^{13}C and ^{15}N NMR Studies on the Interaction between 6,7-Dimethyl-8-ribityllumazine and Lumazine Protein†

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ABSTRACT: The interaction between the prosthetic group 6,7-dimethyl-8-(1′-D-ribityl)lumazine and the lumazine apoproteins from two marine bioluminescent bacteria, one from a relatively thermophilic species, *Photobacterium leiognathi*, and the other from a psychrophilic species, *Photobacterium phosphoreum*, was studied by ^{13}C and ^{15}N NMR using various selectively enriched derivatives. It is shown that the electron distribution in the protein-bound 6,7-dimethyl-8-ribityllumazine differs from that of free 6,7-dimethyl-8-ribityllumazine in buffer. The ^{13}C and ^{15}N chemical shifts indicate that the protein-bound 6,7-dimethyl-8-ribityllumazine is embedded in a polar environment and that the ring system is strongly polarized. It is concluded that the two carbonyl groups play an important role in the polarization of the molecule. The N(3)–H group is not accessible to bulk solvent. The N(8) atom is sp^2 hybridized and has $\delta+$ character. Nuclear Overhauser effect studies indicate that the 6,7-dimethyl-8-ribityllumazine ring is rigidly bound with no internal mobility. The NMR results indicate that the interaction between the ring system and the two apoproteins is almost the same.

Lumazine protein (LumP)[1] is a blue fluorescent protein that has been identified in several strains of *Photobacterium phosphoreum* and *Photobacterium leiognathi* (Gast & Lee, 1978; Lee, 1982; Vervoort et al., 1983; O'Kane et al., 1985a). This protein of 21 200 daltons is called lumazine protein because it contains 6,7-dimethyl-8-ribityllumazine as a tightly bound prosthetic group (Koka & Lee, 1979). The evidence that lumazine protein is functional in the bioluminescence process in vivo includes the fact that its fluorescence spectrum is identical with the in vivo bioluminescence spectrum. Also, in the in vitro reactions carried out with bacterial luciferase, $FMNH_2$, O_2, and aliphatic aldehyde the addition of lumazine protein increases the bioluminescence quantum yield and kinetic rates of the reaction catalyzed by luciferase and shifts the bioluminescence spectrum toward a match to the fluorescence spectrum of lumazine protein (Gast & Lee, 1978; O'Kane et al., 1985a). From these and other studies it was concluded that lumazine protein is the major emitter in bright strains of *Photobacteria* species (O'Kane et al., 1985a).

The protein-bound 6,7-dimethyl-8-ribityllumazine is highly fluorescent (Q_f = 0.6; Visser & Lee, 1980; Lee et al., 1985). When the chromophore binds to the apolumazine protein, the absorbance maximum shifts about 10 nm toward higher wavelength and the fluorescence maximum shifts, in contrast, to lower wavelength with a concomitant increase in fluorescence quantum yield (Lee et al., 1985; O'Kane et al., 1985a). Dynamic fluorescence measurements performed by Visser and Lee (1980) indicated that the protein-bound 6,7-dimethyl-8-ribityllumazine is rigidly held with no mobility independent of the whole protein. Lee et al. (1985) determined dissociation constants of 16 nm for the thermophilic protein and 160 nM for the psychrophilic species. Lee et al. (1985) also showed that lumazine protein has an almost spherical shape, with an axial ratio of about 1.3.

Nuclear magnetic resonance (NMR) has been shown to be a valuable tool for studying the interaction of flavin prosthetic groups with their apoproteins. Numerous studies have been performed on the interaction between apoflavoproteins and their cofactors FAD and FMN by using ^{13}C- and ^{15}N-enriched compounds (Vervoort et al., 1986a,b; Pust et al., 1989). In these studies it was shown that differences in interaction of the flavin prosthetic group and the apoflavoproteins can be monitored by using NMR.

