JOC*Article*

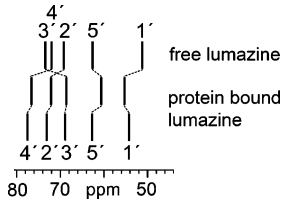
Random Isotopolog Libraries for Protein Perturbation Studies. ^{13}C NMR Studies on Lumazine Protein of *Photobacterium leiognathi*

Boris Illarionov,[†,‡] Chan Yong Lee,[§] Adelbert Bacher,[‡] Markus Fischer,[‡] and Wolfgang Eisenreich*[,‡]

Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany, and Department of Biochemistry, Chungnam National University, Daejeon 305-764, Korea

wolfgang.eisenreich@ch.tum.de

Received August 8, 2005



Lumazine proteins of luminescent bacteria are paralogs of riboflavin synthase which are devoid of catalytic activity but bind the riboflavin synthase substrate, 6,7-dimethyl-8-ribityllumazine, with high affinity and are believed to serve as optical transponders for bioluminescence emission. Lumazine protein of *Photobacterium leiognathi* was expressed in a recombinant *Escherichia coli* host and was reconstituted with mixtures (random libraries) of ^{13}C-labeled isotopologs of 6,7-dimethyl-8-ribityllumazine or riboflavin that had been prepared by biotransformation of [U−$^{13}C_6$]-, [1-$^{13}C_1$]-, [2-$^{13}C_1$]-, and [3-$^{13}C_1$]glucose. ^{13}C NMR analysis of the protein/ligand complexes afforded the assignments of the ^{13}C NMR chemical shifts for all carbon atoms of the protein-bound ligands by isotopolog abundance editing. The carbon atoms of the ribityl groups of both ligands studied were shifted up to 6 ppm upon binding to the protein. Chemical shift modulation of the side chain and chromophore carbon atoms due to protein/ligand interaction is discussed on the basis of the sequence similarity between lumazine protein and riboflavin synthase.

Introduction

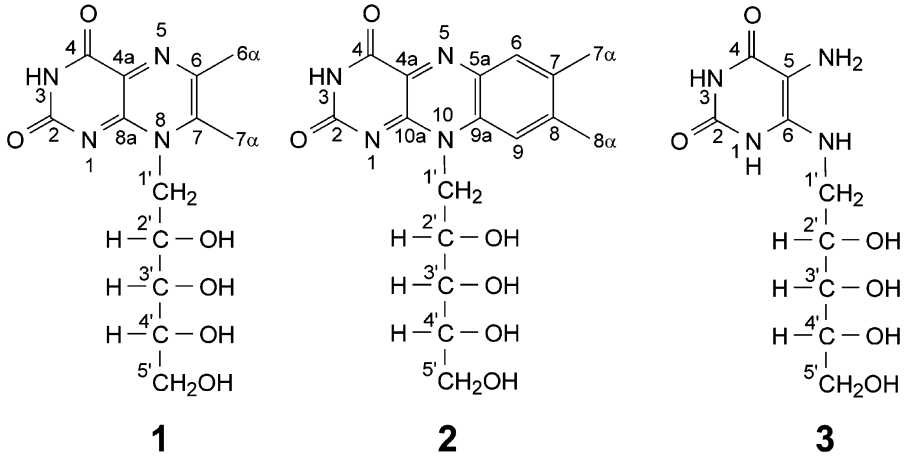
The fluorophore of a fluorescent protein isolated from *Photobacterium phosphoreum* in the late 1970s by Koka and Lee was identified as 6,7-dimethyl-8-ribityllumazine.[1] This protein (later named lumazine protein) binds the ligand with high affinity (up to $K_D = 16$ nM).[2] It is devoid of enzymatic activity and is believed to act as an optical transponder in bioluminescence emission of *P. phosphoreum*.[3] The excited state of lumazine protein has a lifetime in the range of nanoseconds and emits light with a quantum yield in the range of 0.5.[3] The emission maximum of bacterial bioluminescence can be shifted appreciably by lumazine protein acting as an optical transponder.[4]

The fluorophore of lumazine protein, 6,7-dimethyl-8-ribityllumazine, **1** (Figure 1), serves as the terminal intermediate in the biosynthesis of vitamin B_2 (riboflavin, **2**, Figure 1) (for review, see ref 5). The metabolic intermediate **1** is converted into riboflavin by an unusual dismutation affording 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**3**) as a second product.[6] That reaction can proceed spontaneously under relatively mild conditions and is catalyzed by riboflavin synthase, a homotrimeric enzyme with a subunit mass of about 24 kDa.[7] Riboflavin synthase and lumazine protein share consid-

* Corresponding author. Phone: 0049-89-28913336. Fax: 0049-89-289-13363.
† On leave of absence from the Institute of Biophysics, Krasnoyarsk, Russia.
‡ Technische Universität München.
§ Chungnam National University.

(1) Koka, P.; Lee, J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 3068−3072.
(2) Lee, J.; O'Kane, D. J.; Visser, A. J. *Biochemistry* **1985**, *24*, 1476−1483.
(3) Lee, J. *Biophys. Chem.* **1993**, *48*, 149−158.
(4) Petushkov, V. N.; Gibson, B. G.; Lee, J. *Biochemistry* **1995**, *34*, 3300−3309.
(5) Bacher, A.; Eberhardt, S.; Eisenreich, W.; Fischer, M.; Herz, S.; Illarionov, B.; Kis, K.; Richter, G. *Vitam. Horm.* **2001**, *61*, 1−49.
(6) Wacker, H.; Harvey, R. A.; Winestock, C. H.; Plaut, G. W. *J. Biol. Chem.* **1964**, *239*, 3493−3497.
(7) Schott, K.; Kellermann, J.; Lottspeich, F.; Bacher, A. *J. Biol. Chem.* **1990**, *265*, 4204−4209.

