Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 40-46

DIFFERENT STRUCTURE OF THE COMPLEXES OF TWO CYTOCHROME P-450 ISOZYMES WITH ACETANILIDE BY ^{1}H-NMR RELAXATION AND SPECTROPHOTOMETRY

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Received April 8, 1993

SUMMARY: The functional and spectral characteristics of the interaction of acetanilide with phenobarbital- and methylcholanthrene- induced rat liver microsomes, as well as with corresponding major isozymes (cytochromes P-450b and P-450c) have been compared. The magnitude of the reverse 1st type binding spectra proved to be negatively correlated with the acetanilide oxidation on isozymes under study. The data on paramagnetic relaxation of acetanilide protons in the presence of P-450 have shown the structure of the enzyme-substrate complex to be different for two isozymes, acetanilide molecule being closer to Fe ion in the active site in the case of P-450c, which is active towards acetanilide oxidation. For the P-450c - acetanilide complex the group oxidized (phenyl) is the closest to Fe ion. © 1993 Academic Press, Inc.

Since the paper of Schenkman et al. [1] specialists on cytochrome P-450 have been interested in the connection between binding spectra and the oxidation of different compounds on cytochrome P-450. However, some details are still unclear. Furthermore, the problem exists in what way the specificity of substrate oxidation by different isozymes of cytochrome P-450 reflects the structural and kinetic peculiarities of the corresponding enzyme-substrate complex. To this end we have compared the functional and spectral characteristics of the interaction between a definite substrate and two types of microsomes and the main P-450 isozymes involved in them. Acetanilide has been taken as a substrate. For comparison we have taken P-450b and P-450c from rat liver and accordingly liver microsomes from the animals induced with phenobarbital and methylcholanthrene. The information about the geometry of enzyme-substrate complexes can be obtained by measuring the rate of paramagnetic relaxation of substrate protons in the complexes with cytochrome P-450 [2-7]. This approach has also been used in our work.

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0006-291X/93 $4.00
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MATERIALS AND METHODS

Reagents. We have found the yield and stability on storage of cytochrome P-450 and NADPH: cytochrome P-450 reductase depending dramatically on the purity of glycerol used in isolation procedures. The same has been shown for other enzymes [8]. Thus glycerol and 10% solution of detergent (Renex-690, ICI Co., UK) were purified mainly as in [9]. Ethylacetate was redistilled just before usage; acetanilide was recrystallized twice from water. 2,4,6-d_3-acetanilide was kindly given by Dr. O. Usov (Inst. of Chemical Kinetics and Combustion, Novosibirsk) and recrystallized twice from water. Catalase from bovine liver , 46000 u/mg (Sigma, USA) was freed from thymol by gel filtration. NADPH concentration (Reanal, Hungary) was controlled by absorbance at 340 nm. All other chemicals supplied by Reachim (USSR) were of reagent grade and were used without purification.

Animals and enzymes. Male Wistar rats (150-200g) were induced and microsomes were isolated as in [10]. Cytochrome P-450 isozymes were obtained according to [11]. NADPH-cytochrome P-450 reductase was purified as described in [12].

Microsomal oxidation of acetanilide was assayed by 4-hydroxyacetanilide formation being chromatographically determined. Reaction mixture, 0.4 ml, usually consisted of 0.1-2 mg/ml microsomal protein, 0.05 M potassium phosphate buffer, pH 7.5, 3 mM $MgCl_2$, 1-13 mM acetanilide, 1 mM NADPH. On oxidation of acetanilide in the reconstituted system, the sample, 0.4 ml, consisted of the same buffer, NADPH and $MgCl_2$ concentration, 30 μg/ml dilauroylphosphatidylcholine, 2.2 units/ml reductase, 88 pmol/ml P-450, 6000 units/ml catalase, 3.6 mM acetanilide. The mixture of P-450, dilauroylphosphatidilcholine and reductase had been preincubated in the minimum volume for 5 min. at 37°C. In microsomes and the reconstituted system the reaction was started at 37°C by adding NADPH and stopped by pouring out the reaction mixture into the tube with 1ml of cold ethylacetate containing 120 mg of NaCl. The reaction rate was constant at least for 4 min. after starting.

