Halistaurin, phialidin and modified forms of aequorin as Ca^{2+} indicator in biological systems

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(Received 12 February 1985/21 March 1985; accepted 29 March 1985)

Two kinds of aequorin-type photoproteins, i.e., halistaurin and phialidin, and four kinds of modified forms of aequorin, i.e., products of acetylation, ethoxycarbonylation, fluorescamine-modification and fluorescein labelling, were prepared. The modified forms of aequorin were more sensitive to Ca^{2+} than was aequorin in their Ca^{2+} -triggered luminescence reactions, whereas halistaurin and phialidin were less sensitive. The emission maxima of luminescence were all within a wavelength range 450–464 nm, except for fluorescein-labelled aequorin, which emitted yellowish light (λ_{max} , 520 nm). A new technique of measuring Ca^{2+} concentration is suggested.

The photoprotein aequorin isolated from the jellyfish *Aequorea* emits light when a trace of Ca^{2+} is added, regardless of the presence or absence of oxygen. Aequorin has been widely used in the studies of Ca^{2+} in various biological systems, especially in single cells, ever since the first observation, by Ridgway & Ashley (1967), of a Ca^{2+} transient in barnacle muscle cells on micro-injection of aequorin. Although the various photoproteins of coelenterates are all Ca^{2+} -sensitive, only obelin extracted from the hydroid *Obelia* has, in addition to aequorin, been used as a Ca^{2+} indicator (Campbell *et al.*, 1981). The chemical modification of aequorin by acylation was briefly studied previously, and it was found that the acetylation of aequorin causes an enhancement of the sensitivity to Ca^{2+} (Shimomura & Shimomura, 1982).

We recently obtained two kinds of coelenterate photoprotein, namely halistaurin (cf. Shimomura *et al.*, 1963) and phialidin (cf. Levine & Ward, 1982), that have been known but have not hitherto been used as Ca^{2+} indicators. We also successfully prepared three new modified forms of aequorin in addition to the acetylated one. The present paper reports the properties of those photoproteins and the modified forms of aequorin, with special reference to the Ca^{2+} -sensitivities and the emission spectra of Ca^{2+} -triggered luminescence.

Abbreviation used: DTAF, 5-(4,6-dichloro-s-triazin-2-yl)aminofluorescein.

Materials and methods

Chemicals

Aequorin was obtained as previously described (Shimomura & Johnson, 1976). Fluorescamine, fluorescein isothiocyanate (isomer I), EDTA, Hepes, Mops, Tris and all *N*-acetyl-amino acids were purchased from Sigma Chemical Co. Acetic anhydride was from Fisher Scientific Co.; diethyl pyrocarbonate was from Aldrich Chemical Co.; $(\text{NH}_4)_2\text{SO}_4$ (enzyme grade) was from Schwarz-Mann, Cambridge, MA, U.S.A.; DTAF and DTAF on Celite were from Molecular Probes, Junction City, OR, U.S.A.; Ultragel AcA 54 was from LKB Instruments; phenyl-Sepharose CL-4B and Sephadex G-25 (fine grade), G-75 and G-100 were from Pharmacia Fine Chemicals; KCl was Suprapur grade manufactured by E. Merck, Darmstadt, Germany; Celite was an Analytical Filter-Aid manufactured by Johns-Manville Products, Lompoc, CA, U.S.A. Distilled deionized water with a resistance of 18 M Ω at 20°C was used throughout the present work.

General procedures

The specimens of *Halistaura* and *Phialidium* were collected in the vicinity of the Friday Harbor Laboratories, University of Washington, Friday Harbor, WA, U.S.A., by using hand nets made of flat plastic screen (16 mesh). The purification and preparation of photoprotein samples were carried out at 0–4°C, except as noted, and the products

were stored at -75°C until used. The measurements of luminescence and the experiments to determine relationships between pCa and the initial maximum intensity of Ca^{2+} -triggered luminescence were performed as previously described (Shimomura & Shimomura, 1984), except as noted.