10.1021/jo051662f CCC: $30.25
Published on Web 10/28/2005



1 **2** **3**

FIGURE 1. 6,7-Dimethyl-8-ribityllumazine (**1**), riboflavin (**2**), and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**3**).

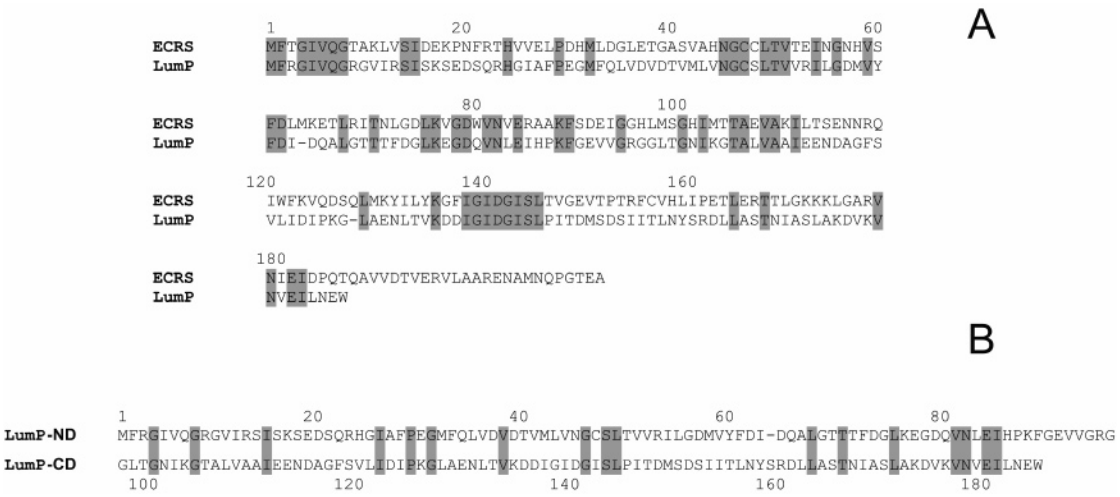


FIGURE 2. Amino acid sequence similarity between lumazine protein from *P. leiognathi* (LumP) and riboflavin synthase from *E. coli* (ECRS) (A) and internal sequence similarity between lumazine protein N-terminal (LumP-ND) and C-terminal (LumP-CD) domains (B). Shadowed boxes indicate identical amino acids. The numbers indicate amino acid positions in the lumazine protein sequence.

erable sequence similarity over their entire length,[7,8] and the sequences of both proteins are characterized by a high degree of intramolecular sequence similarity (Figure 2).

X-ray structure analysis of riboflavin synthases from *Escherichia coli* and *Schizosaccharomyces pombe* showed that each subunit folds into two domains with closely similar folding topology.[9,10] Each of the domains can bind one molecule of 6,7-dimethyl-8-ribityllumazine in a shallow surface cavity. Catalytic dismutation of the substrate is believed to proceed at the interface between the N-terminal domain of one enzyme subunit and the C-terminal domain of an adjacent subunit.[9,10] The two interacting domains form a pseudo-c_2 symmetric ensemble in line with the regiochemical features of the catalytic reaction which require an antiparallel arrangement of the two substrate molecules at the active site.[11,12] Interestingly, the homotrimeric riboflavin synthases are devoid of trigonal symmetry.[9]

In contrast to riboflavin synthase, lumazine protein is a monomer binding a single molecule of 6,7-dimethyl-8-ribityllumazine or its derivatives.[13,14] In all likelihood, this implies that only one of the two homologous subunit domains can bind the ligand, in contrast to riboflavin synthase where both subunit domains bind 6,7-dimethyl-8-ribityllumazine.[14,15]

NMR studies of lumazine protein have been performed with some specifically ^{13}C-labeled isotopologs of 6,7-dimethyl-8-ribityllumazine and with trifluoromethyl-substituted lumazine derivatives,[14,15] but a comprehensive NMR analysis of the protein/ligand interaction was not possible in the past due to the absence of a sufficiently

(8) O'Kane, D. J.; Woodward, B.; Lee, J.; Prasher, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1100−1104.
(9) Liao, D. I.; Wawrzak, Z.; Calabrese, J. C.; Viitanen, P. V.; Jordan, D. B. *Structure (Cambridge, MA, U.S.)* **2001**, *9*, 399−408.
(10) Gerhardt, S.; Schott, A. K.; Kairies, N.; Cushman, M.; Illarionov, B.; Eisenreich, W.; Bacher, A.; Huber, R.; Steinbacher, S.; Fischer, M. *Structure (Cambridge, MA, U.S.)* **2002**, *10*, 1371−1381.
(11) Paterson, T.; Wood, H. C. S. *J. Chem. Soc., Chem. Commun.* **1969**, 290−291.
(12) Plaut, G. W.; Beach, R. L. In *Flavins and Flavoproteins*; Singer, T. P., Ed.; Elsevier: Amsterdam, 1976; pp 737−746.
(13) O'Kane, D. J.; Lee, J. *Biochemistry* **1985**, *24*, 1467−1475.
(14) Scheuring, J.; Lee, J.; Cushman, M.; Patel, H.; Patrick, D. A.; Bacher, A. *Biochemistry* **1994**, *33*, 7634−7640.
(15) Vervoort, J.; O'Kane, D. J.; Muller, F.; Bacher, A.; Strobl, G.; Lee, J. *Biochemistry* **1990**, *29*, 1823−1828.

