Isolation and properties of various molecular forms of aequorin

Osamu SHIMOMURA

*Marine Biological Laboratory, Woods Hole, MA 02543, U.S.A., and Department of Physiology, Boston University School of Medicine, Boston, MA 02118, U.S.A.

The photoprotein aequorin emits light by an intramolecular reaction when a trace of Ca^{2+} is added. The samples of aequorin that were purified by the conventional methods of column chromatography were separated by high-performance liquid chromatography into eight molecular forms (isoequorins), which were designated aequorins A–H. Aequorins A, C and F were obtained in crystalline states. A wide range of properties were studied with aequorins A–F, which were essentially pure. These six isoequorins showed relatively small differences in their spectroscopic properties, but their values of $A_{1\text{ cm}, 280}^{0.1\%}$ were found to be close to 3.0, about 10% more than the previously reported value of 2.70–2.71 that was obtained with the samples of conventionally purified aequorin. The M_r values ranged from 20 100 (aequorin F) to 22 800 (aequorin A), the luminescence activities ranged from 4.35×10^{15} photons/mg (aequorin A) to 5.16×10^{15} photons/mg (aequorin F), and the first-order reaction rate constants of luminescence ranged from 0.95 s^{-1} (aequorin A) to 1.33 s^{-1} (aequorin F). As regards sensitivity to Ca^{2+} , aequorin D was the most sensitive, having a sensitivity about 0.4–0.5 pCa unit above that of the least sensitive kind (aequorin A).

INTRODUCTION

The photoprotein aequorin extracted from the jellyfish *Aequorea* emits light when traces of Ca^{2+} are added in the presence or absence of oxygen (Shimomura *et al.*, 1962). The Ca^{2+} -triggered light emission is caused by an intramolecular reaction, in which aequorin is split into apoequorin, coelenteramide and CO_2 (Shimomura & Johnson, 1978). The product apoequorin can be regenerated into the original active form of aequorin by incubation with coelenterazine in the presence of oxygen and 2-mercaptoethanol (Shimomura & Johnson, 1975). Because of its high sensitivity to Ca^{2+} , aequorin has been widely used as a Ca^{2+} indicator in various biological systems (Ashley & Campbell, 1979; Blinks *et al.*, 1982).

Aequorin is not homogeneous, and it consists of a number of different molecular forms, isoequorins, which have isoelectric points ranging from pH 4.2 to 4.9 (Blinks & Harrer, 1975). The fact that the samples of aequorin consist of various isoequorins and the samples are not uniform by batches has caused difficulties and complications in various studies involving aequorin in the past. For example, efforts to crystallize aequorin were uniformly unsuccessful; data reported on various properties of aequorin were often numerically inconsistent, especially on the peak wavelength of luminescence, the M_r and the sensitivity of Ca^{2+} ; the chemically modified forms of aequorin were virtually impossible to purify. Despite the evident importance of the separation of isoequorins, there has been no practical means to accomplish it on preparative scales until recently. There was a brief description of the isolation of four kinds of isoequorins by preparative gel electrophoresis (Blinks *et al.*, 1982). In our laboratory, however, the use of preparative gel electrophoresis (Poly-Prep 100; Buchler Instruments, Fort Lee, NJ, U.S.A.) was not satisfactory. The separation of various isoequorins on preparative scales has become feasible only recently by progress in the

technology of high-performance liquid chromatography (h.p.l.c.)

EXPERIMENTAL

Materials and general procedures

The specimens of the jellyfish *Aequorea* were collected at Friday Harbor, WA, U.S.A. Aequorin was extracted from 50 000 specimens of *Aequorea* and purified by a series of chromatographies as previously described (Shimomura & Johnson, 1969, 1976). The purest fraction obtained in the last chromatography had a luminescence activity of 4.4×10^{15} photons/mg at 25°C (based on the previously reported value of $A_{1\text{ cm}, 280}^{0.1\%}$ 2.70; Shimomura & Johnson, 1969). The aequorin preparation that was used in the present study contained various, but not all, fractions of the purified aequorin obtained, and it consisted of 85 mg of aequorin preparation with a luminescence specific activity approx. 90% of that of the purest sample just mentioned above.

