# Carbon-13 and Nitrogen-15 Nuclear Magnetic Resonance Study on the Interaction between Riboflavin and Riboflavin-Binding Apoprotein†

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ABSTRACT: Riboflavin-binding apoproteins from egg yolk and egg white have been reconstituted with $^{15}N$- and $^{13}C$-enriched riboflavin derivatives. These protein preparations were investigated in the oxidized and two-electron-reduced states by $^{15}N$ and $^{13}C$ NMR techniques. The chemical shift values of the protein-bound flavin are compared to those of free flavin in water or chloroform. The results are interpreted in terms of interactions between the apoprotein and its prosthetic group and the conformation of bound flavin. In the oxidized and in the reduced states, the chemical shifts and line-broadening effects of the bound riboflavin are extremely similar for the two different proteins, indicating similar binding sites for riboflavin. The solvent accessibility to the bound riboflavin is similar in the reduced and the oxidized complexes; i.e., the N(3), O(2$\alpha$), and N(1) atoms are exposed, whereas the O(4$\alpha$) atom is shielded from solvent water. A hydrogen bond to the N(5) atom exists in the oxidized state. This hydrogen bond is weaker than the corresponding one between free FMN and water. At best, only a weak hydrogen bond exists to the O(4$\alpha$) atom. Binding of oxidized riboflavin forces the N(10) atom of flavin into the molecular plane. The isoalloxazine ring is strongly polarized. No hydrogen bond to the O(4$\alpha$) atom is formed in the reduced neutral state of the complex. Although the N(1) atom is accessible to solvent water, it possesses more $sp^3$ character than in free $FMNH_2$. The data also suggest a rapid motion of the N(1) atom in and out of the molecular plane. The reason for these effects is probably the absence of a hydrogen bond to the O(4$\alpha$) atom, which makes $\pi$-electron delocalization from the N(1) atom unfavorable. The N(10) atom exhibits about the same configuration as in the oxidized state. In contrast, the N(5) atom shows increased $sp^3$ hybridization. The p$K$ value of the deprotonation of the N(1)H group is measured as 7.45, a shift of about 0.8 pH unit to more alkaline pH values as compared to that of free $FMNH_2$. This p$K_a$ shift might be caused by the absence of a hydrogen bond to the O(4$\alpha$) atom. In the anionic reduced state, the N(1) atom shows even more $sp^3$ character than in the neutral complex, which is explained by the absence of a hydrogen bond to the O(4$\alpha$) atom. The $^{15}N$ resonance line due to the N(1) atom is severely broadened, probably due to its motion in and out of the molecular plane. The N(5) atom shows almost the same $^{15}N$ chemical shift as that in the neutral complex, indicating an increased $sp^3$ character. This is confirmed by the coupling constant of 78 ± 5 Hz with the covalently bound hydrogen atom. No coupling was detected in the neutral complex due to rapid hydrogen exchange with solvent water. These results show that the N(5) atom is shielded from solvent at high pH values. The N(10) atom exhibits considerable $sp^2$ character in the anionic complex. The results are discussed in the light of the possible function of the protein.

Riboflavin-binding protein (RBP)[1] is found in the yolks and whites of all avian eggs (Osuga & Feeney, 1968). The function of the protein is still not fully understood, although it is known that it mediates the transport of riboflavin from the maternal system to the developing oocytes, where riboflavin is essential for embryonic growth and development (Winter et al., 1967; Ostrowski et al., 1968). It has also been suggested that RBP in egg whites functions as an inhibitor of bacterial growth (Board & Fuller, 1974).

As flavodoxins (Mayhew & Ludwig, 1975), RBPs are often used as a model system for flavin–protein interactions. The easy availability of large quantities of these proteins makes them especially suitable for a study of the flavin–apoflavoprotein interactions. The importance of these interactions in flavin biochemistry is obvious since it has been suggested that specific interactions in flavin–apoflavoprotein complexes are responsible for the "tuning" of the flavoprotein for its specific catalysis (Müller, 1972; Eweg et al., 1982).

Several physicochemical studies have been reported on RBP and its interaction with riboflavin. It has been shown that RBP from egg yolk (RBPY) resembles RBP from egg white (RBPW) with respect to the thermodynamics of riboflavin binding (Matsui et al., 1982a,b). The two proteins have roughly the same molecular weight (Ostrowski & Krawczyk, 1963; Blankenhorn, 1978; Froehlich et al., 1980), have the same amino acid composition and flavin–protein association constants (Farrell et al., 1969; Ostrowski & Krawczyk, 1963; Steczko & Ostrowski, 1975), and are immunologically indistinguishable (Farrell et al., 1970). They differ however with respect to the content of sialic acid (Jacubczak et al., 1968; Kawabata & Kanamori, 1968). In addition, Matsui et al. (1982a,b) have shown that the volume of the cavity for the binding site of riboflavin differs for the two proteins. This has however no influence on the kinetics of the association between flavin and apoprotein, as shown by Nishina (1977).

