PROTON NMR STUDY OF THE INTERACTION OF RIBOFLAVIN WITH THE EGG-YOLK APOPROTEIN

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1. Introduction

Extensive studies of the flavoprotein complexes by a variety of techniques have not yet given relevant information about the complicated mechanism of flavin–protein interaction. In the interaction of flavins with apoproteins the importance of both the isoalloxazine ring's different reactive sites [1–3] and the ribityl moiety [4] have been suggested. The histidyls [5], tryptophanyls [6–8] and tyrosyls [4] of the polypeptide chain have also been shown or postulated.

As a model for the binding of flavin nucleotides in flavoproteins with redox activity we have studied the interaction of riboflavin with the apoprotein from hen egg-yolk [9]. Following the concept that the mechanism of the flavin–apoprotein interaction should be reflected in the NMR spectra of riboflavin itself when the binding protein is present in the system, we have carried out experiments using NMR spectroscopy.

2. Experimental

The riboflavin-binding protein was isolated from egg-yolk and characterized as described previously [9,10]. The apoprotein was freshly dissolved in 0.1 M Na-phosphate buffer in D_2O (IBJ, Świerk, Poland, 99.8% isotopic purity) at pH 7.0.

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Riboflavin (Koch-Light, England) was freshly dissolved in 0.02 M NaOD (Biorad Labs, USA) in D_2O and the solutions were protected from the light.

The solutions of riboflavin and apoprotein were mixed and diluted to a final concentration just before the NMR run. The pH of the samples was adjusted to 11.3 by adding 0.002 M NaOD using a Mera-Elmat type N-512 pH-meter (Poland). It was found in gel filtration experiments that in these conditions, when protected from light, the full binding activity of riboflavin with apoprotein was preserved.

Proton NMR spectra were recorded at 60 MHz on a VARIAN EM-360 spectrometer, at 28°C with tetramethylsilane (TMS) in $CDCl_3$ as external standard. All measurements with each sample were completed within about 15 min after mixing the solutions.

For the determination of the line-widths at half-heights, the spectra were expanded in the ranges 60–180 Hz, 180–300 Hz or 420–540 Hz.

3. Results

The assignment of the particular lines in the proton NMR spectrum of riboflavin (fig.1) was made by reference to those for FMN and for various lumiflavin derivatives [11], as well as to more recent NMR data [12–14] for substituted or naturally occurring flavins.

With increase of apoprotein concentration the riboflavin spectrum shows several remarkable and selective changes in both the widths and the positions of the

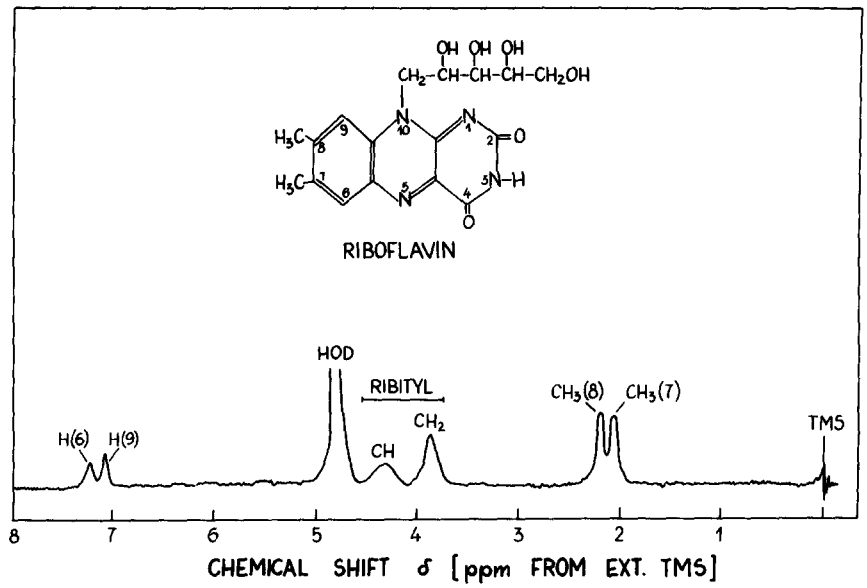

Fig.1. Proton magnetic resonance spectrum of riboflavin, 0.075 M in 0.02 M NaOD.

