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8-FLUORO-8-DEMETHYLRIBOFLAVIN AS A ^{19}F-PROBE FOR FLAVIN-PROTEIN INTERACTION. A ^{19}F NMR STUDY WITH EGG WHITE RIBOFLAVIN BINDING PROTEIN

Retsu Miura, Sabu Kasai,* Kihachiro Horiike,**[1] Kyoko Sugimoto,* Kunio Matsui,* Toshio Yamano,** and Yoshihiro Miyake

Department of Biochemistry, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka 565, Japan
*Division of Biology, Research Institute for Atomic Energy, Osaka City University, Sugimoto-cho, Sumiyoshi-ku, Osaka 558, Japan
**Department of Biochemistry, Osaka University Medical School, Kita-ku, Osaka 530, Japan

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SUMMARY: Binding of 8-fluoro-8-demethylriboflavin to riboflavin binding protein was studied ^{19}F-NMR spectroscopically. The pH dependence of ^{19}F chemical shift of this fluoro derivative both free and bound to the protein revealed a shift of pK_a associated with N(3)-H to alkali when bound to the protein. ^{19}F chemical shift of the fluoro derivative bound to the protein is in a higher magnetic field than that of the free derivative in the neutral to acidic pH region. These results are interpreted in terms of the environmental changes at the 8- and 3-positions of isoalloxazine, indicating the applicability of this derivative for probing flavin-protein interaction at these positions.

Riboflavin binding proteins (RBP) from hen egg have been a subject of various physicochemical studies. The results obtained therein have provided valuable information on the flavin-protein interaction in flavoproteins in general as well as in RBP itself.

In this communication we report on the interaction between 8-fluoro-8-demethylriboflavin (FF) with hen egg white apoRBP by ^{19}F NMR spectroscopy. We provide lines of evidence that this flavin derivative serves as an excellent probe for the exploration of the flavin-protein interaction not only around the 8-position but also in the vicinity of the N(3) of the isoalloxazine nucleus.

1) Present address: Department of Biochemistry, Shiga University of Medical Science, Ohtsu, Shiga 520-21, Japan.

Abbreviations: RBP, riboflavin binding protein; FF, 8-fluoro-8-demethyl-riboflavin.

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MATERIALS AND METHODS

The synthesis of FF has been described elsewhere (1).

ApoRBP from egg white was prepared by the methods described previously (2,3). The purity of the preparation was confirmed by SDS polyacrylamide gel electrophoresis according to Laemmli (4).

Fourier transform ^{19}F NMR spectra were measured at 188.22 MHz on a Varian XL 200 spectrometer. Chemical shifts (δ) were expressed in ppm down field from the external standard, 10 mM sodium fluoride in 0.1 M deuterated sodium phosphate buffer pH 7.0. pH values were direct readings of a pH meter calibrated with standards in light water.

Association constants in the binding of FF to RBP were obtained by fluorometric titration (5,6) with a Hitachi 650-60 fluorescence spectrophotometer.

The concentration of FF was based on the molar extinction coefficient of 1.04×10^4 at 434 nm at pH 7.0 (1). The concentration of apoRBP for the association constant measurements was obtained by fluorometric titration with FF of a known concentration. FF and RBP have been shown previously to bind at the 1:1 ratio (7). The concentration of FF-apoRBP or apoRBP for NMR experiments was estimated by the weight of the lyophilized sample and the molecular weight of 32,000 (8,9).

Samples for NMR measurements were prepared in the following manner. Slight molar excess of FF was mixed with apoRBP. The mixture was thoroughly dialyzed against water and lyophilized. The lyophilized FF-RBP together with lyophilized apoRBP was dissolved in 50 mM deuterated NaH_2PO_4. pH was then adjusted with either DCl or NaOD.

RESULTS

The effect of pH on the binding constant (K in M^{-1}) in FF-apoRBP complex was investigated (Fig. 1). The profile is "bell-shaped" where log K drops sharply at pH below 6 and above 9. This profile not only reflects the mode of binding in the complex but also serves as pertinent data for the NMR experiments described in the subsequent section.

