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Bacterial Luciferase: A Carbon-13, Nitrogen-15, and Phosphorus-31 Nuclear Magnetic Resonance Investigation†

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ABSTRACT: The ^{13}C and ^{15}N NMR spectra of specifically ^{13}C- and ^{15}N-enriched FMN were measured in the presence of bacterial luciferase from *Vibrio harveyi*. In the oxidized state, hydrogen bonds to both carbonyl groups are found, albeit weaker than those of FMN in water. In contrast, the N(1) and N(5) atoms both have stronger hydrogen bonds than FMN in water. The C(8) and C(7) resonances indicate that the isoalloxazine moiety of luciferase-bound FMN is not as strongly polarized as free FMN in aqueous medium and much less than flavodoxin-bound FMN. On reduction of the bound FMN, all ^{13}C resonances, except that due to the C(10a) atom, shift upfield, indicating increased electron density at these carbon centers. The isoalloxazine ring carries a negative charge at the N(1) atom, which possibly interacts with a positively charged group on the protein. The results further indicate that the N(3)H group probably forms a hydrogen bond with the protein, whereas the N(5)H group does not. The N(5) atom of luciferase-bound $FMNH^-$ is highly sp^2 hybridized, indicating an almost planar structure of the reduced prosthetic group, except that the N(10) atom is somewhat placed out of the molecular plane. With highly active luciferase, only one oxidized flavin molecule per luciferase molecule is bound strongly. We have, however, observed that excess reduced flavin is bound to luciferase, probably in an aspecific manner, indicating that luciferase has two different binding sites for reduced flavin.

Bacterial luciferase is a flavoprotein containing riboflavin 5′-phosphate (FMN)[1] as prosthetic group. It catalyzes the oxidation of long-chain aliphatic aldehydes with the emission of light with a spectral maximum around 490 nm. Hastings and Gibson (1963) showed that bacterial luciferase can form a long-lived intermediate after the attack of molecular oxygen on the protein-bound reduced flavin molecule. In the absence of the aldehyde, the intermediate breaks down, forming H_2O_2

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; FMN, oxidized riboflavin 5′-phosphate; $FMNH_2$ and $FMNH^-$, two-electron-reduced riboflavin 5′-phosphate in the neutral and anionic state, respectively; NMR, nuclear magnetic resonance; TARF, oxidized tetraacetylriboflavin; $TARFH_2$, two-electron-reduced tetraacetylriboflavin in the neutral state; TMS, tetramethylsilane; Tris, tris(hydroxymethyl)-aminomethane.

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and oxidized FMN. Numerous studies have been performed on the nature of this intermediate and on the reaction scheme in general (Ziegler & Baldwin, 1981; Lee, 1985). Unfortunately, relatively few studies have been performed on the physical and chemical properties of the protein. The binding between FMN and apoluciferase has been studied by conventional methods in both the oxidized and reduced state (Ziegler & Baldwin, 1981). It was shown that a negative charge on the isoalloxazine side chain at about 0.9 nm away from the N(10) atom is required for good reactivity (Meighen & MacKenzie, 1973). Nielsen et al. (1983) showed that of all phosphorylated riboflavin analogues only the 5′-phosphate analogue (FMN) gives a high bioluminescence activity. A remarkable aspect of the equilibrium binding studies is that FMN hardly binds in the oxidized state, K_d = 0.12 mM, 3 °C, for *Vibrio harveyi* luciferase (Baldwin et al., 1975), while $FMNH_2$ binds about 100 times more tightly, $K_d \sim 1$ μM, 6 °C (Becvar & Hastings, 1975). These dissociation constants are much higher than those of most other flavoproteins.

Various $FMNH_2$ analogues have been used to investigate the flavin reaction site. Meighen and MacKenzie (1973) showed that the N(3)H group of flavin is essential for reaction; alkylation of this group abolishes the bioluminescence activity. Watanabe et al. (1980) and Chen and Baldwin (1984) showed that 8-substituted $FMNH_2$ derivatives produce bioluminescence with *Photobacterium phosphoreum* luciferase. They suggested that the N(5)H group of $FMNH_2$ is involved in the binding to the luciferase molecule. Chen and Baldwin (1984) recently showed that 8-substituted $FMNH_2$ derivatives also produce biolumincescence with *V. harveyi* luciferase and suggested that the methyl group at the 8-position of flavin is exposed to solvent in the complex.

There is some uncertainty about the number of flavins bound to the luciferase molecule and/or involved in the catalytic mechanism. Ziegler and Baldwin (1981) concluded that only one flavin reacts (binds) per luciferase heterodimer from the first-order behavior of the bioluminescence kinetics and the luciferase:flavin quantum yield ratios. Matheson and Lee (1983), however, showed that the kinetics are more complex and that the major bioluminescence pathway is via a bimolecular reaction.

NMR spectrscopy is being used extensively in the study of enzyme–ligand interactions, yielding insight into specific interactions of the ligand with the binding site. In our case, information about the electronic perturbation of flavin upon binding to an apoflavoprotein and the conformation of protein-bound reduced flavin can also be obtained (Vervoort et al., 1985). Although bacterial luciferase is a relatively large biomolecule possessing a molecular mass of ~80 000 Da, it lends itself to NMR study as it is a highly soluble protein produced in quantity by the bioluminescent bacteria. It is also relatively easy to purify (O'Kane et al., 1986). For these reasons, we decided to study luciferase by NMR techniques. In this paper, we report on the interaction of FMN with apoluciferase using ^{13}C- and ^{15}N-enriched FMN derivatives. Since all atoms of the isoalloxazine moiety of FMN have been labeled, a rather detailed insight into the interactions of FMN with apoluciferase is obtained.

Materials and Methods

FMN selectively enriched with ^{13}C at C(2), C(4), C(4a), and C(10a) and with ^{15}N isotopes at all four nitrogen atoms were prepared as described previously (Van Schagen & Muller, 1981; Muller et al., 1983; Franken et al., 1984). The synthesis of FMN derivatives selectively enriched with ^{13}C in the benzene subnucleus will be published elsewhere.

