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Identifications of the True Carbon-13 Nuclear Magnetic Resonance Spectrum of the Stable Intermediate II in Bacterial Luciferase†

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Received February 26, 1986; Revised Manuscript Received June 12, 1986

ABSTRACT: Intermediate II in bacterial luciferase, formed in a reaction of luciferase with $FMNH^-$ and O_2, has been reinvestigated by ^{13}C NMR spectroscopy with ^{13}C-enriched FMN derivatives. It is shown that the previously published spectrum of the intermediate by Ghisla et al. [Ghisla, S., Hastings, J. W., Favaudon, V., & Lhoste, J.-M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5860–5863] does not represent the intermediate but is due to a contamination contained in commercially available [$^2H_6$]ethylene glycol. Relaxation studies show that the resonance line due to the byproduct of [$^2H_6$]ethylene glycol is easier to observe at low temperature than at room temperature, explaining fully the spectral properties of the published artifact. The true C(4a) resonance is found at 82.5 ppm, and this is interpreted as due to considerable sp^2 hybridization at this position, indicating an almost planar configuration by comparison with a model compound.

Bacterial luciferase is a flavoprotein utilizing riboflavin 5′-phosphate (FMN)[1] as a prosthetic group and catalyzing the oxidation of long-chain aliphatic aldehydes. The reaction is accompanied by emission of light. In the course of the bioluminescence reaction, several intermediates are formed, and their involvement in the reaction is still under dispute [e.g., Lee (1985) and Ziegler & Baldwin (1981)]. However, one

† This study was carried out under the auspicies of The Netherlands Foundation for Chemical Research (SON) with financial aid from The Netherlands Organization for the Advancement of Pure Research (ZWO) (to F.M.) and supported in part by National Science Foundation Grant DBM 85-12361 and NATO Grant 835/83 (to J.L.).
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[1] Abbreviations: NMR, nuclear magnetic resonance; TMS, tetramethylsilane; FMN, oxidized riboflavin 5′-phosphate; $FMNH_2$ and $FMNH^-$, two-electron-reduced riboflavin 5′-phosphate in the neutral and anionic state, respectively.

0006-2960/86/0425-8062$01.50/0

of these is a species detected on reaction of O_2 and $FMNH^-$ with luciferase in the absence of aldehyde. This species is commonly termed intermediate II. From a comparison of the UV spectral properties of the intermediate with those of model compounds (Ghisla et al., 1973), it has been suggested that the compound is 4a,5-dihydro-4a-hydroperoxyflavin (Hastings et al., 1973). However, the light absorption technique is far from unambiguous in providing the chemical structure of a new compound, especially in the case of the flavin molecule possessing a complex electronic structure. The fact that intermediate II is rather stable in the protein-bound state makes it feasible to elucidate the structure of intermediate II by ^{13}C NMR, despite the low sensitivity of the technique (Allerhand, 1979), which can be overcome by use of ^{13}C-enriched compounds. Ghisla et al. (1978) have pioneered such a study using [4a-^{13}C]FMN as a prosthetic group for luciferase and claimed to show proof for the substitution at C(4a) of flavin in the intermediate. Since the signal/noise ratio in the experimental spectra was rather low, we suspected that the assignment of the resonance lines could be incorrect. When we repeated the experiments of Ghisla et al. (1978) in the absence of ethylene glycol, we could not observe the line at 74 ppm that they assigned to the intermediate. Since the data of Ghisla et al. (1978) are frequently cited as absolute proof for the existence of a C(4a)-substituted flavin derivative in flavin-dependent hydroxylation reactions, we considered it very important to reinvestigate the structure of the luciferase-bound intermediate (Lee, 1985). It is shown in this paper that the published spectrum for the intermediate is an artifact due to an impurity contained in deuterated ethylene glycol and that the intermediate is indeed a C(4a)-substituted flavin that has a resonance appearing at 82.5 ppm in the ^{13}C NMR spectrum.

Materials and Methods

FMN selectively enriched with ^{13}C was prepared as described previously (Van Schagen & Muller, 1981). The isotopic enrichment was at least 92 atom %. [$^2H_6$]Ethylene glycol was purchased from Merck AG, Darmstadt, West Germany. Bacterial luciferase was from an aldehyde-requiring dark mutant of *Vibrio harveyi* strain 392 (MAV) and was purified to homogeneity (O'Kane et al., 1986). Luciferase was assayed for bioluminescence specific activity (photons s^{-1} A_{280}^{-1}) at room temperature as described (Lee, 1982), with decanal. Photometer calibration was made by reference to the NBS standard lamp and NBS absolute photodiode photometer via the luminol chemiluminescence reactions (Lee & Seliger, 1965; Matheson et al., 1984).

