Cause of spectral variation in the luminescence of semisynthetic aequorins

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Aequorin emits light in the presence of Ca^{2+} , decomposing into apoequorin, coelenteramide and CO_2 . Semisynthetic aequorins, produced by replacing the coelenterazine moiety in aequorin with analogues of coelenterazine, showed widely different sensitivities to Ca^{2+} as well as certain spectral variations. A group of semisynthetic aequorins, *e*-type aequorins, showed bimodal luminescence, with peaks at 400–405 nm and 440–475 nm in various intensity ratios, whereas all other aequorins luminesced with only one peak, in the range 440–475 nm. The cause of the spectral variation was studied by various experiments including: (1) comparison with the fluorescence of the spent solution and the luminescence of the spent solution produced by added coelenterazine; (2) luminescence in $^2\text{H}_2\text{O}$; (3) the rate of conformational change of apoequorin; (4) the rates of regeneration in the presence and absence of O_2 . The results suggested that the

spectrum of Ca^{2+} -triggered luminescence is strongly affected by the ionic charge on the amide N atom of the coelenteramide that is bound to apoequorin. When the amide N atom is negatively charged, light is emitted with a 440–475 nm peak. In the case of *e*-type aequorins, the negative charge on the amide N atom is less because of the structure of *e*-coelenterazine involved, resulting in the emission of a 400–405 nm peak from the uncharged form of coelenteramide; the intensity ratio of 400–405 nm peak to 440–475 nm peak is determined by the amount of negative charge resting on the amide N atom of *e*-coelenteramide at the time of light emission. Most of the spectral variations in luminescence and fluorescence can be explained on the basis of ionic and hydrophobic interaction between a coelenteramide and apoequorin.

INTRODUCTION

The photoprotein aequorin emits light in the presence of a trace amount of Ca^{2+} without requirement of any other cofactor [1]. Because of its sensitivity to Ca^{2+} and harmlessness in biological systems, aequorin has been used as an intracellular Ca^{2+} indicator for the past 25 years. In the presence of Ca^{2+} , it is transformed into a blue fluorescent protein consisting of coelenteramide and apoequorin [2]. Original aequorin can be regenerated by incubating apoequorin with coelenterazine in the presence of O_2 [3]:

Aequorin was originally obtained from the jellyfish *Aequorea* [4]. The samples obtained from the jellyfish were heterogeneous, consisting of a number of isoforms [5]. Thus the composition as well as the properties of natural aequorin varied from preparation to preparation, unless the component isoacquerins were individually isolated in their pure forms [6]. Recently, various kinds of artificial forms of aequorin have been prepared. Recombinant aequorin was obtained by the regeneration of recombinant apoequorin [7–10], which was homogeneous but not completely identical with any of the natural isoacquerins known at present [11]. The replacement of the coelenterazine moiety in natural and recombinant aequorins with various synthetic analogues of coelenterazine yielded a large number of semisynthetic aequorins [11–13] that showed widely different sensitivity to Ca^{2+} . A group of semisynthetic aequorins, *e*-type aequorins, emitted bimodal luminescence, with peaks at 400–405 nm and 440–475 nm,

Scheme 1 Chemiluminescence mechanism of coelenterazine

whereas all other types of aequorin luminesced with a single emission peak (440–475 nm) [11–13]. The intensity ratio of the two peaks of *e*-aequorins varied according to the concentration of Ca^{2+} present.

Aequorin luminescence is essentially the chemiluminescence of coelenterazine that takes place in an enzymic environment. In the chemiluminescence of coelenterazine (Scheme 1), light can be emitted from the excited states of three different forms of coelenteramide, and previous studies on model compounds unambiguously showed that the amide anion (amidate ion) emits a 450–470 nm luminescence peak, and the dianion and neutral species emit a 520–530 and a 380–400 nm peak respectively [14,15]. Thus it is reasonable to consider that the 440–475 nm peak in aequorin luminescence is emitted from the amide anionic form of coelenteramide and the 400–405 nm peak (when it occurs) from the neutral form of the same compound.

