Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 5860–5863, December 1978
Biochemistry

Structure of the oxygen adduct intermediate in the bacterial luciferase reaction: ^{13}C nuclear magnetic resonance determination*

(subzero temperature/oxidation-reduction/substituted flavins/flavoproteins)

SANDRO GHISLA†, J. WOODLAND HASTINGS‡, VINCENT FAVAUDON§, AND JEAN-MARC LHOSTE§

†Fachbereich Biologie, Universität Konstanz, D-775 Konstanz, Germany; ‡Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138; and §Institut Curie, Section de Biologie, 91405 Orsay, France

Communicated by H. S. Gutowsky, September 11, 1978

ABSTRACT **By using FMN enriched in ^{13}C (90%) at position C-4a, we have conclusively shown that the reaction of molecular oxygen with bacterial luciferase-bound $FMNH_2$ forms an adduct at the 4a position. Consistent with this are ^{13}C NMR studies of FMN and other flavin compounds which show that this carbon should be unusually reactive in the reduced 1,5-dihydroflavins with respect to electrophilic attacks.**

An intermediate of the bacterial luciferase reaction, formed upon the addition of molecular oxygen to the reduced flavin mononucleotide/enzyme complex, has been isolated by chromatography at low temperature and characterized spectrally (1, 2). Spectrally related transient intermediates have also been observed in certain flavin-dependent hydroxylases (3). Absorption spectra studies of the oxidation of free 1,5-dihydroflavins by oxygen in aqueous and aprotic media have also given clear indication of the existence of a flavin/oxygen adduct (4, 5). Recent evidence (3, 6) strongly suggests a flavin C-4a peroxide structure for these adducts; however, in view of the great variability of the spectra of reduced flavins depending on substituents and environment (6, 7), the absorption spectra of the intermediates cannot be considered to be sufficiently diagnostic. The structures of these oxygen adducts are thus still disputed, and those of certain other intermediates implicated in the reaction of flavoprotein oxidases are even more speculative (8).

Although the 4a-carbon is now generally considered as the best candidate for the primary electrophilic addition of molecular oxygen, other positions, including C-6, C-8, C-9a, and C-10a, are still discussed (9, 10). The rearrangement of such primary adducts governed by apoprotein conformational changes has also been proposed (9). In this paper we present direct evidence for addition of oxygen at C-4a in the reduced flavin complexed to luciferase by using selectively enriched FMN measured by ^{13}C NMR. These experiments offered also the opportunity to investigate the conditions of application of ^{13}C NMR for the analysis of enzymatic intermediates stabilized at subzero temperatures.

MATERIALS AND METHODS

The luciferase was isolated from the luminous bacterium *Beneckea harveyi*, mutant strain M-17 (11), and purified according to Baldwin *et al.* (12). Samples for the NMR experiments were prepared in mixtures of 20% ethylene glycol-d_4 and phosphate buffer (0.3 M, pH 7.0), which permitted cooling to −20°C without freezing. The concentration of luciferase, measured by absorption using an ϵ_{280} value of 74 mM^{-1} cm^{-1} for the heterodimer (12), was in the range of 1.1–1.6 mM. The buffer was prepared with light water, but deuterated glycol was preferred because it provides a good deuterium lock and exhibits a much lower solvent signal in the ^{13}C NMR experiments. All samples were kept under argon. Aliquots of concentrated [4*a*-^{13}C]FMN and gaseous oxygen were injected through a septum cap.

