

¹H-NMR study of the interaction of aminopyrine with purified rat liver microsomal cytochrome P-450

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Longitudinal relaxation (T_1) measurements for all lines ($N(CH_3)_2$, $N(CH_3)$, $C(CH_3)$, phenyl) in the aminopyrine ¹H-NMR spectrum were used to study the interaction of aminopyrine with purified microsomal cytochrome P-450 from livers of phenobarbital-treated rats. The paramagnetic contribution to the observed T_1^{-1} values was determined from its dependence on aminopyrine concentration. The Solomon-Bloembergen equation was used to calculate between Fe^{3+} and aminopyrine distances in the enzyme-substrate complex.

For all protons these distances are about 8 Å.

Cytochrome P-450 Aminopyrine ¹H-NMR Enzyme-substrate interaction Fe^{3+} -proton distance

1. INTRODUCTION

To understand the mechanism of catalysis of cytochrome P-450, a detailed study of the interaction of this protein with various substrates is needed. This interaction is usually studied by optical methods [1,2] which, however, give no idea of the spatial structure of the enzyme-substrate complexes. In this respect the NMR method, which has been employed, e.g., by Novak et al. [3–6], is more informative. Application of this method to the above complexes is based on the fact that rapid relaxation of the electron spin of Fe^{3+} in the active centre of cyt P-450 enhances relaxation of substrate protons in an enzyme-substrate complex according to the Solomon-Bloembergen equation [7]:

$$\frac{1}{T_{1M}} = \frac{2\hbar^2\gamma_I^2\gamma_S^2S(S+1)}{15r^6} \left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2} \right] + \frac{2S(S+1)A^2}{3\hbar^2} \left[\frac{\tau_c}{1+\omega_S^2\tau_c^2} \right] \quad (1)$$

Abbreviations: AP, aminopyrine; cyt, cytochrome; KP_1 , potassium phosphate buffer

Here T_{1M} is the relaxation time of the nucleus in the vicinity of the paramagnetic iron; r is the distance between the nucleus and the ion; τ_c and τ_e are the correlation times of the dipole-dipole interaction and hyperfine coupling, respectively; ω_I and ω_S are the nucleus and electron Larmor frequencies; $A/\hbar$ is the hyperfine coupling constant (Hz); the remaining symbols are standard (the second term of the equation is so insignificant in comparison with the first that we shall henceforward neglect it. In its turn, T_{1M} is related to the observable relaxation rate of the substrate protons (to be more precise, to its part which is determined by the interaction with the paramagnetic centre), i.e., T_{1A} , to the residence time of the substrate in the complex, τ and molar fraction of the complex, α [8]:

$$\frac{1}{T_{1A}} = \frac{\alpha}{T_{1M} + \tau} \quad (2)$$

Thus, relaxation time (T_1) measurements for various groups of substrate nuclei (R_i) in the presence of the enzyme can be used to evaluate the $Fe-R_i$ distances and hence to deduce the geometrical details of the enzyme-substrate complex. In the present work this NMR technique has

been employed to study the interactions of aminopyrine (AP) with the purified cyt P-450 from liver microsomes of phenobarbital-treated rats.

2. MATERIALS AND METHODS

In our experiments pharmacopoeia AP, KH_2PO_4 (special purity grade), deuteriurea and D_2O (99.8%) (Sojuzkhimreaktiv) were used.

Induction of Wistar male rats (120–140 g) by phenobarbital and isolation of microsomes were carried out as in [9,10]. Highly purified cyt P-450 was prepared using a combination of the methods described in [11] and [12]. Concentrations of the protein and cyt P-450 were determined as suggested in [13] and [14]. The content of cyt P-450 in our preparations ranged from 13 to 15 nmol/mg protein.

Before NMR experiments, the cyt P-450 solution ($\sim 5 \times 10^{-5}$ M) was passed through Sephadex G-50 coarse (Pharmacia) (0.4×20 cm) and Chelex-100 (BioRad) (0.4×2 cm) columns placed in series and equilibrated with 0.1 M KPi , in D_2O (pH_{obs} 7.5). The same buffer was used for elution. All other solutions were also prepared in D_2O and passed through a Chelex-100 column to remove contaminating paramagnetic ions.

Optical measurements were made using Hitachi-556 and Beckman DB-GT spectrophotometers. NMR spectra were recorded using Varian XL-200 and Bruker WP-200 spectrometers. T_1 was determined using a modified inversion-recovery sequence ($T - 90^\circ_x - 240^\circ_y - 90^\circ_x - \tau - 90^\circ - \text{AT}$) [15,16]. The standard deviation of T_1 was 2–5%. The dependence of the relaxation rate on substrate concentration was treated by the least-squares method, the measured K_D being the same for all lines of the substrate.

