1
The Photoproteins

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1.1
Discovery of Photoprotein

In 1961, we found an unusual bioluminescent protein in the jellyfish *Aequorea* and named it "aequorin" after its genus name (Shimomura et al. 1962). The protein had the ability to emit light in aqueous solutions merely by the addition of a trace of Ca^{2+}. Surprisingly, it luminesced even in the absence of oxygen. After some studies, we discovered that the light is emitted by an intramolecular reaction that takes place inside the protein molecule, and that the total light emitted is proportional to the amount of protein luminesced. At that time, we simply thought that aequorin was an exceptional protein accidentally made in nature.

In 1966, however, we found another unusual bioluminescent protein in the parchment tubeworm *Chaetopterus* (Shimomura and Johnson 1966). This protein emitted light when a peroxide and a trace of Fe^{2+} were added in the presence of oxygen, without the participation of any enzyme. The total light emitted was again proportional to the amount of the protein used. These two examples were clearly out of place in the classic concept of the luciferin–luciferase reaction of bioluminescence, wherein luciferin is customarily a relatively heat stable, diffusible organic substrate and luciferase is an enzyme that catalyzes the luminescent oxidation of a luciferin.

Considering the possible existence of many similar bioluminescent proteins in luminous organisms, we have introduced the new term "photoprotein" as a convenient, general term to designate unusual bioluminescent proteins such as aequorin and the *Chaetopterus* bioluminescent protein (Shimomura and Johnson 1966). Thus, "photoprotein" is a general term for the bioluminescent proteins that occur in the light organs of luminous organisms as the major luminescent component and are capable of emitting light in proportion to the amount of protein (Shimomura 1985). The proportionality of the light emission makes a clear distinction between a photoprotein and a luciferase. In a luciferin–luciferase luminescence reaction, the total amount of light emitted is proportional to the amount of luciferin, not to the amount of luciferase. If a luciferin is a protein,

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Table 1.1 Photoproteins that have been isolated.

Source	*Name*	M_r	*Requirements for luminescence*	*Luminescence maximum (nm)*
Protozoa				
Thalassicola sp.[a]	Thalassicolin		Ca^{2+}	440
Coelenterata				
Aequorea aequorea[b]	Aequorin	21 500	Ca^{2+}	465
Halistaura sp.[c]	Halistaurin		Ca^{2+}	470
Phialidium gregarium	Phialidin[d], clytin[e]	23 000 21 600	Ca^{2+} Ca^{2+}	474
Obelia geniculata	Obelin	21 000[f]	Ca^{2+}	475[h]
Obelia geniculata	Obelin	21 000		485[i]
Obelia longissima	Obelin	22 200[g]		495[i]
Ctenophora				
Mnemiopsis sp.[j]	Mnemiopsin-1 Mnemiopsin-2	24 000 27 500	Ca^{2+} Ca^{2+}	485 485
Beroe ovata[j]	Berovin	25 000	Ca^{2+}	485
Annelida				
Chaetopterus variopedatus[k]		120 000 184 000	Fe^{2+}, hydroperoxide, and O_2	455
Hamothoe lunulata[l]	Polynoidin	500 000	Fe^{2+}, H_2O_2, and O_2	510
Mollusca				
Pholas dactylus[m]	Pholasin	34 600	Peroxidase or Fe^{2+}, plus O_2	490
Symplectoteuthis oualaniensis[n]	Symplectin	60 000	Alkaline pH? and O_2	470[o]
Symplectoteuthis luminosa[p]		50 000	Catalase, H_2O_2, and O_2	
Diplopoda				
Luminodesmus sequoia[q]		60 000	ATP, Mg^{2+}, and O_2	496
Echinodermata				
Ophiopsila californica[r]		45 000	H_2O_2	482

a) Campbell et al. 1981
b) Shimomora 1986b
c) Shimomura et al. 1963
d) Levine and Ward 1982
e) Inouye and Tsuji 1993
f) Stephanson and Sutherland 1981
g) Illarionov et al. 1995
h) Morin and Hastings 1971
i) Markova et al. 2002
j) Ward and Seliger 1974
k) Shimomura and Johnson 1969
l) Nicolas et al. 1982
m) Michelson 1978
n) Fujii et al. 2002
o) Tsuji et al. 1981
p) Shimomura unpublished
q) Shimomura 1981, 1984
r) Shimomura 1986a

the luciferin is a photoprotein, regardless of the existence or nonexistence of a specific luciferase.

A photoprotein could be an extraordinarily stable form of enzyme–substrate complex, more stable than its dissociated forms, an enzyme and a substrate. Because of its high stability, a photoprotein, rather than its dissociated forms, occurs as the primary light-emitting component in the light organs. For example, the light organs of the jellyfish *Aequorea* contain aequorin, which is highly stable in the absence of Ca^{2+}, but its components coelenterazine and apoaequorin, both unstable, are hardly detectable in any part of the jellyfish. In the cells of luminous bacteria, the bacterial luciferase forms an intermediate by reacting with $FMNH_2$ and O_2, and this intermediate emits light when a fatty aldehyde is added (Hastings and Gibson 1963; Hastings and Nealson 1977). However, this intermediate is unstable and short-lived; thus, it does not fit the definition of photoprotein.

Presently, there are about 30 different types of bioluminescent systems for which substantial biochemical knowledge is available. About half of these types involve a photoprotein (Table 1.1). These photoproteins include the Ca^{2+}-sensitive type from various coelenterates (aequorin, obelin, etc.); the superoxide-activation types from a scale worm (polynoidin) and the clam *Pholas* (pholasin); the H_2O_2-activation type from a brittle star (*Ophiopsila*); and the ATP-activation type from a Sequoia millipede (*Luminodesmus*). For analytical applications, the photoproteins of the Ca^{2+}-sensitive type and the superoxide-sensitive type (pholasin) have been utilized, and the photoprotein aequorin has been in extensive use in various biological studies for the past 35 years. Each of the various photoproteins are briefly described in the next section, followed by a discussion on the extraction and purification of photoproteins and a more detailed account on the photoprotein aequorin.

1.2 Various Types of Photoproteins Presently Known

1.2.1 Radiolarian (Protozoa) Photoproteins

A Ca^{2+}-sensitive photoprotein that resembles coelenterate photoproteins was isolated from the radiolarian *Thalassicola* sp., but its properties were not investigated in detail (Campbell et al. 1981). It is of interest as the only known example of a Ca^{2+}-sensitive photoprotein other than the coelenterate photoproteins.

