Shining the light: the mechanism of the bioluminescence reaction of calcium-binding photoproteins

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The Ca^{2+}-binding photoproteins from jellyfish have the unique ability to emit blue light in the presence of calcium ions but without molecular oxygen or any other cofactor. Although there is no crystallographic data on the structure of the photoprotein complex, structure–activity studies have elucidated many features of the complex and many aspects of the mechanism of the bioluminescence reaction.

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Introduction

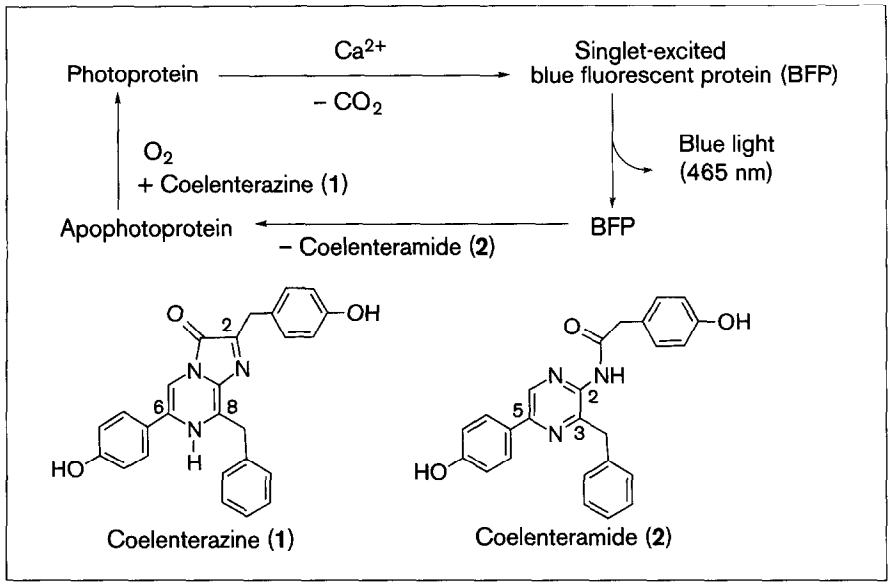
The first calcium-activated photoprotein was extracted and purified from the jellyfish *Aequorea* (Fig. 1), and named aequorin after the genus name [1]. Photoproteins that have since been isolated, such as obelin [2], thalassicolin [3], mitrocomin [4], clytin [5], mnemiopsin and berovin [6,7] are also named after the genus that produces them. The photoproteins consist of an apoprotein and a non-covalently-bound chromophore, generated by a combination of a coelenterate-type luciferin, coelenterazine (compound **1**), and molecular oxygen. In the absence of additional molecular oxygen, the binding of calcium ions triggers the photoprotein to emit visible light. Calcium binding results in an intramolecular reaction in which coelenterazine is oxidized to coelenteramide (compound **2**), yielding light ($\lambda_{max} \approx$ 465 nm), CO_2 and blue fluorescent protein (BFP), which consists of coelenteramide non-covalently bound to apoprotein (Fig. 2) [8]. The emission of light is caused by the decay of the bound coelenteramide from an excited state. BFP can be dissociated into apoprotein and coelenteramide by gel filtration or by treatment with ether [9], and the photoprotein can be regenerated by incubating apoprotein with coelenterazine in the presence of dissolved oxygen [10]. The molecular oxygen has a structural role in formation of the complex between coelenterazine and apoprotein and, upon binding calcium to the apoprotein, is consumed in the oxidation reaction.

The bioluminescent system of photoproteins offers a useful tool for detecting the location and concentration of calcium ions in real time [11,12]. Photoproteins are also useful models for the study of ion–protein interactions. The three-dimensional structure of an apophotoprotein or

Figure 1

The jellyfish, *Aequorea victoria*.

Figure 2



Bioluminescence and regeneration of calcium-binding photoproteins. The photoprotein is a complex of coelenterazine (compound **1**), apophotoprotein and molecular oxygen. Binding of calcium triggers the bioluminescence reaction, producing singlet-excited coelenteramide. The complex of coelenteramide and apoprotein is called blue fluorescent protein (BFP). Light is emitted during decay of the excited molecule to the ground state. Coelenteramide can be removed from BFP by gel filtration or treatment with ether, regenerating the apoprotein.

photoprotein complex has not yet been determined. Here we will review the mechanism of the bioluminescence reaction and the features of the apoprotein active site that have been deciphered through detailed structure–activity studies of both the apoprotein and the chromophore.

Chemistry of coelenterazine

Emission of light results from a reaction of coelenterazine with oxygen. The coelenterazines obtained from luminous jellyfish are derivatives of 3,7-dihydroimidazo[1,2-a]pyrazin-3-one. These derivatives are common substrates in luminous organisms such as the marine crustacean colloquially known as Umi-botaru [13] and the luminous squid Watasenia (also known as Hotaru-ika) [14]. These substrates have an imidazopyrazinone ring, and may be synthesized in the organism from three amino acid residues [15]. In the laboratory, coelenterazine and its analogues are usually prepared by condensation of an aminopyrazine derivative with a glyoxal derivative under acidic conditions [16]. Coelenterazine and its analogues are unstable in aprotic solvents, such as dimethyl sulfoxide (DMSO) or dimethylformamide (DMF), where they chemiluminesce due to the reaction with oxygen.

