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Association of cytochrome P450 enzymes is a determining factor in their catalytic activity

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[†]*In loving memory of David Kupfer*

Abstract

Previously, our laboratory demonstrated that one cytochrome P450 isoenzyme can influence the catalytic properties of another P450 isoenzyme when combined in a reconstituted system. Moreover, our data and that of other investigators indicate that P450 interaction is required for catalytic activity even when one isoenzyme is present. The goal of the current study was to examine the possible mechanism of these interactions in more detail. Analyzing recently published X-ray data of microsomal P450 enzymes and protein docking studies, four types of dimer formations of P450 enzymes were examined in more detail. In case of two dimer types, the aggregating partner was shown to contribute to NADPH cytochrome P450 reductase (CPR) binding-a flavoprotein whose interaction with P450 is required for expressing P450 functional activity of the neighboring P450 moiety. Thus, it was shown that dimerization of P450 enzymes might result in an altered affinity towards the CPR. Two dimer types were shown to exist only in the presence of a substrate, while the other two types exist also without a substrate present. The molecular basis was established for the fact that the presence of a substrate and other P450 enzymes simultaneously determine the catalytic activity. Furthermore, a kinetic model was improved describing the catalytic activity of P450 enzymes as a function of CPR concentration based on equilibrium between different supramolecular organizations of P450 enzymes. This model was successfully applied in order to explain our experimental data and that of other investigators.

Introduction

Cytochrome P450, a terminal component of an electron transport chain located in the endoplasmic reticulum plays a major role in the metabolism of a wide variety of drugs and other xenobiotics as well as endogenous lipid-soluble compounds. The enzyme system converts lipid-soluble substances into more water-soluble products by insertion of an oxygen atom into the substrate molecule. The heme iron of cytochrome P450 has to be reduced via the CPR by the passage of electrons from NADPH in order to have catalytic activity. Therefore, the affinity of a particular P450 towards the CPR is an important factor in expressing catalytic activity. Many other factors have been shown to influence P450 activity:

(1) Characteristics of a particular isoenzyme, since different P450 enzymes were shown to possess different affinity toward the CPR in the absence of a substrate [1]. Additionally,

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alleles of the same P450 enzyme were shown to have dramatic effect on catalytic activity [2].

(2) Substrate binding to P450s has been suggested as a potential mechanism to regulate which P450 will bind most effectively to CPR. Indeed, the presence of an appropriate substrate for a given P450 has been shown to increase both the rate of association [3, 4] and the affinity for the CPR [5].

(3) Many data indicate that P450 enzymes function as aggregates: P450 enzymes were shown to form larger aggregates, e.g. hexamers (CYP2B4, [6]), octamers (CYP2C3) or even 40-mers (CYP1A2, [7]). The catalytic activity as a function of the detergent concentration shows a maximum [7], demonstrating that small aggregates are the most active, while larger aggregates have smaller, monomers have no catalytic activity. Detergent concentration sufficient to produce monomeric P450s was shown to extinguish the monooxygenase activity, although it is difficult to distinguish between effects that are mediated through alterations in aggregation state of P450s and direct inhibitory effect of the detergent [8, 9]. Chemical cross-linking approach provided evidence that some P450 enzymes form complexes in the microsomal membrane [10]. Additionally, specific interactions among multiple P450 isoenzymes have also been shown to affect their catalytic activity [11–13]. In the work of Cawley et al. [11] and Backes et al. [12], using mixtures of CYP2B4, CYP1A2 and CPR, the addition of a second P450 enzyme altered the activity of the original P450 enzyme in a mixed reconstituted system in a substrate dependent manner. CYP2B4 dependent 7-pentoxyresorufin *O*-dealkylation was inhibited in the presence of CYP1A2. In contrast, CYP1A2-dependent 7-ethoxyresorufin *O*-dealkylation was stimulated by the addition of CYP2B4 enzyme. Although these studies were carried out using reconstituted system, the same effects were observed under more natural conditions of the microsomal membrane [14]. Similarly, our laboratory demonstrated that CYP2C19 activated CYP2C9 dependent diclofenac hydroxylation in reconstituted systems although CYP2C19 alone did not efficiently metabolize that compound [15].

Very few study addressed the question whether P450 enzymes functionally interact even when present in a single enzyme containing reconstitution system. However, if a P450 in monomeric form is in complex with one CPR molecule, the catalytic activity of any P450 enzyme as a function of CPR concentration should yield a simple saturating curve in reconstituted system with one isoenzyme and CPR present in phospholipid. This type of curve is rarely observed in the literature: instead, non-saturating curves, S-shaped curves, activities declining at higher CPR concentrations and saturation before 1:1 P450:CPR ratio are often found [13]. Similarly, curves obtained by us also failed to show simple saturation [15] indicating that P450 enzymes do not function in monomeric form even in single enzyme containing expression systems.

These data establish that P450 enzymes function as aggregates, however, the mechanistic details how the catalytic properties of one P450 enzyme is modulated by another P450 has not been clarified. The current study was designed to examine the interactions among P450 enzymes and CPR with molecular modeling. Our study addressed the question how the possible formation of P450 complexes affect their affinity towards the CPR. Additionally, this study focused on the characteristics of the P450–CPR complex. It was anticipated that such an investigation helps explain the reason why oligomeric P450 is required for functional P450 activity.

Experimental procedures

Protein modeling

All crystallographic data were taken from the Protein Data Bank [16]. CYP2B4 X-ray structure (PDB entry: 1PO5) was used for open conformation models, whereas warfarin bound CYP2C9 (PDB entry: 1OG5) was used for closed conformation models. Models of the complex between mammalian microsomal P450s and P450 reductase were built on the basis of experimentally determined structure of the complex between the heme and FMN-binding domains of bacterial cytochrome P450BM-3 (PDB entry: 1BVY). In our calculations, instead of the FMN-binding domain

of cytochrome P450BM-3, human cytochrome P450 reductase (PDB entry: 1B1C) was applied. Similarly, for the heme-binding domain, instead of bacterial cytochrome P450BM-3, human CYP2C9 (PDB entry 1OG5) and CYP2B4 (PDB entry 1PO5) were used. Minimal root mean standard deviation (RMSD) was calculated between the matched monomers. Minimal RMSD fittings were calculated with Swiss-PdbViewer fit command [17]. Complex of cytochrome P450 and reductase was also calculated with Escher program. Dimer structures built from open (CYP2B4, PDB entry: 1PO5) and closed (CYP2C9, PDB entry: 1OG5) P450 monomers were made with the dimeric templates of P450 enzymes (PDB entries: 1OG5 (CYP2C9), 1GWI (CYP154C1) and 1FAH (P450 BM3)) by calculating minimal RMSD. Dimeric model for CYP2C9 was also calculated without constraints by Escher program [18] using default parameters. Further refinements of the resulting complex structures were carried out by Sybyl 6.6 program (Tripos Inc., St. Louis, MO) on a Silicon Graphics Octane workstation under Irix 6.5 operation system. Dimeric structures were fully minimized using AMBER force field without any restriction until the maximum derivatives were less than 0.050 kcal/molÅ. Gasteiger–Huckel partial charges were applied during the calculations. The qualities of the models were verified using 'Procheck', as compared with well-refined structures at the same resolution [19].