Materials and Methods

Lumazine proteins from *P. phosphoreum* strain A-13 and from *P. leiognathi* strain A2D were purified by the methods previously described by O'Kane et al. (1985a). The apoproteins were prepared as described by O'Kane and Lee (1985b). The preparation of [6α,7α-$^{13}C_1$]-6,7-dimethyl-8-ribityllumazine and [6,7-$^{13}C_1$]-6,7-dimethyl-8-ribityllumazine has been reported earlier (Sedlmaier, 1987). [1′-^{13}C]-6,7-Dimethyl-8-ribityllumazine was prepared as follows. [1-^{13}C]Ribose (Omicron, South Bend, IN) was converted to [1-^{13}C]ribitylamine by the procedure of Plaut and Harvey (1971). The product was reacted with 6-chloro-5-nitro-2,4(1*H*,3*H*)-pyrimidinedione (Cresswell & Wood, 1960), yielding [1′-^{13}C]-5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (Nielsen & Bacher, 1988). The product was hydrogenated over palladium on charcoal and condensed with diacetyl, yielding [1′-^{13}C]-6,7-dimethyl-8-ribityllumazine (Bacher,

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[1] Abbreviations: LumP, lumazine protein; NMR, nuclear magnetic resonance; Q_f, fluorescence quantum yield; FMN, flavin mononucleotide; TARF, tetraacetylriboflavin; NOE, nuclear Overhauser effect; DMSO, dimethyl sulfoxide.

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Table I: ^{13}C and ^{15}N Chemical Shifts (in ppm) of 6,7-Dimethyl-8-ribityllumazine Free and Bound to Lumazine Protein[a]

	P. phosphoreum LumP	*P. leiognathi* LumP	6,7-dimethyl-8-ribityllumazine (H_2O)	6,7-dimethyl-8-ribityllumazine (DMSO)	FMN[b]	TARF[b]
N(1)	188.7	nd	183.7	190.9 N(1)	190.8	200.1
N(3)	163.6	nd	161.9	165.4 N(3)	160.5	159.6
N(5)	326.5	nd	333.8	347.0 N(5)	334.7	346.0
N(8)	199.6	nd	198.0	194.6 N(10)	163.5	151.9
C(6)	149.5	149.2	148.1	139.5 C(7)	140.4	136.6
C(6α)	24.8	25.1	24.5	21.7 C(7α)	19.9	19.4
C(7)	159.0	158.7	155.4	148.3 C(8)	151.7	147.5
C(7α)	21.6	21.7	21.2	17.9 C(8α)	22.2	21.4
C(1′)	57.8	nd	54.4	50.7 C(1′)	48.8	44.5

[a] The enzyme was dissolved in 100 mM potassium phosphate, 10 mM β-mercaptoethanol, and 0.5 mM EDTA, pH 7. [b] Taken from Vervoort et al. (1986a).

1986). [8-^{15}N]-6,7-Dimethyl-8-ribityllumazine was prepared by analogous procedures from $^{15}NH_2OH{\cdot}HCl$ (ICN, Cambridge, MA).

[1,3,5,8-$^{15}N_4$]-6,7-Dimethyl-8-ribityllumazine was obtained biosynthetically. The riboflavin-deficient mutant CR5 of *Bacillus subtilis* was grown for 5 days at 37 °C with aeration in 10 L of medium containing, per liter, K_2HPO_4, 10 g; KH_2PO_4, 6 g; trisodium citrate $\times 2H_2O$, 1 g; Na_2SO_4, 2.1 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; L-tryptophan, 25 mg; riboflavin, 70 μg; $^{15}NH_4Cl$, 0.3 g; and glucose, 30 g. The green fluorescence culture fluid was passed through a column of Florisil (8 × 24 cm). The column was washed with water and developed with a mixture of acetone/acetic acid/water (50:2:50). Green fluorescence fractions were combined and evaporated to dryness under reduced pressure. The residue was placed on a column of Dowex 50-WX8 (H^+ form, 4 × 35 cm) which was developed with water. Fractions were combined and evaporated to dryness under reduced pessure. The residue was crystallized from 80% ethanol, yielding 240 mg of yellow needles.

The NMR measurements were performed on a Bruker CXP 300 spectrometer operating at 30.4 MHz for ^{15}N NMR and at 75.6 MHz for ^{13}C NMR. Wilmad 10-mm tubes were used for ^{13}C NMR, and Wilmad 15-mm tubes were used for ^{15}N NMR. The sample volume was 1.6 mL in 10-mm tubes and 4 mL in 15-mm tubes, both containing 10% ^2H_2O for locking the magnetic field. The samples contained 0.5–1.5 mM lumazine protein in 100 mM potassium phosphate, 10 mM β-mercaptoethanol, and 1 mM EDTA, pH 7.0. Broadband decoupling (0.5 W) was achieved with the Waltz 16 pulse sequence (Shaka et al., 1983). Dioxane (3 μL) served as an internal standard for ^{13}C measurements. Chemical shifts are reported relative to TMS ($\delta_{dioxane} - \delta_{TMS} = 67.84$ ppm). During our study small differences in chemical shift values between our results and those of Bown et al. (1986) were observed. It turned out that these differences are due to the different reference compounds used, and for convenience all chemical shift values are corrected to those of Bown et al. (1986).

