Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 312 (2003) 121–130
BBRC
www.elsevier.com/locate/ybbrc

Conformational heterogeneity of cytochrome P450 3A4 revealed by high pressure spectroscopy☆

Dmitri R. Davydov,[a,*] James R. Halpert,[a] Jean-Paul Renaud,[b] and Gaston Hui Bon Hoa[c]

[a] *Department of Pharmacology and Toxicology, The University of Texas Medical Branch, Galveston, TX, USA*
[b] *CNRS UMR 7104, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France*
[c] *INSERM U473, Le Kremlin Bicêtre, France*

Received 25 September 2003

Abstract

We applied hydrostatic pressure perturbation to study substrate-induced transitions in human cytochrome P450 3A4 (CYP3A4) with bromocriptine (BCT) as a substrate. The barotropic behavior of the purified enzyme in solution was compared with that observed in recombinant microsomes of *Saccharomyces cerevisiae* coexpressing CYP3A4, cytochrome b_5, (b_5) and NADPH-cytochrome P450 reductase (CPR). Important barotropic heterogeneity of CYP3A4 was detected in both cases. Only about 70% of CYP3A4 in solution and about 50% of the microsomal enzyme were susceptible to a pressure-induced P450 → P420 transition. The results suggest that both in solution and in the membrane CYP3A4 is represented by two conformers with different positions of spin equilibrium and different barotropic properties. No interconversion between these conformers was observed within the time frame of the experiment. Importantly, a pressure-induced spin shift, which is characteristic of all cytochromes P450 studied to date, was detected in CYP3A4 in solution only; the P450 → P420 transition was the sole pressure-induced process detected in microsomes. This fact suggests unusual stabilization of the high-spin state of CYP3A4, which is assumed to reflect decreased water accessibility of the heme moiety due to specific interactions of the hemoprotein with the protein partners (b_5 and CPR) and/or membrane lipids.

Keywords: Cytochrome P450 3A4; Bromocriptine; Substrate binding; Conformers; Spin equilibrium; Cytochrome P420; Protein hydration; Hydrostatic pressure; Microsomes

Perturbation of chemical equilibria by hydrostatic pressure provides a powerful technique for the study of biochemical transitions accompanied by changes in hydration of biological macromolecules, such as protein–protein, protein–DNA, and protein–ligand interactions [1–3]. This approach is especially informative with heme-containing proteins, where due to the presence of the heme chromophore, pressure-induced transitions can be easily detected through various spectroscopic techniques. Some of the best examples of successful application of hydrostatic-pressure-based methods to mechanistic and structural exploration of hemoproteins involve cytochromes P450 [4,5]. These techniques are of special value for these studies due to the very important role played by protein-bound water in substrate binding and catalytic mechanisms in these heme-thiolate enzymes [6–9]. In the earliest studies, high-pressure-induced transitions were used to study the conformational dynamics of bacterial cytochromes P450cam and P450lin [10–16]. High-pressure spectroscopy has also been applied to mammalian microsomal cytochrome P450 2B4 (CYP2B4) [17–19], P450 1A2 [19,20], P450scc [8], P450 3A4 [21], the heme domain of bacterial P450BM3 [21,22], and cytochrome P450 119 from a thermophilic archaea [23,24]. Application of elevated pressure to ferric cytochromes P450 induces a displacement of the spin equilibrium towards the low-spin state [7–10,18,22]. A second barotropic process has been identified as the conversion of the P450 into the inactive state known as cytochrome P420 [13,16,17].

☆ This research was supported by NIH Grant GM54995 (to J.R.H.), INSERM collaborative Grant 94EO03 (to D.R.D. and G.H.B.H.), and Center Grant ES06676.
* Corresponding author. Fax: 1-409-772-9642.
E-mail address: d.davydov@UTMB.edu (D.R. Davydov).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.09.247

In the case of P450cam, this P450 → P420 transition starts at rather high pressures, when the spin shift is nearly completed [13]. For other cytochromes P450 these two pressure-induced processes take place in overlapping pressure ranges [14,18,22]. When applied to the ferrous carbonyl complexes, high hydrostatic pressure induces only the P450 → P420 transition [13,14,17].

A remarkable observation made in our studies of barotropic behavior of CYP2B4 was its apparent conformational inhomogeneity. We demonstrated that only about 65–70% of the ferrous–carbonyl complex of CYP2B4 is susceptible to a pressure-induced P450 → P420 transition [17], while the rest of the protein appears to be excluded from this inactivation. We attributed this observation to the formation of P450 oligomers in solution, as the dissociation of the oligomers by detergents renders all the P450 sensitive to conversion to P420 [17]. The same non-uniform barotropic behavior was also observed for the oligomers of ferric CYP2B4, where only about 30–35% of the hemoprotein participates in substrate binding and related spin transitions, being at the same time insensitive to the pressure-induced inactivation. The remaining 65–70% appears to be "frozen" in the low-spin state and is apparently unable to bind substrates [18]. An analogous non-uniformity of the P450 pool was also demonstrated in proteoliposomal membranes [25]. This observation led us to hypothesize that there may be a heretofore unrecognized organization in the CYP2B4 oligomers both in solution and in the membranes, where subunits may differ in their spin equilibrium, ability to interact with substrates, and sensitivity to pressure-induced inactivation [18,25]. This heterogeneity was hypothesized to play a role in the regulation of microsomal monooxygenase activity [18,25].

In the current study we used high-pressure spectroscopy to probe conformational heterogeneity in cytochrome P450 3A4 (CYP3A4), the most important human drug-metabolizing P450 enzyme [26]. This enzyme provides the most noteworthy example of P450 cooperativity, as reflected in its activity and substrate interactions [27–30]. The mechanisms of P450 cooperativity are usually explained by the presence of multiple (two–three) substrate-binding sites within the same substrate-binding pocket of the enzyme [27,29,30]. However, there is no apparent reason to exclude the possibility that interactions between subunits in P450 oligomers, which are known to exist in the microsomal membrane [31–36], also play a role in the allosteric phenomena in CYP3A4. In this interpretation conformational inhomogeneity of the enzyme caused by subunit interactions in the oligomer leads to two or more kinetically distinguishable pools of enzyme. The existence of multiple functionally distinct conformers of CYP3A4 in the microsomal membrane was previously inferred from studies of CO-binding kinetics in CYP3A4 expressed in *sf9* cells [37]. In this paper we apply high-pressure spectroscopy to probe and further characterize conformational inhomogeneity of recombinant human CYP3A4 in solution and in microsomes of *Saccharomyces cerevisiae* coexpressing CYP3A4, cytochrome b_5 (b_5), and NADPH-cytochrome P450 reductase (CPR) (NF25-V8-W(R) microsomes [38]) with bromocriptine as a substrate. We chose this ergopeptide alkaloid for this study because it is known to have very high affinity for CYP3A4 and is a potent modulator of the spin equilibrium [38,39]. The fact that CYP3A4 reveals no cooperativity with this substrate simplifies data analysis and interpretation. We therefore consider this study with bromocriptine as a necessary prerequisite for subsequent investigation of the role of CYP3A4 conformers in the mechanisms of cooperativity of this enzyme.

