Interactions among P450 Enzymes When Combined in Reconstituted Systems: Formation of a 2B4−1A2 Complex with a High Affinity for NADPH−Cytochrome P450 Reductase[†]

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ABSTRACT: The purpose of this study is to characterize the interactions among P450 1A2, P450 2B4, and P450 reductase in mixed reconstituted systems. Previously, our laboratory demonstrated that in the presence of certain substrates, 1A2 can influence the catalytic characteristics of 2B4 [Cawley et al. (1995) *Biochemistry 34*, 1244−1247]. The goal of the current study is to distinguish between two models to explain these interactions: one model where substrate increases the affinity of one P450 enzyme for the reductase, and another model where substrate increases the affinity of one P450 for the reductase through the formation of a 1A2−2B4 complex. According to this model, the 1A2 moiety of 1A2−2B4 forms a high-affinity complex with reductase. Reductase, 1A2, and 2B4 were reconstituted with dilauroylphosphatidylcholine, and the effect of reductase concentration on 7-pentoxyresorufin-O-dealkylation was examined with 2B4−reductase and 1A2−reductase binary systems, and in ternary systems containing different 2B4:1A2 ratios. At subsaturating [reductase], there was a dramatic inhibition of the 2B4-dependent activity in the ternary system as compared with the binary systems. These results are consistent with the formation of a ternary (reductase−1A2−2B4) complex where the reductase is bound specifically to 1A2. At higher reductase concentrations where the reductase-binding sites on 1A2 become saturated, the results are consistent with the formation of a quaternary complex in which reductase binds to both P450 enzymes (reductase−1A2−2B4−reductase). Analogous experiments using the 1A2-preferred substrate 7-ethoxyresorufin showed a stimulation of 7-ethoxyresorufin-O-deethylation in the mixed reconstituted system, demonstrating that the high-affinity 2B4−1A2−reductase complex was functionally active and not merely an inhibitory complex.

NADPH-supported catalysis of cytochrome P450 monooxygenase reactions requires a functional interaction between P450 and NADPH−cytochrome P450 reductase. In most systems examining the catalytic activities of purified P450, the P450 enzyme is reconstituted with NADPH−cytochrome P450 reductase in phospholipid. Generally speaking, there is only a single P450 in these reconstituted systems, and the reductase to P450 ratio is 1:1 or greater. Although these reconstituted systems can provide useful information regarding the catalytic behavior of P450 enzymes, the conditions used are far removed from the more native environment of the microsomal membrane where (1) the phospholipid composition is different, (2) more than one P450 enzyme is present in the microsomal membrane, and (3) the total P450 concentration exceeds the reductase concentration by a ratio of at least 10:1 (*1*, *2*).

Despite the interest in the organization of P450s and their relationship to reductase in the microsomal membrane, very few studies have addressed the question of whether one P450 can influence the catalytic characteristics of another P450 when present together in a phospholipid membrane. West and Lu (*3*) originally reported that P450 and P448 competed for reductase when catalyzing 3,4-benzo[*a*]pyrene hydroxylation. However, there was no evidence presented that one P450 altered the kinetic characteristics (e.g., K_m or V_{max}) of the other P450. In a later study, Kaminsky and Guengerich (*4*) observed generalized inhibition of P450-dependent warfarin metabolism with mixtures of eight different P450s in reconstituted systems, with some P450s having a greater potential to act as an inhibitor. The inhibition was not attributed to competition between the P450s for reductase, but was ascribed to P450−P450 aggregation. Based on the conditions used in their assays (low phospholipid concentrations), nonspecific P450−P450 interactions could explain their results. However, these conditions differ significantly from those where the proteins are incorporated into a phospholipid bilayer (*5*−*7*). Dutton et al. (*8*) did not observe the generalized inhibition of monooxygenase activities in mixed reconstituted systems. They reported that testosterone metabolism catalyzed by several P450 enzymes [the same ones used by Kaminsky and Guengerich (*4*)] was not inhibited by the presence of other P450s in complex reconstituted systems. This lack of effect was attributed to the different substrate used.

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Recently, our laboratory demonstrated that the catalytic activity of P450 2B4-dependent 7-pentoxyresorufin-O-dealkylation (PROD)[1] was inhibited by the addition of 1A2 to the reconstituted system (*9*). The inhibition of this 2B4-preferred activity by 1A2 was also shown to be dependent on the substrate used, was more pronounced when NADPH−cytochrome P450 reductase was limiting in the reconstituted system, and appeared to be related to a high-affinity association of 1A2 with NADPH−cytochrome P450 reductase. These results clearly demonstrate that these enzymes function differently in a complex reconstituted system when compared to the simple binary systems. Furthermore, the results demonstrate that the interactions among these proteins involve the formation of a high-affinity reductase−1A2 complex, which draws reductase away from 2B4, especially at limiting reductase concentrations.

Although these data establish that the presence of one P450 enzyme can influence the catalytic properties of another P450, the mechanistic details have not yet been clarified. The present study examines the potential molecular interactions among these proteins in greater detail, focusing on the interactions among reductase, 1A2, and 2B4 in the presence of the substrate 7-pentoxyresorufin (7PR). These experiments were designed to distinguish between two possible models which can describe the formation of the apparent high-affinity complex between reductase and 1A2, the first involving the formation of binary complexes between reductase and P450, and the second involving the formation of P450−P450 complexes with altered catalytic characteristics.

