EDTA-BINDING AND ACYLATION OF THE Ca^{2+}-SENSITIVE PHOTOPROTEIN AEQUORIN

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Received 29 December 1981

1. Introduction

The photoprotein aequorin ($M_r \sim 20\,000$) emits light in aqueous solutions when a trace amount of Ca^{2+} is added, independently of the presence of O_2 [1,2]. Aequorin has been extensively used in detecting and monitoring Ca^{2+} in various biological systems, including single cells [2,3]. EDTA and EGTA have been used to preserve the luminescent activity of aequorin until its use, and they have been also used to prepare calcium-buffer solutions for calibrating the light emission of aequorin.

We now report that EDTA can inhibit the luminescence of aequorin by the binding of EDTA to the photoprotein, in addition to the removal of Ca^{2+} by chelation. Similar effects are expected for EGTA which is functionally similar to EDTA.

We have also found that acylation of aequorin can change the luminescent kinetics of this photoprotein, thus opening possibilities for creating modified forms of aequorin that are more suitable than native aequorin for the specific purposes of analysis.

2. Materials and methods

Aequorin was extracted and purified as in [4,5] and made to 0.1–0.2 mM stock solution in 5 mM MOPS (4-morpholinepropanesulfonic acid)–0.1 mM EDTA (pH 7.0), then stored frozen. EDTA (2 Na-salt), acetic anhydride, and 3-(*p*-hydroxyphenyl)propionic acid *N*-succinimide ester were purchased from Aldrich Chemicals, MOPS was from Sigma, sodium acetate 'Ultrapure' from Alfa, Sephadex G-25-fine from Pharmacia, and ethylenediamine tetra[2-^{14}C]acetic acid (108 mCi/mmol) from Amersham Radiochemical Centre. Deionized distilled water having a resistance of >20 MΩ was used throughout. Ca-EDTA (pH 7.0) 50 mM, was prepared by titrating 100 mM EDTA with 100 mM CaCl using arsenazo III (Sigma, grade I) as the indicator, with continuous adjustment of the pH with 5 M NaOH. Only plastics came into contact with solutions throughout the experiments, except a 5 μl Hamilton syringe to measure aequorin samples and the glass tip of a combination pH electrode having a plastic body.

MOPS buffer solutions without added EDTA were prepared with a stock solution of 50 mM MOPS that were pretreated with Chelex 100 (50–100 mesh, Bio-Rad Labs), as follows: 500 ml 50 mM MOPS (pH 10, adjusted with NaOH) was filtered through a column of Chelex 100 (1.8 × 12 cm) pre-washed with 2 l water, and the last 300 ml of the filtrate was kept and used as the stock solution. The pH and pCa of MOPS buffer prepared with this stock solution were adjusted, respectively, with a diluted HCl that was prepared by dissolving HCl gas in water, and with $CaCl_2$.

EDTA (10 mM)–MOPS (5 mM) calcium-buffers (pH 7.1) of pCa 8.05, 7.57, 7.04, 6.56, 6.1, 5.54 and 4.8 contained, respectively, the following percentages of Ca-EDTA in 10 mM of the total EDTA: 18, 40, 69, 87, 95.1, 98.6 and 99.75 [6]. pCa 4 buffer contained 10 mM Ca-EDTA plus 0.1 mM $CaCl_2$ in addition to 5 mM MOPS.

To measure light intensity of aequorin luminescence, a stock solution of aequorin or its derivative in 0.1 mM EDTA was first diluted with water to reduce the EDTA level to 10 μM, then 1–3 μl of the diluted sample was placed in a polycarbonate test tube, and luminescence was initiated by adding 2 ml of a test solution from a plastic syringe, at 23–25°C.

Equilibrium dialysis was performed as follows:

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Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

1 ml 0.1 mM aequorin stock solution was placed in pre-washed cellulose tubing (inflated diam. 6.3 mm; A. H. Thomas Co.), and dialyzed against 50 ml 3 mM MOPS–50 mM NaCl–0.2, 1 or 5 mM EDTA (pH 7.0) that contained 6 μCi [^{14}C]EDTA, with a magnetic stirrer, at 0–3°C. After 24 h, 0.3 ml each of the inside solution and the outside solution were taken out and each was mixed with 20 μl 0.1 M $CaCl_2$ and 15 ml 'Aquafluor' (New England Nuclear). The radioactivities were then measured in a Packard liquid scintillation counter at 8°C. The equilibration was complete in a control experiment in which aequorin was omitted, although no effort was made to confirm the completeness in the presence of aequorin.