The starting material, [^{15}N]-3,4-dimethylnitrobenzene, for the synthesis of [1,3,5,10-$^{15}N_4$]riboflavin was obtained in the following way. A mixture of 16 g of concentrated H_2SO_4 and 11.2 g of 14 M [^{15}N]HNO_3 was slowly added under vigorous stirring to 12 g of 1,2-dimethylbenzene at 0–5 °C. A total of 11.2 g of 14 M [^{15}N]HNO_3 was prepared from 14.4 mL of 9 M [^{15}N]HNO_3 (Amersham, U.K.) by distillation to a constant-boiling point of 121.7 °C. The reaction mixture was kept for 3 h at 0–5 °C and then for 1 h at 5–10 °C. Then ice was added, and the reaction mixture neutralized with solid Na_2CO_3. The mixture was extracted 3 times each with 50 mL CH_2Cl_2, and the organic phase was dried over Na_2SO_4 and evaporated to dryness. The yield of nitration products was 13.0 g. The oily product was taken up in petroleum ether (fp 40–60 °C) and applied to a silica gel 60 (70–230 mesh, product of Merck AG, FRG) column (2.5 × 30 cm). Washing the column with petroleum ether eluted a pale yellow product, which was identified as [^{15}N]-2,3-dimethylnitrobenzene by ^{13}C NMR (yield 5.0 g). An intense yellow band was eluted with petroleum ether containing 1% ether. The product was shown to be [^{15}N]-3,4-dimethylnitrobenzene by ^{13}C NMR (yield 6.2 g). By use of petroleum ether containing a much higher percentage of ether (up to 30%), relatively small amounts of dinitroxylene and other nitration products were eluted. The purity of the two mononitro compounds can be monitored by thin-layer chromatography using plastic sheets impregnated with a fluorescent indicator (Merck, FRG) and a mixture of isopropyl ether and petroleum ether (1:1.5 by volume) as mobile phase. The desired product, [^{15}N]-3,4-dimethylnitrobenzene was then dissolved in methanol and reduced catalytically with H_2 and Pd/C as catalyst at atmospheric pressure. The aniline derivative thus obtained was crystallized from *n*-hexane after removing the catalyst from the solution by filtration and evaporating the solvent. The subsequent steps in the synthesis of [1,3,5,10-$^{15}N_4$]riboflavin were identical with those described for the synthesis of 7-methyl-10-ribityliso-alloxazine 5′-phosphate (Muller et al., 1983; Franken et al., 1984).

Bacterial luciferase was from an aldehyde-requiring dark mutant of *V. harveyi* strain 392 (MAV) and was purified to homogeneity (O'Kane et al., 1986). The luciferase was assayed for bioluminescence specific activity (photons s^{-1} A_{280}^{-1}) at room temperature as described (Lee, 1982), with decanal. Photometer calibration used the NBS standard lamp and the NBS absolute photodiode photometer with the luminol chemiluminescence reactions (Lee & Seliger, 1965; Matheson et al., 1984).

The NMR samples contained luciferase, which had at least 80% of the maximum obtainable specific activity. For reconstitution experiments, ^{13}C- and ^{15}N-enriched FMN were added on a molar ratio with respect to luciferase protein (80 kDa), unless indicated otherwise. The protein concentration was determined by absorbance [$\epsilon(280)$ = 85 000 M^{-1} cm^{-1}; O'Kane et al., 1986]. All ^{13}C and ^{15}N NMR measurements were done in 50 mM potassium phosphate buffer, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.0, unless indicated otherwise. ^{13}P NMR measurements were done in 200 mM Tris, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.5. The temperature was kept at 5 °C during the measurements.

Wilmad 10-mm precision NMR tubes were used for ^{13}C and ^{31}P NMR experiments, and Wilmad 15-mm precision NMR tubes were used for ^{15}N NMR experiments. The sample volume was 1.6 mL for ^{13}C and ^{31}P NMR and 4.0 mL for ^{15}N NMR measurements. The samples contained 10% ^2H_2O to

Table I: Carbon-13 Chemical Shifts (in ppm) of [^{15}N]-1-Nitro-2,3-dimethylbenzene (**1**) and [^{15}N]-1-Nitro-3,4-dimethylbenzene (**2**) in Chloroform[a]

	chemical shifts							
compd	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(2α/3α)	C(3α/4α)
1	151.1	130.2	139.4	133.5	125.9	121.3	15.3	20.0
2	146.1	123.9	138.0	144.7	130.0	120.7	19.4	19.7

[a] The chemical shifts are relative to TMS. [b] Doublet.

lock the magnetic field. Broad-band decoupling of 0.5 W was used for ^{13}C NMR experiments except for the C(6) and C(9) atoms, where 2.0 W was used. All spectra were recorded with 30° pulses and a repetition time of 1.0 s. The measurements were done on a Bruker CXP 300 NMR spectrometer operating at 30.4 MHz for ^{15}N NMR, at 75.6 MHz for ^{13}C NMR, and at 120.5 MHz for ^{31}P NMR spectra.

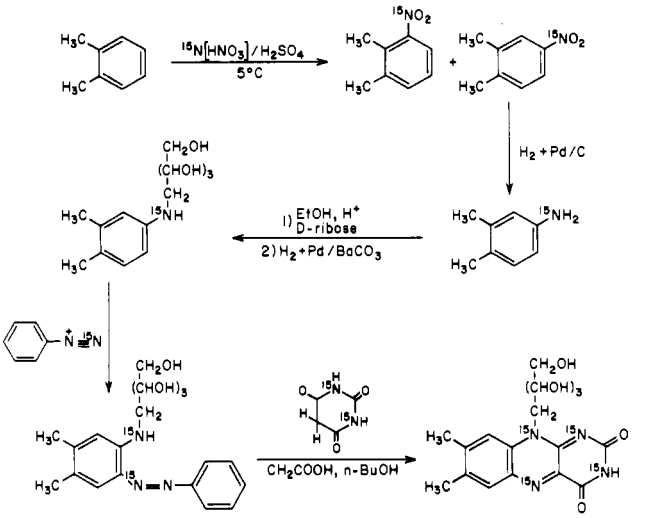
Dioxane (3 μL) served as an internal standard for ^{13}C NMR measurements. Chemical shift values are reported relative to TMS [δ(dioxane) − δ(TMS) = 67.84 ppm]. Neat [^{15}N]CH_3NO_2 was used as an external reference for ^{15}N NMR measurements with a coaxial cylindrical capillary as recommended by Witanowski et al. (1981). Chemical shift values are reported relative to liquid NH_3 at 25 °C [$\delta(CH_3NO_2)$ − $\delta(NH_3)$ = 381.9 ppm for the magnetic field parallel to the sample tube]. For ^{31}P NMR measurements, 85% H_3PO_4 was used as an external reference. Broad-band decoupling of 0.5 W was used for ^{31}P NMR experiments. Values are reported as true shieldings, i.e., corrected for bulk volume susceptibilities. The accuracy of the reported values is about 0.2 ppm for ^{13}C NMR, 0.4 ppm for ^{15}N NMR, and 0.1 ppm for ^{31}P NMR chemical shift values.

Reduction of the sample was made by the addition of the desired amount of a dithionite solution to the anaerobic solution of luciferase and flavin. Anaerobic conditions were achieved by carefully flushing the solution in the NMR tube with argon for about 10 min. The NMR tube was sealed with a serum cap.