Mathematical modeling

The model is based on the following approach: P450–P450 interactions modulate their affinity for CPR. An equilibrium exists between two types (A and B) of P450–P450 aggregates with different affinity and stoichiometry to the CPR. (Scheme 1)

$$A + R = A * R, K_1, k_1 \qquad \textit{Scheme}\ 1$$
$$\mathrm{A} * \mathrm{R} + \mathrm{R} = \mathrm{B} * \mathrm{R} * \mathrm{R}, K_2, k_2$$

Where A symbolizes the A-type oligomer, A*R is 2:1 P450-reductase complex, while B*R*R means 1:1 P450–CPR complex. *K*'s are affinity constants, k_1 and k_2 are proportionality constants between the catalytic activity and A*R or B*R*R concentrations, respectively. In the model the followings are assumed (a modification of a model in [12]): (1) The activity of a P450 will be directly proportional to the fraction of P450 molecules that associate with the CPR. (2) CPR molecules have only one P450-binding site that will support electron transfer to cytochrome P450. (3) P450–CPR complexes function in *A* oligomerization form as P450:CPR 2:1 complexes, in *B* form as 1:1 complexes. (4) There is an equlibrium between CPR bound A- and B-type oligomers (A*R and B*R*R). (5) *A**R and *B**R*R complexes have different association constants. (5) *A*R* and *B*R*R* complexes might have different k_1 and k_2 proportionality constants. As the equations derived from Scheme 1 can be handled implicitly more easily, a Fortran 90 program was written to evaluate the roots at given K_1, K_2, k_1 and k_2 values. An iterative procedure was applied, using the following steps. (1) Initial estimates of K_1, K_2, k_1 and k_2 were made. (2) The predicted concentrations of each product were calculated by an iterative brute-force procedure to find the global minimum solution at a given total reductase and P450 concentration. (3) The algorithm repeatedly calculates the equilibrium concentrations at different total concentrations. (4) The concentration of A*R and B*R complexes was multiplied by k_1 and k_2, respectively, and the predicted velocity was obtained as the sum of these products. (5) The whole procedure was repeated with different manually adjusted *K*'s and *k*'s to improve the fit with experimental data.

Preparation of P450 reconstituted system and incubations with P450 enzymes

The experiments were the same as described in [15]. Briefly, the incubation contained the following constituents**:** 10 mM magnesium chloride in 50 mM sodium phosphate buffer (pH 7.4), substrate (methoxychlor, *S*-mephenytoin or diclofenac at a concentration specified below), cytochrome P450, cytochrome b_5 and CPR (amounts specified below).

Methoxychlor demethylation

The final incubation volume was 1 ml. The incubation mixture contained: 50**.**0 μM methoxychlor (final concentration) added in10 μl ethanol, 25 pmol CYP2C19 or 125 pmol CYP2C9, cytochrome b_5 and varying concentrations of CPR.

S-Mephenytoin 4-hydroxylase
The final incubation volume was 500 µl. The incubation mixture contained: *S*-mephenytoin (100 µM final concentration) added in 10 µl ethanol, 25 pmol CYP2C19 cytochrome b_5 and varying concentration of CPR.

Diclofenac hydroxylase.
The incubation volume was 500 µl. The incubation mixture contained: 100 µM diclofenac (final concentration, added in 50 µl 10% ethanol/water), CYP2C9 (25 pmol), cytochrome b_5 and varying concentration of CPR in the reconstituted systems.

Metabolite analysis. Analyses of the metabolites generated by the reconstitution systems were conducted in the following HPLC apparatus: Waters 1525 binary HPLC pump, 2996 diode array UV detector and 717 plus Autosampler and Packard 500 TR flow scintillation radioactivity detector. Analyses utilized a 5-µm Phenomenex Prodigy ODS3 column (250 × 4.6 mm). The following chromatographic condition was used for the detection of **methoxychlor metabolites** the flow rate was 1 ml/min; the eluates were monitored by their UV absorption at 230 nm and by their radioactivity. The following HPLC conditions were utilized: pump A = 50% acetonitrile/water, pump B = 90% acetonitrile water; a stepwise gradient was applied: 100% pump A from 0 to 4 min followed by 30% pump A, until 8 min. From 8 min a linear gradient of 100% pump B was used over 6 min. The following chromatographic condition was used for the detection of **4-hydroxymephenytoin**: The flow rate was 1 ml/min, and the metabolites were detected at 204 nm (determined to be the λ_{max}). The HPLC solvent system consisted of the following: pump A = 20% acetonitrile/water; pump B = 40% acetonitrile/water. The following stepwise gradient program was utilized: 100% pump A for 0–5 min, 75% pump A and 25 % pump B from 5 to 8 min, 50% pump A and 50% pump B until 11 min followed by 100% pump B. Retention times were 12 min for 4-OH mephenytoin and 18 min for *S*-mephenytoin. The following chromatographic condition was used for the detection of **4-hydroxy-diclofenac** (modification of a method described in the Gentest Co. website): The flow rate was 1 ml/min, and the metabolite was detected at 267 nm (determined to be the λ_{max}). The HPLC solvent system consisted of the following: mobile phase A = 20% acetonitrile, 79.9 % water, and 0.1% HCl, and mobile phase B: 100% acetonitrile. Initial conditions were: 100% A with a linear gradient to 100% B over 12 min.

Results

Experimental data using reconstituted systems

Reconstituted system with a single enzyme present
The rate of both methoxychlor *O*-demethylation and diclofenac 4′-hydroxylation catalyzed by purified CYP2C9 in a reconstituted system reached saturation around CYP2C9: CPR 1:1 ratio (Figure 6e, f). Whereas the rate of diclofenac 4′-hydroxylation remained constant at higher CPR concentration (Figure 6e), further increase in CPR concentration caused a decline in CYP2C9-dependent methoxychlor *O*-demethylation (Figure 6f). In contrast, the rate of methoxychlor *O*-demethylation catalyzed and *S*-mephenytoin 4-hydroxylation by CYP2C19 could not be saturated even at a twofold excess of CPR to CYP2C19 (Figure 6d).

