Molecular Organization of the Microsomal Oxidative System: a New Connotation for an Old Term[1]

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Abstract—The central role that cytochromes P450 play in the metabolism of drugs and other xenobiotics makes these enzymes a major subject for studies of drug disposition, adverse drug effects and drug-drug interactions. Despite tremendous success in elucidating structures and mechanisms of cytochrome P450 function, the concept of the drug-metabolizing ensemble as a functionally integrated system remains undeveloped. However, eukaryotic cells typically possess a multitude of different cytochromes P450 that are co-localized in the membrane of endoplasmic reticulum (ER); they interact with each other through the formation of dynamic heteromeric complexes (mixed oligomers). There has been growing appreciation of the importance of developing an approach to study the ensemble of cytochromes P450 as an integral system inspired growing interest of researchers to the principles of molecular organization of the microsomal monooxygenase system. Academician Archakov and his colleagues made important contributions to this field during the initial period of studies. Subsequent exploration of the molecular organization of the microsomal monooxygenase system as an integral multienzyme and multifunctional system have had an essential impact on our understanding of the key factors that determine the changes in human drug metabolism and other cytochrome P450-related functions in development and aging, as well as under the influence of various pathologies and environmental factors.

Keywords: cytochrome P450, endoplasmic reticulum, multienzyme system, allostery, protein-protein interactions, oligomerization

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INTRODUCTION

Cytochromes P450, the heme-thiolate enzymes found in all domains of life, from Eubacteria and Archaea to Eukarya, are one of the oldest heme-containing proteins. Cytochromes P450 probably appeared about 3.5 billion years ago [1], when the oxygen content in the atmosphere was negligible. It is suggested that ancient cytochromes P450 acted as reducing enzymes, and could play the role of NO-reductases [2, 3]. However, evolutionary development of the cytochrome P450 family intensified about 2 billion years ago, soon after green plants began to release oxygen into the atmosphere, leading cytochromes P450 to acquire oxygen binding capacity [4–6]. Some authors believe that the function of these early cytochromes P450 was to bind oxygen in order to protect cells against uncontrolled oxidative destruction (i.e. "detoxification") [7]. Soon, however, cytochromes P450 acquired an important new function. Appearance of the first P450-containing monooxygenases involved in the synthesis and oxidative metabolism of fatty acids and steroids played an important role in the origin of eukaryotes [8]. Later, the catalysis of hydrophobic compound oxidation became the main function of cytochromes P450.

Over the course of evolution, the cytochrome P450 family has become the largest family of enzymes with a highly conserved polypeptide chain fold and a common catalytic mechanism, which is employed for implementation of various physiological functions. Nature has utilized the cytochrome P450 construct for various purposes; cytochromes P450 act as terminal oxidases in monooxygenase systems, oxidizing various exogenous and endogenous compounds. Known functions of cytochromes P450 include: synthesis of pigments, hormones, second messengers, antibiotics, and toxins, as well as oxidative conversion and detoxification of low molecular weight foreign compounds (xenobiotics).

Present-day cytochromes P450 proteins are comprised of one polypeptide chain, 400–550 amino acid residues in length. All known eukaryotic cytochromes P450, as well as most of their bacterial analogues are not self-sufficient in the context of their catalytic function. The monooxygenase reaction requires two

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electrons, which are usually transferred to cytochrome P450 from a protein partner. Most commonly, the role of such a partner is played by non-heme iron proteins-ferredoxins (in so-called type I monooxygenase system), or flavoproteins (in the type II systems) [9]. Although all cytochromes P450 share a similar polypeptide chain fold and a common catalytic mechanism, eukaryotic cytochromes P450 differ from their prokaryotic analogues in their cellular localization: prokaryotic cytochromes P450 are water-soluble, protoplasmic enzymes, while eukaryotic cytochromes P450 are predominately associated with biological membranes. Although eukaryotic cell mitochondria also possess their own, quite distinctive cytochrome P450 system, the most significant fraction of cellular cytochromes P450 is localized in the membranes of endoplasmic reticulum (ER),

The system of ER cytochromes P450 and their partners, also known as the microsomal monooxygenase system (MMO), has been the focus academician A.I. Archakov's scientific interest for more than 40 years. Studies performed under his leadership became important milestones toward understanding the mechanisms of MMO function as an integrated system. This review summarizes modern knowledge about these mechanisms.

1. MOLECULAR ORGANIZATION OF MICROSOMAL MONOOXYGENASES: HISTORICAL PERSPECTIVES AND THE CURRENT UNDERSTANDING OF THE PROBLEM

The MMO has been found in the ER of most animal tissues. Although the highest content of microsomal cytochromes P450 has been detected in liver cells, they are also present in lung, kidney, brain, vascular smooth muscle, as well as in the intestinal epithelium, nasal mucosa, mammary gland, lymphocytes, etc. The MMO catalyzes oxidation of foreign substances (drugs, carcinogens and other xenobiotics), as well as endogenous substrates (hormones, steroids, fatty acids, prostaglandins, etc.). The central role that cytochromes P450 play in the metabolism of drugs and other xenobiotics makes these enzymes a major subject for studies of drug disposition, adverse drug effects and drug-drug interactions. The MMO can metabolize a wide variety of substrates because it is comprised of multiple forms of cytochrome P450 with different substrate specificities. For instance, the human genome encodes 57 functional cytochromes P450 in addition to 58 non-functional pseudogenes of these enzymes [10].

The major electron donor for cytochromes P450 in MMO is represented by NADPH-dependent cytochrome P450 reductase (CPR), a flavoprotein. Although the P450-CPR pair is functionally competent, the complete system also contains cytochrome b_5, which increases the catalytic efficiency of the system and serves as an alternate electron donor. A very important feature of all known eukaryotic MMO systems is that they contain a significant excess of cytochrome P450 versus reductase. For example, the average molar ratio of cytochrome P450 : CPR in human liver is 7.1 : 1 (the range of this ratio varies from about 2 : 1 to 27 : 1) [11].