1.2.2 Coelenterate Photoproteins

Several kinds of photoprotein, including aequorin and obelin, were isolated from hydrozoan jellyfishes and hydroids. All of them emit blue light when Ca^{2+} is added, regardless of the presence or absence of oxygen. The coelenterate photoproteins

are suitable for use in the detection and measurement of trace amounts of Ca^{2+}, and aequorin has been widely used in the studies of Ca^{2+} in various biological systems, including single cells (Blinks et al. 1976; Ashley and Campbell 1979). The overwhelming popularity of this type of photoprotein compared with the other types sometimes leads to the misconception that the photoproteins are Ca^{2+}-sensitive bioluminescent proteins.

Detailed studies have been made with only three kinds of photoproteins: aequorin obtained from *Aequorea*, obelin obtained from *Obelia*, and phialidin (clytin) obtained from *Phialidium*. Aequorin was isolated from *Aequorea aequorea* by Shimomura et al. (1962), and its purification was described in several papers (Shimomura and Johnson 1969, 1976; Blinks et al. 1978). The recombinant form of aequorin has been made (Inouye et al. 1985, 1986; Prasher et al. 1985, 1987). Obelin was isolated from *Obelia geniculata* (Campbell 1974) and also from *O. australis* and *O. geniculata* (Stephenson and Sutherland 1981). Its recombinant form was prepared by Illarionov et al. (2000). Phialidin was isolated from *Phialidium gregarium* by Levine and Ward (1982) and was cloned by Inouye and Tsuji (1993); the recombinant protein was named clytin.

All coelenterate photoproteins have a molecular weight close to 20 000. The concentrated solutions of purified photoproteins are slightly yellowish (weak absorption at about 460 nm) and non-fluorescent except for ordinary protein fluorescence. After Ca^{2+}-triggered luminescence, the solutions turn colorless and become fluorescent in blue (emission λ_{max} 460 nm). The intensity of the blue fluorescence is dependent on the concentrations of the spent protein and Ca^{2+}; however, the fluorescence intensity is not proportional to the concentration of the protein (Morise et al. 1974). In the case of aequorin, the emission spectrum of blue fluorescence is almost superimposable on the emission spectrum of Ca^{2+}-triggered luminescence, suggesting that the blue fluorescent chromophore formed in the luminescence reaction is probably the light emitter (Shimomura and Johnson 1970).

As a Ca^{2+} indicator, aequorin is useful at a concentration range of Ca^{2+} between $10^{-7.5}$ M and $10^{-4.5}$ M (Blinks et al. 1978), whereas obelin is useful at a range between $10^{-6.5}$ M and $10^{-3.5}$ M under similar conditions (Stephenson and Sutherland 1981). The Ca^{2+} sensitivity of phialidin is about equal to that of obelin (Shimomura and Shimomura 1985). It should be noted here that the Ca^{2+} sensitivity and certain other properties of aequorin, and probably of all coelenterate photoproteins, can be modified by replacing the coelenterazine moiety of the photoprotein with its analogues (explained later).

The chemistry of the bioluminescence reaction of aequorin has been elucidated in considerable detail and will be described later in this chapter. The reaction mechanisms of all hydrozoan photoproteins are believed to be essentially identical with that of aequorin. However, the luminescence reaction differs in luminous anthozoans, which are taxonomically closely related to hydrozoan. The luminous species of anthozoans contain a luciferin (coelenterazine) and a species-specific luciferase instead of a photoprotein, although the presence of a small amount of Ca^{2+}-sensitive photoprotein is suspected in some species, such as the sea

pen *Ptilosarcus gurneyi* and the sea cactus *Cavernularia obesa* (Shimomura and Johnson 1979b).

Spent aequorin that has been luminesced with Ca^{2+} can be regenerated into the active original form by incubation with coelenterazine in the presence of O_2 and a low concentration of 2-mercaptoethanol (Shimomura and Johnson 1975a). The regenerated aequorin is indistinguishable from the original aequorin in every aspect of its properties. The yield of the regeneration is practically 100% when the protein concentration is over 0.1 mg mL^{-1} (Shimomura and Shimomura 1981). Thus, a sample of aequorin can be luminesced and recharged repeatedly. The regeneration of spent photoprotein takes place also with obelin (Campbell et al. 1981), as well as with halistaurin and phialidin (unpublished results).

1.2.3
Ctenophore Photoproteins

The photoproteins mnemiopsin and berovin were isolated from *Mnemiopsis* sp. and *Beroe lovata*, respectively (Ward and Seliger 1974). They are Ca^{2+}-sensitive photoproteins that are similar to aequorin, except that these photoproteins are photosensitive. The absorption maximum of mnemiopsin-2 is 435 nm, which is about 20 nm shorter than that of aequorin. The photosensitivity of ctenophore photoproteins is strikingly different from that of aequorin. Mnemiopsin and berovin are extremely sensitive to light (Hastings and Morin 1968), being easily inactivated by a broad spectral range of light (wavelength 230–570 nm) (Ward and Seliger 1976). Aequorin and other hydrozoan photoproteins are not affected by light.

Photoinactivated mnemiopsin, as well as spent mnemiopsin after Ca^{2+}-triggered luminescence, can be regenerated into its active form by incubation with coelenterazine in the presence of oxygen, like aequorin; however, the regeneration takes place only at a narrow pH range around 9.0 (Anctil and Shimomura 1984).

1.2.4
Pholasin (*Pholas* Luciferin)

The boring clam *Pholas dactylus* is historically important in the field of bioluminescence because it was one of the two luminous species with which Dubois first demonstrated luciferin–luciferase luminescence in 1887. Thus, the luminescence of *Pholas* was originally considered to be a luciferin–luciferase reaction involving *Pholas* luciferin and *Pholas* luciferase. However, *Pholas* luciferin is a glycoprotein with a molecular weight of 34 600 (Henry et al. 1973a, 1973b; Michelson 1978). Therefore, it is appropriate to call this luciferin a photoprotein. The name "pholasin" was first used by Roberts et al. (1987).

The ultraviolet absorption spectrum of pholasin shows a bulge at about 325 nm, in addition to the protein peak at 280 nm. Pholasin emits light (λ_{max} 490 nm) in the presence of various substances such as *Pholas* luciferase, ferrous ions, H_2O_2,

peroxidase, superoxide anions, hypochlorite, and certain other oxidants, all in the presence of molecular oxygen (Henry and Michelson 1970; Henry et al. 1970, 1973a, 1973b; Müller and Campbell 1990). Thus, *Pholas* luciferase is clearly not an essential component for the luminescence of pholasin. The luminescence reaction of pholasin with *Pholas* luciferase is optimum at pH 8–9 and at an ionic strength of about 0.5 M, giving a quantum yield of 0.09 for pholasin (Michelson 1978). According to Reichl et al. (2000), the addition of horseradish peroxidase compounds I and II to pholasin induces an intense luminescence. Moreover, the addition of H_2O_2 to a mixture of myeloperoxidase and pholasin gives an intense burst of light. The chromophore of pholasin is still not chemically identified.

