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LOCALIZATION OF THE ACTIVE CENTER OF MICROSOMAL CYTOCHROME P-450

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SUMMARY To solve the problem of localization of the active center of cytochrome P-450 in microsomal membranes, new bifunctional compounds (I-IV), which contain pyridine radical, aliphatic chain of variable length and diphosphonic acid ("floating" molecules) have been applied. These compounds inhibit oxidation and binding of the substrates of cytochrome P-450 (aminopyrine and aniline), inhibition being of a competitive character. Measurements of distribution coefficients between water and membranes of microsomes and liposomes from egg phosphatidylcholine evidence that the microsomal proteins are necessary for providing effective interaction of I-IV with microsomal membrane. The ^{1}H-NMR method has demonstrated compounds to be incorporated into lipid bilayer so that the non-polar part is in the inner membrane volume. The results obtained confirm our previous conclusion (Krainev A.G., Weiner L.M., Alferyev I.S., Slynko N.M.(1985) Biochim. Biophys. Acta, 818, 96-104) about localization of the active center of microsomal cytochrome P-450 at the depth of $\sim$18 Å from the hydrophilic surface of a membrane.

The electron-transfer complex of liver microsomes catalyzes conversions of numerous exogenous and endogenous substrates /1/. Localization of the active center of cytochrome P-450 still remains one of the most important questions of the functioning of the above complex. Some attempts have been made to solve this problem using different techniques.

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A limited proteolysis of a microsomal fraction does not result in a water-soluble catalytically active fragment of P-450 /2/. The residual activity of the P-450 fragment associated with the membrane is ca. 40% /3/. Upon treating cytochrome $P\text{-}450_{11\beta}$ ($M_r \sim 47000$) built in liposomes with trypsin the membrane serves as a protection. The enzyme transforms into a peptide fragment ($M_r \sim 34000$) which contains heme and is catalytically active /4/. Unfortunately, this approach does not provide information on the depth of location of the enzyme active center in a lipid bilayer.

An alternative approach is application of physical methods to the solution of the problem under consideration. For example, the authors /5/ have measured the distance between spin-labeled methyrapone bound to the active center of microsomal P-450 and water-soluble ion $Fe(CN)_6^{3-}$. However, they reported only the lower estimate of the distance (r) between the hydrophilic surface of the membrane and $\rangle N\text{-}O^{\bullet}$ probe fragment: $r > 8$ Å. Yudanova et al. /6/, who examined the interaction of the heme of microsomal P-450 with water- and membrane-soluble fluorescent probes, and Rich et al. /7/, who used the method of stationary saturation of ESR spectra of P-450 heme also failed to obtain any structural information.

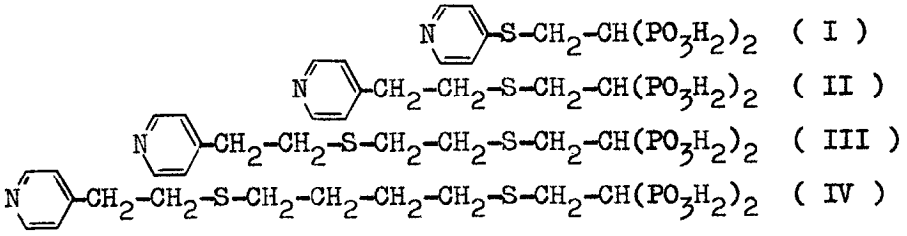
Based on the dependence of the parameters of P-450 interaction with the substrates on their hydrophobic nature /8,9/, the authors /10/ have proved the enzyme active center to be immersed into a phospholipid bilayer.

To solve the problem of localization of the active center of P-450 in the membrane, we have proposed a method of "floating" molecules /11-13/, which is based on the use of bifunctional compounds containing a hydrophilic "head", a variable aliphatic chain with cytochrome P-450 substrate on its end. In /11-13/ a naphthalene residue served as substrate.

In this work we have used compounds (I-IV), involving pyridine residue - a substrate of P-450, which can directly interact with Fe^{3+} in the enzyme active center /14/, as well as an aliphatic chain and a residue of diphosphonic acid. These compounds of amphiphylic nature were introduced into a microsomal membrane, where they interacted with the active center of P-450 with different efficiency. Based on the chemical structure of the compounds, the distance from hydrophilic surface to Fe^{3+} has been estimated.

EXPERIMENTAL

Microsomes from livers of Wistar rats (150-200 g) were prepared as described in/15/.The contents of microsomal proteins and P-450 were determined by methods /16,17/, the activity of microsomal NADPH-cytochrome P-450 reductase was measured as in /18/. Compounds (I-IV) had the following structure*:



Oxidation of aniline and aminopyrine was tested according to /19,20/. The equilibrium distribution of compounds (I)-(IV) between aqueous and lipid phases was determined from absorption in UV-region of aqueous solutions of (I-IV) ($\epsilon_{295} = 1.1 \cdot 10^3$ $M^{-1}cm^{-1}$ (I), $\epsilon_{250} = 6.2 \cdot 10^2$ $M^{-1}cm^{-1}$ (II-IV)) after centrifugation (100000g, 60 min) of samples containing compounds (I-IV), microsomes or liposomes from egg phosphatidylcholine /21/.