Molecular and kinetic modeling of Cyp enzymes

Recent X-ray results of mammalian P450 enzymes demonstrated that P450s exist in two conformations (open and closed). Large scale comformational changes (from open to closed conformation) are generally due to relocation of helices F, G, B′, C, and I and β4 region [20]. As a result of substrate binding usually the closed conformation is favoured [21–23].

In our study, the experimentally available CYP2C9 (1OG5) was used for representing the closed conformation of P450 monomer, while the open structure of this enzyme was built based on the crystallographic template of CYP2B4.

Structure of the 1:1 P450–CPR complex
Two methods were applied to predict the structure of the P450-CPR complex. (A) model structures were built on the basis of the published X-ray structure of a cytochrome P450-redox partner electron transfer complex [24]. (B) Escher protein docking program was utilized for docking CPR to the P450 structure without constraints.

(A) According to the calculations based on the X-ray structure, the optimized RMSD of the cytochrome P450 part is 2.170 Å, the RMSD between the FMN binding domain of cytochrome P450BM-3 and the FMN-binding domain of human P450 reductase ([25], PDB code: 1B1C) is as low as 1.238 Å. In the P450–CPR 1:1 model the following charged residues of CYP2C9 (differing side chains for CYP2B4 are indicated in brackets) have sidechain atoms within 4 Å from the reductase: K (R)84 [26–29], D89 [27, 28, 30], E92, K (R)121 [31–34], R125 [32, 35, 36], R132 [32], K (R)270 [33, 37], K273 [33, 37], K (R)421 [32, 38], K432 [30, 32, 38, 39], E438. With the exception of E92 and E438, all of these residues were demonstrated to be involved in redox partner binding of different P450 enzymes, see references above and a recent review on the topology of redox-partner interacting regions in P450s [40].

(B) Esher protein docking program was also used to calculate the quaternary structure of the P450–CPR complex. The CPR binding region of P450 was calculated to be similar to the calculation based on the complex structure of the heme- and FMN-binding domains of P450BM-3 [24]. Namely, the following P450 residues are involved in the complex formation: E92, R125, R132, K421, K423, K432. All of these residues (with the exception of K423) were involved in the interface interactions as calculated from the complex structure of the heme- and FMN–binding domains of P450BM-3.

To summarize, the results of the Escher modeling supported the results built upon the crystallographic data, which was used for further studies.

Dimer formations of P450 enzymes

Next, it was examined how interactions among P450 enzymes affect their affinity towards the CPR. For possible interaction interfaces, the X-ray structures available as dimers were examined. Additionally, protein docking calculations without preconceptions were used.

Among the reported mammalian P450 structures, CYP2C9 enzyme has been crystallized as a dimer (PDB entry: 1OG5, [23]). Although published in the PDB as monomer, CYP2B4 enzyme has also been shown to form dimers in solution and crystal [41]. Additionally, a bacterial enzyme, CYP154C1 ([42] PDB entry 1GWI) has been crystallized as a dimer. In the crystal structure of CYP2C9 and CYP154C1, the P450 moieties associate in a different way resulting in different dimer types. A third type dimerization motif (PDB entry: 1FAH) can be observed in the crystal structure of bacterial cytochrome P450 BM-3 (PDB entries: 2BMH, [43]; 1FAH, [44]).

The characteristics of these dimerization types (1OG5, 1GWI and 1FAH as labeled according to their PDB entries and Escher model from the docking calculations) are discussed below.

Characteristics of the 1OG5-type dimer.

Affinity towards the neighboring CYP. The 1OG5-type dimer built from open conformation monomers is shown on Figure 1. Characteristic of this type of dimerization is that the acidic D414 and E415 (present in CYP2C9 enzyme) residues point towards the heme of the neighboring P450. In the case of 1OG5-type dimer the substrate induced conformational changes from open to closed structure should not change the affinity of a particular P450 for association with another P450, as the protein-protein interface remains unchanged.

Affinity towards the CPR. Since the CPR binding cleft of one molecule is partially filled by the second P450, this type of dimer binds only one CPR molecule. Besides the CPR binding site of the first enzyme, amino acids of the helices J and K of the second P450 enzyme also contribute to CPR binding. Therefore, 1OG5-type association of P450 enzymes results in an increased affinity towards the CPR as compared to the P450 monomer.

Characteristics of the 1GWI-type dimer.

Affinity towards the neighbouring CYP. Another possible arrangement of a dimer is that of 1GWI-type. The dimer is built up from closed conformation CYP2C9 monomers as shown in Figure 2. This arrangement produces a very tight contact, where the long helix G points toward the same helix of the second protein. Modelling of the open conformation of the 1GWI-type dimer results in overlapping chains at the interface and cannot