Results and Discussion

In previous papers (Franken et al., 1984; Vervoort et al., 1985; Beinert et al., 1985a), we have used [1,3,5,10-$^{15}N_4$]-7-methyl-10-ribitylisoalloxazine 5′-phosphate as a prosthetic group to study the ^{15}N NMR characteristics of FMN-containing flavoproteins. In the case of "old yellow enzyme" (Beinert et al., 1985a), it was noticed that the activity of the reconstituted enzyme was only one-third that of the apoenzyme recombined with FMN. These results indicate that a small modification of the prosthetic group can drastically influence the enzymatic properties of a flavoprotein while the affinity of the apoprotein for the modified flavin is not affected relative to that of FMN. To avoid possible ambiguities in our studies, [1,3,5,10-$^{15}N_4$]FMN was synthesized with *o*-xylene as a starting material (see Materials and Methods). Under our experimental conditions a relatively high amount of mononitroxylenes (65% yield) was obtained on nitration of xylene (Scheme I). The two isomeric nitro compounds, [^{15}N]-1-nitro-2,3-dimethylbenzene and [^{15}N]-1-nitro-3,4-dimethylbenzene, can be quantitatively separated by column chromatography, yielding 45% of the former and 55% of the latter compound. It should be noted that the nitrating mixture must be added slowly and the temperature maintained at 0–5 °C at the initial stage of the synthesis; otherwise, a higher amount of dinitroxylene is formed at the expense of the desired product. The purified mononitro isomers have been unambiguously identified by ^{13}C NMR (Table I). It is obvious that only [^{15}N]-1-nitro-3,4-dimethylbenzene can be used to synthesize

Scheme I: Chemical Synthesis of [1,3,5,10-$^{15}N_4$]Riboflavin



flavin (Scheme I). This was achieved by catalytic reduction of the nitro compound to the corresponding aniline derivative and condensation of this product with D-ribose. This and the subsequent steps of the synthesis (Scheme I) are identical with those described previously for [1,3,5,10-$^{15}N_4$]-7-methyl-10-ribitylisoalloxazine 5′-phosphate (Muller et al., 1983; Franken et al., 1984).

^{31}P NMR of Luciferase. The ^{31}P NMR chemical shift values of the phosphate group of FMN bound to *V. harveyi* luciferase are 5.2 ppm in the oxidized and 5.6 ppm in the reduced state. Both resonances are downfield shifted as compared to that of free FMN in the dianionic state (δ = 5.1 ppm, pH 9.0). The ^{31}P chemical shifts for luciferase therefore suggest that the phosphate group of FMN in luciferase is bound in the dianionic state. The small downfield shift of 0.4 ppm in reduced luciferase as compared to oxidized luciferase could reflect the higher affinity of luciferase for $FMNH^-$ (Becvar & Hastings, 1975), but a conformation change of the protein and/or a change of the environment of the phosphate could also be responsible for the shift. The ^{31}P NMR spectra show no indication for a fast or slow exchange reaction, indicating that flavin is strongly bound by the apoprotein. No covalently bound phosphate groups were observed in the ^{31}P NMR spectrum of luciferase, as observed in some flavoproteins (Edmondson & James, 1979).

^{13}C and ^{15}N NMR of Oxidized Luciferase. Figure 1A shows the ^{15}N NMR spectrum of ^{15}N-enriched FMN bound to luciferase from *V. harveyi*. Several natural abundance lines due to the protein are observed at about 120 and 140 ppm, which represent probably amide groups of the protein (Witanowski et al., 1981). The low-intensity resonances at about 75 ppm could be due to arginine residues (Lapidot & Irving, 1978). Another group of natural abundance resonances appears at about 310 ppm, which remain unassigned.

The nitrogen shieldings of protein-bound FMN are summarized in Table II. According to common practice, the nitrogen atoms in heterocycles are categorized as pyridine or β-type nitrogen and pyrrole or α-type nitrogen (Witanowski

Table II: Carbon-13 and Nitrogen-15 Chemical Shifts (in ppm) of Free FMN and FMN Bound to Luciferase in the Oxidized and in the Reduced State[a]

	chemical shifts in the						
	oxidized state			reduced state			
atom	LUC	FMN[b]	TARF[c]	LUC	$FMNH^-$[b]	$FMNH_2$[c]	$TARFH_2$[d]
C(2)	158.5	159.8	155.2	157.9[d]	158.2	151.1	150.6
C(4)	162.6	163.7	159.8	157.2	157.7	158.3	157.0
C(4a)	137.4	136.2	135.6	103.5	101.4	102.8	105.2
C(5a)	135.7*	136.4	134.6	135.0*	134.2	134.4	136.0
C(6)	130.8	131.8	132.8	116.8	117.3	117.1	116.1
C(7)	139.0	140.4	136.6	132.7*	133.0	134.3	133.6
C(7α)	20.2	19.9	19.4	19.4	19.0	19.0	18.9
C(8)	148.6	151.7	147.5	126.2	130.3	130.4	129.0
C(8α)	21.9	22.2	21.4	19.7	19.4	19.2	18.9
C(9)	119.5	118.3	115.5	115.6	116.8	117.4	118.0
C(9a)	134.6*	133.5	131.2	130.7*	130.9	130.4	128.2
C(10a)	151.3	152.1	149.1	156.2	155.5	144.0	137.1
N(1)	187.1	190.8[e]	200.1[e]	176.8[d]	181.3[e]	128.0[e]	119.9[e]
N(3)	162.3*	160.5[e]	159.6[e]	150.0[d]	150.0[e]	149.7[e]	149.0[e]
N(5)	325.8	334.7[e]	346.0[e]	59.9[d]	58.4[e]	58.0[e]	59.4[e]
N(10)	160.8*	163.5[e]	151.9[e]	94.6	96.5[e]	87.2[e]	76.8[e]

[a] Luciferase and oxidized FMN were measured in 50 mM potassium phosphate, pH 7.0. TARF in the oxidized and reduced state was dissolved in $CHCl_3$. Reduced FMN in the neutral state was measured in 100 mM potassium phosphate, pH 5.0, and in the anionic state in 50 mM potassium pyrophosphate, pH 8.5. All measurements were done at 5 °C. ^{13}C chemical shifts are relative to TMS. ^{15}N chemical shifts are relative to liquid NH^3. Chemical shifts marked with an asterisk are tentative assignments. [b] Taken from Vervoort et al. (1985). [c] Taken from Moonen et al. (1984a). [d] Independent of pH in the range 6.5–8.5. [e] This paper.