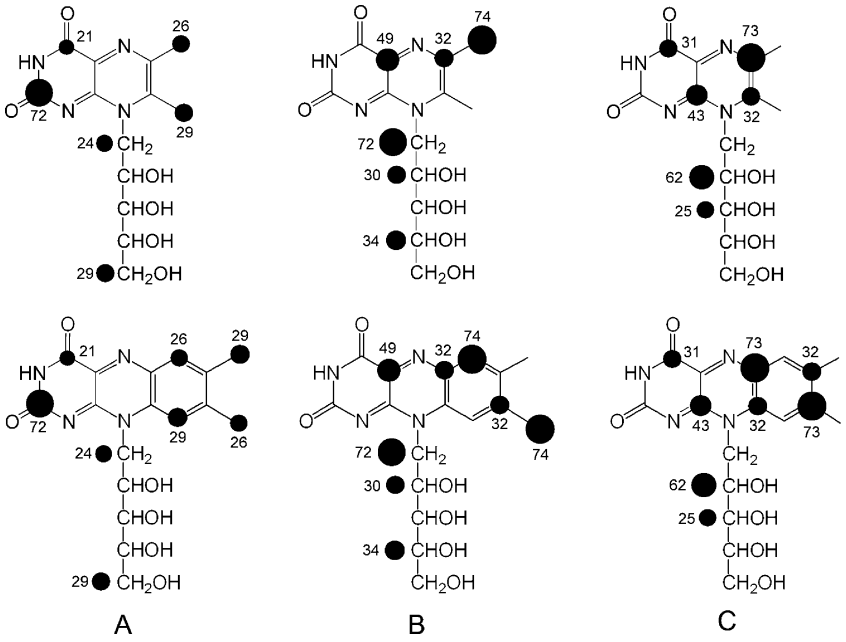


FIGURE 3. Labeling patterns of 6,7-dimethyl-8-ribityllumazine and riboflavin obtained by biotransformation of ^{13}C-labeled glucose samples with an *E. coli* strain M15[pREP4, pRFN4]. (A) From the experiment with [1-$^{13}C_1$]glucose. (B) From the experiment with [2-$^{13}C_1$]glucose. (C) From the experiment with [3-$^{13}C_1$]glucose. ^{13}C enrichments are indicated by scaled dots. The numbers indicate ^{13}C abundances.

comprehensive set of specifically ^{13}C-labeled ligand samples. We have recently developed methods for the preparation of random isotopolog libraries of 6,7-dimethyl-8-ribityllumazine by simple fermentation procedures using ^{13}C-labeled glucose samples as precursors which enable rapid signal assignment in enzyme/ligand complexes by isotopolog abundance editing.[16] This article reports the application of these novel tools to the study of the interaction of lumazine protein from the marine bacterium *Photobacterium leiognathi* with its natural ligand, 6,7-dimethyl-8-ribityllumazine, and with riboflavin. Parallel studies with the recombinant N-terminal domain of riboflavin synthase showed that the ligand binding sites of lumazine protein and riboflavin synthase are similar as judged from the chemical shift modulation of bound ligands. The novel method for the assignment of NMR signals of bound ligands on the basis of isotopolog abundance editing appears suitable as a general tool for the study of protein/ligand interaction.

Results

NMR spectroscopy has been shown earlier to be useful in the study of the interaction between lumazine protein from *P. leiognathi* and its native ligand 6,7-dimethyl-8-ribityllumazine.[15] However, due to technical limitations, this analysis was restricted to the nitrogen atoms and the carbon atoms 6α, 6, 7, 7α, and 1′ of the lumazine chromophore.

More recently, we reported a method for the rapid preparation of libraries of isotopologs of the riboflavin precursor, 6,7-dimethyl-8-ribityllumazine, by in vivo biotransformation of ^{13}C-labeled glucose samples.[16] These isotopolog mixtures are in fact random libraries of isotopologs carrying ^{13}C labels in different positions (Figure 3). Due to the transformation strategy used, the ^{13}C-labeled positions are typically not adjacent to other directly bonded ^{13}C atoms; hence, with few exceptions, the signals appear as singlets of different intensities.

In the present article, we have further extended this approach by converting the libraries of ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine into cognate random libraries of riboflavin isotopologs by treatment with the enzyme, riboflavin synthase, which transfers a four-carbon fragment between two identical substrate molecules with precise regiochemical control.[17−19] In the enzyme-catalyzed reaction, the xylene ring of the riboflavin chromophore is then assembled exclusively from components of 6,7-dimethyl-8-ribityllumazine; the reaction is best described as an unusual dismutation. Since the regiochemistry of the reaction is known in detail, the labeling pattern of the resulting riboflavin libraries can be deduced easily (Figure 3).

Lumazine protein was prepared from a recombinant *E. coli* strain engineered for overexpression of the *lumP* gene of *P. leiognathi*.[20] The recombinant protein was treated with 6 M urea to release the bound cofactor and was then reconstituted with the random isotopolog libraries of 6,7-dimethyl-8-ribityllumazine or riboflavin shown in Figure 3 or with [U−^{13}C]-labeled samples.

^{13}C NMR signals of [U−$^{13}C_{13}$]6,7-dimethyl-8-ribityllumazine in complex with lumazine protein are shown in Figure 4A. The spectrum displays one singlet (156.6

(16) Illarionov, B.; Fischer, M.; Lee, C. Y.; Bacher, A.; Eisenreich, W. *J. Org. Chem.* **2004**, *69*, 5588−5594.

(17) Beach, R. L.; Plaut, G. W. *J. Am. Chem. Soc.* **1970**, *92*, 2913−2916.

(18) Bacher, A.; Le Van, Q.; Keller, P. J.; Floss, H. G. *J. Biol. Chem.* **1983**, *258*, 13431−13437.

(19) Illarionov, B.; Eisenreich, W.; Bacher, A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 7224−7229.

(20) Illarionov, B.; Illarionova, V.; Lee, J.; van Dongen, W.; Vervoort, J. *Biochim. Biophys. Acta* **1994**, *1201*, 251−258.