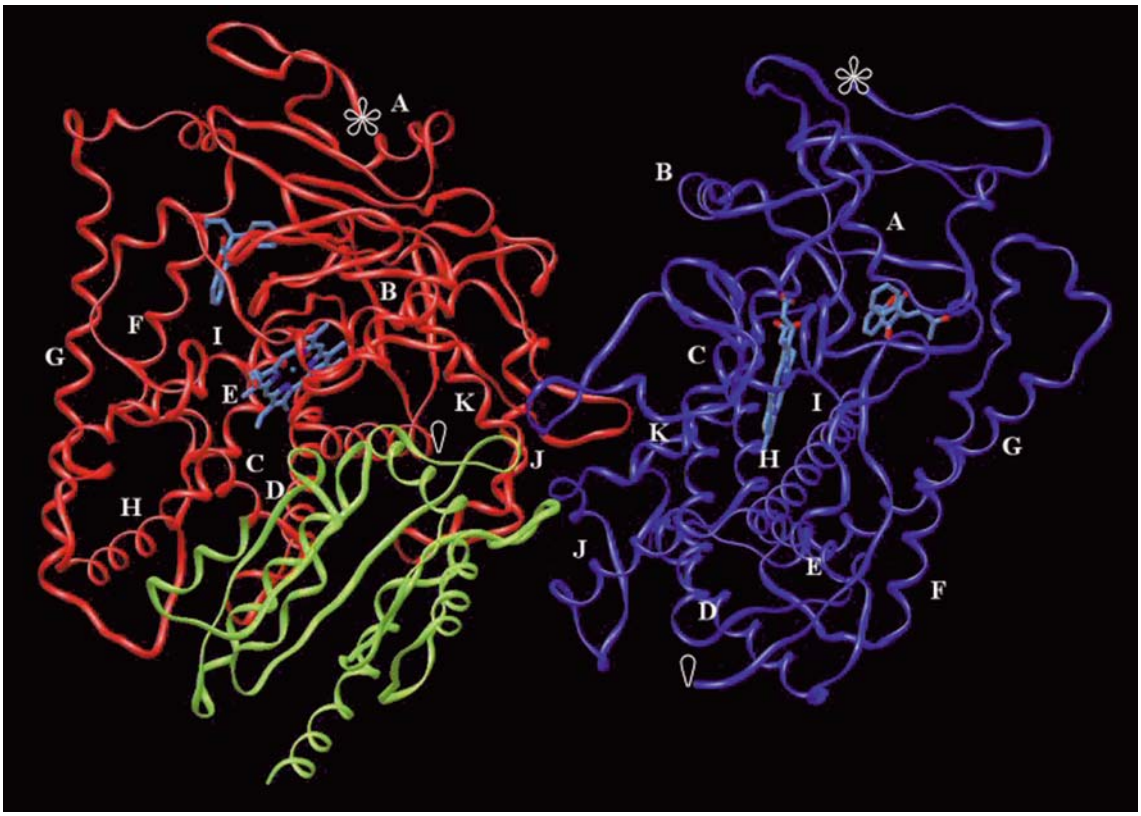

Figure 1. 1OG5-type dimer formation of CYP enzymes built from open conformation of CYP2B4 monomers. Green color means CPR (PDB entry: 1B1C). '*' Indicates the N-terminal region while C-terminal region is indicated with '' sign. The N-terminal region has been suggested to play a role in association with the membrane.

produce a reasonable dimeric structure for this conformation of a P450 enzyme. This observation strongly suggest that no open conformation of 1GWI-type dimerization exists. Although CYP154C1 was crystallized without a bound ligand, its structure resembles the closed conformation of mammalian cytochrome P450 (RMSD of 154C1 with closed 2C9: 2.1 Å for 307 residues, 3.3 Å for all residues, RMSD with open 2B4: 2.6 Å for 237 residues, 5.4 Å for all residues).

Affinity towards the CPR. The closed 1GWI-type dimer binds two CPR molecules. The binding of a CPR by one CYP is again strengthened by the neighboring monomer, but the residues involved in this secondary binding differ from that of the 1OG5-type: amino acids around positions 70 and 225 contribute to the redox-partner binding. Therefore, 1GWI-type dimer possesses altered affinity towards the CPR than P450 monomer and different affinity compared to that of 1OG5.

Characteristics of the 1FAH-type dimer.

Affinity towards the neighboring CYP. Residues from the end of helix D and before helix F are in the proximity of same helices of the other chain, producing a small contact surface. The structure of the interface is very similar in open (Figure 3) and closed forms as the contact surfaces remain unchanged.

Affinity towards the CPR. The 1FAH-type dimer differs from the previously presented two types in that the second CYP does not provide a secondary interface for the CPR. The affinity toward the CPR does not change as a result of aggregation of CYP enzymes and thus this type of dimer exhibits lower affinity towards the CPR than any of the 1OG5 and 1GWI dimers.

Characteristics of the Escher-type dimer.

Affinity towards the neighboring CYP. Escher calculations were carried out to explore alternative

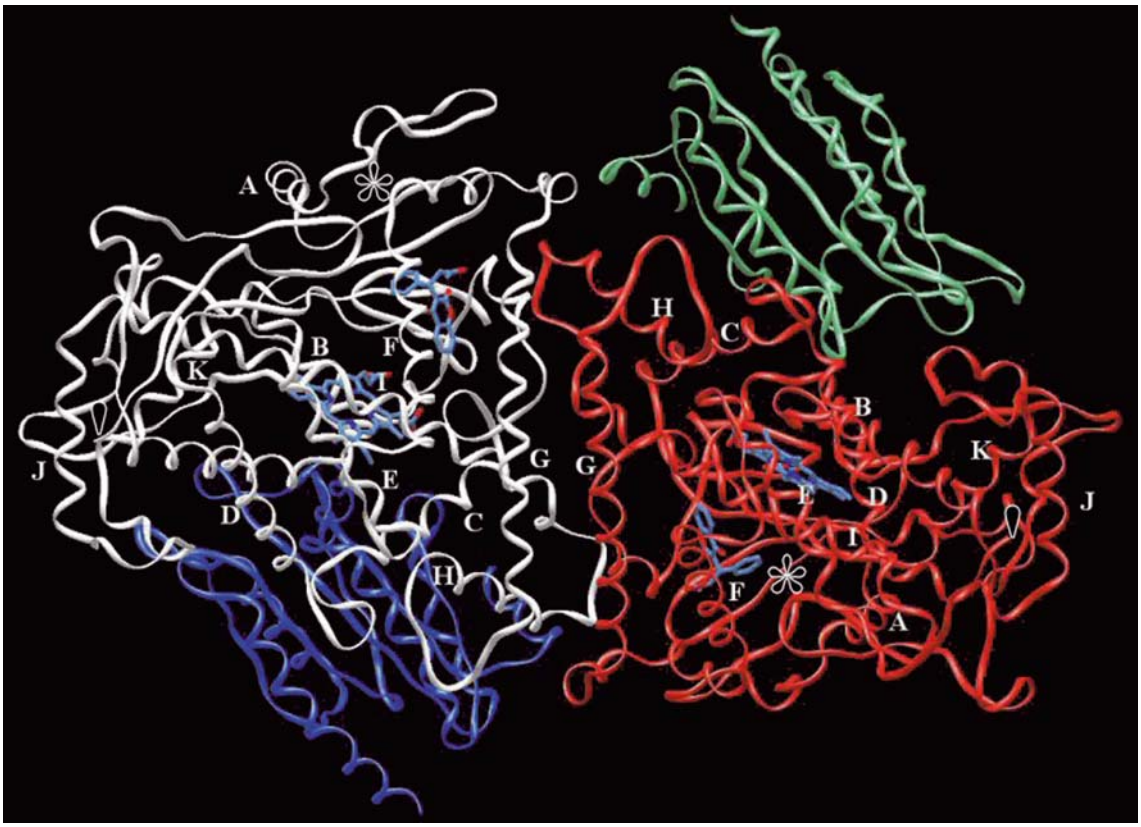

Figure 2. 1GWI-type dimer formation of CYP enzymes built from closed conformation of CYP2C9 monomers. Green and blue colors indicate CPRs (PDB entry: 1B1C). '*' Indicates the N-terminal region while C-terminal region is indicated with '' sign.

supramolecular arrangement for closed state monomers. The best score docking result is shown on Figure 4. In this symmetrical arrangement, the F helices of the two enzymes point towards each other producing large interface. Similarly to 1GWI-type dimer, this arrangement only exists in closed conformation, since F helix was shown to rearrange as a result of conformational change (from open to closed conformation). The interface regions are not favorable for interaction in open conformation.

