Recombinant aequorin and recombinant semi-synthetic aequorins

Cellular Ca^{2+} ion indicators

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Properties of a recombinant aequorin were investigated in comparison with those of natural aequorin. In chromatographic behaviour the recombinant aequorin did not match any of ten isoequorins tested, although it was very similar to aequorin J. Its sensitivity to Ca^{2+} was found to be higher than that of any isoequorin except aequorin D. The recombinant aequorin exhibited no toxicity when tested in various kinds of cells, even where samples of natural aequorin had been found to be toxic. Properties of four recombinant semi-synthetic aequorins (*fch*-, *hcp*-, *e*- and *n*-types), prepared from the recombinant apo-aequorin and synthetic analogues of coelenterazine, were approximately parallel with those of corresponding semi-synthetic aequorins prepared from natural apo-aequorin. Both recombinant *e*-aequorin and natural *e*-aequorin J luminesced with high values of the luminescence intensity ratio I_{400}/I_{465} , although the ratios were not pCa-dependent. The recombinant aequorin and recombinant semi-synthetic aequorins are highly suited for monitoring cellular Ca^{2+} .

INTRODUCTION

The photoprotein aequorin emits light in the presence of Ca^{2+} (Shimomura *et al.*, 1962). Because of its high sensitivity to Ca^{2+} and other favourable properties, aequorin has widely been used as a Ca^{2+} indicator in various biological systems for the past 20 years (cf. Ashley & Campbell, 1979; Blinks *et al.*, 1982).

Aequorin samples previously used were mixtures of many isoequorins (M_r approx. 20000–22000); the composition of isoequorins varies with batches of aequorin preparation and their properties may also vary. The separation and isolation of homogeneous isoequorins from heterogeneous aequorin has been accomplished, resulting in ten types of isoequorin preparations, namely aequorins A–H (Shimomura, 1986) and aequorins I and J (the present paper).

Recently a variety of semi-synthetic aequorins have been prepared by replacing the coelenterazine moiety of aequorin with synthetic analogues of coelenterazine. Some of the semi-synthetic aequorins greatly increased their Ca^{2+} -sensitivity compared with natural aequorin, whereas some decreased their sensitivity, expanding the detection range of Ca^{2+} concentration (Shimomura *et al.*, 1989). One of the semi-synthetic aequorins, designated *e*-aequorin, showed a bimodal luminescence spectrum, with peaks at 405 nm and 465 nm, and the ratio of their peak heights was dependent on the concentration of Ca^{2+} in the range pCa 5–7, thus allowing the determination of Ca^{2+} concentration directly from the ratio of two peak intensities (Shimomura *et al.*, 1988).

Another significant achievement made in recent years is the successful production of aequorin by using the technique of molecular cloning. The cDNA for apo-aequorin was cloned and expressed in *Escherichia coli* (Inouye *et al.*, 1985, 1986; Prasher *et al.*, 1985). The apo-aequorin produced by *E. coli* was extracted and purified (Inouye *et al.*, 1989), and then it was converted into active aequorin, 'recombinant aequorin', by treating it with coelenterazine in the presence of O_2 and 2-mercaptoethanol.

Some properties of recombinant aequorin and recombinant semi-synthetic aequorins are described in the present paper in comparison with those of natural isoequorins.

MATERIALS AND METHODS

Recombinant aequorin

Recombinant apo-aequorin was obtained by the method previously reported (Inouye *et al.*, 1989), which consisted of fusing the apo-aequorin cDNA to the signal-peptide coding sequence of the outer-membrane Protein A of *E. coli*, subsequent expression of the cDNA in *E. coli* and purification of the recombinant apo-aequorin released into the culture medium by acid precipitation and DEAE-cellulose chromatography. The product was free from the signal-peptide sequence (Inouye *et al.*, 1989).

