Semi-synthetic aequorins with improved sensitivity to Ca^{2+} ions

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Thirty-seven coelenterazine analogues were synthesized and incorporated into apo-aequorin, yielding 30 semi-synthetic aequorins that have the capacity to emit a significant amount of light in the presence of Ca^{2+} . The properties of resultant photoproteins were investigated. The most prominent feature of those photoproteins was the wide range in their sensitivities to Ca^{2+} concentration. The relative intensity of Ca^{2+} -triggered luminescence of the photoproteins ranged from 0.01 to 190 when compared with natural aequorin (relative intensity 1.0) at pCa 6 for the cases where the relative intensity is less than 1 and at pCa 7 for the cases where the relative intensity is higher than 1. Eight of the semi-synthetic aequorins belonged to the class of *e*-aequorin. With two of those photoproteins, the degree of dependence of the luminescence intensity ratio I_{400}/I_{465} on pCa was greater than that with *e*-aequorin, suggesting that these two photoproteins are possibly superior to *e*-aequorin in measuring Ca^{2+} concentration by the ratio method.

INTRODUCTION

The photoprotein aequorin (M_r 20 000–21 000) isolated from the luminescent jellyfish *Aequorea* emits blue light when a trace of Ca^{2+} is added under the physiological conditions (Shimomura *et al.*, 1962; Blinks *et al.*, 1976). Because of its sensitivity, specificity and harmlessness, the photoprotein has widely been used as a Ca^{2+} indicator in various biological systems for the past 20 years (Ashley & Campbell, 1979; Blinks *et al.*, 1982). Despite its popular use, the sample of aequorin used in the past was a mixture of heterogeneous photoproteins (Blinks & Harrer, 1975), consisting of at least eight isoequorins. The separation and purification of the heterogeneous aequorin into pure homogeneous isoequorins (aequorins A–H) has been achieved only recently (Shimomura, 1986). For measuring Ca^{2+} concentration in biological systems, the use of pure isoequorin is definitely preferable to the use of an undefined sample of heterogeneous aequorin.

As shown in Scheme 1, the Ca^{2+} -triggered luminescence reaction of aequorin is known to yield coelenteramide, CO_2 and apo-aequorin, accompanied by the emission of light (Shimomura & Johnson, 1978). Apo-aequorin can be reconstituted into the original aequorin quantitatively by incubation with coelenterazine in the presence of O_2 and 2-mercaptoethanol (Shimomura & Johnson, 1975; Shimomura & Shimomura, 1981).

In the luminescence reaction, the total light emitted is proportional to the amount of aequorin that has reacted. The total light elicitable from 1 mg of aequorin was reported to be 4.5×10^{15} photons at 25 °C (Shimomura &

Johnson, 1969). Thus the quantum yield of the heterogeneous aequorin can be estimated to be 0.16 (at 25 °C), assuming its mean M_r to be 21 500, based on the M_r values and the contents of isoequorins in the heterogeneous aequorin reported previously (Shimomura, 1986). When the luminescence is elicited with a low concentration of Ca^{2+} (0.1–1 μM - Ca^{2+}) in a low-ionic-strength buffer, the intensity of light is proportional to the square of the Ca^{2+} concentration. Thus a double-logarithmic plot of the intensity against the Ca^{2+} concentration gives a slope of approx. 2.0 (Shimomura & Johnson, 1976; Shimomura & Shimomura, 1984). The luminescence is inhibited by the high concentration of various salts, including NaCl and KCl (Prendergast *et al.*, 1977). EDTA and EGTA inhibit the luminescence not only by chelation with Ca^{2+} but also by direct interaction with aequorin molecules (Shimomura & Shimomura, 1984). The inhibition, however, is insignificant in the presence of a high concentration of a salt such as 0.1 M-KCl, presumably because the luminescence is already inhibited by the salt.

We expected the reconstitution reaction of aequorin from apo-aequorin to provide a convenient and useful method of preparing a semi-synthetic aequorin containing a functional chromophore different from that of natural aequorin when a synthetic analogue of coelenterazine is used instead of coelenterazine. Indeed, we have successfully prepared four semi-synthetic aequorins and studied their properties (Shimomura *et al.*, 1988). One of the semi-synthetic aequorins, prepared with the coelenterazine analogue bearing a dimethylene ($-\text{CH}_2-\text{CH}_2-$) bridge and designated as *e*-aequorin, showed highly

Scheme 1.

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promising properties for measuring Ca^{2+} . We found that the rise time of luminescence of *e*-aequorin in response to Ca^{2+} was approx. 4 times faster than that of natural aequorin. In addition, the luminescence spectrum showed two peaks (405 nm and 465 nm) whose peak height ratio was dependent on Ca^{2+} concentration in the range of pCa 5–7, thus allowing the determination of Ca^{2+} concentration directly from the ratio of two peak intensities. In the present paper we report the preparation and properties of 30 new semi-synthetic aequorins.

MATERIALS AND METHODS

Aequorin

Aequorin was extracted and purified as previously described (Shimomura & Johnson, 1969, 1976; Shimomura *et al.*, 1988). All the experiments were performed with a single batch of heterogeneous aequorin (not purified by h.p.l.c.) having a typical composition of isoaequorins (the content of aequorin C is approx. 32%). Such a preparation of heterogeneous aequorin is routinely called 'aequorin' in this paper.