Affinity towards the CPR. Similarly to 1FAH-type dimer, Escher-type dimer formation does not influence the affinity of the CYPs towards the CPR comparing to the monomeric form, as the second CYP does not provide secondary interface for CPR binding.

Comparing 1FAH, 1OG5, 1GWI and Escher dimers, no overlapping structural elements are found. This means that larger aggregates can be built from these dimeric motifs. A possible arrangement built from the dimers examined by us is shown in Figure 5. Consecutive oligomerization from the same dimer type is not possible, as they do not have accessible surface for the same type of aggregation. However, a 1GWI-contact can be continued with 1OG5-type and Escher-type contact, while 1FAH-type motif can produce a turn in the aggregation. From this point, the whole aggregation process can continue in the same order and the motifs can be repeated infinitely.

Kinetic modeling

Based on the observation that dimerization might result in an increased affinity of the aggregates toward the CPR, a mathematical model was improved in order to explain the catalytic behavior of cytochrome P450 enzymes. If a P450 in monomeric form is in complex with one CPR molecule, the catalytic activity of any P450 enzyme as a function of CPR concentration should yield a simple saturating curve with saturation at 1:1

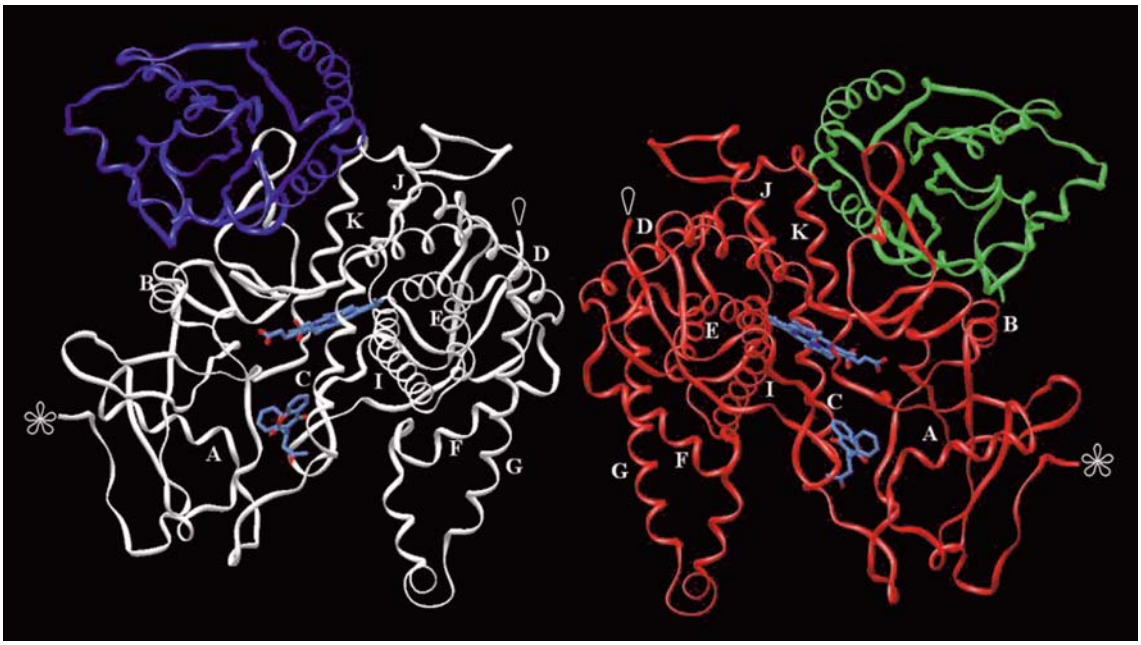

Figure 3. 1FAH-type dimer formation of CYP enzymes built from open conformation of CYP2B4 monomers. Green and blue colors indicate CPR (PDB entry: 1B1C). '*' Indicates the N-terminal region while C-terminal region is indicated with '' sign.

P450: CPR ratio in single enzyme containing systems – such a simple behavior is rarely observed in the literature. In turn, our hypothesis based on our modeling studies that P450–P450 interactions alter their affinity towards the CPR predicts more complex behavior of the reconstituted system as a function of CPR concentration even with one isoenzyme present.

The following assumptions were made from our modeling studies:

(1) There is more than one oligomerization type of P450 which influences the affinity of the P450 moiety towards the CPR. The different oligomerization types are in equilibrium. A-type oligomers built from open state monomers having a 2:1 P450:CPR stoichiometry; B-type for closed state dimers/oligomers possessing 1:1 P450:CPR stoichiometry.
(2) Since different oligomerization types have different stoichiometry for the CPR, the equilibrium is influenced by the amount of CPR present in the system.
In the presence of a substrate, an equilibrium between A- and B-type oligomers, possessing similar characteristics as 1OG5- and 1GWI-type dimers, respectively, were assumed with different affinity towards the CPR and possessing different catalytic activity (Scheme 1). It was examined whether our mathematical model is able to describe the experimental curves obtained by us and found in the relevant literature.

Figure 6a presents catalytic activity as a function of CPR concentration, where saturation of catalytic activity is observed at P450: CPR 2:1 ratio. Such a curve is presented in the work Kaminsky and Guengerich [13], where the formation of metabolites from R-warfarin by $P450_{UT\text{-}A}$ was investigated. This type of curve is simulated when it is hypothesized that B-type oligomer is not formed; e.g. if the ligand cannot induce the appropriate conformational changes for the closed structure or the interface contacts of the particular P450 have repulsive character ($K_2 = 0$). Since A-type oligomer binds CPR in 2:1 stoichiometry, saturation of catalytic activity is observed at P450: CPR 2:1 ratio.