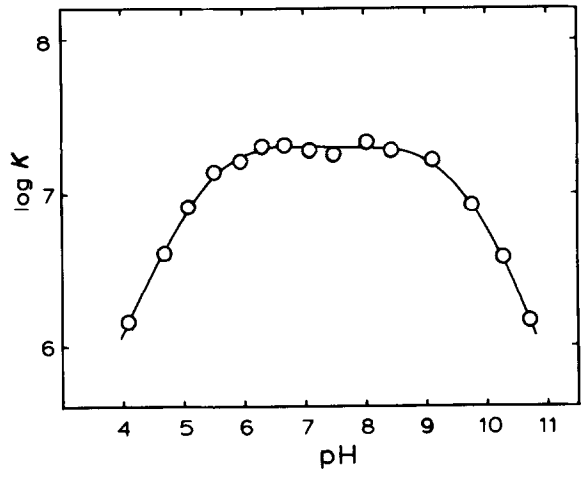

Figure 1. Effect of pH on the association constant (K, in M^{-1}) in 50 mM sodium phosphate. The concentration of apoRBP used was 4.0×10^{-6} M. Temperature was kept at 22°.

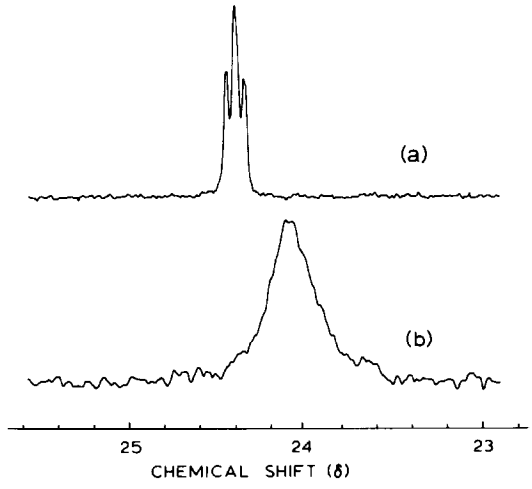

Figure 2. ^{19}F NMR spectra of FF free in solution (a) and bound to apoRBP (b). Concentrations were: (a) FF, 2 mM, (b) FF-apoRBP, 1.1 mM; apoRBP, 0.5 mM. pH: (a) 6.00, (b) 6.18. Pulse width and sampling time were 4 μs (34°) and 0.8 s, respectively. Temperature was kept at 23 °. Numbers of accumulations were: (a) 9,000, (b) 39,400. Delay time of 2 s was taken for (b) between samplings.

Figure 2 shows the ^{19}F NMR spectra of FF free in solution and bound to apoRBP. F(8) of free FF (Fig. 2a) exhibits a sharp triplet signal due to couplings with H(6) and H(9). On the other hand, F(8) of the bound FF gives a broad peak (Fig. 2b). The coupling pattern is no longer apparent being obscured by broadening. Diluting the sample of Fig. 2b did not sharpen the F(8) signal, indicating that the broadening is not due to the viscosity effect but is to be attributed mainly to the motional restriction of FF as the result of complex formation. The F(8) signal of bound FF appears at a slightly higher field than that of free FF at pH about 6, reflecting the difference in the magnetic environment of F(8) in the two species. To investigate this difference in detail, we performed pH titration of the ^{19}F chemical shifts of the two species (Fig. 3). In free FF, raising pH to alkali shifts the signal appreciably to a higher field, giving a simple titration profile (Fig. 3a). From this profile a pK_a value was estimated at about 9.6 in agreement with that obtained otherwise (1). This pK_a corresponds to dissociation of N(3)-H. It is noteworthy that the chemical shift of F(8) is remarkably sensitive to the electronic environment at N(3) of