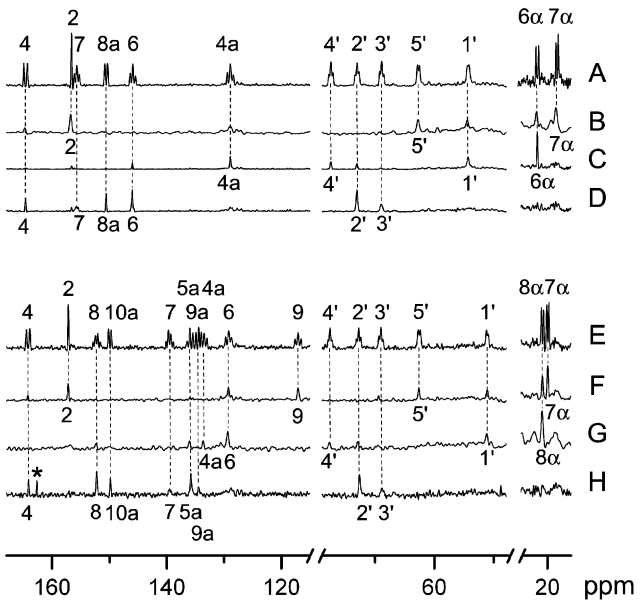


FIGURE 4. ^{13}C NMR spectra of 6,7-dimethyl-8-ribityllumazine (A−D) or riboflavin (E−H) isotopologs in complex with lumazine protein. Ligand isotopologs were obtained from [U−$^{13}C_{13}$]glucose (A, E), [1-$^{13}C_1$]glucose (B, F), [2-$^{13}C_1$]glucose (C, G), or [3-$^{13}C_1$]glucose (D, H). * = Impurities.

ppm), six doublets (164.6, 150.6, 62.7, 54.3, 21.4, and 18.4 ppm), and six pseudo-triplets (155.7, 145.9, 128.9, 77.5, 73.1, and 68.9 ppm). The multiplets are due to scalar coupling between adjacent ^{13}C atoms in the multiply ^{13}C-labeled lumazine ligand. On the basis of chemical shift considerations, the two doublet signals at 21.4 and 18.4 ppm reflect the methyl carbons 6α and 7α, the signals between 50 and 80 ppm are due to the ribityl carbons, and the downfield-shifted signals between 120 and 170 ppm are due to carbons of the heteroaromatic ring system. The signal at 156.6 ppm can be assigned unequivocally to C-2 due to its singlet signature. The assignments of the other signals can be obtained by the use of random isotopolog libraries from single-labeled glucose precursors as described below.

The signal amplitudes of individual ligand carbon atoms in Figure 4 (traces B−D) are modulated in a characteristic way reflecting the abundances of different isotopologs with isolated ^{13}C labels (Table 1). This intensity profile is highly characteristic and can serve as the basis for signal assignments in the protein complexes. The ^{13}C abundances of the lumazine carbon atoms in traces B−D can be calculated by comparison with the signal intensities in trace A (Table 1).

On the basis of the known isotopolog composition of the three 6,7-dimethyl-8-ribityllumazine libraries (Figure 3), all signals can now be assigned unequivocally with the value of their signal intensities shown in Figure 4. For example, both methyl groups are ^{13}C-enriched in the mixture from [1-$^{13}C_1$]glucose and give rise to two signals in the methyl region (Figure 4B). Only the 6α methyl group acquires label from [2-$^{13}C_1$]glucose, and therefore, the signal detected at 21.4 ppm in the spectrum with the isotopolog library from [2-$^{13}C_1$]glucose can be clearly assigned to C-6α.

Due to the specific ^{13}C enrichments in the ribityl moiety of the lumazine samples, the signals for carbons 1′ and 5′ are detected in the isotopolog mixture from [1-$^{13}C_1$]-glucose, signals for 1′, 2′, and 4′ are observed in the isotopolog mixture from [2-$^{13}C_1$]glucose, and signals for 2′ and 3′ can be gleaned in the spectrum of the isotopolog mixture from [3-$^{13}C_1$]glucose. On this basis and in accordance to the observed coupling pattern in the spectrum with [U−$^{13}C_{13}$]6,7-dimethyl-8-ribityllumazine (i.e., doublets for the 1′ and 5′ signals and pseudo triplets for 2′, 3′, and 4′ signals), all ribityl signals can be unequivocally assigned (Table 1 and Figure 4).

It follows that some chemical shifts of the ribityl side chain experience relatively large changes upon binding to lumazine protein (Figure 5). As a consequence, it would have been impossible to assign carbon atoms 2′ through 4′ just on the basis of a chemical shift comparison with the free ligand. It should also be noted that the carbon atoms of the heterocyclic ring system are subject to considerable chemical shift changes upon binding to the protein. Again, unequivocal assignment would have been time-consuming without the use of the isotopolog abundance editing technique.

The present technique allows the unequivocal assignment of all 13 carbon atoms of the ligand using only four ^{13}C-labeled ligand samples (i.e., the universally ^{13}C-labeled ligand and the isotopolog mixtures obtained by biotransformation of three differently labeled glucose samples). The assignments obtained for 6α, 6, 7, 7α, and 1′ in the present experiments agree with the assignments reported earlier.[16]

NMR signals of [U−$^{13}C_{17}$]riboflavin in complex with lumazine protein are shown in Figure 4E. Most signals appear as multiplets due to $^{13}C^{13}C$ coupling in the universally labeled ligand. Figure 4F−H shows signals of the protein with isotopolog mixtures of riboflavin obtained by in vivo biotransformation of [1-$^{13}C_1$]-, [2-$^{13}C_1$]-, and [3-$^{13}C_1$]glucose, respectively. On the basis of the isotopolog editing approach described above in detail for 6,7-dimethyl-8-ribityllumazine, all chemical shifts of bound riboflavin are unequivocally assigned (Table 2 and Figure 4). Again, the chemical shifts of the ribityl side chain undergo considerable modulation upon binding to the protein (Figure 5). Notably, however, the signals of carbon atoms 2′−4′ are almost exactly in register with the corresponding signals of the ribityl side chain of 6,7-dimethyl-8-ribityllumazine bound to lumazine protein. It should also be noted that the pyrimidine carbon atoms C-2 and C-4 of riboflavin and 6,7-dimethyl-8-ribityllumazine in complex with lumazine protein have similar shifts (Figure 5).