VIS and UV absorption spectra were registered on a "Beckman" DB-GD spectrophotometer and spectra of microsomal P-450 bounded with (I-IV) on a "Hitachi-557" spectrophotometer. Reversed phase chromatography on a column of 0.2 x 6.2 cm, filled with sorbent "Nucleosil 5 C_{18}"(Macherey-Nagel, FRG) was employed. A chromatographic analysis was made using "Millichrom"(USSR) with step gradient MeOH mixed with 0.5 LiCl in 0.05 M tris-HCl (pH 7.6) buffer. The ^{1}H-NMR spectra were taken on a JEOL-FX-90Q NMR-spectrometer.

*Synthesis and properties of compounds (I-IV) will be published elsewhere.

RESULTS AND DISCUSSION

Compounds(II-IV) were bound to microsomal P-450 as typical type II substrates (λ_{max} = 425 nm, λ_{min} = 390 nm)/14/. For compound(I)spectral variations in the differential spectrum of P-450 have not been registered. The binding parameters of (I-IV) and those of pyridine as a standard are listed in table 1. From the table it is seen that the efficiency of the interaction of (I-IV) with the P-450 active center is different being maximum for (III). (i.e. comparable in K_s and ΔA_{max} with pyridine).

A study of the influence of (I-IV) on the interaction of the enzyme with typical substrates (aminopyrine (type I) and aniline (type II)) is an alternative method for testing the penetration of an aromatic part of compounds (I-IV) into the active center of P-450. Compounds (I-IV) inhibit the microsomal oxidation of the substrates of cytochrome P-450 according to a competitive type, with the compound (III) being the most effective inhibitor in all cases (see table 2). As shown by control experiments, at concentrations of (I-IV) of up to 3 mM at 3-5 mg/ml of the microsomal protein cytochrome P-450 does not transform into its inactive form (P-420) and the activity of microsomal NADPH-cytochrome P-450 reductase remains unchanged.

Table 1. Parameters of binding of compounds (I-IV) to microsomal cytochrome P-450

Compounds	I	II	III	IV	Pyridine
K_s, mM	-	0.59	0.15	0.6	0.09
ΔA_{max}, o.d.	-	0.011	0.045	0.01	0.046

Experimental conditions: 2.3 mg/ml of microsomal protein, 1.4 μM P-450, 0.1 M tris-HCl (pH 7.6) buffer. Temperature 25°C.

Table 2. Influence of compounds (I-IV) on the interaction of aniline and aminopyrine with microsomal cytochrome P-450

Compounds	I	II	III	IV
Inhibition constants for microsomal oxidation of aminopyrine, K_i, mM	1.2	1.25	0.12	2.4
Inhibition constants for microsomal oxidation of aniline, K_i, mM	10	0.85	0.17	1.5
Effect on $\triangle A_{max}$ (o.d.) at aminopyrine binding (control $\triangle A_{max}$ = 0.013)	0.012	0.010	0.006	0.011
Effect on $\triangle A_{max}$ (o.d.) at aniline binding (control $\triangle A_{max}$ = 0.022)	0.021	0.017	0.008*	0.017

In all experiments the content of microsomal protein was 1.75 mg/ml (1.1 μM P-450) in 0.1 M tris-HCl (pH 7.6) buffer. Compounds (I-IV) (if any) were introduced into the same buffer before substrate addition.

*Spectral variations characteristic for type I substrates (see text).

Compounds (I-IV) inhibited aniline and aminopyrine binding to microsomal cytochrome P-450 (see table 2). As in the case of inhibition of the oxidation of P-450 substrates, the compounds can be arranged in the following order: III > II ∿ IV > I, with the compound (III) having the largest inhibitory effect. Interestingly, in the presence of compound (III) addition of aniline produces changes in the differential spectrum of cytochrome P-450 which are characteristic of the binding of type I substrates (λ_{max} = 390 nm, λ_{min} = = 420 nm). The similar effect for the aniline binding has been observed in the presence of 0.5 mM of pyridine. These facts indicate

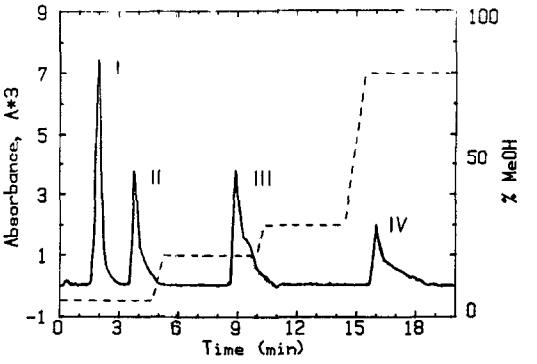


Fig.1. Reversed phase chromatography of compounds (I-IV). 4 μl of 0.01 M solution of compound were introduced. Solid line represents absorption at 250 nm, dashed line - concentration of MeOH. Flow rate is 100 μl/min.

that compound (III) occupies all coordination sites of type II substrates, therefore, aniline binds to P-450 on the sites of type I substrates.