The use of riboflavin analogues provided new insights into the relative importance of the different atoms of the isoalloxazine ring for the binding by RBPY and RBPW (Matsui

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[1] Abbreviations: RBP, riboflavin-binding protein; RBPY, riboflavin-binding protein from egg yolk; RBPW, riboflavin-binding protein from egg white; NMR, nuclear magnetic resonance; FMN, riboflavin 5′-phosphate; TARF, tetraacetylriboflavin; $FMNH_2$ and $FMNH^-$, neutral and anionic 1,5-dihydroflavin mononucleotide; $TARFH_2$, 1,5-dihydrotetraacetylriboflavin; MeIMN, $MeIMNH_2$, and $MeIMNH^-$, 7-methyl-10-ribitylisoalloxazine 5′-phosphate in the oxidized, the neutral, and the anionic 1,5-dihydro state, respectively; MeTARI and $MeTARIH_2$, oxidized and reduced neutral 7-methyl-10-(tetraacetylribityl)isoalloxazine, respectively.

0006-2960/84/0423-4873$01.50/0 

et al., 1982a,b; Choi & McCormick, 1980; Becvar & Palmer, 1982; Nishikimi & Kyogoku, 1973). These studies resulted in the conclusion that the dimethylbenzene ring of flavin is buried in the interior of the protein, i.e., bound differently than in flavodoxins (Mayhew & Ludwig, 1975). Thermodynamic studies on RBPW support this conclusion (Matsui et al., 1982a). In addition, it was shown by chemical modification studies that a tryptophan and a tyrosine residue are involved in the binding of riboflavin by RBPW (Blankenhorn, 1978) and probably also by RBPY (Steczko & Ostrowski, 1975). Kumosinski et al. (1982) suggested for RBPW that an aromatic-rich cleft opens at pH values $3.7 < pH < 7.0$ and concomitantly releases the riboflavin.

The $^{13}C$ and $^{15}N$ NMR techniques are powerful methods for detecting subtle differences in hydrogen bonding and conformation of the flavin in both the oxidized and the reduced state (Moonen et al., 1984) and for elucidation of dynamic processes (Moonen & Müller, 1983). These methods have successfully been applied, with specifically $^{13}C$- and $^{15}N$-enriched flavins, to flavodoxins from *Megasphaera elsdenii* and *Azotobacter vinelandii* (Van Schagen & Müller, 1981; Franken et al., 1984), where specific hydrogen bonding was detected. Some $^{13}C$ NMR experiments on oxidized RBPW were carried out by Yagi et al. (1976). No theory for the interpretation of the data was developed at that time. We now report on a detailed study on the complex between RBPY and riboflavin and RBPW and riboflavin using specifically labeled [$^{13}C$]- and [$^{15}N$]riboflavin derivatives. Several specific interactions are detected in both the oxidized and the reduced systems. In addition, novel information is revealed concerning the hydrogen exchange of hydrogen-carrying nitrogen atoms, which show an unexpectedly strong dependence on pH. Dynamic information about the riboflavin binding site is deduced from these results.

## Materials and Methods

Apo-RBPY was isolated and purified according to Murthy et al. (1979) with the exception that an Amicon concentrator was used instead of aquacide treatment. Apo-RBPW was isolated and purified according to Farrell et al. (1969). Specific $^{13}C$ enrichment of riboflavin was performed according to Van Schagen & Müller (1981). Specific $^{15}N$ enrichment was performed as described by Müller et al. (1983).

The relative concentration of riboflavin-binding apoprotein and riboflavin was 1:1 in all samples. These were prepared as follows. Reconstitution of riboflavin-binding apoprotein with riboflavin was carried out with 0.1 mM apoprotein and an excess of riboflavin in 50 mM potassium phosphate (pH 7.0). Excess of riboflavin was removed by exhaustive dialysis against the same buffer. In the final dialysis, the buffer concentration was decreased to 1 mM. The protein–riboflavin complex was then concentrated by lyophilization.

Wilmad 10-mm precision NMR tubes were used. The samples contained 1–3 mM RBP and 100 mM potassium phosphate buffer of varying pH (see Results and Discussion). Depending on the kind of experiment, the samples contained 10 or 100% $^2H_2O$. The sample volume was 1.6 mL. The pH measurements were carried out before and after the NMR measurements and are reported without correction for the deuteron isotope effect. Reduction was conducted by addition of the desired amount of a dithionite solution to the anaerobic solution of RBP. Anaerobiosis was achieved by carefully flushing the solutions in the NMR tube with argon for about 20 min. The NMR tube was sealed with a serum cap.