Attempts to determine the three-dimensional structure of lumazine protein have been unsuccessful up to now. However, the crystal structures of riboflavin synthases from *E. coli* and *Schizosaccharomyces cerevisiae* have been determined.[9,10] Moreover, the structure of an artifactual dimer of the recombinant N-terminal domain of riboflavin synthase of *E. coli* has been determined by NMR analysis[21] and by X-ray crystallography.[22] In all reported structures, the binding mode of ribityl-substituted ligands as observed by X-ray crystallography was

(21) Truffault, V.; Coles, M.; Diercks, T.; Abelmann, K.; Eberhardt, S.; Luttgen, H.; Bacher, A.; Kessler, H. *J. Mol. Biol.* **2001**, *309*, 949−960.

(22) Meining, W.; Eberhardt, S.; Bacher, A.; Ladenstein, R. *J. Mol. Biol.* **2003**, *331*, 1053−1063.

TABLE 1. NMR Data of ^{13}C-Labeled 6,7-Dimethyl-8-ribityllumazine in Solution and Bound to the Lumazine Protein[a]

	free [U−$^{13}C_{13}$] 6,7-dimethyl-8-ribityllumazine		6,7-dimethyl-8-ribityllumazine isotopologs bound to lumazine protein				
			isotopolog libraries obtained from:				
			[U−$^{13}C_6$]glucose		[1-$^{13}C_1$]glucose	[2-$^{13}C_1$]glucose	[3-$^{13}C_1$]glucose
position	δ ^{13}C ppm	J_{CC} Hz	δ^{13}C ppm	J_{CC} Hz	% ^{13}C	% ^{13}C	% ^{13}C
7α	18.1	46.5(7), 3.3(6), 1.3(6α)	18.4	46	35		
6α	21.4	54.0(6), 7.5(7), 5.3(4a), 1.3(7α)	21.4	55	37	81	
1′	51.2	39.4(2′)	54.3	39	29	**72**	
5′	62.6	40.9(4′), 2, 2	62.7	42	**29**		
2′	69.2	40.0(3′), 39.4(1′)	73.1	43		20	66
4′	72.0	41.0(3′), 41.0(5′)	77.5	42		26	
3′	73.4	41.0(2′), 41.0(4′)	68.9	43			28
4a	130.9	77.8(4), 61.3(8a), 9(6), 9(7), 5.1(6α)	128.9	65, 75		46	
6	144.9	54.2(6α), 59.5(7), 3.3(7α)	145.9	56		22	**73**
8a	150.4	61.7(4a), 9(4), 9(6)	150.6	63			42
7	152.3	46.7(7α), 59.5(6), 7.5(6α), 7.5(4a)	155.7	52			27
2	157.9	3.0(4), 3.0(4a), 3.0(8a)	156.6		56	11	
4	163.1	77.8(4a), 9.3(8a), 6.4(2), 1.3, 1.3	164.6	75	17		32

[a] Numbers in bold indicate reference values[16] that have been used to calculate ^{13}C abundance at respective positions of 6,7-dimethyl-8-ribityllumazine.

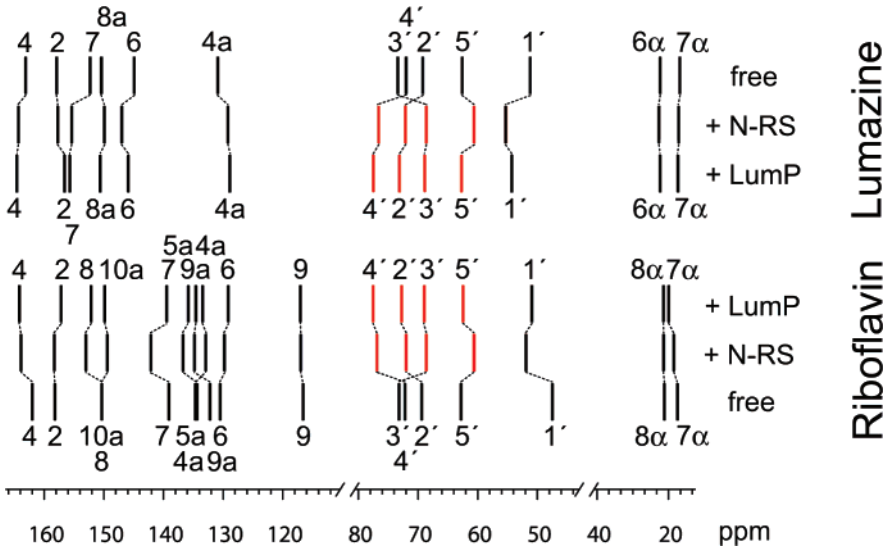


FIGURE 5. Correlation diagram of ^{13}C NMR chemical shifts of riboflavin and [U−$^{13}C_{13}$]6,7-dimethyl-8-ribityllumazine, free or bound to the N-terminal domain of riboflavin synthase (N-RS) or lumazine protein (LumP).

closely similar. Notably, the conformation of the ribityl side chains was similar in all complexes studied.

The sequences of *E. coli* riboflavin synthase and lumazine protein show close similarity: 31% of their amino acid residues are identical (Figure 2) and leave no doubt that lumazine protein folds into two topologically similar domains. Hence, it appears plausible to use the riboflavin synthase structures as a template for the interpretation of the NMR features of ligands bound to lumazine protein. To verify the justification of this approach, we decided to study the chemical shift modulation of 6,7-dimethyl-8-ribityllumazine and of riboflavin upon binding to the artificial N-terminal domain dimer of riboflavin synthase. Experiments were performed as described above for lumazine protein.