Materials and methods

Materials. Bromocriptine mesylate is the product of Sigma Chemicals (USA). Emulgen-913 was obtained from Kao Atlas Co. (Japan). All other chemicals were of ACS grade and were used without further purification.

Expression and purification of CYP3A4. Wild-type CYP3A4 was expressed as a His-tagged protein in *Escherichia coli* TOPP3 cells and purified using Talon metal affinity resin (BD Biosciences Clontech) as described earlier [40,41].

*Yeast transformation and microsome preparation were done as previously described [*38,42*].* In this study we used microsomes containing CYP3A4, reductase, and human liver cytochrome b_5 (b_5) in the W(R) strain, which overexpresses endogenous yeast NADPH-P450 reductase [42]. The CYP3A4:b_5 molar ratio in these microsomal preparations termed NF25-V8-E(R) was about 3:1.

Experimental. High-pressure spectroscopic studies and titration experiments on purified recombinant CYP3A4 were performed using a rapid-scanning multi-channel MC2000-2 spectrophotometer (Ocean Optics, Dunedin, FL, USA) equipped with an L7893 UV–Vis fiber optics light source (Hamamatsu Photonics K.K., Japan). In the high-pressure experiments the instrument was connected by a flexible optic cable to the high-pressure cell [11] connected to a manual pressure generator capable of generating a pressure of 6000 bar. In the experiments with CYP3A4-containing microsomes the spectra were measured on a Cary-3 spectrophotometer (Varian, USA) with a high-pressure cell directly placed into the sample compartment, as described [11]. All experiments were carried out at 25 °C in 100 mM Na–Hepes buffer, pH 7.4, containing 1 mM dithiothreitol and 1 mM EDTA.

Data processing. To interpret the experiments in terms of pressure-induced changes in concentration of P450 and P420 we used principal component analysis (PCA) as described earlier [18,39]. This approach allowed us to significantly increase the signal-to-noise ratio and to remove the spectral perturbations due to the changes in turbidity of the system during the experiments [39]. To interpret the pressure-induced transitions in terms of changes in concentration of P450 species, we used a least-squares fitting of the spectra of principal components to the set of spectral standards of pure P450 and P420 species of the hemoprotein [18,39]. Prior to the analysis all spectra were corrected for the compression of the solvent, as described [17].

Analysis of pressure-induced transitions. Our interpretation of the pressure-induced changes is based on the equation for the pressure dependence of the equilibrium constant [43]

$$K_{eq}(P) = K^0_{eq} \cdot e^{-P\Delta V^0/RT} = e^{(P_{1/2}-P)\Delta V^0/RT}. \quad (1)$$

Here $K_{eq}(P)$ and K^0_{eq} are the equilibrium constants of the reaction at pressure P and at zero pressure, respectively, $P_{1/2}$ is the pressure at which $K_{eq} = 1$ ("half pressure" of the conversion), ΔV^0 is the molar reaction volume, and K^0_{eq} is the equilibrium constant extrapolated to zero pressure, $K^0_{eq} = e^{P_{1/2}\Delta V^0/RT}$. For the equilibrium $A \rightleftharpoons B$ and $K^0_{eq} = [B]/[A]$ Eq. (1) produces the following relationship:

$$[A] = \frac{C_0}{1 + K^0_{eq} \cdot e^{-P\Delta V^0/RT}} = \frac{C_0}{1 + e^{(P_{1/2}-P)\Delta V^0/RT}}, \quad (2)$$

where $C_0 = [A] + [B]$. The experimental data on the pressure dependencies of the concentrations of P450 species were fitted to Eq. (2) with an adjustable offset, A_0, added to the right part. The fitting was made by non-linear regression algorithm as described [17], where A_0, C_0, K^0_{eq}, and ΔV^0 were the target parameters of the optimization. Curve fitting was performed using our SPECTRALAB software package [18].

Results and discussion

Interactions of CYP3A4 with bromocriptine and refinement of CYP3A4 spectral standards

Binding of bromocriptine to CYP3A4 in yeast microsomes was characterized in detail in our previous publications [38,39]. Here we studied the purified enzyme in solution. Spectrophotometric titration experiments showed that the binding of bromocriptine to CYP3A4 results in a type-I spectral change, revealing an important substrate-induced high-to-low spin shift (Fig. 1). The titration curves reveal a simple bimolecular

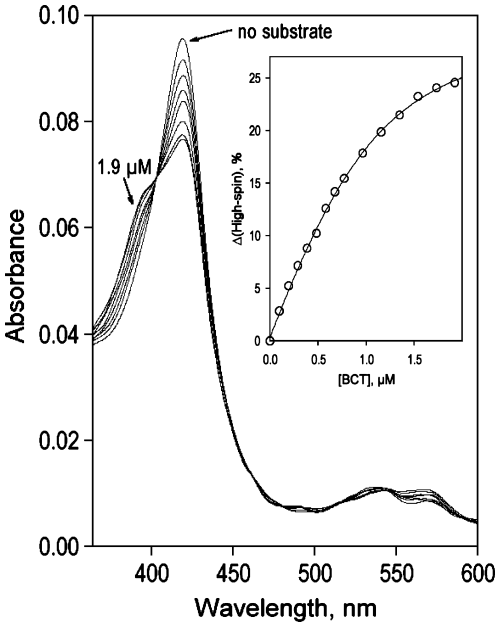

Fig. 1. Titration of CYP3A4 in solution with bromocriptine. The absorbance spectra of 1 μM CYP3A4 recorded with no substrate added and in the presence of 0.2, 0.4, 0.6, 0.8, 1.2, 1.5, and 1.9 μM bromocriptine. The inset shows the titration curve obtained from the first principal component of PCA deconvolution. The solid line shows the results of fitting this data set with the equation for the equilibrium of binary association with $K_D = 0.3\,\mu M$ and the maximal increase in the high-spin content of 31%.

process with no evidence of cooperativity. Interestingly, the affinity of the enzyme for bromocriptine detected in these experiments ($K_D = 0.31 \pm 0.07\,\mu M$) is higher than that reported earlier for CYP3A4 in NF25-V8-E(R) microsomes [38,39].

The titration of CYP3A4 by bromocriptine was used to refine the spectral standards of pure high- and low-spin forms of the enzyme using the spectra of high- and low-spin CYP2B4 [39] as prototypes. The procedure for this refinement was based on the principal component analysis, as described earlier [17,39]. The refined spectrum of the low-spin CYP3A4 is characterized by the α-, β-, and Soret-absorbance bands located at 569 nm ($\varepsilon = 14.1\,mM^{-1}\,cm^{-1}$), 535 nm ($\varepsilon = 12.0\,mM^{-1}\,cm^{-1}$), and 418 nm ($\varepsilon = 107\,mM^{-1}\,cm^{-1}$), respectively. High-spin CYP3A4 has the Soret band at 391 nm ($\varepsilon = 93.2\,mM^{-1}\,cm^{-1}$); the barely resolved α- and β-bands are located at 524–543 nm ($\varepsilon = 8.8\,mM^{-1}\,cm^{-1}$) and the charge-transfer band at 650 nm has an extinction coefficient of $5.0\,mM^{-1}\,cm^{-1}$. These spectra were then used in the analysis of pressure-induced transitions of the enzyme.