All measurements were performed on a Bruker CXP 300 spectrometer operating at 30.4 MHz for $^{15}N$ and 75.6 MHz for $^{13}C$ NMR experiments. Broad-band decoupling of only 0.5 W was applied for $^{13}C$ NMR measurements to prevent heating up of the sample. There was no need for a higher decoupling power as we were mainly interested in quaternary carbon atoms. No decoupling was used for $^{15}N$ NMR measurements in order to be able to observe the exchange rate of the NH protons. Dioxane (3 $\mu$L) was used as an internal reference for $^{13}C$ NMR. Chemical shift values are reported relative to $Me_4Si$ ($\delta_{dioxane} - \delta_{Me_4Si} = 67.84$ ppm). Neat $CH_3{}^{15}NO_2$ was used as an external reference for $^{15}N$ NMR with a coaxial cylindrical capillary as recommended by Witanowski et al. (1981). Chemical shift values are reported relative to liquid $NH_3$ at 25 °C as recommended by Levy & Lichter (1979) [$\delta_{CH_3NO_2} - \delta_{NH_3} = 381.9$ ppm for the magnetic field parallel to the sample tube (Witanowski et al., 1981)]. Values are reported as true shieldings, i.e., corrected for bulk volume susceptibilities as described by Witanowski et al. (1981). The accuracy of the reported values is ±0.1 ppm for $^{13}C$ and ±0.3 ppm for $^{15}N$ chemical shift values, unless otherwise stated. The temperature of all samples was kept constant at 26 ± 2 °C. All spectra were recorded with 30° pulses and a repetition time of 0.5–1 s.

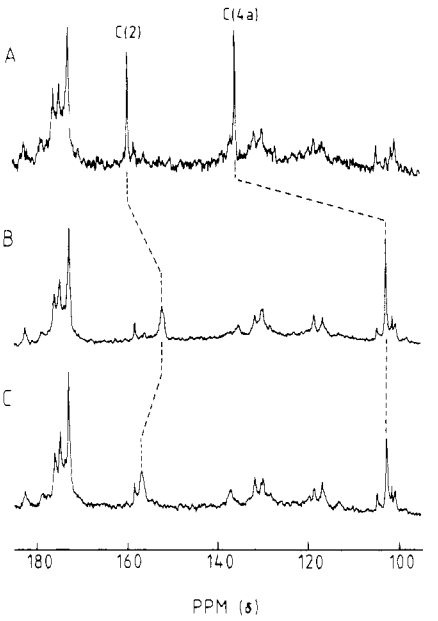


FIGURE 1: $^{13}C$ NMR spectra of riboflavin-binding apoprotein from egg yolk reconstituted with [2,4a-$^{13}C_2$]riboflavin: (spectrum A) oxidized protein, in 100 mM potassium phosphate, pH 8.5; (spectra B and C) two-electron-reduced protein in 100 mM potassium phosphate, pH 6.0, and in 100 mM sodium borate, pH 9.0, respectively.

## Results and Discussion

*Studies on Oxidized State.* The dissociation constant of RBPW and RBPY with riboflavin is about 50 nM (Choi & McCormick, 1980; Zak et al., 1972). For the samples used in this study, it is calculated that only about 0.1% riboflavin is free in solution. Consequently, the reported data are not distorted by free riboflavin. Spectrum A of Figure 1 shows the low-field part of the $^{13}C$ NMR spectrum of RBPY complexed with riboflavin. The possible interference with protein resonances for the determination of the chemical shift values of bound riboflavin was eliminated by the use of difference spectra (data not shown). The resonances due to natural abundance $^{13}C$ of the protein in the spectra can partly be assigned according to Allerhand (1979) and Gratwohl & Wüthrich (1974). All peptide carbonyl and carboxyl resonances appear between 170 and 180 ppm. The Arg $C_\zeta$ atoms are found at 158.2 ppm. Aromatic carbon atoms of Trp, Tyr,

Table I: Carbon-13 and Nitrogen-15 Chemical Shifts of Oxidized Riboflavin Bound to Riboflavin-Binding Apoprotein from Egg Yolk (RBPY) and from Egg White (RBPW)[a]

| | | chemical shift (ppm) of carbon atoms | | | |
|---|---|---|---|---|---|
| compd | solvent | C(2) | C(4) | C(4a) | C(10a) |
| RBPW[b] | pH 8.5 | 159.7 | 162.1 | 135.9 | 152.3 |
| RBPY[b] | pH 8.5 | 159.6 | 162.0 | 135.9 | 152.4 |
| FMN[c,d] | pH 8.0 | 159.8 | 163.7 | 136.2 | 152.1 |
| TARF | $C^2HCl_3$ | 155.2 | 159.8 | 135.5 | 149.1 |
| | | chemical shift (ppm) of nitrogen atoms | | | |
| compd | solvent | N(1) | N(3) | N(5) | N(10) |
| RBPW[b,e] | pH 9.0 | 191.5 | 159.2 | 338.2 | 165.4 |
| RBPW[e,f] | pH 6.4 | 191.5 | 158.5[g] | 337.7[h] | 165.1 |
| RBPY[b,e] | pH 6.2 | 191.6 | 159.4[g] | 338.2[h] | 165.3 |
| MeIMN[c-e] | pH 8.0 | 190.5 | 160.4 | 335.5 | 164.6 |
| MeTARI[e] | $C^2HCl_3$ | 201.1 | 160.7 | 346.7 | 150.4 |