Dutton and Parkinson [45] examined ethoxyresorufin turnover by P450-c (Figure 6b), and saturation was not reached even at a molar

Figure 4. Escher-type dimer formation of CYP enzymes built from closed conformation of CYP2C9 monomers. '*' Indicates the N-terminal region while C-terminal region is indicated with '' sign.

excess of CPR. In turn, sharp saturation was observed at P450: CPR 1:1 ratio when Kaminsky and Guengerich [13] investigated the rate of metabolite formation from R-warfarin by $P450_{PB\text{-}C}$ (Figure 6c). These two types of curves are obtained if the following hypothesis is made: A- and B-type oligomers possess the same catalytic rate ($k_2/k_1 = 1$) and formation of A-type oligomer is similarly favorable in both cases ($K_1 = 10^9$).Using these k_2/k_1 and K_1 values,

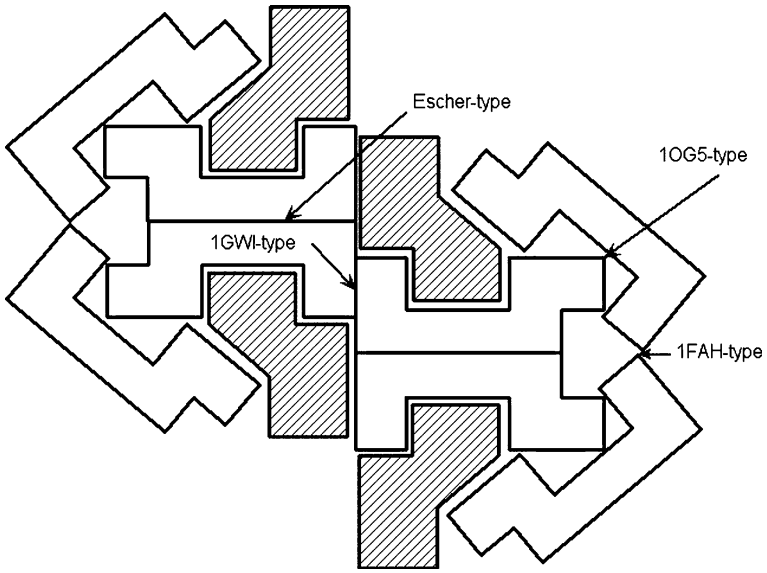

Figure 5. Model of oligomer formation of CYP enzymes from closed conformation monomers. The striped pattern shape CPR molecules, whereas P450 enzymes are indicated with white forms.

lower K_2 value ($K_2 = 10^7$) yields no saturation (Figure 6b), whereas higher K_2 value ($K_2 = 10^9$) results in sharp saturation (Figure 6c).

S-shaped curves were obtained when we examined CYP2C19 dependent methoxychlor *O*-demethylation and *S*-mephenytoin hydroxylation (Figure 6d) and CYP2C9-dependent diclofenac hydroxylation as a function of CPR concentration (Figure 6e**)**. Whereas no saturation was reached in the former case (Figure 6d), saturation could be reached at P450: CPR 1:1 ratio in the latter case (Figure 6e). These curves are simulated if in both cases B-type oligomers are assumed to possess higher catalytic rate than the A-type ($k_2/k_1 = 5$) and formation of A-type oligomer is similarly favorable in both cases ($K_1 = 10^9$). Using these k_2/k_1 and K_1 values, lower K_2 value ($K_2 = 10^7$) yields no saturation (Figure 6d), whereas higher K_2 value – strong B-type interface – ($K_2 = 10^9$) results in saturation (Figure 6e).

Figure 6f shows methoxychlor *O*-demethylation by CYP2C9**,** on Figure 6g 4′-hydroxywarfarin formation from R-warfarin by $P450_{PB\text{-}B}$ can be seen. In both cases, activities decline at higher CPR concentration. These two types of curves are simulated if the followings are assumed: In both cases, A-type oligomers possess higher catalytic rate than the B-type ($k_2/k_1 = 0.2$) and formation of A-type oligomer is similarly favorable in both cases ($K_1 = 10^9$). Using these k_2/k_1 and K_1 values, lower K_2 value ($K_2 = 10^7$) yields slow decline (Figure 6f), whereas higher K_2 value – strong B-type interface – ($K_2 = 10^9$) results in sudden decrease in monooxygenase activity (Figure 6g).

Discussion

Cytochrome P450 system is responsible for the oxidative metabolism of many xenobiotics and endogenous compounds. Cytochrome P450 monooxygenase activity requires an interaction between P450 enzymes and NADPH cytochrome P450 reductase. Although these proteins have been identified to form a 1:1 functional complex *in vitro* [46], experimental data indicate that P450 enzymes form complex in the microsomal membrane [10, 47]. The goal of the current study was to examine the mechanism of P450–P450 interactions in more detail and to investigate how these interactions influence the catalytic activity. It was anticipated that P450–P450 interactions at the surface far from the active center do not cause major conformational changes in the active center of the protein. Thus, the change in the catalytic activity as a result of P450–P450 interaction was assumed to be the result of the altered affinity of the oligomers towards the CPR as compared to the monomer rather than of changes in the active center.