Application of the newly refined spectral standards to analyze the spin state of CYP3A4 showed that in the absence of substrate this enzyme shows a spin equilibrium more shifted towards the high-spin state ($19 \pm 4\%$ high-spin P450 at 25 °C) than CYP2B4 (8–9% high-spin at 25 °C) [17,39]. The amplitude of the bromocriptine-induced spin shift in solution was estimated to be $31 \pm 6\%$, which is similar to that reported earlier for CYP3A4 in the microsomal membrane [39].

Pressure-induced transitions in CYP3A4 in solution

Pressure-induced changes in absorbance of CYP3A4 in solution in the absence of substrate and at a saturating concentration of bromocriptine are shown in Figs. 2A and B. These barotropic spectral changes were quite similar to those previously observed in other cytochromes P450 [5,7–22]. Application of PCA to this spectral series resolved two distinct pressure-induced transitions. The least prominent one, taking place at relatively low pressures and characterized by the second principal component, represents disappearance of the band at 391 nm and increase in the intensity of the 418 nm band (Fig. 2C, dashed line). This transition is readily identified as a pressure-induced high-to-low-spin shift. Pressure-jump experiments showed this process to be quite rapid ($\tau < 100$ ms at 600 bar in the absence of substrate). This process is clearly predominant below 1 kbar. All spectral changes observed below this pressure were immediately and completely reversible upon decompression.

Another pressure-induced transition, which is given by the first principal component, reflects a decrease of both the high- and low-spin P450 bands concomitant

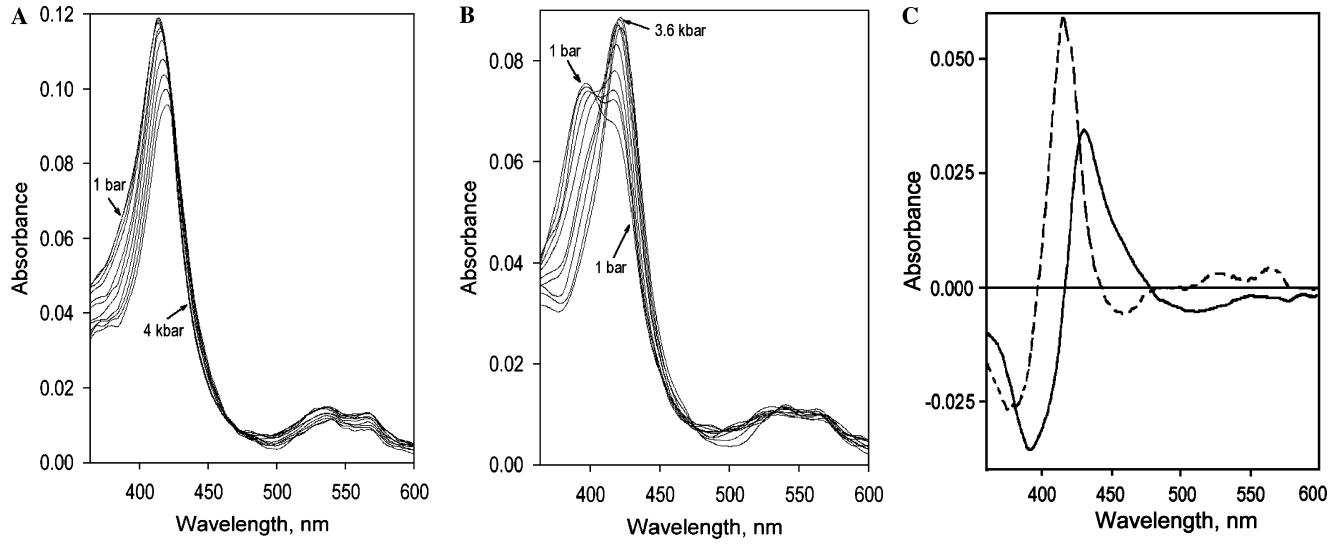

Fig. 2. Pressure-induced spectral changes in CYP3A4 in solution. (A) A series of absorbance spectra of 2.5 μM CYP3A4 with no substrate present recorded in the 1–4000 bar pressure range with 400 bar increments. (B) A series of spectra of 2.1 μM CYP3A4 in the presence of 26 μM BCT recorded in the 1–3600 bar pressure range in 400 bar increments. (C) Spectra of the first (solid line) and the second (dashed line) principal components found by PCA of the series shown in (A). The principal component spectra were scaled to correspond to the transitions in 1 μM protein.

with the appearance of an intense absorbance band at 426 nm (Fig. 2C, solid line). This transition, which was reported earlier for P450cam [13,14], P450lin [14], CYP2B4 [17,25], and P450BM3 [22], is commonly attributed to the pressure-induced conversion of the low-spin P450 into P420 [10,13,14,17]. Pressure-jump experiments indicated that the kinetics of this apparent P450 → P420 transition in CYP3A4 is biphasic (data not shown). The fast phase has the characteristic time (τ) of about 10 s at 1800 bar. This phase is the only one observed below 1500 bar. At higher pressures this fast process is followed by a slower transition ($\tau \geqslant 13$ min at 1800 bar). The slow part of the spectral changes involves only about 30–40% of the total CYP3A4 content. Interestingly, once allowed to reach its limit, the slow phase never appears again upon further pressure increases. In this case all further P450 → P420 transition is represented by the fast phase only. The fast phase of the P450 → P420 conversion was reversible upon decompression. This reconversion takes about 5–10 min to complete. However, if the sample has been incubated at high pressure long enough to allow the slow P450 → P420 conversion, the P420 fraction generated in this phase ($\leqslant$35% of the total CYP3A4 content) remains in P420 state, as verified by the spectra of the ferrous–carbonyl complex.

As evident from the above description, this apparent P450 → P420 transition of the ferric enzyme is rather complex. It reflects at least two distinct processes involving different sub-populations of the enzyme, where only one process is reversible. The amount of P420 determined from the spectrum of the ferrous–carbonyl complex upon decompression was always proportional to the intensity of the pressure-generated absorbance band at 426 nm in the ferric enzyme. It should be noted, however, that the conversion to P420 might be complex, such that the pressure-generated band at 426 nm may correspond to some precursor of the actual P420 state.

The series of CYP3A4 spectra obtained at increasing pressures was then used to refine the spectral standard of the apparent ferric P420 state of the enzyme. The spectra of pressure-generated ferric P420 CYP2B4 [18] and P450BM3 heme domain [22] were used as prototypes. The extinction coefficients were adjusted from the requirement that the total concentration of CYP3A4 be constant, at least in the initial 0–1500 bar interval of the compression experiments. The refined spectral standard of CYP3A4 P420 closely resembles the spectrum reported earlier for the P420 state of the P450BM3 heme domain [22]. The α-, β-, and Soret-bands are located at 579, 553, and 426 nm with extinction coefficients of 7.7, 11.5, and 94.4 $mM^{-1}\,cm^{-1}$, respectively. The amount of P450 and P420 in CYP3A4 preparations determined using the set of refined spectral standards of the low-spin, high-spin, and P420 states of ferric CYP3A4 was always in strict correspondence with the amount determined from the spectra of the ferrous–carbonyl complex of the enzyme.