The cloning and expression of apopholasin was achieved by Dunstan et al. (2000), but attempts to reconstitute the recombinant apopholasin into pholasin by the addition of an acidic methanol extract of *Pholas* failed, although the mixture gave luminescence by the addition of sodium hypochlorite. Pholasin is commercially available from Knight Scientific, Plymouth, UK. The main application of pholasin is the measurement of oxygen radicals.

1.2.5 *Chaetopterus* Photoprotein

The photoprotein of the parchment tubeworm *Chaetopterus variopedatus* purified by chromatography has a molecular mass of approx. 120–130 kDa (Shimomura and Johnson 1966, 1968). The protein is amorphous when precipitated with ammonium sulfate, but it can be converted into a crystalline form with an increased molecular mass of 184 kDa by slow crystallization with ammonium sulfate. The photoprotein emits light in the presence of Fe^{2+}, a peroxide, and molecular oxygen. As a peroxide, H_2O_2 can be used, but an unidentified hydroperoxide existing in old dioxane or tetrahydrofuran was far more effective. Two kinds of additional activators were found to give brighter luminescence, but they were not identified. The light emission of this photoprotein is strongly affected by the pH of the medium, showing a peak at pH 7.7 with a sharp decrease at both sides (50% decreases at pH 6.5 and pH 8.3); the light intensity is not significantly influenced by the salt concentration up to 1 M when tested with NaCl. The optimum temperature for the luminescence intensity is 22 °C. With this photoprotein, a concentration of Fe^{2+} as low as 0.1 μM can be detected.

The purified photoprotein is practically colorless, and its absorption spectrum shows, in addition to the 280-nm protein absorption peak, a very slight absorption in the region of 330–380 nm, although its significance is unclear. A solution of the photoprotein is moderately blue fluorescent, with a fluorescence emission maximum at 453–455 nm and an excitation maximum at 375 nm, and these peaks do not significantly change after the luminescence reaction. The luminescence spectrum of purified photoprotein (λ_{max} 453–455 nm) closely matched with the fluorescence emission spectrum.

1.2.6
Polynoidin

A membrane photoprotein isolated from the scales of the scale worm *Harmothoe lunulata* was named "polynoidin" (Nicolas et al. 1982). The purified photoprotein (M_r 500 000) emits light in the presence of molecular oxygen (λ_{max} 510 nm) by the action of sodium hydrosulfite, the xanthine–xanthine oxidase system, Fenton's reagent (H_2O_2 plus Fe^{2+}), or other reagents that produce superoxide radicals. The photoprotein luminescence was 30% brighter in phosphate buffer than in Tris buffer, and the luminescence response was significantly increased by including a complexing agent such as EGTA. However, the injection of polynoidin solution into the mixture of H_2O_2 and Fe^{2+} failed to produce light; Fe^{2+} must be added last to initiate light emission.

The photoprotein is not fluorescent (except for usual protein fluorescence) after the bioluminescence reaction or before the reaction. The requirements for its luminescence reaction are similar to that of the bioluminescence systems of *Pholas* and *Chaetopterus*, suggesting the involvement of a common basic mechanism in these luminescence systems.

1.2.7
Symplectin

The luminescent substance of the squid *Symplectoteuthis oualaniensis* was first obtained in the form of insoluble particles by Tsuji et al. (1981). The suspension of the particles emitted light in the presence of monovalent cations such as K^+, Rb^+, Na^+, Cs^+, NH_4^+, and Li^+ (in decreasing order of effect). Molecular oxygen was needed for the luminescence. Divalent ions such as Ca^{2+} and Mg^{2+} did not trigger light emission. The light emission (λ_{max} 470 nm) was optimal in the presence of 0.6 M KCl or NaCl and at a pH of 7.8.

The soluble form of the *Symplectoteuthis* photoprotein was isolated and purified from the granular light organs of the squid and was named "symplectin" (Takahashi and Isobe 1994; Fujii et al 2002). The light organs were first extracted with a pH 6 buffer containing 0.4 M KCl to remove impurities, and then symplectin was extracted from the residue with a pH 6 buffer containing 0.6 M KCl. All solutions used in the experiments contained 0.25 M sucrose, 1 mM dithiothreitol, and 1 mM EDTA. The 0.6 M KCl extract was chromatographed by size-exclusion HPLC on a TSK G3000SW column. Symplectin was eluted as two major components of oligomers, having molecular masses of 200 kDa or more, and a minor component of monomer (60 kDa). All processes of extraction and purification were carried out at 4 °C. Warming up a solution of symplectin, adjusted to pH 8, to room temperature causes the luminescence reaction to begin, and the light emission lasts for hours.

A tryptic digestion of the KCl extract increased the content of the 60-kDa species at the expense of the two high-molecular-weight species, accompanied by the formation of 40-kDa and 16-kDa species. SDS-PAGE analysis of the two

high-molecular-weight oligomers revealed that they consist mainly of the 60-kDa protein. The 60-kDa protein and the 40-kDa protein were fluorescent in the SDS-PAGE analysis. The spent protein of symplectin after luminescence (aposymplectin) could be reconstituted into original symplectin by treatment with dehydrocoelenterazine (Isobe et al. 2002).

1.2.8 *Luminodesmus* Photoprotein

This is presently the only example of a photoprotein of terrestrial origin. The millipede *Luminodesmus sequoia* (Loomis and Davenport 1951) emits light from the surface of its whole body continuously day and night. The photoprotein extracted and purified from this organism emits light (λ_{max} 496 nm) when ATP and Mg^{2+} are added in the presence of molecular oxygen (Hastings and Davenport 1957; Shimomura 1981). Thus, the luminescence system of *Luminodesmus* resembles that of the fireflies in that it requires ATP and Mg^{2+}, but it differs in that it needs only the photoprotein rather than the luciferin and luciferase required in the firefly system. The molecular weight of the photoprotein is 104 000, which is close to the molecular weight of firefly luciferase reported earlier (100 000) but larger than its newer value (62 000; Wood et al. 1984). Although it was suspected that the photoprotein might be a complex of a firefly-type luciferase and firefly luciferin, firefly luciferin itself was not detected in this photoprotein. Recently, the possible presence of a porphyrin chromophore in the photoprotein has been suggested, although the role of this chromophore in the light-emitting reaction is unclear (Shimomura 1984). Using the luminescence system of *Luminodesmus*, 0.01 μM ATP and 1 μM Mg^{2+} can be detected.