FMN enriched in ^{13}C (90%) at position C-4a was prepared by condensation of [2-^{13}C]diethylmalonate (Merck, Sharp and Dohme) with urea according to the method of Grande *et al.* (13), leading to barbituric acid with yields of 85–90%. [4*a*-^{13}C]Riboflavin was then obtained by condensation of the enriched barbituric acid with 2-[4-carboxyphenylazo]-4,5-dimethyl-*N*-D-ribitylaniline (13) in 85% yield. The phosphorylation of the riboflavin was carried out by a described method (14), adapted to small scale, with average yield of 60–65% of the crude phosphorylation product. The content of this material in riboflavin 5′-phosphate was determined by fluorometric titration with apoflavodoxin (15). Pure [4*a*-^{13}C]FMN was obtained by affinity chromatography over an apoflavodoxin column (16). The enriched carbon resonance in the ^{13}C NMR spectrum appears at the same frequency as that assigned to C-4a in the complete spectrum of FMN with ^{13}C at natural abundance (see *Results*), thus confirming the correctness and the selectivity of the enriched preparation. The pseudobase 3-methyl-4a-hydroxy-5-ethyl-4a,5-dihydrolumiflavin was prepared directly from the corresponding 5-ethylflavoquinonium perchlorate (17) upon addition of HO^- in deuterated acetonitrile. The stable 4a,5-bridged pseudobase, 4a,5-epoxyethano-3-methyl(or phenyl)-4a,5-dihydrolumiflavin, was synthetized as described (18). Other oxidized flavins were from commercial sources (Fluka, Buchs, Switzerland); riboflavin was acetylated according to McCormick (19). Reductions in aqueous media were carried out by titration with dithionite, but catalytic reduction by hydrogen on palladium was used for the solutions in organic solvents. 3-Methyl-4a-benzyl-4a,5-dihydrolumiflavin and 5-deazaflavins were generous gifts from P. Hemmerich, University of Constance. 5-Deazariboflavin and 5-deaza-7,8-nor-3-propanesulfonatelumiflavin were reduced by dithionite with a trace of borohydride in order to complete the reduction. All compounds were checked by proton magnetic resonance.

The ^{13}C NMR experiments were carried out in 10-mm sample tubes by using a Varian XL-100 spectrometer operated in the Fourier transform mode at 25 MHz and equipped with a calibrated variable temperature accessory. Wide band proton noise decoupling was generally used along with a 90° (45 μs) or 30° excitation pulse for the solutions with or without protein, respectively. The severe line broadening in the low temperature experiments with luciferase-bound flavins was compensated by the isotopic enrichment at C-4a. The spectral accumulation for these samples, as well as for the flavin models with ^{13}C at

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* This work was presented at the Sixth International Symposium on Flavins and Flavoproteins, Kobe, Japan, Mar. 13–17, 1978.

natural abundance, was generally achieved in 2–4 hr (10,000–20,000 transients). Chemical shifts in aqueous solvents are referred to the methyl carbon resonance of trimethylsilylpropanesulfonate-d_4 (four deuteriums in propane). The reference in organic solvents is tetramethylsilane.

RESULTS

Luciferase bound flavins

When oxidized [4a-^{13}C]FMN is added stoichiometrically to the luciferase in solution in phosphate buffer, the resonance of the enriched carbon appears at 137 ppm superimposed on the absorption background of the protein carbons. This line shifts upfield by 34 ppm (Fig. 1, IIa) upon reduction with dithionite, monitored by the loss of fluorescence of the oxidized form. In spite of the low affinity of luciferase for oxidized FMN [$K_a = 3 \times 10^3\ M^{-1}$ (12)], tight binding, with no contribution to the linewidth from exchange reaction at the NMR time scale, can be assumed because the linewidth in the oxidized complex is identical ($\Delta\nu_{1/2} = 10$ Hz at 25°C) to that in the strongly bound reduced complex [$K_a = 1.7 \times 10^6\ M^{-1}$ (20)]. Because of the dipolar contribution to the linewidths, which is inversely proportional to the sixth power of the carbon-hydrogen distance (21), only the quaternary carbons can exhibit in proteins relatively narrow resonances, except for groups having a large internal mobility (22). We observed for [4*a*-^{13}C]FMN bound to flavodoxin apoproteins (from *Desulfovibrio vulgaris* and *D. gigas*) at 30°C in phosphate buffer (pH 9) a linewidth of the proton-decoupled resonance of the enriched carbon-13 as narrow as 1.5–2 Hz in both the oxidized and the fully reduced forms. This linewidth compares favorably with that observed in the luciferase complex in phosphate buffer (Fig. 1, IIa). They are roughly in the ratio of the molecular weights of the proteins (16,000 and 79,000, respectively)—i.e., of the isotropic rotational correlation times of rigid molecules. This indicates the absence of noticeable internal motion of the quaternary carbon and no limitation of the overall tumbling rate by protein aggregation.