All further experiments were designed to study the reversible pressure-induced transitions only. The combination of a rapid-scanning CCD spectrometer with the high-pressure optical cell used in our experiments allowed us to measure a series of spectra in the 1–4000 bar range in 200 bar steps within 5 min. In this experimental setup the slow irreversible P450 → P420 inactivation could be neglected. This design allowed us to interpret all observed pressure-induced changes as reversible, applying the equation for the pressure dependence of the equilibrium constant to interpret the transitions.

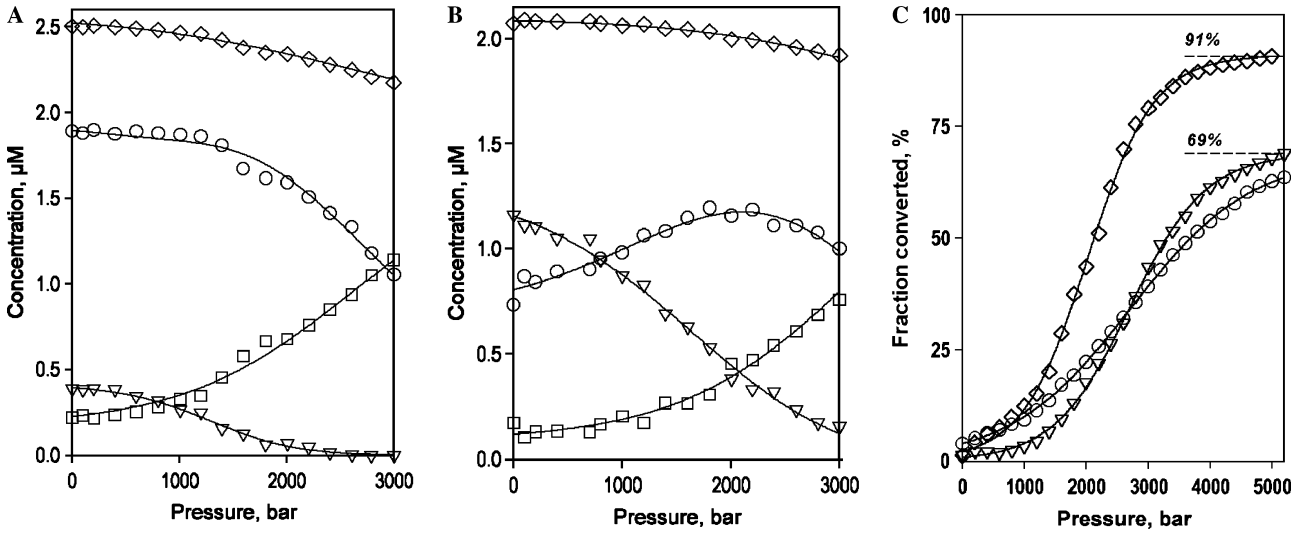

Fig. 3. Pressure-induced transitions in CYP3A4 in solution. (A,B) The changes in the concentration of the high-spin (triangles), low-spin (circles), and P420 (squares) states of CYP3A4 and the total hemoprotein concentration (diamonds) with no substrate added (A) and at 23 μM BCT (B). (C) The course of the pressure-induced P450 → P420 transition with no substrate added (circles), at 23 μM bromocriptine (triangles), and in the presence of 0.8% Emulgen-913 (diamonds). The results of fitting are shown by solid lines corresponding to the following sets of parameters: $\Delta V^0 = -30$ ml/mol, $P_{1/2} = 2790$ bar, $C_0 = 69\%$ (no substrate); $\Delta V^0 = -43$ ml/mol, $P_{1/2} = 2600$, $C_0 = 69\%$ (+ bromocriptine), and $\Delta V^0 = -47$ ml/mol, $P_{1/2} = 2020$ bar, $C_0 = 91\%$ (+ Emulgen).

The pressure-induced changes in the concentration of the high-spin, low-spin, and P420 states of CYP3A4 in the substrate-free state and at saturation with bromocriptine are shown in Figs. 3A and B. Both the spin shift and P450 P420 transitions are clearly visible in these plots. (The pressure-induced spin transition in CYP3A4 will be analyzed in detail elsewhere.) Here we focus on the P450 → P420 transition only. Fig. 3C shows the pressure-induced P450 → P420 transition as a function of the P420 fraction versus pressure with no substrate present and at saturating concentrations of bromocriptine. Fitting these curves to Eq. (2) shows that the total amount of hemoprotein susceptible to this reversible P450 → P420 transition corresponds to $72 \pm 6\%$ of the total P450 content, as given by the average of 12 individual measurements. This value was not dependent on the concentration of bromocriptine over the whole 0–26 μM range studied. The rest of the hemoprotein appears to be excluded from this rapid reversible P450 → P420 transition. Addition of 0.8% non-ionic detergent Emulgen-913, which is supposed to cause dissociation of P450 oligomers [44,45], essentially eliminates this heterogeneity, increasing the maximal degree of P450 → P420 transition to 91% (Fig. 3C).

Interestingly, CYP3A4 interactions with bromocriptine slightly enhance the sensitivity of the enzyme to pressure-induced inactivation, increasing the absolute value of the activation volume and decreasing in the value of $P_{1/2}$. In the absence of substrate the activation volume of the transition (ΔV^0_{P420}) was equal to -25 ± 4 ml/mol with $P_{1/2} = 2710 \pm 180$ bar. At 26 μM bromocriptine ΔV^0_{P420} was -41 ± 3 ml/mol, while $P_{1/2}$ decreases to 2590 ± 30 bar. This observation contrasts with that reported earlier for P450BM3 [22], where the binding of substrate decreases the absolute value of ΔV^0_{P420} and displaces the P450 → P420 transition to higher pressures. In P450BM3 the stabilizing effect of substrate binding was interpreted as evidence that the P450 → P420 transition ultimately requires prior dissociation of the substrate complex [22]. The effect of substrate on P450 → P420 transition in CYP3A4 reported above suggests that, in contrast to P450cam, P450BM3, and CYP2B4 [18,22], hydrostatic pressure does not cause any dissociation of the CYP3A4-substrate complex, so that the substrate dissociation volume (ΔV^0_D) is not negative.