1.2.9 *Ophiopsila* Photoprotein

The brittle star *Ophiopsila californica* is abundant around Catalina Island, off the coast of Los Angeles (Shimomura 1986a). An animal of average size weighs about 3–4 g, and has five arms of about 10 cm long. The purified photoprotein luminesces in the presence of H_2O_2, emitting a greenish-blue light (λ_{max} 482 nm). Molecular oxygen is probably not needed for the luminescence reaction. The molecular weight of *Ophiopsila* photoprotein is estimated to be about 45 000 by gel filtration. The absorption spectrum of a solution of the photoprotein showed a small peak (λ_{max} 423 nm, with a shoulder at about 450 nm) in addition to the 280-nm protein peak. The 423-nm peak decreased slightly through the H_2O_2-triggered luminescence reaction, accompanied by a slight red shift of the peak. The photoprotein was fluorescent in greenish-blue (emission λ_{max} 482 nm; excitation λ_{max} 437 nm), and the fluorescence emission spectrum exactly coincided with the luminescence spectrum of photoprotein in the presence of H_2O_2, suggesting the possibility that the fluorescent chromophore might be the light emitter. However, the fluorescence emission of the photoprotein did not show any detectable change

after the H_2O_2-triggered luminescence reaction; an anticipated increase in the 482-nm fluorescence did not occur.

1.3
Basic Strategy of Extracting and Purifying Photoproteins

Photoproteins are usually highly reactive, unstable substances, like luciferins. Their luminescence activities are easily lost by spontaneous light emission and various other causes. In isolating active photoproteins, it is extremely important to pay special attention to prevent the loss of the luminescence activity. Compared with the isolation of luciferins, however, techniques available for isolating photoproteins are somewhat limited because of their protein nature.

The basic principle is to extract a photoprotein in an aqueous solution and purify the photoprotein by various means of protein purification, all under conditions that prevent the luminescence and denaturation of the protein molecules. Thus, the luminescence system must be reversibly inhibited during the extraction and purification of a photoprotein. The method of reversible inhibition differs depending on the nature and cofactor requirement of the system to be isolated. For example, the calcium chelator EDTA or EGTA is used to inhibit the luminescence of the Ca^{2+}-sensitive photoproteins of coelenterates and ctenophores such as aequorin, obelin, and mnemiopsin (Shimomura et al.,1962; Campbell 1974; Hastings and Morin 1968; Ward and Seliger 1974). Before the discovery of the Ca^{2+} requirement, however, aequorin was extracted with a pH 4 buffer that reversibly inactivated the photoprotein (Shimomura et al. 1962; Shimomura 1995c). In the case of the luminescence systems of *Chaetopterus* and *Pholas,* the metal ion inhibitors 8-hydroxyquinoline and diethyldithiocarbamate, respectively, were used (Shimomura and Johnson 1966; Henry and Monny 1977).

The ionic strength and the pH of buffers are also important, and these conditions should be chosen to optimize the yield of active photoprotein. The use of acidic buffers, pH 5.6–5.8, was effective in suppressing spontaneous luminescence during the extraction of the photoproteins of euphausiids and *Luminodesmus* (Shimomura and Johnson 1967; Shimomura 1981). In the case of the membrane photoprotein polynoidin and the squid photoprotein symplectin, easily soluble impurities were all washed out and the substances that cause the luminescence of the photoprotein are completely removed before the solubilization of the photoproteins; thus, inhibitors were not needed (Nicolas et al. 1982; Fujii et al. 2002).

1.4
The Photoprotein Aequorin

1.4.1
Extraction and Purification of Aequorin

Aequorin is the best-known photoprotein and has been used widely in various applications. The first step in the extraction of aequorin from the jellyfish *Aequorea* (average body weight 50 g) is to cut off the circumferential margin of umbrella that contains light organs, making about 2-mm-wide strips commonly called "rings". This process is important because it eliminates about 99% of unnecessary body parts that do not contain aequorin. The rings can be made efficiently by using specially made cutting devices (Johnson and Shimomura 1978; Blinks et al. 1978) or, much less efficiently, with scissors. The rings (about 0.5 g each) containing light organs are kept in cold seawater. Then, about 500 rings are shaken vigorously by hand with cold, saturated ammonium sulfate solution containing 50 mM EDTA (Johnson and Shimomura 1972) or with cold seawater (Blinks et al. 1978) to dislodge the particles of light organs from the rings. Then, the rings are removed by filtering through a net of Dacron or Nylon (50–100 mesh), and the light organ particles suspended in the filtrate are collected by filtration on a Büchner funnel with the aid of some Celite. The light organ particles in the filter cake are cytolyzed and the aequorin therein is extracted by shaking with cold 50 mM EDTA (pH 6.5). After filtration, crude aequorin is precipitated by saturation with ammonium sulfate.

Of the two methods of shaking the rings mentioned above, using seawater results in much cleaner crude extracts, with a little less yield, than are obtainable by shaking in saturated ammonium sulfate containing EDTA. On the other hand, saturated ammonium sulfate strongly inhibits the luminescence response of the photogenic particles to mechanical stimulation such as shaking and stirring, and it also salts out and stabilizes aequorin, thus resulting in a better yield of aequorin and less effect on the isoform composition of aequorin extracted, compared with that obtainable by shaking in seawater.

With regard to the purification of aequorin, Blinks et al. (1978) described a well-designed method for purifying an aequorin extract that has been obtained by the "seawater shaking method". The method included gel filtration on Sephadex G-50 and ion-exchange chromatography on DEAE-Sephadex A-50 and QAE-Sephadex A-50. The ion exchangers effectively separated the green fluorescent protein from aequorin. For the purification of the extract obtained by the "saturated ammonium sulfate shaking method", gel filtration on a column of Sephadex G-75 or G-100, using buffers containing 1 M ammonium sulfate and not containing ammonium sulfate, and ion-exchange chromatography on DEAE cellulose have been used (Johnson and Shimomura 1972; Shimomura and Johnson 1969, 1976). Aequorin in 1 M ammonium sulfate aggregates to a larger size (M_r > 50 000). Thus, crude aequorin is first chromatographed on Sephadex G-100 with a low-salt buffer not containing ammonium sulfate, then the aequorin fraction obtained is re-

chromatographed on the same column using a buffer containing 1 M ammonium sulfate to obtain purified aequorin. Using Sephadex G-50 is not recommended in this case, at least for the initial step, because of the presence of a large amount of the aggregated form of impurities.

1.4.1.1 Hydrophobic Interaction Chromatography

Butyl-Sepharose 4 Fast Flow (Pharmacia) is an excellent medium for purifying aequorin, supplementing the methods described above. Aequorin in a buffer solution containing 5–10 mM EDTA and 1.8 M ammonium sulfate is adsorbed on the column, and then aequorin is eluted with a buffer containing decreasing concentrations of ammonium sulfate and 5 mM EDTA. Aequorin elutes at an ammonium sulfate concentration between 1 M and 0.5 M.