We used a slight stoichiometric excess of [4*a*-^{13}C]FMN for experiments carried out with luciferase in cryogenic ethylene glycol/aqeuous buffer in order to compare the behavior of free and bound FMN (Fig. 1). In fact the line at 137 ppm in the oxidized complex is then the superimposition of a narrow component, corresponding to the excess of free FMN, and of a broad component for the protein complex (Fig. 1, I). After reduction (IIb) the two lines are no longer superimposed but are separated by approximately 1 ppm. At pH 7 the free FMN is only partly anionic ($pK_a \simeq 6.7$) and a more complete ionization in the complex could explain this difference because a high field shift is observed for this carbon resonance upon alkaline titration of reduced FMN. Long time signal averaging could not be used with the cryogenic solutions at room temperature because of the slow denaturating effect of the added glycol. The estimated doubling of the linewidth upon addition of glycol at 25°C (IIb), as well as the temperature dependence (IIc), is, however, easily explained by the increasing viscosity of the solvent (23). It should be noticed however that the linewidth of the C-4a resonance in bound FMN does not broaden significantly below 0°C. This suggests that the tumbling rate of the whole luciferase complex does not control any longer the linewidth (probably because of molecular association), but rather that microscopic motions less sensitive to the solvent viscosity are then responsible for the spin-spin relaxation times.

When oxygen was added to the reduced solution at −15°C (Fig. 1, III) the C-4a resonances at 103 and 104 ppm disappeared. A narrow line of reoxidized free FMN reappeared at