3. RESULTS

As indicated by the optical spectra, AP binds to purified cyt P-450 as a type I substrate ($\lambda_{\text{max}} = 387$, $\lambda_{\text{min}} = 422$ nm, $K_S = 5$ mM). The ^1H -NMR spectrum of AP is shown in fig.1. Cyt P-450 addition enhances the relaxation of all protons of AP to nearly the same extent (fig.2.). To evaluate the paramagnetic contribution, it has been suggested [3] that the protein should be converted into a diamagnetic reduced carbonyl form. Then the dif-

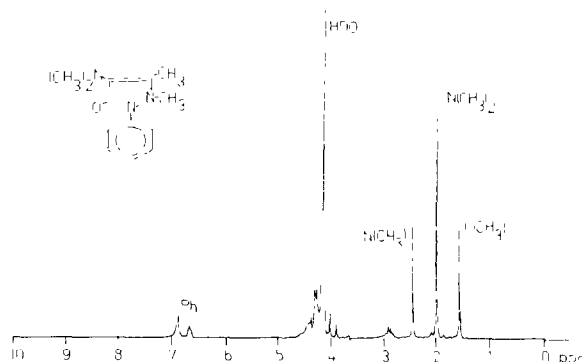


Fig. 1. NMR spectrum of 0.1 M aminopyrine solution in KPi in D_2O , pH_{obs} 7.5.

ference between the 'paramagnetic' and 'diamagnetic' relaxation rates is the paramagnetic contribution:

$$T_{1P}^{-1} = T_{1P450ox}^{-1} - T_{1P450red-CO}^{-1} \quad (3)$$

The corresponding results are presented in table 1.

Similar experiments were carried out in the presence of 7 M urea which leads to complete conversion of the protein to cyt P-420 (optical tests) and destroys its tertiary structure. The results are listed in table 2. As can be seen (tables 1 and 2), in

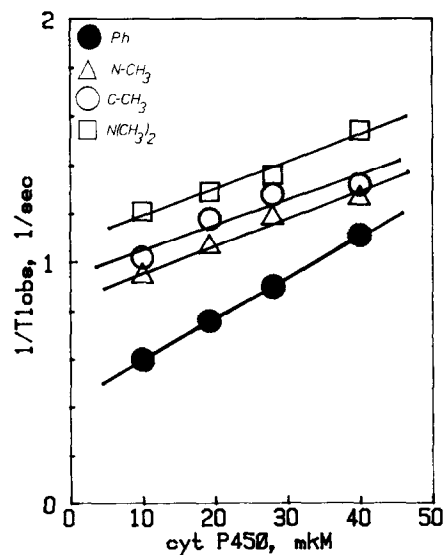


Fig. 2. Relaxation rate of aminopyrine protons (0.012 M) vs concentration of cytochrome P-450. 0.1 M KPi in D_2O , pH_{obs} 7.5.

Table 1

Relaxation rates of aminopyrine protons observed in the presence of the oxidized and CO-reduced cytochrome P-450

Group	$1/T_1$ (s^{-1})		
	P-450-ox	P-450-red + CO	$1/T_{1P}$
C-CH ₃	1.29	1.15	0.14
N(CH ₃) ₂	1.45	1.29	0.16
N(CH ₃)	1.09	0.93	0.16
Ph	0.94	0.74	0.20

[P-450] = 4.8×10^{-5} M, [aminopyrine] = 0.06 M, 0.05 M KP_i in D₂O, pH_{obs} = 7.4, temperature 21°C

the presence of 7 M urea (when specific interactions are hardly probable) T_{1P}^{-1} remains fairly high. Consequently, the T_{1P}^{-1} obtained in this way is not associated only with specific enzyme-substrate interactions. In other words, T_{1P}^{-1} thus obtained includes a substantial nonspecific contribution (due, for example, to outer sphere relaxation) and can be used only as an upper limit for the relaxation rate in a complex. The alternative approach resulting directly from eqn. 2 seems to be more adequate.

Let us consider the relaxation of the protons of the low-molecular-mass substrate in the presence of protein with which this substrate can form a complex. Then,

$$\frac{1}{T_{1obs}} = \frac{1}{T_{1d}} + \frac{P}{T_{1B} + \tau} \times \frac{1}{K_D + S} \quad (4)$$

where T_{1obs}^{-1} is the observed relaxation rate of the

Table 2

Relaxation rates of the groups of aminopyrine protons observed in the presence of cytochrome P-420 and urea

Group	$1/T_1$ (s^{-1})		
	P-420-ox-	P-420-red + CO	$1/T_{1P}$
C-CH ₃	1.40	1.25	0.15
N(CH ₃) ₂	1.53	1.44	0.11
N(CH ₃)	1.29	1.16	0.13
Ph	1.02	0.86	0.16

[P-420] = 5.6×10^{-5} M, [aminopyrine] = 0.06 M, [urea] = 7 M, KP_i in D₂O (0.05 M), pH_{obs} 7.4, temperature 21°C

substrate protons, T_{1d}^{-1} is the relaxation rate of the substrate protons which does not depend on its concentration, P is the protein concentration, T_{1B} is the relaxation time in the enzyme-substrate complex, τ is the residence time in the complex, K_D is the dissociation constant for the complex, and S is the substrate concentration. The T_{1obs}^{-1} vs S^{-1} plot is shown in fig.3. Based on similar dependences for all lines of the ¹H-NMR spectrum of AP, T_{1d}^{-1} , $P/(T_{1B} + \tau)$ and K_D were calculated (table 3). A similar dependence for formylphenylalanine, which, according to our data, produces no changes in optical spectra characteristic of binding to cyt P450, is illustrated in fig.3B. Test experiments demonstrate that over the concentration range employed (6×10^{-2} – 5×10^{-4} M) the intrinsic relaxation rates of all AP protons depend insignificantly on its concentration (not shown).

