 2B4, rabbit liver microsomal cytochrome P450 2B4, CYP2B4; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CPM, 7-(ethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin maleimide (subscript CPM indicates labeling with this reagent); CPR, microsomal nicotinamide adenine dinucleotide phosphate (NADPH)−cytochrome P450 reductase; BMR, reductase domain of P450BM-3; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PCA, principal component analysis.

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substrates, and the degree of coupling of hydroxylation with NADPH oxidation (*10*−*15*). Although the interactions of P450s with NADPH−P450 reductase and cytochrome b_5 have been extensively studied, the mechanism of the complex formation between these proteins remains unclear. Generally, this is because the interactions were followed by indirect methods—by the association-induced spin shift of the P450 heme iron or by the kinetics of P450 reduction and substrate hydroxylation. Such indirect observations are difficult to interpret in terms of molecular mechanisms.

The question of the role of electrostatic interactions in complex formation between P450s and their partners has been extensively discussed in the literature over the past decade. In the case of P450 interactions with cytochrome b_5, the involvement of interprotein charge pairing is well-established (*14*, *16*−*21*). However, there are unresolved contradictions in the literature on the role of electrostatic interactions in the association of P450 with CPR. The importance of charge pairing in the association of microsomal P450 with CPR is supported by a large number of experimental observations, including those employing the techniques of chemical modification and cross-linking (*22*−*27*), competitive binding with cytochrome *c* (*28*), synthetic peptides (*19*, *20*), and anti-peptide antibodies (*29*, *30*), as well as site-directed mutagenesis (*12*, *21*, *31*, *32*). In contrast, Voznesensky and Schenkman, who investigated the dependence of the electron transfer and hydroxylation kinetics on ionic strength (*33*, *34*) and polyol concentration (*35*), interpreted their results as due to the electrostatic repulsion between P450 and CPR even with the complexity of the micellar reconstitution system and the indirect character of the observations (*35*). Nevertheless, a stimulatory effect on the rate of electron transfer and hydroxylation activity in the microsomal monooxygenase system due to ionic strength is well-established in these and other publications (*13*). Thus, a direct examination of the effect of ionic strength on the formation of the CPR−P450 complex is required to resolve these conflicting conclusions regarding the electrostatic nature of the interactions.

An important difficulty in the studies of intermolecular interactions in the microsomal monooxygenase system arises from its complexity. In reality, this membrane-bound multienzyme system consists of multiple isoforms of P450 competing for the same limiting electron donor, CPR. The importance of P450−P450 interactions and the reciprocal modulatory effects of different P450s in the membrane are now becoming evident (*8*, *9*, *36*). In addition, the monooxygenase system also includes cytochrome b_5 that physically and functionally interacts with different P450s in a isoform-specific way (*11*, *37*−*39*). The composition of the lipids of the microsomal membrane is also very important for the intermolecular interactions in the monooxygenase (*40*−*42*). Reconstitution of the microsomal monooxygenase system is almost impossible in vitro in a manner suitable for studies of intermolecular interactions. From our perspective, micellar systems with added phospholipids, which are commonly used to reconstitute the activity of monooxygenases (*13*, *14*, *33*−*35*), are inappropriate for studies of protein−protein interaction inasmuch as the proteins are compartmentalized in small micelles of poorly defined structure. The interactions between the electron carriers in these systems appear to be hindered by a slow protein exchange between these negatively charged micelles, each containing only a few protein molecules. We believe that a soluble system containing a low concentration (0.02−0.05%) of a nonionic detergent (such as Emulgen-913 or Triton N-101) is more relevant for these experiments. Although the system with Emulgen is sometimes referred to as "monomeric" (*43*, *44*), the microsomal proteins are in a rapid equilibrium between monomers and oligomers (*45*, *46*). Using the fluorescence energy transfer technique, we have estimated the characteristic time of decay ($t_{1/2}$) of P450 2B4 oligomers at 0.025% Emulgen-913 to be about 18 s with the dissociation constant being about 0.07 μM at 0.06 M ionic strength (unpublished observations). Our studies on the kinetics of electron transfer in these conditions showed that the formation of the electron transfer complex between P450 2B4 and the reductase under these conditions is extremely rapid and does not limit the rate of electron transfer (*44*). Although this system is still far from being completely adequate and the possible displacement of the oligomerization equilibrium has to be considered while the results are interpreted, the reconstitution of this monooxygenase system with nonionic detergent apparently offers the most transparent strategy for mechanistic studies on protein interactions.

A valuable simplification in the interpretation of the experimental results may be obtained from a comparative approach with the use of a soluble bacterial monooxygenase as a reference system. The only well-characterized bacterial monooxygenase that belongs to class II P450s, where the P450 receives electrons from a flavin adenine dinucleotide/flavin mononucleotide- (FAD/FMN-) containing reductase, is P450BM-3 from *Bacillus megaterium* (*47*). This enzyme consists of a single polypeptide chain and is catalytically self-sufficient in the monooxygenation of fatty acids (*48*). The holoenzyme can be proteolytically cleaved into the NH_2-terminal P450 domain (BMP) and the COOH-terminal reductase domain (BMR), which contains FAD and FMN (*49*). The activity of the holoenzyme may be partially reconstituted by combining purified recombinant BMP and BMR domains (*50*), which have been genetically engineered and expressed as individual enzymes in *Escherichia coli* (*50*, *51*).

