Structure of the Functional Part of Photoprotein Aequorin

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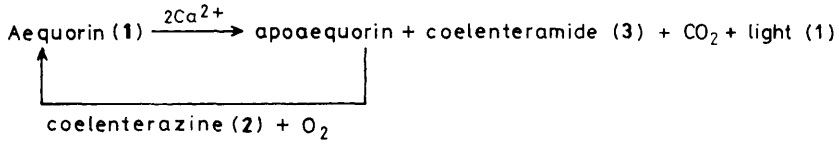
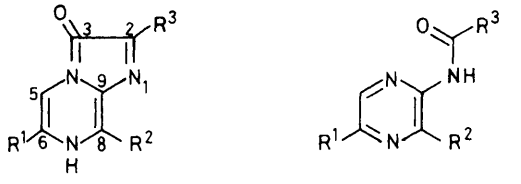
The structure of the functional part of photoprotein aequorin is discussed based on the ^{13}C n.m.r. spectra of aequorins prepared by incubation of specifically ^{13}C-enriched coelenterazines into apoaequorin in the presence of $^{16}O_2$ and $^{18}O_2$, respectively.

The photoprotein aequorin (molecular weight: *ca.* 20,000), isolated from bioluminescent jellyfish Aequorea, emits blue light in aqueous solution when Ca^{2+} or Sr^{2+} is added in either the presence or absence of molecular oxygen. This process has been shown to involve the chemical changes shown by equation (1). Active aequorin can be regenerated by incuba-