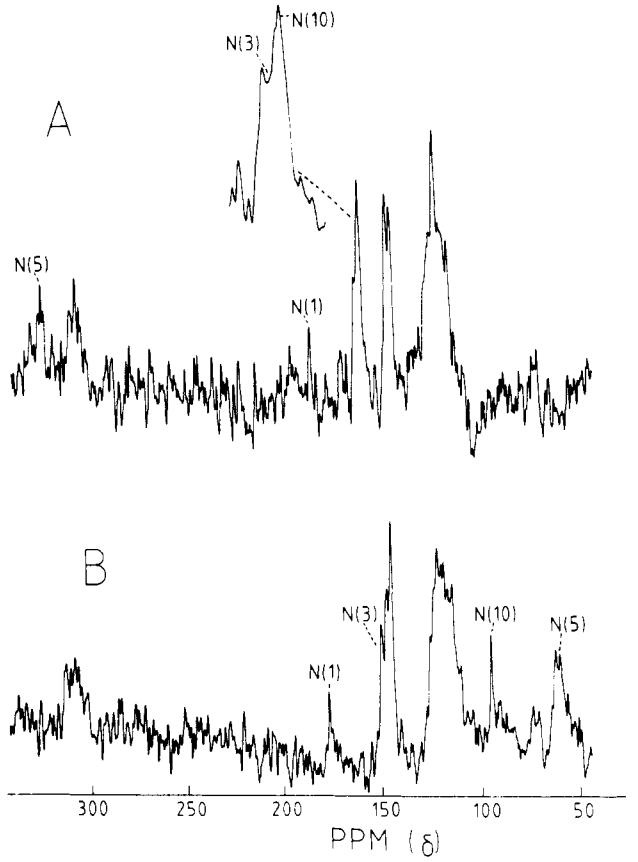


FIGURE 1: ^{15}N NMR spectrum of *V. harveyi* luciferase (2.5 mM) reconstituted with [1,3,5,10-$^{15}N_4$]FMN in the (A) oxidized state (90 800 acquisitions) and in the (B) reduced state (140 300 acquisitions). All samples were in a buffer of 50 mM potassium phosphate, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.0. The temperature was 5 °C.

et al., 1981). Generally, the β-type nitrogens resonate at lower field than the α-type nitrogens. As can be seen from Figure 1A and Table II, the nitrogen atoms of flavin fit well into this categorization. However, a more important characteristic of the two types of nitrogen atoms in our study is that the chemical shifts of pyridine-type nitrogen atoms are much more sensitive to hydrogen-bond formation, leading to an increased shielding of the nitrogen atoms, than those of the pyrrole-type nitrogen atoms (Witanowski et al., 1981).

The resonances of the N atoms of protein-bound FMN appear at fields where no natural abundance peaks are present in the spectrum (Figure 1A). The resonances of N(1) and N(5) of flavin, on the one hand, and those of N(3) and N(10), on the other hand, are easily distinguished by their intensities. No hydrogen atom is attached directly or to a neighboring atom in the former group, in contrast to the latter one. The resonance lines at 325.8 and 187.1 ppm in Figure 1A are therefore assigned to N(5) and N(1), respectively, in accordance with model studies (Moonen et al., 1984a) (Table II). For convenience, the chemical shifts of free and protein-bound flavin are presented schematically in Figure 2.

The resonances of N(10) and N(3) are observed at about 160 ppm (Table II). Since the spectrum in Figure 1A was not obtained under proton-decoupling conditions, the line due to N(10) is expected to be more intense than that due to N(3), because the latter contains a covalently bound proton yielding a doublet of the corresponding resonance line. An expansion of the resonance line at about 160 ppm demonstrates that the line is composed of more than one line differing in intensity (Figure 1A). Therefore, we assign the low-field part tentatively to the N(3) atom and estimate the $^1J(^{15}N(3)-^1H)$ coupling constant to be about 90 ± 10 Hz. The observation of a coupling constant implies that the exchange rate for the proton at N(3) must be slow. If it were fast, one would observe a single sharp and more intense line for N(3) than is the case. The results therefore suggest that the N(3)H group in luciferase-bound FMN is not accessible to bulk water.

The resonance signal due to the N(5) atom of luciferase-bound FMN (Table II, Figure 2) appears at much higher field than that of both FMN and TARF. This result indicates a rather strong hydrogen bond between the N(5) atom and the protein. A similar but weaker effect is observed with the N(1) atom. In both cases, the hydrogen bond is stronger than that formed with FMN in aqueous solution (Table II, Figure 2).

The chemical shift value of N(3) of luciferase-bound FMN is further downfield shifted than that of FMN. This indicates that the N(3)H group of flavin probably forms a hydrogen