Analogues of coelenterazine

Coelenterazine analogues were synthesized by adopting, with small modifications, the synthetic route developed for natural coelenterazine (Kishi *et al.*, 1972; Inoue *et al.*, 1975). The following procedures were representative.

Aminopyrazine *N*-oxides (Scheme 2). To a stirred solution of α -oxo oxime (A) (2.0 mmol) and α -amino nitrile hydrochloride (B) (3.0 mmol) in pyridine (10 ml) was added dropwise TiCl_4 (80 μl) over 2 min at 0 °C. After being heated for 2 h at 80 °C, the mixture was cooled to room temperature, the solvent was removed under high vacuum and the black residue was partitioned between methylene dichloride/methanol (47:3, v/v) and water. The organic phase was washed once with saturated NaCl, dried over MgSO_4 , then filtered, and the solvents were removed under reduced pressure. The residue was

purified by column chromatography [silica gel, with methylene dichloride, methylene dichloride/methanol (99:1, v/v) and methylene dichloride/methanol (49:1, v/v)], affording aminopyrazine *N*-oxide (C) (approx. 50% yield based on the oxime). For the cases of the bridged aminopyrazine *N*-oxides, a second column chromatography [silica gel, with hexanes/ethyl acetate (1:1, v/v), hexanes/ethyl acetate (1:4, v/v) and hexanes/ethyl acetate (1:9, v/v)] was necessary.

Aminopyrazines (Scheme 3). A mixture of aminopyrazine *N*-oxide (C) (1.0 mmol), ethanol (40 ml) and Raney nickel (W-2, 400 mg) was refluxed under H_2 atmosphere (0.1 MPa) for 4 h. After cooling to room temperature, the mixture was filtered through Celite, the Celite was washed thoroughly with ethanol and the solvent was removed under reduced pressure, to give aminopyrazine (D) (80–90% yield). For the cases of bridged aminopyrazine *N*-oxide (C), ethanol/tetrahydrofuran (9:1, v/v) was used as solvent, and a large amount of catalyst and longer reaction time were required.

In the synthesis of natural aminopyrazine, cleavage of the methyl ether was performed by pyridine hydrochloride at elevated temperature (Kishi *et al.*, 1972). However, these conditions were not applicable for the synthesis of the aminopyrazine analogues 24–32 because of concomitant dehydrogenation at the dimethylene bridge. This problem was overcome by using ethanethiol in the presence of a Lewis acid (Node *et al.*, 1980), which gave better results for non-bridged substrates as well. To a stirred solution of aminopyrazine (D) (7.0 mmol) in a mixture of methylene dichloride (800 ml) and ethanethiol (80 ml) was added AlCl_3 (9.70 g) in one portion at 0 °C. On the addition of AlCl_3 , the colour of the solution changed to purple. After stirring for 2 h at room temperature, t.l.c. [with methylene dichloride/methanol (47:3, v/v)] indicated disappearance of the starting material. The mixture was cooled at 0 °C and ice/water (700 ml) was added. The methylene dichloride layer was separated, and the aqueous phase was saturated with

Scheme 2.

Scheme 3.

Scheme 4.

NaCl and extracted several times with tetrahydrofuran. The organic layers were washed once with saturated NaCl, dried over MgSO_4 , filtered and concentrated under reduced pressure. The residue was passed through a short column [silica gel, with hexanes/ethyl acetate (2:1, v/v)] to remove sulphur-containing impurities, giving aminopyrazine (E) (50–70 % yield).

Coelenterazine analogues (Scheme 4). A solution of aminopyrazine (E) (0.20 mmol) and α -oxo aldehyde (F) (0.30 mmol), prepared by adopting the method known in the literature (Kornblum & Frazier, 1966), in ethanol (3.0 ml), water (300 μl) and ethanolic 3 % HCl (620 μl) was heated at 80 $^\circ\text{C}$ for 3 h. For the cases of bridged substrates, a longer reaction time (8–10 h) was required. After cooling to room temperature, the solvent was removed under reduced pressure. The residue was dissolved in methanol and purified by preparative t.l.c. [with methylene dichloride/methanol (23:2, v/v) or methylene dichloride/methanol (9:1, v/v)], yielding coelenterazine analogues. The chemical yield of non-bridged coelenterazine analogues ranged from 30 to 50 % whereas the chemical yield of bridged coelenterazine analogues was about 10 %. The following ^1H -n.m.r. data [δ (p.p.m.), 300 MHz for analogues 2–24 and 500 MHz for analogues 25–32 ($^2\text{H}_4$ methanol)] were observed for coelenterazine analogues.

Analogue 2, 4.17 (2H, s), 4.40 (2H, s), 6.87 (2H, d, $J = 8.7$ Hz), 7.14–7.39 (10H, m), 7.46 (2H, d, $J = 7.5$ Hz) and 7.58 (1H, br s).

Analogue 3, 4.14 (2H, s), 4.39 (2H, s), 6.87 (2H, d, $J = 8.7$ Hz), 6.98 (2H, t, $J = 8.7$ Hz), 7.19–7.39 (7H, m), 7.45 (2H, d, $J = 8.4$ Hz) and 7.56 (1H, br s).

Analogue 4, 4.14 (2H, s), 4.40 (2H, s), 6.88 (2H, d, $J = 8.6$ Hz), 7.12–7.32 (6H, m), 7.39 (2H, d, $J = 8.5$ Hz), 7.45 (2H, d, $J = 8.4$ Hz) and 7.57 (1H, br s).