The differences in hydrophobic properties of the compounds (I-IV) were confirmed by the HPLC method (reversed phase chromatography). As can be seen in Fig.1, the retention time of the compounds (I-IV) increases with increasing aliphatic chain length n. This is evidence that lipophility of compounds increases in the order: I<II<III<IV. Thus, from the chemical structure of the compounds under study and HPLC data it can be assumed that the efficiency of the incorporation of (I-IV) into membranes increases in the order: I<II<III<IV. However, for the ratios studied between the(I-IV) concentrations and lipid for native microsomes, the distribution coefficients do not depend on the length of an aliphatic chain length (see table 3). For boiled microsomes (complete conversion of cytochrome P-450 into cytochrome P-420) and liposomes from egg phosphatidylcholine, such dependence takes place, which is the common fact for interactions of compounds with different hydrophobicity with hydrophobic media /22,23/. This result indicates that the native microsomal proteins play an important role in the interaction of compounds (I-IV) with membranes.

Table 3. Distribution coefficients (K = concentration in a lipid phase/concentration in an aqueous phase) of compounds (I-IV) for different preparations

Compounds	I	II	III	IV
Microsomes (3)	263±70	264±68	338±63	239±71
Boiled microsomes (3)	106±51	315±120	499±131	589±146
Liposomes (4-5)	64±13	310±78	423±116	1222±352

Table gives values averaged over several experiments (number in brackets) ± standard deviation. The initial mixture contained $2 \cdot 10^{-4}$ M of compounds (I-IV) and from 1 to 5 mg/ml of lipid in 0.1 M tris-HCl (pH 7.6) buffer. Temperature 25°C.

It is known /25/ that amphiphilic compounds with an aliphatic chain exceeding 5-6 carbon atoms can incorporate into phospholipid membranes. We have conducted additional investigation of the interaction of (I-IV) with phospholipid membranes. Fig.2 presents our experimental results on the effect of the $Fe(CN)_6^{3-}$ paramagnetic ion on the intensity of the ^{1}H-NMR spectra of compound (III) pyridine α-protons (δ = 8.5 ppm). It should be noted that the data in table 3 indicate that at a lipid concentration of 100 mg/ml (the volume of one lipid molecule is about 1200 Å^3, hence the volume ratio of aqueous and lipid phases is 91:9) the concentration of (III) in lipid will be about $1.27 \cdot 10^{-1}$ M and in water - $3 \cdot 10^{-4}$ M. Thus, in the presence of lipid the main contribution to the line intensity will be made by (III) included in the membrane (the ^{1}H-NMR linewidth in both phases differs negligibly). It is obvious that in solution $Fe(CN)_6^{3-}$ interacts effectively with (III), leading to the intensity decrease. At the same time the membrane exhibits a protecting effect: the pyridine radical of (III) appears to be inaccessible for $Fe(CN)_6^{3-}$. This result shows that the com-

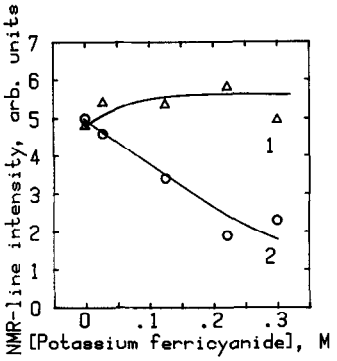


Fig.2. Membrane protecting effect for interaction between $Fe(CN)_6^{3-}$ and pyridine radical of molecule (III)($1.175 \cdot 10^{-2}$ M in 0.05 M K-phosphate (pH 7.6) buffer, 99% D_2O, lipid (if any) = 100 mg/ml) in the presence (1) and absence (2) of liposomes. Temperature 25°C.

pounds(I-IV)are built in phospholipid membrane in such a way that a non-polar part is in the inner membrane volume.

Thus, from all of the above it follows that compounds (I-IV) penetrate into the active center of P-450 with different efficiency (see tables 1 and 2), but it cannot be attributed to the difference in concentrations of (I-IV) in the lipid phase at the ratios between lipid and compounds (I-IV) employed (see table 3). In all cases,the maximum effect of(I-IV) on a microsomal system has been achieved using compound (III). As has already been mentioned, type II substrates of P-450 coordinate directly to the iron of the enzyme heme /14,24/. Based on this and on the assumption that the distance from the hydrophilic "head" to a pyridine radical in molecule (III) at a maximum length conformation accounts to about 17 Å, one can draw the conclusion that the heme of microsomal P-450 seems to be located at the depth of ca. 18 Å from the membrane surface.

However, the results obtained can be interpreted otherwise. By taking into account that the molecule of cytochrome P-450 is quite large (radius of about 24 Å) and is partly above the membrane surface /4/, it is possible to suppose that compounds (I-IV) are built in the hydrophobic entrance of the crevice of the P-450

active center rather than in the lipid bilayer. In this situation, one should evaluate the distance between the active center and the charged surface of the molecule of cytochrome P-450 which can react with a hydrophilic part of compound (III). This distance is ca. 18 Å.*

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*An independent evidence for the active center localization at a distance of ∿14 Å from the membrane surface has been obtained recently by Kulikov et al./26/.

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