The excess of cytochrome P450 over its reductase, as well as the presence of the alternative electron donor, cytochrome b_5, prompted researchers to raise the question of "molecular organization" of the MMO in the early 1970s. At that time, the presence of multiple cytochrome P450 isoforms in the membrane was not yet firmly established, and the problem of "molecular organization" was considered in the context of existence of stable, non-dissociable complexes of cytochrome P450 with its electron donors [12]. According to the so-called "cluster hypothesis" the catalytic unit of the MMO is represented by a non-dissociable cluster composed of one reductase molecule interacting with several molecules of cytochrome P450 [13—16]. This hypothetical model became a subject of extensive discussion and active research in the late 1970s and early 1980s. These early ideas about the "molecular organization of microsomal monooxygenases" are exemplified in Fig. 1, which reproduces illustrations from the two most cited articles of that period devoted to this problem [12, 16].

Subsequent studies did not confirm the presence of electron carrier clusters in the ER membrane, instead they demonstrated that the interaction of cytochrome P450 with its partners to form complexes involves lateral diffusion in the membrane. The study by Archakov and his colleagues demonstrating that the kinetics of electron transfer to cytochrome P450 in the membrane follows the law of mass action provided decisive support for this concept [17].

However, the finding that the cytochrome P450 pool in the membrane is composed of multiple species that differ in their substrate specificity and physiological functions re-emphasized the importance of obtaining an in-depth understanding of molecular organization of the MMO. Competition between various cytochromes P450 for interaction with CPR, as demonstrated by many researchers [18—23], may have significant impact on the activity of each individual cytochrome P450 and therefore may constitute an important element of MMO regulatory mechanisms. However, integrative relationships in the MMO are not limited to this competition. There are a number of convincing demonstrations of interactions between multiple cytochrome P450 species, resulting in the formation of mixed oligomers (heterooligomers) in the ER membrane. The body of evidence regarding modification of the functional properties of individual P450 enzymes by these interactions continues to grow [24, 25]. There is increasing consensus that the mechanisms governing cytochromes P450 ensemble forma-
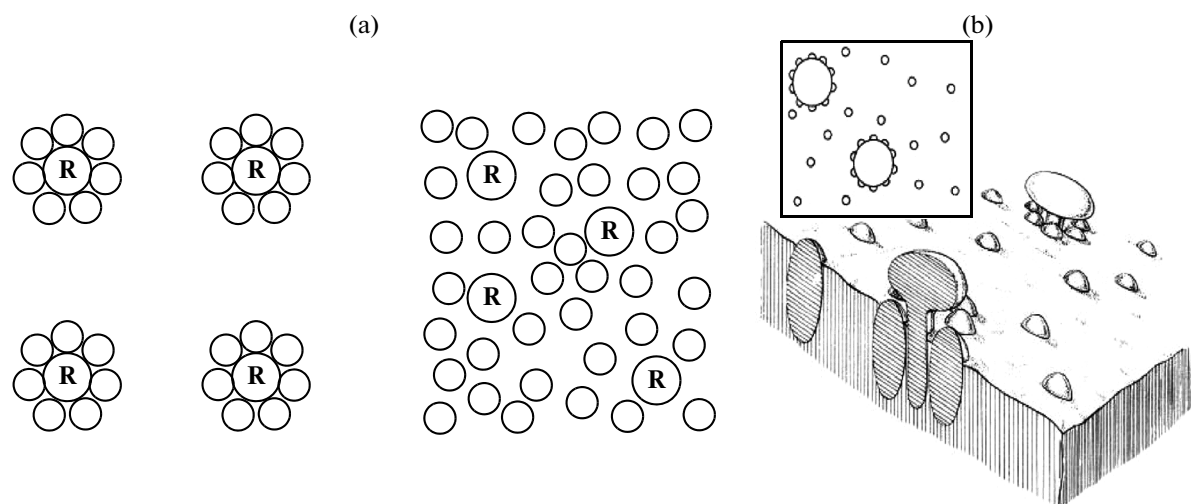
(a)   (b)

Fig. 1. Early models of the molecular organization of microsomal monooxygenase. (a) A contraposing of "rigid" or "cluster" (left) and "non-rigid" (right) models of MMO organization. The illustration was adapted from the article by Yang [12]; (b) the model of MMO organization adapted from the article by Peterson et al. [16]. This model suggested distribution of the pool of cytochrome P450 (ellipsoids embedded into the membrane) into two fractions: the fraction of clusters readily reduced by reductase (molecules anchored in the membrane) and the fraction of free hemoprotein molecules diffusing in the membrane and slowly reducible by the reductase.

tion in the microsomal membrane cannot be understood from studies of each cytochrome P450 form investigated in isolation [26]. Conversely, this understanding requires examination of the ensemble of cytochrome P450 in its entirety, taking into consideration both competition between various cytochromes P450 for interactions with their redox partners, and their interactions with each other to form heterooligomers.

Due to the complex network of intermolecular interactions, the integral properties of the P450 ensemble cannot be predicted by simple summation of the properties of each contributing P450 species. Any change in the P450 expression profile, including those occurring during development and aging, thus alters the protein-protein interaction landscape in the MMO and modifies its functional properties in a complex, hard-to-predict manner.

Thus, the term "molecular organization of microsomal monooxygenases" acquires a new connotation. Further study of the molecular organization of the MMO, an area in which academician Archakov and his colleagues G.I. Bachmanova, I.P. Kanaeva, I.I. Karuzina, A.V. Karyakin, A.A. Zhukov, Yu.D. Ivanov and others made important contributions, is essential for establishing a system-wide approach to the cytochrome P450 ensemble in the ER membrane. The development of such an approach is a prerequisite for in-depth understanding of function of the cytochromes P450 ensemble as a multienzyme system, both in drug metabolism and in signaling pathways that control cell proliferation, differentiation, apoptosis and other processes.