Because apoaequorin elutes at ammonium sulfate concentrations lower than 0.1 M, aequorin is cleanly separated from apoaequorin. Thus, it is possible to prepare virtually pure samples of aequorin using a single column as follows. The aequorin sample is first luminesced by the addition of a sufficient amount of Ca^{2+}. The spent solution, after dissolving 1 M ammonium sulfate, is adsorbed on a column of butyl-Sepharose 4. The apoaequorin adsorbed on the column is eluted with decreasing concentration of ammonium sulfate starting from 1 M; apoaequorin elutes at an ammonium sulfate concentration lower than 0.1 M. The apoaequorin eluted is regenerated with coelenterazine in the presence of 5 mM EDTA and 5 mM 2-mercaptoethanol (see the section 1.4.5.3). The solution of regenerated aequorin in 1.8 M ammonium sulfate is adsorbed on the same butyl-Sepharose 4 column. Aequorin adsorbed on the column is eluted with a decreasing concentration of ammonium sulfate, yielding highly purified aequorin.

1.4.2 Properties of Aequorin

Aequorin is a conjugated protein that has a relative molecular mass of approximately 20 000–21 000 (Blinks et al. 1976), and it contains a functional chromophore corresponding to roughly 2% of the total weight. A concentrated solution of aequorin is yellowish because of its absorption peak (λ_{max} about 460 nm), in addition to a protein absorption peak at 280 nm ($A_{1\%,1cm}$ 30.0; Shimomura 1986b). Aequorin is non-fluorescent, except for a weak ultraviolet fluorescence that is due to its protein moiety. Natural aequorin is a mixture of isoforms, containing more than a dozen of them, designated aequorins A, B, C, etc. (Blinks et al. 1976; Shimomura 1986b). The isoelectric points of these isoforms lie between 4.2 and 4.9 (Blinks and Harrer 1975). The solubility of aequorin in aqueous buffers is generally greater than 30 mg mL^{-1} (Shimomura and Johnson 1979a). Aequorin can be salted out from aqueous buffers with ammonium sulfate, although the salting out is not complete even after the complete saturation of ammonium sulfate. Usually 1–2% of aequorin remains in the solution.

One milligram of aequorin emits 4.3–5.0 × 10^{15} photons at 25 °C when Ca^{2+} is added, at a quantum yield of 0.16 (Shimomura and Johnson 1969, 1970, 1979a; Shimomura 1986b). In the presence of an excess of Ca^{2+}, the luminescence reaction of aequorin has a rate constant of 100–500 s^{-1} for the rise and 0.6–1.25 s^{-1} for the decay (Loschen and Chance 1971; Hastings et al. 1969).

1.4.2.1 **Stability**

Aequorin is always emitting a low level of luminescence, spontaneously deteriorating by itself. Thus, the information concerning its stability is important when aequorin is used as a calcium probe. The stability of aequorin in aqueous solutions containing EDTA or EGTA varies widely by temperature, pH, concentration of salts, and impurities. To minimize the deterioration of aequorin, it is most important to keep the temperature as low as possible. The half-life of aequorin in 10 mM EDTA, pH 6.5, is about 7 days at 25 °C. At room temperature, aequorin is most stable in solutions containing 2 M ammonium sulfate or when it is precipitated from saturated ammonium sulfate. Freeze-dried aequorin is also stable, but the process of drying always causes a loss of luminescence activity (see below). All forms of aequorin are satisfactorily stable for many years at –50 °C or below, but all deteriorate rapidly at temperatures above 30–35 °C. A solution of aequorin should be stored frozen whenever possible because repeated freeze–thaw cycles do not harm aequorin activity.

1.4.2.2 **Freeze-drying**

A note on freeze-dried aequorin may be appropriate here, because most commercial preparations of aequorin are sold in a dried form. The process of freeze-drying aequorin always results in some loss of luminescence activity. Therefore, aequorin should not be dried if a fully active aequorin is required. The loss is about 5% at the minimum, typically about 10%. The loss can be slightly lessened by certain additives; the addition of 50–100 mM KCl and some sugar (50–100 mM) in the buffer seems to be beneficial. The buffer composition used for the freeze-drying of aequorin at the author's laboratory is as follows: 100 mM KCl, 50 mM glucose, 3 mM HEPES, 3 mM Bis-Tris, and 0.05 mM EDTA, pH 7.0.

1.4.3
Specificity to Ca^{2+}

Several kinds of cations other than Ca^{2+} elicit the light emission of aequorin. Some lanthanide ions (such as La^{3+} and Y^{3+}) trigger the luminescence of aequorin as efficiently as Ca^{2+}. In addition, Sr^{2+}, Pb^{2+}, and Cd^{2+} cause significant levels of luminescence; Cu^{2+} and Co^{2+} give some luminescence only in slightly alkaline buffer. However, Be^{2+}, Ba^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, and Ni^{2+} do not elicit any light from aequorin (Shimomura and Johnson 1973). In testing biological systems, however, aequorin is considered to be highly specific to Ca^{2+}, because the occurrence of a significant amount of metal ions other than Ca^{2+} is unlikely. In an *in vitro* test, all of these metal ions except Ca^{2+}, Sr^{2+}, and lanthanoids could be completely

masked by including 1 mM sodium diethyldithiocarbamate in the test solution (Shimomura and Johnson 1975b).

1.4.4 Luminescence of Aequorin by Substances Other Than Divalent Cations

As already mentioned, all forms of aequorin emit photons spontaneously and constantly, regardless of its molecular status or environment conditions, even in the absence of Ca^{2+} or in the presence of a large excess of EDTA. The light emission results in a gradual deterioration of the luminescence capability of aequorin. A luminescence intensity of this type is quite low at 0 °C, though it can be easily measured with a light meter. The intensity is temperature dependent and steeply increases with rising temperature, reaching a maximum intensity at around 60 °C (Shimomura and Johnson 1979a). Such a temperature-dependent luminescence occurs with aequorin dissolved in aqueous solutions, as well as with freeze-dried aequorin and its suspension in a certain organic solvents, such as toluene, acetone, and diglyme (bis-2-methoxyethyl ether). The quantum yield of the spontaneous luminescence of dried aequorin, when warmed with or without an organic solvent, is generally in the range of 0.003–0.005, whereas that of aequorin in aqueous solutions is considerably less (about 0.001 at 43 °C).