Neat [^{15}N]CH_3NO_2 was used as an external reference for ^{15}N NMR using a coaxial cylindrical capillary. Chemical shifts 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 (Witanowski et al., 1981)].

Quadrature phase detection was used. The instrumental settings were 30° pulse, 1 s repetition time, and 8K data points. For determination of nuclear Overhauser effects (NOE) a repetition time of 10 s was used. The temperature was 15 °C.

Results and Discussion

The structure of the prosthetic group of lumazine protein, 6,7-dimethyl-8-ribityllumazine, is shown in Figure 1. This

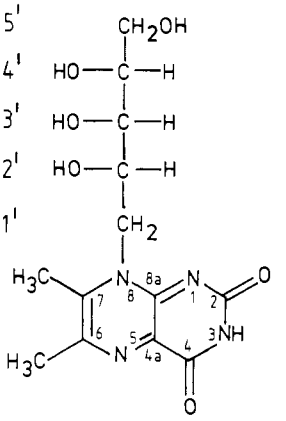

FIGURE 1: Structure of 6,7-dimethyl-8-ribityllumazine.

molecule closely resembles riboflavin, which contains an additional benzene nucleus as compared to the molecule under study. Because of the great resemblance of the two molecules the principles applied earlier for the interpretation of free and protein bound flavins (Moonen et al., 1984) will also be used here. That this is a reasonable approach will become evident. Briefly, this interpretation is based on the fact that there exists a linear relationship between the calculated π electron density of a particular carbon atom and its chemical shift; i.e., a π electron density increase on a particular carbon atom will lead to an upfield shift, and a decrease in π electron density will show an opposite effect on the ^{13}C chemical shift. The nitrogen atoms in 6,7-dimethyl-8-ribityllumazine can be characterized in analogy to the flavin molecule: the N(1) and N(5) atoms are pyridine-type nitrogen atoms, and the N(3) and N(8) atoms [N(10) in flavin] are pyrrole-type nitrogen atoms (Witanowski et al., 1981). The ^{15}N chemical shifts of pyridine-type nitrogen atoms are very sensitive to hydrogen-bonding interactions, leading to strong upfield shifts, whereas the ^{15}N chemical shifts of pyrrole-type nitrogen atoms are much less sensitive and shift only slightly downfield on hydrogen bonding.

Figure 2A shows the ^{15}N NMR spectrum of free [1,3,5,8-$^{15}N_4$]-6,7-dimethyl-8-ribityllumazine in aqueous solution at pH 7. The expected four resonances for the four nitrogen atoms are observed. The resonance at 161.9 ppm (Table I) can be assigned to N(3) on the basis of the fact that its line is split into a doublet in DMSO due to the coupling interaction with the attached proton. On decoupling of the proton only a single resonance line is observed (data not shown), and a NOE value of almost −5 is obtained for the N(3) atom.

The most downfield shifted resonance (333.8 ppm) can be assigned to the N(5) atom on the basis of the close correspondence to the ^{15}N chemical shift of pyrazine (Witanowski