The NMR samples contained luciferase, which was at least 80% of the maximum obtainable specific activity, i.e., 120×10^{12} photons s^{-1} A_{280}^{-1}. For reconstitution experiments, ^{13}C-enriched FMN was added in a slight excess over apoluciferase. The concentration of luciferase was determined from $\epsilon(280) = 85000\ M^{-1}\ cm^{-1}$ (O'Kane et al., 1986). All measurements were done in 50 mM potassium phosphate buffer, pH 7, containing 10 mM β-mercaptoethanol. Intermediate II was prepared from reconstituted luciferase in the same buffer system mentioned above but saturated with dodecanol (Tu, 1979). The sample was then reduced and poured over a short Bio-Gel P6-DG column (2.7 × 7.5 cm), previously equilibrated with the same buffer. After elution of the luciferase-bound intermediate, the sample was concentration to about 1.5 mL by ultrafiltration (Amicon). All procedures were done at about 0 °C.

The decay of the luciferase-bound intermediate was followed spectrophotometrically with time on an Aminco DW2A spectrophotometer and by assays of the bioluminescence reaction. The temperature was kept at 4 °C.

Table I: ^{13}C Chemical Shifts (in ppm) of FMN Bound to Luciferase from *V. harveyi* in the Oxidized, Reduced, and Intermediate II State[a]

	redox state of luciferase				
carbon atom	oxidized	reduced	intermediate II	I[b]	II[b]
C(2)	158.5	157.9	156.5	162.1	154.7
C(4)	162.6	157.2	166.9	167.7	164.9
C(4a)	137.4	103.5	82.5	63.0	83.5
C(10a)	151.3	156.2	157.7	160.0	156.4

[a] All experiments were done in 50 mM potassium phosphate buffer, pH 7.0, in the presence of 10 mM β-mercaptoethanol. The temperature in all cases was 0 °C. For comparison, the relevant ^{13}C chemical shifts of 3-methyl-4a-propenyl-4a,5-dihydrolumiflavin (I) and 3-methyl-4a,5-(epoxyethano)-4a,5-dihydrolumiflavin (II) are also given. The ^{13}C chemical shifts are reported relative to TMS. [b] From Lhoste et al. (1980).

Wilmad 10-mm precision NMR tubes were used for ^{13}C NMR measurements. The sample volume was 1.6 mL, containing 10% ^2H_2O to lock the magnetic field. Broad-band decoupling of 0.5 W was used. All spectra were recorded with 30° pulses and a repetition time of 1.0 s. The temperature was 0 ± 2 °C. Dioxane (3 μL) served as an internal standard. The ^{13}C chemical shift values are reported relative to tetramethylsilane [δ(dioxane) − δ(TMS) = 67.87 ppm). The accuracy of the reported values is about 0.2 ppm. All measurements were done on a Bruker CXP300 NMR spectrometer operating at 75.6 MHz.

When luciferase recombined with FMN was studied in the reduced state, reduction was done by addition of a dithionite solution to the anaerobic solution in the NMR tube. Anaerobiosis was 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 constrast to Ghisla et al. (1978), we used doubly labeled ^{13}C-enriched FMN derivatives throughout this study. This approach has a great advantage in the identification and assignments of the resonances in a complex ^{13}C NMR spectrum of a relatively large protein as luciferase. Since the ^{13}C chemical shifts in oxidized and reduced free flavin have been shown to be directly related to the π-electron density on the corresponding carbon atoms in the molecule (Van Schagen & Muller, 1980), the chemical shifts change in a predictable manner on reduction or modification of flavin. Therefore, a second label in the molecule is a reliable reference point for the other one in a ^{13}C NMR spectrum.