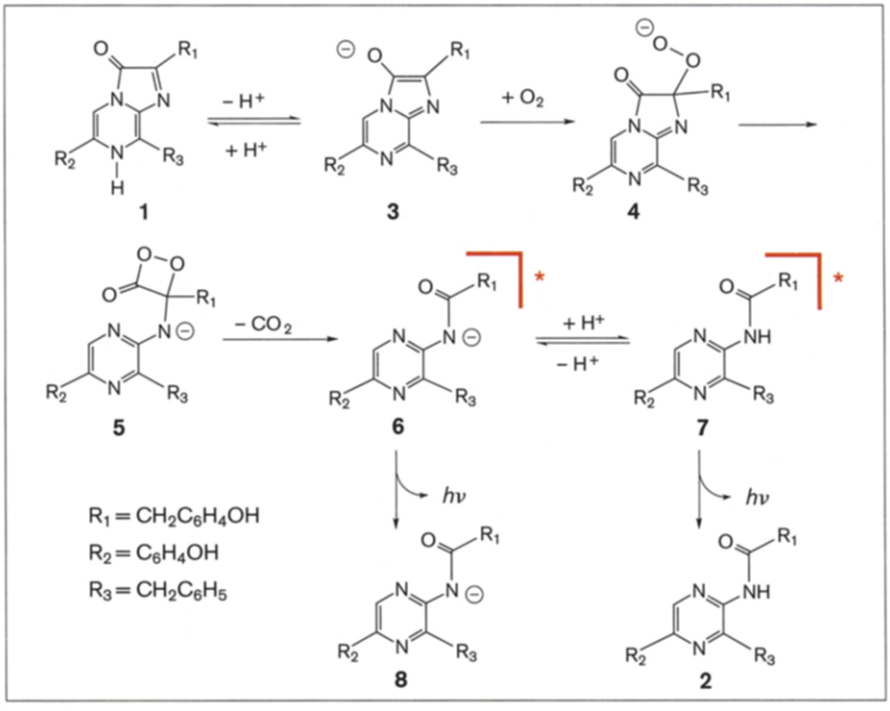
The proposed pathway of the chemiluminescence reaction of coelenterazine is shown in Figure 3 [17,18], and it is assumed that the bioluminescence reaction follows the same mechanism. The products of the chemi- and bioluminescence reactions of coelenterazine are coelenteramide and CO_2. A proton dissociates from the N7 position of coelenterazine, giving anion **3**. This species reacts with oxygen at the C2 position, producing peroxide anion **4**, which cyclizes to give the dioxetanone intermediate **5**. The key step in producing a light-emitting species is the retro[2+2]cyclization reaction of **5**, generating a singlet-excited state of amide anion **6**. The singlet-excited amide anion **6** emits light or is protonated to give a singlet-excited state of neutral coelenteramide **7**, which also emits light.

There is considerable experimental evidence supporting this proposed mechanism. An imidazopyrazinone derivative of coelenterazine was shown to have a pK_a value of 7.6 in water and a peak oxidation potential at 0.4 V relative to a saturated calomel electrode in DMSO [19], suggesting that coelenterazine reacts with molecular oxygen after deprotonation. The chemiluminescence reaction of the imidazopyrazinone derivative under $^{18}O_2$ gave CO_2 containing one ^{18}O atom, indicating that the molecular oxygen participates in the chemiluminescence reaction [20]. The structure of the imidazolone moiety of the imidazopyrazinone ring is such that it reacts easily with molecular oxygen, producing the dioxetanone intermediate.

To establish the structures of the intermediates **4** and **5** in the chemiluminescence reaction, 2-hydroperoxide **10** was prepared by a singlet oxygenation of the coelenterazine analogue **9** in CH_2Cl_2 at –95 °C [21] (Fig. 4). The position of the peroxide moiety in compound **10** and in the stable silyl peroxide derivative **11** established the structure of intermediate **4**. The 2-hydroperoxide **10** emitted light when the temperature was increased to –50 °C, giving coelenteramide analogue **12**. Photo-oxygenation of a ^{13}C-enriched analogue **9** in CF_3CH_2OH/CH_3OH (7:3 v/v) at –78 °C produced dioxetanone **13**, which was detected by ^{13}C NMR [22], establishing the structure of intermediate **5**. Dioxetanone **13** also emitted light at higher temperature

Figure 3

Chemiluminescence reaction of coelenterazine (compound **1**). In aprotic solvent, anion **3** is produced, which reacts with molecular oxygen to produce peroxide **4**. Cyclization of intermediate **4** gives the dioxetanone intermediate **5**. Loss of CO_2 produces singlet-excited anion **6**, which emits light or is protonated to produce singlet-excited neutral coelenteramide **7**, which also emits light.



to give compound **12**. The generation of coelenteramide analogue **12** from compounds **10** and **13** indicated that 2-peroxide **4** and dioxetanone **5** are indeed the intermediates in chemiluminescence.

The efficiency of light production

The efficiency of light production (i.e. quantum yield or ΦL) in a chemi- or bioluminescence reaction is described by the equation:

$$\Phi L = \Phi R \times \Phi S \times \Phi F \qquad (1)$$

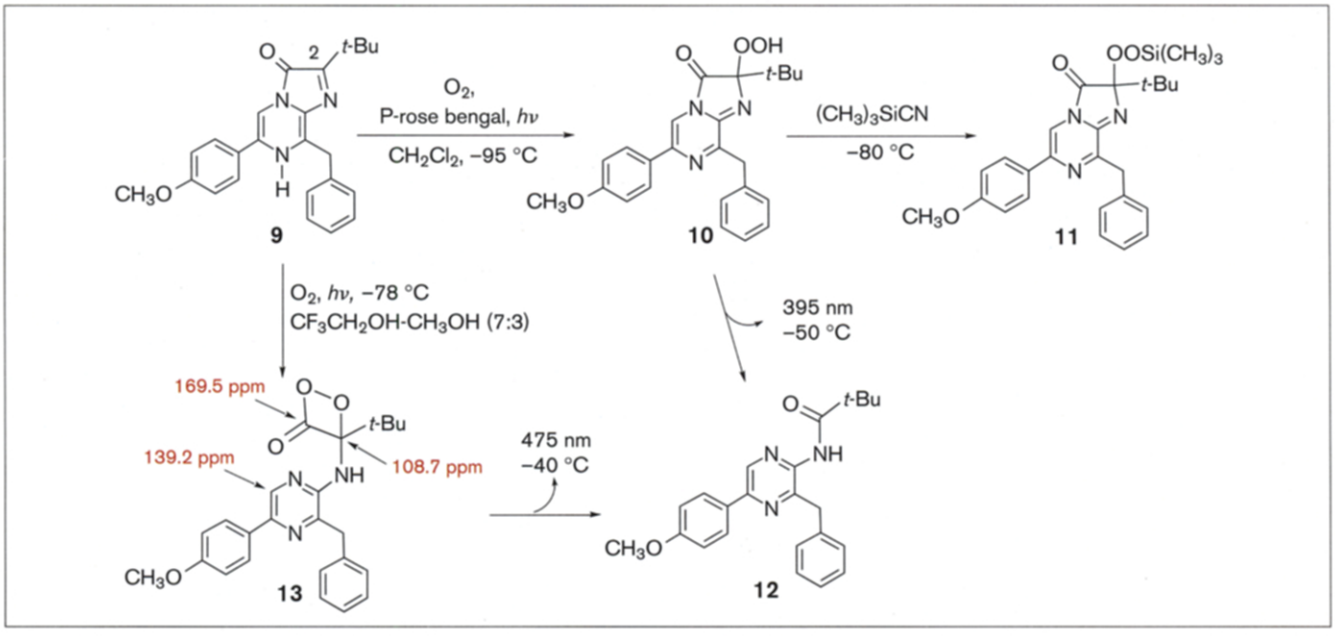
where ΦR is the yield of the reaction, ΦS is a measure of the efficiency of generating a singlet-excited state of a light emitter from an activated intermediate, and ΦF is the fluorescence quantum yield of the light emitter. The ΦL of coelenterazine for the bio- and chemiluminescence reactions are ~0.2 [23] and 0.002 [24], respectively.

Both the bio- and chemiluminescence reactions of coelenterazine result in high reaction yields (ΦR) [25]. The higher ΦL value for the bioluminescence reaction results from higher ΦS and ΦF values, as is discussed below. The values of ΦF for the two forms of coelenteramide produced (amide anion and neutral) are both over 0.2 in DMSO (R. Saito, T.H., H. Niwa & M. Ohashi, unpublished data). Thus the ΦS value for the decomposition of the dioxetanone intermediate, producing the singlet-excited coelenteramide, must be high.

The high ΦS value of coelenterazine cannot be deduced from the chemiluminescence reaction of a dioxetane derivative substituted by alkyl groups, such as 3,3,4,4-tetramethyldioxetane **14**, because its decomposition produces more triplet-excited than singlet-excited molecules ([26], Fig. 5). More informative is dioxetane **15**, which has a phenolate anion and undergoes a chemiluminescence reaction with a ΦS value of 0.57 in DMSO, although the ΦS value of the silylated derivative **16** [27] is 10^{-5} in DMSO. This indicates that the presence of an anionic moiety near the dioxetane ring is important for producing a high ΦS value.

A chemically initiated electron exchange luminescence (CIEEL) hypothesis has been invoked to explain the efficiency of bio- and chemiluminescence reactions [28]. The CIEEL hypothesis was proposed by Schuster [29,30] on the basis of the chemiluminescence reaction of diphenoyl peroxide **17** in the presence of a catalytic chemiluminescence activator. According to this hypothesis, a singlet-excited molecule is produced by the annihilation of a radical ion pair. In the case of coelenterazine, transfer of one electron from the pyradinylamine anion of the dioxetanone **5** to the O–O σ-bond is followed by the decomposition of the dioxetane ring to give a carbon dioxide radical anion and pyradinylamine radical **19** (see Fig. 6, top pathway). Annihilation of the radical ion pair produces CO_2 and the excited amide anion **6** [28]. The CIEEL hypothesis has been called into question [31], however, because the singlet-excited molecule would not be generated selectively in the

Figure 4



Reactions of coelenterazine analogue **9** establish the structure of the intermediates in the chemiluminescence reaction. Singlet oxygenation of compound **9** produces compound **10**, indicating that intermediate **4** is the 2-peroxide of coelenterazine. Photooxygenation of compound **9** in the presence of CF_3CH_2OH/CH_3OH (7:3 v/v) produces dioxetanone **13**, establishing the structure of intermediate **5**. The coelenteramide analogue **12** is produced by raising the temperature of either compound **10** or **13**, indicating that compounds **4** and **5** are indeed intermediates on the reaction pathway to coelenteramide.

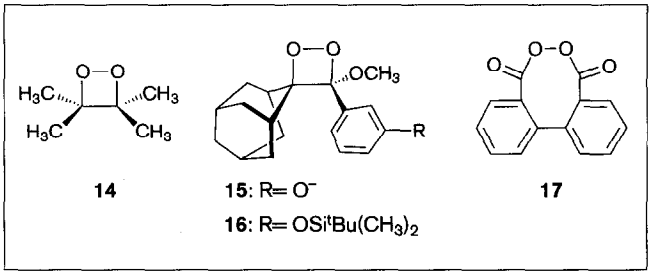
annihilation process. McCapra [32] proposed an alternative pathway in which dioxetanone **5** decomposes to compound **6** through transition state **20** with an intramolecular charge transfer (Fig. 6, bottom pathway) [32]. The repulsion between the negative charge and the strained σ-bond of the dioxetanone intermediate will accelerate the dissociation of the strained bond to produce compound **6** before formation of a triplet molecule occurs. Transition state **20** has characteristics closer to those of compound **6** than to the ground state of coelenteramide, promoting formation of the singlet-excited state.

Structure of the apophotoprotein

In the first quarter of this century, two photoproteins, aequorin and mitrocomin (also called halistaurin), from the class Hydrozoa, were extracted, isolated and characterized [1,33]. Other photoproteins such as obelin [2], thalassicolin [3], clytin (also called phialidin) [5], mnemiopsin [6] and berovin [7], have subsequently been isolated from various genuses within this class of jellyfish. All of these photoproteins have similar characteristics, indicating that the bioluminescence mechanisms of these photoproteins are fundamentally alike.