[a] Line width of $^{13}C$ resonances is 10 ■ 4 Hz and that of $^{15}N$ resonances 15 ± 5 Hz, unless otherwise stated. For comparison also, $^{13}C$ and $^{15}N$ chemical shifts of FMN in water and of TARF in $C^2HCl_3$ are included. [b] Solvent is 90% $H_2O$/10% $^2H_2O$. [c] FMN or MeIMN was used for the comparison because of its higher solubility in $H_2O$ than riboflavin analogues. [d] Independent of pH in the range $5 < pH < 9$. [e] See Figure 3 for structure of the flavin. [f] Solvent is 100% $^2H_2O$. [g] The resonance signal of $^{15}N(3)$ is broadened at a pH value of about 6; line width ~ 40 Hz. Accuracy of the chemical shift given is ±1 ppm (see text). [h] Line width is about 30 Hz.

Phe, and His resonate between 110 and 140 ppm. An interesting region lies between 95 and 105 ppm where normally no carbon atoms of amino acids resonate. However, several natural abundance resonances of RBPY are found in this region. As already pointed out in the introduction, RBPY contains sialic acid. The C(1) atoms of some carbohydrate components of sialic acid appear in the region between 95 and 105 ppm. The resonance position should in principle allow a rough determination of the different stereoisomers (Bock & Thogerson, 1982). In addition, several lines due to other carbohydrate residues were observed in the spectral region between 60 and 80 ppm (data not shown). It should be mentioned that these regions differ between RBPY and RBPW, as would be expected from the difference in sialic acid content. However, these spectral regions are not further considered here as this lies beyond the scope of this study.

The flavin $^{13}C$ chemical shifts for the complexes of RBPY and RBPW are summarized in Table I. Spectrum A of Figure 2 shows a $^{15}N$ NMR spectrum of $^{15}N$-enriched riboflavin bound to RBPW. Only small natural abundance resonances near 120 ppm are observed, which are due to peptide nitrogens (Levy & Lichter, 1979). The $^{15}N$ chemical shifts are also summarized in Table I. The structure of the flavin labeled at all four nitrogen atoms with $^{15}N$ is given in Figure 3.

In the accompanying paper (Moonen et al., 1984), we have shown that the $^{13}C$ and $^{15}N$ chemical shifts are very sensitive to hydrogen bonding, conformation of the flavin, and the polarity of the solvent used. The extreme situations of strong hydrogen bonds and high polarity around the isoalloxazine ring (e.g., FMN in water) as against no hydrogen bonds and low polarity (e.g., TARF in chloroform) are also listed in Table I in order to facilitate the analysis.

A rough comparison between the data of riboflavin bound to RBPY and RBPW shows (Table I) that the chemical shifts for the two proteins hardly differ. The differences are all within the experimental accuracy of the data. This leads to the conclusion that the binding site for the oxidized isoalloxazine ring must be very similar in the two proteins. Consequently, we can discuss the interactions for both complexes together.

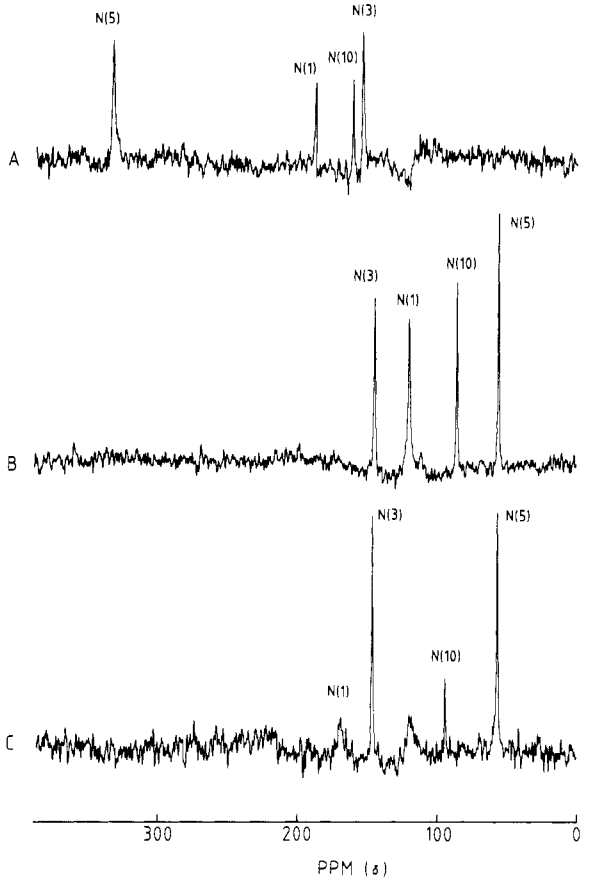


FIGURE 2: $^{15}N$ NMR spectra of riboflavin-binding apoprotein from egg white reconstituted with [1,3,5,10-$^{15}N_4$]-7-methyl-10-ribityl-isoalloxazine: (spectrum A) oxidized protein in 100 mM potassium phosphate, pH 6.4; (spectra B and C) two-electron-reduced protein in 100 mM potassium phosphate, pH 6.3, and 100 mM sodium borate, pH 9.0, respectively.