Aequorin also emits luminescence in the presence of thiol-modification reagents such as *p*-benzoquinone, Br_2, 1_2, *N*-bromosuccinimide, *N*-ethylmaleimide, iodoacetic acid, and *p*-hydroxymercuribenzoate (Shimomura et al. 1974). The luminescence is probably caused by the conformational change of the protein that results from the modification of cysteine residues (by causing the decomposition of the coelenterazine peroxide moiety). The luminescence is weak but lasts for more than one hour. The quantum yields in this type of luminescence never exceed 0.02 (about 15% of Ca^{2+}-triggered luminescence) at 23–25 °C. To prevent this type of luminescence, any reagents that might react with an SH group should be avoided.

1.4.5 Mechanism of Aequorin Luminescence and Regeneration of Aequorin

1.4.5.1 Structure of Aequorin

Aequorin is a globular protein with three "EF-hand" domains to bind Ca^{2+}, and it accommodates a peroxidized coelenterazine in the central cavity of the protein (Head et al. 2000). The presence of a peroxy group bound to position 2 of the coelenterazine moiety was previously suggested (Shimomura and Johnson 1978) and confirmed by ^{13}C nuclear magnetic resonance spectroscopy (Musicki et al. 1986). The protein conformation of aequorin is much more compact and rigid than that of apoaequorin, consistent with the results of the fluorescence polarization studies and the papain digestion of those proteins (La and Shimomura 1982). The functional group, peroxidized coelenterazine, is shielded from outside solvent. Therefore, no reagent can react with this group without first reacting with the

residues of the protein, and any reaction with the protein residues triggers the breakdown of the peroxidized coelenterazine.

1.4.5.2 **Luminescence Reaction**

In the case of aequorin reacting with Ca^{2+}, a conformational change of protein takes place when one molecule of aequorin is bound with two Ca^{2+} ions (Shimomura 1995b). The conformational change results in the cyclization of the peroxide of coelenterazine into the corresponding dioxetanone, which instantly decomposes and produces the excited state of coelenteramide and CO_2 (Shimomura et al. 1974; Shimomura and Johnson 1978). When the energy level of the excited state of coelenteramide falls to ground state, light is emitted. A simplified mechanism of the luminescence reaction is illustrated in Fig. 1.1.

The spent solution of the luminescence reaction of aequorin is a mixture of coelenteramide, apoaequorin, and Ca^{2+} that forms a complex called "blue fluorescent protein" (fluorescence emission maximum about 465–470 nm). The dissociation constant of the complex into coelenteramide plus apoaequorin in the presence of 0.5 mM Ca^{2+} is 7×10^{-6} M at pH 7.4 and 25 °C (Morise et al. 1974; based on the molecular weight of aequorin 21 000). Thus, the luminescence

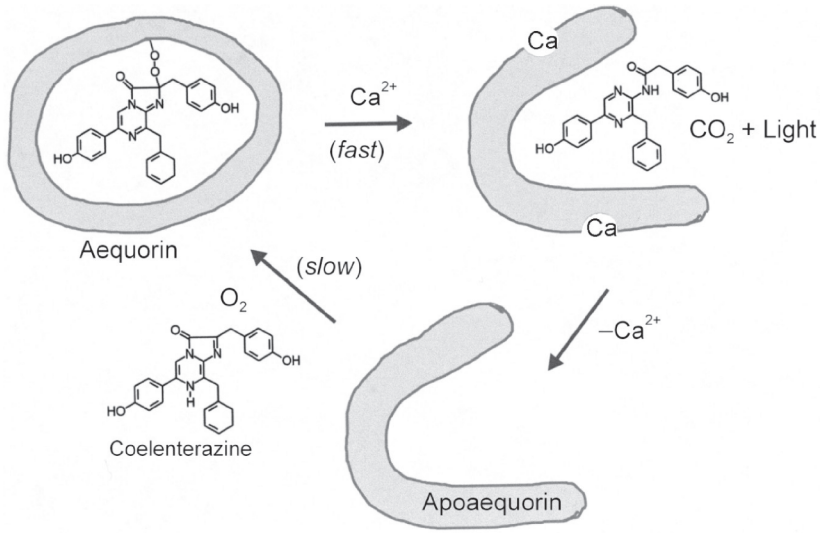


Fig. 1.1 Schematic illustration of a simplified mechanism of the luminescence and regeneration of aequorin. Aequorin (upper left) is a globular protein that contains peroxidized coelenterazine sealed in its central cavity and has three EF-hand Ca^{2+}-binding sites on the outside. When the protein is bound with two Ca^{2+} ions, an intramolecular reaction starts, resulting in the formation of coelenteramide and CO_2, accompanied by the emission of blue light (λ_{max} 460 nm) and opening of the protein shell (upper right). The protein part, apoaequorin (bottom), can be regenerated into the original aequorin by incubation with coelenterazine and molecular oxygen in the absence of Ca^{2+}. In the regeneration reaction, addition of a low concentration of 2-mercaptoethanol increases the yield of regenerated aequorin by protecting the functional cysteine residues of apoprotein.

reaction product of aequorin is usually blue fluorescent, unless the concentration of aequorin used is too low (much less than 1 μM) to form the fluorescent complex. The blue fluorescence of the complex (λ_{max} 465–470 nm) closely matches the bioluminescence emission of aequorin, giving a basis to the postulation that the fluorescent complex is the light emitter of aequorin bioluminescence (Shimomura and Johnson 1970), although it now seems an oversimplification considering that the conformation of apoaequorin continues to change for several minutes after the light emission.

When the light emission of aequorin is measured in low-ionic-strength buffers containing no inhibitor, the log–log plot of the luminescence intensity versus Ca^{2+} concentration gives a sigmoid curve having a maximum slope of about 2.0 for its middle part (Shimomura and Johnson 1976; Shimomura and Shimomura 1982), indicating that the binding of two Ca^{2+} ions to one molecule of aequorin is required to trigger the luminescence of aequorin.

1.4.5.3 **Regeneration**

Apoaequorin can be reconstituted into aequorin by incubation with coelenterazine in the presence of O_2 and 2-mercaptoethanol, which the role of the latter substance is to protect the functional sulfhydryl groups of apoaequorin during the regeneration reaction (Shimomura and Johnson 1975a). For the regeneration reaction to occur, there is no need to separate coelenteramide from apoaequorin if the material contains it. Usually, the product of the luminescence reaction is incubated at 0–5 °C in a pH 7.5 buffer solution containing 5 mM EDTA, 3 mM 2-mercaptoethanol, and an excess of coelenterazine (at least 2 μg mL^{-1} more than the calculated amount). The regeneration is usually 50% complete within 30 min and practically 100% complete after 3 h.

