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# NMR study of the interaction of P-450 with 4-methoxypyridine

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Longitudinal relaxation ($T_1$) measurements for all lines (α-CH, β-CH, O-$CH_3$) in the 4-methoxypyridine $^1$H-NMR spectrum were used to study the interaction of 4-methoxypyridine with purified microsomal cytochrome P-450 from livers of phenobarbital-treated rats. The paramagnetic contribution to the observed $T_1^{-1}$ value was determined from its dependence on 4-methoxypyridine concentration. In the P-450–4-methoxypyridine complex the latter is oriented so that the nitrogen of pyridine is directed towards the $Fe^{3+}$.

Cytochrome P-450; 4-Methoxypyridine; $^1$H-NMR; Enzyme-substrate interaction

## 1. INTRODUCTION

Among the problems associated with the study of the mechanism of action of cytochrome P-450 of great interest is that of the structure of P-450–substrate complexes which can be studied by optical [1,2] and NMR [3,4] methods. Owing to the presence of a paramagnetic ion, namely $Fe^{3+}$, in the enzyme active center, NMR can provide direct information about the kinetic and structural properties of the P-450–substrate complexes [5]. We have studied the interaction of P-450 from phenobarbital-induced rat liver with 4-methoxypyridine (4-MeOPy). This latter compound was chosen because: (i) as for other pyridine derivatives, when interacting with P-450 it produces type II spectra (see below), and (ii) 4-substituted pyridines produce $^1$H-NMR spectra having well resolved signals from α- and β-protons, which are very convenient for relaxation measurements.

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## 2. MATERIALS AND METHODS

The treatment of animals, and preparation and analysis of samples were as described in [6]. Cytochrome P-450 (main phenobarbital-induced form) was isolated as in [7] and released from detergents on a hydroxyapatite column [8]. The preparations contained 13–16 nmol P-450 per mg protein. According to the electrophoresis data the preparations appeared to be a mixture of b (~70%) and e (~30%) forms of P-450 (nomenclature of Ryan et al. [9]). $D_2O$ (Sojuzkhimreaktiv) was 99.8%. Deuterourea was obtained by evaporation (three times) of a urea solution (special purity grade, Sojuzkhimreaktiv) in $D_2O$. 4-MeOPy was synthesized by O.P. Shkurko (Novosibirsk Institute of Organic Chemistry). Optical measurements were made using Hitachi 557 and Beckman DB-GD spectrophotometers. NMR spectra were recorded using Varian XL-200 and Bruker AM-250 spectrometers. $T_1$ was determined using a modified inversion-recovery sequence ($T$–90$^\circ_x$–240$^\circ_y$–90$^\circ_x$–$\tau$–90°–AT) [10,11]. The data were analysed by a least-squares method generalized for the nonlinear dependence on parameters [12].

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## 3. THEORY

According to the Solomon-Blombergen equation, $T_1$ for a nucleus near a paramagnetic ion [13] is:

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

where $T_{1M}$ is the time of longitudinal relaxation for the nucleus near the paramagnetic ion; $r$ is the distance between the ion and the nucleus; $\tau_c$ and $\tau_e$ are the correlation times for the dipole and hyperfine interaction, respectively (in our case both are equal to the time of electron spin relaxation for $Fe^{3+}$, $\tau_S$, see, e.g. [3]); $\omega_I$ and $\omega_S$ are nuclear and electron Larmor frequencies; and $A/\hbar$ is the hyperfine coupling constant (rad·s$^{-1}$), all other designations being standard.

For the substrate in solution in the presence of the paramagnetic protein there will be exchange between complexed and free states; besides, there can also be some other nonparamagnetic mechanisms of relaxation. Taking this into account, one can write:

$$\frac{1}{T_{1obs}} = \frac{1}{T_{1d}} + \frac{P}{T_{1M}+\tau}\cdot\frac{1}{K_d+S} \quad (2)$$

where $1/T_{1obs}$ is the observed relaxation rate; $1/T_{1d}$ considers all diamagnetic contributions to the relaxation; $P$ is the protein concentration; $\tau$ is the residence time of the substrate in the complex; $K_d$ is the dissociation constant of the complex; and $S$ is the substrate concentration. From the dependence of $1/T_{1obs}$ on $S$ (at a constant protein concentration) it is possible to obtain $(T_{1M}+\tau)$ and $K_d$ as parameters.