Analogue 5, 4.26 (2H, s), 4.32 (2H, s), 6.88 (2H, d, $J = 8.6$ Hz), 7.21–7.35 (5H, m), 7.44 (2H, d, $J = 8.5$ Hz) and 7.53 (1H, br s).

Analogue 6, 4.12 (2H, s), 4.39 (2H, s), 6.87 (2H, d, $J = 8.6$ Hz), 7.19–7.42 (9H, m), 7.47 (2H, d, $J = 8.2$ Hz) and 7.61 (1H, br s).

Analogue 7, 4.15 (2H, s), 4.40 (2H, s), 6.88 (2H, d, $J = 8.7$ Hz), 7.22–7.39 (9H, m), 7.46 (2H, d, $J = 8.3$ Hz) and 7.59 (1H, br s).

Analogue 8, 4.11 (2H, s), 4.39 (2H, s), 6.87 (1H, d, $J = 8.6$ Hz), 7.11 (2H, d, $J = 8.3$ Hz), 7.19 (1H, t, $J = 7.0$ Hz), 7.26 (2H, t, $J = 7.0$ Hz), 7.38 (2H, d, $J = 6.9$ Hz), 7.52 (2H, d, $J = 8.2$ Hz), 7.59 (2H, d, $J = 8.2$ Hz) and 7.71 (1H, br s).

Analogue 9, 4.34 (2H, s), 4.40 (2H, s), 6.87 (2H, d, $J = 8.7$ Hz), 7.21–7.30 (3H, m), 7.37–7.52 (7H, m), 7.60 (1H, br s) and 7.76–7.80 (4H, m).

Analogue 10, 0.91 (3H, t, $J = 6.9$ Hz), 1.39–1.41 (4H, m), 1.83 (2H, quintet, $J = 7.4$ Hz), 3.07 (2H, t, $J = 7.6$ Hz), 4.04 (2H, s), 6.67 (2H, d, $J = 8.5$ Hz), 6.91 (2H, d, $J = 8.7$ Hz), 7.13 (2H, d, $J = 8.6$ Hz), 7.52 (2H, d, $J = 8.1$ Hz) and 7.52 (1H, br s).

Analogue 11, 1.01 (6H, d, $J = 6.6$ Hz), 2.24 (1H, heptet, $J = 6.6$ Hz), 2.95 (2H, d, $J = 7.4$ Hz), 4.04 (2H, s), 6.67 (2H, d, $J = 8.5$ Hz), 6.91 (2H, d, $J = 8.6$ Hz), 7.12 (2H, d, $J = 8.5$ Hz), 7.52 (2H, d, $J = 8.5$ Hz) and 7.58 (1H, br s).

Analogue 12, 1.18–1.47 (2H, m), 1.47–1.90 (6H, m), 2.40 (1H, heptet, $J = 7.5$ Hz), 3.07 (2H, d, $J = 7.5$ Hz), 4.04 (2H, s), 6.67 (2H, d, $J = 8.2$ Hz), 6.91 (2H, d, $J = 8.6$ Hz), 7.12 (2H, d, $J = 8.3$ Hz), 7.53 (2H, d, $J = 8.1$ Hz) and 7.54 (1H, br s).

Analogue 13, 1.08–1.26 (5H, m), 1.68–1.72 (5H, m), 1.91 (1H, br s), 2.95 (2H, d, $J = 7.4$ Hz), 4.04 (2H, s), 6.67 (2H, d, $J = 8.5$ Hz), 6.91 (2H, d, $J = 8.6$ Hz), 7.13 (2H, d, $J = 8.5$ Hz), 7.51 (2H, d, $J = 8.5$ Hz) and 7.56 (1H, br s).

Analogue 14, 0.97–1.38 (5H, m), 1.70–1.86 (6H, m), 3.10 (2H, t, $J = 7.8$ Hz), 4.03 (2H, s), 6.67 (2H, d, $J = 8.5$ Hz), 6.90 (2H, d, $J = 8.6$ Hz), 7.13 (2H, d, $J = 8.5$ Hz), 7.52 (2H, d, $J = 8.1$ Hz) and 7.53 (1H, br s).

Analogue 15, 3.12 (2H, t, $J = 7.2$ Hz), 3.36 (2H, t, $J = 7.1$ Hz), 4.02 (2H, s), 6.68 (2H, d, $J = 8.5$ Hz), 6.80 (1H, d, $J = 8.8$ Hz), 6.89 (2H, d, $J = 8.7$ Hz), 7.10 (2H, d, $J = 8.4$ Hz), 7.20 (5H, m), 7.42 (1H, br s), and 7.86 (1H, d, $J = 8.8$ Hz).

Analogue 16, 4.06 (2H, s), 4.37 (2H, s), 6.69 (2H, d, $J = 8.5$ Hz), 6.87 (2H, d, $J = 8.7$ Hz), 7.02 (2H, t, $J = 8.8$ Hz), 7.14 (2H, d, $J = 8.5$ Hz), 7.40 (2H, dd, $J = 5.3, 8.8$ Hz), 7.47 (2H, d, $J = 8.0$ Hz) and 7.59 (1H, br s).

