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Semi-synthetic aequorin

An improved tool for the measurement of calcium ion concentration

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The photoprotein aequorin isolated from the jellyfish *Aequorea* emits blue light in the presence of Ca^{2+} by an intramolecular process that involves chemical transformation of the coelenterazine moiety into coelenteramide and CO_2. Because of its high sensitivity to Ca^{2+}, aequorin has widely been used as a Ca^{2+} indicator in various biological systems. We have replaced the coelenterazine moiety in the protein with several synthetic coelenterazine analogues, providing semi-synthetic Ca^{2+}-sensitive photoproteins. One of the semi-synthetic photoproteins, derived from coelenterazine analogue (II) (with an extra ethano group), showed highly promising properties for the measurement of Ca^{2+}, namely (1) the rise time of luminescence in response to Ca^{2+} was shortened by approx. 4-fold compared with native aequorin and (2) the luminescence spectrum showed two peaks at 405 nm and 465 nm and the ratio of their peak heights was dependent on Ca^{2+} concentration in the range of pCa 5–7, thus allowing the determination of $[Ca^{2+}]$ directly from the ratio of two peak intensities. Coelenterazine analogue (I) (with a hydroxy group replaced by an amino group) was also incorporated into apo-aequorin, yielding a Ca^{2+}-sensitive photoprotein, which indicates that an electrostatic interaction between the phenolate group in the coelenterazine moiety and some cationic centre in apo-aequorin is not important in native aequorin, contrary to a previous suggestion.

INTRODUCTION

The photoprotein aequorin, isolated from the jellyfish *Aequorea*, emits blue light in the presence of Ca^{2+} (Shimomura *et al.*, 1962) through an intramolecular process in which the photoprotein is transformed into apo-aequorin, coelenteramide and CO_2 (Shimomura & Johnson, 1978). Aequorin can be regenerated from apo-aequorin by incubation with coelenterazine in the presence of O_2 (Shimomura & Johnson, 1975; Shimomura & Shimomura, 1981). In aequorin, the coelenterazine moiety is oxygenated (Shimomura & Johnson, 1978; Musicki *et al.*, 1986). These reactions are schematically summarized in Scheme 1.

The standard isolation procedure used in the past gave aequorin consisting of a mixture (Blinks & Harrer, 1975) of closely related photoproteins, i.e. isoaequorins, with M_r values of approx. 20000. Eight of these isoaequorins have been successfully separated and characterized (Shimomura, 1986). Each of the chromatographically homogeneous isoaequorins was distinguished from heterogeneous aequorin by adding a capital letter after aequorin (e.g. aequorin C). Some of the isoaequorins were crystallized (Shimomura, 1986). One type of apo-isoaequorin has been cloned (Prasher *et al.*, 1985; Inouye *et al.*, 1985).

The light-emitting reaction of aequorin is selectively triggered by Ca^{2+}; thus aequorin has been used as a Ca^{2+} indicator in a wide variety of biological systems, including living single cells (Ashley & Campbell, 1979; Blinks *et al.*, 1976, 1982). Chemical modification of the protein part of aequorin was attempted in order to improve the ability of aequorin to measure Ca^{2+}, resulting in modified aequorins with slightly better sensitivities to Ca^{2+} and with yellow luminescence (Shimomura & Shimomura, 1985). The present study was concerned with the chemical modification of the functional part of aequorin, resulting in a highly promising semi-synthetic aequorin for measuring Ca^{2+} concentration.

EXPERIMENTAL

Photoprotein aequorin

Aequorin was extracted and purified as previously described (Shimomura & Johnson, 1969, 1976) with a few minor modifications, including the use of DEAE-Sephacel (Pharmacia) instead of DEAE-cellulose for ion-exchange chromatography and the use of phenyl-Sepharose CL-4B (Pharmacia) for gradient chromatography with a decreasing concentration of $(NH_4)_2SO_4$. The latter was also used for concentrating dilute aequorin solutions by adsorption in the presence of 1 M-$(NH_4)_2SO_4$ and elution with a dilute buffer. All the experiments performed with aequorin were done with a single preparation of the photoprotein; it consisted of a typical composition of isoaequorins (approx. 50% aequorin C). Aequorins A–F were separated and purified by h.p.l.c. in accordance with the procedure previously reported (Shimomura, 1986).