The functional similarity of P450BM-3 to the microsomal P450 enzymes is also reflected in the amino acid sequences. The sequences of the BMR domain of P450BM-3 and those of the microsomal CPR enzymes are about 35% identical (*52*). Similarly, the BMP heme domain reveals 25−30% sequence identity to the closest microsomal members of the P450 superfamily (*53*). We believe that the mechanisms of the interactions of microsomal P450s with the reductase might be notably clarified by comparison with the interdomain interactions in the P450BM-3 monooxygenase. Such a comparison is especially important in view of the availability of high-resolution X-ray crystallography data for the heme domain of P450BM-3. Moreover, the X-ray structure of the BMP complex with the FMN domain of BMR has become available recently (*54*). The structural data are expected to considerably facilitate the analysis of the intermolecular interactions in microsomal monooxygenases when studied in comparison with the interdomain interactions in P450BM-3.

In the present study, we have examined the effect of ionic strength on the interaction of rabbit liver microsomal P450 2B4 with microsomal CPR and compared the results with

BMP−BMR association and the interactions in the heterologous pairs of electron carriers (BMR−P450 2B4 and CPR−BMP).

EXPERIMENTAL PROCEDURES

Materials. 7-(ethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin maleimide (CPM) was from Molecular Probes Inc., and Emulgen-913 was from Kao Atlas Co. Ltd., Japan. All other chemicals were of ACS grade and were used without further purification. The purification of the heme protein domain of P450BM-3 (BMP), from *E. coli* clones containing a plasmid encoding this protein, has been described previously. Purification of P450 2B4 and microsomal NADPH−P450 reductase from livers of phenobarbital-treated rabbits was done by published procedures (*43*, *55*).

Preparation of CPM-Labeled Flavoproteins. Labeling of NADPH−P450 reductase by CPM was performed by a previously described method (*27*) with some minor modifications. Briefly, a 2-fold molar excess of CPM (as a 3 mM solution in acetone−methanol 1:1) was added to a 20 μM solution of CPR in 0.1 M Na-Hepes buffer containing 1 mM EDTA, 1 mM $NADP^+$, 1% sodium cholate, and 20% glycerol. The reaction was followed by the increase in fluorescence at 455 nm (excitation at 385 nm). The time of incubation (about 4 h at 25 °C) was adjusted to reach 95% conversion in the initial kinetic phase of the modification. The reaction was stopped by addition of 1 mM DTT. Removal of the excess label and detergent was achieved by dialysis against 500 volumes of 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM DTT, 1 mM EDTA, and 20% glycerol. Dialysis was continued for 36 h with three changes of buffer. In the last change, the 0.1 M potassium phosphate was replaced by 0.1 M Na-Hepes buffer (pH 7.4). The flavoprotein was then concentrated with an Amicon Minicon-50 concentrator. CPR_{CPM} preparations were stored at −70 °C as 20−50 μM solution. The concentration of CPR_{CPM} and the degree of modification were determined as previously described (*27*). To determine the concentration of CPM, the extinction coefficient of 31 mM^{-1} cm^{-1} (*56*) was used. The molar ratio of CPM to CPR in our preparations was in the range of 0.6−0.9. As reported earlier (*57*), modification of CPR by CPM has no effect on the activity of the enzyme. The modification of BMR by CPM was carried out essentially in the same way except that the addition of 1% cholate was omitted and the CPM was added in 10% (instead of 2-fold) molar excess over BMR. The modification of BMR was considerably faster than that of the microsomal reductase: the equimolar modification of BMR required only 50 min of incubation at room temperature.

Experimental. Fluorescence quenching experiments were done on PTI (Photon Technology Instruments) or Perkin-Elmer LS50B (Perkin-Elmer, U.K.) fluorescence spectrophotometers. Polarization of fluorescence was also measured on a Perkin-Elmer LS50B instrument. The titration experiments were performed with a 5 × 5 mm fluorescence cell. To follow the quenching of CPR_{CPM} fluorescence (λ_{max} = 455 nm), emission spectra in the range of 400−550 nm were recorded with excitation at 385 nm. As both P450 2B4 and BMP absorb at the wavelength of excitation (ϵ_{385} for the low-spin form of these hemoproteins was estimated to be 35 and 38 mM^{-1} cm^{-1}, respectively), the emission intensities were corrected to take into account the "internal filter effect". This arithmetic correction was made by taking into account transmittance of the hemeprotein solution at 385 nm and the path length of the exciting light. The degree of quenching was determined by the least-squares fitting of each spectrum in the series by the first spectrum recorded at the initial conditions (without quencher) combined with the first-order polynomial to compensate for unspecific fluctuation of the baseline. Alternately, a PCA decomposition was used to increase the accuracy of the analysis in the cases when quenching was associated with a shift of the emission band. All data treatment and fitting was done with our SpectraLab software package (*57*).