p-Hydroxyphenylpropionyl aequorin (HPP-aequorin) was prepared as follows: To 1 ml 0.15 mM aequorin stock solution, 10 mg $NaHCO_3$ was added, followed by a stepwise addition of 2% 3-(*p*-hydroxyphenyl)propionic acid *N*-succinimide ester (in dioxane) in 5 μl portions at 0°C, monitoring the total luminescent capacity of aequorin by testing 2 μl fractions of the reaction mixture with 10 mM calcium acetate. When four 5 μl portions of the reagent were added in ~20 min, the luminescent capacity of aequorin decreased to 50% of the initial value. The mixture was immediately filtered through a column of Sephadex G-25 fine (1 × 8 cm; prepared with 0.1 mM EDTA–5 mM MOPS (pH 7.0)) to separate the aequorin product from the reagent and its decomposition products. When needed, the protein was concentrated through precipitation with $(NH_4)_2SO_4$.

Acetylated aequorin was prepared as follows: To 0.6 ml 0.2 mM aequorin stock solution, 0.3 ml saturated aqueous solution of sodium acetate was added, followed by the addition of three 3 μl portions of acetic anhydride at 0°C. When the luminescent capacity of aequorin decreased to ~60% of the initial value, the mixture was filtered through a column of Sephadex G-25 in the same manner as described above for HPP-aequorin.

3. Results and discussion

The relation between light emission of aequorin and [Ca^{2+}] in various buffers is shown in fig.1. The maximum slope of the log–log plot of light emission as a function of [Ca^{2+}] was 2–3, in agreement with [5,7–9]; the data suggest that the inclusion of EDTA in the buffers might have caused the increase of the maximum slope.

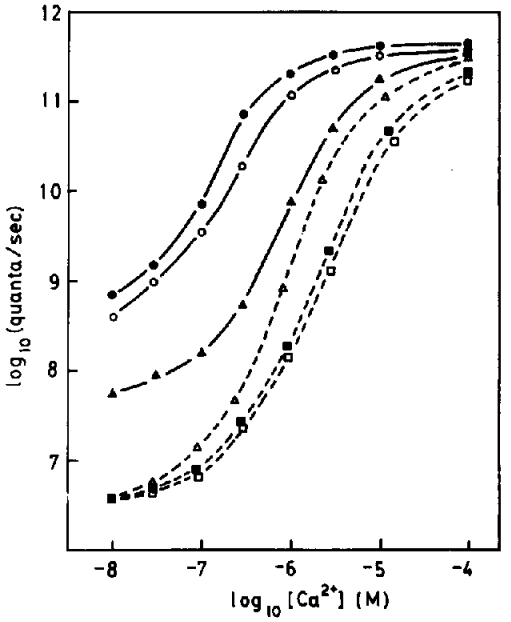

Fig.1. Relationship between [Ca^{2+}] and initial maximum light intensity when 1 μl 10 μM aequorin was luminesced in buffers without added EDTA (——) and with EDTA (– – –). The buffers used and the maximum slope of the curves are (from the top curve): 1 mM sodium acetate (pH 5.8), slope 2.05 (●); 5 mM MOPS (pH 7.1), slope 1.9 (○); 50 mM MOPS (pH 7.1), slope 2.05 (▲); 0.1 mM EDTA–5 mM MOPS (pH 7.1), slope 2.8 (△); 1 mM EDTA–5 mM MOPS (pH 7.1), slope 2.3 (■); 10 mM EDTA–5 mM MOPS (pH 7.1), slope 2.2 (□). The lower light intensities in Chelex 100-treated 50 mM MOPS than in 5 mM MOPS suggest that Chelex 100 had leached some calcium-chelating substance into the 50 mM MOPS, as observed in [5]. The leached substance, as well as the EDTA initially present in the aequorin sample and undetermined traces of contaminating calcium in the buffers, may cause errors at pCa >7 in the absence of added EDTA, although this inaccuracy does not affect these conclusions.