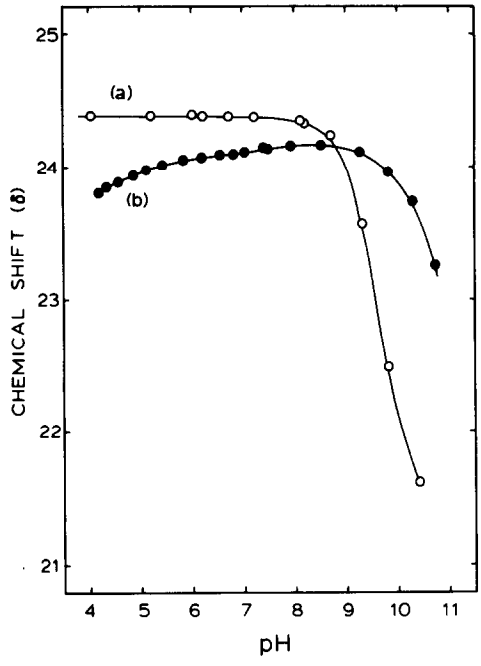

Figure 3. pH titration of ^{19}F chemical shift of FF free in solution (a) and bound to apoRBP (b). The conditions were the same as those in Fig. 1 except pH and numbers of accumulations, which varied from 1,000 to 13,000 in (a) and from 15,000 to 60,000 in (b).

the isoalloxazine nucleus. In contrast to free FF, bound FF exhibits a complex titration profile (Fig. 3b). Taking the concentrations of FF-apoRBP (1.1 mM) and apoRBP (0.5 mM) in the NMR experiments and the pH dependence of the association constant (Fig. 1), we emphasize that the chemical shifts in Fig. 3b in the whole pH range examined represent those of bound FF and not of an equilibrium between free and bound FF. The pK_a corresponding to N(3)-H dissociation in bound FF deviates to more alkali by more than one pH unit. This deviation reflects the characteristic environment of the N(3)-H region of FF surrounded by apoRBP. The F(8) chemical shift of the bound FF is at a distinctly higher magnetic field than that of free FF in the neutral pH region. This chemical shift difference should be derived from the interaction of FF with apoRBP around the 8-position of the isoalloxazine nucleus. Moreover, the F(8) chemical shift of bound FF deviates to an even higher field in a more acidic pH region. Unfortunately, the pK_a value in this

pH region could not be estimated due to a sharp drop in the binding constant at pH below 4.

DISCUSSION

We have demonstrated here that the chemical shift of F(8) of FF is sensitive to the electronic environment of N(3)-H and that F(8) can be a reporter group on the environment around the N(3)-H as well as the 8-position of the isoalloxazine nucleus. The increase of the pK_a value associated with N(3)-H of FF when bound to apoRBP may be an indication that the N(3) proton of FF interacts with a proton acceptor of the apoprotein forming a N(3)-H protein hydrogen bond (10-12). Alternatively, the increase of the pK_a may be explained by the hydrophobic environment around the 3-position or in the overall binding domain (6,13-15) or the presence of negative charge in the vicinity of N(3)-H. Kitagawa *et al.* (11) have pointed out that one of the resonance Raman lines of a flavoprotein can be an indicator of N(3)-H protein interaction which differs from protein to protein. Although the unequivocal interpretation of the pK_a shift must await further investigation, the results obtained here indicate that ^{19}F NMR of FF can be expected to monitor the flavin-protein interaction at N(3)-H in various flavoproteins.

The mode of the contact between riboflavin and apoRBP at the 8-position has been elaborately studied with a variety of riboflavin derivatives with different substituents at the 8-position (7,14-17). These studies revealed different aspects of the bindig mode. The chemical shift difference between ^{19}F of free and bound FF in the neutral pH region should reflect the environment produced by the surrounding apoRBP. The difference is not contradictory to the presence of aromatic residues (6,13,15) or negative charge (7,18) in the vicinity of the 8-position. The chemical shift change of ^{19}F of bound FF to a higher field by lowering pH may be interpreted in terms of a conformational change, since mere protonation of the negative charge

would not explain the behavior. This conformational change is compatible with the suggestion that an aromatic-rich cleft of the riboflavin binding site opens as a result of lowered pH (9). Further investigation to explore the environment surrounding the F(8) in FF-apoRBP by ^{19}F NMR is in progress.

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