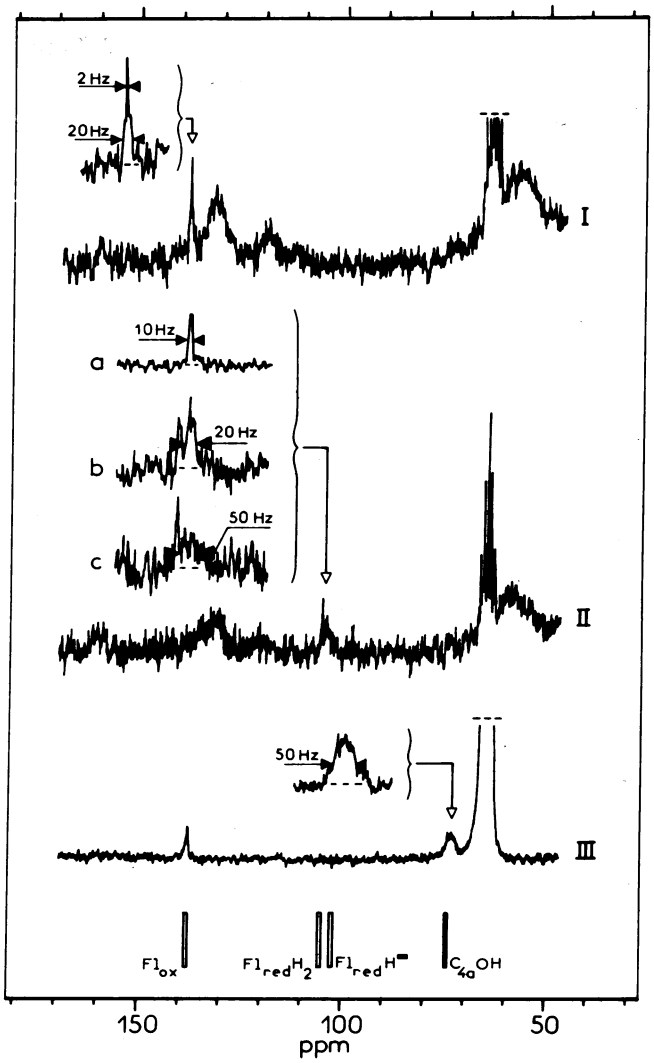

FIG. 1. Carbon-13 NMR spectra of the bacterial luciferase complex with FMN isotopically enriched (90%) at position C-4a. The spectra of the oxidized (I) and fully reduced (II) complexes were recorded at 25°C in a solvent mixture of ethylene glycol-d_4 (20%) and 0.3 M aqueous phosphate buffer (pH 7) in the presence of a slight excess of free FMN. The protein concentration was 1.2 mM. Molecular oxygen was added to the reduced system at −15°C before spectrum III was recorded; at that temperature the protein background absorption was broadened beyond observation and the resolution of the deuterated solvent peak at 64.5 ppm was lost. Four-fold expansions of the spectral region corresponding to the C-4a resonances are presented for the oxidized system at 25°C (I), for the reduced system at 25°C (IIb) and 0°C (IIc), and for the oxygen-containing sample at −15°C (III), as well as for a spectrum of the reduced complex obtained in the absence of an excess of free FMN and recorded at 25°C in phosphate buffer without ethylene glycol (IIa). The NMR spectra were recorded at 25 MHz in the Fourier transform mode by using broad band noise decoupling of the protons, 90° (45 μs) pulses, and accumulation of 10,000–20,000 (chemically stable systems at low temperature or in the absence of ethylene glycol) transients altogether in an 0.8-sec acquisition time.

137 ppm and a new resonance appeared at a rather high field value (74 ppm) having a linewidth (50–60 Hz) comparable to that of the "bound" C-4a resonance in the reduced complex observed at the same temperature.

When the sample was subsequently warmed up to room temperature for a time sufficient for complete reformation of oxidized FMN, this broad resonance disappeared irreversibly. The resonance at 137 ppm corresponding to FMN could then be observed at room or at low temperature. Actually the signal

at 74 ppm did not disappear completely when the samples were kept at room temperature for only a few minutes. This is attributed to the fact that at high concentrations of buffer and luciferase the intermediate is far more stable than in the more dilute conditions previously used in characterizing the lifetime (1, 2). The same experiment was repeated three times with the same results, using different luciferase samples. None of the peaks assigned to C-4a was observed in experiments with luciferase using FMN with ^{13}C at natural abundance.

Flavin models

Fig. 2 presents the chemical shift values for the ^{13}C NMR spectra at natural abundance of flavin models selected from a large series of oxidized flavins and reduced 1,5- and 4a,5-dihydroflavins which will be reported in detail elsewhere. Tetraacetylriboflavin and its 1,5-dihydro form are presented instead of FMN because their higher solubility in dimethyl sulfoxide permits easier recording of the proton-coupled spectra. FMN in heavy water exhibits similar ^{13}C chemical shifts for both the oxidized and reduced states, but the ionic state has to be controlled carefully because of the near neutral pK_a value of the reduced flavin. The assignment of the quaternary carbons of the flavin ring is straightforward according to the proton-coupled spectra. Due to the preeminence of the three-bond couplings ($^3J_{CH}$ = 7–12 Hz) over the two-bond couplings ($^2J_{CH}$ < 3–5 Hz) in conjugated cyclic and heterocyclic compounds, the resonances of C-4a, C-5a, C-9a, and C-10a appear, respectively, as a singlet, a doublet, a doublet of triplets, and a triplet when all exhangeable protons are substituted by deuterium ions because of resolved couplings with H-6, H-9, and CH_2-1′. C-7 and C-8 exhibit a complex, nonresolved structure because of the presence of substituted methyls. These assignments are further confirmed by the spin-lattice relaxation times as measured by the inversion-recovery method (24). The corresponding values for the quaternary carbons of 3-methyltetraacetylriboflavin in deuterated and degassed chloroform are spread in a time range extending from 7 sec (C-7 and C-8) to 36 sec (C-4a). These and the other values (C-2, 20 sec; C-4, 25 sec; C-5a, 12 sec; C-9a, 11 sec; C-10a, 15 sec) are in good agreement with the r^{-6} dependence on the distance between these centers and the various protons of the isoalloxazine ring and of the ribityl side chain. Such assignments can be extended to other substituted flavins (Fig. 2) by introducing the coupling constants expected for the protons introduced upon substitution. A previous report on oxidized riboflavin, FMN, and FAD by Breitmaier and Voelter (25) is partly erroneous, as already noted by Grande *et al.* (13) and by Yagi *et al.* (26), who assigned the pyrimidine ring carbons by selective ^{13}C enrichment. Our assignments, although not requiring extensive chemical synthesis of enriched model compounds, agree with the last two publications. A complete assignment of the 3-methyl-4a-hydroxy-5-ethyl-4a,5-dihydroflavin pseudobase (Fig. 2) spectrum could not be carried out because of the chemical instability of the system at the concentration used for the NMR experiments and the rapid formation of flavin radicals that broadened the ^{13}C resonance (already noticeable when 0.01% of the radical is present) due to fast electron exchange.

