Interaction of Naphthalene-d_8 with Cytochromes P-450*: A ^2H Nuclear Magnetic Resonance Study

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Received April 24, 1985

Abstract

We have used ^2H NMR of naphthalene-d_8 to study the interaction of naphthalene with rabbit liver cytochromes P-450_1, P-450_2 and P-448_1. Line-broadenings of ^2H NMR of naphthalene-d_8 caused by adding cytochromes P-450 were much more marked than those caused by adding other hemoproteins such as cytochrome c, metMb and horseradish peroxidase. It was found that the paramagnetic effect of iron to the ^2H NMR of naphthalene-d_8–P-450 solution could be negligible but a specific binding of naphthalene-d_8 to P-450 contributes to the line-broadening of the ^2H NMR of naphthalene-d_8 caused by adding P-450's. Under the assumption that fast chemical exchange dominates the line-broadening of ^2H NMR of naphthalene-d_8, we have estimated binding constants of naphthalene to cytochromes P-450. The binding constants of naphthalene to the ferric forms of P-450 are 180–240 M^{-1}, which are a little lower than those, 310–400 M^{-1}, to the ferrous forms of P-450. Thus, it was found that the binding constant of naphthalene to ferrous P-450's is a little higher than that to ferric P-450's, although it seems likely that protein conformation at the heme environment is largely changed by the reduction of the heme iron in terms of CD spectra.

Introduction

A family of protoheme-containing monooxygenases, named cytochrome P-450(P-450), is widely distributed in nature and plays an important role in a variety of metabolic reactions [1]. Naphthalene is one of the great number of substrates which are hydroxylated through an electron-transfer system involving P-450 [2, 3]. The first step of the hydroxylation reaction of naphthalene is probably the binding of the substrate to the ferric form of P-450 and, subsequently, P-450 is reduced to the ferrous form through cytochrome P-450 reductase [1]. Hitherto, the binding of the substrate to P-450 has been semi-quantitatively studied from the small change of the Soret absorption band of P-450 caused by adding the substrate [4]. Furthermore, the binding of the substrate to the reduced form of P-450 cannot be quantitatively detected by conventional spectral methods.

NMR spectroscopy of the ^2H ($I = 1$) nucleus has some advantages over the ^1H NMR spectroscopy in studying interactions of substrate with hemoproteins [5–8]:

(1) ^2H NMR spectra of paramagnetic compounds give superior resolution to ^1H NMR spectra. Thus, ^2H NMR signals in a paramagnetic environment are narrower than ^1H NMR signals under the same conditions by a factor of $(\gamma_{1H})^2/(\gamma_{2H})^2 = 42.5$, where γ_{1H} and γ_{2H} are the magnetogyric ratios of the ^1H and ^2H, respectively.

(2) ^2H NMR spectra of deuterated compounds interacting with proteins are not hampered by the numerous signals coming from the water solvent and the protein.

We have used ^2H NMR of naphthalene-d_8 to study the interaction of naphthalene with rabbit liver P-450_1, P-450_2 and P-448_1. Under the assumption that the fast exchange mechanism controls the line-broadening of ^2H NMR of naphthalene-d_8 caused by adding the hemoproteins, we have estimated the binding constant of naphthalene to rabbit liver P-450's. Estimated binding constants were relatively lower compared with those of other enzymes. The binding constants of the substrate to the ferric form of P-450's were lower than those of ferrous form of P-450's.

*Abbreviations used are: P-450, cytochrome P-450; P-450_1, a major component of low-spin type of cytochrome P-450 purified from phenobarbital-induced rabbit liver microsomes; P-450_2, a minor component of low-spin type of cytochrome P-450 purified from phenobarbital-induced rabbit liver microsomes; P-448_1, high-spin type of cytochrome P-450 purified from phenobarbital-induced rabbit liver microsomes.

0020-1693/85/$3.30

Experimental

Isozymes of P-450, P-450_1, P-450_2 and P-448_1, were purified homogenously from phenobarbital-induced rabbit liver microsomes as previously described [9–12]. All P-450 solutions consisted of 0.1 M potassium phosphate (pH 7.25), 20% (v/v) glycerol and 0.2% (w/v) Emulgen 913. Ferric forms of P-450's were reduced to the ferrous forms by sodium dithionite. Reagents used were of the highest guaranteed grade and were used without further purification.