Chromatographic analysis. The samples were treated with 2×1 ml of ethylacetate, then the extract was evaporated to dryness under inert gas flow. The residue was dissolved in 50 or 100 μl of eluent; an aliquot (4-10 μl) was loaded on the 60×2 mm column with Nucleosil 100-5C18 (Machery-Nagel, FRG). The column efficiency was 2000-4000 theoretical plates (by 4-OH-acetanilide), eluent 22% methanol-water (v/v). Chromatography was carried out under isocratic conditions on a Milichrom HPLC instrument (Nauchpribor, Orel, Russia) with UV-detection at 240 nm. Quantitative analysis was performed by either calibration or internal standard. The samples for calibration with all the components of reaction mixture and a certain quantity of 4-hydroxyacetanilide were treated as described above, but NADPH was added just before the termination of the reaction. When analyzing by internal standard, 10 μl of 3-hydroxyacetanilide aqueous solution of definite concentration was added to the samples before the extraction procedure.

Binding spectra were recorded on Hitachi-557 spectrophotometer (Japan) by adding aliquots of acetanilide buffered solution to the cuvette containing P-450 or microsomes. The buffer was added to the reference cuvette. The dependence of spectral amplitude on substrate concentration was treated using the least-square fit.

NMR. AM-250 and WP-200 spectrometers (Bruker, FRG) were used. T_1 was measured using inversion-recovery procedure with the composite 180° pulse and suppression of HDO signal to avoid dynamic range problem during FID digitization. T_1 was calculated by our own program (least squares non-linear three parameter fit), because spectrometers software gave incorrect value of the standard deviation of T_1. The dependence of T_1 on concentration was treated in the similar way. To prepare the samples for NMR, cytochrome P-450 was transferred to D_2O-potassium phosphate buffer, pH_{obs} 7.5 by gel

filtration through the column with Toyopearl HW-40 (Toyo-Soda, Japan). All the solutions were passed through the column with Chelex-100 resin (Bio-Rad Lab., USA) to remove paramagnetic ions impurities.

RESULTS AND DISCUSSION

In the two above mentioned types of microsomes acetanilide was oxidized to yield 4-hydroxyacetanilide, no other oxidation products were found. The reaction rate hyperbolically depends on the substrate concentration, so apparent Michaelis (K_M) and catalytic (k_2) constants are determined (Table 1). Acetanilide exhibits the binding spectra of the reverse 1st type with both microsomes types, λ_{max}=422 nm, λ_{min}=388 nm. Interestingly, the correlation of K_S for two microsomes types is reverse compared to K_M, i.e. K_S is less for phenobarbital microsomes, the amplitudes calculated per mol of P-450 being close to each other. The maximum amplitude decreases twofold, compared to microsomes, for P-450b, whereas for P-450c it decreases by a factor of twenty, so the binding spectrum actually vanishes. It is, however, P-450c that oxidizes acetanilide in the reconstituted system. For P-450b no oxidation product was found in the same conditions, only the upper limit of specific activity being obtained. Hence it might be concluded that the optically observed complex is nonproductive and no oxidation is possible on its formation. Not only 4-hydroxy- , but 3-hydroxyacetanilide was found as oxidation product for P-450d (molecular activity 21 min^{-1} and 4 min^{-1}, correspondingly, not shown in the Table). This 3-hydroxy- product was not observed for other isozymes within the analysis sensitivity.