EDTA, Hepes, Mops and carbonic anhydrase were purchased from Sigma Chemical Co.; ammonium acetate (h.p.l.c. grade), sodium acetate (h.p.l.c. grade), and calcium acetate were from Fisher Scientific Co.; $(\text{NH}_4)_2\text{SO}_4$ (Ultra Pure grade), guanidine hydrochloride (Ultra Pure grade), cytochrome *c*, myoglobin and ovalbumin were from Schwarz/Mann, Cambridge, MA, U.S.A.; DEAE-Sephacel, phenyl-Sepharose CL-4B, Sephadex G-100, Sephadex G-25 (fine grade), ribonuclease A and aldolase were from Pharmacia; Ultrogel AcA 54 was from LKB.

All measurements of the properties of aequorin were carried out at $23\text{--}25^\circ\text{C}$, except as noted. The storage of aequorin samples before and after the measurements was at 0°C for short periods of time, and at -75°C for periods longer than a few hours.

* Address for requests for reprints.

Separation of various molecular forms of aequorin

Various isoequorins were separated by h.p.l.c. on a column of Bio-Gel TSK DEAE-5PW (2.15 cm $\times$ 15 cm; Bio-Rad Laboratories, Richmond, CA, U.S.A.) by elution with 10 mM-Hepes, pH 7.0, containing 2 mM-EDTA and various concentrations of sodium acetate, at a flow rate of 8 ml/min, at 25 °C.

The aequorin sample was first separated into six fractions, each of which predominantly contained one of the six most abundant forms, i.e. aequorins A, B, C, D, E and F, that were eluted successively from the column. The elution was done by the linear increase of sodium acetate concentration from 0.28 M to 0.35 M in 15 min after the injection of a sample, and by holding the sodium acetate concentration at 0.35 M for the next 10 min to improve the separation of aequorins E and F. Only 3 mg of aequorin, or less, was injected in each run, but separations between aequorins A, B and C were sometimes incomplete. All eluate fractions of the same peak were combined, and the resulting six combined solutions, 250 ml–1 litre each, were concentrated as follows.

The solutions were made 1 M in $(\text{NH}_4)_2\text{SO}_4$, then passed through a column of phenyl-Sepharose CL-4B (1.6 cm $\times$ 3–5 cm). Aequorin was quantitatively adsorbed on the column, and then eluted with 5 mM-Hepes, pH 7.3, containing 2 mM-EDTA. Six concentrated solutions, 10–20 ml each, of aequorins A, B, C, D, E and F were obtained.

The concentrated solutions of aequorins A, B, C and E were further purified on the same TSK DEAE-5PW column by isocratic elution with 0.25 M-sodium acetate (for A and B), or with 0.27 M-sodium acetate (for C), or with 0.32 M-sodium acetate (for E), all in 10 mM-Hepes (pH 7.0)/2 mM-EDTA. The maximum amount of sample injected in each h.p.l.c. run was about 0.6 mg. Isoaequorins were well resolved under these conditions, resulting in the separation of two new peaks, i.e. aequorin G (between aequorins A and B) and aequorin H (between aequorins C and D). Solutions of aequorins A, B, C, E, G and H were concentrated separately with a column of phenyl-Sepharose CL-4B by the same procedure as described above. The solutions of aequorins D and F were sufficiently pure without further purification.

Isoaequorins in the solutions were salted out by the stepwise addition of $(\text{NH}_4)_2\text{SO}_4$. The precipitate of isoaequorin was collected by centrifugation and dissolved in a small amount of 10 mM-Hepes buffer, pH 7.1, containing 0.1 mM-EDTA, then the solution was passed through a small column of Sephadex G-25 which had been equilibrated with the same buffer. The concentrated solutions of purified aequorins A–H (the stock solutions) prepared in this manner, each in a volume of 0.3–2.0 ml, were stored at -75°C until used.