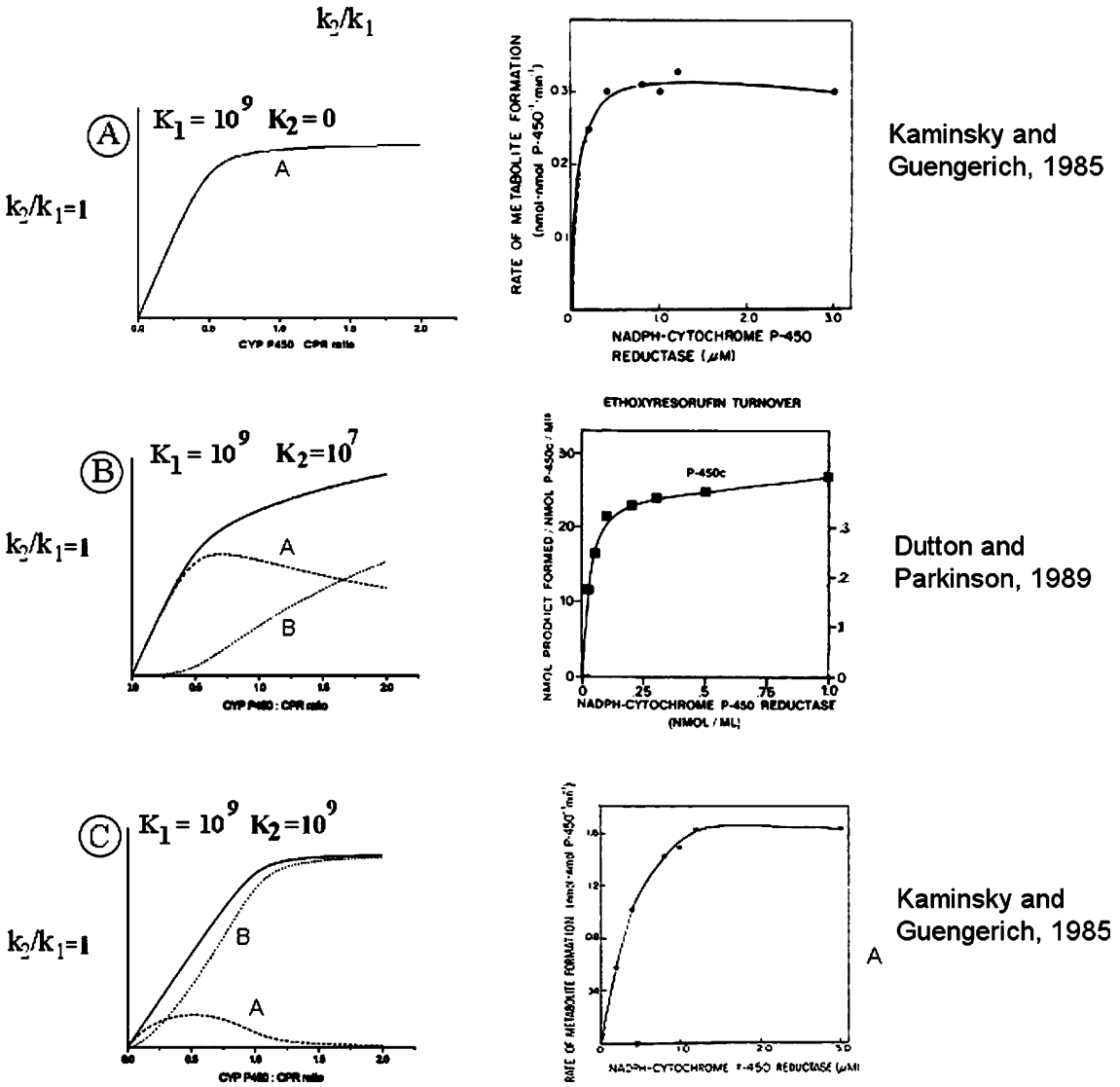

Figure 6. Behavior of cytochrome P450-dependent monooxygenase activity as a function of CPR concentration. Figures on the left panel show results obtained with mathematical modeling which describe the observed experimental curves shown on the right panel. K_1 value (formation of A-type oligomer–reductase complex) was 10^9 in all the calculations. K_2 values (formation of B-type oligomer–reductase complex) and k_2/k_1values (where k_2 and k_1 are proportional constants of the complex concentration and catalytic activity of B- and A-type oligomers, respectively) are indicated on the figures. The dotted lines indicate the contribution of the hypothesized A*R and B*R*R complexes to the total P450 activity. (a): Rates of 6-hydroxywarfarin formation from R-warfarin as a function of increasing NADPH-reductase concentration. P-$450_{UT\text{-}A}$ was used in a concentration of 1.0 μM; (b): *O*-dealkylation of ethoxyresorufin by cytochrome P-450c as a function of increasing NADPH-reductase concentration. P-450c was used in a concentration of 0.02 nmol/ml; Reprinted from [45] with permission from Elsevier. (c): Rates of 7-hydroxywarfarin formation from R-warfarin as a function of increasing NADPH-reductase concentration. P-$450_{PB\text{-}C}$ was used in a concentration of 1.0 μM; (d): Methoxychlor *O*-demethylation (25 nM CYP2C19) and *S*-Mephenytoin hydroxylation (50 nM CYP2C19) by CYP2C19 as a function of increasing NADPH-reductase concentration. On the *X*-axis the CYP2C19: CPR ratio is indicate; (e): Diclofenac hydroxylation (50 nM CYP2C9) by CYP2C9 as a function of increasing NADPH-reductase concentration. On the *X*-axis the CYP2C9: CPR ratio is indicated; (f): Methoxychlor *O*-demethylation (125 nM CYP2C9) by CYP2C9 as a function of increasing NADPH-reductase concentration. On the *X*-axis the CYP2C9: CPR ratio is indicated; and (g): Rates of 4′-hydroxywarfarin formation from R-warfarin as a function of increasing NADPH-reductase concentration. P-$450_{PB\text{-}B}$ was used in a concentration of 1.0 μM.