The spectral variation of semisynthetic aequorin must be caused by the chemical structure of coelenterazine involved and the interaction between the coelenterazine and apoequorin. The 400–405 nm luminescence peak of *e*-aequorins could be emitted as the result of various mechanisms, such as an enhanced rate of protonation at the amide anion of coelenteramide, the presence of an electronegative centre in the structure of *e*-coelenterazine, and a change in apoequorin caused by binding *e*-coelenterazine. The aim of the present study was to understand the spectral variation of aequorin luminescence, including that of the emission of the 400–405 nm peak in the luminescence of *e*-aequorins.

MATERIALS AND METHODS

Aequorin materials, coelenterazine analogues and general conditions

Natural aequorin [1], recombinant aequorin [11], apoequorin [16] and semisynthetic aequorins [11–13] were prepared as reported. All experiments with natural aequorin were performed using a single aequorin preparation. The coelenterazine analogues used (Table 1) were samples prepared for previous studies [11–13]. Most measurements were performed at 23–25 °C; exceptions were noted.

General methods of measuring luminescence and fluorescence

Luminescence intensity and total light emitted were measured with an integrating photometer equipped with an R-928 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ, U.S.A.), which had been calibrated using *Cypridina* luminescence [17]. To measure the total light-emitting capacity of aequorin, 3 ml of 10 mM calcium acetate was added to a small portion (2–100 μl) of aequorin solution. Luminescence and fluorescence spectra were measured with a model MPF-44B fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Luminescence spectra of aequorins

Luminescence spectra of aequorins (40–80 μg) triggered by a low concentration of Ca^{2+} were measured in 0.2 ml of 1 mM EGTA/ Ca^{2+} buffer (pCa 6.5 and pH 7.1) containing 2 mM Mops and 0.1 M KCl; a trace amount of 10 mM calcium acetate was added when the intensity of luminescence was too low for measurement. Luminescence spectra of an aequorin being regenerated in the presence of Ca^{2+} were measured with the spent solution of the aequorin (100–200 μg) in 0.2 ml of 20 mM Mops

Table 1 Structures of coelenterazines used

Coelenterazine	R ²	R ³	
Unmodified	C ₆ H ₅	OH	
<i>h</i>	C ₆ H ₅	H	
<i>f</i>	C ₆ H ₅	F	
<i>cl</i>	C ₆ H ₅	Cl	
<i>br</i>	C ₆ H ₅	Br	
<i>ch</i>	C ₆ H ₁₁	OH	
<i>cp</i>	C ₅ H ₉	OH	
<i>hch</i>	C ₆ H ₁₁	H	
<i>hcp</i>	C ₅ H ₉	H	
<i>tip</i>	CH(CH ₃) ₂	F	
<i>fch</i>	C ₆ H ₁₁	F	
<i>e</i> -type			
<i>e</i>	C ₆ H ₅	OH	
<i>eh</i>	C ₆ H ₅	H	
<i>ef</i>	C ₆ H ₅	F	
<i>ech</i>	C ₆ H ₁₁	OH	
<i>ehch</i>	C ₆ H ₁₁	H	
<i>efcp</i>	C ₅ H ₉	F	

buffer, pH 7.1, containing 2 mM calcium acetate; light emission was started by the addition of a coelenterazine (approx. 5 μg in 5–10 μl of methanol) that was identical with that initially present in the aequorin. The rate of light emission parallels the rate of regeneration of aequorin molecules.

Reactivity of coelenterazine with apoequorin in the absence of O₂

A glass reaction vessel was especially constructed and used; it consisted of two parts connected by a greased ground joint; the upper part was equipped with a stopcock and the lower part had two reservoirs [18]. Apoequorin (0.5 mg) in 3 ml of 10 mM Mops buffer, pH 7.0, containing 0.1 M KCl, 2 mM EDTA and 1 mM 2-mercaptoethanol was placed in one of the reservoirs, and coelenterazine (20 μg in 50 μl of methanol) diluted with water (1 ml) was placed in the other. The vessel was evacuated through the stopcock with a mechanical vacuum pump for about 30 s, avoiding sudden boiling of the solutions inside. The evacuation was repeated six or seven times over a period of 20 min, then the two solutions were mixed by tilting the vessel. After the vessel had been left at 25 °C for 1 h, the stopcock was opened to introduce air and measurement was started. Total aequorin activity in the solution was measured every 2 min using 10 μl of the solution each time.