Table 3

Parameters of aminopyrine binding to cytochrome P-450

Temperature (°C)	Group	C-CH ₃		N(CH ₃) ₂		N-CH ₃		Ph	
		Value	SD	Value	SD	Value	SD	Value	SD
14.0	$1/T_{1d}$ (s^{-1})	1.38	0.013	1.55	0.17	1.33	0.051	1.02	0.03
	$1/(T_{1B} + \tau)$ (s^{-1})	123	13	229	170	70	52	168	31
	K_D (M)	0.020							
21	$1/T_{1d}$ (s^{-1})	1.28	0.014	1.37	0.013	1.08	0.018	0.93	0.03
	$1/(T_{1B} + \tau)$ (s^{-1})	62	10	128	10	131	14	136	25
	K_D (M)	0.013							

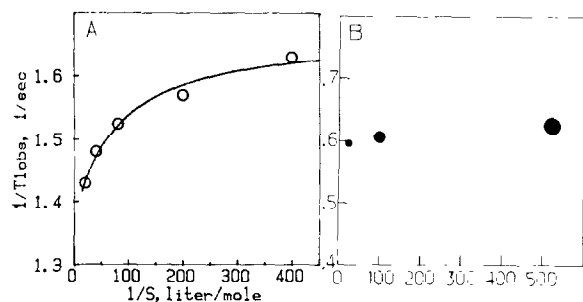


Fig. 3.(A): Relaxation rate of $\text{N}(\text{CH}_3)_2$ protons of aminopyrine vs inverse of aminopyrine concentration. Open circles, experimental points; curve, calculated from the parameters listed in table 3. 0.1 M KPi in D_2O , pH_{obs} 7.5, 21°C; $[\text{P-450}] = 3 \times 10^{-5}$ M. (B): Relaxation rate of phenyl protons of formylphenylalanine. Same buffer and temperature; $[\text{P-450}] = 4.5 \times 10^{-5}$ M.

4. DISCUSSION

To verify the idea that the concentration dependence of T_{obs} for AP is caused by the formation of specific enzyme-substrate complexes, we used a molecule of lipophilicity comparable to that of AP, namely formylphenylalanine. (The distribution coefficient for octanol-water buffer (pH 7.4) system is equal to 1.5 (formyl-phe) and 6.7 (AP) (our data).) This substance, as mentioned above, produced no optical binding spectra with cyt P450. Fig.3b shows that in this case the concentration-dependent change in T_{obs} practically does not occur, in contrast to AP (fig.3A). This confirms the supposition that the observed changes in T_{obs} for AP are associated with the formation of specific P-450-substrate complexes. The K_D value, obtained in NMR experiments (table 3), differs substantially from that calculated from optical spectra (i.e., K_S). There is no satisfactory explanation of this phenomenon but it has been observed in several laboratories (see, for example [17]). The use of optically determined K_S for interpretation of NMR experiments (as in [3–6]) will lead to worse agreement of the experimental points with the calculated curve, and to a decrease in $(T_{\text{IB}} + \tau)^{-1}$, which nevertheless will not alter the main conclusion, that there is a considerable distance between haem iron and the type I substrate binding site (see below).

A decrease in $(T_{\text{IB}} + \tau)^{-1}$ with increasing

temperature (table 3) indicates that $\tau < T_{\text{IB}}$, i.e., the fast exchange case [8]. Thus, the $(T_{\text{IB}} + \tau)^{-1}$ values (table 3) are T_{M}^{-1} (eqn1). For the calculation of Fe-R_i distances it is necessary to know τ_S (for complexes of substrates with cyt P-450 $\tau_c = \tau_S$, where τ_S is the electron spin relaxation time of Fe^{3+} [3]) and the Fe^{3+} electron spin. The P450-AP complex is known to be mostly a low-spin one [18]. This makes it possible to use $\tau_S = 5 \times 10^{-10}$ s obtained in [19] for low-spin P450_{cam} and to derive from eqn 1 $r \approx 8$ Å for all the AP protons. For the high-spin complex (total electron spin $S = 5/2$) the estimate made by Griffin and Peterson [20] gives $\tau_S = 3 \times 10^{-10}$ s and correspondingly $r \approx 10$ Å. In either case these distances are rather large on the molecular scale; they could be somewhat reduced if actually $\tau_S < 5 \times 10^{-10}$ s and/or if only a part of the protein in the solution is capable of binding to AP. If it is true that $r \approx 8$ Å it is reasonable to suggest either the existence of an active oxygen-containing species which diffuses into the substrate binding site or the approach of the substrate to haem upon reduction of cyt P-450. In this connection it seems of interest to determine the mutual arrangement of substrates and haem for different functional states of protein in the presence of lipids.

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