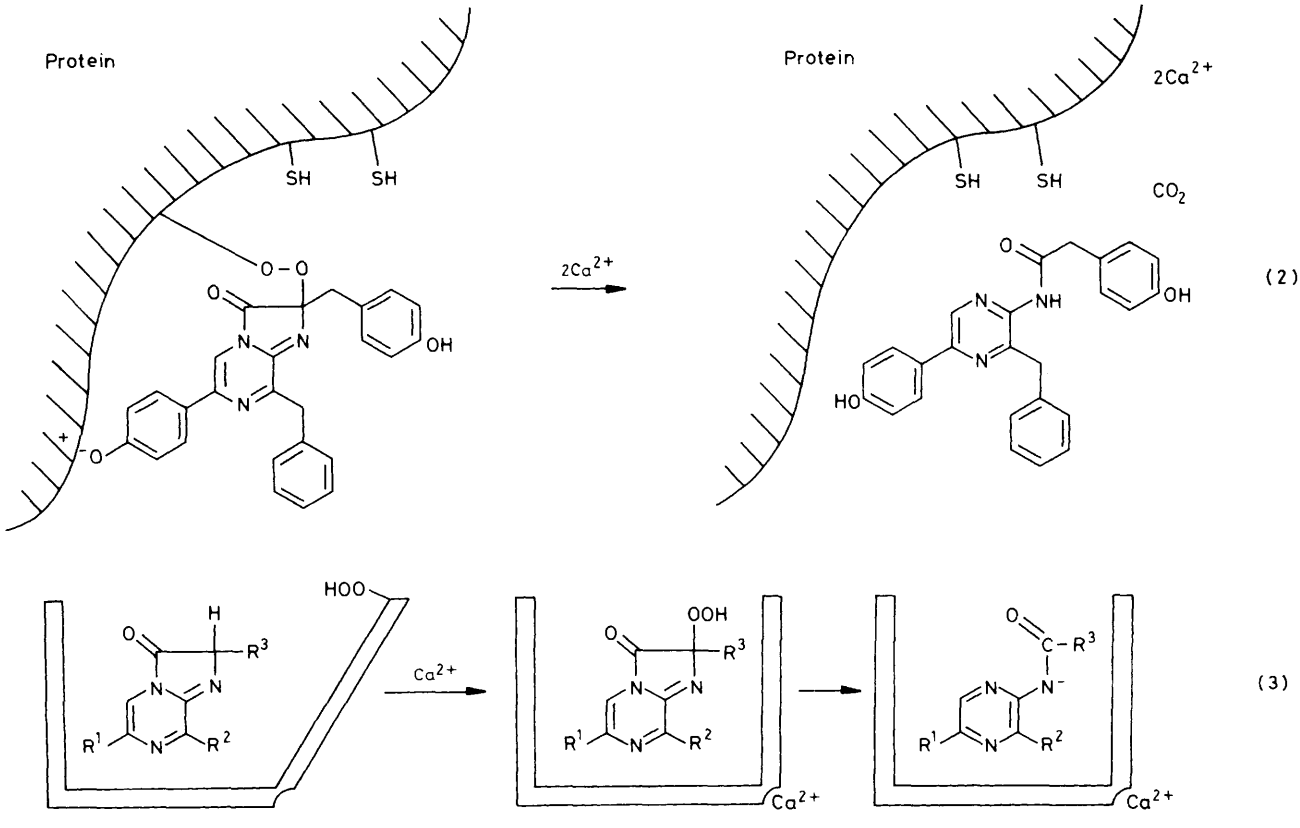




(**2**) coelenterazine (**3**) coelenteramide

$R^1 = p\text{-}C_6H_4OH$, $R^2 = CH_2Ph$, $R^3 = p\text{-}CH_2C_6H_4OH$

a; doubly ^{13}C-enriched at the C-2 and C-9 positions
b; singly ^{13}C-enriched at the C-2 position
c; singly ^{13}C-enriched at the C-9 position



Scheme 1. Equation (2) is the proposal by Shimomura and Johnson and equation (3) is the structure proposed by Cormier *et al.*

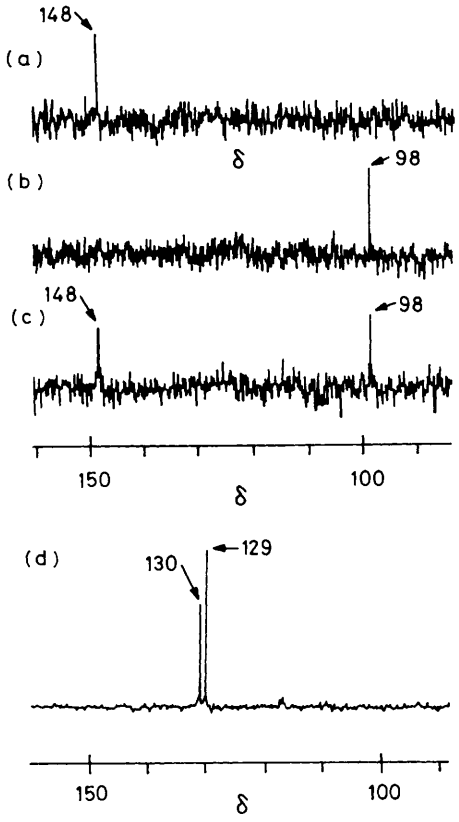


Figure 1. ^{13}C N.m.r. spectra of ^{13}C-enriched aequorins and coelenterazine. (a) Aequorin (**1c**) (16 mg) in 2.2 ml of 10 mM KH_2PO_4 buffer (pH 7.2) made with 50% D_2O containing 0.2 mM edta. The spectrum was recorded on a Bruker 300WB spectrometer equipped with a wide-bore probe at 10 °C overnight; (b) aequorin (**1b**) (18 mg) in 2.2 ml of the buffer; (c) aequorin (**1a**) (12 mg) in 2.2 ml of the buffer; (d) coelenterazine (**2a**) (1.5 mg) in 2.2 ml of a 1 : 1 mixture of CD_3OD and the buffer.