Current studies of the molecular organization of the MMO are focused on analyzing the mechanisms and functional consequences of interactions between cytochromes P450 (P450-P450 interactions). Below I present the major experimental findings accumulated in this area and discuss current concepts on possible physiological role of these interactions.

2. OLIGOMERIC STATE OF CYTOCHROMES P450 IN SOLUTION AND IN THE MEMBRANE

It is well known that microsomal cytochromes P450 tend to form supramolecular complexes (oligomers) in solution [27−33]. The main role in the association of cytochromes P450 in solution is attributed to hydrophobic interactions between their N-terminal fragments [34−38]. Although the removal of these transmembrane fragments reduces the tendency of microsomal cytochromes P450 to aggregate in solution, in most cases this truncation is insufficient to convert microsomal cytochromes P450 into soluble monomeric proteins [34−42].

Oligomerization of cytochromes P450 takes place not only in solutions of purified proteins, but in model membranes, microsomes and living cells, as demonstrated by various methods ranging from measuring the rate of rotational diffusion [43−50], cross-linking with bifunctional reagents [44, 51−53], and freeze-fracture electron microscopy [50], to approaches using fluorescence resonance energy transfer (FRET) [54, 55], bimolecular fluorescence complementation (BiFC) analysis [56] and bioluminescence resonance energy transfer (BRET) [57].

Besides abundant data concerning oligomerization of a range of different cytochrome P450 species in membranes [46, 50, 52, 55, 57−62], there is also an important body of evidence regarding intermolecular interactions between different cytochrome P450 species. Formation of their heterooligomers has been demonstrated in proteoliposomal membranes [53] and microsomes [51], as well as in living cells [56].

There are numerous indications of striking functional consequences of interactions between multiple P450 species (detailed discussion of the relevant data can be found in reviews [24, 25]). Most frequently, these interactions cause activation of one of two interacting enzymes, whereas activity of the partner remains either unchanged or inhibited. This type of interaction has been demonstrated for pairs such as CYP3A4/CYP1A2 [63], CYP2C19/CYP2C9 [64], CYP2D6/CYP2C9 [65], and CYP3A4/CYP2C9 [66].

The most studied combination is the pair of rabbit cytochromes P450 1A2 (CYP1A2) and 2B4 (CYP2B4) [20, 23, 53, 67−71]. The presence of CYP2B4 in mixed systems with CYP1A2 results in activation of CYP1A2 [23, 67], while the activity of CYP2B4 is inhibited [20]. A similar relationship has also been demonstrated in another rabbit pair, CYP1A2 and CYP2E1 [71].

Based on these data, Backes et al. concluded that the effects of CYP2B4 and CYP2E1 on CYP1A2 activity are associated with the formation of heterooligomers, in which the interactions between CPR and CYP1A2 are promoted, while the association of the reductase with paired heme proteins (CYP2B4 and CYP2E1) is hampered [53, 67, 72].

This conclusion is also supported by our studies of CPR interactions with CYP1A2/ CYP2B4 heterooligomers in the soluble reconstituted system developed under academician Archakov's leadership [73]. Using this system we studied the formation of the complex of P450 with CPR, employing fluorescence resonance energy transfer (FRET) from a fluorescent label covalently bound to CPR to the P450 heme [23]. We showed that the dissociation constants (K_D) of CYP1A2 and CYP2B4 forming complexes with CPR were essentially indistinguishable while the titration of the reductase with mixtures of these two proteins in the presence of 7-ethoxyresorufin (7-ER), a substrate of CYP1A2, revealed a multifold decrease in effective affinity for the reductase in the mixtures with a high relative content of CYP2B4 [23]. These results suggest that CYP2B4 association with CYP1A2 in the presence of 7-ER "hides" CYP2B4 from its interactions with CPR [23].

A pronounced effect of P450-P450 interactions has been demonstrated in studies of pressure-induced transitions in CYP1A2/CYP2B4 heterooligomers [69]. When studied separately, these two enzymes revealed quite distinct responses to increasing hydrostatic pressure; while the complex of CYP2B4(Fe^{2+})

with carbon monoxide underwent transition to the inactive P420 state at relatively low pressures, the carbonyl complex of CYP1A2(Fe^{2+}) was essentially resistant to such inactivation. However, CYP2B4 became protected against pressure-dependent inactivation after co-incubation with CYP1A2; moreover, it exhibited a significant decrease in the compressibility of the heme pocket [69].

Although the data considered above reveal a significant impact of P450-P450 interactions on the characteristic features of interacting enzymes, most of these studies were performed in non-membranous systems in vitro. This fact complicates assessment of the physiological significance of the observed effects and evokes a need for new approaches to systematic study of P450-P450 interactions and their consequences in membranous systems where the composition of the monooxygenase system and the properties of its membrane environment are maximally close to those observed in the ER under physiological conditions.

3. STUDIES OF P450-P450 INTERACTIONS IN MICROSOMAL MEMBRANES

3.1. A New System for Studies of Microsomal P450-P450 Interactions

Development of an appropriate model system suitable for the studies of protein-protein interactions in ER membranes is a rather complex task. Such a system should mimic a native membrane environment where the proteins interact by lateral diffusion and undergo reversible complex formation. It should also provide a means to change the composition of a multienzyme ensemble, as well as its concentration in the membrane. In a search for such a model we have developed an approach based on the incorporation of purified enzymes into microsomes of insect cells containing recombinant CPR, but lacking cytochromes P450 (Fig. 2a). Such microsomes are commercially available from Corning Life Sciences (USA) as Control Supersomes™. Incubation of such preparations, which in the context of this review are designated as SS(R), with purified human CYP3A4, CYP3A5 or CYP2E1 results in effective incorporation of these proteins into the membrane. This approach yields catalytically competent microsomes with the desired concentration and composition of the P450 ensemble [62, 74].