Halistaurin

Specimens of *Halistaura* sp. (100) were mixed with 1.5 litres of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 6.5, containing 50 mM-EDTA, stirred for 5 min, then briefly homogenized in a blender. The mixture was filtered on a Büchner funnel with some Celite. The filter cake was shaken with 100 ml of 50 mM-EDTA, pH 6.5, and filtered. The filtrate was saturated with $(\text{NH}_4)_2\text{SO}_4$ to precipitate crude halistaurin. The precipitate collected by centrifugation was dissolved in 15 ml of 10 mM-EDTA, pH 6.5. This material, having a total light-emitting capacity of 7×10^{15} quanta, was purified by two steps of gel filtration: the first step with a column (2.5 cm $\times$ 90 cm) of Sephadex G-75 and the second step with a column (1.5 cm $\times$ 90 cm) of Ultragel AcA 54, in both cases by using 10 mM-Mops buffer, pH 6.5, containing 10 mM-EDTA and 0.4 M-NaCl. The yield of light-emitting capacity was about 90% for each step of the gel filtration.

Phialidin

Specimens of *Phialidium gregorium* (600) were shaken with 1.2 litres of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 6.5, for a few minutes. After adding Celite the mixture was filtered on a Büchner funnel. The filter cake was shaken with 100 ml of 50 mM-EDTA, pH 6.5, then filtered. The filtrate, having a total light-emitting capacity of 3.8×10^5 quanta, was saturated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate of crude phialidin formed was collected by centrifugation, and dissolved in 15 ml of 10 mM-EDTA, pH 6.5, then purified by gel filtration as described above for halistaurin.

Ethoxycarbonylation of aequorin

The pH value of 1 ml of 5 mM-Mops buffer, pH 7.0, containing 2 mg of aequorin and 0.1 mM-EDTA was raised to 7.5 by adding about 10 mg of NaHCO_3 ; then 5% diethyl pyrocarbonate in dioxan was added to this solution in stepwise fashion, 5 μl at first, then followed by 1 μl portions every 8–10 min, with frequent measurement of luminescence activity with 2 μl of the reaction mixture each time. After 30–60 min of reaction time and with a total addition of 9–12 μl of the diethyl pyrocarbonate solution, the luminescence activity decreased to 80–90% of the initial value. The reaction mixture was passed through a column (0.9 cm $\times$ 10 cm) of Sephadex G-25 equilibrated with 5 mM-Mops buffer, pH 7.0, containing 0.1 mM-

EDTA to remove reagent. The fractions containing ethoxycarbonylated aequorin, which were eluted at the void volume, were stored frozen at -75°C until used. The product before freezing showed a high level of spontaneous luminescence, which significantly decreased over a few days at -75°C .

Acetylation of aequorin

To 1 ml of 5 mM-Mops buffer, pH 7.0, containing 2–3 mg of aequorin and 0.1 mM EDTA, about 10 mg of NaHCO_3 was added to raise the pH to 7.5. Acetylation was started by the addition of 2.5 μl of 5% acetic anhydride in dioxan to the aequorin solution. The total light-emitting capacity of the reaction mixture (measured with 2 μl portions) decreased to approx. 85% of the initial value at about 7 min after starting the reaction. The mixture was immediately filtered through a column of Sephadex G-25 as described above for the ethoxycarbonylation of aequorin.

Modification of aequorin with fluorescamine

To 1 ml of 5 mM-Mops buffer, pH 7.0, containing 2 mg of aequorin and 0.1 mM-EDTA, 3–4 μl of 2% fluorescamine in acetone was added. The total light-emitting capacity of the reaction mixture decreased to about 75–80% of the initial value at about 15 min after the addition of fluorescamine solution. The reaction mixture was promptly filtered through a column of Sephadex G-25 as described above for ethoxycarbonyl-aequorin.