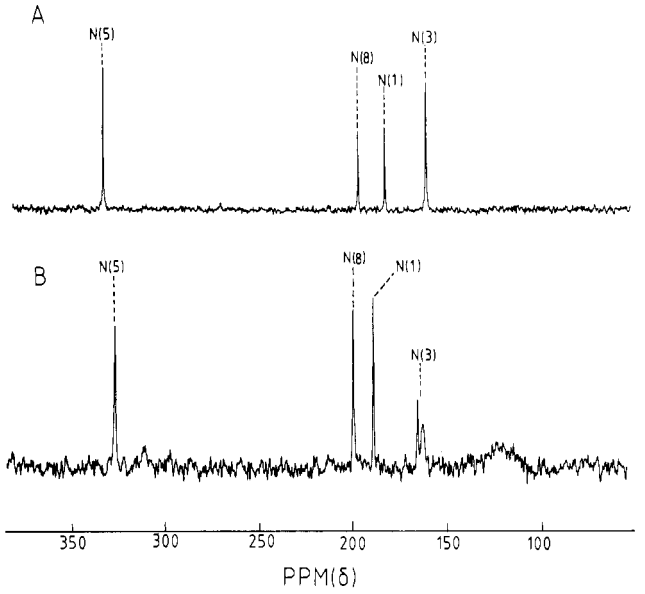

FIGURE 2: ^{15}N NMR spectra of [1,3,5,8-$^{15}N_4$]-6,7-dimethyl-8-ribityllumazine free (A, 1 mM, number of transients = 3400) and bound to the apoprotein of *P. phosphoreum* lumazine protein (B, 1 mM, number of transients = 38 000). Both spectra were in a buffer of 50 mM potassium phosphate, 1 mM EDTA, and 10 μM β-mercaptoethanol, pH 7.0. The temperature was 15 °C.

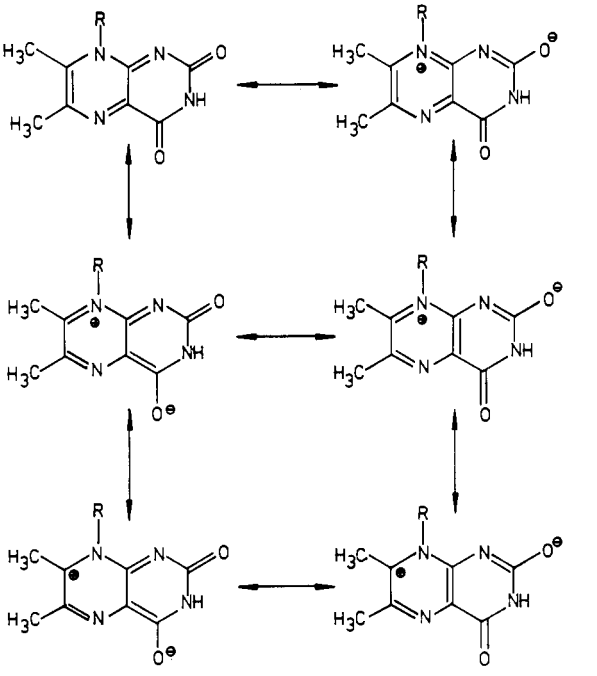

FIGURE 3: Possible mesomeric structures of 6,7-dimethyl-8-ribityllumazine as deduced from NMR results.

et al., 1981). This assignment is further supported by the close correspondence of the ^{15}N chemical shift with that observed in flavin (Moonen et al., 1984).

The remaining two resonances of the N(1) and N(8) atoms in Figure 2A are very close to each other, and a discrimination between these two is not possible on the basis of the spectrum shown. Therefore, we decided to prepare [8-^{15}N]-6,7-dimethyl-8-ribityllumazine. With the aid of this compound the resonance at 198.0 ppm could be assigned unequivocally to that of the N(8) atom, and therefore the resonance at 183.7 ppm can be attributed to the N(1) atom.

The ^{15}N NMR chemical shifts are shown in Table I. Also shown in Table I are the ^{15}N NMR chemical shifts of 6,7-dimethyl-8-ribityllumazine in H_2O and DMSO. The N(1) and N(5) resonances are strongly downfield shifted in DMSO as can be expected for pyridine-type nitrogen atoms in an aprotic solvent. The N(3) resonance shifts by 3.5 ppm downfield on going from water to DMSO, and the N(8) resonance shifts by 3.4 ppm upfield. The downfield shift of the N(3) atom is opposite to what would be expected on going from a polar protic solvent toward a more polar aprotic solvent. However, it is well-known that DMSO is a good hydrogen-bond acceptor (Joris et al., 1972), and therefore it is possible that the N(3) atom in DMSO has an even stronger hydrogen bond than in water. The upfield shift of the N(8) resonance is stronger than expected and is probably the result of a slight change in hybridization toward a slightly sp^3 hybridized atom.

The ^{13}C chemical shifts of selectively ^{13}C-enriched free 6,7-dimethyl-8-ribityllumazine are also given in Table I and for convenience compared with the equivalent ^{13}C chemical shifts of flavin. Unambiguous assignments could be done by use of 6,7-^{13}C-, 6α,7α-^{13}C-, and 1′-^{13}C-labeled molecules. The chemical shifts for the aqueous solution are in agreement with those published by Bown et al. (1986) for the neutral molecule. In going from DMSO to aqueous solution, all five carbon atoms shift downfield, indicating that the π electron density at these centers is decreased (Table I). The largest downfield shifts are found for C(6) (8.6 ppm) and C(7) (7.1 ppm). The equivalent carbon atoms in flavin exhibit a similar behavior on going from polar to apolar solvent (Moonen et al., 1984).