3.2. New Methods of Registration of P450-P450 Interactions

In our studies of P450-P450 interactions we employ methods based on fluorescence or luminescence resonance energy transfer and (FRET and LRET, respectively) between fluorophores covalently attached to cysteine residues of interacting proteins.
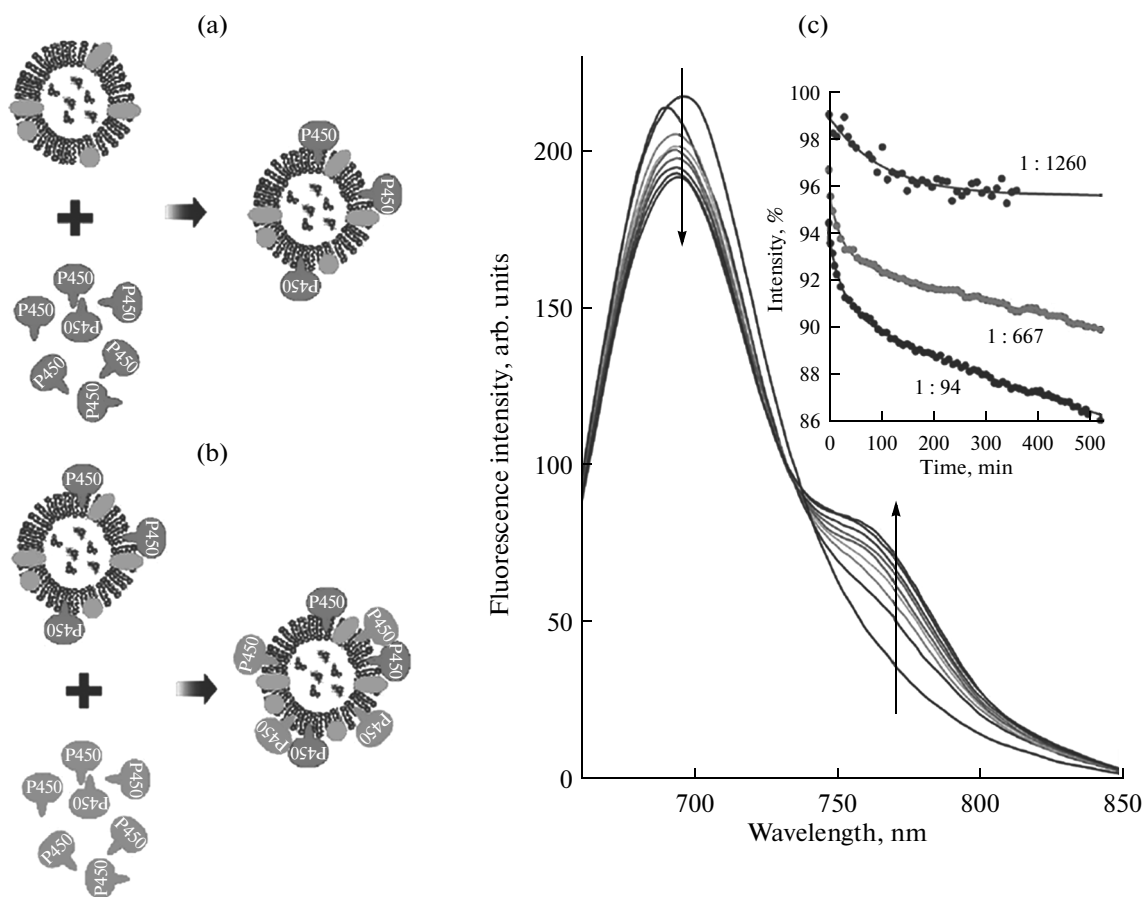
(a)

(b)

(c)

Fig. 2. Studies of P450-P450 interactions in the membrane of model microsomes. (a) The figure illustrates the process of reconstitution of catalytically competent system by inserting purified recombinant cytochrome P450 into the microsomal membranes containing recombinant NADPH-cytochrome P450 reductase; (b) the scheme of experimental determination of the degree of P450 oligomerization in the microsomal membrane by means of LRET, which monitrs the formation of heterooligomers upon incorporation of cytochrome P450 molecules containing the acceptor probe (DYM) into the microsomal membrane with incorporated protein that carries the donor fluorophore (ERIA); (c) changes in the spectrum of delayed fluorescence observed upon incorporation of CYP3A4(C166)ERIA into microsomes containing CYP3A4(C468)DYM (modified from [74]). Direction of changes is shown with gray arrows. The insert shows kinetic curves of changes in the donor emission intensity registered at different P450 : lipid (P/L) molar ratios.

We have recently developed a new sensitive and specific method for registration of P450-P450 interactions applicable either to homooligomers of one P450 or to heterooligomers formed by two different cytochrome P450 species [62, 74]. It is based on LRET, where a fluorophore with a long lifetime of the excited state is used as a donor. The thiol-reactive phosphorescent dye erythrosine iodoacetamide (ERIA) was chosen as a donor, while fluorescent DY-731 maleimide (DYM), which emits in the far-red region, serves as an acceptor. The Förster distance (R_0) for this pair is about 34 Å, providing high efficiency of LRET between samples located in adjacent subunits of the oligomer. Positioning of the excitation and emission bands of the probes in the far red region ensures lack of their overlap with the absorption bands of the heme, resulting in a highly reliable and selective method.

