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Conformational Changes near the Cytochrome P450 Active Site upon Binding of Two Different Ligands

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Abstract—It is shown that a stable nitroxyl radical, 4-cyano-2,2,6,6-tetramethylpiperidine-1-oxyl, forms a complex with cytochrome P4502B4 by analogy with the second type substrates by joining directly to pentacoordinate heme iron. The bound radical is inaccessible to water-soluble paramagnetic ions, which confirms its localization in a hydrophobic pocket near the heme. Benzphetamine and N,N-dimethylaniline, the first-type nonpolar substrates, induce conformational changes of the spin-labeled hemoprotein which are evidently accompanied by an increase in the volume of the pocket resulting in emergence of contact with aqueous phase, and the heme-bound spin label becomes accessible to water-soluble paramagnetics. In this case potassium ferricyanide broadens the spin-labeled cytochrome signal and, as a result, lowers the amplitudes of the spectral components. Similar changes were registered at non-micellar concentrations of nonionic detergent Emulgen 913, whose activating effect on hydroxylation reactions is associated, as we showed previously, with its presence in the CYP2B4 active site simultaneously with substrates.

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Cytochromes P450 comprise a large family of autooxidizable hemoproteins found in all organisms. Human CYP (over 50 genes) are present in all organs and tissues except skeletal muscle, and they are usually incorporated in intracellular membranes. The name of this hemoprotein group refers to a specific absorption band of reduced CYP complex with carbon monoxide at 450 nm.

Human CYP and those of higher animals are involved in biosynthesis and metabolism of a number of physiologically active substances. CYP of endoplasmic reticulum of liver cells exhibit a unique ability to catalyze biotransformation of xenobiotics (drugs, toxins, various environmental pollutants, etc.). This function is associated with unusual enzyme properties of these hemoproteins. The main reaction catalyzed by CYP is monooxygenase-type hydroxylation of hydrophobic molecules using reducing equivalents of NADPH (in eukaryotes) or NADH (in bacteria) and incorporation of one oxygen atom into the substrate and the other into water. However, the catalytic abilities of CYP in hepatocytes are not restricted to hydroxylation. Along with the latter, these CYP can catalyze reactions of N-, S-, and O-dealkylation, epoxidation, heteroatom oxygenation, oxidative cleavage of ester and amide bonds, peroxidation, isomerization, desaturation, etc. [1-3] and exhibit extremely broad substrate specificity. The reaction kinetics are complex and do not obey Michaelis–Menten kinetics. Hypotheses concerning the two-step mechanism of substrate binding, the possibility of simultaneous binding of two and more ligand molecules in the cytochrome active site, and finally, the variability of conformational rearrangements in the heme pocket region have been suggested. Facts supporting two last ideas have been obtained in recent works [4-7].

The CYP catalytic cycle begins from substrate fixation in a hydrophobic pocket near the heme with the heme iron state in the form of Fe^{3+} and mainly in the low-spin electron configuration. In this case, changes in the region of the sixth heme ligand (the sixth coordination position is occupied by water) allow the system to acquire the high-spin electron configuration. The formed enzyme–substrate complexes exhibit certain optical

Abbreviations: 4-cyano-TEMPO, 4-cyano-2,2,6,6-tetramethylpiperidine-1-oxyl; CYP, cytochrome P450 (accepted nomenclature of isoforms of this hemoprotein with addition of alphanumeric code for each individual isoform (e.g. CYP2B4)).

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properties. The shape of their differential absorption spectra (i.e. complex without free hemoprotein) makes it possible to differentiate two main types. The first type spectra are characterized by absorption maximum at 385-390 nm and minimum at 420-425 nm. The first type spectral changes are caused by almost all nonpolar compounds irrespective of their chemical nature [8-10]. The second type substrates (many nitrogen-containing compounds, mainly different amines) and some inhibitors of hydroxylation reactions join directly to pentacoordinate heme iron, thus inducing spin shift towards the low-spin state. Binding is accompanied by emergence of an absorption maximum at 425-435 nm and minimum at 390-411 nm [8-11]. The change in intensities can be characterized by maximal amplitude (ΔA_{max}) on conversion to 1 mol of cytochrome or to 1 mol of protein, and by spectral binding constant (K_s) numerically equal to substrate concentration necessary for obtaining the amplitude of spectral changes equal to half ΔA_{max}. Among the second type ligands, studied in classical works, there are cyanides and isocyanides that are known to exhibit high affinity to ferroporphyrins [12, 13]. We used free radical alkyls of these compounds, registered EPR spectra of spin-labeled complexes with hemoprotein, and expected to obtain information concerning events in the region of the hepatocyte CYP2B4 active site in the presence of substrates and some other factors known to be able to bind in the pocket near the heme of this CYP form.