The preparation of recombinant aequorin from the recombinant apo-aequorin was carried out by adopting the method previously established (Shimomura & Johnson, 1975). Thus freeze-dried recombinant apo-aequorin (72 mg) was dissolved in 35 ml of 10 mM-Hepes/NaOH buffer, pH 8.5, containing 5 mM-EDTA, 0.5 M-KCl and 50 mM-2-mercaptoethanol. After adjustment of the pH to 7.5 with 1 M-HCl, the solution was cooled to 0 °C. To this solution was added an aqueous solution of coelenterazine (2 mg) in three portions over 8 h, then the mixture was left standing for an additional 12 h at 0 °C to complete the incorporation. The coelenterazine solution used was prepared by first dissolving coelenterazine in a small amount of water containing 1 drop of 1 M-NaOH, then adjusting its pH to 9.0 with 1 M-HCl. The extent of incorporation achieved was 75 % of the apo-aequorin used, on the basis of the Ca^{2+} -triggered light emission. The solution was saturated with $(\text{NH}_4)_2\text{SO}_4$, then centrifuged. The precipitate was dissolved in 5 ml of 10 mM-Mops/NaOH buffer, pH 7.0, containing 2 mM-EDTA,

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and purified by h.p.l.c. on a TSK DEAE-5PW column (21.5 mm × 15 cm; Toso-Haas, Philadelphia, PA, U.S.A.) with 10 mM-Mops/NaOH buffer, pH 7.0, containing 2 mM EDTA and 0.32 M-sodium acetate at 23 °C; about 2 mg of the protein was injected in each run and the elution was monitored by the absorption at 280 nm.

The eluate fractions containing aequorin were combined and concentrated by the following procedure. The solution was made 20% (w/v) in (NH₄)₂SO₄ and passed through a column of phenyl-Sepharose CL-4B (1.6 cm × 5 cm; Pharmacia Fine Chemicals) at 0 °C. Aequorin tightly adsorbed at the top part of the column was eluted with 10 mM-Hepes/NaOH buffer, pH 7.2, containing 5 mM EDTA, to give 42 mg of the recombinant aequorin.

Natural isoeaquorins

Aequorin was extracted from the jellyfish *Aequorea* collected in the vicinity of Friday Harbor, WA, U.S.A., in July 1983, August 1985 and August–September 1988. The crude extract of aequorin obtained each year was separately purified into heterogeneous aequorin by the procedure previously reported (Shimomura & Johnson, 1969, 1976; Shimomura *et al.*, 1988), with some minor modifications. The purification steps employed for the 1988 extract were: (1) gel filtration on Sephadex G-100 (Pharmacia Fine Chemicals) in the presence of 0.25 M-NaCl; (2) hydrophobic-interaction chromatography on phenyl-Sepharose CL-4B with decreasing gradient of (NH₄)₂SO₄ concentration; (3) gel filtration on Ultrogel AcA 54 (LKB Instruments); (4) anion-exchange chromatography on DEAE-Sepharose (Pharmacia Fine Chemicals). On the basis of luminescent activity, the yield of heterogeneous aequorin from the crude extract was approx. 50% for this experiment. Heterogeneous aequorin thus obtained was separated into isoeaquorins by the method previously described (Shimomura, 1986).

Semi-synthetic aequorins

Coelenterazine analogues were synthesized and incorporated into apo-aequorin as previously described (Shimomura *et al.*, 1989), with one modification in the reaction medium: 0.2 M-KCl was included in the buffer solution for the incorporation. The recombinant apo-aequorin used for preparation of recombinant semi-synthetic aequorins was prepared from the recombinant aequorin by luminescing with Ca²⁺ rather than using the recombinant apo-aequorin originally isolated, since the recombinant apo-aequorin prepared in this manner was significantly more effective in the incorporation experiment.

Comparison of chromatographic behaviours

Comparison was made by analytical h.p.l.c. at room temperature under two sets of conditions, as follows.

The first set of conditions involved anion-exchange chromatography on a TSK DEAE-5PW column (7.5 mm × 7.5 cm) with elution buffers (A) 0.24 M-sodium acetate/2 mM-EDTA/5 mM-Mops, pH 7.0, and (B) 0.38 M-sodium acetate/2 mM-EDTA/5 mM-Mops, pH 7.0. Elution (flow rate 1 ml/min) was programmed to start with 100% buffer A, changing to 40% buffer A/60% buffer B at 14 min, and to 100% buffer B at 18 min, both by linear gradient.