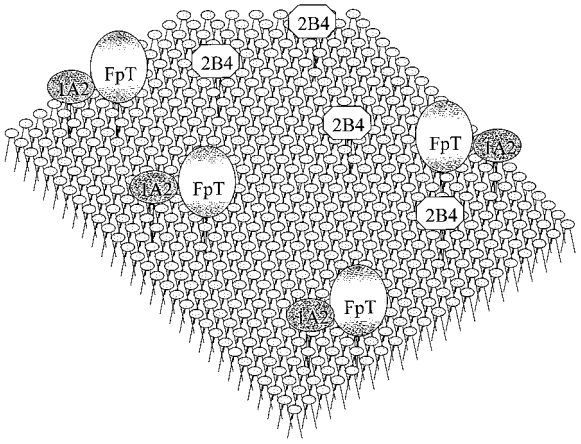
A second question to be addressed by these studies involves the catalytic characteristics of this high-affinity reductase−1A2 complex. 7PR is a substrate that is much more efficiently metabolized by 2B4, and formation of the reductase−1A2 complex causes a dramatic inhibition of this reaction. This leads to the question of whether the high-affinity reductase−1A2 complex is catalytically functional or simply inhibitory in nature. The question will be addressed by examining the potential interactions among reductase and P450 enzymes in the presence of 7-ethoxyresorufin (7ER) which is preferentially metabolized by 1A2.

MATERIALS AND METHODS

Cytochrome P450 1A2 was purified from β-naphthoflavone-treated rabbits (*10*). P450 2B4 and NADPH−cytochrome P450 reductase were purified from phenobarbital-treated rabbits as described (*10*−*12*).

Catalytic activities of P450 1A2 and 2B4 were determined using reconstituted systems where (1) reductase and P450 1A2, (2) reductase and P450 2B4, or (3) reductase, P450 1A2, and P450 2B4 were combined in DLPC. DLPC was prepared to a concentration of 8 mM in 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 M NaCl, and 5 mM EDTA and sonicated until clarification (approximately 30 min). After mixing of the proteins into the liposomes, the reconstituted systems were preincubated for 2 h prior to the addition of the other assay components as described previously (*6*). The P450 concentrations during this preincubation step were in excess of 2 μM. After preincubation, the reconstituted systems were diluted with buffer and other assay components, and assayed within 1 min. These preincubation conditions permit the formation of stable interactions among the microsomal proteins. Incubation for a longer duration (1 h) after dilution with the buffer gives results that are identical with those obtained using the standard assay conditions. The final assay conditions for PROD and 7-ethoxyresorufin-O-deethylation (EROD) were the reconstituted system, substrate (1.3 μM 7PR or 1.3 μM 7ER), 0.1 mM EDTA, and 15 mM magnesium chloride in 50 mM Hepes buffer, pH 7.5 (*9*). The experimental values shown are the average of triplicate determinations. In general, the SEM for a reconstituted system at given concentrations of P450 and reductase is ±5%.

Scheme 1: Potential Mechanism Describing the Interactions among P450s and Reductase Leading to High-Affinity Binding of Reductase to 1A2[a]



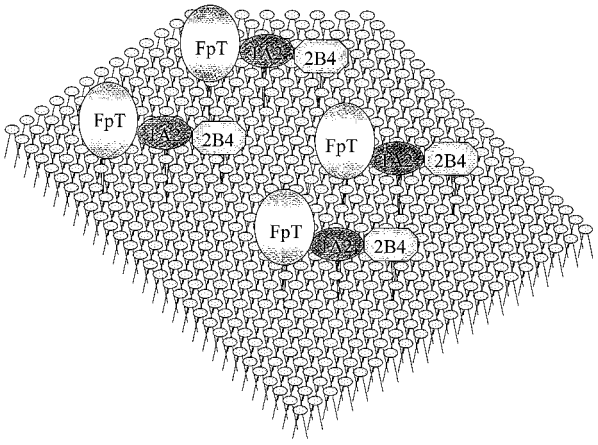
[a] In this model, substrate (e.g., 7PR) causes an increase in the affinity of 1A2 for the reductase (FpT). Consequently, the reductase is not available for binding to 2B4, and becomes metabolically underrepresented.

Mathematical Modeling. The purpose of the simulations was to distinguish between two general models of interactions: (1) binary complex model with simple competition between P450s for reductase; and (2) ternary complex models in which P450−P450 interactions modulate their affinity for reductase. Specific models of interactions among P450s and reductase were tested by comparing how well they could fit the experimental data. In each model, substrate has the potential to affect the K_m and k_{cat} values for the interactions between reductase and P450.

To make a robust analysis, it is necessary to use Occam's razor ruthlessly; it is a truism that adding terms to a fitting equation will almost always result in an improved fit to the data. In the present case, the null hypothesis is Scheme 1, which is the simplest biochemically reasonable model for a system involving two P450s; thus, the kinetic parameters (k_{cat} and K_m) determined in the binary experiments (only one P450 and reductase) should predict results of experiments with both P450s present. Scheme 2 adds one additional factor, interaction between the two different P450s which alter K_m's and k_{cat}'s.

[1] Abbreviations: 1A2, cytochrome P450 1A2; 2B4, cytochrome P450 2B4; reductase, NADPH−cytochrome P450 reductase; FpT (*in Figures*), NADPH−cytochrome P450 reductase; DLPC, dilauroylphosphatidylcholine; 7PR, 7-pentoxyresorufin; PROD, 7-pentoxyresorufin-O-dealkylation; 7ER, 7-ethoxyresorufin; EROD, 7-ethoxyresorufin-O-deethylation; SEM, standard error of the mean.