Comparison between the rate of light emission and the rate of conformational change in apoequorin

The conformational change was monitored by measuring the fluorescence of tryptophan residues in apoequorin. Using a constant-rate syringe CR 700-200 (Hamilton Co., Reno, NV,

U.S.A.), 40 mM calcium acetate (75 μ l) was injected into 1.5 ml of 5 mM Mops buffer, pH 7.1, containing 0.1 M KCl, 0.1 mM EDTA and natural aequorin (150 μ g) at 25 °C, and light emission and the increase in fluorescence intensity at 333 nm (excitation at 280 nm) were recorded separately.

Relative rate of regeneration of aequorins

The regeneration rate in the absence of Ca^{2+} was measured with apoaequorin (approx. 40 μ g) in 0.5 ml of 10 mM Hepes buffer, pH 7.5, containing 2 mM EDTA, 2 mM 2-mercaptoethanol and 0.1 M KCl. A coelenterazine (2.5 μ g in 5 μ l of methanol) was added to the solution, and the total light-emitting capacity of regenerated aequorin was monitored at intervals of 2 min, using a 5 μ l portion of the solution each time. When regeneration rate in the presence of Ca^{2+} was being measured, 2 mM EDTA in the above solution was replaced by 2 mM CaCl_2 , and the light intensity, which indicates regeneration rate, was continuously recorded after the addition of a coelenterazine. Data were corrected for the spontaneous chemiluminescence of the coelenterazine used, by carrying out a control run from which apoaequorin was omitted.

Disassociation of coelenteramide from apoaequorin in the spent solution of the luminescence reaction

Measurements were by gel filtration. An aequorin sample (0.5 mg) in 0.5 ml of a pH 7 buffer solution was exhaustively luminesced by the addition of an excess of Ca^{2+} . One-half of the spent solution was passed through a column (0.7 cm $\times$ 13 cm) of Sephadex G-25 (fine grade) that had been equilibrated with 10 mM Mops buffer, pH 7.1, containing 10 mM EDTA and 50 mM NaCl. Protein fractions (monitored by measuring A_{280}) were saved and combined. The remaining half of the spent solution was diluted with equilibration buffer, making its volume equal to that of the gel-filtered sample (approx. 1.2 ml). Fluorescence intensities of the two solutions were measured on excitation at 330 nm. The ratio of intensities was assumed to reflect the ratio of the amounts of coelenteramide in the two solutions.

RESULTS

Spectral data of aequorins

The Ca^{2+} -triggered luminescence spectra of various semisynthetic aequorins were compared with fluorescence emission of their spent solutions after light emission, as well as with the luminescence of the spent solutions caused by the addition of a coelenterazine. The results are summarized in Table 2. The luminescence of spent solutions is caused by instantaneous reaction of regenerated aequorin with existing Ca^{2+} (2 mM), therefore it will be called 'regeneration luminescence' in this paper. The data showed a previously unnoticed anomaly besides the bimodal spectra of *e*-type aequorins: the regeneration luminescence peaks of *cl*- and *br*-aequorins were significantly blue-shifted from the normal Ca^{2+} -triggered luminescence peaks.