MATERIALS AND METHODS

CYP2B4, induced by *in vivo* phenobarbital treatment of rabbits and isolated from membranes of endoplasmic reticulum of liver as described earlier, was used in this work [14, 15]. The method included solubilization of microsome membranes by sodium cholate, fractionation with polyethylene glycol, and consecutive purification on columns with aminooctyl-Sepharose, hydroxyapatite, and CM-Sephadex. The purification of CYP using aminooctyl-Sepharose was proposed earlier by Imai [16]. In our case high-capacity aminooctyl-Sepharose synthesized in our laboratory and enabling separation of the 2B4 form from concomitant CYP isoforms was used at this stage. The detergent Emulgen 913, used at all column stages of the procedure, was removed by washing the hemoprotein sorbed on CM-Sephadex column by detergent-free buffer. All solutions contained 20% glycerol. Electrophoretically pure final enzyme preparations were practically detergent-free, monodisperse according to analytical ultracentrifugation, and contained only CYP2B4 hexamers. Cytochrome concentration in preparations was determined by absorption of reduced CYP complex with carbon monoxide using the differential scheme of Omura and Sato [17]. Stable nitroxyl radical 4-cyano-2,2,6,6-tetramethylpiperidine-1-oxyl (4-cyano-TEMPO) was chosen as spin label. A weighed portion of compound was dissolved in a minimal volume of ethanol. The label was added to final concentration 2.5-3.0 mM to the hemoprotein portion (1-2 ml, 1-2 μM protein) in phosphate buffer containing 20% glycerol, and the mixture was incubated for 5 min at 25°C. To remove unbound label, the mixture was placed into a dialysis bag and dialyzed at 4°C against several changes of 100 mM phosphate buffer, 10% glycerol, to complete disappearance of UV absorption caused by the filtered label. To register EPR spectra of spin-labeled cytochrome, an RE-1307 radiospectrometer of 3-cm range was used (production of experimental plant of the USSR Academy of Sciences, Chernogolovka). Samples of 35 μl were placed into a flat quartz cell. The "spin probe/spin label" method was used to establish supposed structural rearrangements in the region of the CYP heme pocket [18]. Paramagnetic ions served as a probe; the effect of these ions was revealed in broadening the spin label EPR signal and resulting lowering of the spectral component amplitudes. Potassium ferricyanide at initial concentration $5 \cdot 10^{-1}$ M, allowing the use of minimal volume ratios of ferricyanide solution to that of spin-labeled hemoprotein, was used as a broadening agent. Concentrations of substrates added to spin-labeled CYP2B4 were within the limits of a series usually used for demonstration of CYP binding spectra and measurements of enzymic activity (on the basis of data in the literature and our previous works [5, 19-21]). Spectrophotometric measurements were carried out using Aminco-2W (USA) and Hitachi-557 (Japan) spectrophotometers. When spectra of spin label binding to hemoprotein were registered, the four-cell scheme was used due to the presence of absorption band in the free radical spectrum in the Soret band; in this case free label was added to the control cell of the second pair containing only buffer solution.

All substrates were from Sigma (USA), and 4-cyano-TEMPO was from Aldrich (USA).

RESULTS AND DISCUSSION

Among possible ligands able to join directly to the CYP2B4 heme iron, there are both substrates of monooxygenase reactions converted to hydroxylated products (such as the known substrate aniline) and compounds that do not undergo visible transformations but form complexes with the same spectral properties (like imidazole inhibitors). Characteristics of over 20 nitrogen-containing compounds of different chemical structure studied with respect to the type of their binding in the active site of two CYP forms and the probability of inhibitory effect are described in a recent work [11]. By spectral characteristics of their complexes with CYP, alkylcyanides and isocyanides occupy exactly the position among the second type ligands with absorption maximum