Scheme 2: Potential Mechanism Describing the Interactions among P450s and Reductase Where the Formation of a 1A2−2B4 Complex Leads to High-Affinity Reductase Binding[a]



[a] According to this model, the presence of a particular substrate causes the formation of heteromeric P450 complexes (e.g., 1A2−2B4) with altered catalytic characteristics. In this particular model, the 1A2 moiety of the 1A2−2B4 complex is capable of forming a high-affinity complex with reductase. After the 1A2 sites become saturated (which will be observed at higher reductase concentrations), reductase can then associate with the 2B4 moiety of the complex as well as any free 2B4.

We used the Michaelis−Menten kinetic model as the basis for our simulations of the variation of P450 activity with reductase concentration. Implicit in this model are the following assumptions: (a) the activity of a P450 will be directly proportional to the fraction of P450 molecules that associate with a reductase; (b) cytochrome P450 reductase has only one P450-binding site that will support productive electron transfer to P450; (c) P450 reductase equilibrates among available P450s; (d) K_m = dissociation constant (an assumption in Michaelis−Menten). Examination of Figures 1 and 3 reveals that 50% maximal activity is obtained at reductase concentrations near the P450 concentrations; hence, any analysis of the variation of PROD with [reductase] must take into account the amount of bound reductase (in other words $[\text{reductase}]_{free} < [\text{reductase}]_{total}$).

For Scheme 1 and Scheme 2, the same iterative procedure was used, written in Turbo Pascal. (1) Initial estimates of K_m and k_{cat} were made. (2) The predicted concentrations of each complex were calculated by the procedure of Storer and Cornish-Bowden (*13*). For each data point, the total concentrations of reductase and P450(s) were input to a subprogram implementing this algorithm which computes the concentrations of complexes given dissociation constants (K_m's), stoichiometries, and total concentrations of the constituents. This routine was used because it reliably computes complex equilibria and it is easy to input different models and calculated the predicted result. (3) Then the concentration of each complex was multiplied by its respective k_{cat}, and the predicted velocity obtained as the sum of the products (velocities of each individual complex). (4) At this point, K_m's and k_{cat}'s were adjusted manually until a good fit was obtained to the data set.

In the case of Scheme 1, K_m and k_{cat} for each P450 were obtained by fits to the data from binary experiments. Mathematically Scheme 2 only adds the assumption that 1A2 and 2B4 can form a heterodimer and that the catalytic properties (K_m and k_{cat}) of the P450s in the heterodimer may differ from monomeric P450. Thus, simulations of Scheme 2 involve the same the K_m's and k_{cat}'s for Fpt−1A2 and Fpt−2B4 complexes as well as K_m's and k_{cat}'s for the interaction of reductase with 1A2−2B4 and with Fpt−1A2−2B4 complexes. The constants derived from the binary experiments ($K_m^{FpT-2B4} = 0.01\ \mu M$; $k_{cat}^{FpT-2B4} = 620\ s^{-1}$; $K_m^{FpT-1A2} = 0.15\ \mu M$; $k_{cat}^{FpT-1A2} = 95\ s^{-1}$) were not altered in fitting Scheme 2 to the data, to constrain the fits to well-established kinetic parameters.

The procedure of Cornish-Bowden was chosen for this application because it is a robust algorithm that has been successfully applied to systems involving equilibria among competing complexes. The values of K_m's given in the figures are the constants for dissociation of the indicated complexes into monomeric units. These constants are, of course, the product of stepwise dissociation constants and can be expressed as such; for example, in Figure 2C, $K_m^{FpT-1A2-2B4-FpT}$ is the constant for dissociation of reductase from 2B4 (0.0625 μM) times $K_m^{FpT-1A2-2B4}$ (0.00008 μM) which in turn can be expressed as the product of the constant for dissociation of 2B4 (0.0005 μM) times the dissociation constant for the Fpt−1A2 complex (0.15 μM). Although such microscopic dissociation constants may be calculated, our present understanding of the system is insufficient to give much credence to specific values, but rather to the extent to which different models can approximate the data.

RESULTS

In our previous study, the formation of a high-affinity reductase−1A2 complex was hypothesized based on evidence that small amounts of P450 1A2 were able to strongly inhibit PROD, which is preferentially catalyzed by P450 2B4. This inhibition was not observed with all substrates, and supported the idea that one P450 can influence the catalysis of another P450 under specific conditions. The following studies are designed to examine the mechanism of this interaction in more detail, by comparing experimental data to those obtained using defined models. In the first model (Scheme 1), the presence of certain substrates (i.e., 7PR) causes an increase in the affinity of 1A2 for the reductase. Consequently, at subsaturating reductase concentrations, the reductase is bound almost exclusively to 1A2. As the concentration of reductase is increased further and the high-affinity binding sites on 1A2 become saturated, the reductase can then bind to 2B4. According to the second model (Scheme 2), the presence of 7PR causes the formation of a 1A2−2B4 complex that has a high affinity for reductase. At limiting reductase concentrations, the reductase binds only to the 1A2 moiety of the 1A2−2B4 complex. Furthermore, this complex would have different functional characteristics when compared to the simple binary reductase−1A2 complex which could be manifested as a change in either the catalytic activity of the complex and/or the affinity of reductase for the complex. As the reductase concentration is increased and the binding sites on 1A2 become saturated, the available reductase can now bind to the 2B4 moiety of the complex.