The spectral curves of four aequorins (natural, recombinant *cl*-, *e*- and *ehch*-) are shown in Figure 1 as representatives. The curves for natural aequorin (Figure 1a) represent those of aequorins other than *e*-type aequorins and *cl/br*-aequorins, with closely matched spectra under the three conditions. The data for *br*-aequorin resembled those of *cl*-aequorin, and are represented by the latter (Figure 1b). The luminescence of *e*-type aequorins

Table 2 Luminescence maxima of various aequorins and the fluorescence emission maxima of the spent solutions of the luminescence reaction

Aequorin type	Luminescence with a trace of Ca^{2+} (nm)	Fluorescence emission of spent solution, excited at 330 nm (nm)*	Luminescence of spent solution with added coelenterazine (nm)*
Natural (heterogeneous)			
Unmodified	465	458	458
<i>e</i>	405 and 460	428	402 and 450
Recombinant			
Unmodified	462	460	460
<i>h</i>	467	460	460
<i>f</i>	473	465	468
<i>cl</i>	473	470	441†
<i>br</i>	472	470	447†
<i>ch</i>	453	454	446
<i>lip</i>	452	450	456
<i>fch</i>	464	460	458
<i>e</i>	405 and 465	420	400 and 460†
<i>ef</i>	404 and 472	428	400 and 470†
<i>ech</i>	400 and 440	418	401†
<i>ehch</i>	400 and 440	418	400†

* In the presence of 2 mM Ca^{2+} .

† Low-intensity luminescence.

was bimodal, but their fluorescence spectra always showed a single peak. The regeneration luminescence of *e*- and *ef*-aequorins was bimodal (Figure 1c) but *ech*- and *ehch*-aequorins showed single peaks (Figure 1d).

The fluorescence emission maximum appeared to be slightly dependent on the concentration of Ca^{2+} . Thus the fluorescence emission maximum of the spent solution of an aequorin (except *e*-types), which was exactly the same as, or slightly the blue side of, the luminescence maximum of the aequorin originally used, shifted to the red by 5–10 nm in EDTA (i.e. Ca^{2+} -free) medium. Thus it was possible to have exactly the same fluorescence maximum as the luminescence maximum by simply adjusting the Ca^{2+} concentration of the solution. The results suggest that the emission maxima of Ca^{2+} -triggered luminescence and regeneration luminescence might be similarly dependent on Ca^{2+} concentration.

Luminescence reaction in $^2\text{H}_2\text{O}$

In order to examine the possibility that the protonation of the amide anion might be faster in the luminescence of *e*-type aequorins than in the other types of aequorin, the ratio of luminescence intensities I_{405}/I_{465} (*e*-aequorin) and I_{400}/I_{440} (*ech*- and *ehch*-aequorins) in H_2O medium and $^2\text{H}_2\text{O}$ medium (99% atom enrichment) were compared. Against expectation, the ratio was found to be higher in $^2\text{H}_2\text{O}$ than in H_2O by about 15% for all the aequorins tested. Thus it appears that an effect of $^2\text{H}_2\text{O}$ on the protein part of aequorin predominated over the rate of protonation at the amide anion of coelenteramide.

Comparison between the rate of light emission and the rate of conformational change

Tryptophan fluorescence increased about 2.5-fold when Ca^{2+} was added to aequorin. The half-completion times of the increase

Figure 1 Comparison of the emission spectra of four representative kinds of aequorin: (a) natural, (b) recombinant *cl*-aequorin, (c) recombinant *e*-aequorin and (d) recombinant *eheh*-aequorin

—, Ca^{2+} -triggered luminescence; ·····, fluorescence of the spent solution of luminescence reaction containing 2 mM Ca^{2+} ; —, luminescence of the spent solution caused by the addition of a coelenterazine. The intensity scales are arbitrary for each curve. A minor peak at 375 nm in (b) is due to Raman scattering.

Figure 2 Comparison between the rate of light emission (○) and the rate of conformational change in the protein part of aequorin measured by tryptophan fluorescence emitted at 333 nm (□)

Calcium acetate (40 mM, 75 μl) was injected into 1.5 ml of a pH 7.1 buffer solution containing natural aequorin (150 μg) at zero time. Total light and fluorescence intensity are shown in arbitrary units.

in fluorescence and light emission were 1.6 s and 0.75 s respectively, showing that conformational change was significantly slower than light emission (Figure 2).