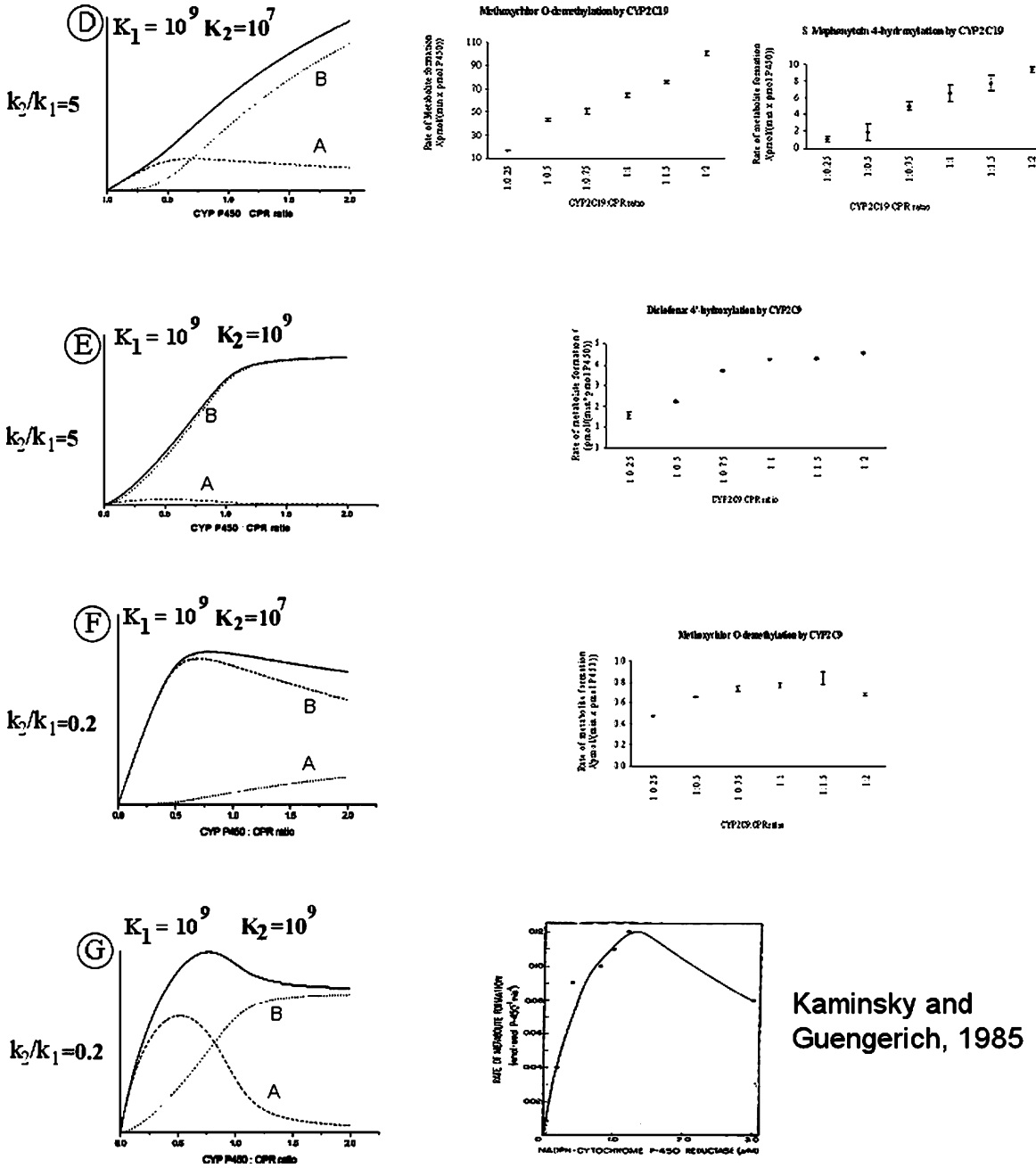

Figure 6. (Continued)

Analyzing possible aggregation states of cytochrome P450 enzymes based on X-ray structures available in Protein Data Bank and protein docking calculations, four different P450 dimer types were examined in more detail, namely 1OG5-, 1GWI-, 1FAH (as named after their PDB entry) and Escher-type (named after the modeling program used for calculation). 1FAH and Escher-type dimer formation of P450 enzymes were found not to influence the affinity of any P450 for the CPR as compared to the monomer. In contrast, 1OG5- and 1GWI-type dimers were shown to have altered affinity towards the CPR as compared to the monomer. The reason for altered affinity is that

in the case of 1OG5- and 1GWI-type dimers the aggregating partner contributes to the CPR binding of the first P450. That means that in case of the latter two dimers the aggregation state influences the affinity towards the CPR and thus the catalytic activity of the P450 aggregates as compared to the P450 monomer. Therefore, these two types of dimerization forms were subject to further investigations.

As P450 enzymes are known to exist in open and closed conformation, where substrate induced conformational changes were shown to make the closed conformation possible [21], it was examined whether the dimer types exist in open (i.e. in the absence of a substrate) and closed (i.e. in the presence of a substrate) conformation. 1OG5-type dimerization seems to occur both in open and closed monomer conformation, whereas 1GWI-type dimerization was shown to be possible from closed conformation monomers only. It means that a conformational change in the monomer as a result of substrate binding might also alter possible interactions with other P450 moieties; and thus new patterns of supramolecular organization with altered affinity towards the CPR might come to existence. Our model is in accordance with the experimental data, namely, that both the presence of a substrate [3, 4] (closed conformation) and the presence of another P450 [13, 15, 45] (supramolecular organizations) simultaneously determine the catalytic activity of the cytochrome P450 enzyme.

It should be emphasized that the 1OG5 and 1GWI dimers possess different stoichiometry towards the CPR in our molecular model. This fact was used when improving our mathematical model attempting to describe the behavior of single enzyme containing reconstituted systems as a function of CPR concentration, namely, two oligomers (*A*- and *B*-type) with different stoichiometry towards the CPR were hypothesized to be in equilibrium. The catalytic activity of the P450 in question depends on the relative stability and catalytic activity of the A- and B-oligomers in equilibrium. As the oligomers possess different stoichiometry towards the CPR, the equilibrium between the oligomers is dependent on the CPR concentration in our model. The oligomer type possessing higher stoichiometry (i.e. capable of binding more CPR) towards the CPR is favored at higher CPR concentration.

It should be emphasized that –as our model is based on P450 oligomers in equilibrium– it can be applied to both homo-(single enzyme containing system) and hetero-oligomers (binary systems with two or more isoenzymes present). Homo- and hetero-oligomer formation are both expected in the case of complex (more than one P450 isoenzyme is present) systems. Heterooligomeric P450 activity is influenced by the interface between P450 enzymes – shifting the equilibrium between oligomerization types – which in turn affects the CPR binding of the particular isoenzymes in our model. Therefore, our model is in accordance with the available experimental evidence showing that as a result of heteromeric P450–P450 interactions in more than one isoenzyme containing reconstitution systems both activation and inhibition of catalytic activity can be expected as compared to catalytic activities in single enzyme containing reconstitution systems [12, 15].

Furthermore, our model was successfully applied to explain the experimentally observed declining activities at higher CPR concentration as well as the S-shaped and non-saturating catalytic activities as a function of CPR concentration in a single enzyme containing systems. If the A- and B-type oligomers were assumed to possess the same catalytic rate, simple saturating-shape curves were obtained (Figure 6a–c). If the B-type oligomers were assumed to possess higher catalytic rate than the A-type, than S-shaped curves were simulated (Figure 6d, e). If A-type oligomer is catalytically more active than the B-type, than curves declining at higher CPR concentration could be simulated (Figure 6f, g).

It should be noted that although different P450s have already been shown to interact in reconstitution systems and this interaction was shown to affect their catalytic activity [12, 13, 15, 45], the hypothesis of P450 isoenzymes interacting even in single enzyme-containing reconstitution systems needs to be examined in more detail.

Moreover, the hypothesis of different homomeric oligomer types in equilibrium offers an alternative explanation for the observed biphasic kinetics in the rate of the first electron transfer, which is obtained even with a single purified P450 containing system with saturating reductase concentration. Peterson et al. [47] found that 50–90% of the P450 enzymes present in microsomes is

reducible in the slow phase. This fact is in accordance with the time consuming rearrangement of oligomers – as compared to the rate of electron transfer – of P450 oligomers in our model.

To summarize, the purpose of this paper was to propose that formation of both homo- and heteromeric P450 complexes are an important factor in expressing their catalytic activity. Homomeric P450 interactions are hypothesized to be responsible for the unusual kinetic behavior observed when P450 enzyme activities are examined as a function of reductase concentration in single enzyme-containing reconstitution systems. Four potential schemes were examined by which P450 complexes can be formed. Mathematical modeling data was provided to support our hypothesis to explain the unusual behavior of P450 activity as a function of CPR concentration measured by our own and other research groups that can be mediated by homomeric P450 interactions in singe enzyme containing reconstitution systems. Although experimental data indicate that P450s aggregate in solution, supramolecular organizations of P450s at the atomic level in the microsomal membrane are yet to be explored.

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