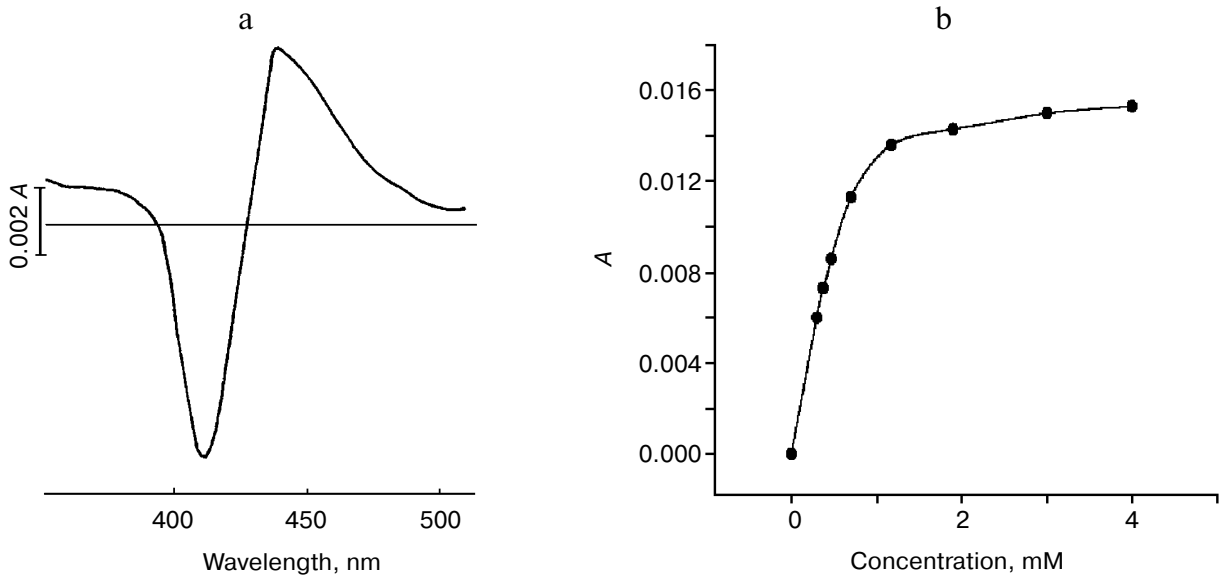

Fig. 1. Differential absorption spectra of CYP2B4 induced by spin label. a) Absorption spectrum of radical complex with CYP2B4 minus absorption of free hemoprotein in 100 mM phosphate buffer, 20% glycerol. b) Dependence of amplitude of spectral change of CYP2B4 (1 μM) in the interval 411-435 nm on radical concentration.

in differential spectrum at 435-440 nm and minimum at 409-411 nm [8, 12, 13]. Cytochrome P4502B4 in the presence of an alkylcyanide radical exhibits characteristic second-type spectrum with absorption maximum at 435 nm and minimum at 411 nm (Fig. 1a) similar to that induced by the second-type substrate aniline (Fig. 2). The dependence of amplitude of spectral changes on concentration of added spin label is shown in Fig. 1b.

The EPR spectrum of stable nitroxyl radical 4-cyano-TEMPO in aqueous solution (Fig. 3, curve *1a*) is a triplet whose components are of equal intensity. Broadening of the high-field component of the EPR spectrum is observed upon complex formation of the spin label with CYP2B4 (Fig. 3, curves *2a* and *2c*), which points to decreased label mobility in the bound condition. The amount of label in the spin-labeled protein was estimated by the intensity of the EPR signal of the preparation compared to the spectrum of free label in standard solutions. The protein and label were present in preparations in approximately equimolar amounts, i.e. there was no noticeable bound label washing off upon dialysis. In this connection, previous works on microsomal fraction using a similar free radical compound for labeling the membrane-bound cytochrome should be mentioned, in which the excess of label was removed upon pelleting and re-pelleting of the microsomes from label-free solution. After additional re-pelleting procedures, the amount of bound label in the preparations practically did not change [9, 22].

The fact that the radical occupies the site of the second-type substrates is also confirmed in addition to the typical binding spectrum by the absence of changes in the spectrum of the spin-labeled hemoprotein upon addition of aniline substrate (Fig. 2).