Equilibrium dialysis of 0.1 mM aequorin in pH 7.1 buffers containing 5 mM, 1 mM and 0.2 mM [^{14}C]-EDTA resulted in the binding of 2.3, 1.9 and 1.0 mol EDTA/mol aequorin, respectively. The data clearly indicate the ability of aequorin to bind EDTA. No effort was made, however, to obtain the binding constant between EDTA and aequorin by this technique, due to the decreased stability of aequorin in EDTA solutions of lower concentration.

The effect of Ca-EDTA on aequorin luminescence appears insignificant: luminescence of aequorin in 5 mM MOPS buffer (pH 7.1) containing 0.1 mM $CaCl_2$ was not appreciably inhibited by up to 50 mM Ca-EDTA buffer.

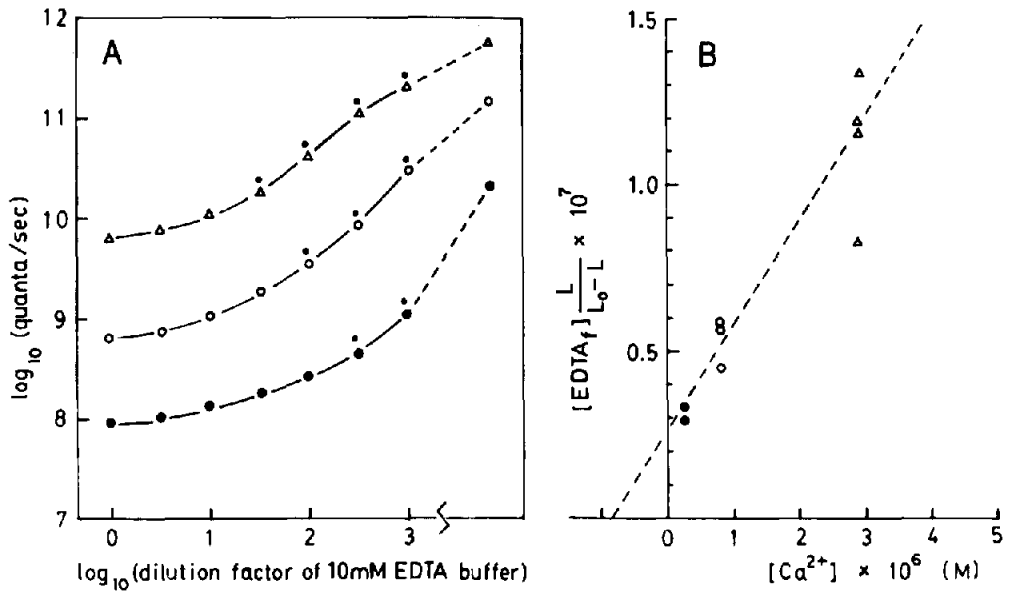

Fig.2. (A) Effect of the dilution of 10 mM EDTA–5 mM MOPS (pH 7.1) [pCa 5.54 (△); 6.1 (○); and 6.56 (●)] with 5 mM MOPS (pH 7.1) on the initial light intensity of 3 μl 10 μM aequorin. The 3 points on the right, connected with broken lines, were obtained with 5 mM MOPS containing $CaCl_2$ in amounts corresponding to the pCa values. (B) The data obtained with the solutions that contained <10 μM free EDTA (symbols with superscript dot in A) are plotted as in [11]. *Abbreviations:* $EDTA_f$, free EDTA; L, initial light intensity in the presence of EDTA; L_0, initial light intensity in absence of EDTA.

When 10 mM EDTA calcium-buffers (pCa 5.54, 6.1 and 6.56) each made in 5 mM MOPS buffer (pH 7.1) were stepwise diluted with 5 mM MOPS buffer (pH 7.1), a progressive increase in the luminescence intensity of added aequorin was noted (fig.2A). These data provide direct proof for the luminescent inhibitory effect of free EDTA through direct interaction with aequorin. Deviation of pCa-values by dilution (significant only at high dilutions) does not affect this conclusion. The inhibition of aequorin luminescence by EDTA is thus due to:

(i) Removal of free Ca^{2+} by chelation;

(ii) Inhibition by directly binding to aequorin.

The data in fig.2A have been replotted by the Dixon method [10] taking the [free EDTA] *vs* the reciprocal of light intensity ($1/L$). Although aequorin is not an enzyme, such treatment should be justified if only initial light emission is considered. The plot, however, did not give the anticipated straight line; at >10 μM free EDTA, $1/L$ increased much lower in proportion to the increase of [free EDTA]. The deviation from a straight line could be due to competition of free EDTA and non-inhibitory Ca-EDTA, although other explanations are possible. Extrapolation of the curve to the zero concentration of free EDTA to obtain the inhibitor constant is not justified because the error of data becomes greatest at the lowest [free EDTA].