Figure 1A shows the ^{13}C NMR spectrum of luciferase-bound [2,4a-$^{13}C_2$]FMN in the two-electron reduced state. The spectrum shows one resonance line at 103.5 ppm for the C(4a) atom. The resonance line is shifted downfield by 2.1 ppm as compared with free $FMNH^-$ (Moonen et al., 1984). This is in contradiction with the value reported by Ghisla et al. (1978) for the free flavin, i.e., 104 ppm. The resonance line due to C(2) (Table I) is hidden under the natural abundance lines of the C^ζ atom of arginine residues (Vervoort et al., 1986), the latter resonating at 158.2 ppm (Allerhand, 1979). However, a difference spectrum between the spectra of reduced and oxidized luciferase-bound FMN reveals that the C(2) atom resonates at 157.9 ppm in the reduced and at 158.5 ppm in the oxidized state (Vervoort et al., 1986) (Table I). These results indicate that luciferase-bound reduced FMN is ionized, i.e., carrying a negative charge at N(1) (Van Schagen & Muller, 1981). The broad lines between about 120 and 140

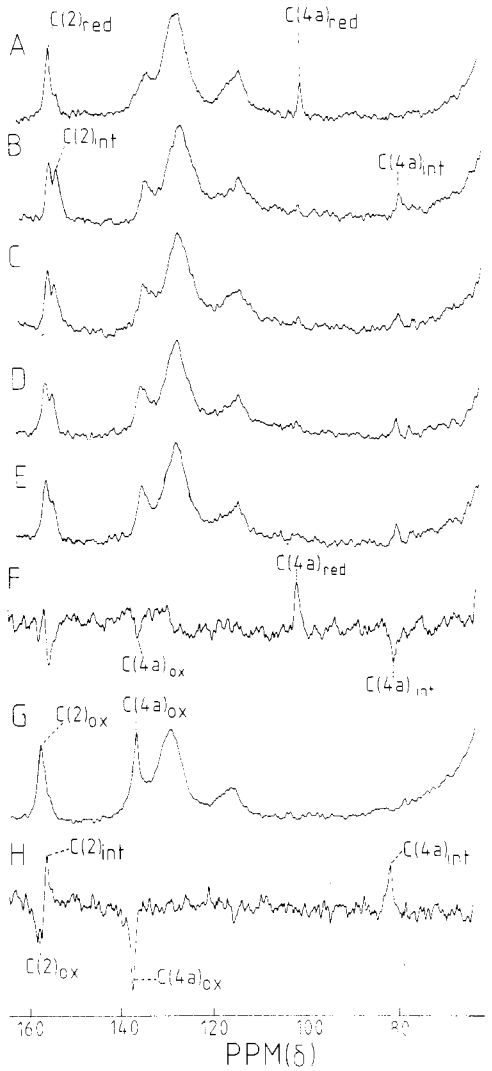

FIGURE 1: Carbon-13 NMR spectra of 3 mM luciferase in the presence of 2.4 mM [2,4a-$^{13}C_2$]FMN in 50 mM potassium phosphate, pH 7.0, containing 10 mM β-mercaptoethanol and a saturating concentration of dodecanol. The sample also contained 10% ^2H_2O to lock the magnetic field. (A) The anaerobic sample was reduced with a slight excess of dithionite and kept under argon. The NMR tube was sealed with a serum cap. A total of 10000 transients were accumulated. (B) Spectrum of intermediate II prepared by column chromatography and subsequent concentration by ultrafiltration (see Materials and Methods). Then, the spectrum was recorded immediately. A total of 7200 transients were collected, taking 120 min. (C–E) Subsequent spectra of intermediate II, collecting 7200 transients for each. (F) Difference spectrum between that of (A) and that of (B). (G) The sample of (E) was allowed to stand for 2 h at room temperature to achieve full reoxidation. After the sample was recooled to 0 °C, the spectrum was recorded (16700 transients). (H) Difference spectrum between that of intermediate II (B) and that of the reoxidized sample (F). All spectra were obtained at 0 °C.

ppm are due to the natural abundance aromatic carbon atoms of tyrosine, tryptophan, phenylalanine, and histidine residues (Allerhand, 1979).