Reaction between apoaequorin and coelenterazine in the absence of O_2

The rate of regeneration of aequorin when apoaequorin and coelenterazine were mixed in the presence of air was found to be identical with that when apoaequorin and coelenterazine were first mixed in the absence of O_2 in a sealed container, then the container was left standing for 1 h, and finally air was introduced into the mixture at zero time of regeneration (see the Materials and methods section). The experiment indicated that no significant chemical reaction between apoaequorin and coelenterazine in the absence of O_2 is detectable by the technique used.

Comparison of the rate of regeneration of semisynthetic aequorins in the absence and presence of Ca^{2+}

Under constant O_2 pressure, the regeneration rate may be affected by the chemical structure of the coelenterazine, as well as by the

Table 3 Relative rate of regeneration of semisynthetic aequorins in the absence and presence of Ca^{2+} at 24 °C

The initial relative rate of regeneration is presented in terms of the total light-emitting capacity of regenerated aequorin in arbitrary units, when 40 μg of apoaequorin in 0.5 ml of pH 7.5 buffer was allowed to react with 2.5 μg of coelenterazine analogues. One unit corresponds to approx. 2.2×10^{11} quanta/s.

Coelenterazine analogue	Initial relative rate of regeneration	
	– Ca^{2+} (with EDTA)	+ Ca^{2+}
Unmodified	7.5	2.7
<i>h</i>	1.3	4.5
<i>f</i>	1.2	4.0
<i>cl</i>	5.4	0.5
<i>ch</i>	6.6	12.0
<i>hch</i>	1.7	21.8
<i>hcp</i>	1.2	14.2
<i>fch</i>	1.3	9.0
<i>e</i>	12.4	0.13
<i>eh</i>	2.4	0.04
<i>ehch</i>	1.8	0.07
<i>etcp</i>	1.4	0.13

Table 4 Comparison of the amount of coelenteramide remaining bound to apoaequorin after gel filtration of luminescence-spent products

Gel filtration was on a Sephadex G-25 column (0.7 cm $\times$ 13 cm) equilibrated with 10 mM Mops buffer, pH 7.1, containing 10 mM EDTA and 50 mM NaCl. Aequorin C is an isoaequorin isolated from natural aequorin [6], which was later found to be a mixture of three molecular species [11].

Type of aequorin	Coelenteramide on apoaequorin (%)
Natural aequorin (heterogeneous)	17
Natural ϵ -aequorin (heterogeneous)	33
Aequorin C	14
ϵ -aequorin C	39
Recombinant aequorin (unmodified)	33
Recombinant ϵ -aequorin	67
Recombinant f -aequorin	42
Recombinant ϵf -aequorin	79

conformation of apoaequorin bound. Thus the relative rate of regeneration of semisynthetic aequorins in the presence and absence of Ca^{2+} was measured as a means of studying apoaequorin conformation. The results (Table 3) clearly indicate extremely low regeneration rates of *e*-type aequorins in the presence of Ca^{2+} compared with other types of aequorin.

Relative affinity between apoaequorin and coelenteramides

The affinity between apoaequorin and a coelenteramide formed in the light-emitting reaction may influence luminescence spectra because the conformation of apoaequorin can be altered by the coelenteramide bound to it. The method used here (gel filtration of the spent solution of the luminescence reaction) may provide information on the binding affinity of apoaequorin, but only for apoaequorin in its completely stabilized state; it would be difficult to obtain information on apoaequorin at the moment of light emission. The results presented in Table 4, obtained in EDTA medium, clearly show that *e*-type coelenteramides have stronger affinities for apoaequorin than do other types. In media con-

taining Ca^{2+} , similar results were obtained except that natural apoaequorin showed a considerably stronger affinity for each coelenteramide.

DISCUSSION

Aequorin is a highly unusual protein in two respects. First, it is a rare example of a high-energy-charged protein that can be viewed as an extremely stable enzyme–substrate complex. Secondly, the functional coelenterazine moiety can be replaced with a wide range of coelenterazine analogues without loss of its ability to luminesce in the presence of Ca^{2+} , indicating a high degree of conformability at the coelenterazine-binding site. The present subject of spectral variation is closely related to this second point.