The apoproteins in the photoprotein complex are composed of a single polypeptide chain with a molecular weight of 21–28 kDa. In the last decade, the apoproteins from aequorin [34,35], clytin [36], mitrocomin [37] and obelin [38] have been cloned and sequenced. The amino acid sequences of the proteins are aligned in Figure 7 with that of human calmodulin, a typical Ca^{2+}-binding protein [39]. The apoproteins are 189 amino acid residues in length (190 for apomitrocomin) and contain three Ca^{2+}-binding sites, referred to as EF-hand structures. Calmodulin is 148 amino-acids long and contains four EF-hand structures. The sequence of apoaequorin is 61.9 %, 68.8 % and 68.8 % identical to those of apoclytin, apomitrocomin and apoobelin, respectively [40]. The Ca^{2+}-binding sites

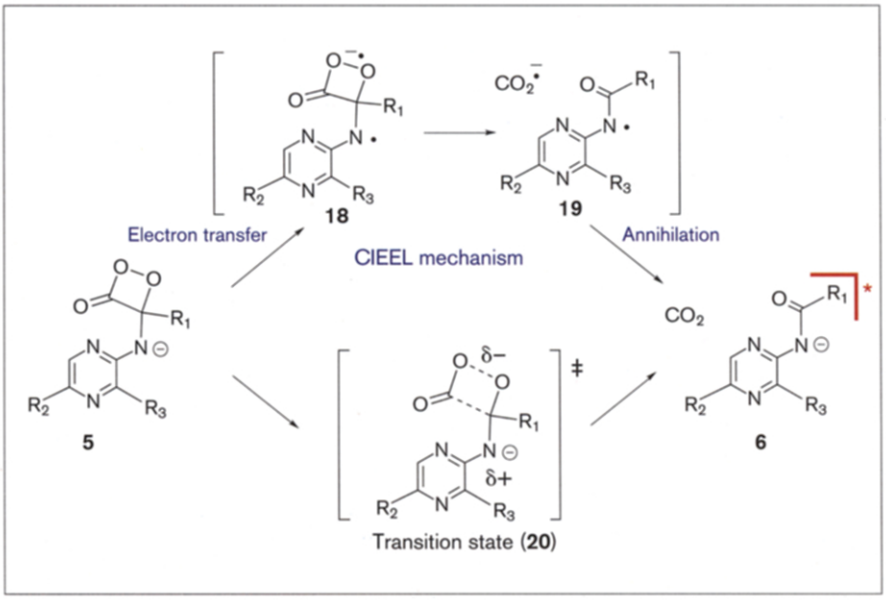
Figure 5



Chemiluminescent dioxetane derivatives. The ΦS values of these derivatives indicate that the phenolate anion (present in compound **15**) is an important determinant of the high ΦS value of singlet-excited coelenteramide.

Figure 6

Two possible mechanisms for decomposition of dioxetanone **5**, generating the excited amide anion **6**. The CIEEL mechanism (top) has been called into question, because it would not selectively produce the singlet-excited molecule. Decomposition through transition state **20** (bottom) would promote formation of the singlet-excited state (see text).



of apoproteins are homologous to EF-hand structures of the other Ca^{2+}-binding proteins, such as paralbumin, troponin C and calmodulin, and the distance between the second (third in calmodulin) and third (fourth in calmodulin) EF-hand domains is identical in the apoproteins and calmodulin. The apophotoproteins may thus have a common evolutionary origin with other Ca^{2+}-binding proteins.

The function of the apophotoproteins is, however, completely different from those of other Ca^{2+}-binding proteins. There are three differences between the sequences of the apophotoproteins and those of other Ca^{2+}-binding proteins that may be relevant to the bioluminescence reaction: the relatively high cysteine, histidine, tryptophan and proline content in the apoproteins, the missing EF-hand structure corresponding to the second Ca^{2+}-binding site of calmodulin, and the extended carboxy-terminal region of the apoproteins (Fig. 7). Removal of the carboxy-terminal proline residue from apoaequorin abolished the bioluminescence activity

Figure 7

Amino acid sequence alignment of the apophotoproteins aequorin (AQ) [8], clytin (CL) [10], mitrocomin (MI) [11] and obelin (OL) [12]. The standard single-letter code is used. Amino-acid residues that are identical in the four apophotoproteins are highlighted in blue. A red letter indicates an amino-acid residue that is essential for bioluminscence activity and a green letter indicates a cysteine residue that may participate in a disulfide bond. The Ca^{2+}-binding sites (EF-hand structures) are highlighted with yellow boxes.

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apoAQ   VKLTSDFDNPRWIGRHKHMFNFLDVNHNGKISLDEMVYKASDIVINNL    (48)
apoCL   VKLRPNFDNPKWVNRHKFMFNFLDINGDGKITLDEIVSKASDDICAKL    (48)
apoMI   VKLTTDFDNPKWIARHKHMFNFLDINSNGQINLNEMVHKASNIICKKL    (48)
apoOL   VKLTPDFDNPRWIKRHKHMFDFLDINGNGKITLDEIVSKASDDICAKL    (48)
Cal.       ADQLTEEQ IAEFKEAFSLFDKDGDGTITTKELGTVM     RSL    (39)

apoAQ   GATPEQAKRHKDAVEAFFGGA GMKYGVETDWPAYIEGWKKLATDELEK   (96)
apoCL   GATPEQTKRHQDAVEAFFKKI GMDYGKEVEFPAFVDGWKELANYDLKL   (96)
apoMI   GATEEQTKRHQKCVEDFFGGA GLEYDKDTTWPEYIEGWKRLAKTELER   (96)
apoOL   EATPEQTKRHQVCVEAFFRGC GMEYGKEIAEPQFLDGWKQLATSELKK   (96)
Cal.    GQNPTEAE LQDMINEVDADGNG    TIDFPEFLTMMAR     KMKD   (78)

