NUCLEAR MAGNETIC RESONANCE STUDIES OF HEMOPROTEINS

Interaction of horseradish peroxidase with indolepropionic acid

Isao MORISHIMA, Satoshi OGAWA, Toshiro INUBUSHI, and Teijiro YONEZAWA
Department of Hydrocarbon Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606, Japan

Received 9 June 1977

1. Introduction

It has been assumed from the kinetic studies of peroxidase that the compound II of the enzyme reacts with a hydrogen donor molecule to form an active ternary complex [1,2]. Optical studies of difference spectrum (HRP-donor vs. HRP) indicated that the enzyme in the native form also interacts with the donors such as aromatic amines and phenols to form a 1 : 1 complex [3,4]. Recently proton NMR technique has been used to delineate the interaction between the native enzyme and the donor molecule [4–6]. In these studies, nuclear magnetic relaxation enhancement of the substrate protons has been employed in search for the distance between substrate and paramagnetic heme iron. It appears, however, that the mode of interaction, especially the effect of donors on the structural change of heme environment of the enzyme, is still unclear.

Although hyperfine shifted proton resonances of heme peripheral groups have been proved to be potentially useful for the studies of the electronic structure and conformational surroundings of the heme in hemoproteins, such studies on ferric high spin hemoproteins have been quite limited [7,8]. We have currently been investigating largely hyperfine shifted resonances of various hemoproteins in ferric high-spin state ($S = 5/2$), which is quite sensitive to the heme environmental structure. In order to gain insight into structure of the donor–peroxidase complex, we have studied here the effect of indolepropionic acid (IPA) on the hyperfine shifted resonances of horseradish peroxidase (HRP) in ferric high-spin state.

North-Holland Publishing Company – Amsterdam

2. Materials and methods

Sigma type VI HRP was purified by the method of Shannon et al. [9] except for the elution of the enzyme from the CM-cellulose column by deuterated phosphate buffer (0.5 M) at p^2H 7.0. All experiments were performed in this buffer. The enzyme concentration was 2 to 3 mM in the ^2H_2O buffer and were measured optically at 403 nm by using a millimolar extinction coefficient of 102 at pH 7.0. The cyanide complex of HRP was prepared by adding an excess of KCN to the enzyme solution until its NMR spectral change is saturated.

The proton high resolution NMR spectra were recorded in pulsed Fourier transform mode (PFT) on Varian Associate HR-220 spectrometer equipped with Nicolet TT-100 PFT accessary. The quadrature phase detection (QPD) method was used to cover a wide spectral region along with short pulse width (20 μsec = 48° pulse). The spectra were obtained by 4 K points transform of 40 K Hz spectral width. The pulse interval was 0.05 sec and 8192 to 16384 transients were collected. The proton chemical shift is referenced with respect to the residual water proton signal in the sample.

3. Results and discussion

Binding of indolepropionic acid (IPA) to horseradish peroxidase (HRP) was studied at pH 7.0 and 22°C over a wide range of the donor concentration by following the resonance shift of the hyperfine shifted proton NMR signals of the enzyme. The