Pressure-induced transitions in CYP3A4 in microsomes

Pressure-perturbation studies of cytochromes P450 in the microsomal membrane are extremely intricate due to high turbidity and the presence of other proteins (cytochrome b_5 and flavoproteins, in particular), which absorb in the Soret region. However, application of principal component analysis in combination with the set of spectral standards of pure high-, low-spin, and P420 states of CYP3A4 made it possible to analyze the pressure-induced transition even in yeast microsomes. Another important prerequisite for the success of this study was our preliminary detailed thermodynamic analysis of spin transitions and bromocriptine binding in recombinant yeast microsomes [39]. This analysis provided us with the equilibrium constants necessary to calculate the exact spin state of CYP3A4 in microsomes

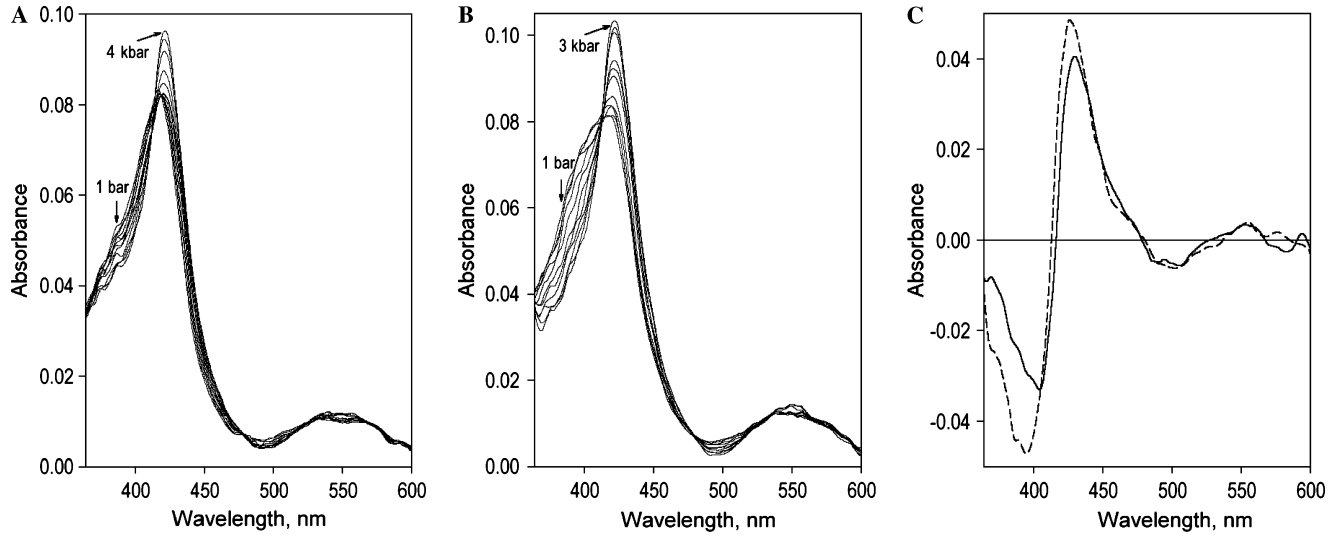

Fig. 4. Pressure-induced spectral changes in CYP3A4 in NF25-V8-E(R) microsomes. (A) A series of spectra with no substrate present recorded in the 1–3000 bar pressure range in 300 bar increments and at 4000 bar. (B) A series of spectra recorded in the presence of 26 μM BCT in 1–3000 bar pressure range at 300 bar increments. The spectra in (A) and (B) were treated by PCA-based polynomial correction algorithm [39] to suppress the turbidity of the microsomal suspension. The absorbance of the ferric cytochrome b_5 present in these preparations is also subtracted. (C) Spectra of the first principal component found by PCA of the series shown in (A) (solid line) and (B) (dashed line). The principal component spectra were scaled to correspond to the transitions in 1 μM protein.

at each given concentration of bromocriptine. Since the exact spin state of P450 in each sample at room pressure was known, the spectrum of P450 in microsomes at each bromocriptine concentration was easily calculated as a linear combination of the CYP3A4 high- and low-spin spectral standards. This approach makes the procedure of pressure correction strictly defined and reproducible. All spectra of CYP3A4 in microsomes presented in this publication are corrected for turbidity of microsomes and absorbance of cytochrome b_5.

Pressure-induced changes in the spectra of CYP3A4 in microsomes with no substrate added and at saturating concentration of bromocriptine are shown in Figs. 4A and B. Even preliminary visual analysis reveals an important difference from CYP3A4 in solution: in contrast to the spectral series shown in Fig. 2, where no isosbestic points were observed, the spectral changes shown in Fig. 4 exhibit clear isosbestic points. This fact suggests that in the microsomes all changes observed in the P450 might be described by a unique pressure-induced process, instead of the two transitions described above for CYP3A4 in solution. Indeed, the principal component analysis of the series of spectra presented in Fig. 4 reveals only one principal component that might be reasonably described by a combination of the CYP3A4 spectral standards. More precisely, a low-amplitude second principal component, which might reflect some spin transition, appears only when no substrate was added. This component, covering about 2% of the total spectral changes, corresponds to a low-amplitude process with $\Delta V^0 = -97$ ml/mol and $P_{1/2} = 2570$ bar, suggesting an equilibrium that is completely shifted towards the high-spin state at 1 bar, which is not plausible. Since this transition takes place at the pressures where the P450 → P420 transition is already close to completion, it probably reflects some secondary effect of P420 interactions with P450 or an effect of hydrostatic pressure on the microsomal membrane.

Thus, the only representative pressure-induced process observed in CYP3A4-containing microsomes corresponds to the P450 → P420 transition. This important peculiarity of the microsomal system considerably simplifies subsequent analysis. All spectral transitions observed in NF25-V8-W(R) microsomes were completely reversible on decompression. No slow phase of the P450 → P420 transition was detected here. Control experiments with W(R) microsomes lacking CYP3A4 (but still containing CPR and b_5) reveal no significant pressure-induced spectral changes other than changes in the turbidity of the microsomal suspension.

The pressure-induced changes in the concentrations of high-spin and low-spin P450 and P420 in microsomes are shown in Fig. 5. The pressure-related behavior illustrated by this figure clearly demonstrates heterogeneity of the pool of CYP3A4 in the microsomes. Only about half of the hemoprotein exhibits a P450 → P420 transition (Fig. 5C). As evident from Fig. 5, the position of the spin equilibrium of the P450 fraction undergoing a P450 → P420 transition is very different from the position of the apparent "overall" spin equilibrium of the whole pool of CYP3A4. In the presence of bromocriptine, the low-spin P450 is almost completely excluded from the pressure-induced transition (Fig. 5B). Fig. 6A shows the dependence of the high-spin content in the