^{2}H NMR spectra were obtained at 46.06 MHz on a Bruker CXP-300 spectrometer in a spinning 10 mm tube at 295 K: repetition time, 1.0 s; pulse width, 23 μs (90° pulse); sweep width, 3000 Hz; size, 4k–8k; exponential line-broadening, 1 Hz; scan number, 3000–10000. Chemical shift values were obtained by reference to an internal standard of ^{2}HHO(DHO) at the natural abundance of the solvent, thereby eliminating any susceptibility effects.

Results and Discussion

Figure 1 shows ^{2}H NMR spectra of naphthalene-d_8 in the absence and presence of various amounts

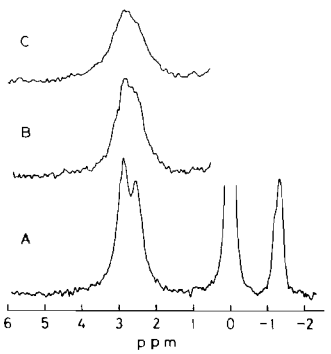

Fig. 1. ^{2}H NMR spectra of 1.5 mM naphthalene-d_8 in the absence (A) and presence of 1.0 μM (B) and 4.5 μM (C) of P-450_1. Signals at around 2–3.5 ppm, 0 ppm and −1.2 ppm are ascribed to naphthalene-d_8, naturally abundant DHO and deuterated glycerol, respectively. Experimental conditions are described under 'Experimental'.

of P-450_1. The line-width of naphthalene-d_8 was increased linearly by adding P-450_1. ^{2}H NMR spectral changes of naphthalene-d_8, caused by adding heme octapeptide of cytochrome c and hemoproteins such as cytochrome c, metMb or horseradish peroxidase, were much lower than those caused by adding P-450_1 by two orders (Fig. 2). To know whether the line-broadening of naphthalene-d_8 caused by adding P-450_1 is associated with the paramagnetic effect of the heme iron in P-450_1, we formed a diamagnetic

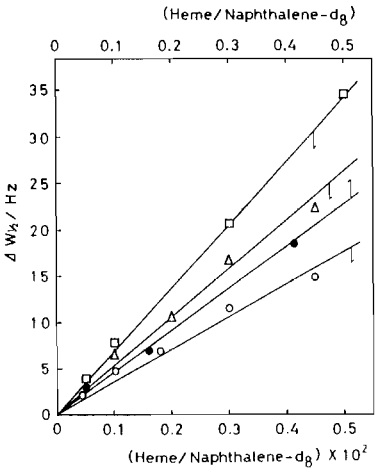

Fig. 2. Line-broadenings of ^{2}H NMR signals of 1.0 mM naphthalene-d_8 caused by adding P-450_1 (–○–), P-450_2 (–△–), P-448_1 (–□–) and metMb (–●–). The ordinate shows the line-width in Hz, while the abscissa shows the concentration ratio of heme to naphthalene-d_8. The upper abscissa scale is for metMb and the lower abscissa scale is for P-450's.

compound, a CO complex of ferrous P-450_1. The line-width of the naphthalene (1.5 mM)-P-450_1 (5.0 μM) solution was increased by approx. 10% upon reduction by sodium dithionite and subsequently bubbling of CO gas. Since disappearance of the paramagnetism must narrow the line-width of naphthalene-d_8 [6–8, 13], it seems likely that the paramagnetic iron may not influence the line-width of the ^{2}H NMR of the naphthalene-d_8-P-450_1 solution. A structural change at the substrate binding site of P-450_1 caused by reduction of P-450_1 will broaden the line-width of the naphthalene-d_8–P-450_1 solution. This structural change at the substrate binding site of P-450_1 will reflect the binding constant of naphthalene to P-450_1, as will be mentioned later. The ^{2}H NMR findings observed for P-450_1 were essentially the same as those observed for P-450_2 and P-448_1. Thus, it is suggested that the line-broadenings of ^{2}H NMR of naphthalene-d_8 caused by adding P-450's will reflect the binding of naphthalene to the substrate binding site of P-450's. It seemed likely that the chemical exchange mechanism dominates the line-width of the naphthalene-d_8–P-450 solutions.