## 4. RESULTS AND DISCUSSION

4-MeOPy binds to P-450 as a type II substrate ($\lambda_{max}$ = 431 nm, $\lambda_{min}$ = 396 nm). The curve of P-450 titration with 4-MeOPy is not described by a single $K_s$, namely $K_s$ ~50 $\mu$M for [4-MeOPy] ≃ 8–500 $\mu$M and $K_s$ = 1 mM for [4-MeOPy] ≃ 3–20 mM.

The $^1$H-NMR spectrum of 4-MeOPy has 3 lines, namely $\delta$ 7.36 ($\alpha$-protons, doublet), $\delta$ 6.32 ($\beta$-protons, doublet), and $\delta$ 3.20 ($OCH_3$, singlet). Fig.1 shows the dependence of the relaxation rate of 4-MeOPy protons on the concentration of P-450. It can be seen that in contrast to the behaviour of a type I substrate [6], there is a pronounced selectivity in the P-450-induced relaxation, i.e. with increasing concentration of protein $T_1$ is decreased more for $\alpha$-protons than for $\beta$-protons and $OCH_3$. This is evidence that the 4-MeOPy molecule is oriented relative to $Fe^{3+}$ in the active center of the enzyme. The plot of relaxation rates vs substrate concentration is given in fig.2, the parameters obtained from this plot using eqn 2 being listed in table 1.

To determine the type of exchange in the complex, i.e. the $T_{1M}$ to $\tau$ relation (eqn 2), we have measured the temperature dependence of the relaxation rates of the 4-MeOPy protons (fig.3). An opposite behaviour of the dependence for $\alpha$ and $OCH_3$ groups and an intermediate type for $\beta$-protons is clearly seen. This fact suggests a slow exchange [13] in the complex for $\alpha$-protons [$T_{1M} \ll \tau$ and hence $(T_{1M}+\tau) \simeq \tau$] and allows one to calculate the value of $T_{1M}$ for $\beta$ and $OCH_3$ groups (table 2). The selectivity of the change of $T_{1obs}$ (figs 1,2) and the different behaviour of the temperature

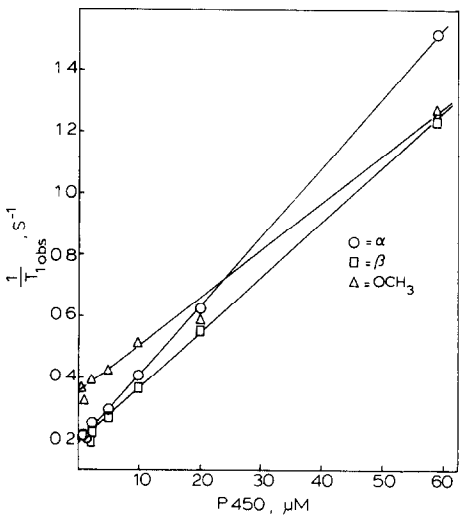


Fig.1. Plot of the rate of longitudinal relaxation of protons of 4-MeOPy vs concentration of P-450. 0.1 M $KP_i$ in $D_2O$, $pH_{obs}$ 7.5, 21°C; [4-MeOPy] = 6.7 mM.

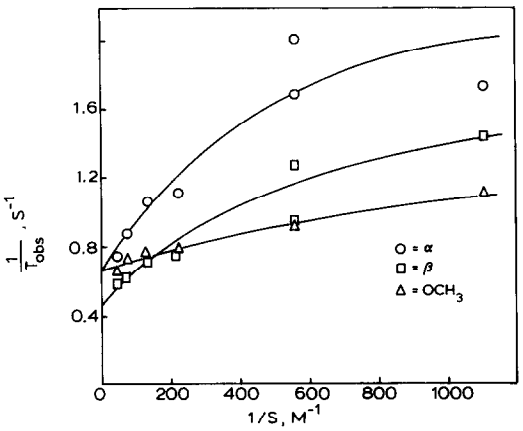


Fig.2. Plot of the rate of longitudinal relaxation of protons of 4-MeOPy vs concentration of the substrate in the presence of P-450. Buffer and temperature as in fig.1; [P-450] = $2.3 \times 10^{-5}$ M. The curves are constructed with the use of eqn 2 and parameters listed in table 1.