Before continuing to the ^{13}C NMR spectra of the intermediate, a few points deserve a brief discussion. In an accompanying paper (Vervoort et al., 1986), we will show that inactive luciferase also binds reduced FMN. In this case the C(4a) atom of $FMNH^-$ resonates at 101.4 ppm. Figure 1A shows that this resonance line is absent from the spectrum in agreement with the fact that only a slight excess of flavin over (dimeric) apoluciferase (see Materials and Methods) has been added in the reconstitution reaction in order to avoid further spectral complexity and side reactions during the preparation of the intermediate (see below). This means that the concentration of active reconstituted luciferase in the sample used to obtain Figure 1A is about 2.4 mM. Furthermore, we used a different buffer system and a different procedure to prepare the intermediate than Ghisla et al. (1978). We used 50 mM phosphate buffer (pH 7) in the presence of 10 mM β-mercaptoethanol and a saturating concentration of dodecanol instead of 300 mM phosphate buffer (pH 7.0) in the presence of 20% [$^2H_6$]ethylene glycol (Ghisla et al., 1978). β-Mercaptoethanol is needed to preserve the activity of luciferase, especially in long-lasting experiments (O'Kane et al., 1986). Dodecanol has been shown to stabilize intermediate II in luciferase (Tu, 1979). On the other hand, high concentrations of phosphate buffer inhibit the bioluminescence reaction and possibly the interaction between luciferase and $FMNH^-$, competitively, K_i = 220 mM (Meighen & MacKenzie, 1973), leading possibly also to a decrease in the concentration of the intermediate. Furthermore, rather than preparing the intermediate directly in the NMR tube as done by Ghisla et al. (1978), we isolated the intermediate by column chromatography (Hastings et al., 1973; Becvar et al., 1978) at 0 °C and concentrated the sample subsequently by ultrafiltration. Although these steps take about 90 min, the loss of intermediate is small considering the very long lifetime of the intermediate even at 0 °C (see below). An advantage of the chromatographic step is that excess flavin, which was present in the sample used by Ghisla et al. (1978), is removed, which if present leads to the formation of a considerable amount of luciferase-bound flavosemiquinone (Kurfurst et al., 1982), also resulting in an additional decrease of the concentration of the intermediate. In order to be sure that we are measuring and following the decay of the intermediate, we withdrew an aliquot of the prepared intermediate prior to the start of the NMR measurements and followed its decay spectrophotometrically and by its bioluminescence activity with time, parallel to the registration of the ^{13}C NMR spectra.

The first spectrum of the intermediate accumulated after the preparation is shown in Figure 1B. The initial concentration of the intermediate in the sample was about 2 mM as judged by UV spectrophotometry (Becvar et al., 1978). The accumulation of this spectrum took 2 h. Additional spectra of the same sample were collected under identical instrumental settings (Figure 1C–E). Each spectrum represents a 2-h accumulation time. The spectra of the intermediate exhibit lines at 82.5 ppm for the C(4a) atom and at 156.5 ppm for the C(2) atom, the latter overlapping slightly with the high-field edge of the line due to C^ζ atom of arginine residues. The intensities of both lines decrease slowly with time. Concomitantly, the resonance lines at 137.4 and 158.5 ppm due to oxidized flavin increase in intensity. The changes observed in the ^{13}C NMR spectra parallel those of activity measurements and the UV spectra. The slow formation of oxidized luciferase-bound FMN is not accompanied by the formation of other possible species, at least not in concentrations high enough or lifetimes long enough to be observed in the ^{13}C NMR spectrum. The spectrophotometric experiments and the assay measurements indicate that the half-life of the intermediate under our experimental conditions is 13 h at 0 °C, a time long enough not to lead to a great loss of the intermediate during the chromatographic procedure and subsequent concentration of the sample (90 min). This is demonstrated in Figure 1F, showing the difference spectrum between that in Figure 1A and that in Figure 1B. The low intensity of the C(4a) resonance of oxidized flavin indicates again the slow decomposition of the intermediate.

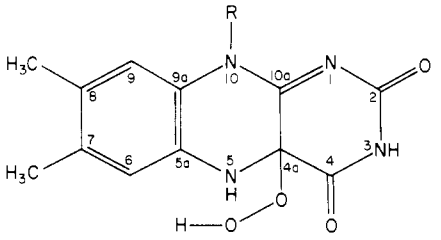

FIGURE 2: Structure of 4a,5-dihydro-4a-hydroperoxyflavin.

After registration of the spectrum shown in Figure 1E, the sample was brought to room temperature and allowed to stand for 2 h at this temperature. Then the sample was cooled again to 0 °C and its spectrum obtained (Figure 1G). The high-field peak at 82.5 ppm has disappeared completely from the spectrum, indicating the full decomposition of the intermediate (Hastings et al., 1973; Becvar et al., 1978). This was also ascertained by spectrophotometry and assay measurements. Figure 1G clearly shows now the increased intensity of the lines due to oxidized flavin at 137.4 and 158.5 ppm and the decrease of intensity at about 156.5 and at 82.5 ppm. Figure 1H shows the difference spectrum between that of the intermediate (Figure 1B) and that of the oxidized sample (Figure 1G). This difference spectrum shows the resonance lines of the intermediate very clearly and allows an unambiguous assignment of all resonance lines. The experiments described above have been repeated several times with freshly prepared luciferase. The results were identical.