Ultraviolet and visible absorption data

All measurements were performed on a Shimadzu u.v.–visible recording spectrophotometer (model UV-240; Shimadzu Corp., Kyoto, Japan), by using self-masking-type micro cells (0.5 ml volume). The stock solutions of aequorins were diluted with 10 mM-Hepes, pH 7.1, containing 0.1 mM-EDTA to give A_{280} values of 1.2–1.4, then the 280 nm absorption peak and the 455 nm absorption peak were recorded with the full-scale absorbance settings of 2.0 and 0.2 respectively.

To measure the ratio of A_{205}/A_{280} , the stock solutions of aequorins were first diluted about 100-fold with 2 mM-sodium phosphate buffer, pH 7.1, containing 0.1 mM-EDTA and 20 mM- Na_2SO_4 . The A_{280} values of these solutions were 0.1–0.2. After further dilution of exactly 5-fold with the same buffer, the values of A_{205} were measured.

To determine the ratio of A_{332}/A_{280} , the stock solutions were diluted with 10 mM-Mops, pH 7.1, containing 0.1 mM-EDTA to give A_{280} values of 1.0–1.7, then exactly 1 ml of each diluted solution was mixed with 0.1 ml of 10 mM-calcium acetate. When the emission of light completely ceased, three drops of 1 M-HCl were added and the mixture was extracted with 6 $\times$ 1 ml of diethyl ether (freshly redistilled). The ether extracts were combined and evaporated to dryness under reduced pressure. The residue was dissolved in exactly 1 ml of ethanol, then A_{332} was measured.

Determination of dry weight

Approx. 0.15 ml of the stock solution (0.6–1.0 mg of aequorin) was filtered through a column of Sephadex G-25 (0.4 cm $\times$ 10 cm) equilibrated with 0.1 mM-EDTA/50 mM-ammonium acetate, pH 7.15. Then the eluate that contained aequorin (about 0.4 ml) was diluted to 1.1–1.2 ml with the same EDTA solution. After measurement of A_{280} (which was 2.0 ± 0.4), exactly 1 ml of the solution was first freeze-dried, then dried further over P_2O_5 and NaOH in a vacuum desiccator at 90 °C for 4 h. The dry weight of the protein was obtained from the weight of the residue minus the weight of a control from which aequorin was omitted.

The dry weight obtained was corrected for the presence of extrinsic substances bound to the protein molecule, i.e. (1) approx. 1 mol of EDTA (M_r 326 for diammonium salt; Shimomura & Shimomura, 1982) and (2) ammonium and acetate groups bound as salts. Aequorin contains 35 carboxy groups and 21 basic groups (Inouye *et al.*, 1985), of which the excess 14 carboxy groups must be in the form of the NH_4^+ salt. It was assumed that 85% of the rest (21 each of acidic and basic groups) were paired when dried. The increase in M_r caused by (1) and (2) is 795 for aequorin, which has a reported M_r of approx. 20000–21000 (Blinks *et al.*, 1976; Shimomura & Johnson, 1979). Therefore the dry weight obtained above was decreased by 4%. If the acid–base pairing in (2) were complete, the values thus obtained would be 1.2% too low.

Measurements of luminescence

The luminescence activities of aequorin samples were measured with an integrating photometer (Chase, 1960), which had been calibrated by *Cypridina* luminescence (λ_{max} 462 nm; Shimomura & Johnson, 1970a) within 1 month of the measurements. Luminescence was elicited by adding 3 ml of 10 mM-calcium acetate solution to a small volume (2–10 μl) of sample.

Luminescence spectra were measured on a model MPF-44B fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT, U.S.A.) without turning on its xenon lamp and chopper. A portion (0.1 ml) of the solution of aequorin that had been used for the measurement of A_{455}/A_{280} ratio was diluted with 1 ml of 10 mM-Hepes, pH 7.2, containing 0.1 mM-EDTA, then weak luminescence lasting at least 10 min was elicited by careful addition of 10 mM-calcium acetate (about 10 μl).