When the regeneration reaction of apoaequorin is carried out in the presence of an excess of free Ca^{2+}, rather than in 5 mM EDTA, the result is a continuous, weak light emission from the reaction mixture. This weak luminescence lasts many hours, differing from the short, bright flash of the Ca^{2+}-triggered luminescence of aequorin. The weak luminescence of the regeneration mixture in the presence of Ca^{2+} can be intensified several times by including 0.5% diethylmalonate in the reaction medium (Shimomura and Shimomura 1981).

During the regeneration in the presence of Ca^{2+} described above, apoaequorin appears to be acting as an enzyme that catalyzes the luminescent oxidation of coelenterazine. The mechanism involved might be a simple, straightforward one: aequorin is first formed, and then it instantly reacts with Ca^{2+} to emit light. This simple mechanism, however, has no experimental support at present; the regeneration reaction of aequorin in the presence of EDTA was not activated by diethylmalonate, suggesting either that Ca^{2+} is needed in the activation by diethylmalonate or that aequorin is not an intermediate in the luminescence reaction in the presence of Ca^{2+} (Shimomura and Shimomura 1981). Whatever the mechanism, apoaequorin must be a very sluggish enzyme if it is an enzyme. Apoaequorin has a turnover number of 1–2 per hour (Shimomura and Johnson 1976).

1.4.6
Inhibitors of Aequorin Luminescence

All thiol-modification reagents cause weak, spontaneous luminescence of aequorin in the absence of Ca^{2+}, as already mentioned. They are in effect inhibitors of the Ca^{2+}-triggered luminescence of aequorin, because the quantum yields of aequorin in the luminescence caused by these reagents (~0.008) are much lower than that of the Ca^{2+}-triggered luminescence of aequorin (Shimomura et al. 1974).

Bisulfite, dithionite, and *p*-dimethyaminobenzaldehyde are all strongly inhibitory even at micromolar concentrations (Shimomura et al. 1962). It has been found that the functional group of aequorin, i.e., a peroxide of coelenterazine, decomposes without light emission when the photoprotein is treated with bisulfite or dithionite, resulting in the formation of a corresponding hydroxy-coelenterazine or coelenterazine (Shimomura and Johnson 1978).

A number of inorganic and organic substances at high concentrations (> 50 mM) suppress the luminescence intensity of the Ca^{2+}-triggered light emission. Thus, KCl (100–150 mM) used in physiological buffers is significantly inhibitory. Magnesium ions are inhibitory at millimolar concentrations, probably by competing with Ca^{2+} (cf. Blinks et al. 1976).

EDTA and EGTA can inhibit the Ca^{2+}-triggered luminescence of aequorin in two ways: (1) when free Ca^{2+} is removed from the reaction medium by chelation, the luminescence reaction is practically stopped; and (2) when the free (unchelated) forms of these chelators directly bind with the molecules of aequorin, inhibition results (Shimomura and Shimomura 1982; Ridgway and Snow 1983). The second type of inhibition is strong in solutions of low ionic strength and in the absence of other inhibitor ions such as Mg^{2+}, but it is relatively weak in the presence of 0.1 M KCl (Shimomura and Shimomura 1984), presumably because aequorin is already inhibited by KCl. Therefore, great care must be taken if EDTA or EGTA is to be used in the calibration of the Ca^{2+} sensitivity of aequorin; this is especially important in the case of low-ionic-strength calcium buffers. It should also be noted that in usual calcium buffers, the lower the Ca^{2+} concentration, the higher the inhibitory free chelator concentration, resulting in a slope steeper than the true slope in the log–log plot of luminescence intensity versus Ca^{2+} concentration.

1.4.7
Recombinant Aequorin

The cloning and expression of apoaequorin cDNA was accomplished by two independent groups in 1985. One of these groups analyzed the cDNA clone AQ440 they obtained and reported that apoaequorin is composed of 189 amino acid residues (M_r 21 400) with an NH_2-terminal valine and a COOH-terminus proline (Inouye et al. 1985, 1986), which is consistent with the results of the amino acid sequence analysis of native aequorin reported by Charbonneau et al. (1985). In contrast, the other group reported that the cDNA AEQ1 they obtained contains

the entire protein-coding region of 196 amino acid residues, which includes seven additional residues attached to the N-terminus, and the apoaequorin expressed in *Escherichia coli* showed a molecular weight of 20 600 (Prasher et al. 1985, 1987). The recombinant aequorin of the former group did not exactly match any of the isoforms of natural aequorin in the HPLC mobilities and the properties of Ca^{2+}-triggered luminescence (Shimomura et al. 1990). No detailed comparison has been made with the recombinant aequorin obtained by the latter group, although a brief test indicated that the recombinant aequorins from both sources are practically identical.

1.4.8 Semi-synthetic Aequorins

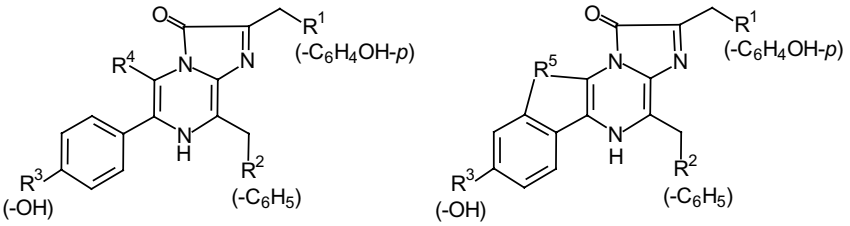
The core cavity of the aequorin molecule can accommodate various synthetic analogues of coelenterazine in place of coelenterazine. The coelenterazine moiety in native aequorin can be replaced by a simple process. First, aequorin is luminesced by the addition of Ca^{2+}, and then the apoaequorin produced is regenerated with an analogue of coelenterazine in the presence of EDTA, 2-mercaptoethanol (or DTT), and molecular oxygen. The products are called semi-synthetic aequorins and are identified with an italic prefix (see Table 1.2). Semi-synthetic aequorins can be prepared from both native aequorin and recombinant aequorin, using various synthetic analogues of coelenterazine. A large number of coelenterazine analogues were synthesized, and about 50 kinds of semi-synthetic aequorins have been prepared and tested (Shimomura et al. 1988, 1989, 1990, 1993). Some semi-synthetic aequorins are significantly different from the native type of aequorin in various properties, including spectral characteristics (Shimomura 1995a) and sensitivity to Ca^{2+}, the rate of luminescence reaction, and the rise time of luminescence (Table 1.2). The relationship between Ca^{2+} concentration and the initial light intensity of various semi-synthetic aequorins is shown in Fig. 1.2.

As is apparent from the data of Table 1.2 and Fig. 1.2, the tolerance of the central cavity of the aequorin molecule in accommodating the coelenterazine moiety is surprisingly wide. The apparent limitations in the substitution of the coelenterazine moiety, and the changes in the Ca^{2+} sensitivity caused by the substitution, are as follows.