The experiments were carried out at 25 °C in 100 mM Na-Hepes buffer, pH 7.4, containing 1 mM dithiothreitol (DTT) and 1 mM EDTA, if not otherwise indicated. The ionic strength (I) of this buffer was determined to be 0.058 M. In the experiments with variable ionic strength, 10 mM Na-Hepes buffer, pH 7.4 (I = 0.006 M), was used and the desired ionic strength was reached by addition of KCl. The experiments with microsomal proteins (P450 2B4 and CPR) were carried out in the presence of 0.025% Emulgen-913. In this case the proteins were preincubated with detergent overnight as described (*43*).

RESULTS

Interactions of P450 2B4 with microsomal NADPH−P450 Reductase and the Flavin Domain of P450BM-3. As reported earlier (*27*), formation of the complex of CPR_{CPM} with P450 2B4 results in quenching of the fluorescence of the label. In the present work, we also attempted to monitor the interactions by the changes in the polarization of the fluorescence of CPM by recording a series of polarization scans in the 410−550 nm region (excitation at 385 nm). To increase the signal-to-noise ratio, the series was then subjected to the PCA procedure and only the first principal component (60−70% of the total changes) was considered. The changes in degree of polarization given by the first principal component were then averaged over the whole spectral region. As shown in Figure 1, titration of CPR_{CPM} results in an increase of the degree of polarization of the CPM fluorescence. Titration curves obtained by this method were in good agreement with those recorded by the fluorescence quenching technique (Figure 1). Both methods give the same estimates for the dissociation constant of P450 2B4−CPR complex (about 0.035 μM in 100 mM Na-Hepes buffer, pH 7.4, I = 0.058 M), which is in good agreement with previously reported values (*27*).

Titration of CPR_{CPM} by P450 2B4 at variable ionic strength clearly demonstrates the electrostatic nature of the interactions of these proteins. A series of the titration curves obtained at various ionic strengths is shown in Figure 2a. As can be seen from these data, raising the ionic strength causes a considerable increase of the K_d of the complex. Therefore, our results are consistent with the conclusion that the interaction between microsomal P450 2B4 and CPR is primarily electrostatic (*3*, *21*, *25*, *28*, *58*, *59*). Along with the change in K_d, there was a decrease in the amplitude of quenching as the ionic strength was increased. This observation indicates that an increase in the ionic strength causes a

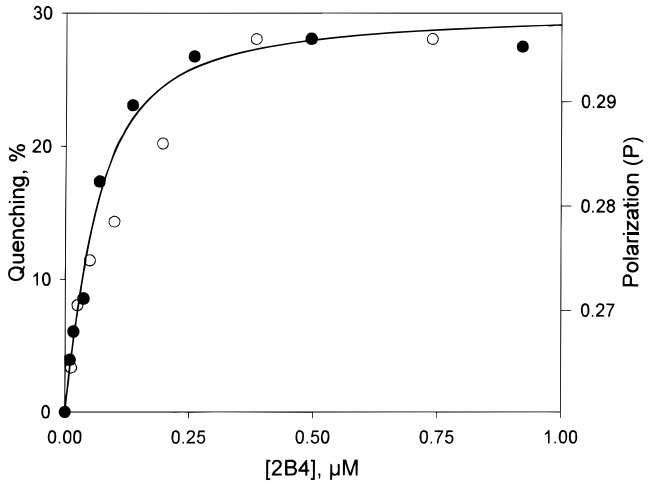

FIGURE 1: Titration of microsomal CPR by cytochrome P450 2B4. Formation of the complexes was monitored by quenching of CPM fluorescence (○) or by changes in polarization of CPM fluorescence (●). Conditions: 100 mM Na-Hepes, pH 7.4 (I = 0.058 M), 1 mM DTT, 1 mM EDTA, and 0.025% Emulgen-913, 25 °C. Concentration of CPR was 0.05 μM.

conformational transition in the interacting proteins, which expands the distance between the label (CPM probe in the molecule of CPR_{CPM}) and the quencher (heme of P450 2B4).

We also monitored the interactions between P450 2B4 and the flavin domain of P450BM-3. In this case, the quenching of fluorescence of BMR_{CPM} was also observed (Figure 2b). These titration curves were similar to those obtained by the polarization of the fluorescence of CPM (data not shown). The titration curves obey the equation for the equilibrium formation of a binary complex between P450 2B4 and BMR_{CPM} with a K_d of the same magnitude as was found for the complex of P450 2B4 with CPR_{CPM}. Similar to that reported above for P450 2B4−CPR interactions, an increase in ionic strength causes a decrease in the amplitude of quenching in the BMR_{CPM}−P450 2B4 complex, suggesting that there is an ionic strength-dependent conformational transition in the proteins.

As described in the introduction, in the conditions used in the present study (0.025% Emulgen-913), P450 2B4 exists in a rapid equilibrium between monomers and oligomers. Therefore, there is a chance that the results on P450 2B4 interactions with the flavoproteins are affected by the ionic strength-induced changes in the oligomerization of P450. However, we have not observed any effect of ionic strength on the shape of the titration curves, which strictly obey the equation for the equilibrium of binary association. There were no systematic deviations of the experimental data from the fitting curves, which would be expected if the P450 2B4 interactions with flavoproteins are affected by the formation of oligomers. Therefore, the effect of ionic strength observed here appears to reflect changes in the association of P450 2B4 with the flavoproteins rather than the displacement of the oligomerization equilibrium.