Preparation of fluorescein-labelled aequorin

Approx. 0.5 mg portions of DTAF on Celite were added every 40–60 min to 3 ml of 10 mM-Hepes buffer, pH 7.5, containing 1.5 mg of aequorin, 2 mM-EDTA and 20 mM- $(\text{NH}_4)_2\text{SO}_4$, with occasional stirring. The mixture was frozen at -75°C in the evening, and the next morning it was thawed and the reaction allowed to resume. The progress of the reaction was monitored by the peak intensities of luminescence at 460 nm and 540 nm that were emitted when a 2 μl portion of the reaction mixture was allowed to luminesce by adding 2 ml of 10 mM-calcium acetate. The intensities of luminescence were measured with a Perkin-Elmer fluorescence spectrophotometer model MPF-44B in DC mode, without turning on its xenon lamp. The 460 nm/540 nm intensity ratio before any addition of DTAF was approx. 4. The 460 nm intensity decreased and the 540 nm intensity increased progressively. On the third day, when the 460 nm intensity decreased to one half of the initial value and the 540 nm intensity doubled its height (i.e., the 460 nm intensity equalled the 540 nm intensity), the reaction mixture was filtered through a column (1.5 cm $\times$ 10 cm) of Sephadex G-

25 equilibrated with 10mM-Hepes/2mM-EDTA buffer, pH 7.0, containing 0.2M- $(\text{NH}_4)_2\text{SO}_4$. The protein band, having greenish-yellow fluorescence, was collected (about 4ml) and was allowed to adsorb on a column (0.9cm $\times$ 2cm) of phenyl-Sephadex CL-4B. This second column was washed with 2ml of the Hepes/EDTA buffer containing 0.15M- $(\text{NH}_4)_2\text{SO}_4$, followed by 2ml of the buffer containing 0.1M- $(\text{NH}_4)_2\text{SO}_4$, then fluorescein-bound aequorin was eluted with the buffer alone. Eluate fractions collected (0.5ml each) were tested for the peak intensities at 460nm and 520nm, and the fractions having the intensity ratios (520nm/460nm) of more than 8 were pooled, and finally the product solution was passed through a column of Sephadex G-25 to decrease the concentration of EDTA, as described for ethoxycarbonylated aequorin. The yields, with reference to the amount of protein and to the light-emitting capacity, were 40 and 12% respectively, indicating a considerable decrease in the quantum yield due to the binding of fluorescein chromophore.

In the Phenyl-Sephadex chromatography described above, the absorption spectrum of the eluate fraction that had the highest intensity ratio (520nm/460nm) showed the presence of 1.07 fluorescein group per molecule of aequorin on the basis of the following values for the molar absorption coefficients: aequorin, 54000 litre·mol⁻¹·cm⁻¹ at 280nm (cf. Shimomura & Johnson, 1976); DTAF allowed to react with a large excess of *N* α -acetyl-L-lysine at pH 7.5, 58000 litre·mol⁻¹·cm⁻¹ at 492nm and 29900 litre·mol⁻¹·cm⁻¹ at 280nm (present work).

The preparation of fluorescein-bound aequorin by using fluorescein isothiocyanate resulted in a lower yield than that obtained by using DTAF. In the modification of aequorin with DTAF, the use of $(\text{NH}_4)_2\text{SO}_4$ (20mM) noticeably improved the yield of the final product, although it slightly retarded the reaction, as expected. Increasing the concentration of $(\text{NH}_4)_2\text{SO}_4$ to 100mM, or replacing $(\text{NH}_4)_2\text{SO}_4$ with triethanolamine, did not affect the reaction significantly; the addition of 5mM-2-mercaptoethanol caused an accelerated inactivation of the luminescence activity.

Identification of the binding site of the fluorescein group

Approx. 50 μg of fluorescein-bound aequorin was heated with 0.1 ml of 6M-HCl in a sealed glass tube at 105°C for 18 h. The hydrolysate was dried in a vacuum desiccator over P_2O_5 and NaOH, dissolved in 50% (v/v) methanol, then compared with control samples by t.l.c. on a Gelman ITLC type SA sheet. The control samples were prepared by allowing 2mg each of *N*-acetyl-L-tyrosine, *N* α -acetyl-L-lysine, *N* α -acetyl-L-histidine and *N*-acet-

yl-L-cysteine to react with 1 mg of DTAF in 0.4 ml of 2% NaHCO_3 , followed by HCl hydrolysis in the same manner as for the sample of fluorescein-bound aequorin. The R_F value of the fluorescent spot of the protein sample coincided only with that of the control sample made from *N* α -acetyl-L-lysine, indicating that the ϵ -amino group of a lysine residue is the binding-site [R_F 0.40 with benzene/pyridine/acetic acid (4:1:2, by vol.); R_F 0.05 with benzene/pyridine/acetic acid (12:3:4, by vol.)].