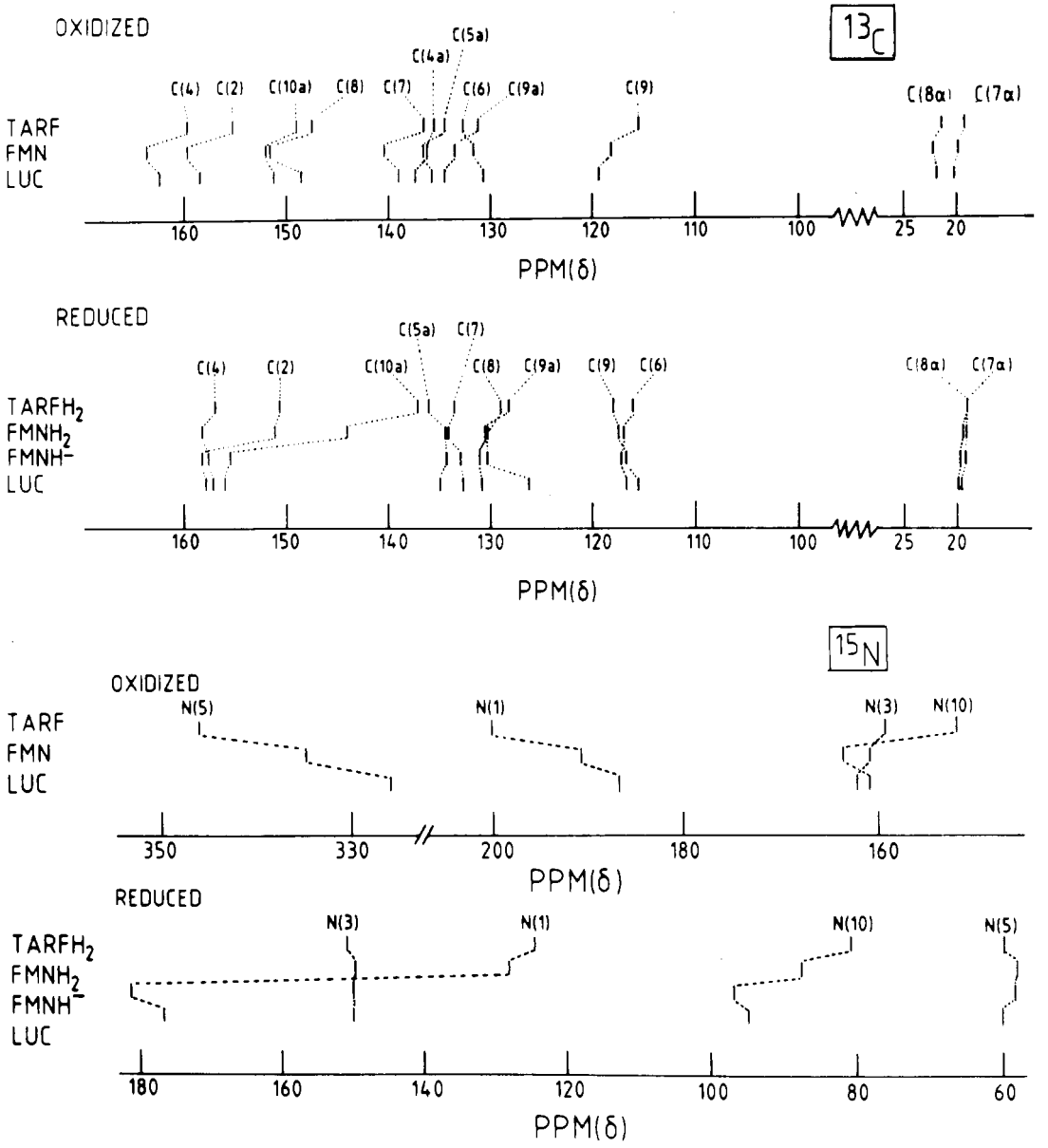


FIGURE 2: Correlation diagram of ^{13}C and ^{15}N NMR chemical shifts of flavin free and bound to *V. harveyi* apoluciferase in the oxidized and in the reduced state.

bond with the protein. This suggests also coupling between $^{15}N(3)$ and its proton. This interpretation is consistent with the finding that replacement of the proton at N(3) by a methyl group abolishes completely the bioluminescence activity of flavin and luciferase (Meighen & MacKenzie, 1973).

The resonance line of the N(10) atom is upfield from that of free FMN but considerably downfield from that of TARF (Table II, Figure 2), indicating that the N(10) atom in luciferase-bound FMN is mostly sp^2 hybridized (see also below), but less than that in free FMN (Moonen et al., 1984a). This means that the N(10) atom of protein-bound flavin is slightly out of the molecular plane [for an explanation, see Moonen et al. (1984a)].

A typical ^{13}C NMR spectrum of luciferase, recombined with [4,10a-$^{13}C_2$]FMN, is shown in Figure 3. In addition to the two resonances due to the flavin, the usual spectral features expected for the natural abundance ^{13}C in a protein of this size are observed. The peptide carbonyl and carboxyl resonances appear between 170 and 185 ppm. The arginine C^{ζ} atoms are observed at 158.2 ppm. Aromatic carbon atoms of histidine, tyrosine, phenylalanine, and tryptophan residues resonate between 110 and 140 ppm. No resonances appear

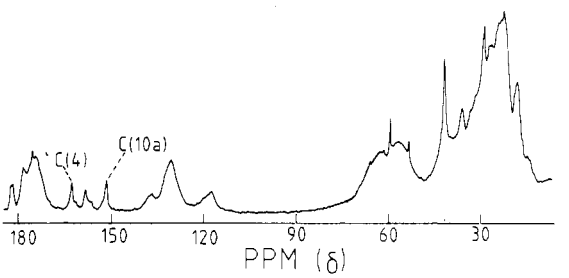


FIGURE 3: ^{13}C NMR spectrum of oxidized *V. harveyi* luciferase (2 mM) recombined with [4,10a-$^{13}C_2$]FMN in 50 mM potassium phosphate, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.0 (36 000 acquisitions). The temperature was 5 °C.

in the spectral region from 70 to 110 ppm. The C^{α} atoms resonate at about 60 ppm, and all other aliphatic carbon atoms appear at higher field.

The natural abundance resonance lines can interfer with the observation of the resonance lines of certain atoms in protein-bound flavin, even when highly enriched flavin derivatives are used. To improve the assignment, difference spectra have been recorded where necessary. Figure 4A shows, for example,

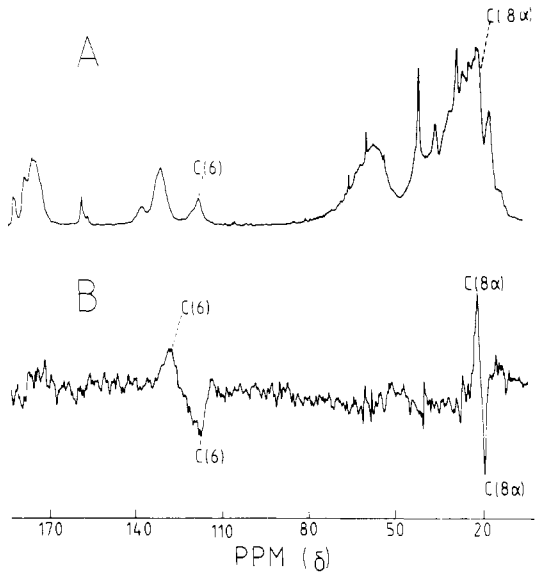


FIGURE 4: ^{13}C NMR spectra of *V. harveyi* luciferase (2 mM) recombined with [6,8α-$^{13}C_2$]FMN in the reduced state (A) (44 100 acquisitions) in 50 mM potassium phosphate, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.0. (B) is a difference spectrum between the spectrum in the oxidized (26 500 acquisitions) minus that in the reduced state of luciferase.

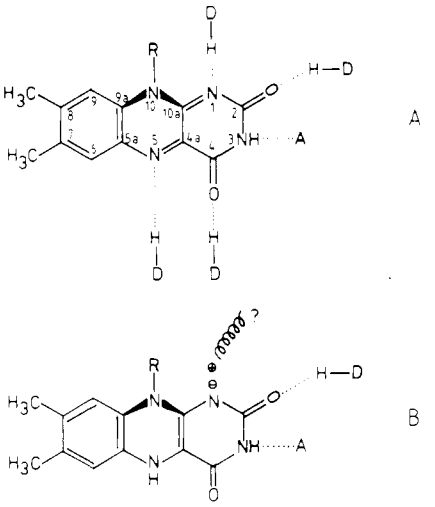


FIGURE 5: Structure and flavin–apoenzyme interactions of FMN bound to apoluciferase in the oxidized (A) and reduced (B) state as revealed by ^{13}C and ^{15}N NMR data. A = hydrogen bond acceptor; D = hydrogen bond donor.

the difficulty of observing directly the resonances of the protein-bound [6,8α-$^{13}C_2$]FMN. We have first recorded the spectrum of the oxidized FMN–luciferase complex and then reduced the complex by an excess of dithionite in the same sample tube, then recording the spectrum of the reduced complex under identical instrumental settings and taking the difference spectrum. Figure 4B demonstrates the good quality of such difference spectra.