The dramatic upfield shift observed for the C-4a carbon resonance upon reduction and substitution was confirmed directly by using the [4a-^{13}C]FMN compound in which a single resonance was clearly observed. Indeed, the upfield shift observed in any flavin compound upon reduction to the 1,5-dihydro form is at least twice that measured for the other carbons of the isoalloxazine ring. Such a behavior was also observed with 5-deazaflavins in which the C-4a resonance shifts upon reduction from 113.7 to 83.5 ppm for 5-deazariboflavin in dimethyl sulfoxide-d_6, and from 115.2 to 86.2 ppm for the water-soluble 3-propanesulfonate derivative.

DISCUSSION

The behavior of the C-4a resonance in the flavin models illustrates the possibilities offered by ^{13}C NMR for the analysis of biochemical reaction mechanisms involving substitution of a prosthetic group or large changes in charge densities such as those observed in oxidation–reduction reactions. The large upfield shift (33 ppm) observed for that resonance upon two-electron reduction without substitution of the corresponding atom is rather unusual, even in heterocyclic systems. It indicates a large increase in π-electron charge on that atom and probably a change in conformation to a more sp^3-type orbital configuration. By contrast, the C-10a resonance exhibits a much smaller shift (*ca.* 12 ppm), comparable to those of the benzenoid ring carbons. Such a shift can be attributed simply to the hydrogenation of N-1. As expected, the charge distribution in the benzenoid ring appears much more symmetrically distributed after

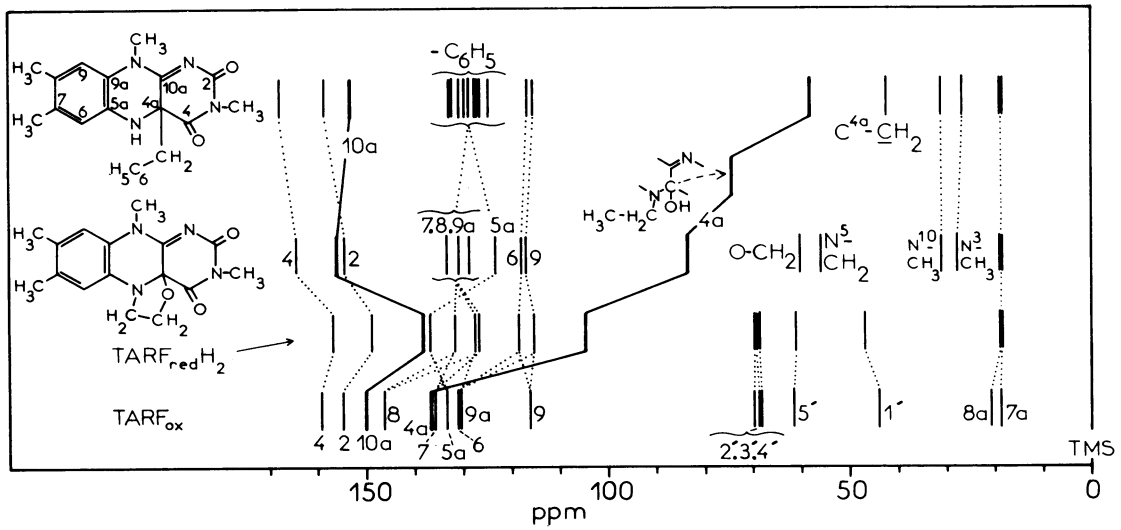

FIG. 2. Correlation diagram of the ^{13}C NMR chemical shifts of the oxidized, reduced, and substituted flavin derivatives: tetraacetylriboflavin (TARF), 1,5-dihydrotetraacetylriboflavin, 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin (and the C-4a resonance in the open pseudobase analog), and 4a-benzyl-3-methyl-4a,5-dihydrolumiflavin in dimethyl sulfoxide-d_6 (50–100 mM). The corresponding spectra were recorded at 25 MHz and assigned as described in the text by using the proton-coupled spectra. TMS, trimethylsilane.