As it is known, the heme of P450 cytochromes is localized in the cavity formed by hydrophobic regions of the protein molecule, a kind of hydrophobic pocket that is closed by a turn of polypeptide helix from direct con-

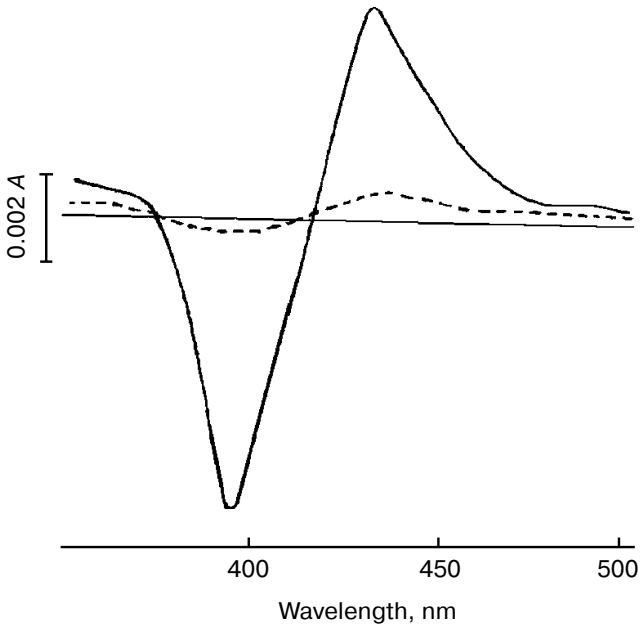

Fig. 2. Spectra of aniline substrate (15 mM) binding with free (1 μM) and spin-labeled CYP2B4 (1 μM) (solid and dashed lines, respectively).