The use of this new donor-acceptor pair makes it possible to monitor the process of P450 oligomerization by appearance of LRET caused by the formation of heterooligomers observed after addition of the acceptor-labeled protein to the SS(R) microsomes containing a protein labeled with donor (Fig. 2b) [62]. The amplitude of LRET observed in these experiments is proportional to the concentration of heterooligomers. According to the law of mass action this amplitude increases at higher surface concentrations of proteins (i.e., at low molar ratios of lipid : P450) (see insert at Fig. 2c). In a series of experiments on incorporation of labeled proteins at different lipid : P450 ratios it is possible to characterize the dependence of oligomerization on the surface concentration of P450 and to determine the dissociation constant of oligo-

mers, as well as LRET efficiency in the formed complex.

3.3. Oligomerization of Cytochromes CYP3A4, CYP3A5, and CYP2E1 in Model Microsomes and Its Functional Consequences

The use of the above described approaches in studies of human cytochromes P450 CYP3A4, CYP3A5, and CYP2E1 incorporated into microsomal membrane allowed us to demonstrate that these proteins have a high propensity to oligomerize in microsomal membranes [62, 74]. Remarkably, our studies demonstrated a high degree of oligomerization of all three P450 species studied at concentrations commensurate with those observed in the ER of hepatocytes. According to our measurements, the concentrations (surface densities) at which 50% of cytochrome P450 molecules are oligomerized, ranged from 0.16 (for CYP2E1) to 0.47 pmol/cm^2 (for CYP3A4). Corresponding values for the probed combinations of two dissimilar cytochrome P450 species varied from 0.07 to 0.30 pmol/cm^2. For comparison, measurements performed by Watanabe et al. suggest that the surface density of cytochromes P450 in hepatocyte ER varies from 0.6 to 2.8 pmol/cm^2 [75]. Thus, we can conclude that at the physiological concentrations of CYP3A4, CYP3A5, and CYP2E1 in the ER membrane all three heme proteins are strongly oligomerized and, for ,part, involved in the formation of heterooligomers.

We supplemented the above results on the interactions between CYP3A4, CYP3A5 and CYP2E1 by a study of their functional effects. Our results demonstrate that the oligomeric organization of cytochrome CYP3A4 is a central element in the mechanism of its activation by such ligands as α-naphthoflavone (ANF) or steroids. For example, we demonstrated that the stimulation of CYP3A4 by its prototypical activator ANF is observed only in the oligomers of this enzyme and disappears upon their dissociation [62].

The study of the effects of P450-P450 interactions in the pairs CYP3A4/CYP3A5, CYP3A4/CYP2E1, and CYP3A5/CYP2E1 on the catalytic properties of each of the interacting enzymes has shown that the interaction of CYP3A4 with CYP2E1 or CYP3A5 was accompanied by a greater than 2-fold increase in the k_{cat} value for O-demethylation of 7-methoxy-4-(trifluoromethyl) coumarin (7-MFC), a specific substrate of CYP2E1, while CYP2E1 had insignificant effect on CYP3A activity assayed with its substrate, 7-benzyloxyquinoline (7-BQ) [74].

Studying 7-BQ O-debenzylation catalyzed by cytochromes CYP3A, we found that the k_{cat} value of the reaction catalyzed by CYP3A5 was approximately 2-fold higher than that observed with CYP3A4. At the same time, addition of ANF significantly (about 1.7 times) increased the rate of reaction catalyzed by CYP3A4, but had no effect on the activity of CYP3A5.

Interestingly, co-incorporation of both enzymes into the membrane caused further increase in the rate of catalysis and completely eliminated the activating effect of ANF [74]. Taken together, these data convincingly demonstrate that the formation of heterooligomers of dissimilar P450 species causes significant modulation in the catalytic properties of the microsomal ensemble of drug metabolizing enzymes.

4. STUDIES OF ARCHITECTURE OF CYP3A4 OLIGOMERS

Recently, we have introduced a hypothesis concerning the relationship between CYP3A4 oligomerization and its susceptibility to activation by allosteric ligands (such as ANF or testosterone). This hypothesis was based on the observation of a peripheral ligand binding site at the interface between subunits in the X-ray structure of the crystallographic dimer of the CYP3A4 complex with progesterone (PDB 1W0F) [76]. We suggested that the intersubunit interface of CYP3A4 oligomers in the membrane is similar to that observed in the X-ray structure (Fig. 3a). In this case the position of bound progesterone molecules in 1W0F may point to the location of the allosteric ligand binding site, which is expected to exist only in the dimeric enzyme and disappear upon its dissociation [62]. To probe this hypothesis, we combined LRET measurements of the distances between the site-specific labels with cross-linking of CYP3A4 oligomers by bifunctional reagents.