We estimated binding constants of naphthalene to P-450 under the fast exchange assumption as follows [13]:

Assuming that [E] is the concentration of P-450 and [S] is the concentration of naphthalene; when one molar of naphthalene binds to one molar of P-450, the binding constant, K_b, of naphthalene to P-450 can be described as:

$$K_b = [ES]/[E][S] \quad (1)$$

When $[E]_o$ is the concentration of added P-450 and $[S]_o$ is the concentration of added naphthalene, the proportion, P, of naphthalene bound to P-450 is:

$$P = [ES]/[S]_o \quad (2)$$

The eqn. (1) can be converted to eqn. (3) by using the eqn. (2) as follows:

$$K_b = \frac{P[S]_o}{([E]_o - P[S]_o)([S]_o - P[S]_o)} \quad (3)$$

Since $[S]_o \cong$ mM, $[E]_o \cong \mu$M and $[ES] \cong \mu$M, then $[S]_o \gg [E]_o$ and $P = [ES]/[S]_o \ll 1$.

Thus, eqn. (3) can be reduced to eqn. (4):

$$K_b \simeq \frac{P}{([E]_o - P[S]_o)} \quad (4)$$

Under the assumption of the fast chemical exchange regime, P can be correlated with transverse relaxation time T_2 as follows [13]:

$$1/T_{2o} = P/T_{2b} + (1 - P)/T_{2f} \quad (5)$$

Here T_{2o} is the observed T_2, T_{2b} is T_2 of bound naphthalene and T_{2f} is T_2 of free naphthalene.

By using the line-width $W_{1/2} = (1/\pi){T_2}^{1-}$, the eqn. (4) can be converted to eqn. (6):

$$\Delta W_{1/2}[S]_o = (W_{1/2b} - W_{1/2f})[E]_o - (1/K_b)\Delta W_{1/2} \quad (6)$$

where $\Delta W_{1/2}$ is the difference (in Hz) between the line-width, $W_{1/2b}$, of the bound form and that, $W_{1/2f}$, of the free form of naphthalene.

Since the first term of the right part of the eqn. (6) is constant, the eqn. (6) should form a straight line with a slope of $-1/K_b$ on a graph describing the change of $\Delta W_{1/2}$ as abscissa and $\Delta W_{1/2}[S]_o$ as ordinate. From the slope of the straight line, one can evaluate the binding constant, K_b.

To evaluate K_b values, we measured the line-width of various amounts of naphthalene-d_8 in the presence of 3.26 μM P-450 (the left figures in Fig. 3). Since the signal of naphthalene-d_8 consists of two magnetically inequivalent signals, the signal of naphthalene-d_8 was resolved into two signals by using the Lorentzian function (the right figures in Fig. 3). The signal 1 at lower magnetic field (the right figures in Fig. 3) was used for the evaluation of K_b. As can be seen in Figs. 4 and 5, $\Delta W_{1/2}[S]_o$ *vs.* $\Delta W_{1/2}$ of naphthalene-d_8–P-450 solutions depicted straight lines under our experimental conditions. Thus, the assumption mentioned above can be validly applicable to the ^{2}H NMR of naphthalene-d_8–P-450 systems. The ^{2}H NMR technique was applied for the evaluation of K_b of naphthalene to

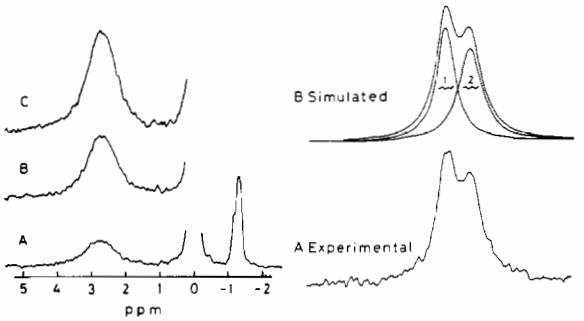

Fig. 3. Left figures: ^{2}H NMR spectra of 3.26 μM P-450$_1$ in the presence of 0.41 mM (A), 0.84 mM (B) and 1.29 mM (C) naphthalene-d_8. The signal at around 2.8 ppm is ascribed to naphthalene-d_8. Right figures: experimental (A) and simulated (B) ^{2}H NMR spectra of naphthalene-d_8 in the absence of P-450.

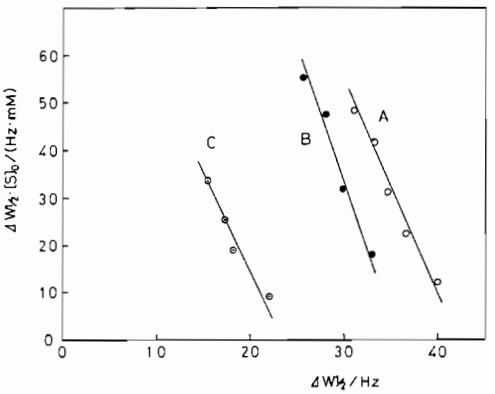

Fig. 4. Experimental plots of the eqn. (6) for naphthalene-d_8-ferric P-450$_1$ (A), P-450$_2$ (B) and P-448$_1$ (C) solutions. The ordinate shows $\Delta W_{1/2}[S]o$ in Hz. mM, while the abscissa shows the line-width in Hz.