The second set of conditions involved a reverse-phase chromatography on a Vydac Protein C4 (10 µm particle size) column (4.6 mm × 25 cm; Separations Group, Hesperia, CA, U.S.A.) with elution solvents (A) 40% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid and (B) 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Elution (flow rate 1.5 ml/min) was programmed to start with 100% solvent A and change to 30% solvent A/70% solvent B in 20 min by linear gradient.

The amount of aequorin injected in each run was about 5 µg for the anion-exchange h.p.l.c. and 10 µg for the reverse-phase

Table 1. Properties of natural isoeaquorins A–J and recombinant aequorin

See the Materials and methods section for experimental details. The reversed-phase h.p.l.c. (Vydac C4 column) of apo-aequorins B, C and D showed two or more peaks, indicating that each sample consisted of multiple components. All other samples, including the recombinant aequorin, showed sharp single peaks under both h.p.l.c. conditions.

Type of aequorin*	Retention time (min)		Luminescence of 1 µg of photoprotein		
	TSK DEAE-5PW column	Vydac Protein C4 column	Light-emitting capacity (10 ¹² quanta)	Intensity at pCa 7 (10 ⁸ quanta/s)	Intensity Ca ²⁺ not added† (10 ⁸ quanta/s)
A	5.26	16.30	4.35	0.52	0.08
B	8.18	12.00 and 16.40	4.46	1.32	0.10
C	11.27	11.88, 12.70 and 15.70	4.95	1.14	0.08
D	15.80	12.30 and 15.70	4.79	5.60	0.10
E	20.30	11.20	4.82	0.80	0.04
F	21.48	11.60	5.16	1.36	0.05
G	6.12	15.68		2.14	0.15
H	12.40	12.20		2.30	0.07
I	12.00	11.00	4.27	0.45	0.02
J	13.80	11.44	4.55	0.56	0.03
Recombinant	13.50	11.50	4.80‡	2.32	0.04

* The isoeaquorin composition of heterogeneous aequorin, obtained from the jellyfish collected in August–September, 1988, was significantly different from that obtained from the jellyfish collected in July, 1983. The 1983 material did not contain detectable amounts of aequorins I and J (Shimomura, 1986), whereas the 1988 material did not contain aequorin H. The isoeaquorin composition for the 1983 material was as follows: A 15.9%, B 12.0%, C 31.8%, D 8.2%, E 6.3%, F 9.4%, G 5.3% and H 5.0%; that for the 1988 material was as follows: A 18.6%, B 16.3%, C 31.4%, D 6.8%, E 1.7%, F 4.6%, G 3.0%, I 7.1% and J 5.4%. Heterogeneous aequorin obtained from the jellyfish collected in August 1985 was similar to the 1988 material in isoeaquorin composition.

† The pCa value is approx. 9.5 based on the levels of calcium contamination in the chemicals used.

‡ The $A_{1\text{cm}}^{0.1\%}$ value at 280 nm was assumed to be 3.0, based on the data reported for isoeaquorins (Shimomura, 1986).

Table 2. Properties of semi-synthetic aequorins derived from recombinant aequorin and natural aequorin J

Results are given on the basis of the weights of recombinant aequorin and aequorin J used to prepare semi-synthetic aequorins. Coelenterazine analogues used in preparing semi-synthetic aequorins are as follows:

Semi-synthetic aequorin	Light-emitting capacity (10 ¹² quanta/μg)	Light intensity (10 ⁸ quanta/s μg)		
		pCa7	pCa6	Without added Ca ²⁺
Recombinant aequorin (control)	4.80	2.32	240.0	0.04
Recombinant <i>fch</i> -aequorin	3.05	210.0		0.5
Recombinant <i>hcp</i> -aequorin	2.23	445.0		0.7
Recombinant <i>n</i> -aequorin	1.34	0.60	3.8	0.01
Recombinant <i>e</i> -aequorin	2.23	4.1		0.2
Aequorin J (control)	4.50	0.56	50.0	0.03
<i>fch</i> -Aequorin J	3.52	52.0		0.15
<i>hcp</i> -Aequorin J	2.88	120.0		0.24
<i>n</i> -Aequorin J	1.21	0.10	0.6	0.01
<i>e</i> -Aequorin J	2.08	2.1		0.16

h.p.l.c. In the latter case the aequorin sample was mixed with a slight excess (to luminesce) of 10 mM-calcium acetate immediately before the injection. Eluate was monitored by u.v. absorption at 280 nm.