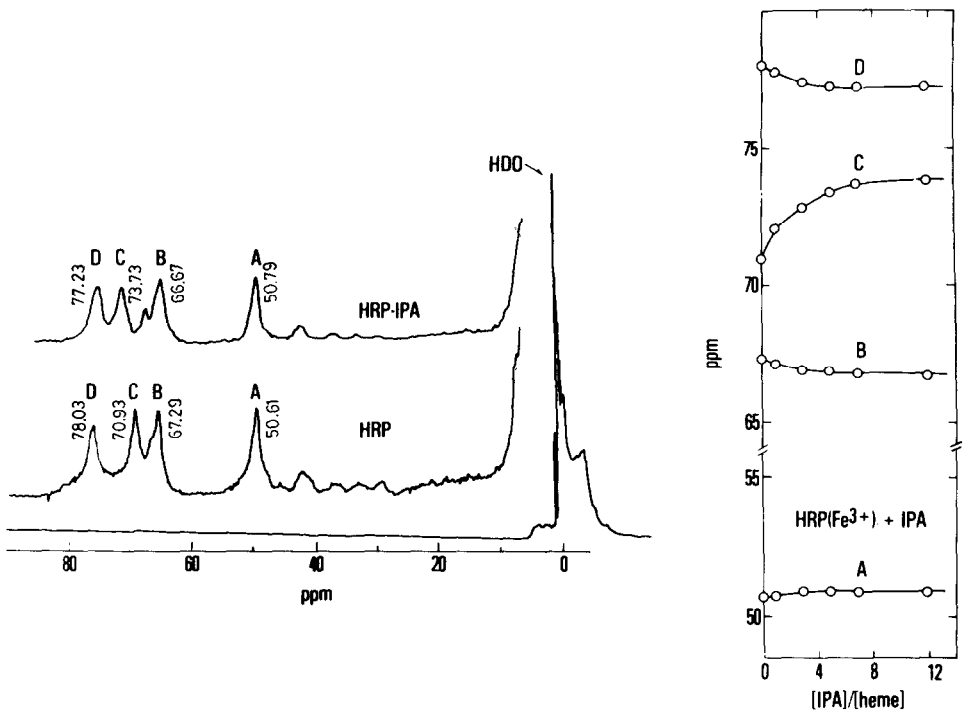

Fig.1. Proton NMR spectra of HRP free and bound to IPA at 22°C and pH 7.0. The heme peripheral methyl signals (A–D) exhibited a small but significant shift to the upper or lower field side with addition of the donor. Dependence of the hyperfine shifts of these methyl signals on the enzyme-donor concentration ratio is also illustrated.

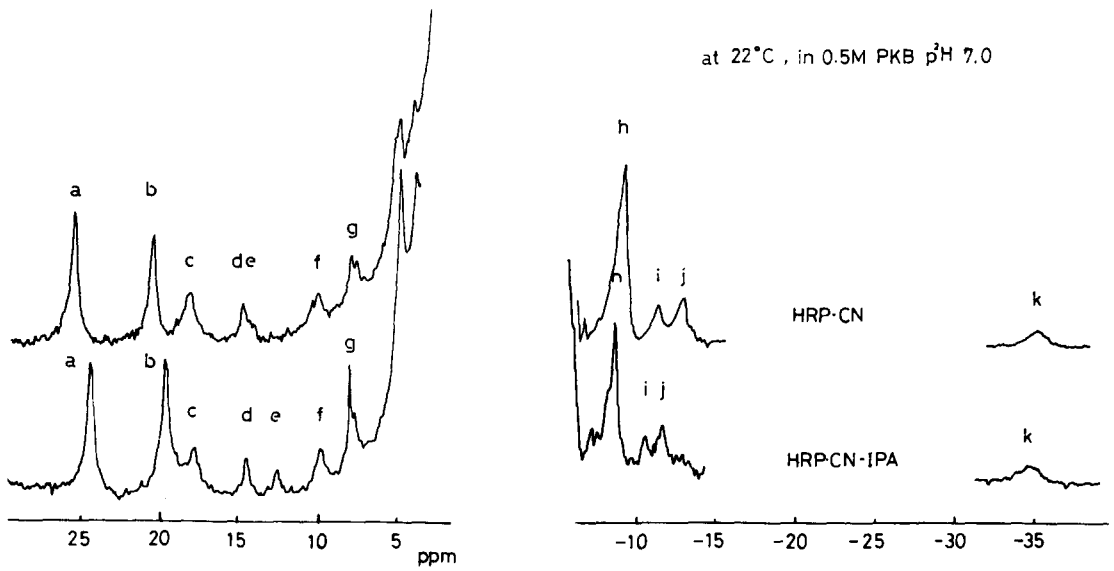

Fig.2. Hyperfine shifted proton NMR spectra of the cyanide complex of HRP at 22°C and pH 7.0. The spectra in the presence and absence of IPA are compared. All the peaks (a–k) show sizable shifts with stepwise addition of the donor.

presence of the electron donor in HRP solution caused a small but significant change in the spectrum of the enzyme. Figure 1 shows the representative proton NMR spectra of HRP in the presence and absence of IPA, together with dependence of the signal positions on IPA-concentration of the four heme peripheral methyl peaks (signal A–D). The heme ring methyl at 50.6, 67.2, 70.9 and 78.0 ppm in the native enzyme show a sizable shift to the upper or lower field side with the stepwise additions of the donor. The shift is especially appreciable for the heme methyl peak C at 70.9 ppm which exhibits downfield shift by about 3 ppm from its original position. This spectral change encountered in HRP is quite distinguishable from the solvent perturbation of IPA, since no substantial change in the spectrum of metmyoglobin was detected upon addition of the donor.