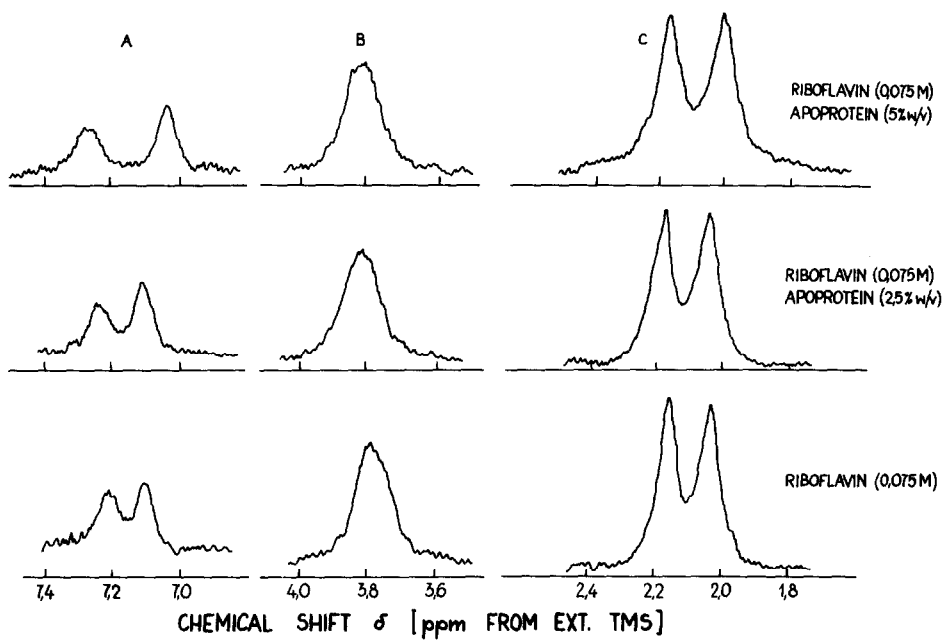

Fig.2. Changes in the riboflavin proton magnetic resonance spectra with increasing apoprotein concentration. (A) Acryl protons H(6) and H(9). (B) CH_2 peak in ribityl moiety. (C) Methyl protons at C(7) and C(8).

individual lines (fig.2). The lines attributable to the aryl proton H(6) (fig.2A) as well as to both methyl groups at C(7) and C(8) (fig.2C) are likewise broadened by comparison to either the H(9) peak (fig.2A) or to the ribityl methylenes (fig.2C). Also, characteristic upfield shifting of the H(9) peaks can be noticed whereas other peaks are only slightly shifted to lower or higher fields as compared to their position in free riboflavin.

The dependences of line-width of the riboflavin peaks on apoprotein concentration gave the detailed quantitative comparison (fig.3) between the selective broadenings of some isoalloxazine peaks and the essential lack of broadening of the CH_2 signal of the side chain. The respective dependence of the chemical shifts (fig.4) indicates the only distinct upfield shift for H(9), slight downfield shift for H(6), and the tendency toward the separation of the riboflavin peaks with decreasing riboflavin : apoprotein molar ratio for both aryl protons and to a lesser extent for methyl groups.

In contrast to isoalloxazine proton lines, where strong changes are observed with increase of apoprotein concentration, the ribityl methylene peak shows lack of broadening (fig.3) and only slight variation in the position (fig.4). It is apparent, that combination of both the line-width and the chemical shift data reveals, within the limits of the error involved, relatively slightest differences for ribityl methylene as compared with all isoalloxazine protons.

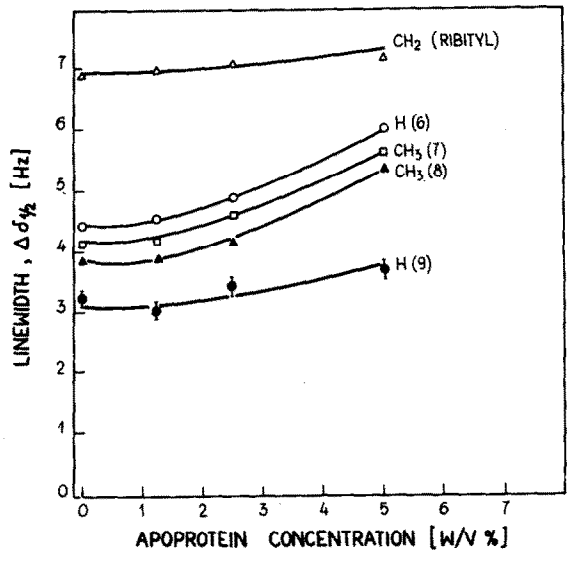

Fig.3. The dependence of the line-widths of the riboflavin lines on the apoprotein concentration. Each point is the mean value from three independently prepared and measured samples. The error, a maximum deviation between the results is not higher than indicated for H(9).