The spectra were not corrected for changes in the sensitivity of the photomultiplier at different wavelengths.

The first-order reaction rate constants of the Ca^{2+} -triggered luminescence were measured with an integrating photometer connected to an oscillographic recorder (model M21; MFE Corp., Salem, NH, U.S.A.). The aequorin solution used for the measurement of A_{455}/A_{280} ratio was diluted 200-fold with 10 mM-Hepes, pH 7.1, containing 0.1 mM-EDTA, then 100 μl of the diluted solution was injected with a constant-rate syringe (model CR 700-200; Hamilton Co., Reno, NV, U.S.A.) into 3 ml of 10 mM-calcium acetate. The light emitted was recorded at a chart speed of 25 mm/s, and time (t) required for the emission of half of the total light after the injection was read from the recording. The rate constant was calculated by $0.69/t$.

Relationship between pCa and the initial maximum intensity of Ca^{2+} -triggered luminescence was measured in 1 mM-citrate buffer, pH 7.0, and also in 1 mM-Mops, pH 7.0, containing 0.1 M-KCl, by the procedure previously reported (Shimomura & Shimomura, 1984).

Estimation of molecular size by size-exclusion chromatography

The molecular size of aequorin was estimated by h.p.l.c. on a Spherogel-TSK 3000SW column (7.5 mm $\times$ 30 cm; Beckman Instruments, Berkeley, CA, U.S.A.) with two different buffer solutions. When 10 mM-Mops buffer, pH 7.1, containing 2 mM-EDTA and 0.15 M-NaCl (flow rate 1 ml/min) was used, the column was calibrated with ribonuclease A (M_r 13 700), ovalbumin (M_r 45 000) and aldolase (M_r 158 000); when 10 mM-sodium phosphate buffer, pH 6.0, containing 6 M-guanidine hydrochloride (flow rate 0.8 ml/min) was used, the column was calibrated with cytochrome c (M_r 13 000), ribonuclease A, myoglobin (M_r 18 000), carbonic anhydrase (M_r 29 000) and ovalbumin.

RESULTS AND DISCUSSION

Separation of various molecular forms of aequorin

Aequorin samples purified by the conventional methods of column chromatography were separated with a sufficient resolution into various molecular forms of aequorin (isoaequorins) by h.p.l.c. on a TSK DEAE-5PW anion-exchange column by using buffers containing sodium acetate (Fig. 1, curve 1). After two steps of h.p.l.c. eight different kinds of the photoprotein, i.e. aequorins A, B, C, D, E, F, G and H, were obtained, and the first six of them were highly pure, judged by the results of analytical h.p.l.c. of these materials (Fig. 1, curves 2–7).

When the separated samples were tested by h.p.l.c. by using elution buffers containing $(\text{NH}_4)_2\text{SO}_4$, instead of sodium acetate, aequorin B, but no other kind, gave a deformed peak, and the peak was split into two peaks when the amount of injected sample was very small (Fig. 1, curve 8). Thus aequorin B probably consists of two molecular species. The separation of them, however, was considered impractical because the amount of aequorin B that could be separated by one h.p.l.c. run was only 10–15 μg on a preparative column (21.5 mm $\times$ 15 cm).

In the present study, 85 mg of the starting material of aequorin (approx. purity 90%) yielded aequorins A, B, C, D, E and F in the amounts shown in Table 1, line o;

the yields of aequorins G and H were respectively 1.3 mg and 1.0 mg. Thus the total yield of the eight kinds of isoaequorins separated from the starting material was approx. 70%. The 30% loss can be attributed to the discarded portions of eluate on both sides of h.p.l.c. peaks (cut at approx. 15% of the peak height) and also to the inactivation of aequorin during h.p.l.c. at room temperature; performing h.p.l.c. at lower temperatures was not advantageous, owing to sharp decreases in the efficiency of the column.