It is interesting to note that the ^{13}C chemical shifts of the two molecules are also similar, as already observed for the ^{15}N chemical shifts (Table I).

The above results suggest that it is indeed reasonable to interpret the data of the lumazine chromophore by analogy with the ^{13}C and ^{15}N chemical shifts of flavin. Using this semiempirical approach, we arrive at the mesomeric structures shown in Figure 3 for 6,7-dimethyl-8-ribityllumazine in aqueous solution. As with flavin, the lumazine molecule becomes highly polarized in water, whereby π electron density from the N(8) atom is reallocated onto the two carbonyl groups. The shifts for these carbon atoms in water are reported by Bown et al. to be at 160.8 (C-2) and 166.8 ppm (C-4). These values are close to the values reported for FMN in water (161.5 ppm for the C-2 carbonyl group and 165.4 ppm for the C-4 carbonyl group) after correction for the different reference systems used (see Materials and Methods) (Vervoort et al., 1986a). This suggests that, as in flavin chemistry, the amide functions in the pyrimidine subnuclei in both molecules are of great importance in the polarization of these molecules; i.e., the mesomeric structures can only be stabilized by localization of charge onto the carbonyl functions (Moonen et al., 1984; Pfleiderer et al., 1966).

The N(8) atom acquires a higher degree of sp^2 hybridization in aqueous than in DMSO solution. The partial positive charge at N(8) exerts an influence on the chemical shift of the C(1′) atom, which shifts downfield as expected (Moonen et al., 1984). In addition, the downfield shift of the resonance of C(6) is in agreement with this interpretation and demonstrates that some of the positive charge on N(8) is allocated to C(6). This is also reflected by a downfield shift of the resonance due to C(6α).

The C(7) and C(7α) resonance lines are also strongly affected in going from DMSO to water. Under our experimental conditions we did not observe a resonance at 90.0 ppm, assigned to the 7α-exo-methylene group by Bown et al. (1986), who showed that an alkaline medium is required for the formation of this structure. As shown in Figure 4 the anion of the 7α-exo-methylene compound (**1**) can form a complex mixture of ring forms, such as **2** and **3**. In the neutral pH