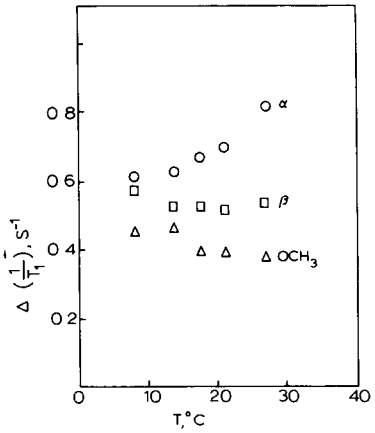


Fig.3. Plot of relaxation rate of protons of 4-MeOPy vs temperature. [P-450] = $2.3 \times 10^{-5}$ M, [4-MeOPy] = 15 mM, 0.1 M $KP_i$ in $D_2O$, $pH_{obs}$ 7.5.

dependence for the different 4-MeOPy groups (fig.3) lead one to suggest that the substrate molecule is oriented in the active center of P-450 in such a way that the pyridine nitrogen forms a bond (possibly indirect) with $Fe^{3+}$. The question naturally arises: what is the contribution of scalar interaction (second term, eqn 1) to the $T_{1M}$ values obtained? To estimate this contribution to the relaxation rate of $\alpha$-protons for the direct coordination of 4-MeOPy to $Fe^{3+}$, we assume that $S = 1/2$ since P-450-type II substrate complexes are low-spin [1], $A/h \simeq 1$ MHz (as follows from the data for ferriprotoporphyrin-imidazole complexes [14]), $\tau_e = \tau_S = 5 \times 10^{-10}$ s (as obtained in [15] for the low-spin $P\text{-}450_{cam}$). The estimated scalar contribution to $1/T_{1M}$ is ~0.05 Hz; in the absence of direct coordination this contribution will be even less. Thus, we can regard the $T_{1M}$ values obtained (table 1) as purely dipolar and use them for calculating distances from the corresponding proton group to $Fe^{3+}$ (table 2). Here we also assume that $\tau_c = \tau_S = 5 \times 10^{-10}$ s. It can be seen in table 2 that the distance obtained does not correspond to those expected for the direct coordination of 4-MeOPy to $Fe^{3+}$. We now analyze the parameters assumed in the calculation which could change the results obtained.

Table 1

Parameters obtained from the dependence of $T_1$ of 4-MeOPy protons vs concentration of 4-MeOPy (± SD)

| N[a] | $K_d$ (mM) | $(T_{1M} + \tau)^{-1}$ (Hz) | | |
|---|---|---|---|---|
| | | α | β | $OCH_3$ |
| 1 | 2.1 ± 0.9 | 169 ± 46 | 76 ± 23 | 50 ± 14 |
| 2 | 5.8 ± 4.7 | 387 ± 208 | 229 ± 131 | 116 ± 77 |
| 3 | 9.9 ± 3.0 | 394 ± 100 | 171 ± 50 | 105 ± 33 |
| Mean[b] | 2.9 ± 0.9 | | | |

[a] Three different preparations of cytochrome P-450
[b] Weighted mean with variances as weights

[P-450] = $2.3 \times 10^{-5}$ M (1); $1.3 \times 10^{-5}$ M (2); $4.6 \times 10^{-5}$ M (3); potassium phosphate $D_2O$ buffer, $pH_{obs}$ = 7.5, temperature 21°C

(i) For part of P-450 in the solution the formation and decomposition of the enzyme-substrate complex might be slow because of steric hindrance (protein aggregation), for example. The estimates indicate that under these experimental conditions the complexes characterized by a dissociation rate constant $k_{-1}$ 50 $s^{-1}$ would not practically make a contribution to the observed acceleration of the relaxation. In this case the effective concentration of the enzyme in eqn 2 could be smaller than that found from the stationary CO-difference spectrum.

Table 2

Residence time in the complex, experimental and model (direct coordination) distances for the P-450–4-MeOPy complex (± SD)

| $N^a$ | $\tau$ (s) | $T_{1M\beta}$ (s) | $T_{1MOCH_3}$ (s) | $r_\beta$ (Å) | $r_{OCH_3}$ (Å) |
|---|---|---|---|---|---|
| 1 | $(5.9 \pm 1.6) \times 10^{-3}$ | $7.3 \times 10^{-3}$ | $1.4 \times 10^{-2}$ | $7.6 \pm 0.75$ | $8.5 \pm 0.6$ |
| 2 | $(2.6 \pm 1.4) \times 10^{-3}$ | $1.8 \times 10^{-3}$ | $6.0 \times 10^{-3}$ | $6.0 \pm 1.6$ | $7.4 \pm 1.2$ |
| 3 | $(2.5 \pm 0.6) \times 10^{-3}$ | $3.3 \times 10^{-3}$ | $7.0 \times 10^{-3}$ | $6.7 \pm 0.6$ | $7.6 \pm 0.6$ |
| Mean[b] | | | | $7.0 \pm 0.5$ | $8.0 \pm 0.4$ |
| Model[c] | | | | 5.1 | 6.4 |