The estimated contents of aequorins A, B, C, D, E and F each in the combined total of purified aequorin obtained from one batch of extraction are given in Table 1, line p. The data, however, do not necessarily represent the composition of isoaequorins in the jellyfish, considering that the yield of purified aequorin from the jellyfish is usually only about 30% (i.e. the loss is 70%) and that the extent of loss might be different for each isoaequorin.

A possibility that isoaequorins are artifacts produced by some kinds of enzyme, such as proteinases, in the course of extraction and purification is not likely, at least for the major kinds of isoaequorins, on the basis of following facts.

(1) According to Blinks *et al.* (1976), the extracts made from individual specimens of *Aequorea* showed the presence of at least eight isoaequorins by isoelectric focusing, in good agreement with the present results.

(2) Aequorin extracted from 15 specimens of *Aequorea* and chromatographed on a column of Ultrogel AcA 54, in a short period of 1 h, clearly revealed the presence of aequorins A, C (the highest peak) and F by h.p.l.c. The presence of other isoaequorins was unclear, owing to the presence of impurities.

(3) When different batches of purified aequorin, each independently extracted and purified in the last several years, were examined by h.p.l.c., at least 90% of protein in each batch was assignable to the eight kinds of isoaequorins now isolated.

The precipitates of aequorins A, C and F salted out with $(\text{NH}_4)_2\text{SO}_4$ (approx. 40% saturation) were crystalline, consisting of fine needles approx. 1 μm in thickness and 10–20 μm in length. The crystalline nature of the needles was confirmed by polarization microscopy.

Ultraviolet and visible absorption

The absorption spectrum of aequorin C is shown in Fig. 2, representing the spectra of all other isoaequorins, which are very similar to each other. The spectrum is essentially the same as that of highly purified aequorin previously reported (Shimomura & Johnson, 1969), although the 456 nm peak was reported as 465 nm in the 1969 report. There are small but clear differences in certain spectroscopic properties of isoaequorins, as summarized in Table 1, lines a–e.

The values of $A_{1\text{ cm}}^{0.1\%, 205}$ (Table 1, line c) calculated by the method of Scopes (1974) coincide with each other within 1%, indicating that A_{205} is a good parameter to be used in measuring small amounts of pure aequorin, although the method is greatly restricted in actual use because EDTA and many other chemicals of common use strongly absorb at 205 nm.

The measurement of A_{280} is one of the two commonly used means to quantify aequorin; the alternative is the measurement of emitted light with a calibrated photometer. The values of $A_{1\text{ cm}}^{0.1\%, 280}$ were obtained by two methods in this study, i.e. (1) by using the values of

Table 1. Properties of various molecular forms of aequorin (isoeaquorins) at 23–25 °C

Property	Aequorin					
	A	B	C	D	E	F
(a) A_{455}/A_{280} *	0.0275 (455)	0.0268 (457)	0.0286 (456)	0.0275 (456)	0.0282 (459)	0.0289 (458)
(b) A_{205}/A_{280} †	11.34	10.82	10.98	11.40	11.00	11.05
(c) $A_{1\text{ cm}, 205}^{0.1\%}$	34.1	34.4	34.3	34.1	34.3	34.3
(d) $A_{1\text{ cm}, 280}^{0.1\%}$ (c/b)	3.00	3.18	3.12	2.98	3.12	3.10
(e) $A_{1\text{ cm}, 280}^{0.1\%}$ (by dry weight)	2.92	3.10	3.02	2.92	3.01	3.00
(f) A_{332}/A_{280} ‡	0.2103	0.2023	0.2179	0.2186	0.2282	0.2327
(g) ϵ_{280} ($\text{M}^{-1}\cdot\text{cm}^{-1}$)§	66600	69200	64200	64000	61300	60200
(h) M_r (g/e)	22800	22300	21300	21900	20400	20100
(i) Luminescence maximum (nm)	460	466	468	468	472	472
(j) Luminescence activity (10^{15} quanta/ml when $A_{1\text{ cm}, 280} = 1$)	1.49	1.44	1.64	1.64	1.60	1.72
(k) Luminescence activity (10^{15} quanta/mg) ($j \times e$)	4.35	4.46	4.95	4.79	4.82	5.16
(l) Quantum yield (einsteins/mol)	0.165	0.165	0.175	0.174	0.163	0.172
(m) First-order reaction rate constant (s^{-1})	0.95	1.21	1.17	1.13	1.30	1.33
(n) Sensitivity to Ca^{2+} Median sensitivity (pCa) at low ionic strength	6.91	7.20	7.15	7.32	7.23	7.28
Median sensitivity (pCa) in the presence of 0.1 M-KCl	5.81	5.96	6.00	6.25	6.01	6.11
(o) Amount isolated (mg)	11	9.4	17	10.7	1.1	3.1
(p) Composition of total purified aequorin (%) ¶	15.9	12.0	31.8	8.2	6.3	9.4