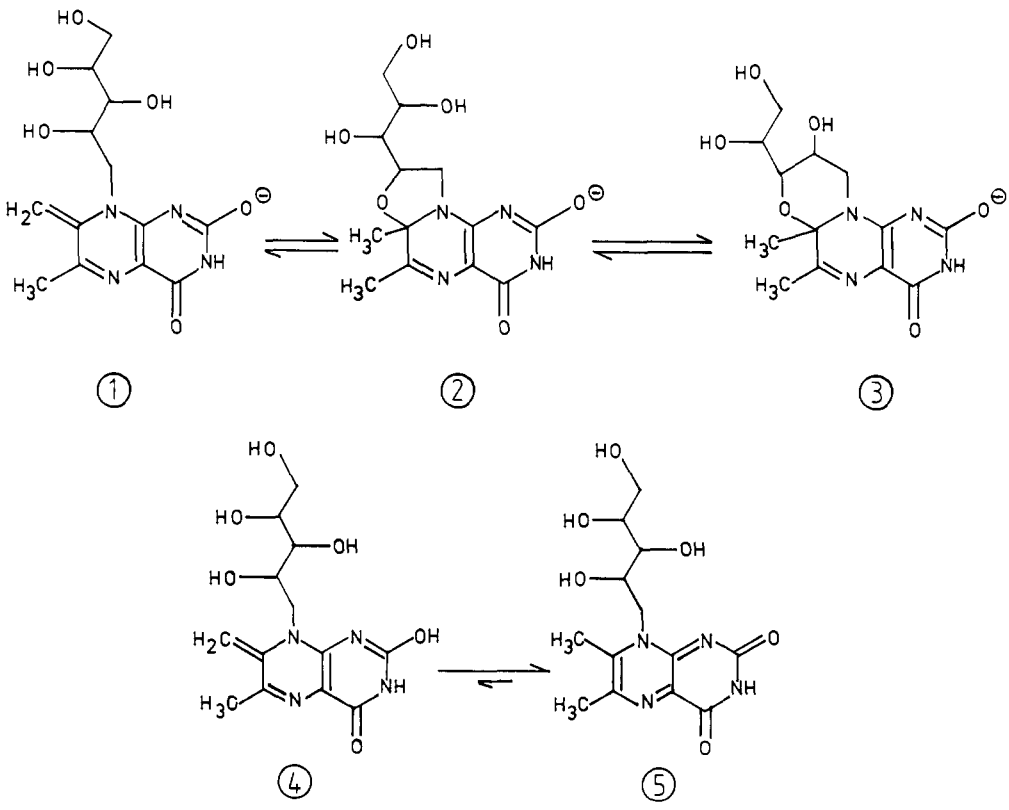

FIGURE 4: Structures of 6,7-dimethyl-8-ribityllumazine formed in alkaline and neutral solution.

region, where our NMR experiments were done, protonation of the ionized oxygen atom (**1**–**3**) could yield the neutral species **4**. This in turn could tautomerize to **5**. If the equilibrium between **4** and **5** is completely shifted in the direction of **5**, then **4** could not be observed by NMR at neutral pH, which is in agreement with our results. Furthermore, since **2** and **3** are colorless (λ_{max} = 320 nm), they can clearly not be the chromophoric species of LumP. On the other hand, the optical properties of **1** (λ_{max} = 360 nm) and **4** (λ_{max} unknown) might be compatible with the optical properties of the LumP chromophore, but they are clearly ruled out by the present NMR data. Therefore, we suggest, as an explanation for the large downfield shift of the resonances due to C(7) and C(7α) in water as compared to DMSO, that the molecule is also polarized by π electron delocalization from C(7) onto the two carbonyl groups as shown schematically in Figure 3. This polarization pattern resembles that deduced for flavin by Moonen et al. (1984) and also, by analogy, means that a solvent with a high dielectric constant is needed to stabilize these structures.

Table I also includes for comparison the corresponding ^{15}N NMR chemical shifts of the isoalloxazine ring of the flavin molecule in a polar (FMN) and in an apolar (TARF) solvent (Vervoort et al., 1986a). It can be seen that the resonances due to the N(3) and N(5) atoms in 6,7-dimethyl-8-ribityllumazine and in FMN, have almost the same chemical shift. The N(1) atom of 6,7-dimethyl-8-ribityllumzine resonates at higher fields (by 7.1 ppm) than that in flavin. Among other effects this difference reflects probably also the difference in basicity of the N(1) atoms in the two molecules. The protonation in flavin occurs at a lower pH value (~0; Dudley et al., 1964) than in 6,7-dimethyl-8-ribityllumazine (0.6; Pfleiderer et al., 1966).

The resonance due to the N(8) atom in 6,7-dimethyl-8-ribityllumazine is quite dissimilar and strongly (34.5 ppm) downfield shifted compared to the equivalent position in FMN. This strong downfield shift of the N(8) atom cannot be explained by differences in hydrogen-bonding character as the N(8), being a pyrrole-type nitrogen, is unlikely to have a strong hydrogen bond. However, in ^{13}C and ^{15}N NMR studies of the flavin molecule, it was shown that the N(10) nitrogen atom [N(8) in 6,7-dimethyl-8-ribityllumazine] can change hybridization from a partly sp^3 hybridized atom toward a completely sp^2 hybridized atom, depending on solvent polarity (Moonen et al., 1984). It was concluded that this change in hybridization is due to electron delocalization from the N(10) atom toward the two carbonyl groups. The strong downfield shift of the N(8) atom in 6,7-dimethyl-8-ribityllumazine compared to the N(10) atom in FMN indicates that the N(8) atom in the former compound is polarized to a greater extent than the N(10) atom in FMN. As a possible explanation we suggest that in 6,7-dimethyl-8-ribityllumazine more electron density from the N(8) atom is reallocated at the two carbonyl groups than from the N(10) atom onto the two carbonyl groups in FMN.

Figure 2B shows the ^{15}N NMR spectra of [1,3,5,8-$^{15}N_4$]-6,7-dimethyl-8-ribityllumazine bound to *P. phosphoreum* lumazine protein. In these spectra the N(3) and N(5) resonances can be assigned unambiguously on the basis of their chemical shifts and on the basis of the splitting of N(3) resonance due to the attached proton. The resonance at 199.6 ppm is assigned to the N(8) atom with the aid of the selectively ^{15}N(8)-labeled compound bound to *P. phosphoreum* lumazine protein. The chemical shifts are collected in Table I.