Interaction of the heme domain of P450BM-3 with the Flavoprotein Partners. Titration of BMR_{CPM} by BMP also results in quenching of the CPM fluorescence, which obeys the equation for a binary association. We also monitored the formation of a complex of BMP with intact (unlabeled) BMR by changes in the polarization of fluorescence of the flavins. Since the lifetime of the excited state of flavins is rather

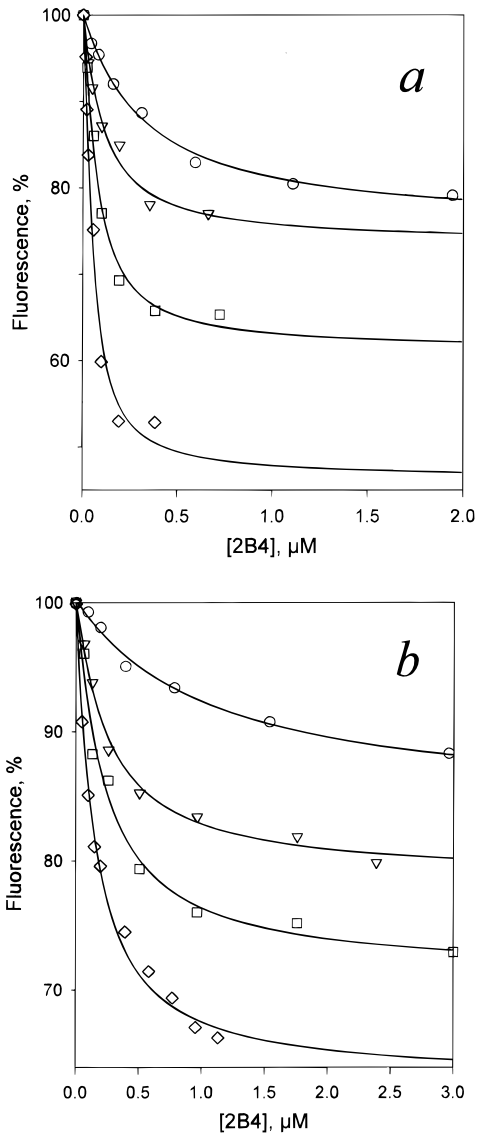

FIGURE 2: Titration of microsomal CPR_{CPM} (a) and BMR_{CPM} (b) by cytochrome P450 2B4 at various ionic strengths. Formation of the complexes was monitored by quenching of the CPM fluorescence. Solid lines show the results of fitting of the data by the equilibrium equation of the bimolecular association. Conditions: 10 mM Na-Hepes buffer, pH 7.4, 1 mM EDTA, 1 mM DTT, and 0.025% Emulgen-913. Ionic strength was adjusted by addition of the appropriate concentration of KCl. (a) Titration of CPR_{CPM} (0.05 μM) by P450 2B4 at I = 0.006 M (◇), 0.03 M (□), 0.06 M (▽), and 0.21 M (○). (b) Titration of BMR_{CPM} (0.1 μM) by P450 2B4 at I = 0.006 M (◇), 0.06 M (□), 0.21 M (▽), and 0.26 M (○).

short, the degree of polarization of flavin fluorescence is low (4−8%), and in addition, the quantum yield of the flavin fluorescence is very sensitive to ionic strength. Nevertheless, we were able to detect the formation of BMR−BMP complex at medium ionic strength (0.05−0.1 M) by this approach (Figure 3). The treatment of the series of polarization scans (470−550 nm with excitation at 380 nm) was done by the PCA technique as it was described above for the polarization of the CPM fluorescence. The value of the dissociation constant obtained by this technique for the complex of unmodified BMR with BMP was similar to that deduced from the fluorescence quenching titration of BMR_{CPM} by BMP. Both methods show the affinity of BMR to BMP to be low (K_d = 3−6 μM at I = 0.06 M) compared with that described above for both BMR and the microsomal flavopro-

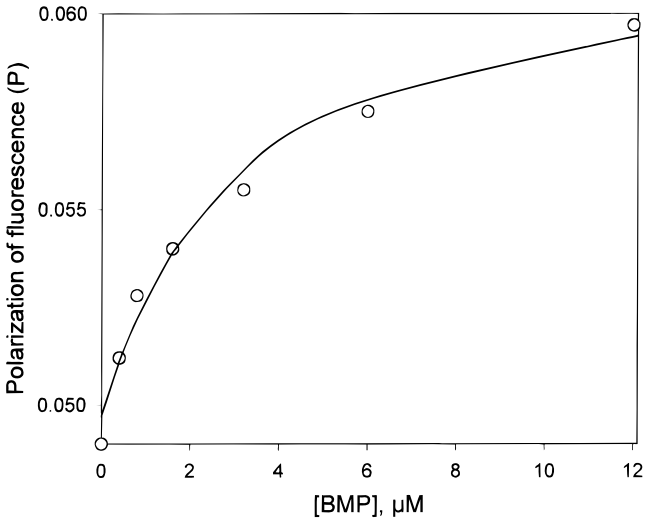

FIGURE 3: Titration of BMR (unlabeled) by BMP. Formation of the complexes was monitored by changes in polarization of the fluorescence of flavins. Conditions: 100 mM Na-Hepes, pH 7.4 (I = 0.058 M), 1 mM DTT, and 1 mM EDTA, 25 °C. Concentration of BMR was 0.1 μM. Solid line shows the results of fitting of the data by the equilibrium equation of the bimolecular association with K_d = 2.65 μM.

tein to P450 2B4. These results suggest that there is an important difference between BMP and P450 2B4 in the structure of the flavoprotein binding site.