Figure 4B shows also interesting features. In both redox states, the line width of the C(6) resonance is much larger than that of the C(8α) resonance. An identical observation (not shown) came from using [7α,9-$^{13}C_2$]FMN as a prosthetic group. This shows that the larger line widths are probably not artifacts. The line width of the resonance lines of these atoms depends also on the rotational correlation time of the molecule under study (Wilbur et al., 1976; Norton et al., 1977). The calculated rotational correlation time for luciferase (M_r 80 000) is about 50 ns at 5 °C. Therefore, larger line widths of the resonances from C(6) and C(9) suggest that the isoalloxazine ring of FMN is rigidly associated with the protein with little independent motion in either redox state.

The ^{13}C chemical shifts of protein-bound FMN are collected in Table II and compared with those of free flavin in solvents of different polarity (see also Figure 2). The chemical shifts of the two carbonyl groups of protein-bound FMN appear at higher field than those of free FMN in aqueous solution, indicating an apparent increase in electron density on these C atoms suggesting that the two functional groups form weaker hydrogen bonds with the protein than FMN does with water and much weaker than those of the prosthetic group in flavodoxins (Van Schagen & Muller, 1981; Moonen et al., 1984a; Vervoort et al., 1985). The hydrogen bond to C(2)=O does not much affect the indirectly polarizable carbon atoms C(8), C(8α), C(6), and C(10a), which resonate at higher field than the corresponding atoms of free FMN in aqueous solution; i.e., there is only a little π electron delocalization from the benzene ring onto the C(2)=O group (Moonen et al., 1984a). It is interesting to note that FMN bound to apoflavodoxin is, by the interaction of C(2)=O with the protein highly polarized (Vervoort et al., 1985), in contrast to FMN bound to luciferase. Crystallographic studies on *Clostridium Mp* flavodoxin showed (Burnett et al., 1974) that part of the benzene subnucleus of flavin is accessible to bulk solvent. In our opinion, this contributes to the stabilization of the highly polarized prosthetic group [for tautomeric structure, see Moonen et al. (1984a)]. Therefore, it is suggested that the benzene ring in luciferase-bound FMN is in a more hydrophobic microenvironment than FMN bound to apoflavodoxin. This conclusion is in agreement with the chemical shifts of the C(8) atom and that of the 8α-methyl group and with published circular dichroism and absorbance spectra (Baldwin et al., 1975).

The decreased sp^2 character of N(10) of protein-bound FMN as compared with that of N(10) of free FMN should lead to a downfield shift of C(9), C(7), C(7α), and C(4a) (Moonen et al., 1984a). This is found and is in accordance with the interpretation of the N(10) chemical shift. In Figure 5A a schematic representation of the structure and hydrogen-bond interaction of FMN with apoluciferase is given.

^{13}C and ^{15}N NMR of Two-Electron-Reduced Luciferase. The ^{15}N NMR spectrum of protein-bound luciferase is shown in Figure 1B. The resonances due to N(1) and N(10) appear as sharp lines whereas those due to N(3) and N(5) are broader and less intense, in agreement with the fact that both nitrogen atoms carry a covalently bound proton. Owing to the lack of resolution of the resonance of N(3), which is obscured partially by natural abundance resonances, no definite statement can be made with respect to the presence or absence of a coupling. At any rate, the ^{15}N chemical shift of the N(3) atom suggests that the N(3)H group is involved in hydrogen bonding similar to that of $FMNH^-$ in aqueous solution (Table II, Figure 2). On the other hand, the resonance line of N(5) is possibly split into a doublet with an estimated $^1J(^{15}N(5)-^1H)$ of about 85 ± 10 Hz, suggesting that no fast proton exchange reaction occurs. The N(5) chemical shift (Table II) strongly indicates that the N(5)H group is not forming a hydrogen bond with the protein and that the N(5) atom possesses a high degree of sp^2 hybridization (Moonen et al., 1984a) (see also below). These results also indicate that the N(5)H group of luciferase-bound FMN experiences a rather hydrophobic microenvironment.

The N(1) atom in luciferase-bound flavin resonates at 176.8 ppm, a value very close to that of the corresponding atom in free $FMNH^-$ (Table II, Figure 2). It has been shown by ^{13}C and ^{15}N NMR (Van Schagen & Muller, 1981; Franken et al.,

1984) that N(1)H of reduced free flavin deprotonates with a pK_a of 6.7. The chemical shift of N(1) of protein-bound flavin is pH-independent in the range 7.0–8.5. Therefore, it is concluded that the N(1) atom in luciferase-bound reduced FMN is ionized. This conclusion is supported by ^{13}C NMR data (see below). The N(1) ionization has also been observed for all flavodoxins studied (Van Schagen & Muller, 1981; Franken et al., 1984; Vervoort et al., 1985), for "old yellow enzyme" (Beinert et al., 1985a,b), and for lipoamide dehydrogenase and glutathione reductase (Van den Berg et al., 1984). The only exception found up to now, in what seems to be a rule in flavoproteins, is riboflavin-binding protein (Moonen et al., 1984b).

The resonance of the N(1) atom in luciferase appears about 5 ppm upfield from that of free $FMNH^-$ (Table II, Figure 2). This suggests that the negative charge on N(1) is either somewhat delocalized or is counteracted by a nearby positively charged group, which could also be due to the positively charged pole of an α-helix. It has been found that two ionizable groups (pK_a values of 6.2 and 6.8) are involved in the bioluminescence of $FMNH_2$ with *V. harveyi* luciferase (Nicoli et al., 1974). It was suggested that these pK_a values could be due either to $FMNH_2$ (phosphate and isoalloxazine moieties) or to cysteine (Nicoli et al., 1974) or histidine residues (Cousineau & Meighen, 1976). Our results indicate that the pK_a of 6.8 is probably due to $FMNH_2$, which must be ionized to bind.

Also, the resonance due to the N(10) atom of luciferase-bound $FMNH^-$ is observed at higher field than that of free $FMNH^-$ (Table II, Figure 2). From this we conclude that the N(10) atom of protein-bound $FMNH^-$ possesses a slightly lower degree of sp^2 hybridization than that of free $FMNH^-$. Consequently, this atom is placed slightly out of the molecular plane of protein-bound $FMNH^-$ as compared with free $FMNH^-$ [for a detailed explanation, see Moonen et al. (1984a)].