The effect of reductase concentration on PROD was measured in simple binary reconstituted systems with a single P450 (either 1A2−reductase or 2B4−reductase), or the ternary reconstituted system containing reductase, 1A2, and

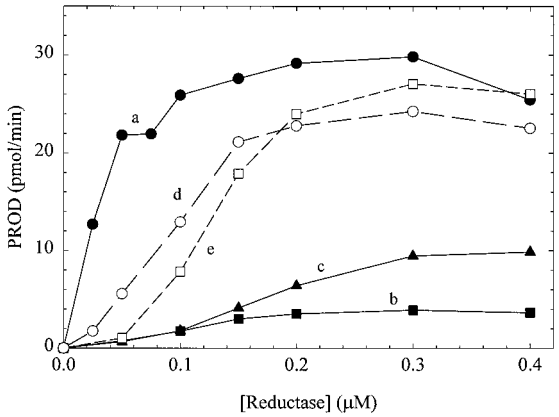


FIGURE 1: Effect of 1A2 on 2B4-mediated PROD metabolism as a function of reductase concentration. PROD was determined as a function of reductase concentration in both simple and mixed reconstituted systems. Each reconstituted system contained 16 μM DLPC, and (a) 0.05 μM 2B4 (●), (b) 0.05 μM 1A2 (■), (c) 0.15 μM 1A2 (▲), (d) 0.05 μM 2B4 plus 0.05 μM 1A2 (○), and (e) 0.05 μM 2B4 plus 0.15 μM 1A2 (□). The reductase concentration was varied as indicated in the figure. Each reconstituted system was preincubated for 2 h as described under *Materials and Methods*. Each point represents the average of 3 different reconstituted systems.

2B4. The results are shown in Figure 1. P450 2B4 was shown to be the more effective enzyme for PROD when compared to 1A2. The apparent K_m for reductase with 2B4 was estimated to be about 0.03 μM (Figure 1), and the reaction appeared to exhibit some inhibition at the highest reductase concentration. P450 1A2 did not exhibit a typical saturation curve, but appeared to be saturated as the reductase concentration reached 0.3 μM. Interestingly, the binary system containing reductase and 1A2 did not exhibit a high affinity for the reductase in the presence of 7PR as predicted by Scheme 1. When 1A2 and 2B4 were present together in the ternary reconstituted systems, a dramatic inhibition of PROD was observed that was more pronounced at subsaturating reductase concentrations. As the concentration of reductase was increased, less inhibition was observed, producing an "S"-shaped curve. Furthermore, the "S"-shaped curves for the mixed reconstituted systems were shifted to the right as the 1A2:2B4 ratio was increased (Figure 1).

As mentioned in our previous report, these results were consistent with formation of a high-affinity reductase−1A2 complex that was more effective at competing for the reductase than was 2B4. Beginning with the simplest possible models, these data could potentially be described by either the two competing binary complex model (Scheme 1) or the model where a complex between 1A2 and 2B4 was formed (Scheme 2). Based on these models, simulations of the experimental data were determined (Figure 2). With the two binary complex model (Scheme 1), the $K_m^{reductase}$ and k_{cat} values for each of the enzymes were estimated from the simple reconstituted systems and used to simulate the potential reductase dependence for the mixed reconstituted systems. Using these conditions, an extremely poor fit of the experimental data was obtained (Figure 2A), with the mixed reconstituted systems exhibiting a small degree of inhibition at low reductase and an additive effect at saturating reductase concentrations.

One of the assumptions inherent in the model described in Scheme 1 is that the affinity of the complex in the presence