apoAQ   YSKNQITLIRLWGDALFDIIDKDQNGAISLDEWKAYTKSDGIIQSSED   (146)
apoCL   WSQNKKSLIRDWGEAVFDIFDKDGSGSISLDEWKAYGRISGICSSDED   (146)
apoMI   HSKNQVTLIRLWGDALFDIIDKDRNGSVSLDEWIQYTHCAGIQQSRGQ   (146)
apoOL   WARNEPTLIREWGDAVFDIFDKDGSGTITLDEWKAYGKISGISPSQED   (146)
Cal.    TDS EEE IR   EA FRVFDKDGNGYISAAELRHVMTNLGEKLTDEE   (110)

apoAQ   CEETFRVCDIDESGQLDVDEMTRQHLGFWYTMDPACEKLYGGAVP      (189)
apoCL   AEKTFKHCDLDNSGKLDVDEMTRQHLGFWYTLDPNADGLYGNFVP      (189)
apoMI   CEATFAHCDLDGDGKLDVDEMTRQHLGFWYSVDPTCEGLYGGAVPY     (190)
apoOL   CEATFRHCDLDNSGDLDVDEMTRQHLGFWYTLDPEADGLYGNGVP      (190)
Cal.    VDEMIREADIDGDGQVNYEEFVQMMTAK                       (148)
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of the reconstituted photoprotein [41,42]. The role of this residue is not yet clear, although it may be important for the stability of the protein [42], the formation of the catalytic site, or the assembly of the apoprotein with coelenterazine and molecular oxygen [41].

Site-directed mutagenesis studies of the conserved histidine and tryptophan residues indicated that five specific amino acid residues in apoaequorin contribute to the bioluminescence activity of the photoprotein: these are His16, His58, His169, Trp108 and Trp173 [43,44]. Circular dichroism spectra of the mutants indicated that mutation of His16, Trp108 or Trp173 altered the secondary structure of the apoprotein. Mutation of His58 and His169 affected the assembly of the apoprotein, coelenterazine and molecular oxygen [44].

Cysteine residues appear to be important for the regeneration of the photoprotein following light emission [10]. Only one cysteine residue (Cys152) is conserved in the sequences of the four apoproteins, although apoaequorin, apoclytin, apomitrocomin and apoobelin contain three, three, six and five cysteine residues, respectively. Mutation of all three cysteines in apoaequorin does not affect luminescence activity, indicating that cysteine residues are not essential for the bioluminescence reaction [45]. The regeneration reaction with wild-type apoaequorin requires the presence of dithiothreitol or 2-mercaptoethanol, indicating that the formation of disulphide bonds may be important. The fact that regeneration of the photoprotein from the mutant apoaequorin took three times as long as with the wild-type protein [45] supports this conclusion. Apoaequorin contains a disulfide bond between Cys145 and Cys152 as indicated by fast atom bombardment mass spectrometry of the reduced and oxidized protein [46].

The three-dimensional structure of an apophotoprotein has not been determined although the crystallization of recombinant aequorin has been reported [47]. A model of the three-dimensional structure of obelin, based on the X-ray crystal structures of four different Ca^{2+}-binding proteins, suggests that it has a compact globular shape with a hydrophobic core that harbors the coelenterazine [48].

Structure of the photoprotein complex

A photoprotein is a high energy, extremely stable, charged complex, formed from apoprotein, coelenterazine and molecular oxygen during the regeneration process.

The peroxide moiety of the chromophore

Two models have been proposed for the structure of the coelenterazine and molecular oxygen at the active site of apoaequorin. On the basis of the absorption spectra for aequorin and for one of the imidazopyrazine tautomers, Cormier and coworkers [49] predicted that the incorporated coelenterazine has a tautomeric structure and that molecular oxygen is incorporated into an amino acid residue of apoaequorin. Shimomura and Johnson [50,51], however, obtained an unstable yellow compound (**21**) by treating aequorin with $NaHSO_3$. The structure of compound **21** was assigned as the 2-hydroxy derivative of coelenterazine (Fig. 8), and Shimomura and Johnson proposed that coelenterazine exists as the 2-peroxide **22** in aequorin. Teranishi *et al.* [52] suggested that the structure of compound **21** should be revised to the 5-peroxide of coelenterazine; however the existence of the 5-peroxide in aequorin has been excluded by the observation that 5-hydroperoxide **23** did not give the coelenteramide on thermal reaction (K. Teranishi, unpublished data).

To establish the structure of compound **21**, ^{13}C NMR spectra of aequorin, prepared using coelenterazines enriched with ^{13}C at the C2 and C9 positions, were measured in the presence of either $^{16}O_2$ or $^{18}O_2$ [53]. The shifts of the C2 and C9 carbons were observed at 98 and 148 ppm, respectively, as quaternary carbons. The chemical shift at the C2 position could correspond to the value for a carbon centre with the structure, –CO–C(OR)(NH–)R'. Recently, Teranishi *et al.* [54] were able to establish the chemical shifts of the 2-peroxide **24** and the 2,2-dialkyl derivative **25** (Fig. 8). The assignment of the chemical shift for the C2 carbon of the incorporated coelenterazine was confirmed by the observation that the chemical shift of 105 ppm for the C2 carbon of **24** was close to that of the incorporated coelenterazine, whereas the chemical shift for the C2 carbon of **25** (85 ppm) did not correspond as closely. The chemical shifts at ~150 ppm for the C9 carbon of these compounds could not give any structural information. These ^{13}C-NMR investigations clarified the fact that the 2-peroxide of coelenterazine is the structure at the active site of apoaequorin, corroborating Shimomura and Johnson's model.