We have also studied the effect of the donor on the spectral perturbation of the cyanide complex of HRP in ferric low spin state. As fig.2 shows, the donor molecule causes a sizable shift of the hyperfine shifted proton NMR signals even for the cyanide complex. In this figure the signals at 26.45 and 21.40 ppm of the two heme methyl peaks and the one at 15.2 ppm (signal e) are noted to shift more appreciably than other peaks with increasing the donor concentration.

These donor-induced spectral perturbations of HRP and its cyanide complex suggest not only that the donor binds at specific site of the enzyme, close to the heme ring enough to affect the heme electronic structure, but also that the chemical exchange between the free and the IPA-complexed forms of HRP is fast on the 220 MHz proton NMR time scale (10^{-4} sec). Hence we have estimated the association constant (K_a) and fully IPA-bound shift (limiting shift, δH) of the hyperfine shifted signals from the above titration experiments, assuming a 1 : 1 complex formation and rapid chemical exchange between HRP and the HRP–IPA complex. Here we used a computer simulation method of least square fit procedure to obtain $K_a = 580\ M^{-1}$ and $510\ M^{-1}$ for the HRP–IPA and the cyano HRP–IPA complexes, respectively by following the shifts of the heme methyl peak C (HRP) and single proton signal e (cyano HRP). The association constants obtained here are in good agreement with the one reported for *p*-cresol ($K_a = 390\ M^{-1}$) obtained from the result of usual difference spectroscopic method at pH 7.0 [4]. The limiting shifts of these signals C (HRP) and e (cyano HRP) amount to 3.2 and 2.3 ppm with respect to their original position, respectively. The fully IPA-bound chemical shifts of the hyperfine shifted resonances are illustrated for the native enzyme and the cyanide complex in table 1.

Having confirmed the binding of IPA to the enzyme, let us now consider why the shift is remarkable for the heme methyl peak C (HRP) and for the two heme ring methyls and single proton peak e (cyano HRP) upon addition of the donor. If the donor binds to the heme iron directly (sixth coordination position) or to the proximal histidine by forming a hydrogen bond as suggested previously [3,4], more striking change in the hyperfine shifted spectrum of the enzyme should be expected to occur since a little change in the binding nature of the iron axial ligand leads to substantial alternation in the unpaired electron spin distribution on the heme ring to move

Table 1
Proton hyperfine shifts of HRP and cyano-HRP and limiting shifts for their IPA bound complexes

	Resonance	Hyperfine[a] shift (ppm)	IPA-induced[b] shift (ppm)
HRP	A	50.61	−0.21
	B	67.29	+0.71
	C	70.93	−3.21
	D	78.03	+0.90
Cyano-HRP	a	25.38	+1.22
	b	20.66	+0.84
	c	18.99	+0.19
	d	15.18	+0.09
	e	13.22	+2.25
	f	10.82	−0.08
	g	8.69	−0.19
	h	− 8.37	+0.25
	i	−10.46	+0.18
	j	−11.30	−0.60
	k	−35.40	+0.51

[a] Hyperfine shifts of the heme peripheral groups in the absence of substrate. Chemical shift is referenced with respect to the residual water proton signal in the sample with positive sign for the lower field resonances.

[b] Limiting shift (fully IPA bound shift) obtained by assuming 1 : 1 complex with $K_a = 580\ M^{-1}$ and $510\ M^{-1}$ for HRP–IPA and cyano HRP–IPA, respectively. Plus and minus designate upfield and downfield shifts, respectively from the original position.

all the hyperfine shifted signals more drastically [7,8]. It is also unlikely that the binding of the donor to the enzyme causes serious alternation of the protein conformation of HRP. If the donor induces a drastic change in the protein conformation of the enzyme, the exchange rate between HRP and the HRP–IPA complex is expected to be slow on the NMR time scale. It then follows that the sizable shift of the heme methyl signals and the single proton resonance in the native enzyme and cyano HRP are attributable to a local conformational change occurring near these groups in the heme periphery.