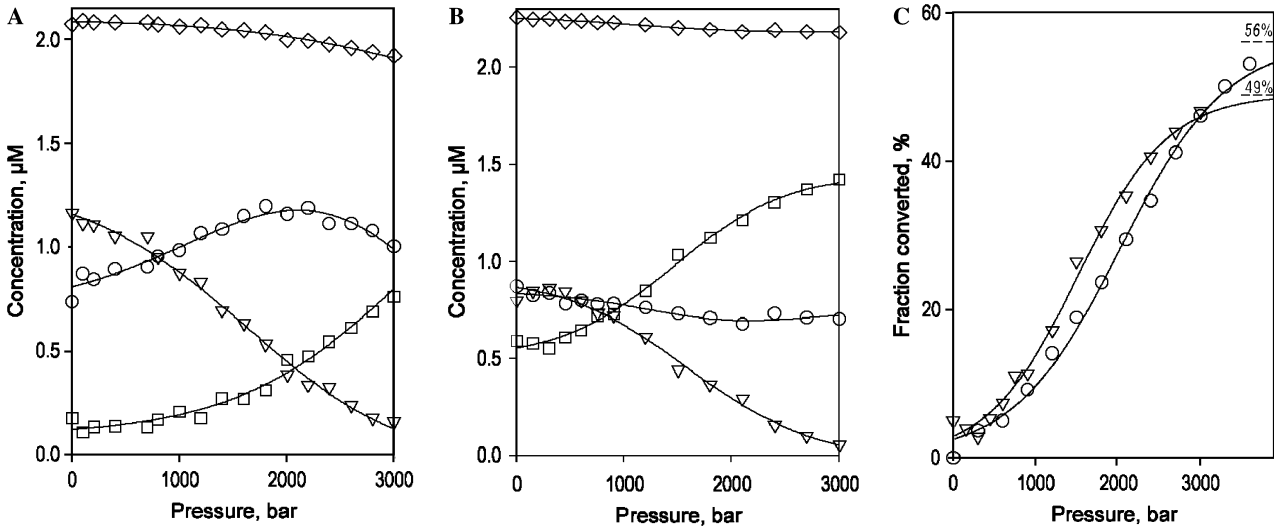

Fig. 5. Pressure-induced transitions in CYP3A4 in NF25-V8-E(R) microsomes. (A,B) The changes in the concentration of the high-spin (triangles), low-spin (circles), and P420 (squares) states of CYP3A4 and the total hemoprotein concentration (diamonds) with no substrate added (A) and at 200 μM BCT (B). (C) The course of the pressure-induced P450 → P420 transition with no substrate added (circles) and at 200 μM BCT (triangles). The results of fitting are shown by solid lines corresponding to $\Delta V^0 = -30$ ml/mol, $P_{1/2} = 1580$ bar, $C_0 = 56\%$ (no substrate), and $\Delta V^0 = -61$ ml/mol, $P_{1/2} = 1410$, $C_0 = 49\%$ (200 μM bromocriptine).

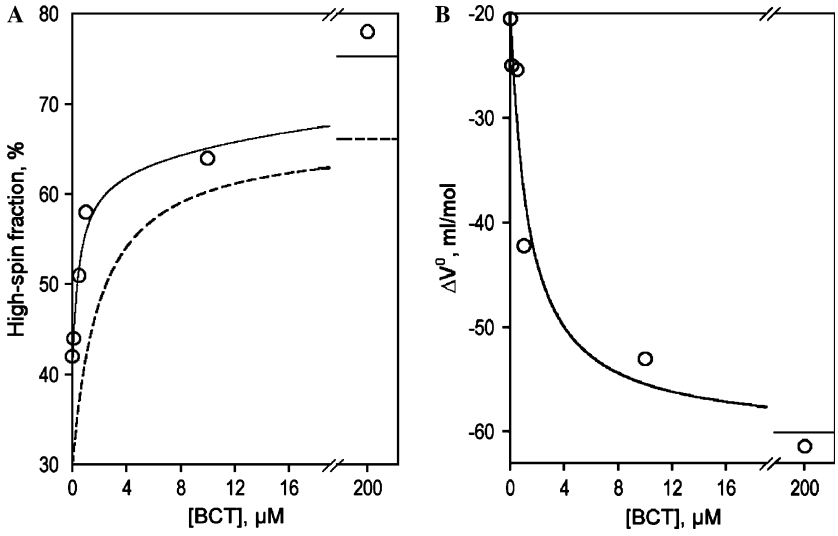

Fig. 6. Parameters of pressure-induced P450 → P420 transitions in NF25-V8-E(R) microsomes at various concentrations of bromocriptine. (A) Effect of bromocriptine on the content of the high-spin state in pressure-susceptible fraction of CYP3A4, as determined from the spectrum of the first principal component. The dashed line shows the content of the high-spin state in the total pool of CYP3A4, as calculated from the equilibrium constants of spin transition and substrate binding in CYP3A4 in NF25-V8-E(R) microsomes. (B) Effect of bromocriptine on the activation volume (ΔV^0) of the pressure-induced P450 → P420 transition.

pressure-sensitive fraction of CYP3A4 (F_{h1}) on the concentration of bromocriptine. As seen from this figure, this content is always higher than the percentage of the high-spin state in the entire pool of CYP3A4 (F_{h0}). The best fitting of this curve is given by the sum of two Michaelis–Menten equations with $K_{M1} = 0.52\,\mu M$, $K_{M2} = 77\,\mu M$, and a 1:2 ratio of amplitudes. This may indicate the presence of two distinct substrate binding sites, which were not resolved in our titration experiments [38,39].

The value of ΔV^0_{P420} is also dependent on the concentration of bromocriptine, changing from −21.5 ml/mol with no substrate present to −66.5 at 200 μM bromocriptine (Fig. 6B). These values are in good agreement with those reported above for CYP3A4 in solution. The dependence of ΔV^0_{P420} also obeys the sum of two Michaelis–Menten equations with $K_{M1} = 0.77\,\mu M$ and $K_{M2} = 67\,\mu M$, which are in reasonable agreement with the values obtained from the substrate dependence of F_{h1}.

These results clearly demonstrate heterogeneity of the CYP3A4 pool in the microsomes. Only about 50% of the total hemoprotein undergoes a pressure-induced P450 → P420 transition. Only this fraction of the hemoprotein is exposed to substrate-dependent spin equilibrium, while the pressure-resistant part of CYP3A4 appears to be represented by the low-spin state only.

Conclusions

The data presented here clearly demonstrate conformational heterogeneity of CYP3A4 both in solution and in microsomal membranes, confirming the previous observation of Koley et al. [37]. This inhomogeneity closely resembles the situation reported previously for CYP2B4 in solution and in proteoliposomal membranes [17,18,25]. However, there are several important differences. For CYP2B4, the fraction susceptible to a pressure-induced P450 → P420 transition appears to be excluded from substrate-modulated spin equilibrium and is represented by the low-spin state only. In the case of CYP3A4 both the P450 → P420 transition and substrate-sensitive spin shift are shown to take place in the same fraction of the enzyme, while the remaining part is represented by the predominantly low-spin state. This latter fraction constitutes about 1/3 of the enzyme in solution and about 50% of CYP3A4 in microsomes. In solution it undergoes slow irreversible inactivation at pressures higher than 1500 bar. By analogy with our interpretation of CYP2B4 heterogeneity, we propose that the heterogeneity in CYP3A4 is caused by the architecture of P450 oligomers. The simplest explanation is that the subunits in the oligomer have different orientations of the substrate access channel, resulting in different substrate and water accessibility of the heme moiety. This interpretation is supported by the X-ray crystal structure of the heme-containing domain of P450BM3 (BMP). Thus, the two BMP monomers that form the dimeric crystallization unit have significantly different conformations, one with more open substrate and water access to the heme moiety than the other [46]. Although P450BM3 is believed to be monomeric in solution, the architecture of its dimeric crystallization unit may be related to the architecture of oligomers of microsomal cytochromes P450.