The same type of experiments were done with luciferase reconstituted with [4,10a-$^{13}C_2$]FMN under identical conditions as described above. The results are collected in Table I. The resonance line of C(4) of the intermediate can be directly observed since at this resonance position no protein background is present. The C(10a) resonance of the intermediate on the other hand is strongly overlapping with the natural abundance lines of the C^{ζ} atom of arginine residues at about 158 ppm and can only be identified by difference spectroscopy, which has been done.

The strong upfield shift by 21 ppm of the C(4a) atom in the intermediate as compared with that in the luciferase–$FMNH^-$ complex indicates that the C(4a) in the latter changes from a sp^2 hybridized one to a more sp^3 hybridized one in the intermediate. In fact, the ^{13}C chemical shift of C(4a) in the intermediate is within 1 ppm of that of 3-methyl-4a,5-(epoxyethano)-4a,5-dihydrolumiflavin (Lhoste et al., 1980) (Table I). The crystal structure of this compound is known (Bolognesi et al., 1978) and shows that the molecule is almost planar, forming an angle of 5.5° between the two planes intersecting along the N(5)–N(10) axis of the molecule. The largest displacement was found for the C(4a) atom, indicating its increased sp^3 character. It is interesting to note that the C(4a) atom in C(4a)-alkylated flavins resonates at about 60 ppm (Ghisla et al., 1978; Van Schagen & Muller, 1980) (Table I), indicating its fully sp^3-hybridized character. Substituting the C(4a) atom in flavin by a hydroxyl group as in 4a-hydroxy-5-alkyl-4a,5-dihydroflavins (pseudobase) shifts the resonance downfield by about 10 ppm (Ghisla et al., 1978) due to the electronegative character of the oxygen atom. The fact that the C(4a) atom in the intermediate resonates about a further 10 ppm downfield from that in the pseudobase (δ = 74.5 ppm) is in agreement with the C(4a)-hydroperoxide structure (Figure 2). The close resemblance of the chemical shifts of intermediate II with those of 3-methyl-4a,5-(epoxyethano)-4a,5-dihydrolumiflavin strongly suggests that the intermediate possesses a rather planar structure and that therefore C(4a) in the intermediate is more sp^2 hybridized than that in the pseudobase, as can be deduced from the fact that the chemical shifts of intermediate II are intermediate between those of compound I and compound II (Table I). The rather planar structure of the intermediate and consequently the decreased sp^3 hybridization of its C(4a) atom allows a certain conjugation between the residual system of the isoalloxazine ring and the C(4a) atom. This interpretation is in agreement with the fact that 5-ethyl-4a-hydroxyperoxy-4a,5-dihydrolumiflavin shows a pK_a of 9.2 for the hydroperoxy function and that the pK_a in 1,10-ethano-5-ethyl-4a-hydroperoxy-4a,5-dihydrolumiflavinium is lowered to about 7 (Eberlein & Bruice, 1983). Therefore it is postulated that the degree of planarity of the C(4a)-hydroperoxy intermediate in the various flavoprotein hydroxylases determines its stability and consequently its hydroxylation power. This interpretation could offer an explanation for the observation that intermediate II is more stable in some flavoproteins and less stable in others.

The chemical shifts due to C(2) and C(10a) (Table I) indicate that the π electron density does not change much on formation of the intermediate, which also proves that oxygen is not attached to the C(10a) atom, which has also been proposed (Massey & Hemmerich, 1975).

The chemical shift of C(4) also indicates that oxygen is attached to C(4a) in the intermediate. On formation of the intermediate, C(4) undergoes a relatively large downfield shift, which can be explained as a change toward an electronically isolated sp^2-hybridized carbon atom with a vicinal nitrogen atom, resembling a peptide bond.

The line width of the resonance at 82.5 ppm ($\Delta\nu_{1/2}$ = 50 Hz) in the intermediate (Figure 1B) is slightly larger than that of the line at 103.5 ppm (Figure 1A) or that of the line at 137.4 ppm (Figure 1H) ($\Delta\nu_{1/2}$ = 40 Hz). Furthermore, it is obvious that the intensity of the line of C(4a) in the intermediate is slightly less that of the lines of C(4a) in the oxidized and reduced state, a result observed in all experiments. This reflects probable differences in T_1 of the C(4a) atom in the different states. It is not unlikely that the spin–lattice relaxation time (T_1) of the C(4a) atom in the intermediate is larger than in the oxidized and reduced state.