When the data involving <10 μM free EDTA in fig.2A were replotted as in [11], an inhibitor constant, K_i, of $\sim 2.7 \times 10^{-8}$ M was obtained for free EDTA, as shown in fig.2B. In plotting this graph, corrections were not made for the shifts of pCa-values nor for the errors in [free EDTA] caused by greatly diluting the original 10 mM EDTA buffer solutions. This should not cause a large error in the K_i as the dilution effects are negligibly small for pCa 6.56 buffer, although discernible for pCa 6.1 buffer and significantly large for pCa 5.54 buffer.

We have also found that acylation of aequorin can modify its luminescent kinetics (fig.3). The maximum slope of the log–log plot of light *vs* $[Ca^{2+}]$ for *p*-hydroxyphenylpropionyl aequorin (HPP-aequorin) was 1.1 in contrast to 1.9 for native aequorin, both in 5 mM MOPS buffer (pH 7.1) without added EDTA. In the same buffer, luminescence of acetylated aequorin was ≥15-times higher than that of native aequorin over 10^{-8}–10^{-7} M Ca^{2+} and reached a maximum at $>10^{-7}$ M Ca^{2+}.

In the presence of 0.1 mM EDTA buffer, lumines-

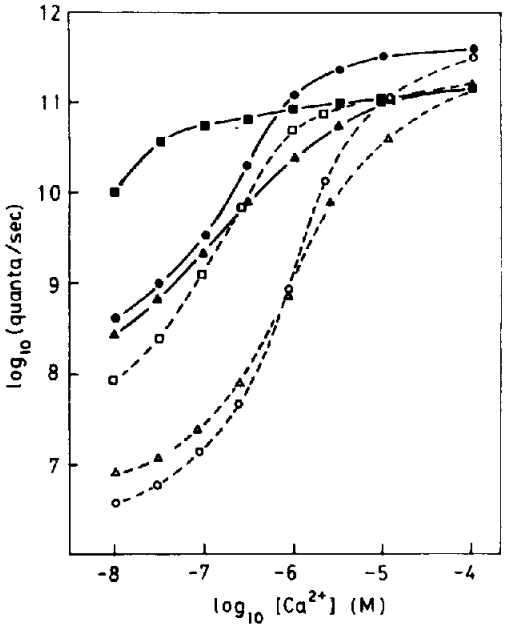

Fig.3. Relationship between $[Ca^{2+}]$ and the initial light intensities of aequorin (●,○), HPP-aequorin (▲,△), and acetylated aequorin (■,□) in 5 mM MOPS (pH 7.1) (——) and in 0.1 mM EDTA–5 mM MOPS (pH 7.1) (– – –). The amounts of photoprotein samples used were the same as that of aequorin in fig.1. The maximum slopes of the curves are: 1.9 (●); 1.1 (▲); 1.1 (■); 2.9 (○); 2.05 (△); and 1.7 (□).

cence at low $[Ca^{2+}]$ was strongly suppressed, thereby showing a maximum slope of 2.05 for the curve of HPP-aequorin, in comparison to 2.9 for native aequorin. In the same buffer, luminescence of acetylated aequorin was ≥20-times higher than native aequorin over 10^{-8}–10^{-6} M Ca^{2+}. Other types of acylated aequorin, such as succinyl aequorin and butyryl aequorin, also showed distinctive characteristics in luminescence (not shown).

The evidence described indicates that acylation is an effective means of modifying the Ca^{2+}-dependent luminescence of aequorin. In detecting and monitoring Ca^{2+} with aequorin, a specific acylated aequorin should be chosen.

Acknowledgements

This work was initiated by the suggestion of Dr E. B. Ridgway to create a 'linear aequorin' which would luminesce in direct proportion to $[Ca^{2+}]$. We thank Dr A. Fabiato for computing the compositions of some of the EDTA buffers used, Dr F. H. Johnson and Dr E. B. Ridgway for reading the manuscript, and B. Bamman for measuring radioactivities. This work was supported by grants from the National Science Foundation (PCM-7822959) and the National Institutes of Health (GM-25093).

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