Analogue 17, 0.90 (3H, t, $J = 7.0$ Hz), 1.38–1.44 (4H, m), 1.83 (2H, quintet, $J = 7.3$ Hz), 3.07 (2H, t, $J = 7.5$ Hz), 4.11 (2H, s), 6.91 (2H, d, $J = 8.7$ Hz), 6.97 (2H, t, $J = 8.8$ Hz), 7.32 (2H, dd, $J = 5.4, 8.5$ Hz), 7.51 (2H, d, $J = 8.3$ Hz) and 7.57 (1H, br s).

Analogue 18, 1.01 (6H, d, $J = 6.6$ Hz), 2.24 (1H, heptet, $J = 6.4$ Hz), 2.95 (2H, d, $J = 7.5$ Hz), 4.12 (2H, s), 6.91 (2H, d, $J = 8.6$ Hz), 6.98 (2H, t, $J = 8.8$ Hz), 7.31 (2H, dd, $J = 5.4, 8.6$ Hz), 7.51 (2H, d, $J = 8.1$ Hz) and 7.57 (1H, br s).

Analogue 19, 1.26–1.41 (2H, m), 1.50–1.67 (2H, m), 1.67–1.85 (4H, m), 2.40 (1H, heptet, $J = 7.1$ Hz), 3.07 (2H, d, $J = 7.6$ Hz), 4.14 (2H, s), 6.91 (2H, d, $J = 8.7$ Hz), 7.15 (1H, t, $J = 7.2$ Hz), 7.24 (2H, t, $J = 7.2$ Hz), 7.31 (2H, d, $J = 7.1$ Hz), 7.52 (2H, d, $J = 7.9$ Hz) and 7.52 (1H, br s).

Analogue 20, 1.31–1.40 (2H, m), 1.56–1.60 (2H, m), 1.69–1.74 (4H, m), 2.40 (1H, heptet, $J = 7.5$ Hz), 3.07 (2H, d, $J = 7.7$ Hz), 4.12 (2H, s), 6.90 (2H, d,

$J = 8.7$ Hz) 6.98 (2H, t, $J = 8.8$ Hz), 7.32 (2H, dd, $J = 5.5, 8.7$ Hz), 7.51 (2H, d, $J = 8.4$ Hz) and 7.57 (1H, br s).

Analogue 21, 1.03–1.39 (5H, m), 1.61–1.82 (5H, m), 1.92 (1H, br s), 2.96 (2H, d, $J = 7.4$ Hz), 4.14 (2H, s), 6.91 (2H, d, $J = 8.7$ Hz), 7.15 (1H, t, $J = 7.1$ Hz), 7.24 (2H, t, $J = 7.2$ Hz), 7.31 (2H, d, $J = 7.0$ Hz), 7.51 (2H, d, $J = 8.2$ Hz) and 7.57 (1H, br s).

Analogue 22, 1.07–1.29 (5H, m), 1.68–1.72 (5H, m), 1.92 (1H, br s), 2.96 (2H, d, $J = 7.4$ Hz), 4.12 (2H, s), 6.91 (2H, d, $J = 8.7$ Hz), 6.98 (2H, t, $J = 8.8$ Hz), 7.32 (2H, dd, $J = 5.4, 8.6$ Hz), 7.50 (2H, d, $J = 8.5$ Hz) and 7.55 (1H, br s).

Analogue 23, 2.64 (3H, s), 4.00 (2H, s), 4.26 (2H, s), 6.69 (2H, d, $J = 8.5$ Hz), 6.86 (2H, d, $J = 8.6$ Hz), 7.14 (2H, d, $J = 8.5$ Hz), 7.19 (2H, d, $J = 8.5$ Hz), 7.22–7.29 (3H, m) and 7.35 (2H, d, $J = 6.8$ Hz).

Analogue 25, 2.90 (2H, t, $J = 6.8$ Hz), 3.71 (2H, br s), 4.08 (2H, s), 4.39 (2H, s), 6.69–6.71 (2H, m), 7.13–7.31 (9H, m) and 7.36 (2H, d, $J = 7.3$ Hz).

Analogue 26, 2.91 (2H, t, $J = 7.7$ Hz), 3.71 (2H, br s), 4.06 (2H, s), 4.40 (2H, s), 6.70–6.71 (2H, m), 6.98 (2H, t, $J = 8.8$ Hz), 7.18–7.32 (6H, m), 7.36 (2H, d, $J = 7.4$ Hz).

Analogue 27, 1.09–1.30 (5H, m), 1.68–1.74 (5H, m), 1.90 (1H, br s), 2.90 (2H, t, $J = 7.6$ Hz), 2.94 (2H, d, $J = 7.3$ Hz), 3.72 (2H, br s), 3.96 (2H, s), 6.67 (2H, d, $J = 8.5$ Hz), 6.71 (1H, d, $J = 2.1$ Hz), 6.74 (1H, dd, $J = 2.5, 8.4$ Hz), 7.11 (2H, d, $J = 8.5$ Hz) and 7.52 (1H, br s).

Analogue 28, 1.06–1.29 (5H, m), 1.57–1.73 (5H, m), 1.90 (1H, br s), 2.90 (2H, t, $J = 7.6$ Hz), 2.94 (2H, d, $J = 7.3$ Hz), 3.71 (2H, br s), 4.06 (2H, s), 6.71 (1H, s), 6.74 (1H, dd, $J = 2.5, 8.4$ Hz), 7.14 (1H, t, $J = 7.3$ Hz), 7.23 (2H, t, $J = 7.7$ Hz), 7.29 (2H, d, $J = 7.5$ Hz) and 7.51 (1H, br s).