[a] Three different preparations of P-450

[b] Weighted average with weights equal to variances

[c] For the direct coordination of the substrate; $r$ was taken as the distance between $Fe^{3+}$ and the point closest to it of the cone circumscribed by the protons of the $OCH_3$ group when rotating around the O–C bond. A strong dependence of $T_{1M}$ on the distance $r^6$ causes this 'averaging'

To verify this idea, we measured the kinetics of CO binding with reduced P-450 using the stopped-flow method. This system was chosen because the kinetic constant for the $P\text{-}450^{2+}$ + CO interaction is high ($\sim 10^5$ $M^{-1} \cdot s^{-1}$ [16]) and thus a stationary level should be achieved over about 100 ms. If this does not happen one can conclude that part of the enzyme is inaccessible to fast binding with the substrate, namely CO. According to our stopped-flow experiments 100% binding of CO with P-450 occurs in $\leqslant$100 ms, which allows us to reject possibility (i).

(ii) The value of $\tau_c$ for the 4-MeOPy–P-450 complex could be different from the $5 \times 10^{-10}$ s used for estimating the value of $r$. However, from eqn 1 it follows that $T_{1MCH_3}/T_{1M\beta} = r_{CH_3}^6/r_\beta^6$. This is equal to 3.8 for direct coordination at an Fe–N bond length of 2.0 Å [17]. The experimental value of 2.1 ± 0.6 is evidence that direct coordination is absent for the complexes observed by NMR. This fact together with the difference between the equilibrium constants obtained from NMR and optical data indicate that most probably we observed different P-450–substrate complexes in NMR and spectrophotometric experiments on binding.

Let us estimate the lifetime of the complexes observed by optical methods: $\tau = 1/K_S k_1$, where $k_1$ is the kinetic constant of the interaction of P-450 with the substrate. Using $k_1 = 10^4$ $M^{-1} \cdot s^{-1}$ for P-450 binding with pyridine [16] and $K_S = 5 \times 10^{-5}$–$10^{-3}$ M (our data) we obtain $\tau \simeq 0.1$–2 s. Such long-lived complexes cannot be observed under NMR conditions. The complexes observed in NMR are likely to be the precursors of the optically observable complexes, for instance due to: $E + S \rightleftharpoons ES \rightleftharpoons ES^*$ where ES is the rapidly exchanging complex, observed by NMR, and ES* is the tighter 'optical' complex. However, such a scheme fails to account for the character of the amplitude of differential spectral dependence on the substrate concentration in spectrophotometric experiments; further investigations to elucidate the problem are essential.

A problem which remains unclear is the difference in structures of the active centers of P-450 and of its inactivated forms P-420 [5]. It is assumed, for example, that the mercaptide ligand of the heme is substituted by histidine when passing from P-450 to P-420 [18]. This problem is also of importance from the methodical point of view by conducting NMR experiments, since in the preparations of purified P-450 a quantity of P-420 is often present. We obtained P-420 by adding urea in high concentrations (6 M) to the solution of P-450. The preparations produced no differential spectra for binding with 4-MeOPy. On adding P-420 to 4-MeOPy the relaxation rate of the substrate protons tends to increase; however, there is no selectivity such as that observed for the native P-450. Besides, we observed no dependence of $T_1$ on the concentration of substrate in the presence of P-420. This fact seems to indicate that the dissociation constant of the P-420–4-MeOPy complex is

high and beyond the concentration range used.

Thus, the NMR data obtained support the idea that 4-MeOPy in the active center of P-450 is oriented so that the nitrogen of pyridine is directed towards the $Fe^{3+}$. However, comparison of the NMR and optical data provides evidence for a complex mechanism of the interaction of substrates with P-450 and does not allow us to state with certainty the existence of complexes with direct coordination of (P-450)$Fe^{3+}$ to the type II substrate. [It should be noted that [5] presents preliminary results which allowed one to draw the conclusion of the possibility of direct coordination of 4-MeOPy with $Fe^{3+}$. The contradiction with the present results can be explained by the fact that the P-450 preparation used in [5] was not sufficiently purified and contained detergent. This aspect requires further investigation.]

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