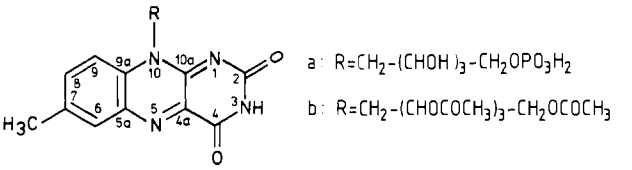


FIGURE 3: Structure of flavin derivatives enriched with $^{15}N$ at positions 1, 3, 5, and 10. R = ribityl, tetraacetylribityl, and ribityl 5′-monophosphate.

Contrary to $^1H$ NMR where ring current effects of neighboring aromatic amino acids influence chemical shifts, such effects are of relative negligible importance in $^{13}C$ and $^{15}N$ NMR. Therefore, the data in Table I can be analyzed directly in terms of hydrogen bonding, specific interactions, and conformation of the flavin in the complexes following the semiempirical rules of the accompanying paper (Moonen et al., 1984).

In the oxidized state, the $^{15}N(3)$ atom in riboflavin bound to the two proteins is the only nitrogen atom carrying a covalently bound hydrogen atom. A $^1J_{^{15}N(3)-^1H}$ of 92 Hz is expected (Franken et al., 1984). No splitting of the $^{15}N$ signal due to the N(3) atom of flavin is observed on use of $H_2O$ as a solvent. This indicates that the exchange rate of the proton is high (lifetime $\tau \ll 1/^1J$, i.e., $\tau \ll 10$ ms). This result strongly suggests that the N(3) atom is exposed to solvent and also explains why RBP can be easily purified by using riboflavin coupled via the N(3) position to a matrix (Blankenhorn et al., 1975). Choi & McCormick (1980) arrived at the same

conclusion by studying the interaction between RBP and flavin analogues.

The resonance position of C(2) is almost the same as that of FMN in water. This suggests that O(2$\alpha$) is also exposed to solvent water, besides N(3), again in accordance with the binding studies of flavin analogues. The resonance position of C(4) clearly shows that the hydrogen bond to O(4$\alpha$) is considerably weaker than in free FMN. The upfield shift of N(3) is probably a consequence of the weak hydrogen bond to O(4$\alpha$), as by approximation the N(3)H–C(4)–O(4$\alpha$) group in oxidized flavin can be regarded as a "peptide bond". It is known that the nitrogen resonance of peptides is mainly governed by the hydrogen bond to the carbonyl function (Witanowski et al., 1981). The resonance position of N(1) indicates that a strong hydrogen bond is formed to the N(1) atom of bound riboflavin. This hydrogen bond is comparable to that of FMN in water. Thus, with regard to hydrogen bonding and solvent exposure, the data indicate that N(3), O(2$\alpha$), and N(1) are exposed to solvent, whereas N(5) exhibits a somewhat weaker hydrogen bond as compared with free FMN.

It has been observed that the p$K_a$ value of the deprotonation of N(3)H of protein-bound riboflavin is increased (Miura et al., 1983) as compared to that of free riboflavin. It is suggested that the weak hydrogen bond to O(4$\alpha$) of the protein-bound riboflavin is responsible for the increased p$K_a$ value.

The chemical shifts due to N(10) and C(10a) can be used as monitors with regard to the polarization of the isoalloxazine ring and the configuration of the N(10) atom. The N(10) resonance position is shifted even further downfield than in free FMN. The same holds for C(10a), although to a lesser extent. This clearly demonstrates that N(10) is forced into the molecular plane and the isoalloxazine ring is strongly polarized. These results suggest that riboflavin interacts strongly with the apoprotein and that the neighborhoods of the N(10) atom and the N(10) side chain of riboflavin are strongly involved in the interaction. This interpretation is in accord with binding studies of flavin analogues (Choi & McCormick, 1980).

The $^{15}N$ NMR data at lower pH values show that polarization or hydrogen bonding is hardly altered. The resonance line of N(3) is broadened however (line width ~30 Hz) at pH 6.2 compared to that at higher pH values. This broadening is absent if $^2H_2O$ is used as the solvent. This strongly indicates that we are dealing with a hydrogen-exchange reaction, which is slower at low than at high pH values, in agreement with base-catalyzed hydrogen exchange.