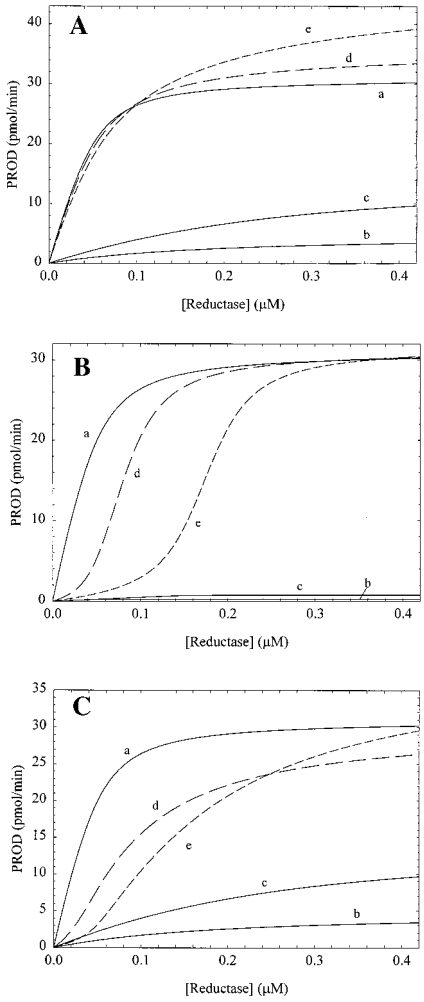


FIGURE 2: Simulations of the interactions among reductase and P450 enzymes which can result in the formation of a high-affinity reductase−P450 complex: Effect of 1A2 on 2B4-mediated PROD metabolism. (A) Simulated data using the model described in Scheme 1 where only binary complexes between reductase and P450 can be formed. The simulations for the mixed reconstituted systems were based on the K_m and k_{cat} values for the binary systems. The following values were used in the fitting routines: $K_m^{FpT-2B4} = 0.01$ μM; $k_{cat}^{FpT-2B4} = 620$ s^{-1}; $K_m^{FpT-1A2} = 0.15$ μM; $k_{cat}^{FpT-1A2} = 95$ s^{-1}. The curves are identified as follows: (a) 0.05 μM 2B4, (b) 0.05 μM 1A2, (c) 0.15 μM 1A2, (d) 0.05 μM 2B4 plus 0.05 μM 1A2, and (e) 0.05 μM 2B4 plus 0.15 μM 1A2. (*B*) Simulated data using the model described in Scheme 1 where a high-affinity binary complex is formed between reductase and 1A2. The following values were used in the fitting routines: $K_m^{FpT-2B4} = 0.01$ μM; $k_{cat}^{FpT-2B4} = 620$ s^{-1}; $K_m^{FpT-1A2} = 0.0005$ μM; $k_{cat}^{FpT-1A2} = 5$ s^{-1}. These simulations were produced by assuming a high-affinity reductase−1A2 complex which ignored the experimental data showing lower affinity complex formation. The curves are identified as follows: (a) 0.05 μM 2B4, (b) 0.05 μM 1A2, (c) 0.15 μM 1A2, (d) 0.05 μM 2B4 plus 0.05 μM 1A2, and (e) 0.05 μM 2B4 plus 0.15 μM 1A2. (*C*) Simulated data using the model described in Scheme 2 where a complex between 1A2 and 2B4 is formed. The following values were used in the fitting routines: $K_m^{FpT-2B4} = 0.01$ μM; $k_{cat}^{FpT-2B4} = 620$ s^{-1}; $K_m^{FpT-1A2} = 0.15$ μM; $k_{cat}^{FpT-1A2} = 95$ s^{-1} (which were determined experimentally from the binary systems), and $K_m^{FpT-1A2-2B4} = 0.00008$ μM; $k_{cat}^{FpT-1A2-2B4} = 0$ s^{-1}; $K_m^{FpT-1A2-2B4-FpT} = 0.000005$ μM; $k_{cat}^{FpT-1A2-2B4-FpT} = 570$ s^{-1}. The 1A2 moiety of the 1A2−2B4 complex has an increased affinity for the reductase as described by Scheme 2. The curves are identified as follows: (a) 0.05 μM 2B4, (b) 0.05 μM 1A2, (c) 0.15 μM 1A2, (d) 0.05 μM 2B4 plus 0.05 μM 1A2, and (e) 0.05 μM 2B4 plus 0.15 μM 1A2.

of the second enzyme is the same as in its absence (i.e., the P450s do not interact with each other in a way that influences function). The experimental data from the binary reconstituted systems indicate that the affinity of the reductase−1A2 complex is about 0.15 μM (Figure 1). If we use this K_m, we cannot obtain reasonable fits of the experimental data (Figure 2A). Next, we wanted to determine if there were *any* conditions where "S"-shaped curves could be obtained with the mixed reconstituted systems using the model described in Scheme 1. We were able to obtain "S"-shaped curves under conditions where reductase and 1A2 formed a very high-affinity complex having very low catalytic activity (Figure 2B). Although we were able to obtain "S"-shaped curves, this model suffers because it must ignore the experimental data from the binary systems (Figure 1). Thus, using the model described in Scheme 1, we can only fit the general characteristics of either the mixed reconstituted systems or the simple binary systems, but we cannot fit the entire set of experimental data. Such analysis of the experimental data allows us to eliminate Scheme 1 as a reasonable model to explain the interactions of these P450s when in mixed reconstituted systems.

In contrast to the poor agreement observed with the binary complex model, the characteristic features of each curve are retained when the formation of a 1A2−2B4 complex is invoked (Figure 2C). These results strongly support the view that simple binary complexes (reductase−1A2 and reductase−2B4 as described in Scheme 1) alone cannot account for the experimental data. The data are also consistent with the formation of a 1A2−2B4 complex (as described in Scheme 2) which preferentially binds reductase to the 1A2 moiety of this heteromeric P450 complex.

These results support the conclusion that in complex reconstituted systems containing 1A2, 2B4, and reductase, 7PR causes the formation of a heteromeric 1A2−2B4 complex with altered kinetic properties. The 1A2 enzyme of this complex has a high-affinity for reductase—to such a degree, that it takes reductase away from the 2B4 moiety, resulting in the inhibition of PROD metabolism. However, some basic characteristics of the 2B4−1A2−reductase complex remain to be elucidated. Since addition of 1A2 to the reconstituted systems containing 2B4 causes an inhibition of PROD, it is uncertain whether the reductase−1A2−2B4 complex is catalytically active or if the complex is inhibitory in nature. In an effort to determine whether the reductase−1A2−2B4 complex is inhibitory, we switched to 7ER as the substrate. The advantages to the use of this substrate are that (1) EROD is preferentially catalyzed by 1A2 and that (2) it is structurally similar to 7PR, presumably capable of producing similar conformational effects. Consequently, EROD would be expected to be stimulated in the mixed reconstituted systems if the reductase−1A2−2B4 complex were functional. On the other hand, if this complex was without catalytic activity, inhibition of EROD would be expected with the mixed reconstituted system. The results (Figure 3) clearly demonstrate that the addition of 2B4 to the reconstituted system containing reductase and 1A2 leads to a stimulation of EROD, and are consistent with the formation of a catalytically functional reductase−1A2−2B4 complex.