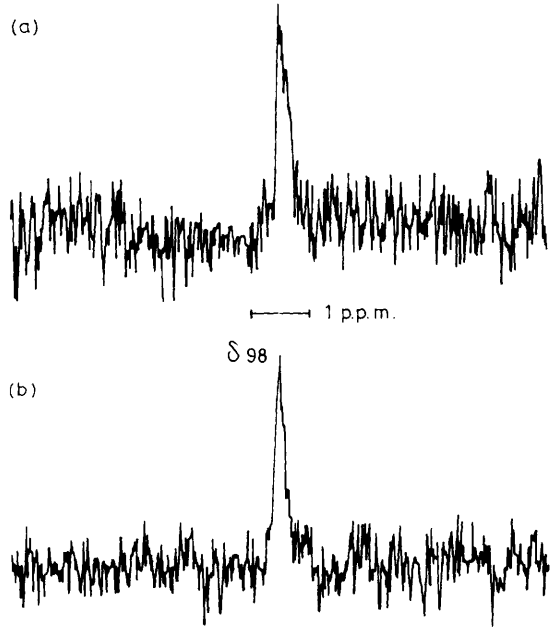


Figure 2. ^{13}C N.m.r. spectra of [^{18}O]- and [^{16}O]-aequorins. (a) A 1 : 2 mixture of [^{18}O]- and [^{16}O]-aequorins prepared from coelenterazine ^{13}C-enriched specifically at the C-2 position. For the conditions of n.m.r. measurements, see Figure 1. (b) [^{16}O]-aequorin prepared from coelenterazine ^{13}C enriched specifically at the C-2 position.

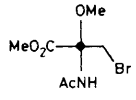
tion of apoaequorin with coelenterazine (**2**) in the presence of molecular oxygen.[1] Based on the u.v.–visible absorption spectrum and the degradation products obtained by treatment with $NaHSO_3$ or $Na_2S_2O_4$, Shimomura and Johnson suggested the structure of the functional part of aequorin and its luminescent reaction as shown in Scheme 1, equation (2).[2] Contrary to Shimomura's results, Cormier and his co-workers have proposed the noncovalently bound structure for aequorin [Scheme 1, equation (3)].[3] In this communication, we present evidence which establishes the functional part of aequorin.

Using $Na^{13}CN$ as the ^{13}C-source, three specifically ^{13}C-enriched coelenterazines (**2a—c**) were synthesized by a slight modification of the published method[4] and then incorporated into apoaequorin to yield three ^{13}C-enriched aequorins (**1a—c**).[5] The ^{13}C n.m.r. spectra of (**1a—c**) and (**2a—c**) were recorded using a Bruker 300WB spectrometer equipped with a wide-bore probe at 10 °C in 10 mM KH_2PO_4 buffer made with 50% D_2O containing 0.2 mM ethylenediaminetetra-acetic acid (edta) and the pH adjusted to 7.2 with 2 M NaOH. The stability of aequorin under the n.m.r. conditions was confirmed by comparing the bioluminescent activity before and after the n.m.r. measurements.

The completely proton-decoupled ^{13}C n.m.r. spectra of (**1a—c**) and (**2a**) are shown in Figure 1. During the incubation of coelenterazine (**2a**) into apoaequorin to form aequorin (**1a**), the C-2 carbon atom undergoes a hybridizational change from sp^2 to sp^3 in either Shimomura's partial structure or Cormier's, so that the chemical shift of this carbon of aequorin must be shifted upfield, compared with that of coelenterazine (**2a**).† In fact, the result is consistent with this expectation. Since the chemical shift of –CO–*C*(H)(NH–)R usually occurs in the range δ_C 40—60[7] and that of –CO–*C*(OR^1)(NH–)R in the range 80—100,‡ our n.m.r. experiments support Shimomura's partial structure.§ In order to obtain more direct evidence for this, an experiment using $^{18}O_2$ was carried out.

† Chemical shifts for carbon atoms similar to C-9 in (**1**) are known to occur around δ_C 145. For examples, see ref. 6.

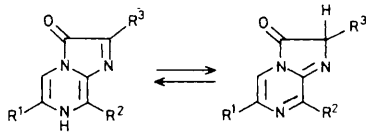
‡ Several examples for the chemical shift of this type of functional group are known in the indole alkaloid area. For example, see N. Neuss, H. E. Boaz, J. L. Occolowitz, E. Wenkert, F. M. Schell, P. Potier, L. Kan, M. M. Plat, and M. Plat, *Helv. Chim. Acta*, 1973, **56**, 2660. In addition, the synthetic model compound (**4**) showed a chemical shift at δ_C 87.



(**4**)

§ The possibility that tautomerization between (**5**) and (**6**) occurs faster than the n.m.r. time scale cannot be excluded. However, even if this is the case, the equilibrium between (**5**) and (**6**) in aequorin must be heavily oriented toward (**6**) since the u.v.–visible absorption corresponding to (**5**) is not detected in the spectra of aequorin (*cf.* the u.v. time scale). Consequently, the observed chemical shift of the C-2 carbon atom must be very close to the real value.