A series of titration curves obtained at various ionic strengths for the BMP−BMR_{CPM} pair is shown in Figure 4a. As can be seen, the changes induced by increasing the ionic strength were rather minor in this case, in contrast to that found for the interaction of P450 2B4 with either BMR or CPR. However, a slight decrease in the K_d of BMR_{CPM}−BMP complex was detected at increased ionic strength. Therefore, the effect of ionic strength on the association of BMR and BMP is different from that observed for the complexes of P450 2B4 with the flavoproteins. The effect of ionic strength on the amplitude of quenching in the BMR_{CPM}−BMP complex was also small, again in contrast to that observed for P450 2B4.

Studying the interactions of BMP with CPR_{CPM} (Figure 4b), we observed almost the same results as for the complex of BMP with its native partner. Here the affinity of the BMP to flavoprotein is also very low and the value of K_d decreases with increasing ionic strength. The effect of ionic strength on the amplitude of quenching was also insignificant.

Therefore, our data reveal an important difference between P450 2B4 and BMP in their modes of interactions with the flavoprotein partners. If the interactions of P450 2B4 with both BMR and CPR are apparently driven by the attraction of opposite charges, the BMP hemoprotein exhibits an electrostatic repulsion with both flavoproteins. At the same time, there were no significant differences between BMR and CPR in their interactions with the heme proteins. Similarly, the ionic strength-dependent changes in the amplitude of quenching appear to be specific to P450 2B4, as they have not been observed in the association of BMP with either flavoprotein.

Kinetics of Electron Transfer in BMR−P450 2B4 and CPR−BMP Pairs. The data reported above demonstrate the ability of BMP and P450 2B4 to form the complexes with both BMR and CPR. However, if the complexes of BMP

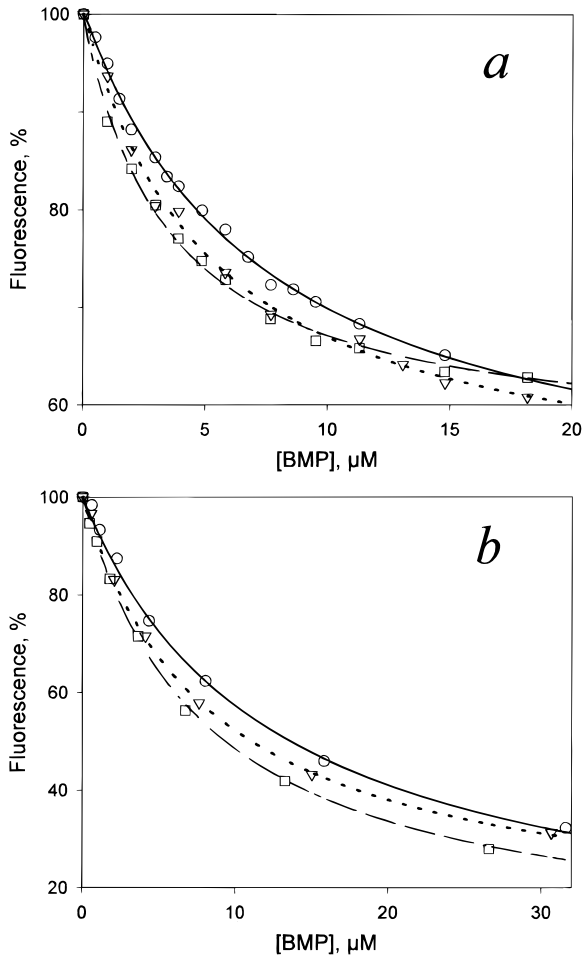

FIGURE 4: Titration of microsomal CPR_{CPM} (a) and BMR_{CPM} (b) by the heme domain of P450BM-3 (BMP). Formation of the complexes was monitored by quenching of the CPM fluorescence. Lines show the results of fitting of the data by the equilibrium equation of the bimolecular association. Conditions: 10 mM Na-Hepes buffer, pH 7.4, 1 mM EDTA, 1 mM DTT, and 0.025% Emulgen-913. Ionic strength was adjusted by addition of the appropriate concentration of KCl. (a) Titration of CPR_{CPM} (0.05 μM) by BMP at I = 0.006 M (○), 0.06 M (▽), and 0.5 M (□). (b) Titration of BMR_{CPM} (0.1 μM) by BMP at I = 0.006 M (○), 0.06 M (▽), and 0.5 M (□).