Analogue 29, 0.97–1.30 (5H, m), 1.59–1.82 (5H, m), 1.90 (1H, br s), 2.91 (2H, t, $J = 7.3$ Hz), 2.95 (2H, d, $J = 7.0$ Hz), 3.70 (2H, br s), 4.04 (2H, s), 6.72 (1H, s), 6.74 (1H, dd, $J = 2.4, 8.4$ Hz), 6.97 (2H, t, $J = 8.8$ Hz), 7.31 (2H, dd, $J = 5.6, 8.2$ Hz) and 7.49 (1H, br s).

Analogue 30, 1.29–1.41 (2H, m), 1.52–1.65 (2H, m), 1.65–1.81 (4H, m), 2.38 (1H, heptet, $J = 7.5$ Hz), 2.90 (2H, t, $J = 7.6$ Hz), 3.05 (2H, d, $J = 7.6$ Hz), 3.72 (2H, br s), 3.96 (2H, s), 6.67 (2H, d, $J = 8.6$ Hz), 6.71 (1H, d, $J = 2.3$ Hz), 6.73 (1H, dd, $J = 2.5, 8.4$ Hz), 7.11 (2H, d, $J = 8.5$ Hz) and 7.52 (1H, br s).

Analogue 31, 1.29–1.41 (2H, m), 1.49–1.64 (2H, m), 1.64–1.81 (4H, m), 2.39 (1H, m), 2.91 (2H, t, $J = 7.6$ Hz), 3.06 (2H, d, $J = 7.0$ Hz), 3.70 (2H, br s), 4.06 (2H, s), 6.72 (1H, s), 6.74 (1H, dd, $J = 2.4, 8.4$ Hz), 7.15 (1H, t, $J = 7.2$ Hz), 7.24 (2H, t, $J = 7.5$ Hz), 7.29 (2H, d, $J = 7.4$ Hz) and 7.49 (1H, br s).

Analogue 32, 1.29–1.43 (2H, m), 1.51–1.65 (2H, m), 1.65–1.83 (4H, m), 2.38 (1H, heptet, $J = 7.1$ Hz), 2.91 (2H, t, $J = 7.5$ Hz), 3.05 (2H, d, $J = 7.4$ Hz), 3.71 (2H, br s), 4.04 (2H, s), 6.72 (1H, s), 6.74 (1H, dd, $J = 2.5, 8.4$ Hz), 6.97 (2H, t, $J = 8.8$ Hz), 7.31 (2H, dd, $J = 5.6, 8.4$ Hz) and 7.49 (1H, br s).

Incorporation of coelenterazine analogues into apo-aequorin

A solution of aequorin (1 mg/ml) was luminesced by the dropwise addition of 10 mM-calcium acetate at room temperature and its light-emitting capacity was completely exhausted in a period of about 30 min. We avoided

the addition of any more calcium acetate than necessary. The spent solution was cooled at 0 °C and saturated with $(\text{NH}_4)_2\text{SO}_4$. After centrifugation, the supernatant was discarded and the pellet containing apo-aequorin was dissolved in 10 mM-Hepes/NaOH buffer, pH 7.5, containing 5 mM-EDTA, at a protein concentration of about 2 mg/ml. The apo-aequorin solution was used immediately or stored frozen at –70 °C if it had to be left for more than 1 h.

The incorporation reaction was started by mixing 75 μl of the apo-aequorin solution, 500 μl of the buffer solution (10 mM-Hepes/NaOH buffer, pH 7.5, containing 5 mM-EDTA and 6 mM-2-mercaptoethanol) and 10–30 μl of saturated methanolic solution of coelenterazine analogue at 0 °C. Because an excess of coelenterazine analogue (yellow) was used, the reaction mixture remained yellow after completion of the incorporation reaction. The progress of the incorporation was monitored by the increase in the total luminescence capacity present in the reaction mixture. The luminescence capacity was measured by integrating the light emitted when 3 ml of 10 mM-calcium acetate was added to 5 μl of the reaction mixture. The reaction was judged to be complete when the luminescence capacity reached a plateau or maximum and a further addition of coelenterazine analogue did not increase the luminescence capacity. The time required for completion of the incorporation was 4–12 h in most cases. However, it took more than 24 h when analogues 2, 3, 8, 9, 22 and 23 were used. Immediately after the completion of the incorporation reaction, the solution containing semi-synthetic aequorin was used in the measurements of luminescence properties.

Measurement of luminescence properties

Luminescence capacity was measured with a MacNichol integrating photometer (Chase, 1960) that had been calibrated with *Cypridina* bioluminescence having an emission maximum at 462 nm (Shimomura & Johnson, 1970). Luminescence was elicited by adding 3 ml of 10 mM-calcium acetate to 5 μl of a photoprotein solution to be tested at room temperature. Luminescence intensity was measured when 3 ml of EGTA/ Ca^{2+} buffer, pH 7.0 and pCa 7.0 or 6.0, containing 100 mM-KCl, 2 mM-Mops and 1 mM free EGTA (Shimomura & Shimomura, 1984) was added to 5 μl of a photoprotein solution to be tested. The intensity of Ca^{2+} -independent luminescence was measured with the same EGTA buffer without added Ca^{2+} ; it should be noted that the buffer solution was not completely free of Ca^{2+} (probably pCa approx. 9.5) owing to the presence of contaminating Ca^{2+} originating mainly from KCl (ACS grade; Fisher Scientific).