Coelenterazine analogues

With the necessary modifications of the synthetic route to coelenterazine (Kishi *et al.*, 1972; Inoue *et al.*, 1975),

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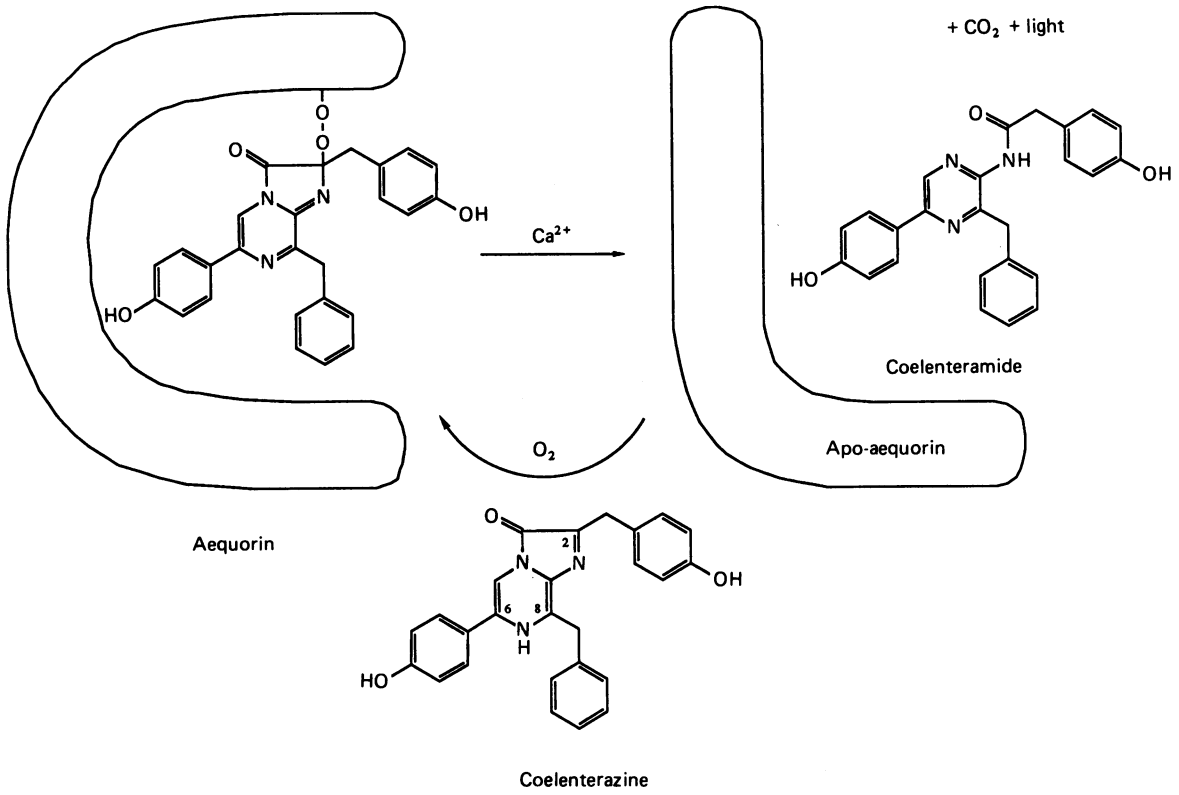