In myoglobin and hemoglobin, the hemes are visualized by the X-ray studies to be embedded in a hydrophobic crevice of the protein chains with the vinyl groups inserted into the interior. Their central heme irons are accessible to many external ligands such as amines and imidazoles in ferric state, indicating that the heme crevice of these hemoproteins are roomy to accomodate the exogenous ligands. In HRP, however, the heme is not so susceptible to these ligands compared with hemoglobin and myoglobin, suggesting that the heme of HRP is more firmly buried in a hydrophobic crevice than those of oxygen carrying hemoproteins [10].

From above results and discussion it is tempting to speculate that the binding site of the electron donor to the enzyme is not the heme axial position but the heme periphery, probably near the heme propionate group. The spectral similarity of the proton NMR between the cyanide complexes of HRP and metmyoglobin may allow us to expect that the peak e at 15.2 ppm of cyano HRP is arising from the heme vinyl group ($-CH{=}CH_2$)* and also that the two heme methyl peaks at 26.4 and 21.4 ppm are due to the methyl groups at ring position 5 and 1, respectively [7,11]. The fact that the heme ring methyls and the heme vinyl peaks are concomitantly shifted upon addition of the donor may be interpreted in terms of pairing effect which is manifested in the simultaneous shift of the heme peripheral groups (1 and 5 methyls, for example) at the diagonal position [7,12]. It is thus reasonable to see that the binding of IPA to HRP, possibly at the heme edge, is accompanied by local conformational change such as the change in the heme ring planarity, eventually resulting in appreciable shifts of these proton NMR signals. The specific shift of the heme methyl signal at 70.9 ppm of the native ferric high spin enzyme is also probably resulting from this local conformational change occurring at this methyl group. The slight shift of other signals of the hyperfine shifted resonances encountered for HRP and cyano HRP may be a second order effect of this conformational change effected by the donor.

Here we have attempted to speculate the model of interaction between IPA and HRP in which the model previously proposed for the binding of donor to the enzyme is criticized. We have also examined here other donors such as aminotriazole and resorcinol and obtained similar spectral shifts in favor of the present model of the heme edge interaction between the enzyme and the donor. It seems, however, premature to draw a firm conclusion on the orientation of the donor molecule in the HRP–donor complex until the exact assignments of the NMR signals of the enzyme are performed, which is currently under progress in our laboratory.

* This was confirmed by our preliminary studies of heme reconstitution of HRP from protoheme to mesoheme (I. Morishima and T. Inubushi, unpublished result).

Acknowledgements

The authors wish to thank Drs T. Hosoya and J. S. Leigh for their helpful suggestions. We are also grateful to Drs T. Yonetani and T. Iizuka for their encouragement. A part of this investigation was supported by a grant from the Ministry of Education, Japan, and Toray Research Fund for Science and Technology.

References

[1] Hosoya, T. and Morrison, M. (1967) J. Biochem. 242, 2828–2836.
[2] Critchlow, J. E. and Dunford, H. B. (1972) J. Biol. Chem. 247, 3714–3725.
[3] Shonbaum, G. R. (1973) J. Biol. Chem. 248, 502–511.
[4] Schejter, A., Lanir, A. and Epstein, N. (1976) Arch. Biochem. Biophys 174, 36–44.
[5] Bruns, P. S., Williams, R. J. P. and Wright, P. E. (1975) J. C. S. Chem. Comm. 1975, 795–796.

[6] Leigh, J. S., Maltempo, M. M., Ohlsson, P. I. and Paul, K. G. (1975) FEBS Lett. 51, 304–308.
[7] Wuthrich, K. (1970) Struct. Bonding 8, 53–121.
[8] Morishima, I. and Iizuka, T. (1974) J. Am. Chem. Soc. 96, 7365–7367.
[9] Shannon, L. M., Kay, E. and Lew, J. Y. (1966) J. Biol. Chem. 241, 2166–2172.
[10] Morishima, I., Ogawa, S., Inubushi, T., Yonezawa, T. and Iizuka, T. (1977) Biochemistry, submitted for publication.
[11] Mayer, A., Ogawa, S., Shulman, R. G., Yamane, T., Cavaleiro, J. A. S., Gonsalres, A. M. d'A.R., Kenner, G. W. (1974) J. Mol. Biol. 86, 749–756.
[12] Shulman, R. G., Glarum, S. H. and Karplus, M. (1971) J. Mol. Biol. 57, 93–115.