LRET-based studies of CYP3A4 oligomerization were performed using single cysteine mutants for site-specific introduction of fluorescent labels. In the initial experiments, we used the mutants CYP3A4 (C64) and CYP3A4 (C468). The LRET efficiency in the complex of CYP3A4(C64)ERIA with CYP3A4 (C468) DYM was equal to $10 \pm 1\%$, suggesting the distance between labels of about 49 Å. Considering the large size of the ERIA and DYM molecules (the distances between the cysteine sulfur atom to the most distant atom of the labels are 15 and 18 Å, respectively) this distance is comparable to the distance between the sulfur atoms in the residues C468 and C64 in the neighboring subunits of the 1W0F dimer (67.7 Å). Later we created a single cysteine construct CYP3A4 (C166), in which the mutation T166C was used to place the only modification-accessible cysteine residue at position 166. According to the distance between the T166 β-carbon atom and C468 sulfur atom of the dimeric structure (46 Å), this relocation of the label should be accompanied by a significant increase in LRET efficiency. In good agreement with these expectations, the use of CYP3A4(C166)DYM in the pair with CYP3A4(C448)ERIA resulted in an increase in LRET efficiency of up to $27.5 \pm 1.7\%$. This value corresponds to the distance between the labels of about 40 Å [74]. This result indicates that subunit orienta-
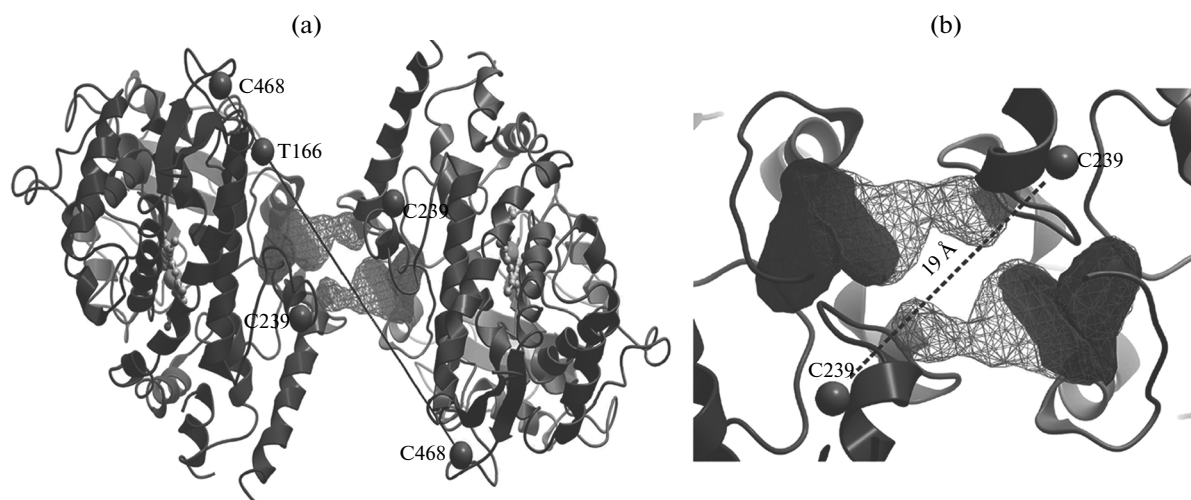
(a)   (b)

Fig. 3. Geometry of the CYP3A4 dimer in the structure of the CYP3A4 complex with progesterone (PDB 1W0F). (a) General view in the direction perpendicular to the plane of the membrane; (b) zoom-in view of the region of the intermolecular interface and the ligand-binding site. The ligand binding region shown as a grid surface was determined using the ICM PocketFinder program [104]. The amino acid residues T166, C239 and C468 are shown as spheres.

tion in the oligomer CYP3A4 is consistent with that observed in 1W0F.

Analyzing the crystallographic structure of the dimer, we found that the interacting surfaces of two CYP3A4 molecules include cysteine residues C239, which are separated by a distance of 19.7 Å (Fig. 3b). This relatively short distance allowed for the introduction of a cross-link between the C239 residues using thiol-reactive bifunctional reagents of an appropriate length. Taking advantage of this feature of CYP3A4 for mapping the site of inter-subunit interactions, we were able to eliminate residue C239 by the C239S mutation and compare the resulting mutant with wild-type CYP3A4 in cross-linking experiments. In these experiments we used dibromobimane (bBBr, optimal distance for cross-linking is 5 Å), *o*-dimaleimidyl benzene (o-DMB, 9.6 Å) and bis(maleimidophenyl) methane (bMPM, 15 Å) as bifunctional thiol-reactive reagents. SDS-PAGE electrophoresis showed that the cross-linking of the wild type protein oligomers in solution by any of these three reagents resulted in the appearance of two bands of cross-linked aggregates with molecular weights of about 120 and 190 kDa, that corresponds to the dimer and trimer, respectively. The efficiency of cross-linking increased as the optimal distance of cross-linking typical for each of the reagents increased. It reached its maximum in the case of bMPM (permissible range of cross-linking distances of 9.4–17.3 Å [77]). The most important observation was that the C239S mutation eliminated the band at 190 kDa, while the band at 120 kDa became more intense [74]. Similar results were obtained in the case of cross-linking of CYP3A4 and CYP3A4(C239) inserted in the proteoliposomal membranes [74].

According to these results, the most probable size of the minimal organization of the CYP3A4 oligomer is a trimer, in which the interactions between subunits involve two different interfaces. One of these interfaces is identical to that observed in the structure of 1W0F and includes two C239 residues, located in close proximity to each other. Thus, the peripheral ligand binding site detected in the 1W0F structure is located in the region of helices F' and G' of two interacting molecules of CYP3A4 and is probably physiologically relevant as it represents an allosteric ligand binding site specific for oligomers. This site may be involved in modulation of MMO by physiological ligands (e.g. steroids or their derivatives).

5. RELATIONSHIP BETWEEN OLIGOMERIZATION AND "PERSISTENT HETEROGENEITY" OF CYTOCHROMES P450

Important conclusions regarding the mechanisms of the functional effects of P450-P450 interactions may be drawn from considering the above discussed results together with numerous indications of a "persistent conformational heterogeneity" of cytochromes P450 in the membrane. We use this term to refer to an unusual "non-equilibrating" distribution of the population of membrane-bound cytochrome P450 into fractions that differ in their functional properties. Such persistent heterogeneity has been observed both in solution and in membranes by various methods. These data are reviewed in [78].

One of the early indications of a stable, persistent distribution of oligomeric cytochromes P450 into two non-interconverting fractions with differing properties was obtained from our study of pressure-induced tran-

sitions in cytochromes P450. We have found that only 65–70% CYP2B4 in oligomers in solution are susceptible to inactivation of the heme protein by its conversion into the P420 state, which is induced by high pressures (>2 kbar) [79–81]. The fact that this heterogeneity is eliminated by protein monomerization [79, 80, 82] suggests that it is determined by molecular organization of the protein oligomers, where subunits forming the oligomer may differ in their conformation, orientation and conformational mobility. This behavior has also been demonstrated in human cytochrome CYP3A4 both in solution and in recombinant microsomes [83]. Heterogeneity in protein response to application of high hydrostatic pressure was also observed in CYP2E1 [84] and mitochondrial P450scc [85].