* Precise peak wavelength of A_{455} peak is shown in parentheses.

† Calculated by: $A_{1\text{ cm}, 205}^{0.1\%} = 27.0 + 120 \times A_{280}/A_{205}$ (Scopes, 1974). Correction for the presence of chromophore was made by multiplying the measured value of A_{280} by 0.67, i.e. the ratio of A_{280} of apoeaquorin to A_{280} of aequorin (Shimomura & Johnson, 1969); the measured value of A_{205} was not corrected because the effect of chromophore appears to be small (less than 5%), judged from absorption by coelenterazine.

‡ This is the ratio of A_{332} of coelenteramide formed from aequorin by the luminescence reaction to A_{280} of aequorin. See the Experimental section.

§ ϵ_{280} of coelenteramide was 14050 ± 140 . Thus ϵ_{280} of aequorin was calculated by: $14000/f$.

|| Median sensitivity is defined in the last paragraph of the text. The low-ionic-strength 1 mM-sodium citrate buffer, pH 7.0, contained various amounts of calcium citrate to set pCa values; 0.1 M-KCl was in 1 mM-Mops, pH 7.0, containing various amounts of CaCl_2 to set pCa values.

¶ Estimated from the h.p.l.c. elution curve of the sample that was prepared by mixing 0.05% each of all fractions of purified aequorin obtained in the present extraction, by the integration of each peak. The conditions of h.p.l.c. were the same as used in the experiments of Fig. 1, curves 1–7. Contents of aequorins G and H were 5.3% and 5.0% respectively.

A_{205}/A_{280} and $A_{1\text{ cm}, 205}^{0.1\%}$ and (2) by using A_{280} and the dry weight of aequorin (Table 1, lines d and e). Method 1 gave slightly larger values than did Method 2. The range of variation with various isoeaquorins was roughly 6% by both methods. The data obtained by Method 2 are

probably more reliable than those obtained by Method 1, because Method 2 involves a lesser extent of assumption. The present data suggest that the value of $A_{1\text{ cm}, 280}^{0.1\%}$ for purified aequorin (mixtures of isoeaquorins) should be close to 3.0, rather than the previously reported

Fig. 1. Examples of h.p.l.c. elution curves

Plots are shown for: a typical example of purified aequorin [30 μg ; (1)], aequorin A [6 μg ; (2)], aequorin B [6 μg ; (3)], aequorin C [5 μg ; (4)], aequorin D [6 μg ; (5)], aequorin E [5 μg ; (6)] and aequorin F [6 μg ; (7)] on a Bio-Gel TSK DEAE-5PW column (7.5 mm $\times$ 7.5 cm) eluted with 10 mM-Mops, pH 7.1, containing 2 mM-EDTA and sodium acetate linearly increased from 0.25 to 0.34 M in 14 min after the injection of sample, recorded at 0.02 A full-scale; aequorin B [1 μg (8)] on the same column and eluted with 10 mM-Mops, pH 7.1, containing 2 mM-EDTA and 85 mM- $(\text{NH}_4)_2\text{SO}_4$, recorded at 0.005 A full-scale; the same aequorin sample as in (1) [5 μg (9)] and aequorin F [5 μg (10)], on a Spherogel TSK 3000SW column (7.5 mm $\times$ 30 cm) eluted with 10 mM-Mops, pH 7.1, containing 2 mM-EDTA and 0.15 M-sodium acetate, recorded at 0.02 A full-scale. Flow rate was 1 ml/min, chart speed was 0.5 cm/min, and A_{280} (ordinate) was measured with a 5 mm-light-path cell. The left-hand ends of the horizontal scales coincide with the injection marks, and each section of the scale represents 2 min.