Scheme 1. Schematic illustration of the luminescence and regeneration of aequorin

four coelenterazine analogues were synthesized from 4'-nitroacetophenone (analogue I), 6-methoxy-1-tetralone (analogues II and IV) and 5-methoxy-1-indanone (analogue III), and fully characterized. Analogue (I): ^{1}H n.m.r. δ ([$^2H_4$]methanol) 4.05 (2H, s), 4.37 (2H, s), 6.67 (2H, d, $J = 8.6$ Hz), 6.71 (2H, d, $J = 8.6$) 7.15 (2H, d, $J = 8.5$), 7.19–7.40 (7H, m) and 7.48 p.p.m. (1H, s); u.v. (methanol) $\lambda_{max.}$ 205 ($\log\epsilon$ 4.55), 277 ($\log\epsilon$ 4.41) and 428 nm ($\log\epsilon$ 3.91). Analogue (II): ^{1}H n.m.r. δ ([$^2H_4$]-methanol) 2.90 (2H, t, $J = 7.6$ Hz), 3.73 (2H, broad t), 3.98 (2H, s), 4.39 (2H, s), 6.67–6.71 (4H, m), 7.13 (2H, d, $J = 8.5$) and 7.21–7.60 p.p.m. (6H, m); u.v. (methanol) $\lambda_{max.}$ 207 ($\log\epsilon$ 4.47), 280 ($\log\epsilon$ 4.34) and 433 nm ($\log\epsilon$ 3.89). Analogue (III): ^{1}H n.m.r. δ ([$^2H_4$]methanol) 4.05 (2H, s), 4.29 (2H, s), 4.43 (2H, s), 6.69 (2H, d, $J = 8.5$ Hz), 6.79 (1H, dd, $J = 2.3$, 8.3), 7.02 (1H, d, $J = 2.0$), 7.17 (2H, d, $J = 8.5$), 7.19–7.39 (5H, m) and 7.53 p.p.m. (1H, d, $J = 8.3$); u.v. (methanol) $\lambda_{max.}$ 208 ($\log\epsilon$ 4.46), 272 ($\log\epsilon$ 4.29) and 430 nm ($\log\epsilon$ 3.87). Analogue (IV): ^{1}H n.m.r. δ ([$^2H_4$]methanol) 4.06 (2H, s), 4.50 (2H, s), 6.70 (2H, d, $J = 8.6$ Hz), 7.17–7.23 (5H, m), 7.29 (2H, t, $J = 7.6$), 7.42 (2H, d, $J = 7.3$), 7.62 (1H, d, $J = 8.5$), 8.40 (1H, broad s) and 9.25 p.p.m. (1H, broad s); u.v. (methanol) $\lambda_{max.}$ 238 ($\log\epsilon$ 4.76), 285 ($\log\epsilon$ 4.24), 306 ($\log\epsilon$ 4.15) and 447 nm ($\log\epsilon$ 4.05).

Incorporation of coelenterazine analogues into apoaequorin

A solution of photoprotein (1 mg/ml) was luminesced by dropwise addition of 10 mM-calcium acetate until its light-emitting capacity was completely exhausted. The spent solution was saturated with $(NH_4)_2SO_4$ and then centrifuged. After the supernatant was discarded, the pellet was dissolved in 10 mM-Hepes/NaOH buffer, pH 7.5, containing 2 mM-EDTA and 4 mM-2-mercaptoethanol at a protein concentration of 1–3 mg/ml. After cooling the solution with an ice/water bath, a saturated methanolic solution of a coelenterazine analogue was slowly added until the combined sum of the methanolic solution became about 5–6% of the total volume. The progress of the incorporation was monitored by the luminescence activity of the solution. The luminescence activity was recorded by adding 3 ml of 10 mM-calcium acetate to 1 μl of the solution. The time required to complete the incorporation was approx. 1 h for coelenterazine analogues (II) and (III) and 1–2 days for analogues (I) and (IV). When the reaction was completed, excess reagents were removed by gel filtration (Sephadex G-25; 5 mM-Mops/NaOH buffer, pH 7.0, containing 0.1 mM-EDTA and 100 mM-KCl). The solutions of photoproteins thus prepared were stored at -75 °C and thawed at least 30 min in advance of use to allow conformational relaxation of the protein (cf. La & Shimomura, 1982).

Designation of semi-synthetic aequorins containing coelenterazine analogues

The structural modification made in the coelenterazine moiety of the aequorin is indicated by the prefix of a lower-case italic letter. Thus aequorins derived from coelenterazine analogues (I), (II), (III) and (IV) are referred to as *a*-, *e*-, *m*- and *v*-aequorin respectively.