The off-resonance ^{13}C n.m.r. spectra of aequorins derived from coelenterazines (**1a**) and (**1b**) showed no splitting for the signal of the C-2 carbon atom. These experiments exclude the possibility of Cormier's partial structure, unless rapid tautomerization between (**5**) and (**6**) exists.



(**5**) (**6**)

The ^{13}C n.m.r. spectrum of a 1:2 mixture¶ of aequorins obtained by incubation of (**2b**) in the presence of $^{18}O_2$ and $^{16}O_2$, respectively, was compared with that of (**1b**) (Figure 2). As expected, the C-2 signal in the ^{13}C n.m.r. spectrum of the 1:2 mixture is broader than the corresponding signal of (**1b**). Furthermore, a shoulder is apparent approximately 0.07 p.p.m. upfield from the peak at δ_C 98. The signal is rather broad ($w_{\frac{1}{2}}$ *ca.* 12 Hz on a 75 MHz instrument) probably because of the nitrogen atom. It is tempting to attribute this shoulder to the ^{18}O isotope effect, although the chemical shift difference observed is slightly larger than known cases.[8]

Based on the experiments reported here, there is little doubt that an oxygen atom originating from molecular oxygen is attached to the C-2 carbon of aequorin. Furthermore, taking into account the observations made by Shimomura and Johnson,[2] it seems evident that this oxygen must exist as a peroxide or hydroperoxide group.

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¶ This experiment was carried out by first recording a spectrum of $[^{16}O_2]$-aequorin and then recording a spectrum of $[^{16}O_2]$-aequorin diluted with $[^{18}O_2]$-aequorin. A 1:1 mixture of $[^{18}O_2]$- and $[^{16}O_2]$-aequorins also gave a spectrum consistent with this explanation.

References

1 For reviews see: T. Goto, 'Marine Natural Products,' vol. 3, ed. P. J. Scheuer, Academic Press, New York, 1980, pp. 179–222; O. Shimomura, 'Chemical and Biological Generation of Excited States,' ed. W. Adams and G. Cilento, Academic Press, New York, 1982, pp. 249—256; O. Shimomura, 'Natural Products and Biological Activities,' ed. H. Imura, T. Goto, T. Murachi, and T. Nakajima, University of Tokyo Press, Tokyo, 1986, pp. 33—42.

2 O. Shimomura and F. H. Johnson, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 2611.

3 K. Hori, J. M. Anderson, W. W. Ward, and M. J. Cormier, *Biochemistry*, 1975, **14**, 2371; for the latest publication on this subject from Cormier and his co-workers, see D. Prasher, R. O. McCann, and M. J. Cormier, *Biochem. Biophys. Res. Commun.*, 1985, **126**, 1259.

4 For the synthesis of coelenteramine, see Y. Kishi, H. Tanino, and T. Goto, *Tetrahedron Lett.*, 1972, 2747; for the synthesis of coelenterazine from coelenteramine, see S. Inoue, S. Sugriura, H. Kakoi, T. Hashizume, T. Goto, and H. Iio, *Chem. Lett.*, 1975, 141.

5 O. Shimomura and F. H. Johnson, *Nature*, 1975, **256**, 236; O. Shimomura and A. Shimomura, *Biochem. J.*, 1981, **199**, 825.

6 R. J. Pugmire, J. C. Smith, D. M. Grant, B. Stanovnik, and M. Tisler, *J. Heterocycl. Chem.*, 1976, **13**, 1057; M-T. Chenon, R. J. Pugmire, D. M. Grant, R. P. Panzica, and L. B. Townsend, *J. Am. Chem. Soc.*, 1975, **97**, 4627.

7 For example, see C. Grathwohl and K. Wuthrich, *J. Magn. Reson.*, 1974, **13**, 217; W. Voelter, G. Jung, E. Breitmaier, and E. Bayer, *Z. Naturforsch., Teil B*, 1971, **26**, 213; W. Voelter, St. Fuchs, R. H. Seuffer, and K. Zech, *Montash. Chem.*, 1974, **105**, 1110.

8 For example, see J. M. Risley and R. L. van Etten, *J. Am. Chem. Soc.*, 1980, **102**, 6699; J. Diakur, T. T. Nakashima, and J. C. Vederas, *Can. J. Chem.*, 1980, **58**, 1311.