*Studies on Two-Electron-Reduced State.* The dissociation constant of RBPY with reduced riboflavin is about 1 $\mu$M (Scheller et al., 1979). Consequently, our samples investigated contain about 2% riboflavin free in solution. The dissociation constant of RBPW with riboflavin is about 100 times higher in the reduced than in the oxidized state (Blankenhorn, 1978), i.e., 8% of free riboflavin is calculated to be present in our samples. Our chemical shift data will therefore mainly reflect the complex between RBP and riboflavin. Spectra B and C of Figure 1 show $^{13}C$ NMR spectra for RBPY at different pH values. Spectra B and C of Figure 2 show $^{15}N$ NMR spectra of RBPW. The chemical shift values are summarized in Table II. The data for $FMNH_2$ and $FMNH^-$ in water and $TARFH_2$ in chloroform [cf. accompanying paper, Moonen et al. (1981)] are included for comparison.

A comparison of the data for RBPY and RBPW shows that in the reduced state the differences in the chemical shift values are also within the experimental error. From this we conclude,

Table II: Carbon-13 and Nitrogen-15 Chemical Shifts of Reduced Riboflavin Bound to RBPY or RBPW[a]

| compd | solvent | chemical shift (ppm) of carbon atoms C(2) | C(4) | C(4a) | C(10a) |
|---|---|---|---|---|---|
| RBPW[b] | pH 6.0 | 151.1 | 156.5 | 102.8 | 143.9[f] |
| RBPY[b] | pH 6.0 | 151.1 | 156.6 | 102.8 | 144.2[f] |
| $FMNH_2$[c] | pH 5.0 | 151.1 | 158.3 | 102.8 | 144.0 |
| $TARFH_2$ | $C^2HCl_3$ | 150.6 | 157.0 | 105.1 | 137.1 |
| RBPW[b] | pH 9.0 | 158.1 | 156.8 | 102.8[f] | 155.0 |
| RBPY[b] | pH 9.0 | 158.0 | 157.0 | 102.4[f] | 155.0 |
| $FMNH^-$[c] | pH 9.0 | 158.2 | 157.7 | 101.4 | 155.5 |

| compd | solvent | chemical shift (ppm) of nitrogen atoms N(1) | N(3) | N(5) | N(10) |
|---|---|---|---|---|---|
| RBPW[d,e] | pH 5.5 | 123.5[g] | 148.3 | 59.1 | 89.9 |
| RBPW[b,d] | pH 6.3 | 128.5[h] | 148.2[i] | 59.8[i] | 89.9 |
| RBPY[b,d] | pH 6.3 | 129.8[h] | 148.9[i] | 59.9[i] | 90.3 |
| $MeIMNH_2$[c,d] | pH 5.0 | 128.1 | 150.7 | 60.6 | 87.3 |
| $MeTARIH_2$ | $C^2HCl_3$ | 116.7 | 145.8 | 60.7 | 72.2 |
| RBPW[b,d] | pH 9.0 | 176.0[j] | 150.3 | 60.1[k] | 97.7 |
| $MeIMNH^-$[c,d] | pH 9.0 | 186.9 | 150.7 | 60.6 | 97.2 |

[a] For comparison the values for $FMNH_2$ in water and $TARFH_2$ in chloroform are also included. Line width of $^{13}C$ resonances is 10 ± 4 Hz and that of $^{15}N$ resonances 13 ± 5 Hz, unless otherwise stated. [b] Solvent is 90% $H_2O$/10% $^2H_2O$. [c] FMN or MeIMN was used for the comparison because of its higher solubility in $H_2O$ than riboflavin analogues. [d] See Figure 3 for structure of the flavin. [e] Solvent is 100% $^2H_2O$. [f] Line width ~ 70 Hz; precision of chemical shift is ±0.6 ppm. [g] Line width ~ 50 Hz; precision of chemical shift is ±0.4 ppm. [h] Line width ~ 80 Hz; precision of chemical shift is ±1.0 ppm. [i] Line width ~ 50 Hz; precision of chemical shift is ±0.8 ppm. [j] Line width ~ 200 Hz; precision of chemical shift is ±2.0 ppm. [k] Doublet with a coupling constant of 78 ± 5 Hz.

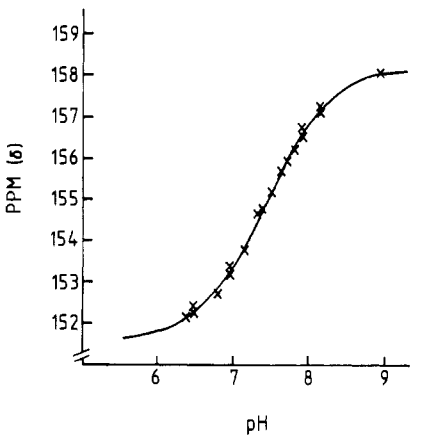


FIGURE 4: pH dependence of the $^{13}C$ chemical shift of 1,5-dihydro-[2-$^{13}C$]riboflavin bound to riboflavin-binding apoprotein from egg white. The solid curve has been calculated with a p$K_a$ value of 7.45.

in agreement with the results on the oxidized state, that the binding site for reduced riboflavin is similar in RBPY and RBPW. Again, our analysis concerns consequently both proteins.