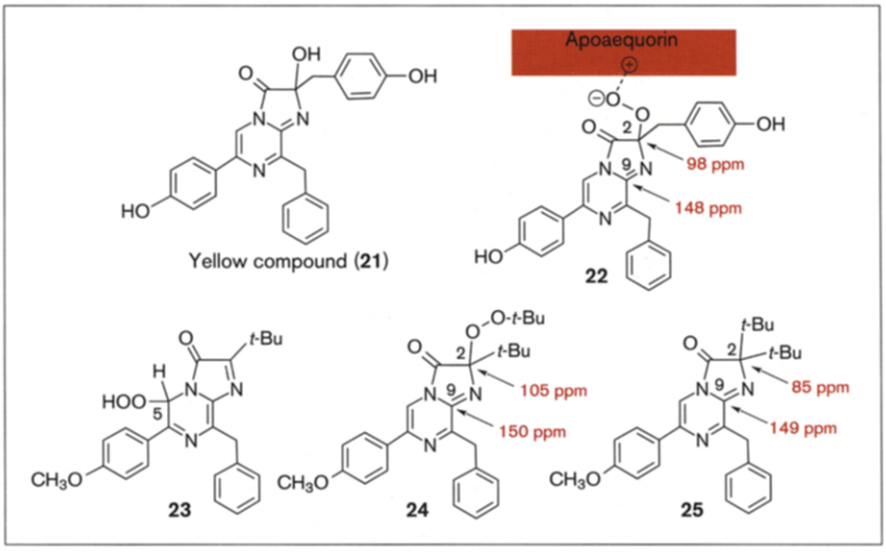
It has been predicted [51] that the peroxidized coelenterazine **22** binds to specific amino acid residues on the photoprotein, although whether the interaction at the binding site has an ionic or hydrophobic character has not been determined. The incorporation of coelenterazine into the photoprotein complex can be inhibited by chemical modification of apoaequorin with diethyl pyrocarbonate, suggesting that one or more histidine residues are essential for forming the structure of the complex and may interact with the oxygenated coelenterazine [40]. Site-directed mutagenesis of histidine residues in apoaequorin showed that one of two residues, either His58 or His169, is important for full activity and is involved in the regeneration of aequorin [45,55]. Molecular modeling studies indicate that both histidine residues may be located at the hydrophobic core of the apoprotein [48].

The side chains of the chromophore

The importance of the substituted phenyl groups at posi-

Figure 8

The 2-peroxide of coelenterazine is the structure at the active site of apoaequorin. The structures of the yellow compound (**21**), coelenterazine incorporated in aequorin (**22**), and their model compounds are shown. The 5-hydroperoxide (**23**) did not give coelenteramide on thermal reaction, excluding this structure for the bound coelenteramide. The C2 chemical shift of analogue **24**, but not analogue **25**, corresponds to that of the bound coelenteramide.



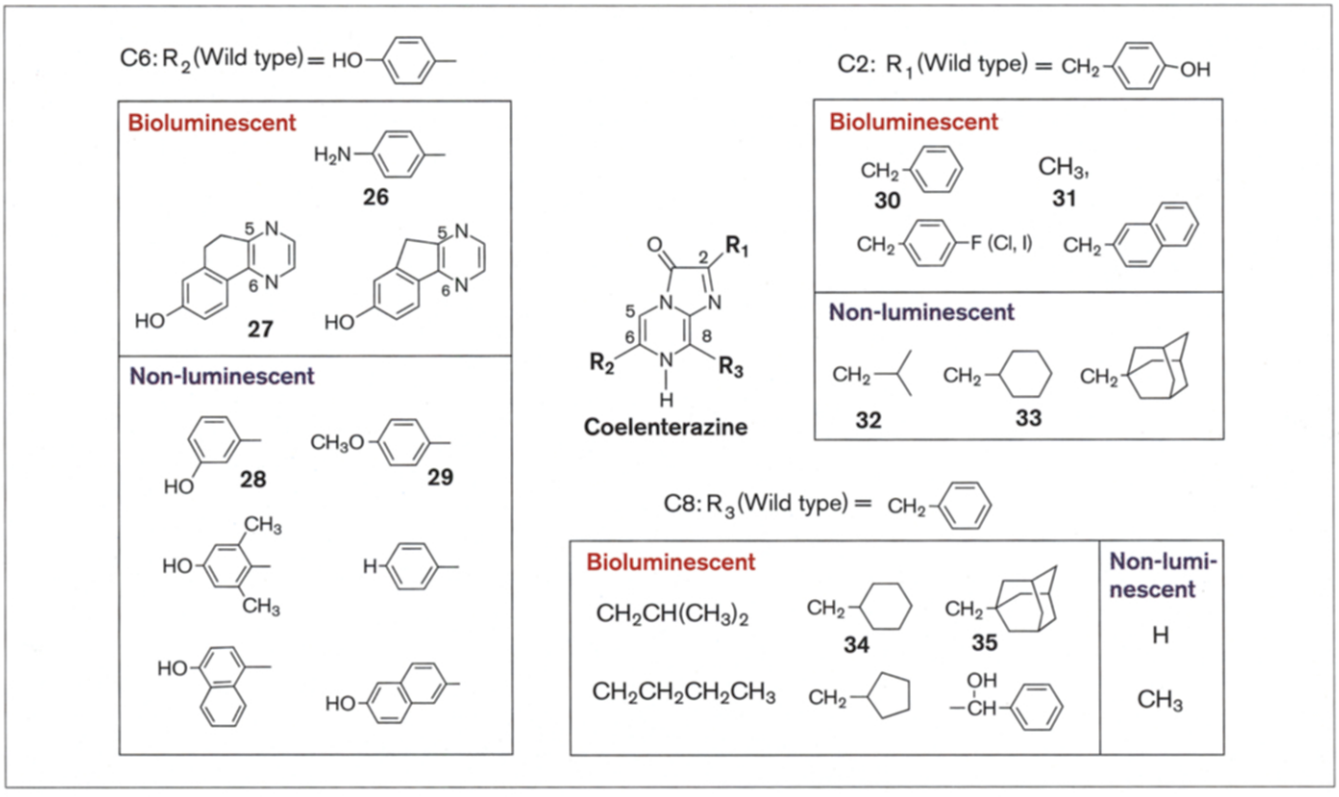
aequorin has been investigated in studies using analogues substituted at these positions. The interaction between the *p*-hydroxyphenyl group at the C6 position of coelenterazine and the apophotoprotein could not be determined definitively, because it was not possible to establish whether the *p*-hydroxyphenyl group was anionic or neutral in the photoprotein [56]. The semi-synthetic aequorins composed of the native apoprotein complexed with the coelenterazine analogues **28** and **29**, which have *m*-hydroxyphenyl and *p*-methoxyphenyl groups at the C6 position, respectively, showed no luminescence activity, although a semi-synthetic aequorin containing the *p*-aminophenyl analogue **26** had low activity (Fig. 9) [57–60]. Thus the active site of apoaequorin may recognize the accurate stereochemistry of the *p*-hydroxyphenyl group at the C6 position and, furthermore, an electrostatic interaction between the hydroxyl group and a cationic centre in apoaequorin may be important for complex formation.