The ^{13}C chemical shifts of reduced flavin bound to luciferase are collected in Table II and shown in Figure 2 (see also Figure 4). These chemical shifts support the interpretation of the ^{15}N chemical shifts and follow the reasoning discussed above for the oxidized luciferase–FMN complex. Therefore, only a few points will be considered here.

The slight upfield shift of the resonance due to C(2), compared to $FMNH^-$, reflects the decreased charge on N(1), because the resonances of C(2) and C(10a) are very sensitive to the field from this negative charge (Van Schagen & Muller, 1981). Therefore, despite the upfield shift of the C(2) resonance, the result indicates that C(2) is still hydrogen bonding. The field effect does not apply to the C(4) atom, and therefore, the C(4)=O hydrogen bond is much weaker than that on C(2)=O.

The downfield shifts of the resonances due to C(4a) and C(5a) also reflect the somewhat decreased sp^2 character of the N(10) atom as compared with that of free $FMNH^-$ (Moonen et al., 1984a). The strong upfield shift of C(8) and the lesser extent of an upfield shift of C(6) reflect the high sp^2 hybridization of N(5) and the π electron reallocation from the last atom onto the first two. In this context, it should be noted that the sp^2 hybridization of N(5) as deduced from the ^{15}N and ^{13}C NMR results is in agreement with the fact that reduced luciferase exhibits an increased molar absorption coefficient at 450 nm as compared with free reduced flavin (Dudley et al., 1964). As discussed previously (Vervoort et al., 1985), both parameters reflect the high coplanarity of the flavin molecule around the N(5) atom, while the N(10) atom is somewhat out of the molecular plane (Figure 5B).

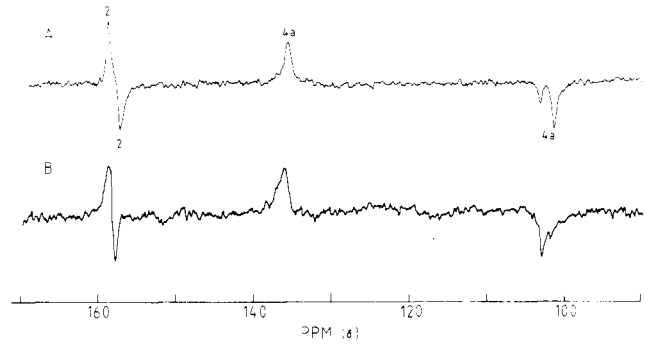


FIGURE 6: ^{13}C NMR spectra of *V. harveyi* luciferase (3 mM) reconstituted with [2,4a-$^{13}C_2$]FMN in 200 mM potassium phosphate, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, pH 8.5. (A) Specific activity of the enzyme was 50×10^{12} photons s^{-1} A_{280}^{-1}. Difference spectrum for the spectrum of oxidized (38 500 acquisitions) minus that of reduced luciferase (42 300 acquisitions). (B) Specific activity of the enzyme was 120×10^{12} photons s^{-1} A_{280}^{-1}. Difference spectrum for the spectrum of oxidized (39 400 acquisitions) minus that of reduced luciferase (45 100 acquisitions).

Luciferase reconstituted with [2,4a-$^{13}C_2$]FMN in the reduced state was also studied in the presence of tetradecanal and dodecanol in order to investigate possible influences of these compounds on the electronic system of the flavin. No influence of these compounds on the ^{13}C chemical shifts was found.

Luciferases are known as proteins binding the prosthetic group relatively weakly in the oxidized state (Ziegler & Baldwin, 1981), in contrast to most flavoproteins. In the reduced state, the prosthetic group in luciferase is more tightly bound than in the oxidized state. For instance, the dissociation constant for the *V. harveyi* luciferase–FMN complex in the oxidized state has been determined to be 0.12 mM at 3 °C in 0.05 M Bis-Tris [2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol], pH 7.0, containing 0.2M NaCl (Baldwin et al., 1975), while the K_d for the reduced complex was found to be about 1 μM at 6 °C and in 0.1 M Bis-Tris, pH 7.0, in the presence of 0.1 M NaCl and 0.2 mM dithiothreitol (Becvar & Hastings, 1975). Considering these facts and the fact that we used about 2 mM solutions of luciferase, we should observe roughly 25% free flavin in the oxidized state and about 3% in the reduced state. The latter value is small enough not to be observed in the ^{13}C and ^{15}N NMR spectra. However, in the oxidized state we should observe a considerable contribution of free FMN in the ^{13}C spectra of luciferase, which is not observed. There are two arguments to explain this apparent discrepancy. First, the luciferase preparations used possessed a high specific activity, in fact an activity exceeding those published before (O'Kane et al., 1986). Second, it has been found that maximal reconstitution and a tighter binding of FMN is obtained in 50 mM phosphate buffer than in other buffer systems such as Tris and Bis-Tris (J. Lee, unpublished results).

It has been proposed that two flavin molecules are involved in the catalytic mechanism of luciferase [see, e.g., Lee (1985)]. These findings are in contradiction to published work by others [see, e.g., Ziegler and Baldwin, (1981)]. Therefore, this issue is worth considering in the light of the present results. In the course of this study we observed that ^{13}C resonances were split into doublets representing two flavin molecules in different environments. These doublets were observed in the reduced rather than in the oxidized state of the protein. Figure 6 shows ^{13}C NMR difference spectra of the spectrum of oxidized luciferase minus that of reduced luciferase. The spectrum of

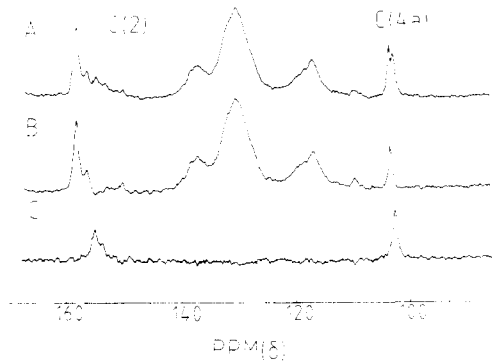


FIGURE 7: ^{13}C NMR spectra of *V. harveyi* luciferase (sp act. 120×10^{12} photons s^{-1} A_{280}^{-1}) (3 mM) reconstituted with [2,4a-$^{13}C_2$]FMN in the reduced state in 50 mM potassium phosphate, 0.5 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.0. (A) The flavin/apoluciferase ratio was 2:1 (44 100 acquisitions). (B) The sample of (A) was chromatographed under anaerobic conditions (see text), and the solution was measured again (33 400 acquisitions). (C) Difference spectrum for spectrum A minus spectrum B.