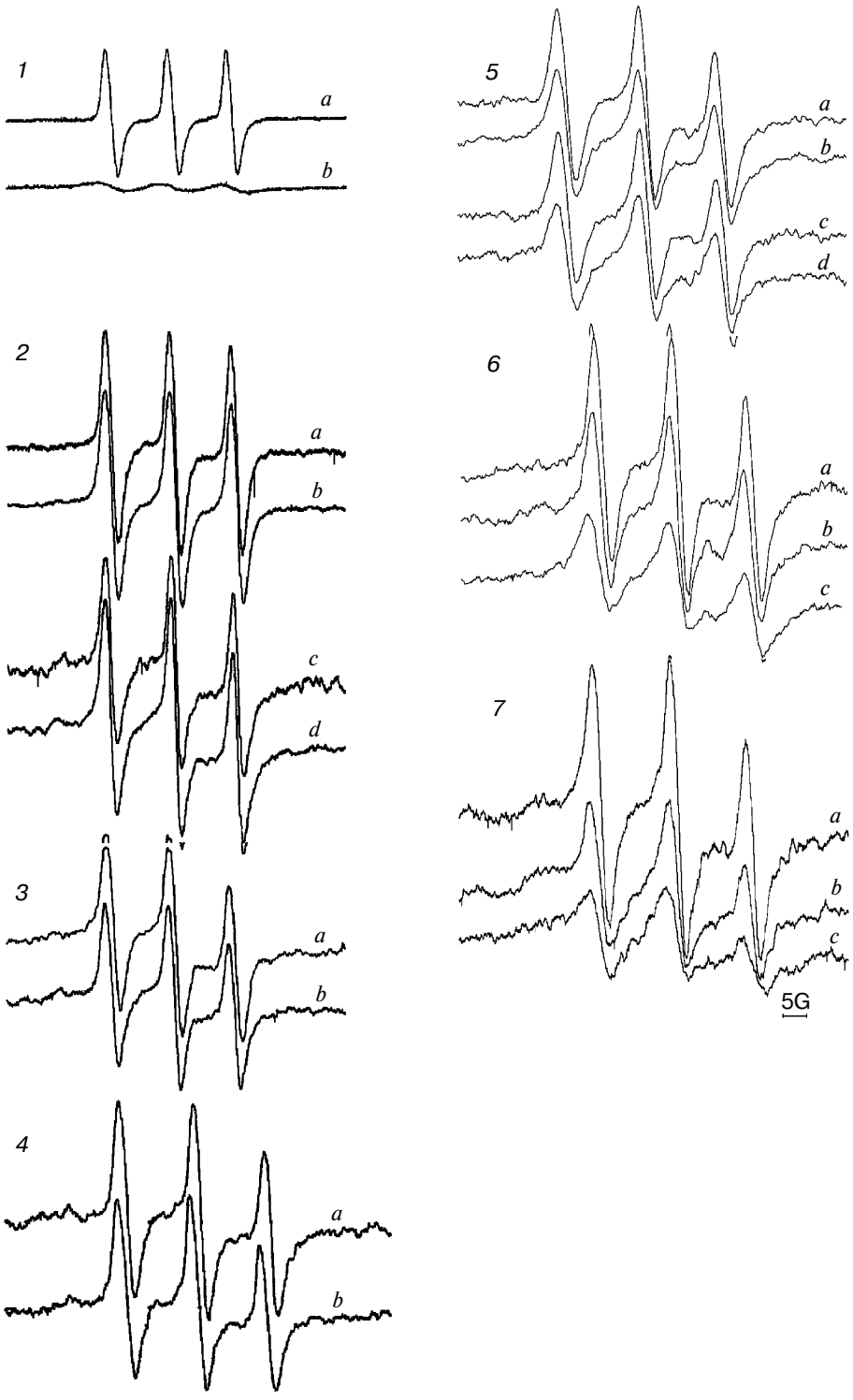

Fig. 3. EPR spectra of spin-labeled CYP2B4 (1 μM) in the presence of different additions: *1a*) free radical in solution without additions; *1b*) in the presence of ferricyanide (0.03 M); *2a*, *2c*) spin-labeled CYP2B4, 1 μM (two different preparations); *2b*, *2d*) the same preparations in the presence of ferricyanide; *3a*) spin-labeled CYP; *3b*) spin-labeled CYP in the presence of Emulgen 913 (0.025%); *4a*) spin-labeled CYP in the presence of aniline (12 mM); *4b*) spin-labeled CYP in the presence of aniline and ferricyanide; *5a*, *5c*) spin-labeled CYP (two different preparations) in the presence of benzphetamine (2 mM); *5b*, *5d*) the same preparations in the presence of benzphetamine and ferricyanide; *6a*) spin-labeled CYP in the presence of N,N-dimethylaniline (5 mM); *6b*, *6c*) spin-labeled CYP in the presence of N,N-dimethylaniline (3 and 5 mM, respectively) and ferricyanide; *7a*) spin-labeled CYP in the presence of Emulgen 913 (0.025%); *7b*, *7c*) spin-labeled CYP in the presence of Emulgen 913 (0.006 and 0.025%, respectively) and ferricyanide.

tact with the aqueous phase [2, 3]. To estimate the accessibility of the bound label to additions in the aqueous solution, paramagnetic ion of potassium ferricyanide was used whose contact with the spin-labeled compound is exhibited in broadening the EPR spectral components and resulting noticeable lowering of peak amplitudes. Broadening of spectra of similar free radical compounds in solution is in direct proportion with ferricyanide concentration [23]. However, in microsomal membranes [22] the dependence on the $K_3Fe(CN)_6$ concentration in the range of 0.01-0.06 M was much weaker because it was defined first of all by the label accessibility. Salt in higher concentrations influences protein condition in solution in an undesirable way. In our experiments ferricyanide added to samples within the same range of concentrations practically "melted" the triplet signal of the radical in solution (Fig. 3, curve *1b*) but had no effect on EPR spectra of spin-labeled CYP2B4 (Fig. 3, curves *2b* and *2d*), which was indicative of the bound label localization in the nonpolar region of the hemoprotein and absence of its contact with the aqueous phase.

All additions to the spin-labeled CYP2B4 preparations maintained equal final protein concentration and minimal volumes. When effects of studied compounds are registered in all series of experiments shown in Fig. 3, the same preparation of spin-labeled hemoprotein always served as control, and the EPR spectrum of control sample was measured immediately before the experimental sample. Solutions and additions were free of any foreign ingredients able to reduce radical.