Additional information concerning the structure of P-450-substrate complexes may be extracted using NMR technique [2-7]. In this case the problem appears to distinguish the paramagnetic contribution to the relaxation observed and contribution of the relaxation time and lifetime in the complex to their sum experimentally determined. The problem was discussed in detail earlier [2,5,6]. To simplify the spectrum in NMR experiments 2,4,6-d_3-acetanilide was used, with two narrow singlets due to methyl (δ=2.15) and phenyl (δ=7.40) protons. In the presence of cytochrome P-450b and P-450c the observable acetanilide protons longitudinal relaxation time (T_{1obs}) depends on acetanilide concentration due to P-450 - acetanilide complex formation (fig. 1). This dependence is not observed for the solution without P-450 within the range of concentration used. From those dependences one can calculate the dissociation constant of the complex (K_D) and the effective linewidth in complex $(T_{1M}+\tau)^{-1}$ using the formula

$$1/T_{1obs}=1/T_{1d}+P/((T_{1M}+\tau)\times(K_D+S)),$$

TABLE 1

Kinetic constants of acetanilide oxidation by microsomes and isolated P-450 isozymes, spectral parameters of acetanilide–P-450 complexes according to data of optical and ^{1}H-NMR spectroscopy

	Phenobarbital microsomes	Methylcholanthrene microsomes	P-450b	P-450c
Oxidation				
[a] $k_2(min^{-1})$	[b] 3.8	8.5	<1.5	67
K_M(mM)	3.1	0.85	-	1.0±0.26
Binding spectra (reverse type 1)				
K_S(mM)	0.58	1.8	1.4	0.74±0.25
[c] $\Delta\varepsilon_M(mM^{-1}cm^{-1})$	48	36	22	1.8

NMR Temperature (K)	Parameters	P-450b	P-450c
296	K_D(mM)	0.65±0.08	1.6±0.16
	$(T_{1M}+\tau)^{-1}$-Methyl (Hz)	113±8	321±19
	$(T_{1M}+\tau)^{-1}$-Phenyl (Hz)	102±8	542±34
308	K_D(mM)	2.1±0.24	1.55±0.23
	$(T_{1M}+\tau)^{-1}$-Methyl (Hz)	172±18	252±28
	$(T_{1M}+\tau)^{-1}$-Phenyl (Hz)	151±17	484±39

[a] V_{max} divided by P-450 concentration.
[b] The standard deviation is less than 10 percent of value unless otherwise indicated.
[c] ΔA_{max} divided by P-450 concentration.

where T_{1d} denotes the relaxation time independent of the substrate concentration (diamagnetic); τ stands for the lifetime of the complex; P designates the paramagnetic protein concentration; S is the substrate concentration [5].

The data obtained for two temperatures are given in Table 1. The decrease of $(T_{1M}+\tau)$ with increasing temperature for P-450c testifies to a fast exchange, i.e. a small contribution of τ to the sum. Hence, this sum is approximately equal to T_{1M}, and accordingly the distance from Fe^{3+} ion in the active site to the corresponding group of substrate protons can be calculated using the Solomon-Bloembergen equation [13]. The distances in