The luminescence spectrum was measured with a Perkin-Elmer model MPF-44B fluorescence spectrophotometer in the d.c. mode with its xenon lamp turned off (Shimomura *et al.*, 1988). Luminescence was elicited by mixing 10–20 μl of a sample with 2 ml of an EGTA/ Ca^{2+} buffer, pCa 5.0–7.5, having the same general composition as the buffer used for the intensity measurement. The ratio of the intensity at 400 nm to the intensity of 465 nm was 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 monochromator scan. At low

Table 1. Properties of semi-synthetic aequorins derived from coelenterazine analogues **Analogue 1-23** **Analogue 24-32**

The suggested prefix to be used to designate each semi-synthetic aequorin of potential usefulness is given in parentheses in the first column. The relative luminescence capacity is the ratio of the light-emitting capacity of a semi-synthetic aequorin to that of natural aequorin, on the basis of equal protein amounts. When the incorporation reaction is quantitative, quantum yield can be given by multiplying 0.16. The relative intensity is the ratio of the luminescence intensity of semi-synthetic aequorin to that of natural aequorin at pCa 7 (> 1) or at pCa 6 (< 1), on the basis of equal protein amounts. The ratio of the intensities of Ca²⁺-independent luminescence is shown in parentheses. The half-total time is the time required to emit 50% of the total light in 10 mM-calcium acetate: F (fast), 0.15–0.3 s; M (medium), 0.4–0.8 s. The half-rise time of luminescence: F, 2–4 ms; all others, 6–20 ms.

				Light emission at 24 °C			
Analogue used				Maximum (nm)	Relative lumines- cence capacity	Relative intensity	Half-total time (s)
No.	R	R'	R''				
1 (coelenter- azine)	C ₆ H ₄ OH- <i>p</i>	C ₆ H ₅	H	465	1.00	1.00 (1.0)	M
2 (<i>h</i>)	C ₆ H ₅	C ₆ H ₅	H	464	0.82	10 (3)	M
3 (<i>f</i>)	C ₆ H ₄ F- <i>p</i>	C ₆ H ₅	H	473	0.80	18 (14)	M
4	C ₆ H ₃ F ₂ - <i>m,p</i>	C ₆ H ₅	H	470	0.68	24 (3)	M
5	C ₆ F ₅	C ₆ H ₅	H	465	0.14	25 (5)	M
6	C ₆ H ₄ Cl- <i>p</i>	C ₆ H ₅	H	463	0.85	0.19	4
7	C ₆ H ₄ Br- <i>p</i>	C ₆ H ₅	H	473	0.80	0.37 (0.25)	2.4
8 (<i>i</i>)	C ₆ H ₄ I- <i>p</i>	C ₆ H ₅	H	476	0.70	0.03	8
9 (<i>n</i>)	β-Naphthyl	C ₆ H ₅	H	467	0.26	0.01 (1)	5
10	C ₆ H ₄ OH- <i>p</i>	(CH ₂) ₃ CH ₃	H	448	0.79	12	M
11 (<i>ip</i>)	C ₆ H ₄ OH- <i>p</i>	CH(CH ₃) ₂	H	441	0.54	47 (17)	1
12 (<i>cp</i>)	C ₇ H ₄ OH- <i>p</i>	C ₅ H ₉ - <i>c</i> *	H	442	0.95	15	F
13 (<i>ch</i>)	C ₆ H ₄ OH- <i>p</i>	C ₆ H ₁₁ - <i>c</i> *	H	452	1.00	4.2 (3.5)	F
14	C ₆ H ₄ OH- <i>p</i>	CH ₂ C ₆ H ₁₁ - <i>c</i> *	H	442	0.77	2	M
15	C ₆ H ₄ OH- <i>p</i>	CH ₂ C ₆ H ₅	H	444	0.60	11	M
16	C ₆ H ₄ OH- <i>p</i>	C ₆ H ₄ F- <i>p</i>	H	466	0.70	2.8 (2)	M
17 †	C ₆ H ₄ F- <i>p</i>	(CH ₂) ₃ CH ₃	H	460	0.37	73 (45)	M
18 †	C ₆ H ₄ F- <i>p</i>	CH(CH ₃) ₂	H	449	0.16	140 (70)	M
19 † (<i>hcp</i>)	C ₆ H ₅	C ₅ H ₉ - <i>c</i> *	H	444	0.67	190 (40)	F
20 † (<i>fcp</i>)	C ₆ H ₄ F- <i>p</i>	C ₅ H ₉ - <i>c</i> *	H	452	0.57	135 (52)	M
21	C ₆ H ₅	C ₆ H ₁₁ - <i>c</i> *	H	450	0.70	45	M
22 (<i>fch</i>)	C ₆ H ₄ F- <i>p</i>	C ₆ H ₁₁ - <i>c</i> *	H	461	0.70	66 (15)	F
23	C ₆ H ₄ OH- <i>p</i>	C ₆ H ₅	CH ₃	438	0.28	0.33	M
24 (<i>e</i>) (<i>e</i> -coelenter- azine)	C ₆ H ₄ OH- <i>p</i>	C ₆ H ₅	H	405 and 465	0.50	4 (15)	F
25	C ₆ H ₅	C ₆ H ₅	H	405 and 465	0.47	9 (10)	F
26	C ₆ H ₄ F- <i>p</i>	C ₆ H ₅	H	410 and 475	0.50	35 (33)	F
27 (<i>ech</i>)	C ₆ H ₄ OH- <i>p</i>	C ₆ H ₁₁ - <i>c</i> *	H	400	0.46	4 (11)	F
28	C ₆ H ₅	C ₆ H ₁₁ - <i>c</i> *	H	400	0.30	14 (9)	F
29	C ₆ H ₄ F- <i>p</i>	C ₆ H ₁₁ - <i>c</i> *	H	400	0.30	55 (30)	F
30 †	C ₆ H ₄ OH- <i>p</i>	C ₅ H ₉ - <i>c</i> *	H	400 and 440	0.29	11	F
31 †	C ₆ H ₅	C ₅ H ₉ - <i>c</i> *	H	400 and 440	0.29	50	F
32 †	C ₆ H ₄ F- <i>p</i>	C ₅ H ₉ - <i>c</i> *	H	440 and 440	0.24	172	F