with BMR and P450 2B4 with CPR are native, the cross-pairs (BMP−CPR and P450 2B4−BMR) are not. The question remains whether these heterogeneous complexes are functional. To probe this point, the kinetics of the electron transfer was studied in four possible combinations. The concentration of the proteins was chosen to reach the apparent saturation of the flavoprotein by P450. Consistent with previously reported results (*28*, *44*), the kinetic curves of P450 2B4 reduction by CPR obey a simple exponential equation and never reach 100% reduction. The maximal amount P450 2B4 reduced was never higher than 70% of the total content of the hemoprotein. In the case of the reduction of BMP by BMR, in agreement with that reported previously (*60*), the kinetics were more complex, having a fast initial burst and a long-lasting final part. Here all of the BMP was reducible by BMR. At apparent saturation of BMR by BMP ([BMR] = 2.6 μM, [BMP] = 11 μM, I = 0.06 M), the maximal reduction level was reached in approximately 30 min after addition of NADPH (data not shown). Reduction of the hemoprotein by the flavoprotein partner was also

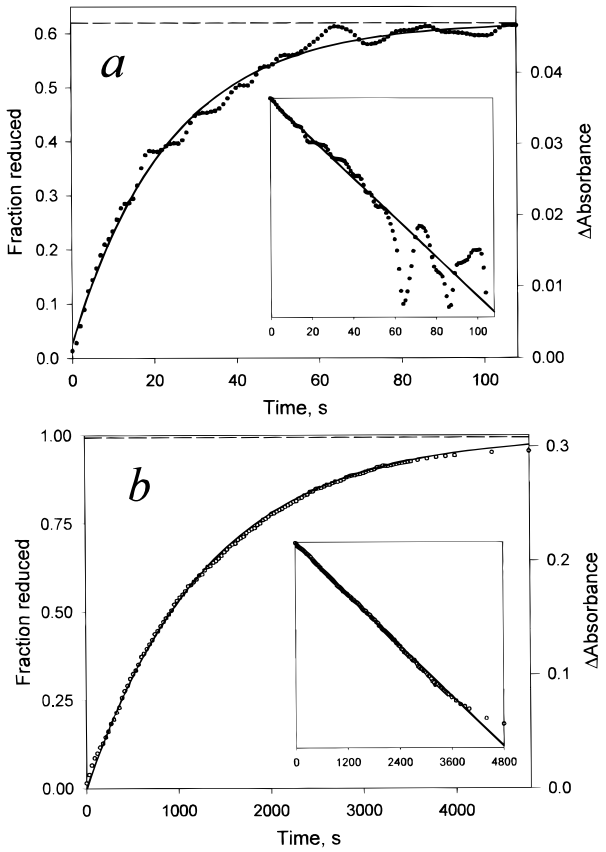

FIGURE 5: Kinetics of reduction of P450 2B4 (a) and BMP (b) by the heterogeneous flavoprotein reducers. Kinetic curves are shown in linear and semilogarithmic coordinates. (a) Reduction of P450 2B4 by BMR; (b) Reduction of BMP by microsomal CPR.

detected in the "hybrid" systems (BMR−P450 2B4 and CPR−BMP). As shown in Figure 5, the kinetic curves of the reduction of P450 2B4 by BMR obey a simple exponential equation. Similar to that observed for P450 2B4 reduction by CPR, the 100% level of reduction of P450 2B4 was unreachable in this case. The maximal reduction level here also tends to be about 70% of the total content of the heme protein. In contrast, BMP could be completely reduced by both CPR (Figure 5b) and BMR.

Thus, all four possible heme protein−flavoprotein combinations appear to be productive in regard to the electron transfer in the complex. The inability of P450 2B4 to be completely reduced in an NADPH-dependent reaction in the conditions used in our experiments appears to be due to some peculiarity of this hemoprotein, as it was observed for both CPR−P450 2B4 and BMR−P450 2B4 pairs.

To compare the rate of the electron transfer in the complexes of the native and "heterologous" pairs of the electron carriers, we calculated the apparent rate constants of the intracomplex electron transfer from the initial velocities of the reduction in the conditions close to apparent saturation of the hemoprotein by its flavoprotein partner (Table 1). The values presented in Table 1 led us to a suggestion that the rate of the electron transfer in the flavoprotein−P450 complex is determined mostly by the nature of the flavoprotein. The intracomplex reduction of both P450 2B4 and BMP by BMR appears to be considerably (3−6-fold) faster than the electron transfer from CPR.

Table 1: Apparent Rate Constants of the Electron Transfer in the Complexes of BMR and CPR Flavoproteins with Cytochromes P450 2B4 and BMP[a]

donor (flavoprotein)	acceptor (hemoprotein)	
	BMP	P450 2B4
CPR	$(4.0 \pm 0.6) \times 10^{-3}$	$(9.1 \pm 0.3) \times 10^{-3}$
BMR	$(23 \pm 2) \times 10^{-3}$	$(28 \pm 9) \times 10^{-3}$

[a] The values of the constants (given in reciprocal seconds) were calculated from the initial velocities of reduction at 0.06 M ionic strength (100 mM Na-Hepes buffer) taking into account the apparent concentration of the complex as calculated from the values of K_d determined in the present study.