Heterogeneity associated with oligomerization was also demonstrated in our study of the kinetics of dithionite-dependent reduction of CYP3A4. In 3A4 oligomers (either in solution or in proteoliposomes) the reduction process followed triexponential kinetics, while monomerization of CYP3A4 by incorporating it into nanodiscs or liposomes with a high lipid-to-protein molar ratio ($R_{L/P}$) altered the kinetics making it monoexponential [32]. The most remarkable observation was that the fraction of oligomeric P450 reducible in the rapid phase was almost completely represented by the low-spin state of the enzyme, whereas the fraction reducible in the slow phase was mostly represented by the high-spin state [32].

Analogous differences in the kinetic properties between the high- and low-spin states of P450 were also found in studies of NADPH-dependent reduction. However, in contrast to the case of reduction by dithionite, the predominant fraction of the heme protein reduced in the fast phase of the NADPH-dependent process was represented by the high-spin (rather than the low-spin) state. Selective reduction of the high-spin state of P450 in the fast phase of the NADPH-dependent process was demonstrated in experiments with CYP2C11 [86], CYP2B4 [87], and eventually with CYP3A4 [61, 88].

The contrast between the high- and low-spin states in the kinetics of their reduction indubitably contradicts the high rate of their interconversion [89–92]. Extremely rapid transitions between the two states imply that the position of equilibrium between them should remain unaffected over the course of reduction. This contradiction suggests that the distribution of cytochrome P450 between the high- and low-spin states can not be described as a simple equilibrating transition in which the whole pool of the heme protein molecules is involved. It appears that the enzyme is instead distributed between separate non-interconverting fractions that differ from one another in their: position of spin equilibrium, affinity for substrates, and ability to form complexes with their electron transfer partners.

Such a non-equilibrating distribution has been convincingly demonstrated in our experiments where we used the soluble, flavin-containing domain of cytochrome-P450-BM3 (BMR) as a surrogate replacement for the membrane-bound reductase. In these studies, we demonstrated that only about 50% of oligomeric CYP3A4 can be reduced by BMR, either in solution or in the membrane. Akin to the case of dithionite-dependent reduction, monomerization of CYP3A4 eliminated such heterogeneity and made the whole hemoprotein pool reducible in the NADPH-dependent process [61, 88].

6. HYPOTHETICAL MODEL OF MOLECULAR ORGANIZATION OF THE MICROSOMAL CYTOCHROME P450 ENSEMBLE

The results pertaining to cytochrome P450 interactions in the model microsomal system discussed above provided a plausible explanation for "stable heterogeneity" and its contribution to the functional effects of hetero-association of multiple P450 species. According to the results of our cross-linking experiments, CYP3A4 oligomers in the membrane are apparently organized as trimers or multiples of trimers. The interactions between the subunits in the basic trimeric block involve two different types of intersubunit interfaces. According to our hypothesis, such architecture leads to orientation and/or conformational differences between the subunits in the trimer, which is apparently organized as a dimer with an associated third subunit. Consequently, the formation of heterooligomers of multiple P450 species of P450 can result in selective activation of a particular P450 enzyme and/or inhibition of its interacting partner (Fig. 4).

According to this hypothesis, two types of subunits in the P450 oligomer differ from each other both in their competence to form functional electron transfer complexes with CPR, and in their ability to interact with substrates. Because of these differences, the presence of a substrate specific for one of the interacting enzymes should cause redistribution of cytochrome P450 species between the active and "hindered" positions in the oligomers, so that the P450 possessing its specific substrate becomes activated (Fig. 4). A physiologically significant role of this regulatory mechanism may be in the maintenance of a functionally-acceptable balance between P450 substrate oxidation and generation of reactive oxygen species (ROS) due to uncoupled NADPH oxidation. Such a mechanism would ensure rapid adaptation of the microsomal oxidative system to any changes in cell exposure to various xenobiotics.

An important feature of the proposed model is that the degree of possible activation via this mechanism is determined by the affinity of each of the interacting P450 subunits for each individual substrate. The most pronounced effect should be observed with substrates
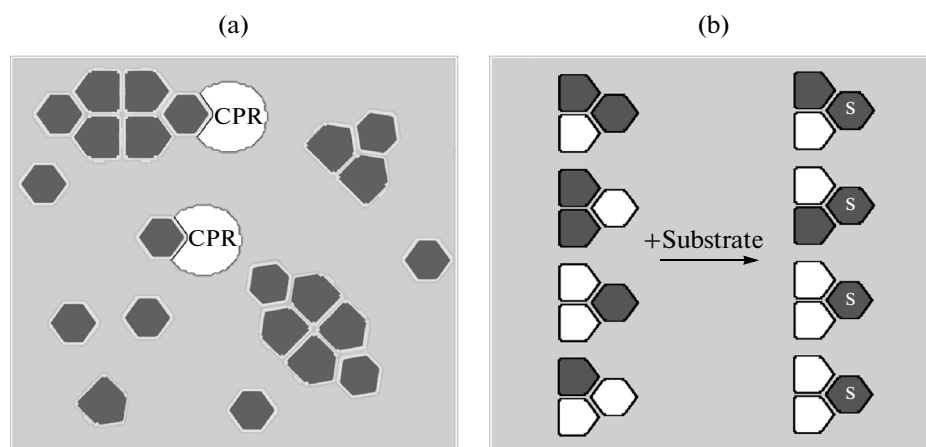
(a)   (b)