Measurement of the rise, decay and quenching time of luminescence

To measure the rise and decay time, a droplet (less than 1 μl) of a photoprotein solution was placed at the centre of the bottom of a flat-bottomed plastic vial

(internal diam. 12 mm), and then 150 μl of 10 mM-Mops/NaOH buffer, pH 7.0, containing 1 mM-calcium acetate and 100 mM-KCl was added with a model CR 700-200 constant-rate syringe (Hamilton Co., Reno, NV, U.S.A.) to the droplet. Luminescence intensity was detected with a photomultiplier assembly and read on a model T912 storage oscilloscope (Tektronix, Beaverton, OR, U.S.A.). The oscilloscope sweep was triggered to start by a switch installed on the plunger shaft of the syringe. The reproducibility of measurements was $\pm 10\%$. Averaged values of several measurements were used.

To measure the quenching time, 20 μl of the pCa 6.0 buffer (see below) was added to 1 μl of sample solution to start weak long-lasting luminescence, and then 150 μl of 10 mM-Mops/NaOH buffer, pH 7.0, containing 10 mM-EGTA and 100 mM-KCl was added by a CR 700-200 syringe to quench the luminescence. The measurement was performed with the same set-up as used for measuring rise and decay times.

To test the mixing time of the methods adopted, the rise time of the luminol chemiluminescence was measured by addition of 150 μl of 10 mM-NaOH containing 2% (v/v) H_2O_2 to 1 μl of 10 mM-$K_3Fe(CN)_6$, containing a trace of luminol, by the same procedure as used for measurement of the rise time of photoprotein luminescence.

Measurement of Ca^{2+}-sensitivity

Luminescence intensities at pCa values from 5 to 8, in 0.5-pCa-unit intervals, were measured in EGTA/calcium buffer, pH 7.0, containing 2 mM-Mops, 1 mM free EGTA and 150 mM-KCl by the method previously described (Shimomura & Shimomura, 1984). The maximum intensity was measured in 10 mM-calcium acetate, pH 7.0, containing 150 mM-KCl, and the intensity of the Ca^{2+}-independent luminescence in 1 mM-EGTA, pH 7.0, containing 150 mM-KCl. The median sensitivity (Shimomura & Shimomura, 1985) represents the value of pCa at which the initial maximum intensity is equal to $\sqrt{(I_0 I_{max.})}$, where I_0 is the initial maximum intensity in the absence of Ca^{2+} and $I_{max.}$ is the initial maximum intensity in the presence of 10 mM-Ca^{2+}.

Spectral measurement of luminescence

Luminescence spectra were measured in the EGTA/calcium buffer of pCa 6.0 or 6.5 prepared for measuring Ca^{2+}-sensitivity (see above), with a Perkin–Elmer model MPF-44B fluorescence spectrophotometer in the d.c. mode and with its xenon light turned off. The I_{400}/I_{465} ratios were measured on the same instrument by manually changing the wavelength setting from 400 nm to 465 nm and vice versa at intervals of a few seconds while the time axis of the recorder was driven at the setting of the excitation monochrometer scan; at pCa values of 4.0 and 4.5, where the decay of the intensity was excessively fast, the ratios were obtained from the initial peak heights of luminescence observed.

Quantum yield

Samples of *e*-aequorins A–F were prepared from coelenterazine analogue (II) and corresponding apo-aequorins A–F and purified by h.p.l.c. under the conditions described previously (Shimomura, 1986). The quantum yield was estimated on the basis of the total light elicitable with 10 mM-calcium acetate per the A_{280} value for the h.p.l.c. eluate of the photoprotein peak, assuming that the $A^{0.1\%}_{1\,cm,280}$ value of each *e*-isoaequorin is identical with that of its native form previously reported (Shimomura, 1986). The integrating photometer used was calibrated with *Cypridina* bioluminescence ($\lambda_{max.}$ 462 nm; Shimomura & Johnson, 1970).