Figure 6A was obtained by using a luciferase preparation that was only about 30% active (sp act. 50×10^{12} photons s^{-1} A_{280}^{-1}), while the preparations used to obtain the results presented in Table II and Figures 1–4 had a specific activity of 160×10^{12} photons s^{-1} A_{280}^{-1}. Nevertheless, the sample used in Figure 6A was reconstituted with [2,4a-$^{13}C_2$]FMN in a molar ratio of 1:1 flavin:protein. The spectrum of oxidized luciferase was then recorded and, thereafter, the protein reduced in the sample tube and the spectrum obtained again. The difference spectrum between the two redox states shows one line each for C(2) and C(4a) in the oxidized state and two lines for C(4a) and one line for C(2) in the reduced state. The major peak appears at 101.5 ppm and the minor peak at 103.5 ppm. The ^{13}C chemical shifts correspond well with those of free and protein-bound $FMNH^-$ (Table II). The C(2) of the $FMNH^-$–luciferase complex appears as a singlet at about 158 ppm and is not split because of the very small difference in the chemical shift between free and protein-bound $FMNH^-$ at the pH value studied (Table II). On the other hand, the difference in the chemical shifts between free and protein-bound flavin in the oxidized state is sufficient to be observed, if present (Table II).

Using an enzyme preparation showing a specific activity of 120×10^{12} photons s^{-1} A_{280}^{-1} and following the same procedure as outlined above gave the difference spectrum shown in Figure 6B. The major peak appears now at 103.5 ppm and the minor one at 101.4 ppm. Furthermore, it was found that the line at 101.4 ppm is pH-dependent, whereas the line at 103.5 ppm is pH-independent (Table II). The pH-dependent line at 101.4 ppm follows exactly the pH titration curve of free $FMNH_2$ (Van Schagen & Muller, 1981). These results show that the ^{13}C spectra of reduced luciferase can be used to estimate the relative content of active luciferase molecules in preparations.

To check the possibility that highly active luciferase can bind more than one molecule of $FMNH^-$, we added excess flavin stepwise to the solution of Figure 6B up to a molar ratio of 2:1 (results not shown). Upon addition of reduced flavin, the intensity of the resonance line at 101.4 ppm (Figure 6B) increases in a concentration-dependent fashion while remaining of the same broad width, and the intensity of the line at 103.5 ppm stays unchanged. The addition of a higher excess of $FMNH^-$ leads to the appearance of sharp resonance lines superimposed on those assigned to the aspecifically bound $FMNH^-$. These sharp lines are due to free $FMNH^-$. These results strongly suggest that luciferase can bind more than one reduced flavin. However, the binding interaction with the second molecule is much less specific than with the first molecule as can be deduced from the chemical shifts. Since the line width of the resonances due to the weakly bound flavin is about 40 Hz as compared with that of free flavin ($\Delta\nu_{1/2}$ = 10 Hz), there is no doubt that the second molecule interacts with luciferase, very much the same way as flavin binds to inactive luciferase. In fact, we have found that the interaction of excess flavin with luciferase resembles that of bovine serum albumin with flavin, although the latter possesses no specific flavin binding site. This result suggests that flavin interacts in both proteins, either with some hydrophobic sites or with charged groups via the phosphate moiety of $FMNH^-$. In both cases, the flavin is accessible to bulk water, this in contrast with the flavin bound specifically by luciferase.

It can easily be demonstrated that the aspecifically bound $FMNH^-$ binds more weakly to luciferase than the other one. Figure 7A shows the ^{13}C NMR spectrum of luciferase reconstituted with [2,4a-$^{13}C_2$]$FMNH^-$. The specific activity of the sample was the same as that shown in Figure 6B, except the pH value of the solution was 7.0. This sample was poured over a Bio-Gel P6-DG column in the presence of dithionite; the enzyme was collected and measured again. The spectrum of Figure 7B shows that the aspecifically bound flavin has been removed completely. As evident from this spectrum, only a very small amount of specifically bound flavin is possibly lost during the chromatographic procedure as revealed by the difference spectrum between the two spectra (Figure 7C). It should also be noted that in Figure 7A the resonance of C(2) of the aspecifically bound flavin can be seen due to the larger chemical shift difference between the two species at pH 7.0 (Table II).

The observation that the ^{13}C NMR spectra of luciferase recombined with oxidized flavin do not show different microenvironments for the strongly and more weakly bound flavin, in contrast to the spectra in the reduced state, indicates that a conformational change of the protein occurs on changing the redox state. Recently it was shown that such a conformational change indeed occurs (Aboukhair et al., 1985). This conformational change affects specifically the microenvironment of the flavin involved in the catalysis. Since the aspecifically bound flavin seems to experience the same microenvironment in both inactive and highly active luciferase, its possible role in the catalysis needs further investigation.

We have also measured the ^{13}C NMR spectra of luciferase from *V. fischeri* and from *Photobacterium phosphoreum* in the presence of [2,4a-$^{13}C_2$]$FMNH^-$. The ^{13}C chemical shifts are the same as those found in *V. harveyi*, so very likely the interaction between these luciferases and flavin is not very different from that observed in *V. harveyi* luciferase.

When comparing our results with those of Ghisla et al. (1978), who used [4a-^{13}C]FMN only, we notice some discrepancies in the interpretation of the ^{13}C NMR spectra. The ^{13}C NMR spectra of Ghisla et al. (1978) had a signal to noise ratio much lower than our spectra. Therefore, the minor differences can be attributed to this fact [see also Vervoort et al. (1986)].

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Registry No. **1**, 104715-29-9; **2**, 104715-30-2; [1,3,5,10-$^{15}N_4$]FMN, 104715-35-7; [4,10a-$^{13}C_2$]FMN, 61390-84-9; [6,8α-$^{13}C_2$]$FMNH^-$,

104715-36-8; [2,4a-$^{13}C_2$]FMN, 104715-37-9; [2,4a-$^{13}C_2$]FMNH$^-$, 104715-38-0; FMN, 146-17-8; reduced FMN, 5666-16-0; *o*-$Me_2C_6H_4$, 95-47-6; $PhN^+{\equiv}{}^{15}N$, 60154-30-5; [1,3,5,10-$^{15}N_4$]riboflavin, 104715-31-3; [^{15}N]-3,4-dimethylaniline, 104715-32-4; D-[^{15}N]-3,4-dimethylanilinoribitol, 104715-33-5; D-5-[6-(phenyldiazo-^{15}N)-3,4-dimethylanilino-^{15}N]-5-deoxyribitol, 104715-34-6; [$^{15}N_2$]-2,4,6-trioxo-5*H*-pyrimidine, 60031-77-8; luciferase, 9014-00-0.

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