Fig. 2. Absorption spectra and luminescence spectrum of aequorin C

(1) Absorption of aequorin C (0.44 mg/ml) in 10 mM-Hepes, pH 7.1, containing 2 mM-EDTA; (2) the same solution measured with the absorbance scale 10 times expanded; (3) after dilution with 14 vol. of the same buffer, shown with the absorbance scale as indicated; (4) emission spectrum of Ca^{2+} -triggered luminescence.

value of about 2.7 (Shimomura & Johnson, 1969; Prendergast & Mann, 1978).

M_r

One molecule of aequorin after the Ca^{2+} -triggered luminescence reaction splits off one molecule each of coelenteramide and CO_2 (Shimomura & Johnson, 1979). The amounts of coelenteramide produced from various isoequorins were quantitatively measured by the peak absorption of coelenteramide at 332 nm (Shimomura & Johnson, 1973), then the M_r values of various aequorins were calculated on the basis of values of $A_{1\text{ cm}, 280}^{0.1\%}$ obtained by Method 2 (Table 1, lines e–h). The values thus obtained ranged from 20100 (aequorin F) to 22800 (aequorin A).

The M_r of aequorin previously determined with various samples of purified aequorin (mixtures of isoequorins) was about 20000–21000 (Blinks *et al.*, 1976; Shimomura & Johnson, 1979). Inouye *et al.* (1985) reported that apoequorin is composed of 189 amino acid residues and has an M_r of 21400, thus indicating an M_r of approx. 21800 for aequorin after adding its chromophore. Although the nature of the aequorin that they analysed is not known, it appears to be significant that the latter value (21800) is in close agreement with the M_r of aequorin C (21300), which is the most abundant component in most preparations of purified aequorin.

The M_r values were examined also by size-exclusion h.p.l.c. on a TSK 3000SW column, although the method is less reliable than the chromophore assay method described above. In a buffer containing 0.15 M-sodium acetate, the peaks of isoequorins were very close to each other, thus a mixture of isoequorins resulted in a single slightly broadened peak (Fig. 1, curve 9). In this buffer, the M_r values were estimated to be about 22800 for aequorins A, B, C and D, and about 25100 for aequorins E and F. In a buffer containing 6 M-guanidine hydro-

chloride, the values were approx. 20200 for aequorins A, B, C and D, and 19200 for aequorins E and F. Note the reversed order of the group of aequorins A, B, C and D, and the group of aequorins E and F in those two buffer systems, which could suggest, among other things, that the molecular conformations of aequorins E and F in native states are looser and more expanded than those of aequorins A, B, C and D.

Properties related to luminescence

The peak wavelengths of the Ca^{2+} -triggered luminescence of isoequorins ranged from 460 nm (aequorin A) to 472 nm (aequorins E and F), as shown in Table 1, line i. Some inconsistent values of peak wavelengths previously reported for the samples of purified aequorin [460 nm (Johnson *et al.*, 1962); 470 nm (Shimomura & Johnson, 1970b); 460 nm (Morin & Hastings, 1971); 465 nm (Shimomura & Shimomura, 1985)] can now be explained on the basis of such variations, at least in part. Significant variations were also found in the values of luminescence activity and the first-order reaction rate constants of luminescence (Table 1, lines j, k and m), but only small variations were found in the values of quantum yields (Table 1, line l).