RESULTS AND DISCUSSION

Four coelenterazine analogues (Fig. 1) were successfully incorporated into apo-aequorin, resulting in semi-synthetic Ca^{2+}-sensitive photoproteins, *a*-, *e*-, *m*- and *v*-aequorin respectively. The properties of the products are summarized in Table 1 in comparison with native aequorin. It may be noted that coelenterazine analogues bearing an *o,o*-dimethyl-*p*-hydroxyphenyl, *p*-methylaminophenyl, 4-α-naphthol or 6-β-naphthol group in place of the *p*-hydroxyphenyl group at the C-6 position of coelenterazine, when incorporated into apo-aequorin, yielded negligible luminescence activity in the presence of Ca^{2+}.

a-Aequorin showed a fast rise of luminescence in response to Ca^{2+}, but its chemical yield from apo-aequorin was only 30% with respect to the amount of protein (based on visible absorption) and 8% with respect to the activity of luminescence. However, the successful formation of Ca^{2+}-sensitive *a*-aequorin indicates that an electrostatic interaction between the phenolate group in the coelenterazine moiety and some cationic centre in apo-aequorin is not important, contrary to a previous suggestion (Shimomura *et al.*, 1974). *m*-Aequorin showed a fast rise of luminescence, but the chemical yield from apo-aequorin with respect to luminescence capacity was too low for practical use. *v*-Aequorin did not show any properties superior to native aequorin as a Ca^{2+} indicator.

e-Aequorin had an approximately 4-fold faster rise of luminescence than native aequorin in response to Ca^{2+}. The formation of this photoprotein from apo-aequorin was fast and virtually quantitative with respect to the amount of protein used. Furthermore, its luminescence spectrum was bimodal, with two emission peaks at 405 nm (emission from the neutral form of coelenteramide) and 465 nm (emission from the anionic form of coelenteramide; Hori *et al.*, 1973), and more importantly, the ratio of their peak intensities was found to be pCa-dependent. Thus the pCa value of solutions can be calculated directly from the ratio of luminescence intensities at 400 nm (400 nm, instead of 405 nm, was chosen to decrease the influence from the 465 nm peak) and 465 nm without knowing the absolute level of the luminescence intensity. *e*-Aequorins A–F, prepared from homogeneous apo-aequorins A–F respectively, showed an approximately 3-fold faster rise of luminescence than the corresponding native isoaequorins, and the I_{400}/I_{465} ratio was found to be pCa-dependent with *e*-aequorins B, C and D, but not with *e*-aequorins A, E and F (Table 2). The relationship between the I_{400}/I_{465} ratio and the pCa value for a freshly prepared sample of *e*-aequorin C is shown in Figs. 2 and 3. The ratio slightly increased with freezing and thawing of the sample, presumably as the result of a small change of the protein conformation. After several cycles of freezing–thawing over a period of 2 months, the ratio increased by about 0.1 at pCa 7.0 and 0.02 at pCa 5.0, but the total luminescence capacity was not affected.