DISCUSSION

The interactions between P450s and their redox partners, whether they be iron sulfur proteins or flavoproteins, is important for electron transfer to the heme iron. Their interactions may modulate, or may be modulated by, substrate binding to the P450. In addition, evidence is accumulating that the uncoupling of electron flow from oxygen activation by the heme iron is strongly influenced by redox partner interactions. In view of the importance of redox partner interaction and conflicting conclusions in the literature, we have developed new approaches to studying this problem.

Our studies of the effect of ionic strength on the association of P450 2B4 with microsomal CPR strongly support the view that the interactions of the complementary charges between the proteins play a very important role in the formation of the electron-transfer complex. This conclusion is in agreement with a large body of data on the mechanism of interaction of CPR with microsomal P450s (*3*, *21*, *25*, *28*, *58*, *59*). This is in conflict with the conclusion of Voznesensky and Schenkman that "the cytochrome P450 2B4−NADPH cytochrome P450 reductase electron-transfer complex is not formed by charge-pairing" (*35*). Unfortunately, in their experiments, comparable concentrations of the interacting proteins were used to indirectly observe the effect of ionic strength on the catalytic activity and on the rate of the electron transfer. From these data, they calculated the dissociation constant of the complex using the Michaelis−Menten equation, giving values of "K_M". Yet the experiments of Voznesensky and Schenkman clearly demonstrate an increase in the activity and the rate of electron transfer in the monooxygenase with increasing ionic strength. As can be deduced from the results presented here, this increase can be explained by facilitation of the intracomplex electron-transfer rather than by the changes in the association of the electron carriers. As mentioned above, the increase in the ionic strength that altered the fluorescence quenching in the CPR−P450 2B4 complex indicates an important rearrangement in the complex where the distance between the CPM label and the heme is increased. These changes must be associated with an important alteration in the rate of intracomplex electron transfer, which is likely to be the cause of the observations of Vosnesensky and Schenkman (*34*, *35*). This hypothesis will be the subject of our further investigations.

In fact, the effect of ionic strength on association/dissociation constants could be due to the changes in rate constants of both the formation (k_{on}) and the decay (k_{off}) of

the complexes. It is impossible to discriminate between these mechanisms without data on the kinetics of the protein interactions. Nevertheless, so that we could analyze our data, we arbitrarily proposed that the observed effects of the ionic strength were due solely to the changes in k_{on}, as this case is rather common for the interactions of the electron carrier proteins (*61*−*63*).

In recent decades, several different approaches have been suggested for the treatment of the ionic strength dependencies on the kinetics of protein association (*64*−*66*). All known formalisms have been obtained by modification of the equation originally deduced by Wherland and Gray (*64*) for the ionic-strength dependence of the kinetic association constant between two spheres having randomly distributed charges. However, the most realistic model was recently proposed by Watkins et al. (*66*). It takes into account not only monopole−monopole interactions but also monopole−dipole and dipole−dipole interactions as well. This model was termed the "parallel plate model" as it considers the interactions of the charged planar sites at the surfaces of the interacting molecules. The main parameters determining the kinetics of association are the radius of the interacting sites, the rate constant at infinite ionic strength [$k(\infty)$], and the energetic potentials of the monopole−monopole ($V_{ii}RT$), monopole−dipole ($V_{id}RT$), and dipole−dipole ($V_{dd}RT$) interactions.

The results from fitting the ionic strength dependencies of the association constants for P450 2B4 and BMP complexes with the flavoproteins to the equation of Watkins et al. are shown in Figure 6. We found our data for all four pairs of electron carriers to be consistent with the simplest implementation of the model, when only the monopole−monopole interactions are taken into account. One can see that the ionic strength dependencies of the association constants reveal a drastic contrast between P450 2B4 and BMP in their modes of interaction with the flavoproteins. Our data on the complexes of P450 2B4 with both CPR and BMR are consistent with strong electrostatic attraction of complementarily charged sites of these proteins ($V_{ii}RT$ has the magnitude of −5 kcal/mol). These values suggest the participation of at least 2−3 charge pairs in the complex. On the other hand, the behavior of BMP−BMR and BMP−CPR complexes reveals rather weak electrostatic repulsion of the interacting site of BMP with those in the flavoproteins ($V_{ii}RT$ is approximately equal to +2 kcal/mol). Thus, BMR and CPR exhibit remarkably similar behavior in their interactions with P450 partners.

Recently the X-ray structure of the complex of BMP with the FMN domain of BMR became available (*54*). The FMN domain (residues 479−630 of P450BM-3) was shown to interact with BMP at the proximal face of the molecule, at the site that includes parts of the C and L α-helices and a portion of the "meander." The association of the proteins involves the formation of one salt bridge, two direct hydrogen bonds, and several water-mediated contacts. This structural analysis shows the interactions of the FMN domain with BMP to be rather weak, which is consistent with our results. However, analyzing the surface potentials of BMP and the FMN domain represented by this structure, Sevriukova et al. (*54*) suggested that the interactions of these molecules might be facilitated by long-range electrostatic attraction; however, our experimental results on the effect of ionic