Results and discussion

The Ca^{2+} -sensitive photoproteins halistaurin and phialidin, which have been known for some time but have not been used in measuring Ca^{2+} hitherto, were extracted and purified from the jellyfishes *Halistaura* and *Phialidium* respectively. The purified photoproteins were not completely pure, but were considered to be sufficiently so for use as Ca^{2+} indicators *in vitro*.

The chemical modification of aequorin with various NH_2 -group-modification reagents always caused some, frequently a very large, loss in the luminescence capacity of the photoprotein, making it difficult to obtain sufficiently active product in a good yield. The NH_2 -group-modification reagents often caused a spontaneous weak luminescence in addition to a large, sometimes complete, loss of the luminescence activity of aequorin, an effect similar to the action of thiol-modification reagents previously reported (Shimomura *et al.*, 1974). Probably the NH_2 -group-modification reagents react with one of functional thiol groups of aequorin in addition to, or instead of, the 'intended' NH_2 group.

Despite this difficulty, we contrived to prepare four kinds of modified aequorin that are sufficiently active and that might be useful as Ca^{2+} indicators in biological systems, by carefully allowing aequorin to react with acetic anhydride, diethyl pyrocarbonate, fluorecamine and DTAF, although the products were difficult to purify, owing to the fact that aequorin consists of several isoproteins having isoelectric points from 4.2 to 4.9 (Blinks & Harrer, 1975).

Fluorescein-labelled aequorin prepared with DTAF contained one fluorescein group per molecule of aequorin, and the fluorescein group was bound at the ϵ -amino group of a lysine residue (or possibly more than one lysine residue) of the modified photoprotein. It may be reasonable to assume that the modification site(s) for other reagents is the same.

Modification with reagents other than the four kinds noted above was less satisfactory, owing to the greater losses of luminescence activity caused or the formation of mixtures of products that were

not amenable to ready characterization; such reagents included benzoic anhydride, succinic anhydride, trichloroacetic anhydride, trifluoroacetic anhydride, dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride, dinitrofluorobenzene, *p*-dimethylaminobenzaldehyde plus NaBH_4 , and various *N*-hydroxysuccinimide esters. The *p*-hydroxyphenylpropionylated aequorin previously prepared (Shimomura & Shimomura, 1982) was probably a mixture of products with a wide range of Ca^{2+} -sensitivity.

The relation between pCa and luminescence intensity of halistaurin, phialidin and the four kinds of modified aequorin is shown in Fig. 1, the luminescence spectra are shown in Fig. 2, and a summary of those results is given in Table 1, all in comparison with the data for aequorin. The results indicate that: (1) all photoprotein samples are less sensitive to Ca^{2+} in the presence of 0.1 M-KCl than in a low-ionic-strength buffer, apparently owing to inhibition by the salt; (2) halistaurin and phialidin are less sensitive than aequorin, whereas the modified forms of aequorin are more sensitive; (3) the spectrum of luminescence is affected by the modifying group, and by the type of photoprotein; (4) fluorescein-labelled aequorin emits greenish-yellow light, apparently by intramolecular energy transfer from the native chromophore of aequorin to the fluorescein group, in contrast with the blue light emitted from the other photoprotein samples.

In the measurement of Ca^{2+} in biological systems with a photoprotein, it is desirable to use a photoprotein that has a range of Ca^{2+} -sensitivity best suited for the pCa to be measured; a sufficiently strong luminescence response is needed, but an excessively fast consumption of a photoprotein by the luminescence reaction should be avoided. The availability of various photoproteins with different Ca^{2+} -sensitivities presently

Fig. 1. Relation between pCa and the initial maximum intensity of various photoproteins at 25°C in low-ionic-strength citrate buffer (—) and in 0.1 M-KCl buffer (----).