* C₅H₉-*c* and C₆H₁₁-*c* represent cyclopentyl and cyclohexyl groups respectively.

† Incorporation was carried out in the presence of 0.5 M-KCl. In the absence of KCl analogue **18** resulted in a relative luminescence capacity of 0.13 and a relative intensity of 110.

pCa values (below pCa 5.5–6.0), where the decay of intensity was excessively fast, the ratio was obtained from the initial peak heights of luminescence at 400 nm and 465 nm separately measured. The pCa 4.5 solution, pH 7.0, contained 0.032 mM-calcium acetate, 100 mM-KCl and 2 mM-Mops.

RESULTS AND DISCUSSION

Thirty-seven new coelenterazine analogues were synthesized and then incorporated into apo-aequorin in order to obtain semi-synthetic aequorins. The products of the incorporation reaction were investigated with regard to their properties of Ca^{2+} -triggered luminescence, including the two basic characteristics, relative luminescence capacity and relative luminescence intensity. The former, relative luminescence capacity, is directly related to the quantum yield except when the incorporation reaction is incomplete with respect to apo-aequorin. The relative luminescence intensity is an indicator of the sensitivity to Ca^{2+} ; an increase in the intensity under the given conditions generally indicates an increase in the Ca^{2+} -sensitivity (Shimomura & Shimomura, 1985). On the basis of the square-law relationship between the intensity of luminescence and the concentration of Ca^{2+} (see the Introduction), it is a rough estimate that a 100-fold difference of the relative intensity corresponds to a 1 pCa unit difference of the Ca^{2+} -sensitivity.

The properties of 30 semi-synthetic aequorins that had a significant luminescence capacity (over 10% of that of natural aequorin) are summarized in Table 1, in comparison with natural aequorin regenerated from apo-aequorin and coelenterazine (1) and also with *e*-aequorin (Shimomura *et al.*, 1988) prepared from apo-aequorin and coelenterazine analogue 24. The coelenterazine analogue 2, in which the phenolic hydroxy group of the R group is replaced by a hydrogen atom, yielded a photoprotein that showed a relative luminescence intensity 10 times higher than that of natural aequorin at pCa 7. Analogue 3, in which the phenolic hydroxy group of the R group is replaced with a fluorine atom, gave a photoprotein having a relative luminescence intensity higher than that of the semi-synthetic aequorin derived from analogue 2. However, further introduction of fluorine atoms on the phenyl groups of the R group, i.e. analogues 4 and 5, yielded photoproteins that showed relatively small increases in the luminescence intensity and some decreases in the luminescence capacity. Substituting the phenolic hydroxy group of the R group with a halogen atom other than fluorine, i.e. to give analogues 6–8, resulted in a decrease of relative luminescence intensity in the resultant photoproteins; the decrease was largest when an iodine atom was introduced. Substituting the R group with a β -naphthyl group yielded a photoprotein that luminesced at only one-hundredth of the luminescence intensity of natural aequorin at pCa 6.

Coelenterazine analogue 11, in which the R' group of coelenterazine was replaced by an isopropyl group, gave semi-synthetic aequorin having a markedly increased relative luminescence intensity at pCa 7. Analogues 12 and 13, bearing a cyclopentyl and cyclohexyl group at R' respectively, yielded photoproteins having a moderately increased relative luminescence intensity, a high reaction rate and a high luminescence capacity that was practically equal to that of natural aequorin.

The semi-synthetic aequorins derived from coelenterazine analogues with both R and R' groups modified showed very high levels of relative luminescence intensity. The photoproteins derived from analogues 19 and 22 appear to be particularly suited for measuring low concentrations of Ca^{2+} for practical purposes, by having relatively high luminescence capacities, high sensitivities to Ca^{2+} , fast responses and relatively low levels of Ca^{2+} -independent luminescence. In the case of these photoproteins, their relative luminescence intensities are close to the multiple of the relative intensity values of the two photoproteins, each of them prepared with the analogues having the corresponding single substitution, i.e. analogues 2 and 12 for the former and analogues 3 and 13 for the latter. Such a relationship, however, does not hold in the cases of the photoproteins derived from analogues 17 and 18, probably because of low yields in the formation of photoprotein.