In reduced free FMN, the deprotonation of N(1)H occurs with a p$K_a$ of about 6.6 (Dudley et al., 1964). By using $^{13}C$ (Van Schagen & Müller, 1981) and $^{15}N$ NMR (Franken et al., 1984), it has been shown that the chemical shifts due to $^{15}N(1)$, $^{13}C(2)$, and $^{13}C(10a)$ reflect this deprotonation clearly. Figure 4 shows the dependence of the chemical shift on the pH for RBPW complexed with [2-$^{13}C$]riboflavin. The curve and the extreme chemical shift at low and high pH values are identical with those of reduced FMN in the same buffer, but the p$K_a$ value is shifted from ~6.6 to 7.45 ± 0.05. The p$K_a$ value for RBPY was determined as 7.35 ± 0.05. This supports our conclusion drawn above that the binding sites in the two

proteins must be very similar. An explanation for the increased $pK_a$ values for the complexes as compared with reduced free FMN will be offered below.

The chemical shift of C(2) in the protein-bound, neutral 1,5-dihydroriboflavin is identical with that of $FMNH_2$, suggesting that O(2$\alpha$) interacts with solvent water. No coupling could be observed with $H_2O$ as a solvent for the $^{15}N$ resonance of the N(3)H group. This indicates the exposure of this site to the solvent. The chemical shift of N(3), however, resembles more that of $TARFH_2$ than that of $FMNH_2$. As in the oxidized molecule, a hydrogen bond to O(4$\alpha$) is absent, clearly demonstrated by the upfield position of C(4). The situation is more complex with regard to the N(1) atom. The fact that a good titration curve for the deprotonation of N(1)H could be obtained demonstrates that the protonation/deprotonation reaction is faster than the chemical shift separation of C(2) in the two states (Binsch, 1975), which is about 500 Hz. Moreover, no sharp resonance line for N(1) could be observed in $H_2O$, indicating hydrogen exchange with the solvent being faster than that expected on the one-bond NH coupling constant. These data clearly indicate exposure of N(1) to solvent water. On the other hand, the chemical shift correlates better with that of $TARFH_2$, suggesting a weak exposure to solvent water. This apparent discrepancy is explained as follows. The accompanying paper (Moonen et al., 1984) shows that N(1) in $TARFH_2$ is not of 100% $sp^2$ character and that the $sp^2$ character of the N(1) and the N(10) atom is increased on polarization of the flavin. Although the N(10) atom thus possesses considerable $sp^2$ character in the complex, as illustrated by its chemical shift, the N(1) atoms shows more $sp^3$ character than in $FMNH_2$. Steric hindrance by apo-RBP could explain this observation, but this explanation is not in agreement with the easy accessibility to solvent water. It is therefore suggested that the absence of a hydrogen bond to O(4$\alpha$) causes the observed effect. As O(4$\alpha$) is an unfavorable $\pi$-electron acceptor in the absence of a hydrogen bond, the delocalization of the $\pi$ electrons of N(10) and N(1) is less favored. The N(10) atom is likely forced into the molecular plane by the apoprotein, which makes $sp^2$ hybridization of the N(1) atom unlikely. This could explain why the resonance lines due to N(1) and also C(10a) are broadened considerably at about pH 6.0 (Table II). This broadening and the other data suggest that the N(1) atom in RBP moves in and out of the molecular plane. The C(10a) atom is probably also influenced by this motion. At pH 5.5, the line width of the N(1) resonance line decreases to about 50 Hz (Table II), indicating that the frequency of this motion is increased. The latter result is compatible with the observed swelling of the protein at low pH values (Kumosinski et al., 1982). The $^{19}F$ NMR data of Miura et al. (1983) can be similarly explained. The N(5) atom is also fairly exposed to solvent as no coupling constant could be detected in $H_2O$.

In the reduced state, the degree of bending around the N(5)–N(10) axis (i.e., the butterfly conformation) is expressed directly by the N(5) and N(10) resonances and indirectly by the C(10a) and C(4a) resonances, which are under the influence of the $\pi$-electron-donating character of the N(5) and N(10) atoms (Moonen et al., 1984). The N(10) atom resonates at about 2 ppm downfield from that of $FMNH_2$, whereas the N(5) atom is shifted upfield. These results indicate strongly that the bending is more pronounced at the N(5) than at the N(10) position, as compared to the conformation of free $FMNH_2$. The C(10a) atom resonates at a position comparable with that in $FMNH_2$, which supports this conclusion. The absence of a hydrogen bond to O(4$\alpha$) disfavors $\pi$-electron delocalization from N(10) and N(1) to the O(4$\alpha$) position. An increase in the $\pi$-electron density is therefore expected at C(4a). Since the N(1) atom is less $sp^2$ hybridized than the N(10) atom, however, the cooperative $\pi$-electron delocalization of the two nitrogen atoms is decreased as compared to that in $FMNH_2$. The overall apparent effect is that the resonance due to C(4a) seems to be unaffected.