1. The group R^1 must be aromatic. A replacement of the original *p*-hydroxyphenyl group with a group of larger size tends to decrease Ca^{2+} sensitivity.
2. The group R^2 must be lager than the ethyl group. The replacement of the original phenyl group with a smaller non-aromatic group increases Ca^{2+} sensitivity.
3. The group R^3 must be an OH group, and no substitution is allowed on the phenyl group bearing this OH.

Table 1.2 Selected semi-synthetic aequorins derived from recombinant aequorin (Shimomura et al. 1993).

No. (Prefix)	*Structural modification of coelenterazine*[a]	*Luminescence max (nm)*	*Relative luminescence capacity*[b]	*Relative intensity at 10^{-6} or 10^{-7} M Ca^{2+}*[c]	*Half-total time(s)*[d]
1	None	466	1.00	1.00	M
2 (*h*)	R^1: C_6H_5	466	0.75	16	M
3 (*f*)	R^1: C_6H_4F(*p*)	472	0.80	20	M
4 (*f2*)	R^1: $C_6H_3F_2$(*m,p*)	470	0.80	30	M
6 (*cl*)	R^1: C_6H_4Cl(*p*)	464	0.92	0.6	5
9 (*n*)	R^1: β-naphthyl	468	0.25	0.15	5
9′ (*n*/J)[e]	R^1: β-naphthyl	467	0.30	0.07	5
12 (*cp*)	R^2: cyclopentyl	442	0.63	28	F
13 (*ch*)	R^2: cyclohexyl	453	1.00	15	F
17 (*fb*)	R^1: C_6H_4F(*p*), R^2: *n*-butyl	460	0.20	1100	2
19 (*hcp*)	R^1: C_6H_5, R^2: cyclopentyl	445	0.65	500	F
21 (*hch*)	R^1: C_6H_5, R^2: cyclohexyl	450	0.52	80	F
22 (*fch*)	R^1: C_6H_4F, R^2: cyclohexyl	462	0.43	73	M
23 (m5)	R^4: methyl	440	0.37	2	M
24 (*e*)	R^5: CH_2CH_2	405, 472	0.50	6	F
26 (*ef*)	R^1: C_6H_4F(*p*), R^5: CH_2CH_2	405, 470	0.35	40	F
27 (*ech*)	R^2: cyclohexyl, R^5: CH_2CH_2	402, 440	0.40	8	F
Fluorescein-labeled[f]		528	1.00	2	M



a) Only the changes from the coelenterazine structure are shown in this column. Those unchanged are shown in parentheses in the above structures.
b) The ratio in luminescence capacity: semi-synthetic aequorin/unmodified aequorin.
c) The ratio in luminescence intensity: semi-synthetic aequorin/unmodified aequorin, in 10^{-7} M Ca^{2+} for a value of 1 and larger and in 10^{-6} M Ca^{2+} for a value of less than 1.
d) The time required to emit 50% of the total light in 10 mM calcium acetate: F, 0.15–0.3 s; M, 0.4–0.8 s. The half-rise time of luminescence: F, 2–4 ms, all others, 6–20 ms.
e) Prepared from aequorin isoform J.
f) Fluorescein was chemically bound to apoaequorin, followed by regeneration using unmodified coelenterazine.

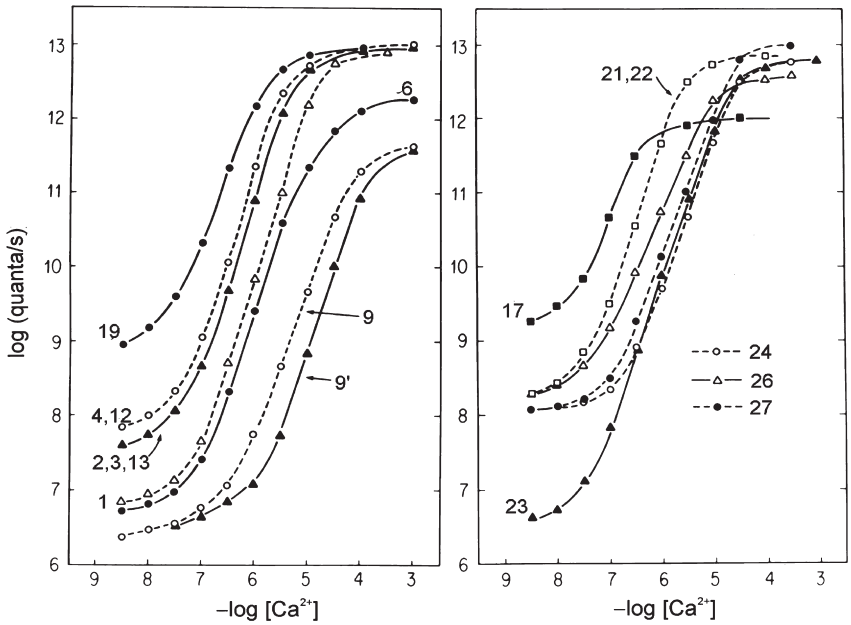


Fig. 1.2 Relationship between Ca^{2+} concentration and the initial light intensity of various recombinant semi-synthetic aequorins and *n*-aequorin J (a semi-synthetic natural aequorin made from an isoform, aequorin J). The curve number corresponds to the photoprotein number used in Table 1.2.
A photoprotein (3 μg) was added to 3 mL of Ca^{2+} buffer with various pCa values (pH 7.0) containing 1 mM total EGTA, 100 mM KCl, 1 mM free Mg^{2+}, and 1 mM MOPS, at 23–24 °C.
The data are taken from Shimomura et al. (1993).

1.4.8.1 *e*-Aequorins

e-Aequorins, containing a ligand of *e*-coelenterazine, show properties significantly different from other aequorins (Shimomura et al. 1988, 1989, 1990, 1993). In the structure of *e*-coelenterazines, the 5 position of the imidazopyrazinone structure is bound with the α position of the 6-(*p*-hydroxyphenyl) group through an ethylene linkage, thus restraining the two ring systems into the same plane. The luminescence reactions of *e*-aequorins are fast, with a half-rise time of 2–4 ms and a half-total time of 0.15–0.3 s, like *ch*-aequorins with an 8-cyclohexylmethyl substituent. The luminescence spectra are bimodal, with peaks at 400–405 nm and 440–475 nm. The ratio of the two peaks is variable not only with the type of aequorin but also with the measurement conditions, such as the concentration of Ca^{2+} and pH. *e*-Coelenterazines scarcely luminesce in the presence of apoaequorin, Ca^{2+}, and 2-mercaptoethanol in air (Shimomura 1995a).

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