The side-chains at both the C2 and C8 positions of coelenterazine are important in fixing the conformation of coelenterazine at the active site of apoaequorin through hydrophobic interactions. The semi-synthetic aequorin prepared using the 2-benzyl analogue **30** has a high level of bioluminescence activity, although those prepared using the 2-isobutyl or 2-cyclohexylmethyl analogues **32** or **33** were inactive (Fig. 9). These results suggest that a cavity on the active site recognizes the planar shape of the benzyl moiety of the C2 side chain [58–61]. Replacement of the benzyl group at C8 with a small group such as a methyl group resulted in a loss of bioluminescence, whereas the semi-synthetic aequorins prepared using analogues **34** and **35**, containing an 8-cyclohexylmethyl or 8-adamantylmethyl group, respectively, have high levels of bioluminescence activity [58–60,62]. The interaction between the benzyl group at the C8 position of coelenterazine and a hydrophobic region of apoaequorin thus seems to stabilize the complex structure. The relative bioluminescence activities of the 8-adamantylmethyl analogues **35**, **36** and **37** matches that of coelenterazine **1** and its analogues **30** and **31**, respectively [62] (Table 1), which have matching groups at the C2 position and a benzyl (wild-type) group at the C8 position, indicating that the effects of substitutions at C2 and C8 are independent. The interactions between the C2, C6, and C8 side chains of coelenterazine with the active site of apophotoprotein are summarized in Figure 10.

The bioluminescence reaction

Upon binding Ca^{2+}, the photoprotein undergoes a conformational change, converting itself into a luciferase, which then catalyzes the luminescence reaction of coelenterazine. A log–log plot of light intensity as a function of calcium concentration over a range of 10^{-9} to 10^{-2} M produces a sigmoidal curve with a log phase, logarithmic phase and saturated phase [63,64]. The logarithmic phase occurs at physiologically relevant concentrations of calcium (10^{-7}–10^{-5} M) and has a slope of 2.25–2.5, suggesting that a molecule of aequorin must bind at least two Ca^{2+} ions. When the EF-hand sites in apoaequorin were individually mutated at position 6 of the motif (Gly→Arg), the molecule with the third site mutated (Gly158) had almost the same level of activity as the wild type, suggesting that the binding of Ca^{2+} to site III may be unnecessary for light emission and that binding of two Ca^{2+} ions may be sufficient for initiating the bioluminescence reaction [55]. This has recently been confirmed experimentally by Shimomura using aequorin [65]. Other divalent cations,

Figure 9



The activities of semisynthetic aequorins, containing analogues of coelenterazine, establish the important structural features of the chromophore. Luminescent and non-luminescent analogues of coelenterazine substituted at C2, C6 or C8 are shown. Apoaequorin recognizes the stereochemistry of the *p*-hydroxyphenyl group at C6; the side chains at C2 and C8 participate in hydrophobic interactions.

including Eu^{2+}, Sr^{2+} and Ba^{2+}, can substitute for Ca^{2+} in initiating the bioluminescence reaction, but Mg^{2+} inhibits the reaction [66]. The relative potencies of the different cations have been estimated as $Eu^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$.

As noted above, a quantum yield of 0.16–0.18 has been determined for the bioluminescence reaction of aequorin [23], whereas the chemiluminescent quantum yield of coelenterazine was 0.002 in DMSO [24]. The higher luminescent yield for the aequorin reaction is due in part to a higher ΦS value for the biological reaction; the apoprotein holds the coelenteramide molecule in a suitable conformation for generating the singlet-excited state. The fluorescent yield (ΦF) of coelenteramide may also be higher in the bioluminescence reaction. A ΦF value of 0.1 has been measured for BFP [67], whereas coelenteramide does not fluoresce in an aqueous buffer [56]. The apoprotein provides a hydrophobic environment and inhibits molecular motion, which may increase the fluorescent yield of coelenteramide in BFP.

If the product coelenteramide is allowed to reassociate with the apoprotein, it is spontaneously excited, leading to a fluorescence peak at 465 nm, the same as the emission peak of aequorin [9,56]. This indicates that the structure of the singlet-excited coelenteramide generated by the bioluminescence reaction of aequorin corresponds to that of the spontaneously generated species in BFP. There are three possible structures for the excited state of coelenteramide: neutral (compound **2**); phenolate anion **38**; and, amide anion 8 (Fig. 11). The structure of the bioluminescent

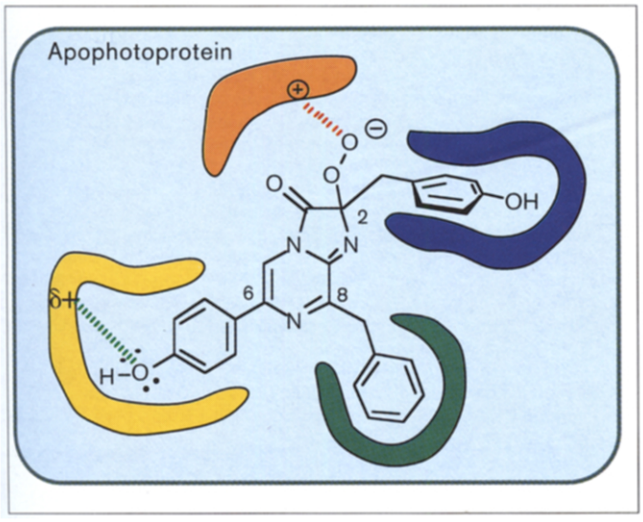
Table 1

Relative bioluminescence intensities of aequorin and semisynthetic aequorins prepared from coelenterazine 1 and analogues 30, 31 and 35–36.