Figure 6A−D shows sections of ^{13}C NMR spectra of isotopolog libraries of 6,7-dimethyl-8-ribityllumazine in complex with the N-terminal domain of riboflavin synthase. The signals were assigned by the isotopolog editing approach described above. Again, all signals could be assigned unequivocally (Table 3 and Figure 6). As shown in Figure 5, the chemical shifts of the lumazine in complex with lumazine protein and with the riboflavin synthase domain are similar.

NMR spectra of the N-terminal domain of riboflavin synthase with [U−$^{13}C_{17}$]riboflavin and the random isotopolog libraries described above are shown in Figure 6E−G. The detailed analysis of the signal intensity profiles affords solid evidence for all asignments (Table 4 and Figure 6). Again, a close correspondence between the chemical shifts of the ribityl carbon atoms and the pyrimidine carbon atoms, respectively, was found for riboflavin bound to lumazine protein or the N-terminal domain of riboflavin synthase (Figure 5).

Discussion

NMR is a powerful technique for the study of protein/ligand interaction. Flavoproteins are particularly interesting examples because redox reactions affect both the electron distribution and the conformation of the isoalloxazine ring. The chemical shifts of flavin cofactor

TABLE 2. NMR Data of ^{13}C-Labeled Riboflavin Isotopologs in Solution and Bound to the Lumazine Protein[a]

		riboflavin isotopologs bound to lumazine protein				
		isotopolog libraries obtained from:				
	free [U−$^{13}C_{17}$] riboflavin	[U−$^{13}C_6$]glucose		[1-$^{13}C_1$]glucose	[2-$^{13}C_1$]glucose	[3-$^{13}C_1$]glucose
position	δ ^{13}C ppm	δ ^{13}C ppm	J_{CC} Hz	% ^{13}C	% ^{13}C	% ^{13}C
7α	18.5	20.0	43.1	38		
8α	20.7	20.9	43.3	34	65	
1′	47.5	51.0	43.0	37	**72**	
5′	62.8	62.5	44.9	**29**		
2′	69.4	72.8	47.0		42	69
4′	72.2	77.6	42.7		24	
3′	73.2	69.0	46.0			27
4a	134.7	133.5	61.3			
6	130.4	129.2		31	60	
10a	150.3	149.9	56.2			40
7	139.1	139.5				25
2	158.2	157.2		64		
4	162.0	164.2	74.3	16		**31**
8	150.3	152.2			22	71
5a	135.1	135.9			25	69
9	116.8	117.1	63.0	37		
9a	132.2	134.6				21

[a] Numbers in bold indicate reference values[16] that have been used to calculate ^{13}C abundance at respective positions of riboflavin.

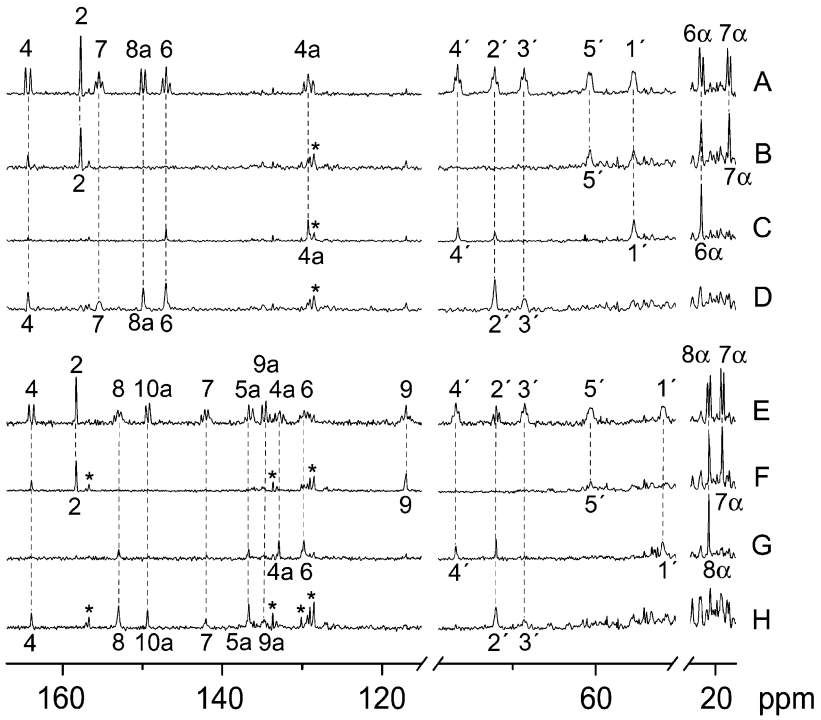


FIGURE 6. ^{13}C NMR spectra of 6,7-dimethyl-8-ribityllumazine (A−C) or riboflavin (E, F) isotopologs in complex with N-terminal domain of riboflavin synthase. Ligand isotopologs were obtained from [U−$^{13}C_{13}$]glucose (A, E), [1-$^{13}C_1$]glucose (B, F), [2-$^{13}C_1$]-glucose (C, G), or [3-$^{13}C_1$]glucose (D, H). * = Impurities.

carbon atoms are sensitive to both types of modulation and can therefore be used to monitor the physical state of the flavin cofactor in considerable detail.[23−25] Obviously, however, the interpretation of the data depends on robust assignment of all flavin NMR signals; this is a nontrivial task because of the large chemical shift variations that can arise as a consequence of protein binding and catalysis.

In earlier studies, the assignment problem was typically addressed by the NMR analysis of numerous samples reconstituted with different flavin isotopologs that had been prepared by chemical synthesis.[26] This approach is laborious as a consequence of the work

(23) Sanner, C.; Macheroux, P.; Ruterjans, H.; Muller, F.; Bacher, A. *Eur. J. Biochem.* **1991**, *196*, 663−672.