Using $K_m^{FpT-1A2}$ values estimated from the simple reconstituted systems (Figure 3), simulations of the experimental data were generated (Figure 4). Again, an extremely poor

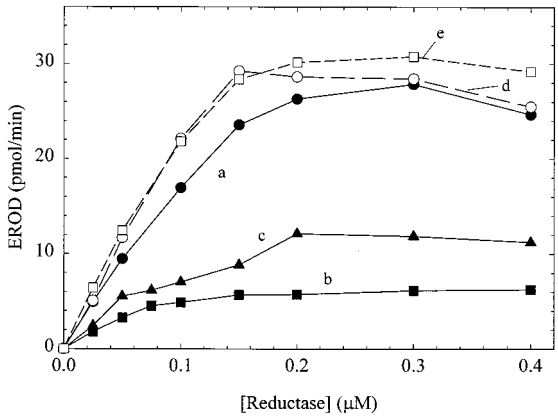


FIGURE 3: Effect of 2B4 on 1A2-mediated EROD metabolism as a function of reductase concentration. EROD was determined as a function of reductase concentration in both simple and mixed reconstituted systems. Each reconstituted system contained 16 μM DLPC, and (a) 0.05 μM 1A2 (●), (b) 0.05 μM 2B4 (■), (c) 0.15 μM 2B4 (▲), (d) 0.05 μM 1A2 plus 0.05 μM 2B4 (○), and (e) 0.05 μM 1A2 plus 0.15 μM 2B4 (□). The reductase concentration was varied as indicated in the figure. Each reconstituted system was preincubated for 2 h as described under Materials and Methods. Each point represents the average of 3 different reconstituted systems.

fit of the experimental data was observed when only the reductase−1A2 and reductase−2B4 binary complexes could be produced as described by Scheme 1 (Figure 4A). According to this model, the rate of EROD from the mixed reconstituted systems (containing both 1A2 and 2B4) would be expected to be lower than the simple reconstituted systems at low reductase concentrations because 2B4 would sequester some of the reductase. In contrast, the experimental data in Figure 3 actually exhibit a stimulation of EROD (by 2B4) at subsaturating reductase levels. This stimulation of EROD cannot be produced with the model described by Scheme 1 even if the formation of a high-affinity reductase−1A2 complex is invoked (Figure 4A). The fit to the experimental data was not improved with the two-complex model (Scheme 1) where reductase forms a high-affinity complex with 1A2 (Figure 4B). In contrast, simulations based on the conditions described by Scheme 2 (where 1A2−2B4 complexes were permitted) produced curves that agreed well with the experimental data (Figure 4C). These results lend further support to the idea that the formation of 1A2−2B4 complexes allows for the high-affinity binding of reductase (at the binding site on the 1A2 moiety).

The goal of the next studies was to determine if EROD was synergistically stimulated by the combination of P450 enzymes in mixed reconstituted systems (Table 1). In these experiments, EROD was measured in simple reconstituted systems and in mixed reconstituted systems at both subsaturating and saturating reductase. If the interactions between reductase and the P450s were unaffected by their presence in a complex reconstituted system, then the rate of EROD in the mixed system would be expected to be the sum of the rates of the simple binary systems. These results clearly demonstrate that a synergistic elevation of EROD was observed at subsaturating reductase, whereas a simple additive effect was observed at higher reductase levels. Taken together, these results are consistent with the metabolic characteristics of one P450 being sensitive to the presence of another P450 enzyme.

DISCUSSION

The oxidative metabolism of xenobiotics as well as many endogenous compounds is dependent on the P450 system, with changes in the expression of P450 enzymes being able to alter the disposition of a compound. Generally speaking, there has been a tacit assumption that the disposition of a particular xenobiotic was simply a reflection of the P450 enzymes present. According to this hypothesis, the relative ability of these enzymes to produce their effects could be predicted by their behavior in simple reconstituted systems (or in expression systems). As a simplified example of this hypothesis, let us assume there are two enzymes that are responsible for the metabolism of a compound, one hydroxylating the substrate to produce metabolite "A" and another enzyme forming metabolite "B". Higher levels of the second enzyme (as might be seen as a result of induction or as a consequence of individual variation) would be expected to increase the production of metabolite "B". In other words, a 2-fold increase in P450 "B" might be expected to produce a proportional increase in formation of "B". The results presented in this and our previous report (*9*) clearly demonstrate that this is not necessarily the case. The presence of one enzyme can cause a substantial alteration in the metabolic characteristics of another enzyme, even if the modulating enzyme does not metabolize that substrate efficiently. Furthermore, the altered interactions result from the formation of P450−P450 complexes (e.g., 1A2−2B4) having catalytic characteristics that differ from those of the separate P450s.

The model used in this study is one of the simplest possible to describe the data, and supports the idea that a complex forms between 1A2 and 2B4. The data are consistent with complex formation leading to an increase in the affinity for reductase at the reductase−1A2 binding site. The data *cannot* be reasonably fit using a model where substrate simply causes an alteration in the affinity of the reductase−1A2 complex without the formation of P450−P450 complexes (Scheme 1). However, the characteristic features of the experimental data are readily obtained when heteromeric P450 complexes are formed as described in Scheme 2. Although extremely good fits of the experimental data were obtained using the model described in Scheme 2, it is only a first approximation. In actuality, much more complex interactions are possible, including the formation of 1A2−1A2 as well as 2B4−2B4 complexes. The formation of such complexes could further influence these monooxygenase activities. The apparent inhibition of both PROD and EROD at high reductase concentrations with the simple reconstituted systems (Figures 1 and 3) is consistent with the possible formation of other as yet unidentified homomeric complexes.