Compound	Relative bioluminescence intensity
1	1.0
30	0.35
31	0.01
35	2.0
36	0.73
37	0.02

30: R_1 = benzyl, R_3 = benzyl
31: R_1 = methyl, R_3 = benzyl
35: R_1 = *p*-hydroxyphenylmethyl, R_3 = adamantylmethyl
36: R_1 = benzyl, R_3 = adamantylmethyl
37: R_1 = methyl, R_3 = adamantylmethyl

Figure 10



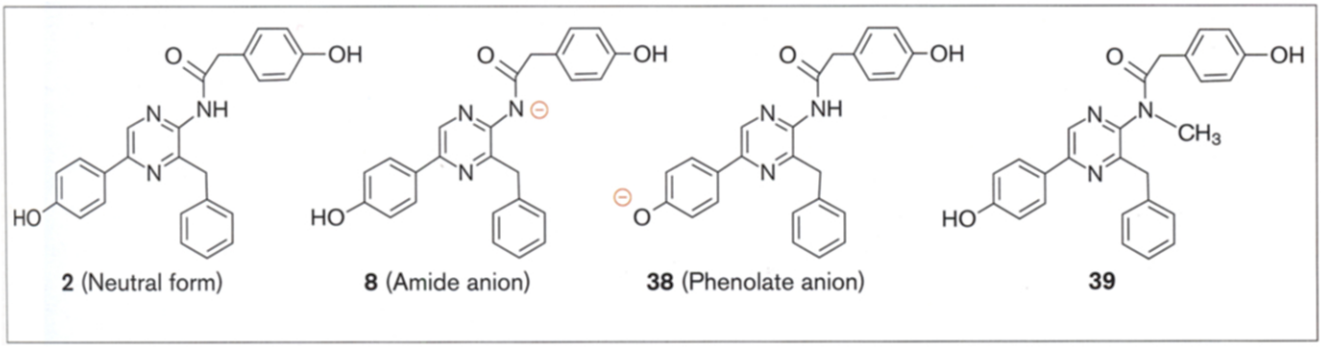
Schematic illustration of the interaction between apophotoprotein and coelenterazine peroxide based on the structure–activity studies of semi-synthetic aequorins containing coelenterazine analogues and mutated aequorins (see Fig. 9, and text).

coelenteramide had been assigned as that of compound **8**, because a coelenterazine analogue had been shown to emit light at 460 nm from the excited amide anion in DMF [23]. It was difficult, however, to explain why coelenteramide would form the amide anion when the acidity of the amide proton is lower than that of the proton in the phenolic hydroxyl group [19,56].

The fluorescence character of the neutral molecule (compound **2**) is dependent on the solvent; it has fluorescence maxima of 420 nm in methanol and 390 nm in dioxane [68]. This solvatochromism is caused by an intramolecular charge transfer in the singlet-excited state, where the 5-hydroxyphenyl group serves as an electron donor and the pyrazine ring as an electron acceptor. It was thus unlikely that the excited state of coelenteramide is the neutral molecule, indicating that it may be the phenolate anion **38**. This conclusion has been confirmed by studying the fluorescence behavior of coelenteramide analogues, having various substituents on the 5-phenyl group (R. Saito, T.H., H. Niwa and M. Ohashi, unpublished data). Combining coelenteramide and apoprotein generates BFP with a fluorescence spectrum (λ_{max} 470 nm) nearly identical to the bioluminescence spectrum [9]. A fluorescence maximum of 480 nm was observed for the semi-synthetic BFP prepared by the incubation of *N*-methyl analogue **39** with apoaequorin [58]. As this fluorescence is close to that of coelenteramide and cannot be due to deprotonation at the amide group, the structure of the excited state of coelenteramide must be that of phenolate anion **38** [9].

The emission spectra of semi-synthetic aequorins can provide information about the interaction between the chromophore and specific residues in the apoprotein. The semi-synthetic complex consisting of the coelenterazine analogue **27** and apoaequorin has two emission maxima (400 and 465 nm) [59–63], indicating that there are two possible structures (ionic or neutral) for the excited state of the resulting coelenteramide analogue. The mutant aequorin (Trp86→Phe) also had two luminescence maxima at 400 and 465 nm [44], but a semi-synthetic aequorin composed of the Trp86→Phe mutant apoprotein and analogue **27** emitted light with only one maximum at 400 nm. The emitting structure at 400 nm in the mutant aequorin was assigned as the neutral form of coelenteramide (compound **2**) generated by the protonation of phenolate anion **38**. Thus Trp86 may be located in the neighborhood of the 6-(*p*-hydroxyphenyl) group of coelenteramide, where it stabilizes the phenolate anion. In the semi-synthetic complex with analogue **27**, this residue may interact with an ethano-bridge of the analogue, possibly affecting the protonation of the excited phenolate anion of the resulting coelenteramid analogue.

Figure 11



Three possible structures of the singlet-excited coelenteramide (compound **2**, **8** or **38**) in BFP. The fluorescence maximum for semisynthetic aequorin containing analogue **39** matches that of native aequorin.

Conclusions

The structure of the complex of apoprotein, coelenterazine and molecular oxygen in the calcium-activated photoproteins determines the characteristics of the bioluminescence reaction. In the absence of crystallographic data on the structure of this complex, structure–activity studies using chromophore analogues and mutant apoproteins have elucidated many features of the complex and of the mechanism of this reaction.

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