Fig. 4. Illustration of the hypothesis on the mechanisms of functional effects of cytochrome P450 oligomerization in the membrane [74]. (a) The pool of cytochrome P450 (dark gray polygons) is in equilibrium between monomers and oligomers. The oligomer subunits exist in two different conformations (hexagons and pentagons), depending on their position in the trimer, which is the minimal unit of the oligomeric structure. One of the two conformations (hexagons) preferentially forms electron-transfer complexes with reductase (white ovals) and interacts with P450 substrates. Thus, the "pentagonal" conformation of the enzyme is completely or partially excluded from the processes of electron transfer and catalysis. (b) Illustration of the hypothesis of the substrate induced rearrangement of heterooligomers formed by two cytochromes P450 (white and gray polygons). In the absence of substrate (left side), the distribution between the two interacting enzyme conformations (hexagons and pentagons) is random. Addition of a substrate specific for one of the two interacting proteins, causes redistribution in the oligomer subunits, so that all positions opened for interaction with the substrate (hexagons) are occupied by the protein for which the system has a substrate (gray polygons).

specific to just one of the interacting species. Such a situation occurred with the CYP2E1/CYP3A pair interacting with the substrate 7-MFC. The proposed model of substrate-dependent rearrangements of oligomers (Fig. 4) well explains the activating effect of CYP2E1 interactions with CYP3A4 and CYP3A5, as CYP2E1 affinity to 7-MFC is much higher than that of CYP3A4 and CYP3A5. In contrast, the effects of the interaction of two closely related P450 species, such as CYP3A4 and CYP3A5, are likely negligible due to their similar substrate specificity.

Additional complexity to the proposed mechanism may be added by differences between the interacting proteins in their preferential occupation of the active or "hindered" positions in the oligomer, which can take place even in the absence of substrates. In addition, oligomerization of P450 isoforms may result in formation of effector binding sites at the subunit interface, a phenomena that apparently occurs in the case of CYP3A4 [62, 74, 76, 93]. Such sites may be involved in allosteric regulation and coordination of the catalytic properties of the cytochrome P450 ensemble as an integrated system. Although this hypothetical model requires further in-depth investigation, it provides a plausible explanation for the effects of all known functional P450-P450 interactions.

7. THE POSSIBLE PHYSIOLOGICAL ROLE OF P450-P450 INTERACTIONS AND PROSPECTS FOR THEIR FURTHER STUDIES

The central role that cytochromes P450 play in metabolism of drugs and other xenobiotics attracts much attention to these enzymes as the main focus of studies concerning the mechanisms of drug disposition, adverse drug effects, and drug-drug interactions. However, the function of microsomal cytochromes P450 in the cell may span far beyond their role in the metabolism of xenobiotics and oxidation of endogenous substrates. The extraordinary complexities, contradictory functional properties, and perplexing regulatory connections of the P450 ensemble suggest that these enzymes may play an important, yet poorly understood role in cellular signaling pathways. A remarkable feature of eukaryotic cytochromes P450, which differentiates them from their bacterial analogues, is that these enzymes are rather catalytically inefficient (their catalytic turnover number is usually below 10 min^{-1}), and energetically wasteful (the degree of coupling of NADPH consumption to substrate oxidation is typically below 50%) [94–97]. These apparent "imperfections" of the MMO represent a potential hazard to the cell, as they lead to the continuous production of reactive oxygen species (ROS) [98]. Even in the absence of substrates these enzymes continue to produce ROS [94–97]. This feature of the P450 ensemble suggests that it might be involved in cellular signaling as a regulated source of

ROS [23, 99−102], particularly in the cascade of cytochrome *c*-dependent initiation of apoptosis [103].

However, the above proposed regulatory function of the P450 ensemble requires the processes of ROS generation, which are so distinctly manifest in vitro, to be under a strict regulatory control in the living cell. We believe that the central regulatory mechanism coordinating the function of microsomal cytochromes P450 is found in the tightly controlled interactions between multiple P450 isoforms co-localized to the ER membrane. According to the hypothesis introduced above (Fig. 4), these interactions block a certain fraction of the pool of cytochromes P450 from their association with the electron-donating partners thus minimizing nonproductive consumption of NADPH and P450-dependent ROS generation. The proposed hypothetical mechanism of regulation provides for selective activation of certain cytochrome P450 species, either in response to appearance of their specific substrates or due to interactions with certain allosteric effectors that regulate production of ROS by the P450 ensemble. According to this hypothesis, the interaction of allosteric effectors with any particular P450 species is capable of modulating the catalytic properties of the whole ensemble.

The main physiological role of this mechanism is maintenance of the balance between P450-dependent substrate oxidation and P450-dependent ROS generation, which can play an important role in cellular signaling. Furthermore, according to the proposed model of the functioning of the P450 machinery (see Fig. 4), this mechanism also ensures rapid adaptation of the cell to any changes in its exposure to a dynamic spectrum of xenobiotics.

CONCLUSIONS

Data considered in this review demonstrate the importance of physical interactions between multiple cytochrome P450 species as one of the most critical factors determining the functional properties of the drug-metabolizing system in the human body. According to modern concepts, any changes in the composition of the P450 ensemble should result in significant alterations in the regulatory properties of the MMO as an integral entity. In light of recent findings, the problem of "molecular organization of microsomal monooxygenases," in which the studies of academician Archakov and his colleagues made such an important contribution, acquires a new connotation, and re-establishes itself as a central problem in studies concerning the role of cytochromes P450 in the living cell. Further detailed investigations of the molecular mechanisms and functional consequences of P450-P450 interactions as well as identification of the signaling pathways that control them will have undisputable significance for modern science. These studies are crucial for developing new approaches for individualized prediction of drug metabolism, and in-depth understanding of changes in xenobiotic oxidation resulting from alterations in cytochrome P450 expression profiles under the influence of environmental factors and various pathological processes, as well as in the course of development and aging.

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