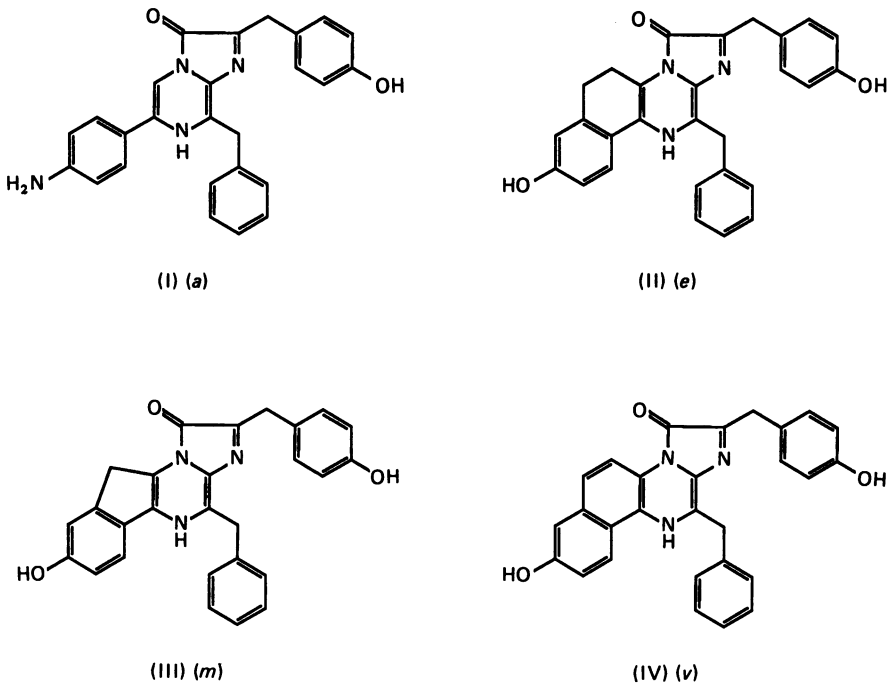

Fig. 1. Coelenterazine analogues incorporated into apo-aequorin, resulting in semi-synthetic photoproteins

Each photoprotein is identified by the prefix shown in parentheses.

Table 1. Properties of semi-synthetic aequorins derived from coelenterazine analogues

All the measurements were made at 23–25 °C with samples prepared from a single preparation of aequorin.

Property	Aequorin	*a*-Aequorin	*e*-Aequorin	*m*-Aequorin	*v*-Aequorin
Visible absorption maximum (nm)	465	425	468	457	462
Luminescence maximum (nm)	465	460	405 and 465*	460	485†
Half-rise time (ms)‡	12	6	2.8	2.8	10
Half-decay time (s)‡	0.65	0.32	0.26	0.15	70
Half-quenching time (ms)§	11	9	8	7	‖
Median sensitivity to Ca^{2+} in the presence of 150 mM-KCl (pCa)	5.95	6.05	5.88	5.90	‖
Luminescence activity (%) relative to aequorin (100%)	100	8	50	1	3.5

* The I_{400}/I_{465} ratio ranged from 0.51 (pCa 7) to 0.85 (pCa 4).
† Slight bulge at 425 nm.
‡ Tested with 1 mM-Ca^{2+} containing 150 mM-KCl.
§ Tested with 10 mM-EGTA.
‖ Values not available.

The rise and quenching times shown in Tables 1 and 2 were considered to be probably longer than the actual times of the events as a consequence of the technique of measurement employed. Although the present method does not suffer from a dead-time of observation (usually 1–2 ms in the stopped-flow method), the mixing of reactant solutions is likely to be slower and less perfect than that in the stopped-flow method. The half-rise time of the luminescence of aequorin by the stopped-flow method under comparable conditions, but in the absence of KCl, was reported as 7 ms in 10 mM-Ca^{2+} (Hastings *et al.*, 1969) and 1.4 ms in 0.5 mM-Ca^{2+} (Loschen & Chance, 1971), whereas the value by the present method in 10 mM-Ca^{2+} was 8.5 ms. The half-rise time of luminol chemiluminescence by the present method was 1.5 ms. These data indicated that the shortest possible time involved in this study was still within the response range of the method employed; however, without any information on true rise time of the luminol reaction, errors in the measurement remain unknown.

In summary, the present studies demonstrate the usefulness of *e*-aequorin and *e*-aequorin C as Ca^{2+} indicators in biological systems for two reasons: (1) the Ca^{2+}-response time is enhanced approximately 4-fold, which should allow this system to be applied to measure a rapid change of $[Ca^{2+}]$, and (2) based on the I_{400}/I_{465}

Table 2. Comparison of the properties of *e*-aequorins A–F and their native forms

The data for native aequorins A–F are shown in parentheses. All the experiments were conducted at 23–24 °C.