Aequorin (20–22 kDa) is fairly stable in the absence of Ca^{2+} . Fluorescence polarization studies indicated that the conformation of aequorin is considerably more rigid than that of apoaequorin, thus aequorin is not affected by papain whereas apoaequorin is readily digested [19]. A molecule of aequorin has three Ca^{2+} -binding sites of E-F hand structure [7], but it can bind many more Ca^{2+} ions at high Ca^{2+} concentrations [20]. For the luminescence reaction to take place, a molecule of aequorin must bind to at least two Ca^{2+} ions [21,22].

One molecule of coelenterazine contains four aromatic groups that can contribute to the hydrophobic binding of this compound to apoaequorin, i.e. an imidazopyrazinone group that is capable of chemiluminescence, a phenyl (phenol) group linked by CH_2 at the 2-position, a phenyl (phenol) group directly bound at the 6-position, and a phenyl group linked by CH_2 at the 8-position. The aromatic groups at the 2- and 8-position have a considerable degree of spatial freedom, but the phenyl group bound at the 6-position can only rotate around its C-1–C-4 axis. The aromatic groups at the 2- and 6-position appear to be essential for the luminescence activity of aequorins, but that at the 8-position does not appear to be [12,13]. In the molecule of *e*-coelenterazine, the plane of the phenyl ring at the 6-position is held in the same plane as that of the imidazopyrazinone group, making a single expanded aromatic-binding group.

On the basis of results obtained here and the information given above, a simplified mechanism of aequorin luminescence is presented in Scheme 2. In aequorin molecules (Stage A), coelenterazine exists as a peroxide (the upper-centre structure in Scheme 1) [23] that is tightly bound to apoaequorin by various forces, including (a) hydrophobic interaction by the four aromatic groups, (b) bonding via the peroxy group and (c) possible ionic interaction. The peroxy group can be bound to the protein part covalently or by ionic interaction. Although nothing is known about the existence of significant ionic interaction, it is likely that the coelenterazine moiety has a negative charge in order to counteract a positive-charge centre that exists in apoaequorin (see below). If the peroxide anion is such a negative charge, (b) and (c) become a single force. There is also a slight possibility that aequorin contains the dioxetanone intermediate (the upper-right structure in Scheme 1) instead of the peroxide.

When Ca^{2+} is added to aequorin (Stage B), the binding of Ca^{2+} may cause a local conformational change in the protein, resulting in the destabilization of the peroxy function. Thus coelenterazine peroxide decomposes instantly to coelenteramide and CO_2 , accompanied by emission of light. The wavelength of the light emitted indicates that the coelenteramide produced must be in the form of a monoanion, with a negative charge on its amide N atom; a lesser possibility, namely that the negative charge is on the O atom, is ignored in the present paper. The Ca^{2+} -triggered luminescence reaction is almost instantaneous and significantly

Scheme 2 A simplified mechanism for the luminescence of aequorin and *e*-aequorin

The protein part of aequorin, apoaequorin, is illustrated in two conformations: the conformation in Ca^{2+} -free (EDTA) medium is shown by shading with oblique lines, and the conformation in Ca^{2+} medium is shown by shading with cross-hatched lines. The four aromatic binding groups in coelenterazine (CZ) and coelenteramide (CA), and the three groups in *e*-coelenterazine (*e*-CZ) and *e*-coelenteramide (*e*-CA), are shown as black rectangular bars, the bar representing the imidazopyrazinone/aminopyrazine group is at or near the bottom of each coelenterazine/coelenteramide representation.