The relative luminescence capacity is, in the present definition, determined not only by the intrinsic nature of the photoprotein produced but also by the yield of the photoprotein in the incorporation reaction. The yield of photoprotein with respect to the amount of apo-aequorin used may become less than 100%, primarily by the following two causes. (1) The decomposition of photoprotein by the Ca^{2+} -independent luminescence (Allen *et al.*, 1977) in competition with the formation of the photoprotein. The Ca^{2+} -independent luminescence is generally greater when the relative luminescence intensity, i.e. sensitivity to Ca^{2+} , is greater, but it also varies significantly with the analogue incorporated. (2) The spontaneous inactivation of apo-aequorin in the course of unendingly repeated turnover of the protein when a high level of Ca^{2+} -independent luminescence is present.

The photoprotein derived from analogue 18 has shown by far the highest level of Ca^{2+} -independent luminescence among all the semi-synthetic photoproteins reported. In the case of this photoprotein, the half-total time of Ca^{2+} -independent luminescence and the half-completion time of the incorporation reaction were 25 min at 24 °C and 60 min at 0 °C respectively, strongly suggesting a reason for the low yields of photoprotein in the incorporation reaction. The overall property of this photoprotein appears to be not favourable in practical use despite its high relative intensity at pCa 7.

The photoproteins of *e*-aequorin type, prepared from the coelenterazine analogues containing a dimethylene bridge, i.e. analogues 25–32, showed fast luminescence reactions, high relative luminescence intensities and, like *e*-aequorin, the dependency of the spectral distribution of luminescence on the concentration of Ca^{2+} . As previously observed for *e*-aequorin, the luminescence spectra of the photoproteins derived from analogues 25 and 26 showed two peaks, whereas those derived from analogues 27, 28 and 29 showed only one apparent peak at 400 nm. The relationship between pCa and the ratio of the luminescence intensity of 400 nm to that at 465 nm for those photoproteins (Fig. 1) indicates that the photoproteins derived from analogues 27 and 28 are possibly superior to *e*-aequorin and *e*-aequorin C in the measurement of Ca^{2+} concentration by the ratio method.

In addition to the compounds listed in Table 1, seven coelenterazine analogues were investigated. Coelenterazine analogues 33–37 gave corresponding incorporation products, but they showed no or very limited luminescence activity when treated with Ca^{2+} . Coelenterazine

Fig. 1. Relationship between pCa and the ratio of intensity at 400 nm to intensity at 465 nm (I_{400}/I_{465} ratio) for semi-synthetic aequorins (continuous lines) derived from analogues 25 (●), 26 (○), 27 (△), 28 (▲) and 29 (×), and for *e*-aequorin (broken line), at pH 7 in the presence of 100 mM-KCl at 24 °C

An irregularity shown on the curve of the photoprotein derived from analogue 29, below pCa 6, might be an artifact caused by the very fast luminescence reaction that is beyond the linear response range of the measuring technique used.

analogues 38 and 39 yielded photoproteins having a low relative luminescence capacity (6 % and 8 % respectively).

In conclusion, the results described prove that the structural modification of the functional moiety of

aequorin is highly effective in improving certain properties of natural aequorin. This approach provides an effective method in increasing or decreasing the sensitivity of the photoprotein to Ca^{2+} concentration. By using an appropriate semi-synthetic aequorin, the range of measuring Ca^{2+} concentration can be extended by at least 1 pCa unit on both sides; namely, compared with the range of pCa 5–7 with natural aequorin, semi-synthetic aequorins could cover the range pCa 4–8. In the case of measuring Ca^{2+} concentration by the ratio method, the semi-synthetic aequorins derived from analogues 27 and 28 are possibly superior to *e*-aequorin or *e*-aequorin C reported previously (Shimomura *et al.*, 1988).

The aequorin used for preparation of semi-synthetic aequorins in this study was a heterogeneous mixture consisting of at least eight kinds of isoequorins, aequorins A–H, which have small, but significant, differences in their properties. As previously observed on *e*-aequorins A–F (Shimomura *et al.*, 1988), semi-synthetic aequorins derived from various isoequorins may show significant differences, and a semi-synthetic aequorin prepared from a certain isoequorin might have more favourable properties than the ones made from a mixture of isoequorins.

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Analogue 33: $\text{R} = \text{C}_6\text{H}_{11}\text{-}c$, $\text{R}' = \text{C}_6\text{H}_5$, $\text{X} = \text{OH}$, $\text{Y} = \text{H}$

Analogue 34: $\text{R} = \text{CH}(\text{CH}_3)_2$, $\text{R}' = \text{C}_6\text{H}_5$, $\text{X} = \text{OH}$, $\text{Y} = \text{H}$

Analogue 35: $\text{R} = \text{C}_6\text{H}_4\text{OH-}p$, $\text{R}' = \text{H}$, $\text{X} = \text{OH}$, $\text{Y} = \text{H}$

Analogue 36: $\text{R} = \text{C}_6\text{H}_4\text{OH-}p$, $\text{R}' = \text{C}_6\text{H}_5$, $\text{X} = \text{F}$, $\text{Y} = \text{H}$

Analogue 37: $\text{R} = \text{C}_6\text{H}_4\text{OH-}p$, $\text{R}' = \text{C}_6\text{H}_5$, $\text{X} = \text{H}$, $\text{Y} = \text{OH}$

Analogue 38: $n = 3$, $\text{X} = \text{OH}$, $\text{Y} = \text{H}$

Analogue 39: $n = 2$, $\text{X} = \text{OH}$, $\text{Y} = \text{OH}$

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