The fact that both CYP3A4 and CYP2B4 reveal a 1:2 distribution of apparent conformers in solution seems remarkable and might indicate the size and type of symmetry of the oligomers in solution. Hexamers predominate in solution with both CYP2B4 and P450 1A2 [44,47], and the hexamers of CYP2B4 were demonstrated to be dimers of trimers [48]. A 1:2 conformer distribution shown here for CYP3A4 in solution suggests a hexameric organization in this enzyme as well, such that two subunits may have a distinct orientation as compared to the other four.

Data presented here clearly demonstrate a remarkable effect of the membrane environment and/or interactions of CYP3A4 with CPR and b_5 on water accessibility of the heme pocket. The fact that no pressure-induced spin transitions were observed in CYP3A4 in NF25-V8-W(R) microsomes may suggest that the high-spin state of this enzyme is stabilized by interactions with CPR and b_5. These interactions apparently diminish water accessibility of the heme pocket, hence preventing the pressure-induced spin shift. Overall, our results suggest that the heterogeneity of CYP3A4 should be considered in further studies of the mechanisms of cooperativity of this enzyme.

Acknowledgments

This paper is dedicated to I.C. Gunsalus in honor of his 90th birthday. Gaston Hui Bon Hoa would like to thank Dr. Gunsalus for fruitful collaboration during his several stays in the laboratory of Pierre Douzou in Paris, especially during his sabbatical year. The authors appreciate the decisive impetus provided by P. Douzou and I.C. Gunsalus to study P450 systems at subzero temperatures. This effort was then logically extended to high pressure spectroscopy of various cytochromes P450 and reached out to other laboratories all over the world.

References

[1] J.A. Kornblatt, M.J. Kornblatt, The effects of osmotic and hydrostatic pressures on macromolecular systems, Biochim. Biophys. Acta 1595 (2002) 30–47.
[2] R.B. Macgregor, The interactions of nucleic acids at elevated hydrostatic pressure, Biochim. Biophys. Acta 1595 (2002) 266–276.
[3] K. Heremans, L. Smeller, Protein structure and dynamics at high pressure, Biochim. Biophys. Acta 1386 (1998) 353–370.
[4] C. Jung, Cytochrome P-450-CO and substrates: lessons from ligand binding under high pressure, Biochim. Biophys. Acta 1595 (2002) 309–328.
[5] G. Hui Bon Hoa, M.A. McLean, S.G. Sligar, High pressure, a tool for exploring heme protein active sites, Biochim. Biophys. Acta 1595 (2002) 297–308.
[6] S.G. Sligar, I.C. Gunsalus, Proton coupling in the cytochrome P-450 spin and redox equilibria, Biochemistry 18 (1979) 2290–2295.
[7] C. Di Primo, S.G. Sligar, G. Hui Bin Hoa, P. Douzou, A critical role of protein-bound water in the catalytic cycle of cytochrome P-450 camphor, FEBS Lett. 312 (1992) 252–254.
[8] F. Bancel, N. Bec, C. Ebel, R. Lange, A central role for water in the control of the spin state of cytochrome P-450(scc), Eur. J. Biochem. 250 (1997) 276–285.
[9] C. Di Primo, E. Deprez, G. Hui Bon Hoa, P. Douzou, Antagonistic effects of hydrostatic pressure and osmotic pressure on cytochrome P-450(cam) spin transition, Biophys. J. 68 (1995) 2056–2061.
[10] M.T. Fisher, S.F. Scarlata, S.G. Sligar, High-pressure investigations of cytochrome P-450 spin and substrate binding equilibria, Arch. Biochem. Biophys. 240 (1985) 456–463.