It is important to note that the interactions among these proteins are clearly substrate-dependent. Despite the strong evidence for the direct interaction between 1A2 and 2B4 in the presence of 7PR, such changes in functional interactions are not apparent when benzphetamine is used as substrate (*9*). The addition of P450 1A2 to a 2B4−reductase reconstituted system in the presence of benzphetamine causes an inhibition consistent with simple competition between 1A2 and 2B4 for the reductase (*9*). However, in the presence of 7PR, 1A2 becomes a very strong inhibitor of 2B4-dependent PROD. The present results support this previous finding and demonstrate that the effect is consistent with the formation of a 1A2−2B4 complex with an altered ability to bind reductase.

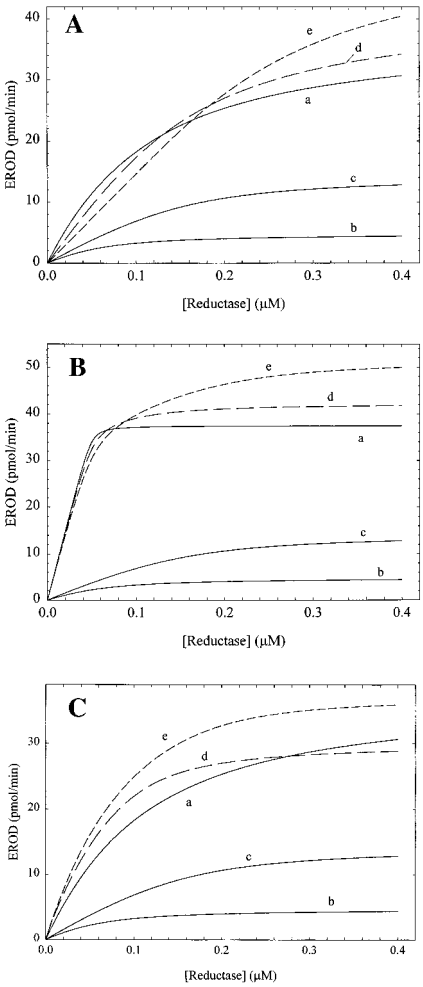


FIGURE 4: Simulations of the interactions among reductase and P450 enzymes which can result in the formation of a high-affinity reductase−P450 complex: Effect of 2B4 on 1A2-mediated EROD metabolism. (A) Simulated data using the model described in Scheme 1 where only binary complexes between reductase and P450 can be formed. The following kinetic constants were used in the fitting routines: $K_m^{FpT-2B4} = 0.03\ \mu M$; $k_{cat}^{FpT-2B4} = 95\ s^{-1}$; $K_m^{FpT-1A2} = 0.08\ \mu M$; $k_{cat}^{FpT-1A2} = 750\ s^{-1}$. The curves are identified as follows: (a) 0.05 μM 1A2, (b) 0.05 μM 2B4, (c) 0.15 μM 2B4, (d) 0.05 μM 1A2 plus 0.05 μM 2B4, and (e) 0.05 μM 1A2 plus 0.15 μM 2B4. (*B*) Simulated data using the model described in Scheme 1 where only binary complexes between reductase and P450 can be formed except that 7ER causes the formation of a high-affinity complex between reductase and P450 1A2. The following kinetic constants were used in the fitting routines: $K_m^{FpT-2B4} = 0.03\ \mu M$; $k_{cat}^{FpT-2B4} = 95\ s^{-1}$; $K_m^{FpT-1A2} = 0.0005\ \mu M$; $k_{cat}^{FpT-1A2} = 750\ s^{-1}$. The curves are identified as follows: (a) 0.05 μM 1A2, (b) 0.05 μM 2B4, (c) 0.15 μM 2B4, (d) 0.05 μM 1A2 plus 0.05 μM 2B4, and (e) 0.05 μM 1A2 plus 0.15 μM 2B4. (*C*) Simulated data using the model described in Scheme 2 where a complex between 1A2 and 2B4 is formed. The following values were used in the fitting routines: $K_m^{FpT-2B4} = 0.03\ \mu M$; $k_{cat}^{FpT-2B4} = 95\ s^{-1}$; $K_m^{FpT-1A2} = 0.08\ \mu M$; $k_{cat}^{FpT-1A2} = 750\ s^{-1}$ (which were determined experimentally from the binary systems), and $K_m^{FpT-1A2-2B4} = 0.0005\ \mu M$; $k_{cat}^{FpT-1A2-2B4} = 550\ s^{-1}$; $K_m^{FpT-1A2-2B4-FpT} = 0.000005\ \mu M$; $k_{cat}^{FpT-1A2-2B4-FpT} = 550\ s^{-1}$. The 1A2 moiety of the 1A2−2B4 complex has an increased affinity for the reductase as described by Scheme 2. The curves are identified as follows: (a) 0.05 μM 1A2, (b) 0.05 μM 2B4, (c) 0.15 μM 2B4, (d) 0.05 μM 1A2 plus 0.05 μM 2B4, and (e) 0.05 μM 1A2 plus 0.15 μM 2B4.