The conclusions remain roughly the same with regard to the anionic 1,5-dihydroriboflavin bound to the protein as given for the neutral reduced form. The chemical shift of C(2) and the absence of a coupling constant for N(3)H again indicate the exposure of these centers to solvent water. The resonance position of C(4) indicates the absence of a hydrogen bond. The upfield shift of the resonance due to N(1), as compared with $FMNH^-$, indicates that the $sp^2$ character of this atom is further decreased as compared to that at the lower pH values. The considerable line width even suggests that the N(1) atom rapidly changes its configuration (i.e., configurational averaging). The broadened resonance of C(4a) might be caused by the configuration averaging of N(1). The results further indicate that the configurations of the N(5) and the N(10) atoms are roughly the same as those in the neutral protein-bound flavin molecule. The small downfield shift of the resonance due to C(4a) as compared with $FMNH^-$ supports this interpretation and indicates that $\pi$-electron delocalization from the N(1) atom is indeed decreased. It can be concluded from these results that most features of the bound neutral reduced riboflavin are maintained in the negatively charged system. There are two exceptions to this conclusion: (i) the $sp^3$ hybridization of the N(1) atom is increased; (ii) the N(5)H group shows a coupling constant of 78 ± 5 Hz. The magnitude of this coupling constant clearly shows that the N(5) atom possesses increased $sp^3$ character, already concluded from its resonance position. The fact that the coupling constant could only be observed at high pH values indicates that the protein undergoes a conformational change when the pH of the solution is increased. This change in conformation shields N(5)H to solvent water at high pH. The result is compatible with a swelling of the protein at low pH and a shrinking at high pH, as proposed recently by Kumosinski et al. (1982).

We return now to the point of the higher $pK_a$ value for the deprotonation of N(1) as compared with reduced free riboflavin. The apparent destabilization of the negative charge on N(1) might be due to a negative charge in the vicinity of N(1) in the complex. Another more likely possibility is the absence of a hydrogen bond to O(4$\alpha$). As we have seen in the accompanying paper (Moonen et al., 1984), the structure of the flavin [especially N(10)] changes upon deprotonation in such a way that the $\pi$-electron delocalization of N(1) is also favored. As has been indicated, the C(4a) and O(4$\alpha$) are important centers for this delocalization. The delocalization to O(4$\alpha$) is not favored in the complex, which probably influences the degree of hybridization of N(1) and thus renders the deprotonation unfavorable.

The apparently decreased $sp^2$ character of N(1) in the reduced state might explain the weaker binding of reduced as compared with oxidized riboflavin. However, at the moment we cannot quantify this effect.

This study has demonstrated that detailed information can be obtained from flavoproteins by using $^{13}C$ and $^{15}N$ NMR techniques and isotopically enriched flavocoenzymes. The information thus obtained is more specific than binding studies with flavin derivatives (Choi & McCormick, 1980). Some results can be compared directly with published results such as the solvent accessibility, which is in excellent agreement

with the binding study of flavin derivatives. Other features such as a strong interaction of O(4$\alpha$) and N(10) of riboflavin with the apoprotein have been suggested from binding studies and are now confirmed by our results. The data on dynamics and strength of hydrogen bonding are novel however and describe the characteristics of binding in a very detailed way.

Finally, in light of our results, we briefly discuss the question of the function of the protein. It is known (McDonnell et al., 1951) that the egg is under anaerobic conditions during the first stage of development. It might well be that riboflavin is in the reduced state in this stage. The relative exposure of RBP-bound riboflavin could suggest that reduced flavin serves as an electron donor for the reduction of the disulfide bonds of ovomucine, a phenomenon that is long known (McDonnell et al., 1951) but still remains unexplained. Our hypothesis further implies that the mentioned redox reaction requires the consumption of one proton if the reduced riboflavin is in the anionic state. This mechanism could also explain the observation that the pH value of the albumen of a fresh egg is 7.6 and increases to 8.5 within the first day of development (Carter, 1968). The initial pH value of a fresh egg is thus above the p$K_a$ value of the reduced complex; i.e., reduced riboflavin is predominantly in the anionic state. Thus, our NMR results and the hypothesis derived from them seem to offer an explanation for the observed pH changes in the developing embryo. The egg yolk is highly structured, and pH gradients are important for the differentiation of the cells. It is therefore not unlikely that the redox reactions mentioned are related to the phenomena of pH gradients.

Acknowledgments

We are thankful to J. C. Toppenberg-Fang for typing the manuscript, to M. M. Bouwmans for preparing the figures, and to Dr. C. A. H. Rasmussen for carefully reading the manuscript.

**Registry No.** FMN, 146-17-8; $FMNH_2$, 5666-16-0; $FMNH^-$, 79703-59-6; TARF, 752-13-6; $TARFH_2$, 18717-85-6; MeIMN, 986-53-8; $MeIMNH_2$, 91632-20-1; $MeIMNH^-$, 91738-67-9; MeTARI, 86431-61-0; $MeTARIH_2$, 91632-21-2; $^{15}N$, 14390-96-6.

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