[11] G. Hui Bon Hoa, M.C. Marden, The pressure dependence of the spin equilibrium in camphor-bound ferric cytochrome P-450, Eur. J. Biochem 124 (1982) 311–315.
[12] M.C. Marden, G. Hui Bon Hoa, P-450 binding to substrates camphor and linalool versus pressure, Arch. Biochem. Biophys. 253 (1987) 100–107.
[13] C. Di Primo, G. Hui Bon Hoa, P. Douzou, S.G. Sligar, Heme-pocket-hydration change during the inactivation of cytochrome P-450 (camphor) by hydrostatic pressure, Eur. J. Biochem. 209 (1992) 583–588.
[14] G. Hui Bon Hoa, C. Di Primo, I. Dondaine, S.G. Sligar, I.C. Gunsalus, P. Douzou, Conformational changes of cytochromes P-450cam and P-450lin induced by high pressure, Biochemistry 28 (1989) 651–656.
[15] C. Di Primo, G. Hui Bon Hoa, P. Douzou, S.G. Sligar, Effect of the tyrosine 96 hydrogen bond on the inactivation of cytochrome P-450cam induced by hydrostatic pressure, Eur. J. Biochem. 193 (1990) 383–386.
[16] R.A. Tschirret-Guth, G. Hui Bon Hoa, R.P. Ortiz de Montellano, Pressure-induced deformation of the cytochrome P450cam active site, J. Am. Chem. Soc. 120 (1998) 3590–3596.
[17] D.R. Davydov, T.V. Knyshko, G. Hui Bon Hoa, High pressure induced inactivation of ferrous cytochrome P-450 LM2 (IIB4) CO-complex: evidence for the presence of two conformers in the oligomer, Biochem. Biophys. Res. Commun. 188 (1992) 216–221.
[18] D.R. Davydov, E. Deprez, G. Hui Bon Hoa, T.V. Knyushko, G.P. Kuznetsova, Y.M. Koen, A.I. Archakov, High-pressure-induced transitions in microsomal cytochrome CYP2B4 in solution—Evidence for conformational inhomogeneity in the oligomers, Arch. Biochem. Biophys. 320 (1995) 330–344.
[19] D.R. Davydov, N.A. Petushkova, A.I. Archakov, G. Hui Bon Hoa, Stabilization of CYP2B4 by its association with P450 1A2 revealed by high-pressure spectroscopy, Biochem. Biophys. Res. Commun. 276 (2000) 1005–1012.
[20] P. Anzenbacher, N. Bec, J. Hudecek, R. Lange, E. Anzenbacherova, High conformational stability of cytochrome P-450 1A2. Evidence from UV absorption spectra, Coll. Czech. Chem. Commun. 63 (1998) 441–448.
[21] E. Anzenbacherova, N. Bec, P. Anzenbacher, J. Hudecek, P. Soucek, C. Jung, A.W. Munro, R. Lange, Flexibility and stability of the structure of cytochromes P450 3A4 and BM-3, Eur. J. Biochem. 267 (2000) 2916–2920.
[22] D.R. Davydov, G. Hui Bon Hoa, J.A. Peterson, Dynamics of protein-bound water in the heme domain of P450BM3 studied by high-pressure spectroscopy: comparison with P450cam and P450 2B4, Biochemistry 38 (1999) 751–761.
[23] M.A. McLean, S.A. Maves, K.E. Weiss, S. Krepich, S.G. Sligar, Characterization of a cytochrome P450 from the acidothermophilic archaea *Sulfolobus solfataricus*, Biochem. Biophys. Res. Commun. 252 (1998) 166–172.
[24] R.A. Tschirret-Guth, L.S. Koo, G. Hui Bon Hoa, P.R. Ortiz de Montellano, Reversible pressure deformation of a thermophilic cytochrome P450 enzyme (CYP119) and its active site mutants, J. Am. Chem. Soc. 123 (2001) 3412–3417.
[25] D.R. Davydov, G. Hui Bon Hoa, Pressure-induced transitions in cytochrome CYP2B4 as an evidence of its functional inhomogeneity in the proteoliposomal membrane, in: K. Heremans (Ed.), High Pressure Research in the Biosciences and Biotechnology, Leuven University Press, 1997, pp. 107–110.
[26] F.P. Guengerich, Cytochrome P-450 CYP3A4: regulation and role in drug metabolism, Annu. Rev. Pharmacol. Toxicol. 39 (1999) 1–17.
[27] S. Ekins, D.M. Stresser, J.A. Williams, In vitro and pharmacophore insights into CYP3A enzymes, Trends Pharm. Sci. 24 (2003) 161–166.
[28] K.K. Khan, H. Liu, J.R. Halpert, Homotropic versus heterotopic cooperativity of cytochrome P450eryF: a substrate oxidation and spectral titration study, Drug Metab. Dispos. 31 (2003) 356–359.
[29] N.A. Hosea, G.P. Miller, F.P. Guengerich, Elucidation of distinct ligand binding sites for cytochrome P450 3A4, Biochemistry 39 (2000) 5929–5939.
[30] M. Shou, R. Dai, D. Cui, K.R. Korzekwa, T.A. Baillie, T.H. Rushmore, A kinetic model for the metabolic interaction of two substrates at the active site of cytochrome P450 3A4, J. Biol. Chem. 276 (2001) 2256–2262.
[31] K. Alston, R.C. Robinson, S.S. Park, H.V. Gelboin, F.K. Friedman, Interactions among cytochromes P-450 in the endoplasmic reticulum. Detection of chemically cross-linked complexes with monoclonal antibodies, J. Biol. Chem. 266 (1991) 735–739.
[32] K.N. Myasoedova, P. Berndt, Cytochrome P-450LM2 oligomers in proteoliposomes, FEBS Lett. 275 (1990) 235–238.
[33] D. Schwarz, J. Pirrwitz, H.W. Meyer, M.J. Coon, K. Ruckpaul, Membrane topology of microsomal cytochrome P-450: saturation transfer EPR and freeze-fracture electron microscopy studies, Biochem. Biophys. Res. Commun. 171 (1990) 175–181.
[34] R. Greinert, S.A. Finch, A. Stier, Cytochrome P-450 rotamers control mixed-function oxygenation in reconstituted membranes. Rotational diffusion studied by delayed fluorescence depolarization, Xenobiotica 12 (1982) 717–726.
[35] S. Kawato, J. Gut, R.J. Cherry, K.H. Winterhalter, C. Richter, Rotation of cytochrome P-450. I. Investigations of protein–protein interactions of cytochrome P-450 in phospholipid vesicles and liver microsomes, J. Biol. Chem. 257 (1982) 7023–7029.
[36] P.R. McIntosh, S. Kawato, R.B. Freedman, R.J. Cherry, Evidence from cross-linking and rotational diffusion studies that cytochrome P450 can form molecular aggregates in rabbit-liver microsomal membranes, FEBS Lett. 122 (1980) 54–58.
[37] A.P. Koley, J.T. Buters, R.C. Robinson, A. Markowitz, F.K. Friedman, CO binding kinetics of human cytochrome P450 3A4. Specific interaction of substrates with kinetically distinguishable conformers, J. Biol. Chem. 270 (1995) 5014–5018.
[38] M.A. Peyronneau, M. Delaforge, R. Riviere, J.P. Renaud, D. Mansuy, High affinity of ergopeptides for cytochromes P450 3A. Importance of their peptide moiety for P450 recognition and hydroxylation of bromocriptine, Eur. J. Biochem. 223 (1994) 947–956.
[39] J.P. Renaud, D.R. Davydov, K.P.M. Heirwegh, D. Mansuy, G. Hui Bon Hoa, Thermodynamic studies of substrate binding and spin transitions in human cytochrome P-450 3A4 expressed in yeast microsomes, Biochem. J. 319 (1996) 675–681.
[40] T.L. Domanski, J. Liu, G.R. Harlow, J.R. Halpert, Analysis of four residues within substrate recognition site of human cytochrome P450 3A4: role in steroid hydroxylase activity and alpha-naphthoflavone stimulation, Arch. Biochem. Biophys. 350 (1998) 223–232.
[41] T.L. Domanski, Y.A. He, K.K. Khan, F. Roussel, Q. Wang, J.R. Halpert, Phenylalanine and tryptophan scanning mutagenesis of CYP3A4 substrate recognition site residues and effect on substrate oxidation and cooperativity, Biochemistry 40 (2001) 10150–10160.
[42] M.A. Peyronneau, J.P. Renaud, G. Truan, P. Urban, D. Pompon, Optimization of yeast-expressed human liver cytochrome P450 3A4 catalytic activities by coexpressing NADPH-cytochrome P450 reductase and cytochrome b_5, Eur. J. Biochem. 207 (1992) 109–116.
[43] G. Weber, Protein Interactions, Chapman and Hall, New York, 1991.
[44] P. Berndt, N.N. Magretova, K.N. Myasoedova, V.Ya. Chernyak, Cytochrome P-450 LM2 hexamer dissociation in the presence of a nonionic detergent Emulgen 913, Biokhimya 54 (1989) 338–341.
[45] R.I. Viner, K.N. Novikov, V.B. Ritov, V.E. Kagan, M.A. Alterman, Effect of different solubilizing agents on the aggregation

state and catalytic activity of two purified rabbit cytochrome P450 isozymes, CYP1A2 (LM4) and CYP2B4 (LM2), Biochem. Biophys. Res. Commun. 217 (1995) 886–891.

[46] K.G. Ravichandran, S.S. Boddupalli, C.A. Hasermann, J.A. Peterson, J. Deisenhofer, Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's, Science 261 (1993) 731–736.

[47] K.N. Myasoedova, V.L. Tsuprun, Cytochrome P-450: hexameric structure of the purified LM4 form, FEBS Lett. 325 (1993) 251–254.

[48] V.L. Tsuprun, K.N. Myasoedova, P. Berndt, O.N. Sograf, E.V. Orlova, V.Ya. Chernyak, A.I. Archakov, V.P. Skulachev, Quaternary structure of the liver microsomal cytochrome P-450, FEBS Lett. 205 (1986) 35–40.