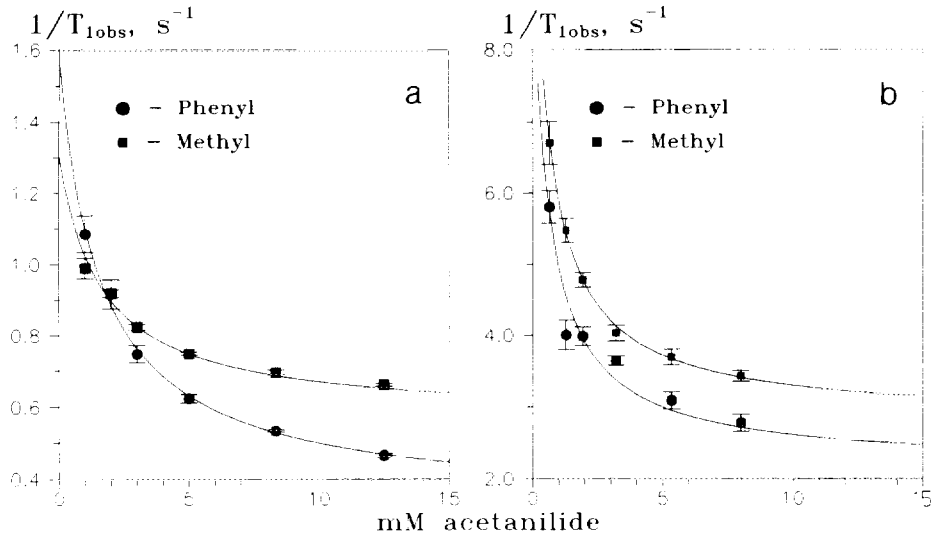

Fig. 1. Dependence of longitudinal relaxation rate of 2,4,6-d_3 acetanilide protons (● -phenyl, ■ -methyl) on d_3-acetanilide concentration in the presence of cytochrome P-450, Temp 296 K
a) [P-450c]=3.73μM
b) [P-450b]=44.6μM.
Solid lines are the least squares fit with parameters listed in Table 1.

Table 2 were calculated for both temperatures using the correlation time of electron-nuclear dipole interaction and its temperature dependence [14]. For details see [5] and [6]. Table 2 shows that the distances are actually the same for different temperatures which confirms a negligible contribution of the lifetime to the (T_{1M}+τ) sum for both temperatures. Further, the activation energy related to the temperature dependence of T_{1M} can be

TABLE 2

The distances from Fe^{3+} ion to acetanilide protons in the acetanilide-P-450 complex

Isozyme	Distance (Å)		Temperature (K)
	Phenyl	Methyl	
P-450c	6.13	6.84	308
P-450c	6.10	6.66	296
P-450b	7.2	7.0	*a*

Standard deviation is below 0.1 Å.

a not indicated due to absence of temperature dependence of T_{1M} suggested (see text).

estimated from the ratio of the apparent linewidths in the complex for two temperatures. The value was determined to be 2.7 kcal/mol which is close to 3 kcal/mol obtained by Philson et al. [14]. The forward rate constant of the complex formation can be calculated by using K_D and τ values as $K_D=1/(k_1*\tau)$. At 296 K, $k_1=(0.3\div1.2)\times10^6$ $M^{-1}s^{-1}$.

For P-450b the apparent linewidth is less and the temperature dependence of K_D is steeper probably due to a strong temperature dependence of τ. This is confirmed by the decrease of $(T_{1M}+\tau)$ with increasing temperature (slow exchange). The increase in K_D may be attributed to the decrease in the lifetime of complex, that corresponds to the activation energy of 18 kcal/mol (for proton exchange - 15 kcal/mol, [14]).Then τ can be calculated assuming the temperature dependence of T_{1M} to be much weaker. This approach gives: τ_{308}= 1.4 ms, τ_{296}= 4.5 ms, T_{1M}(phenyl)= 5.2 ms, T_{1M}(methyl)= 4.4 ms, $k_1= 3\times10^{-5}M^{-1}s^{-1}$.

The calculated distances from the corresponding protons to Fe^{3+} ion at the active site are listed in Table 2. The model constructed using the obtained data gives the structure with the phenyl ring plane is nearly parallel to that of haem.

Thus, a difference in the structure is observed for the complex of acetanilide with cytochromes P-450b and P-450c: (i) The distances to Fe ion in the complex with P-450b is larger; (ii) These distances are actually the same for methyl- and phenyl- protons of acetanilide in the complex with P-450b and are different for P-450c; (iii) The position subject to oxidation (phenyl) is closer to Fe ion. A dramatic dependence of reaction efficacy on the distance can be explained by the mechanism proposed by White et al. [15]: since hydrogen atom is transferred to $(Fe=O)^{3+}$ probably by a tunneling, this dependence must be exponential, i.e. very steep.

Thus, the comparison of the structure of the enzyme-substrate complex with the catalytic activity of the different isozymes of P-450 shows that the substrate specificity of P-450 isozymes depends on the geometrical parameters of the enzyme-substrate complex. The fact that no acetanilide oxidation was observed on P-450b shows that the distance between Fe ion and substrate is probably too large for effective reaction. This reaction in phenobarbital microsomes is likely to be due to the minor forms of P-450.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of Dr A. Denisov (In-t of Organic Chemistry, Novosibirsk) in the experiments on WP-200, and are thankful to S. Bizyaeva (Novosibirsk University) for carrying out some experiments.

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