reduction. Using the generally accepted relationship of 160 ppm ^{13}C chemical shift per added π-electron in conjugated systems (27), the shifts observed upon reduction of flavins correspond to the addition of one-fourth of an electron in this ring, mainly at C-6 and C-8 as already well established for the spin distribution in the semiquinone radical (28, 29). The highest electron density after reduction should occur at the N-5/C-4a region, as also shown by observations of the ^{15}N NMR shifts (30). This large and localized variation of charge upon reduction is however also observed in the 5-deazaflavins.

Direct substitution of C-4a, corresponding to the formation of 4a,5-dihydroflavins, further shifts the resonance to the range of aliphatic carbons with differences associated with the electronegativity of the substituent groups. A small difference between the open and the bridged pseudobases can be interpreted in terms of conformation, with a more tetrahedral configuration of the C-4a atom in the open compound due to the release of intramolecular strain.

The resonance that appears at 74 ppm upon addition of oxygen can be confidently assigned to the enriched C-4a carbon of FMN. It exhibits the same linewidth as in the reduced form at the same temperature. Other sources of ^{13}C could hardly result in such a signal; the protein does not absorb in this spectral range and the possibility of a modified solvent peak or of a spinning side-band from the main peak of the deuterated ethylene glycol was eliminated because there is no symmetrical absorption with respect to the main peak and there is no absorption in that range at any other stage of the experiment or in control experiments without enriched FMN. Direct evidence that this resonance is that of C-4a in the oxygen adduct is provided by the integration of the NMR signals in the experiments carried out with a slight excess of [4*a*-^{13}C]FMN. The ratio of the integrated intensity of the 74-ppm signal to that of the 137-ppm signal (corresponding to reoxidized, free FMN) is equal to the ratio of the signals of bound and free reduced FMN before addition of oxygen. The oxygenated intermediate could indeed be observed by optical absorption (1) in these samples.

It is quite remarkable that the chemical shift value for C-4a in the oxygenated complex is within 1 or 2 ppm of that observed for this carbon in the open pseudobase (Fig. 2). C-4a is substituted by a hydroxyl group in this compound. This is direct evidence that an oxygen atom is directly bound to C-4a in the intermediate, in good agreement with the postulated hydroperoxide structure (1, 8, 9, 31). The exact nature and ionic state of the oxygen-linked substituent as well as substitution of N-5 in the model compound should have an effect on the C-4a shift value, but this effect should be necessarily limited and cannot be predicted at the moment. However, the position of substitution, which is now clearly established to be at C-4a, was more questionable (9) than the nature of the substituent.

In conclusion, the study of flavins in solution already shows that the C-4a carbon in the reduced 1,5-dihydroflavins should be unusually reactive with respect to electrophilic attacks. No other position among the quaternary or proton-substituted carbons of the dihydroisoalloxazine system carries such a large electron density. Thus, this property appears to be inherent to the 1,5-dihydroflavin system and might be at the origin of its catalytic properties. The present results conclusively demonstrate that the oxygen-containing intermediate of the luciferase reaction is a C-4a substituted adduct of reduced FMN. Because of the spectral similarities, the intermediates observed by Entsch and coworkers (3, 6) should have the same structure and this may be of general significance for other flavoprotein oxidases and monooxygenases.

We are grateful to Prof. P. Hemmerich for providing some of the compounds and to Dr. C. Balny for helpful discussions. This research was supported in part by grants from the North Atlantic Treaty Organization (to S.G. and J.W.H.), Institut National de la Santé et de la Recherche Médicale (to V.F. and J.M.L.), and the National Science Foundation (Grants 74-23651 and 77-19917 to J.W.H.).