The chemical shift of the N(5) atom is found upfield from the one in free 6,7-dimethyl-8-ribityllumazine. This upfield shift indicates that the N(5) atom has a stronger hydrogen bond than the free chromophore in a protic solvent (water). The strong downfield shift of the N(1) atom, on the other hand, indicates that this atom has a very weak hydrogen bond as compared to the free chromophore in water. The N(3) atom is rather strongly downfield shifted (for a pyrrole-type nitrogen, vide supra), and this indicates that the N(3) atom must have a strong hydrogen bond with the apoprotein.

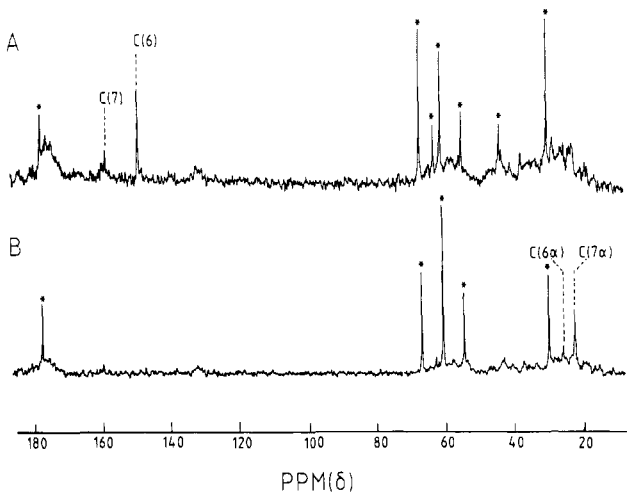

FIGURE 5: ^{13}C NMR spectra of *P. leiognathi* apolumazine protein reconstituted with ^{13}C-enriched 6,7-dimethyl-8-ribityllumazine containing 80% [6-^{13}C]- and 20% [7-^{13}C]-6,7-dimethyl-8-ribityllumazine (A, 0.5 mM, number of transients = 130000) and reconstituted with 80% [7α-^{13}C]- and 20% [6α-^{13}C]-6,7-dimethyl-8-ribityllumazine (B, 0.5 mM, number of transients = 79000) in 50 mM potassium phosphate, 1 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.0. The temperature was 15 °C. Resonances marked with an asterisk are due to the buffer system used.

It can be seen in Figure 2B that the N(3) resonance is split into a doublet with a coupling constant of 86 Hz. The fact that this coupling constant can be observed indicates that the N(3) proton does not exchange with bulk solvent on the NMR time scale ($\upsilon \gg 100$ Hz.) On decoupling of the proton, only a single resonance line is observed with almost no nuclear Overhauser effect. The absence of an NOE on the N(3) resonance of the protein-bound 6,7-dimethyl-8-ribityllumazine indicates that the protein-bound molecule has no or very limited mobility. This is in perfect agreement with the conclusions from other spectroscopic studies (Visser & Lee, 1980). The N(8) resonance is slightly downfield shifted on binding to the protein. This small downfield shift indicates that the N(8) atom is polarized even slightly stronger than the free chromophore in water and that this atom has a strong $\delta+$ character.

Figure 5 shows ^{13}C NMR spectra of selectively ^{13}C-enriched 6,7-dimethyl-8-ribityllumazine bound to lumazine protein from *P. leiognathi*. On comparison of the two spectra the ^{13}C resonances of the protein-bound ^{13}C-labeled molecule can be clearly observed. The ^{13}C NMR chemical shifts are collected in Table I.

All ^{13}C resonances of the protein-bound molecule are shifted downfield as compared to those of the free molecule in water. This indicates a further polarization of the ring system in the protein-bound state. This interpretation is in agreement with the fact that the partial positive charge on the N(8) atom is further increased in the protein-bound state, as evidenced by a downfield shift of the resonance due to the N(8), and is also corroborated by the downfield shift of the resonance due to C(6).

The ^{13}C NMR chemical shifts of the C(6), C(6α), and C(7α) atoms are all slightly downfield shifted when bound to the protein compared to those of free 6,7-dimethyl-8-ribityllumazine. These slight downfield shifts indicate that small positive charges are created at these positions. The C(7) resonance shifts strongly downfield (3.6 ppm), and a relatively large partial positive charge must be developed at this atom on binding to the protein.