A direct comparison between our ^{13}C NMR spectra of the intermediate and that published by Ghisla et al. (1978) reveals a very striking difference; i.e., in our spectra several natural abundance resonances of the protein can be observed in addition to the resonances of the ^{13}C-enriched flavin carbons, whereas no intensity at all was observed for the protein resonances in the published spectrum of Ghisla et al. (1978). This could only be explained by a drastic difference in T_1 and/or T_2 values for the C(4a) of the intermediate in the different experiments. These aspects will be briefly discussed in the following.

For a tight protein–flavin complex, we are dealing with the situation where $\omega\tau_r \gg 1$ for the field strength and viscosities used (Allerhand, 1979); i.e., a linear relationship can be expected for T_2 (and thus line with) and the rotational correlation time. This is ascertained in our study on increasing the viscosity by lowering the temperature. This has been demonstrated also by Ghisla et al. (1978). However, at −15 °C the viscosity is about 15 cP for a mixture of 20% ethylene glycol and 80% water (Douzou, 1977) and ~2 cP at 20 °C. Therefore, an increase in line with by a factor of about 7 can be expected at −15 °C for this mixture, lowering the signal/noise ratio for a single resonance by the same factor.

The viscosity change also has drastic effects on the T_1 values. As T_1 values of about 2 s can be expected for the quaternary carbon atoms of the luciferase flavin complex at 1-cP, esti-

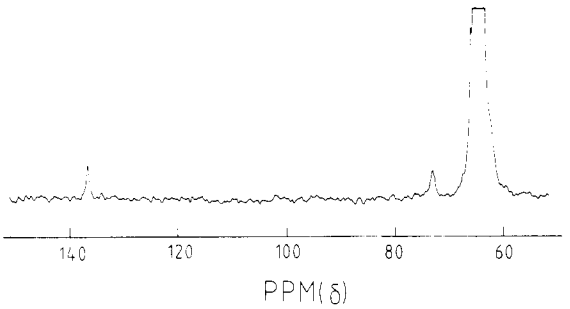

FIGURE 3: Carbon-13 NMR spectrum of 1 mM [4a-^{13}C]FMN in a mixture of 0.3 M potassium phosphate and [$^2H_6$]ethylene glycol (20%), pH 7.0. The temperature was −15 °C.

mated rotational correlation time ~30 ns (Vervoort et al., 1986; Allerhand, 1979), we can expect much higher T_1 values (possibly up to 25 s) at −15 °C in an ethylene glycol/water mixture. With 90° pulses and repetition times of 0.8 s, severe saturation can be expected for these quaternary carbon atoms. On the other hand, the majority of natural abundance carbon resonances are due to carbon atoms with a directly bound hydrogen atom, rendering the T_1 and T_2 relaxation much more effective. Therefore, at high viscosity compared with low viscosity and using the acquisition parameters of Ghisla et al. (1978), one would expect that the natural abundance resonances of the protein appear with a relatively high intensity compared with the quaternary flavin carbon atoms. This is indeed observed in our spectra (data not shown). In the spectrum of the intermediate at −15 °C reported by Ghisla et al. (1978), no protein resonances were observed at all. With the above given analyses, one would expect that the C(4a) atom of the flavin in the intermediate cannot be observed either, due to the increased line width and increased saturation. Yet, the resonance at 74 ppm was observed in their spectrum with a relatively good signal/noise ratio. This fact also suggests strongly that the resonance at 74 ppm in their spectrum cannot originate from the C(4a) carbon atom of the intermediate.