Measurement of luminescence

All luminescence measurements were done by the methods previously described (Shimomura *et al.*, 1989).

RESULTS AND DISCUSSION

The chromatographic comparison revealed that the recombinant aequorin was not identical with any of ten isoequorins hitherto isolated (Table 1). Recombinant aequorin was very similar to aequorin J in the chromatographic behaviour under two sets of conditions, although they were definitely different in the relative luminescence intensity measured at pCa 7.0.

Four types of semi-synthetic aequorins were prepared from the recombinant apo-aequorin and apo-aequorin J respectively, and their properties were compared. The four semi-synthetic types, *fch*-, *hcp*-, *n*- and *e*-aequorins, were chosen as the representatives of more than 30 semi-synthetic aequorins previously prepared (Shimomura *et al.*, 1988, 1989). As shown in Table 2, the light-emitting capacity and luminescence intensity of recombinant semi-synthetic aequorins were approximately parallel to those of semi-synthetic aequorins derived from aequorin J; the same relationship was also observed between recombinant semi-synthetic aequorins and heterogeneous semi-synthetic aequorins previously reported (cf. Shimomura *et al.*, 1988).

The luminescence spectra of recombinant *e*-aequorin and *e*-aequorin J (Fig. 1) showed two emission maxima at 400 nm and 465 nm, with the *I*₄₀₀/*I*₄₆₅ ratios 1.35:1 and 1.30:1 respectively. These ratios are considerably higher than those previously found for aequorins A–F (0.45–0.88:1) under similar conditions (Shimomura *et al.*, 1988). However, the *I*₄₀₀/*I*₄₆₅ ratios of recombinant *e*-aequorin and *e*-aequorin J were not affected by changing the concentration of Ca²⁺.

Three isoequorins, i.e. aequorins A, C and F, have previously been crystallized by slowly salting out with (NH₄)₂SO₄ (Shimomura, 1986). Under the same conditions, however, both the recombinant aequorin and aequorin J have failed to crystallize so far.

As it is a natural protein, aequorin has generally been assumed to be non-toxic when used as a Ca²⁺ indicator in biological systems. In spite of this assumption, various toxic effects have been occasionally encountered when heterogeneous aequorin was injected into certain types of cells (cf. Fabiato, 1985). The toxicity of the recombinant aequorin has recently been tested by four groups. Dr. Lionel Jaffe and Dr. Andrew Miller (Marine Biological Laboratory, Woods Hole, MA, U.S.A.) demonstrated that the recombinant aequorin injected into the eggs of the fucoid alga *Pelvetia* did not affect the germination of eggs, whereas all samples of heterogeneous aequorin tested, as well as most isoequorins, caused the failure of germination. Dr. Richard Payne (University of Maryland, College Park, MD, U.S.A.) found that the recombinant aequorin injected into the *Limulus* (horseshoe-crab) photoreceptors was not toxic, although most samples of heterogeneous aequorin had previously been shown to be toxic. Dr. Robert Silver (Cornell University, Ithaca, NY,

Fig. 1. Luminescence spectra of recombinant *e*-aequorin (—), *e*-aequorin J (---) and *e*-aequorin C (···) at pCa 6.0 and at pH 7.0

The spectra were measured in Ca^{2+} EGTA buffer containing 1 mM free EGTA, 2 mM-Mops and 100 mM-KCl (Shimomura & Shimomura, 1984) at 24 °C with a Perkin-Elmer model MPF-44B fluorescence spectrophotometer without corrections for the sensitivity difference of photomultiplier by wavelength. Intensity units are arbitrary with each curve.

U.S.A.) used the eggs of sea-urchins in the test, and Dr. Raymond Kado (C.N.R.S., Gif-sur-Yvette, France) and Dr. Laurinda Jaffe (University of Connecticut Health Center, Farmington, CT, U.S.A.) used the eggs of starfish. In both cases the eggs that had been microinjected with recombinant aequorin, upon fertilization, developed normally, whereas the eggs that had been injected with heterogeneous aequorin were prevented from cell division. These results demonstrate that recombinant aequorin

has very little toxicity, if any, and may suggest that the toxicity observed for natural aequorin is caused by unknown contaminants in natural aequorin preparations.

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