The initial maximum intensity was measured by adding 2 ml of the buffer solutions of various pCa values to a 2 μl portion of a photoprotein solution containing aequorin ($\blacktriangle$), halistaurin ($\square$), phialidin ($\blacksquare$), acetylated aequorin ($\bullet$), ethoxycarbonylated aequorin ($\circ$) or fluorescein-labelled aequorin ($\triangle$). The data for fluorescamine-modified aequorin were similar to those for fluorescein-labelled aequorin. The solution of photoprotein contained about 10 μM -aequorin, or other photoprotein of roughly equivalent light-emitting capacity. The citrate buffer, pH 7.0, contained 1 mM-sodium citrate plus various concentrations of calcium citrate to set the pCa values [calculated on the basis of a stability constant for calcium citrate of $10^{4.5}$ (cf. Shimomura & Shimomura, 1984)]; the KCl buffer, pH 7.0, contained 1 mM-Mops plus appropriate amounts of CaCl_2 to set the pCa values, in addition to 0.1 M-KCl; both buffers were prepared as previously described (Shimomura & Shimomura, 1984).

Table 1. Sensitivities to Ca^{2+} and luminescence emission maxima of aequorin, halistaurin, phialidin and some modified forms of aequorin

Median sensitivity is shown by the value of pCa at which the initial maximum intensity of luminescence is equal to $\sqrt{I_0 I_{\text{max}}}$, where I_0 is the initial maximum intensity with no Ca^{2+} added and I_{max} is the initial maximum intensity with 0.01 M- Ca^{2+} . The values of the maximum sensitivity are approx. 1.5–2.0 units greater than the values of median sensitivity shown. The citrate buffer and the KCl buffer (both pH 7.0) are the same as those used in the experiments of Fig. 1. All experiments were performed at approx. 25°C.

Photoprotein	Median sensitivity (pCa)		Emission maximum (nm)
	1 mM-Citrate	0.1 M-KCl	
Aequorin	7.15	5.9	465
Halistaurin	6.84	5.6	470
Phialidin	6.2	5.1	474
Acetylated aequorin	7.6	6.2	460
Ethoxycarbonylated aequorin	7.6	6.25	450
Fluorescamine-modified aequorin	7.5	6.1	463
Fluorescein-labelled aequorin	7.4	6.1	520

Fig. 2. Ca^{2+} -triggered luminescence spectra of various photoproteins

Weak luminescence lasting at least 10 min was elicited by careful addition of CaCl_2 solution into 1 ml of 5 mM-Mops buffer, pH 7.0, containing 0.1 mM-EDTA and approx. $10 \mu\text{g}$ of ethoxycarbonylated aequorin (E), aequorin (A), halistaurin (H), phialidin (P), or fluorescein-labelled aequorin (F). The spectrum of fluorescamine-modified aequorin was similar to that of aequorin. The luminescence spectra were recorded with Perkin-Elmer fluorescence spectrophotometer (model MPF-44B) in DC mode, then corrected for the decay of intensity with time, but not corrected for changes in sensitivity of the photomultiplier (R-928) at different wavelengths.

demonstrated, together with other photoproteins to be discovered in the future, will help to meet these requirements.

The use of fluorescein-labelled aequorin in the microinjection method will make it possible to estimate the amount of injected photoprotein in a cell by fluorescence measurement. More importantly, this same material might be useful in a new technique of Ca^{2+} measurement that may virtually

eliminate the necessity of calibration *in situ*. For example, when a mixture of fluorescein-labelled aequorin and halistaurin is microinjected into a cell in which the concentration of KCl would be at least 0.1 M and pCa to be measured is within a range of 6–7.5, the ratio of luminescence intensities at 460 nm and 520 nm emitted from the cell should directly indicate the Ca^{2+} concentration of the light-emitting site, this assumption being based on the fact that the pCa–light-intensity curves of these two photoproteins are not parallel over the pCa range chosen.

This work was supported by grants from the National Institutes of Health (GM 31314) and the National Science Foundation (PCM-8201005). We are indebted for the use of facilities at the Friday Harbor Laboratories, University of Washington.

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