It can be easily demonstrated that the resonance line at 74 ppm originates from a compound other than flavin. We repeated the experiment of Ghisla et al. (1978) in the absence of the protein, i.e., 0.3 M phosphate buffer containing 20% [$^2H_6$]ethylene glycol and 1 mM [4a-^{13}C]FMN at −15 °C. The spectrum is shown in Figure 3. This spectrum is identical with that published by Ghisla et al. (1978). The high-field peak resonates at 73 ppm, 1 ppm upfield of that reported by Ghisla et al. (1978). This can be ascribed to the use of a different reference compound (Lhoste et al., 1980). The resonance of the C(4a) atom of oxidized flavin is found at 136 ppm in our spectrum (Figure 3), also 1 ppm upfield from that of C(4a) in the spectrum of Ghisla et al. (1978). During the deuteration process of ethylene glycol, diethylene glycol can be formed. In our case, was found by gas chromatographic and mass spectral analysis that commercially available [$^2H_6$]ethylene glycol is contaminated with 2% of [$^2H_{10}$]diethylene glycol. Due to the one-bond coupling between the deuterium and the C(2) atoms of [$^2H_{10}$]diethylene glycol, the resonance line at 73 ppm is, under the conditions used in Figure 3, a nonresolved quintet with $^1J(^{13}C-^2H)$ = 22 Hz (Kalinowski et al., 1984). This yields a resonance line with an apparent line width of about 50 Hz, as also observed by Ghisla et al. (1978). The coupling constant is independent of the magnetic field strength. The line width of C(4a) of free flavin is 10 Hz under these conditions. Ghisla et al. (1978) reported that the resonance at about 74 ppm disappeared from the spectrum on heating the sample to room temperature. This is due to the various relaxation effects, discussed briefly below.

The calculated rotational correlation time (τ_r) of diethylene glycol is much smaller than 0.1 ns, so we are dealing with the "extreme narrowing limit", i.e., $\omega\tau_r \ll 1$ (Allerhand, 1979; Kalinowski et al., 1984). Under these conditions the spin–lattice relaxation time (T_1) is dependent on τ_r of the molecule. We determined the T_1 of [$^2H_6$]ethylene glycol at 25 and −15 °C in a sample containing apoluciferase and 20% of [$^2H_6$]-ethylene glycol in 0.3 M phosphate, pH 7.0, to be about 25 and about 7.5 s, respectively. The T_1 of the C(2) atoms of [$^2H_{10}$]diethylene glycol is slightly different owing to the small differences in the τ_r of the two molecules (Kalinowski et al., 1984). In both molecules, however, the ^{13}C nuclei relax primarily through dipole–dipole interactions, and the T_1 is therefore independent of the magnetic field strength. As a result, under the conditions used by Ghisla et al. (1978), it is almost impossible to observe the resonance line of [$^2H_{10}$]-diethylene glycol at 25 °C, especially with the inferior signal/noise ratio reported by Ghisla et al. (1978), but the resonance line is much easier to observe at −15 °C. In fact, a very close inspection of the published spectrum of Ghisla et al. (1978) at 25 °C suggests the presence of the resonance at about 74 ppm as a very broad line.

The spin–lattice relaxation times of the protein, on the other hand, show an opposite behavior. These facts are illustrated by obtaining the spectra at various temperatures and comparing roughly the ratio of the intensity of the line at about 128 ppm due to the protein and that of the line at 73 ppm. Under our instrumental settings (30° pulses and 1.0-s repetition time), the following ratios were determined: 2.2 at 25 °C, 1.0 at 0 °C, and 0.4 at − 15 °C. Under the conditions of Ghisla et al. (1978), a ratio of 5.6 was found at 25 °C. These results definitely prove that the spectrum of the intermediate published by Ghisla et al. (1978) is an artifact due to a contamination in deuterated ethylene glycol, i.e., [$^2H_{10}$]diethylene glycol. This study also demonstrates that great caution must be excercised in the identification and assignments of NMR lines in complex spectra of biological material.

ACKNOWLEDGMENTS

We are indebted to S. Hutcheson for typing the manuscript, to W. H. B. Van Berkel in the assistance of some experiments, to Dr. M. A. Posthumus for the gas chromatographic and mass spectral analysis, and to M. M. Boumans for preparing the figures.

Registry No. [2,4a-$^{13}C_2$]FMN, 104715-37-9; [4,10a-$^{13}C_2$]FMN, 61390-84-9; [4a-^{13}C]FMN, 61390-82-7; luciferase, 9014-00-0.

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

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Received February 26, 1986; Revised Manuscript Received June 12, 1986

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

† This study was carried out under the auspicies of The Netherlands Foundation for Chemical Research (SON) with financial aid from The Netherlands Organization for the Advancement of Pure Research (ZWO) (to F.M.) and supported in part by National Science Foundation Grant DBM85-12361 and NATO Grant 835/83 (to J.L.) and by a grant from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie (to A.B.).
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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.

0006-2960/86/0425-8067$01.50/0