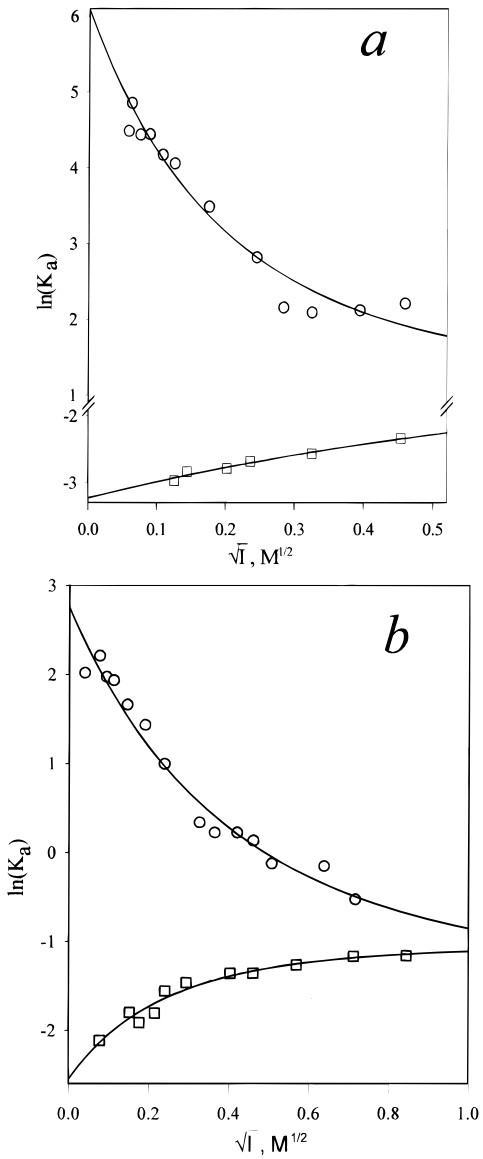

FIGURE 6: Ionic strength dependencies of the dissociation constant for the complexes of microsomal CPR (a) and BMR (b) by cytochrome P450 2B4 (○) and the heme domain of BMP (□). Solid lines show the results of fitting by the "parallel plates" model (*66*).

strength on BMP−BMR interactions do not support this point of view. It should be noted that the FMN domain fragment used in the X-ray structure determination represents only about 19% of the whole BMR molecule, while the remainder of BMR (i.e. the FAD-domain) contains most of the charged amino acid residues. In the rabbit microsomal CPR, the cluster of negative charges located at positions 207−215 in the FAD domain is believed to participate in the interactions of the reductase with cytochrome P450 (*31*), and in BMR, a comparable negatively charged cluster is located at a similar position (residues 647−655 in the FAD domain of the P450BM-3 sequence). Unfortunately, this cluster is not contained within the BMP−FMN domain fragment represented by the X-ray structure. Therefore, this structure does not provide enough information to judge the participation of charged residues in the interaction of BMP with the whole BMR molecule.

Comparing the X-ray structure of the FMN domain of CPR (*67*) with that of FMN domain of P450BM-3, Sevriukova et al. (*54*) also suggested that a strong negative potential on

the CPR surface around the FMN facilitates the formation of the complex between the reductase and the P450. The electrostatic interactions of this site were deemed important in the mode of interaction between CPR and P450 as compared to that of BMR, where no important negative potential was found around the FMN. However, our experimental observations do not support this hypothesis, as the values for K_d and its dependency on ionic strength for the P450 2B4−CPR and P450 2B4−BMR pairs was found to be similar. Therefore, we propose that the charged region around the FMN on the surface of CPR is not important for the interactions of the flavoprotein with P450 2B4, and additionally, the putative P450-recognition site of CPR is expected to be conserved in BMR.

Since the BMP−CPR and P450 2B4−BMR heterogeneous pairs behaved similarly to the pairs of BMP−BMR and P450 2B4−CPR, it seems that the observed diversity in the interaction mechanisms is determined mainly by the heme proteins rather than their flavoprotein counterparts. In comparing the distribution of the charged residues in the amino acid sequences of P450 2B4 and BMP, a preliminary analysis reveals large differences between these heme proteins. If we calculate the overall charge of these hemeproteins by balancing the total number of positively (R, K) and negatively (D, E) charged residues, we can see that the overall charge of P450 2B4 is +6, while the charge of BMP is −11. This is consistent with the observed differences in the electrostatic interactions of these heme proteins with either reductase.

The P450BM-3 monooxygenase is known to be an extremely efficient and highly coupled system, as compared to its microsomal homologue. Thus, the observation of the electrostatic repulsion and low affinity between the electron donor and acceptor in this system was rather unexpected. However, it must be remembered that in P450BM-3, the reductase and the P450 domains are tethered as a consequence of being expressed as a single polypeptide chain. Therefore, very tight binding between these redox partners would be of no value here. Furthermore, it should be kept in mind that in the present paper we deal with the interactions between the two oxidized electron carriers. It would be reasonable to suppose that the association of the electron carrying domains in P450BM-3 is modulated by their state of reduction to provide a firm synchronization of electron transfer with catalytic turnover. This synchronization is thought to be an essential condition for the tight coupling and efficient catalysis in P450BM-3 (*68*). To probe this hypothesis, we have initiated the examination of the effect of the state of reduction of both BMR and BMP domains on their association.

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