4. Discussion

In the analysis of small ligand NMR spectra the selective line broadening or shifting in the presence of a protein-carrier can be interpreted in terms of direct participation of a portion of a ligand molecule in the formation of a molecular complex. The method based on this concept [15] has proved successful in the investigations of many ligand–protein systems and has been well reviewed [16,17].

In our case, the selective broadening of the lines for aryl protons H(6) together with those for methyl protons at C(7) and C(8) can be interpreted in terms

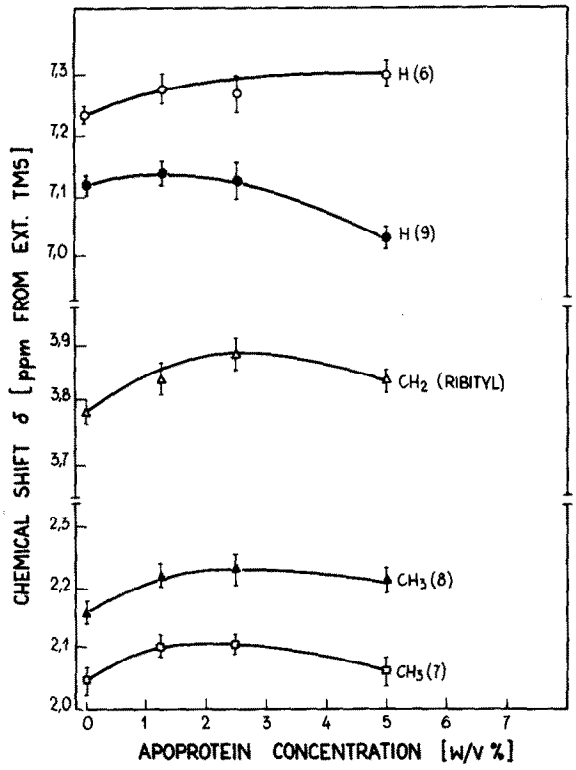

Fig.4. The dependence of the chemical shifts of the riboflavin lines on the apoprotein concentration.

of specific interactions between riboflavin and apoprotein and the strong, but assymmetric immobilization of the aromatic ring of isoalloxazine during flavoprotein complex formation. The lack of broadening for the methylene protons in the ribityl moiety, can suggest the absence of a weak hydrophobic interaction between the side chain of riboflavin and the apoprotein. However, this view needs more experimental work on flavins with modified ribityl groups, since the preliminary results [18] obtained for these showed lower affinities for the apoprotein studied.

Our experiments were carried out at high molar excess (~ 50–200) of riboflavin over the apoprotein, whereas a stoichiometry of 1:1 in the case of this flavoprotein is found [9]. In these conditions 1.5-fold broadening of the selective peaks (fig.3) at about 50-fold molar excess of riboflavin can reflect the strong immobilization of a small fraction of the flavin present in the solution and the fast exchange between the fractions of free and bound riboflavin.

Recently the importance of tryptophan residues and hydrophobic interaction in riboflavin binding has been shown experimentally in egg-white [8] and egg-yolk [7] flavoproteins. Completing the model of riboflavin–apoprotein interaction one can postulate that it could involve hydrophobic or π–π interaction between two identical aromatic rings in the isoalloxazine moiety and a tryptophanyl residue. The hydrophobic interaction [20] is made manifest by increases in the line-widths of H(6) protons and methyl groups. The significant upfield shift for the H(9) proton at the lowest molar riboflavin : apoprotein ratio also suggests stacking of aromatic rings [21] probably by assymmetric π –π interaction in egg-yolk flavoprotein. A similar mechanism for the interaction of flavin with L-lactate apodehydrogenase of yeast was postulated by Risler [22]. However, the sites which interact with apoproteins are known to be also localized at the 1–5 positions in the isoalloxazine moiety [23]. These positions cannot be studied by proton magnetic resonance but rather by ^{13}C and ^{15}N NMR FT spectroscopy which has already supplied some relevant information about the flavin structures [24,25].

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