The light-emitting sensitivity to Ca^{2+} can be best shown by plotting the relationship between the logarithm of luminescence intensities and the pCa values of solutions, as employed in many publications. Because the slopes of the curves drawn in this manner are nearly parallel to each other, we chose to use the pCa value of the middle point of the slope as a convenient, simple way to indicate the sensitivity, and termed the pCa value 'median sensitivity' (Shimomura & Shimomura, 1985). Thus the median sensitivity can be defined as the value of pCa at which the initial maximum intensity is equal to $\sqrt{I_0 I_{\text{max}}}$, 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 0.01 M- Ca^{2+} . The median-sensitivity data for the six kinds of isoequorins (Table 1, line n) indicate that aequorin D is the most sensitive of them and aequorin A is the least sensitive one, with a sensitivity difference of approx. 0.4–0.5 pCa unit between them in both the presence and the absence of 0.1 M-KCl.

I thank Dr. Shinya Inoué for the polarization microscopy of aequorin crystals, and Dr. Yoshito Kishi for a sample of coelenteramide. This work was supported by grants from the National Science Foundation (PCM 8201005 and DMB-8502695), and benefited by the facilities of the Friday Harbor Laboratories, University of Washington.

REFERENCES

- Ashley, C. C. & Campbell, A. K. (eds.) (1979) *Detection and Measurement of Free Ca^{2+} in Cells*, Elsevier/North-Holland Biomedical Press, Amsterdam
- Blinks, J. R. & Harrer, G. C. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 474
- Blinks, J. R., Prendergast, F. G. & Allen, D. G. (1976) *Pharmacol. Rev.* **28**, 1–93
- Blinks, J. R., Wier, W. G., Hess, P. & Prendergast, F. G. (1982) *Prog. Biophys. Mol. Biol.* **40**, 1–114
- Chase, A. M. (1960) *Methods Biochem. Anal.* **8**, 61–117
- Inouye, S., Noguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T. & Tsuji, F. I. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3154–3158

- Johnson, F. H., Shimomura, O., Saiga, Y., Gersham, L. C., Reynolds, G. T. & Waters, J. R. (1962) *J. Cell. Comp. Physiol.* **60**, 85–104
- Morin, J. G. & Hastings, J. W. (1971) *J. Cell. Physiol.* **77**, 305–311
- Prendergast, F. G. & Mann, K. G. (1978) *Biochemistry* **17**, 3448–3453
- Scopes, R. K. (1974) *Anal. Biochem.* **59**, 277–282
- Shimomura, O. & Johnson, F. H. (1969) *Biochemistry* **8**, 3991–3997
- Shimomura, O. & Johnson, F. H. (1970a) *Photochem. Photobiol.* **12**, 291–295
- Shimomura, O. & Johnson, F. H. (1970b) *Nature (London)* **227**, 1356–1357
- Shimomura, O. & Johnson, F. H. (1973) *Tetrahedron Lett.* 2963–2966
- Shimomura, O. & Johnson, F. H. (1975) *Nature (London)* **256**, 236–238
- Shimomura, O. & Johnson, F. H. (1976) *Symp. Soc. Exp. Biol.* **30**, 41–54
- Shimomura, O. & Johnson, F. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2611–2615
- Shimomura, O. & Johnson, F. H. (1979) in *Detection and Measurement of Free Ca^{2+} in Cells* (Ashley, C. C. & Campbell, A. K., eds.), pp. 73–83, Elsevier/North-Holland Biomedical Press, Amsterdam
- Shimomura, O. & Shimomura, A. (1982) *FEBS Lett.* **138**, 201–204
- Shimomura, O. & Shimomura, A. (1984) *Biochem. J.* **221**, 907–910
- Shimomura, O. & Shimomura, A. (1985) *Biochem. J.* **228**, 745–749
- Shimomura, O., Johnson, F. H. & Saiga, Y. (1962) *J. Cell. Comp. Physiol.* **59**, 223–240

Received 8 July 1985/25 September 1985; accepted 23 October 1985