(24) Fleischmann, G.; Lederer, F.; Muller, F.; Bacher, A.; Ruterjans, H. *Eur. J. Biochem.* **2000**, *267*, 5156−5167.

(25) Eisenreich, W.; Kemter, K.; Bacher, A.; Mulrooney, S. B.; Williams, C. H., Jr.; Muller, F. *Eur. J. Biochem.* **2004**, *271*, 1437−1452.

(26) Lee, J.; Matheson, I. B. C.; Müller, F.; O'Kane, D. J.; Vervoort, J.; Visser, A. J. W. G. In *Chemistry and Biochemistry of Flavoenzymes*; Müller, F., Ed.; CRC Press: Boca Raton, FL, 1991; pp 109−151.

TABLE 3. NMR Data of ^{13}C-Labeled 6,7-Dimethyl-8-ribityllumazine Isotopologs Bound to the N-Terminal Domain of Riboflavin Synthase[a]

	6,7-dimethyl-8-ribityllumazine isotopologs bound to riboflavin synthase				
	isotopolog libraries obtained from:				
	[U−$^{13}C_6$]glucose		[1-$^{13}C_1$]glucose	[2-$^{13}C_1$]glucose	[3-$^{13}C_1$]glucose
position	δ ^{13}C	J_{CC}	% ^{13}C	% ^{13}C	% ^{13}C
7α	18.3	47	37		
6α	21.6	54	39	82	
1′	55.3	nd	42	81	
5′	60.6	nd	44		
2′	72.1	41		23	**62**
4′	76.6	41		**34**	
3′	68.6	40			29
4a	129.2	73		56	
6	147.0	56		28	71
8a	149.9	63			48
7	155.4	52			27
2	157.7		**72**		
4	164.3	78	21		33

[a] Numbers in bold indicate reference values[16] that have been used to calculate ^{13}C abundance at respective positions of 6,7-dimethyl-8-ribityllumazine.

TABLE 4. NMR Data of ^{13}C-Labeled Riboflavin Isotopologs Bound to N-Terminal Domain of Riboflavin Synthase

	riboflavin isotopologs bound to riboflavin synthase				
	isotopolog libraries obtained from:				
	[U−$^{13}C_6$]glucose		[1-$^{13}C_1$]glucose	[2-$^{13}C_1$]glucose	[3-$^{13}C_1$]glucose
position	$\delta^{13}C$ ppm	J_{CC} Hz	% ^{13}C	% ^{13}C	% ^{13}C
7α	19.1	44.7	35		
8α	20.8	42.2	27	66	
1′	51.9		28	68	
5′	60.6		31		
2′	72.0	43.2		23	68
4′	76.9	40.6		25	
3′	68.6	40.6			32
4a	132.9			**49**	
6	129.8		23	64	
10a	149.4	57.3			45
7	142.1				25
2	158.3		68		
4	163.9	77.1	15		37
8	153.0			26	78
5a	136.7			23	**73**
9	117.0	69.0	**29**		
9a	134.8				39

[a] Numbers in bold indicate reference values[16] that have been used to calculate ^{13}C abundance at respective positions of riboflavin.

invested in (i) the synthesis of numerous different isotopologs, (ii) the preparation of the numerous cognate protein conjugates, and (iii) the performance of numerous individual NMR measurements. Moreover, only a limited fraction of the isotopologs of interest can be obtained with an acceptable effort. In the case of a flavoprotein, 17 single-labeled isotopologs of riboflavin would be required to unequivocally assign all flavocoenzyme carbon signals. In practical terms, such an approach is hardly feasible.

We have recently reported the rapid preparation of isotopolog mixtures of the riboflavin precursor, 6,7-dimethyl-8-ribityllumazine, by the in vivo biotransformation of selectively ^{13}C-labeled glucose.[16] In the present article, we have extended this approach by converting the libraries of ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine into the respective isotopolog libraries of riboflavin by riboflavin synthase (Figure 3).

The random isotopolog libraries of 6,7-dimethyl-8-ribityllumazine and riboflavin were then used to reconstitute lumazine protein as well as the recombinant N-terminal domain of riboflavin synthase; both proteins had previously been separated from their ligands by mild dissociating treatment. As shown above, the intensity modulation of individual carbon signals could then be used to rapidly assign all NMR lines using a small number of samples. Most notably, it was possible for the first time to unequivocally assign the carbon atoms of the ribityl side chains of both riboflavin and 6,7-dimethyl-8-ribityllumazine in complex with the proteins under study. As shown in more detail above, a rigorous assignment technique was required for that purpose in light of the observed crossovers of certain signals that occurred upon protein binding.

The assignment of all signals of riboflavin and 6,7-dimethyl-8-ribityllumazine in complex with the riboflavin synthase domain and with lumazine protein serves as the basis for the comparison between these respective experimental systems. It is relevant in this context that

the folding pattern of riboflavin synthase is amply documented by two X-ray structures and one NMR structure, whereas the structure of lumazine protein remains to be determined. Only the sequence similarity between the two proteins under study suggests far-reaching folding similarity at the present time.

In aqueous solution, the carbon atoms 2′, 3′, and 4′ of riboflavin and 6,7-dimethyl-8-ribityllumazine, respectively, resonate at similar frequencies in the range of 69−74 ppm. Binding of either ligand to either of the proteins results in a significant spreading of that frequency range (cf. Figure 5). Even more important, the signal of C-3′ in the free ligands resonates at low field as compared to the signals of 4′ and 2′ but is shifted to high field by comparison with these signals upon binding to either of the proteins under study. Clearly, without a rigorous assignment method, this phenomenon would have escaped detection.