Table 1: Demonstration of Synergistic Interaction between Different P450 Enzymes and NADPH−Cytochrome P450 Reductase in Mixed Reconstituted Systems[a]

system components	[reductase]:[total P450]	EROD
2B4 and reductase	1:1	2.73 ± 0.14
1A2 and reductase	1:1	11.5 ± 0.2
2B4, 1A2 and reductase	1:1	22.6 ± 1.1*
2B4 and reductase	3:1	4.4 ± 1.1
1A2 and reductase	3:1	25.9 ± 2.8
2B4, 1A2 and reductase	3:1	29.9 ± 2.8

[a] Purified P450 2B4 and 1A2 were combined with reductase in DLPC as a reconstituted system containing either one or both P450s as described under Materials and Methods. In these reconstituted systems, reductase (at concentrations ranging from 0.05 to 0.15 μM) was combined with either 2B4 (0.05 μM), 1A2 (0.05 μM), or both P450s (0.05 μM each). EROD was examined in the simple binary systems and compared to the results obtained in the mixed reconstituted systems. The reductase concentration in the complex reconstituted system was twice the concentration in the systems containing only a single P450 in order to maintain the [reductase]:[total P450] ratio. These values are the mean ±SEM for 3 determinations. The asterisk denotes that the value is significantly different from the sum of the individual binary systems ($p < 0.001$).

It is important to keep in mind when considering this model that it is likely to be a simplification of the actual interactions that may occur. For example, in the absence of substrate, it is possible that 1A2−1A2, 2B4−2B4, and 1A2−2B4 interactions occur. However, no kinetic changes would be expected from these interactions unless the catalytic characteristics of the complexes differ from those of the monomeric enzymes. These results demonstrate that the presence of the resorufin substrates causes a change in these catalytic characteristics which is consistent with the formation of a heteromeric 1A2−2B4 complex with a high affinity for reductase binding.

The studies with 7ER were used to further examine the interaction between 1A2 and 2B4. The goal of these experiments was to determine if the high-affinity binding of reductase to the 1A2 moiety of the 1A2−2B4 complex was catalytically functional, or if it was merely an inhibitory complex. 7ER was an ideal substrate to use in this regard for two reasons. First, 7ER is much more effectively dealkylated by 1A2 than 2B4 (which is opposite the selectivity found with 7PR). Second, this substrate is structurally similar to 7PR and would be expected to have a similar effect on the interactions between these P450s. Consequently, if the proposed high-affinity reductase−1A2 complex is functionally active, then the mixed reconstituted systems (containing both P450s) would be expected to exhibit a stimulation of EROD activity. On the other hand, if reductase and 1A2 did not form a *functional* high-affinity complex, then EROD activity in the mixed reconstituted systems would be substantially inhibited as compared to the simple binary systems. The elevation of EROD with the mixed reconstituted system (Figure 3) is consistent with the formation of a catalytically functional high-affinity interaction between reductase and the 1A2 moiety of the 1A2−2B4 complex.

There has been a continual interest in the factors controlling the interaction between reductase and P450. When reconstituted into liposomes, these proteins form a functional complex with a K_m of about 0.2 μM (*14*). Several factors influence the characteristics of this complex including the P450 enzymes involved (*14*−*16*) and the ionic strength of the medium (*14*, *15*). Substrate also has a significant influence on the reductase binding characteristics of several P450 enzymes, increasing both the affinity of the reductase−P450 complex (*17*) and the rate at which the two proteins associate (*18*, *19*). Complex formation has been reported to occur via two different processes, complementary charge pairing (*20*−*23*) and hydrophobic interactions (*24*−*27*). These studies illustrate the complexity of the interactions between reductase and P450.

The data in this paper lend further support to the complexity of the interactions between reductase and P450. The results demonstrate that the catalytic activity of a P450 enzyme is dependent on (a) the P450 enzymes present, (b) the potential of these enzymes to form P450−P450 complexes, (c) the substrates present, and (d) the potential for substrate to alter the manner by which the P450 enzymes interact. For example, some substrates (e.g., benzphetamine) do not appear to influence the way P450 1A2 and 2B4 interact (*9*). In contrast, 7PR and 7ER cause changes consistent with the formation of a 1A2−2B4 complex, capable of high-affinity reductase association [(*9*) and this study].

Yamazaki et al. (*28*) have recently reported that P450 1A2 synergistically stimulates P450 3A4-dependent testosterone 6β-hydroxylation. These studies provide added confirmation to the potential for one P450 enzyme to influence the catalytic characteristics of another P450. The results also demonstrate that the potential for altered catalysis resulting from P450−P450 interactions may be a general phenomenon which could significantly modulate the disposition of foreign compounds.

Interactions have also been demonstrated between P450 2A6 and 2E1 for reductase using a Baculovirus expression system (*29*). These data demonstrate that interactions among reductase and P450s can be obtained using microsomal membranes from expressed proteins as well as in reconstituted systems. These studies showed that 2A6 and 2E1 appear to compete for the available reductase, an effect that is more prominent at subsaturating reductase concentrations. Interestingly, the addition of cytochrome b_5 decreases these competitive interactions. Taken together, the results from the literature (*29*, *28*, *9*), and the present study illustrate the importance of considering the metabolism of a substrate by P450 in terms of a multienzyme system where interactions among these proteins can significantly influence their metabolic behavior.

Many interactions are possible when examining the catalytic characteristics of P450s in the microsomal membrane. Not only are there multiple P450s present, but also the reductase levels are much too low for all P450 enzymes to have a reductase bound at all times. Additionally, other microsomal proteins may interact with the reductase (e.g., heme oxygenase, cytochrome b_5, and fatty acid desaturase) as well as other P450 enzymes. The simultaneous presence of multiple substrates for P450 within the cell further complicates our understanding of those factors which control xenobiotic metabolism. Despite these additional complicating factors, the demonstration that P450 enzymes can influence each others metabolism is another step in developing an understanding of the complexities in the in vivo function of the P450 system.

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