Table 5 Fluorescence emission maxima (nm) of coelenteramide and *e*-coelenteramide in various solvents when excited at 330 nm

Solvent	Fluorescence emission maximum (nm)	
	Coelenteramide	<i>e</i> -Coelenteramide
Dioxane	388	394
Bis-(2-methoxyethyl) ether	398	405
Dimethylformamide	403	409
Dimethyl sulphoxide	411	412
Butan-1-ol	417	428
Methanol	425	432
Aqueous buffer with surfactant*	418	422

* 20 mM Hepes buffer, pH 7.5, containing 0.05% hexadecyldimethylammonio-3-propane-1-sulphonate.

faster than the conformational change in the protein, thus the conformation of apoaequorin at the moment of light emission must be largely unchanged in spite of the presence of the Ca^{2+} ions already bound.

If the luminesced aequorin is left in the presence of Ca^{2+} , the protein conformation settles down into the one that is defined by the concentration of Ca^{2+} present (Stage C). The fluorescence maximum of the material obtained from various aequorins (except *e*-types) was close to the luminescence maximum of the aequorin originally used, indicating that coelenteramide is bound as the amide anion, strongly suggesting the existence of a counteracting positive-charge centre in apoaequorin.

When coelenterazine is added to a solution of the C stage, the coelenteramide bound to apoaequorin is replaced by the coelenterazine added if O_2 is present, resulting in the formation of active aequorin. The aequorin formed, however, instantly undergoes luminescence (stage D) caused by Ca^{2+} ions already present. Thus apoaequorin is now acting as an enzyme that catalyses

luminescent oxidation of coelenterazine. It must be pointed out here that the conformation of aequorin momentarily formed cannot be the same, or as rigid, as that in stage A, because of the slow change in protein conformation.

In the case of *e*-aequorin (Scheme 2, lower part), the imidazopyrazinone-binding group in *e*-coelenterazine is expanded in size to include a *p*-hydroxyphenyl group, as already noted. It appears that this expansion causes decreased interaction between *e*-coelenterazine and apoaequorin. Thus, when *e*-aequorin was caused to luminesce by Ca^{2+} , a bimodal spectrum was emitted (Figure 2c), suggesting that the *e*-coelenteramide formed is a mixture of the neutral and monoanionic forms, with only a partial negative charge on the amide N atom (Scheme 2, B stage). The peak at 465 nm was emitted apparently by the amide anion of *e*-coelenteramide and the 404 nm peak by the neutral form; the latter peak corresponds well to the fluorescence emission peak of *e*-coelenteramide in aprotic solvents (Table 5).

The spent solution (Scheme 2, stage C) showed a single fluorescence emission peak at 420 nm, coinciding with the fluorescence maximum of uncharged *e*-coelenteramide in alcohols (Table 5), suggesting that the negative charge on the amide N atom was negligibly small and that the environment of the *e*-coelenteramide molecule was significantly less hydrophobic compared with that at stage B. In the case of the regeneration luminescence of *e*-aequorin (Scheme 2, stage D), the peak wavelengths were similar to those of normal Ca^{2+} -triggered luminescence (stage B) but the intensity ratio of the peaks differed significantly. The difference suggests that the amount of negative charge on the amide N atom was different, probably because of the difference in apoaequorin conformation.

Other *e*-type aequorins are generally similar to *e*-aequorin, except for some differences in peak wavelengths and the intensity ratio of the two peaks; these are apparently caused by the chemical structure of the coelenterazine involved, and by ionic and hydrophobic interaction between the coelenterazine and apoaequorin. With regard to *ech*- and *ehch*-aequorins, both the fluorescence of the spent solution (stage C) and the regeneration

luminescence (stage D) showed a single peak, indicating that the negative charge on the amide N atom is very small, but the peak wavelengths suggest that the environment of the light emitter is significantly more hydrophobic in stage D than in stage C.

In the regeneration luminescence of *cl*-aequorin (and *br*-aequorin), the emission peak (441 nm) was markedly shifted to the blue side of the normal luminescence peak (473 nm). A possible interpretation is that an electron-withdrawing halogen atom weakened the negative charge of the amide N atom in coelenteramide bound to apoaequorin, via field and/or inductive effects, thus making the light emission from the neutral form predominant; the peak wavelength was close to the fluorescence emission maximum of *cl*-coelenteramide in methanol (430 nm).

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