Property	*e*-Aequorin ... A	B	C	D	E	F
Half-rise time (ms)	4 (12)	3.5 (10)	3 (10)	3 (9)	2.8 (9)	2.7 (8)
Half-decay time (s)	0.27 (0.7)	0.2 (0.5)	0.2 (0.55)	0.22 (0.6)	0.16 (0.53)	0.16 (0.55)
Half-quenching time (ms)	10 (12)	8 (11)	9 (11)	8 (11)	8 (12)	7 (9)
I_{400}/I_{465} ratio*	0.56	pCa-dependent 0.53–0.85	pCa-dependent 0.45–0.88	pCa-dependent 0.52–0.85	0.74	0.85
Median sensitivity to Ca^{2+} (pCa)†	5.62 (5.81)	5.75 (5.96)	5.88 (6.00)	5.95 (6.25)	5.87 (6.01)	5.88 (6.11)
Quantum yield (%) relative to native form (100%)	40	45	50	51	51	51

* All native forms showed a single luminescence peak and the I_{400}/I_{465} ratios were less than 0.1.

† The values shown for *e*-aequorins A–F are in the presence of 150 mM-KCl, whereas the values for native forms are in the presence of 100 mM-KCl; the latter values are taken from the data previously reported (Shimomura, 1986).

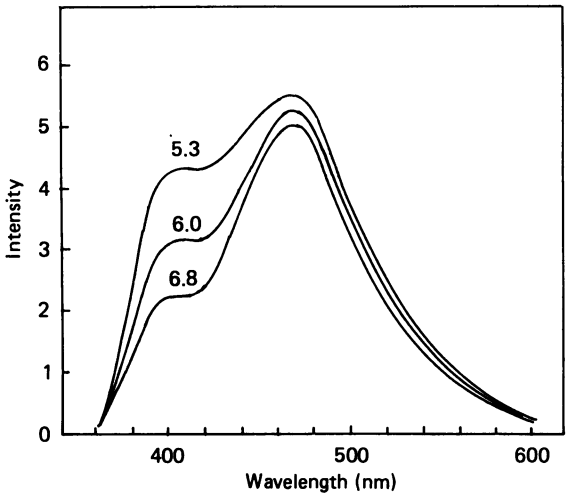

Fig. 2. Luminescence spectra of *e*-aequorin C at pCa 5.3, 6.0 and 6.8 at pH 7.0 in the presence of 150 mM-KCl at 24 °C

For full details see the Experimental section. Corrections were made for the decay of luminescence intensity during recording but not for the sensitivity difference of the photomultiplier by wavelength. Intensity units are arbitrary with each curve.

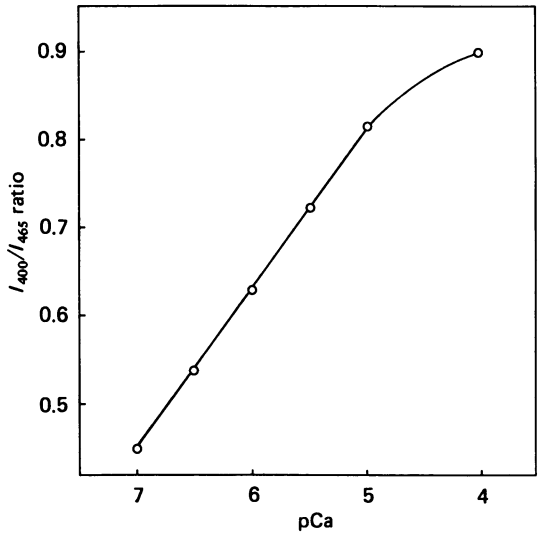

Fig. 3. Relationship between the I_{400}/I_{465} ratio and pCa for *e*-aequorin C at pH 7.0 in the presence of 150 mM-KCl at 23–24 °C

For full details see the Experimental section. The buffer solution of pCa 4 did not contain EGTA.

luminescence ratio, the direct measurement of $[Ca^{2+}]$ is feasible in the range pCa 5–7. For the second purpose, it should be best to use *e*-aequorin C. However, considering the fact that aequorin C is the most abundant isoaequorin and thus preparations of *e*-aequorin often contain more than 50% of *e*-aeuqorin C, it may be acceptable to use *e*-aequorin instead of *e*-aequorin C under certain circumstances.

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