The second-type substrate aniline, whose place in active site of spin-labeled CYP2B4 is occupied by alkylcyanide (see above as well as Fig. 2), at concentrations usually used for demonstration of CYP binding spectrum and enzymic activity had no noticeable effect on the triplet signal of the labeled hemoprotein, which also did not change after addition of ferricyanide (Fig. 3, curves *4a* and *4b*). In N,N-dimethylaniline, i.e. in a derivative of aniline with two methyl groups, the nitrogen atom is blocked for interaction with the heme iron, while the two additional nonpolar groups convert it into a classical first-type substrate. In the presence of dimethylaniline, addition of paramagnetic resulted in broadening the EPR signal, and the more obvious result was lowered amplitudes of spectral components (Fig. 3, curves *6b* and *6c*). Thus, the nonpolar contacts of dimethylaniline initiated conformational changes in the pocket near the heme with bound alkylcyanide already present there, which were most likely accompanied by increase in pocket volume and consequent contact with the aqueous phase, owing to which the radical became accessible for water-soluble paramagnetic. A similar result (Fig. 3, curves *5b* and *5d*) was observed in the case of another first-type substrate, benzphetamine, which usually serves as a test-substrate just for the CYP2B4 form. In the absence of ferricyanide both first-type substrates caused no changes in EPR spectrum of spin-labeled CYP2B4 (Fig. 3, curves *5a*, *5c*, and *6a*). It should be also noted that substrates do not influence sedimentation characteristics and oligomeric structure of the hemoprotein [24]. There is no reason to discuss the possibility of spin label displacement by the first-type substrates from the pocket region, because it is known that the second-type ligands bind the hemoprotein more firmly compared to the first-type ligands, and as follows from the data shown in Figs. 2 and 3, the second-type substrate aniline at concentration significantly exceeding the amount of bound alkylcyanide also has no effect on the label binding.

It is known that some nonionic detergents, including Emulgen 913, within certain concentration limits are able to activate hydroxylation reactions (including those of dimethylaniline and benzphetamine), which was usually explained by mimicking the activating effect of phospholipids. It follows from our previous works [5, 25] that the phenomenon of activation is realized only within the limits of detergent concentrations below its critical micelle concentration, when the detergent is in solution is rather in the form of individual molecules rather than forming large micelles, comparable with small proteins for this type of detergent. Emulgen binds in the CYP active site region, and like substrates it induces changes in the absorption spectra of the hemoprotein following the first type and, within known limits, it does not prevent substrate binding (in this case changes are additive). Detergent binding evidently results in the increase in volume of the pocket near the heme, which is the basis of the activating effect. In this case Emulgen at activating concentrations has no effect on oligomers of the hemoprotein. These data are indicative of the possibility of simultaneous presence of two different ligands in the active site of CYP.

Figure 3 (curves *7b* and *7c*) shows changes in the EPR spectrum in the presence of ferricyanide for two concentrations of Emulgen 913: within the limits below the critical micelle concentration (curve *7b*) and at the concentration corresponding only to a slight detergent effect on CYP oligomers (curve *7c*). In both cases there is an obvious signal broadening and lowering of the triplet component amplitudes in the EPR spectrum of spin-labeled CYP2B4, which is indicative of existence of contacts between the CYP active site region and the aqueous phase caused by conformational changes induced by the second ligand. In the absence of paramagnetic ion the detergent did not cause any change in the EPR spectrum (Fig. 3, curves *3a* and *3b*).

Reichman et al. in their abovementioned work on microsome fraction of rat liver [22] found spin label contact with the aqueous phase upon effects damaging both membranes and proteins (*p*-chloromercuribenzoate, 8 M urea, 4% deoxycholate), but they registered the increase in spin mobility in the presence of cytochrome P450 substrates phenobarbital and aminopyrine, which is consistent with our data.

The possibility of simultaneous existence of two ligand molecules in the cytochrome P4503A4 active site has been recently shown on crystals of a modified shortened form (native hydrophobic CYP forms have not been crystallized) obtained in the presence of large substrate (erythromycin) or inhibitor (ketoconazole) molecules [4]. It was shown that in the hydrophobic pocket near the heme two ketoconazole molecules can be present simultaneously, while erythromycin molecules can be differently bound in the active site. Significant conformational changes were registered in both models compared to the structure of the free hemoprotein, and they were different for the two ligands. The changes were accompanied by increase in volume of the active site region (up to 80% additional volume). Taking into account these results, the variability of conformational changes in the heme pocket region is discussed, which explains in part the broad substrate specificity of CYP. The variability of conformational changes upon ligand binding is confirmed by results of investigations of modified CYP2B4 using X-ray analysis, isothermal calorimetry, and spectroscopic methods. It has been shown that imidazole derivatives of different chemical structure (CYP inhibitors) induce different conformational rearrangements in the active site region [6, 7]. Simultaneous presence of two or more molecules of the same or different ligands in the CYP active site region suggest the possibility of homotropic or heterotropic cooperative effects. Since human CYPs metabolize practically all drugs, the phenomenon of heterotropic cooperativity can be clinically significant, causing unpredictable consequences in the case of simultaneous use of different drugs.

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