The rather drastic chemical shift changes experienced by all carbon atoms of the ribityl side chain of both ligands under study upon binding to either of the proteins indicate a substantial modulation of the environment of the ribityl side chain in the proteins as compared to aqueous solution. In the case of riboflavin synthase, it is known that the ribityl side chain interacts tightly with the protein; more specifically, this interaction has been shown to involve several hydrogen bonds.[22] The close similarity of the chemical shift changes experienced upon binding provides strong evidence for similar environments of the ribityl side chain in lumazine protein and riboflavin synthase, respectively. Thus, even without knowledge of the three-dimensional structure for lumazine protein, it must be assumed not only that the general folding pattern is similar but also, more specifically, that the ligand molecules are bound in a similar mode by both proteins.

Compared to free riboflavin and 6,7-dimethyl-8-ribityllumazine, the ^{13}C chemical shifts of the side chain carbon atoms are variably affected in the protein-bound ligands. The signals for C-2′ and C-4′ undergo large downfield shifts of 3.9 and 5.5 ppm, respectively, implicating strong interactions with the apoprotein by hydrogen bonding. The observed downfield shift of the C-1′ could reflect the specific configuration of this atom in protein-bound riboflavin and 6,7-dimethyl-8-ribityllumazine.

The approach used for NMR signal assignment can be easily transferred to a variety of other natural products. The only requirement is the availability of a microbial or plant cell system that can convert ^{13}C-labeled glucose into a natural product with reasonable efficacy. The resulting isotopolog library of that natural product can then be used for complexation with appropriate binding proteins.

Experimental Section

Materials. Recombinant lumazine protein of *P. leiognathi* was prepared and purified by published procedures.[5,20] Purification of the N-terminal domain of riboflavin synthase has been described earlier.[27] The preparation of [U−$^{13}C_{13}$]6,7-dimethyl-8-ribityllumazine and of random ^{13}C-labeled isotopolog libraries of 6,7-dimethyl-8-ribityllumazine isotopologs is described elsewhere.[16]

Preparation of Random Riboflavin Isotopolog Libraries. Universally and partially ^{13}C-labeled riboflavin isotopologs were prepared from [U−$^{13}C_6$]6,7-dimethyl-8-ribityllumazine and 6,7-dimethyl-8-ribityllumazine isotopolog mixtures using *E. coli* riboflavin synthase.[16] Reaction mixtures contained 100 mM sodium/potassium phosphate, pH 7.0, 1 mM dithiothreitol (DTT), 1.6 mM 6,7-dimethyl-8-ribityllumazine, and 5 μM enzyme. The mixtures were incubated for 30 min at 37 °C and were then passed through columns of Florisil (1 × 1 cm) that were washed with water and developed with water/acetone/ammonium hydroxide, 1000:1000:1 (v/v). Fractions were combined and lyophilized. The residue was dissolved in 2 mL of 50 mM hydrochloric acid and applied to a column of Hypersil RP 18 (4.6 × 250 mm), which was developed with an eluent containing 0.1 M ammonium formate and 40% methanol (v/v). The effluent was monitored by a diode array photometer. The retention volume of riboflavin was 2.3 mL.

Ligand Exchange Procedures. A solution containing 100 mM sodium/potassium phosphate, pH 7.0, and 6 mg of lumazine protein or the N-terminal domain of riboflavin synthase (both preloaded with riboflavin) in 1 mL was dialyzed overnight against 2 L of 100 mM sodium/potassium phosphate, pH 7.0, containing 4 mM DTT and 6 M urea.[28] The riboflavin-free protein solution was centrifuged, and the supernatant was injected into 50 mL of 100 mM phosphate, pH 7.0, containing 0.3 mM DTT and 20 μM of a ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine or riboflavin isotopolog mixture, respectively. The sample was concentrated to 1 mL by ultrafiltration. The solution was dialyzed twice against 200 mL of 100 mM phosphate, pH 7, containing 0.2 mM DTT and 50 μM isotopolog mixtures of 6,7-dimethyl-8-ribityllumazine or riboflavin, respectively, and concentrated to 0.5 mL by ultrafiltration. For NMR analysis, D_2O was added to a final concentration of 5% (v/v). Samples were stored at −80 °C.

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were recorded at 20 °C using a Bruker DRX 500 spectrometer. Typically, ^1H-decoupled ^{13}C NMR spectra were recorded for 4−16 h. The transmitter frequency was 125.1 MHz. Prior to Fourier transformation, the free induction decay was multiplied with a mild Gaussian function (LB = −5; GB = 0.01). The chemical shifts were referenced to internal [1-$^{13}C_1$]glucose (95.8 ppm for the C-1 signal of the β-anomer).

Determination of ^{13}C Enrichments. ^{13}C enrichments were determined by quantitative NMR spectroscopy. For this purpose, ^{13}C NMR spectra of lumazine protein or the N-terminal domain of riboflavin synthase in complex with 6,7-dimethyl-8-ribityllumazine or riboflavin from the experiments with the selectively ^{13}C-labeled glucose specimens (i.e., with random isotope libraries) and of the respective samples in complex with totally ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine or riboflavin were measured under the same experimental conditions. The ratios of the signal integrals of the complexes with the random isotopolog libraries and of the same complexes with totally ^{13}C-labeled ligands were then calculated for each respective carbon atom. Relative ^{13}C abundances were then normalized to the known abundance for certain carbon atoms of the free ligands (cf. Tables 1−4), thus affording absolute ^{13}C abundances for every single carbon atom (% ^{13}C in Tables 1−4).

Acknowledgment. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Korea Science and Engineering Foundation (Project No. 20025-209-07-2 to C.Y.L.), the Fonds der Chemischen Industrie, and the Hans Fischer Gesellschaft e.V.

JO051662F

(27) Eberhardt, S.; Zingler, N.; Kemter, K.; Richter, G.; Cushman, M.; Bacher, A. *Eur. J. Biochem.* **2001**, *268*, 4315−4323.
(28) Petushkov, V. N.; Gibson, B. G.; Visser, A. J.; Lee, J. *Methods Enzymol.* **2000**, *305*, 164−180.