1. Hastings, J. W., Balny, C., Le Peuch, C. & Douzou, P. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3468–3472.
2. Becvar, J. E., Tu, S.-C. & Hastings, J. W. (1978) *Biochemistry* **17**, 1807–1812.
3. Entsch, B., Ballou, D. P. & Massey, V. (1976) *J. Biol. Chem.* **251**, 2550–2563.
4. Kemal, C. & Bruice, T. C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 995–999.
5. Favaudon, V. (1977) *Eur. J. Biochem.* **78**, 293–307.
6. Ghisla, S., Entsch, B., Massey, V. & Husain, M. (1977) *Eur. J. Biochem.* **76**, 139–148.
7. Ghisla, S., Massey, V., Lhoste, J. M. & Mayhew, S. G. (1974) *Biochemistry* **13**, 589–597.
8. Massey, V. & Hemmerich, P. (1975) in *The Enzymes*, ed. Boyer, P. (Academic, New York), 3rd Ed., Vol. 12, pp. 191–252.
9. Hemmerich, P. & Wessiak, A. (1976) in *Flavins and Flavoproteins*, ed. Singer, T. P. (Elsevier, Amsterdam), pp. 9–22.
10. Mager, H. I. X. (1977) *Tetrahedron* **33**, 981–989.
11. Ulitzur, S. & Hastings, J. W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 266–269.
12. Baldwin, T. O., Nicoli, M. Z., Becvar, J. E. & Hastings, J. W. (1975) *J. Biol. Chem.* **250**, 2763–2768.
13. Grande, H. J., Gast, R., van Schagen, C. G., van Berkel, W. J. H. & Müller, F. (1977) *Helv. Chim. Acta* **60**, 367–379.
14. Scola-Nagelschneider, G. & Hemmerich, P. (1976) *Eur. J. Biochem.* **66**, 567–577.
15. Mayhew, S. G. (1971) *Biochim. Biophys. Acta* **235**, 289–302.
16. Mayhew, S. G. & Strating, M. J. J. (1975) *Eur. J. Biochem.* **59**, 539–544.
17. Ghisla, S., Hartman, U., Hemmerich, P. & Müller, F. (1973) *Ann. Chem.* 1388–1415.
18. Bolognesi, M., Ghisla, S. & Incoccia, L. (1978) *Acta Crystallogr. B34*, 821–828.
19. McCormick, D. B. (1970) *J. Heterocycl. Chem.* **7**, 447–450.
20. Meighen, E. A. & Hastings, J. W. (1971) *J. Biol. Chem.* **246**, 7666–7674.
21. Kuhlmann, K. R., Grant, D. M. & Harris, R. K. (1970) *J. Chem. Phys.* **52**, 3439–3448.
22. Allerhand, A., Childers, R. F. & Oldfield, E. (1973) *Biochemistry* **12**, 1335–1341.
23. Douzou, P. (1977) *Cryobiochemistry* (Academic, London), p. 27.
24. Freeman, R. & Hill, H. D. W. (1970) *J. Chem. Phys.* **53**, 4103–4105.
25. Breitmaier, E. & Voelter, W. (1972) *Eur. J. Biochem.* **31**, 234–238.
26. Yagi, K., Ohishi, N., Takai, A., Kawano, K. & Kyogoku, Y. (1976) in *Flavins and Flavoproteins*, ed. Singer, T. P. (Elsevier, Amsterdam), pp. 775–781.
27. Stothers, J. B. (1972) *Carbon-13 NMR Spectroscopy* (Academic, New York), pp. 90–97.
28. Ehrenberg, A., Müller, F. & Hemmerich, P. (1967) *Eur. J. Biochem.* **2**, 286–293.
29. Walker, W. H., Ehrenberg, A. & Lhoste, J. M. (1970) *Biochim. Biophys. Acta* **215**, 166–175.
30. Kawano, K., Ohishi, N., Takai, A., Suzuki, A., Kyogoku, Y. & Yagi, K. (1978) *Biochemistry* **17**, 3854–3859.
31. Hastings, J. W. & Balny, C. (1975) *J. Biol. Chem.* **250**, 7288–7293.