The NOEs of the two methyl groups were determined and found to be 1.04 for the C(7α) and 1.17 for the C(6α) methyl group, indicating that little, if any, enhancement is observed under proton decoupling conditions. These results are in agreement with the result obtained for the N(3)–H group (see above) and support the observation that the molecule is tightly bound to the protein with no internal mobility (Visser & Lee, 1980).

Conclusions

The picture that emerges from the ^{13}C and ^{15}N chemical shifts of the protein-bound 6,7-dimethyl-8-ribityllumazine is that the ring system is polarized to a great extent with creation of partial positive charges mainly at the centers N(8) and C(7). The NMR results presented can be explained by the mesomeric structures shown in Figure 3. The two carbonyl groups play an important role in the polarization of the ring system and are very likely to have strong hydrogen bonds. In flavin models it was shown that for the creation of partial positive charges a medium with a high permittivity is needed (Moonen et al., 1984). In this context it is interesting to note that most chemical shifts reported in this paper resemble rather closely the equivalent chemical shifts in flavin and show a similar behavior in going from an aprotic to a protic and highly polar solvent. The present results indicate that the protein-bound molecule is embedded in a very polar environment, with the pyrimidine ring in a tight binding region due to strong interactions toward the two carbonyl groups and a strong hydrogen bond toward the N(3)–H group. It is very conceivable that the pyrazine part of the molecule is solvent accessible. This would be in agreement with conclusions obtained by Lee et al. (1985). The chemical shifts of C(7) and C(7α) clearly indicate the existence of the neutral species **5** (Figure 4) and not of any of the other structures.

The binding interaction between the ligand and protein is almost the same in the two lumazine proteins examined since the chemical shifts are hardly different. This is in agreement with a coherent anti-Stokes Raman scattering (CARS) study on both types of lumazine proteins in which it was observed that the vibrational modes in the fingerprint region are identical (Vervoort et al., 1983). In this CARS study it was also shown that the vibrational modes of the protein-bound chromophore are not shifted compared to the modes of the free molecule in water. This indicates two things: first, as we also concluded in this paper, the ring system is in a polar environment and, second, the N(8) atom is not directly involved in the vibrational modes of the CARS spectra. If the N(8) atom were directly involved, we would have expected some changes to occur on binding because this is observed in the NMR.

The function of lumazine protein in the bioluminescent bacteria is to efficiently transform the chemical energy from the oxidation of $FMNH_2$ and a fatty aldehyde into the fluorescent state of the protein-bound lumazine derivative. This fluorescence has a maximum at 475 nm, corresponding to an optimal transmission of this light in the ocean environment. The present NMR study supports the conclusion arrived at from fluorescence measurements: the binding site of the protein is such as to allow a highly polar structure for the lumazine, and this favors a high quantum yield of fluorescence. In water the quantum yield is lower than on the protein, and the emission spectrum has a maximum at 490 nm (Lee, 1985). Solvents of lower polarity shift the spectrum to longer wavelength with a lowering of the fluorescence yield. The NMR results are also consistent with rigidity of the bound lumazine on the protein, also indicated by the long correlation time derived from lumazine's emission anisotropy. This rigidity is probably the responsible factor for shifting the fluorescence maximum to a shorter wavelength. This rigidity along with

surface accessibility no doubt also plays a part in the alignment of the lumazine acceptor nearby the site of energy generation on the luciferase enzyme to favor efficient deposition of the reaction exergonicity.

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Registry No. 6,7-Dimethyl-8-(1′-D-ribityl)lumazine, 2535-20-8; [1-^{13}C]ribose, 70849-24-0; [^{15}N]ribitylamine, 124583-07-9; [1-^{13}C]ribitylamine, 124583-08-0; 6-chloro-5-nitro-2,4(1*H*,3*H*)-pyrimidinedione, 6630-30-4; [8-^{15}N]-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, 124583-09-1; [1′-^{13}C]-5-nitro-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione, 124583-10-4; diacetyl, 431-03-8; [1′-^{13}C]-6,7-dimethyl-8-ribityllumazine, 124583-11-5; [8-^{15}N]-6,7-dimethyl-8-ribityllumazine, 124602-70-6; [^{15}N]hydroxylamine, 72960-76-0; [1,3,5,8-^{15}N$_4$]-6,7-dimethyl-8-ribityllumazine, 124583-12-6.

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