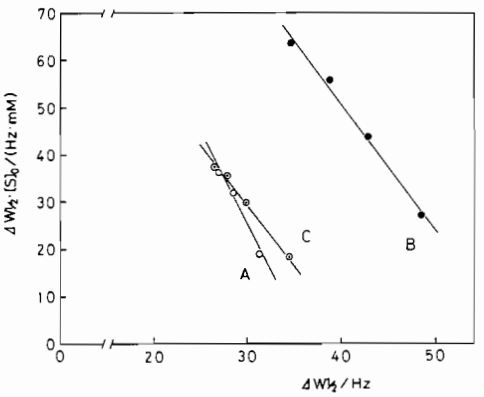

Fig. 5. Experimental plots of the eqn. (6) for naphthalene-d_8-ferrous P-450$_1$ (A), P-450$_2$ (B) and P-448$_1$ (C) solutions.

TABLE I. Binding Constants, K_b, of Naphthalene to Ferric and Ferrous P-450's Determined from Figs. 4 and 5 (*cf.* Text).

P-450	K_b of naphthalene (M^{-1})
ferric P-450$_1$	220
ferrous P-450$_1$	310
ferric P-450$_2$	180
ferrous P-450$_2$	380
ferric P-448$_1$	240
ferrous P-448$_1$	400

both ferric and ferrous P-450's. Table I summarizes binding constants, K_b, of naphthalene to ferric and ferrous forms of P-450$_1$, P-450$_2$ and P-448$_1$. From these results, several important points were inferred as follows:

(1) K_b values of naphthalene to P-450's are relatively small compared with usual enzyme systems;

(2) K_b values of naphthalene to P-450$_1$, P-450$_2$ and P-448$_1$ are nearly the same as each other;

(3) K_b values of naphthalene to ferrous P-450's are higher than those to ferric P-450's, although the differences are not very large.

The estimated K_b values are similar to those, 10^2–10^3 M^{-1}, observed from difference absorption spectra of the Soret region of P-450 [1, 4]. Thus, the K_b values estimated in this study will reflect the binding of naphthalene to a specific binding site in P-450 rather than to non-specific binding sites in P-450. It is interesting to note that apparent K_b values of naphthalene to P-450 increase upon reduction of P-450. The rate-limiting step of the hydroxylation reaction involving P-450 is not the binding of the substrate to P-450 [1]. The enhancement of the substrate binding to P-450 upon reduction may accelerate the hydroxylation reaction. It should be noted, however, that K_b values of naphthalene to ferrous P-450's are not very large compared with those to ferric P-450's, since the heme environments of P-450's are largely changed upon reduction in terms of CD spectra [12]. Thus, it is suggested that the heme environment of P-450 may not be closely correlated structurally to the substrate binding site of P-450.

It should be noted here that a change of viscosity of the medium caused by adding P-450's does not influence the line-width of naphthalene-d_8 because the line-width of DHO, which reflects the viscosity of the solution [14], was not changed at all by adding P-450's. Addition of sodium dithionite to the naphthalene-d_8 solution in the absence of P-450 also retained the same line-width of ^2H NMR signals of naphthalene-d_8. Note that, furthermore, the eqn. (6) could not be applied to hemoproteins other than P-450's since the above-mentioned assumption was not valid for those systems.

In concluding remarks, we have determined K_b values of naphthalene to ferric and ferrous P-450's in terms of ^2H NMR spectroscopy. The K_b values to ferrous P-450's were larger than those to ferric P-450's. It is found here that naphthalene specifically interacts with P-450's in terms of ^2H NMR spectra and that the presence of other hemoproteins hardly influence the ^2H NMR spectra in comparison with that of P-450. The high utility of the ^2H NMR method to study the interaction of the substrate with the paramagnetic hemoprotein should be emphasized here.

Acknowledgements

We are very grateful to Dr. Y. Imai of the Protein Insitute, Osaka University and Prof. M. Watanabe and his associates of the Research Institute for Tuberculosis and Cancer, Tohoku University, for their kind instruction on the purification of P-450's. This work was supported in part by Nissan Science Foundation and by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan.

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