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COMPARATIVE ASPECTS OF THE CALCIUM-SENSITIVE PHOTOPROTEINS AEQUORIN AND OBELIN

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SUMMARY

1. The calcium-dependency of the process of light emission has been investigated for the photoproteins aequorin and obelin.

2. The experimental curves of light production, expressed as a percentage of the maximal rate of utilisation, versus p*Ca* are accurately predicted by the cooperative action of at least $2Ca^{2+}$ for aequorin and at least $3Ca^{2+}$ for obelin.

3. At low total monovalent cation concentrations, a pH change from 6.8 to 7.1 shifts the light production vs p*Ca* curve by approx. 0.2 p*Ca* units to the right for aequorin, while that for obelin is shifted by some 0.37 p*Ca* units.

4. Other monovalent cations, such as Na^+ are able to compete with Ca^{2+} for the active sites of aequorin and also shift the light production vs p*Ca* curve to the right. There is no apparent change in the calcium stoichiometry for light production under these conditions.

5. The same calcium stoichiometry for light emission was also obtained for aequorin or obelin in the presence of either unbuffered Ca^{2+} solutions or of calcium/EGTA buffers.

INTRODUCTION

The use of the calcium-sensitive photoprotein, aequorin, isolated from the hydromedusa *Aequorea forskalea* [1], proved to be an important step towards the better understanding of a number of cellular processes dependent upon low levels of ionized calcium (Ca^{2+}) [2–12]. Further investigations have led to the discovery of similar calcium-activated luminescent proteins [13–15, 26] recently it has been reported that the photoprotein, obelin, extracted from the hydroid *Obelia geniculata*, would also be available in sufficient quantities for physiological experiments [13, 16–18]. In order to decide to what extent the two photoproteins are similar, it was of importance to compare the dependency of the light emission process upon Ca^{2+} concentration for both proteins assayed under the same experimental conditions.

Abbreviations: EGTA, ethanedioxy bis(ethylamine)tetraacetic acid; TES buffer, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

MATERIALS AND METHODS

In these experiments a simple but effective mixing technique was used which provided mixing times of about 0.1 s. This method permitted an accurate estimate to be made of the apparent overall rate of photoprotein utilization (see Eqns 2 and 3). Generally a small aliquot of diluted photoprotein (30 or 60 nl) was added onto a shaped glass stirrer which was driven by a small electric motor. The stirrer was then introduced in the dark into 1–3 ml of a calcium solution buffered with EGTA which was contained in a vial on top of the face-plate of an EMI 9635 photo-multiplier tube (cathode sensitivity, 100 μA/lumen, EHT: −900 V). The apparent overall rate of utilization was calculated from the half-decay times of the light emission,

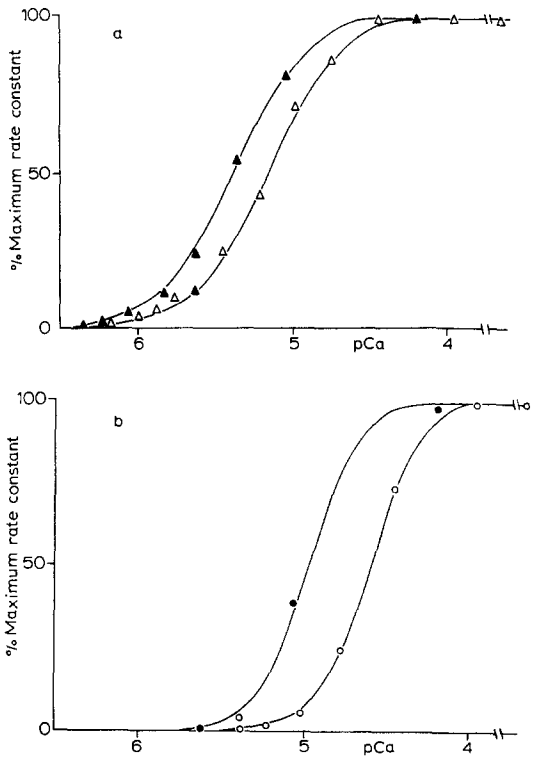

Fig. 1. Apparent relative rate (k_r) of the photoprotein utilization as function of pCa (= $-\log[Ca^{2+}]$) in a calcium-buffered medium of the following composition (mM): K_2EGTA+Ca-K_2EGTA, 20; KCl, 40; K-TES, 10. The solid lines represent the theoretical predictions from a consecutive scheme of reaction [5] involving two Ca^{2+} for aequorin ($k_r^A = K_1^A K_2^A Ca^2/(1+K_1^A K_2^A Ca^2)$) and three Ca^{2+} for obelin ($k_r^0 = K_1^0 K_2^0 K_3^0 Ca^3/(1+K_1^0 Ca+K_1^0 K_2^0 Ca^2+K_1^0 K_2^0 K_3^0 Ca^3)$). The superscripts A and 0 refer to aequorin and obelin respectively and K_i represents the binding constant of the photoprotein for the ith Ca^{2+}. (a) Aequorin, △, pH 6.8±0.01; theoretical fit for $K_1^A < 1000 K_2^A$, $K_1^A K_2^A = 2.3 \cdot 10^{10}$ M^{-2}. ▲, pH 7.1±0.01; theoretical fit for $K_1^A < 1000\ K_2^A$; $K_1^A K_2^A = 6.46 \cdot 10^{10}$ M^{-2}. (b) Obelin, ○, pH 6.8±0.01; theoretical fit for $K_1^0 < 0.001\sqrt{K_2^0 K_3^0}$ and $K_3^0 > 1000\sqrt{K_1^0 K_2^0}$; $K_1^0 K_2^0 K_3^0 = 7.95 \cdot 10^{13}$ M^{-3}. ●, pH 7.1±0.01; theoretical fit for $K_1^0 < 0.001\sqrt{K_2^0 K_3^0}$ and $K_3^0 > 1000\sqrt{K_1^0 K_2^0}$; $K_1^0 K_2^0 K_3^0 = 1 \cdot 10^{15}$ M^{-3}. The maximal overall rate of utilization, k for aequorin was 1.5 s^{-1} and for obelin 3.9 s^{-1} at 23 °C, pH 6.5–7.5.

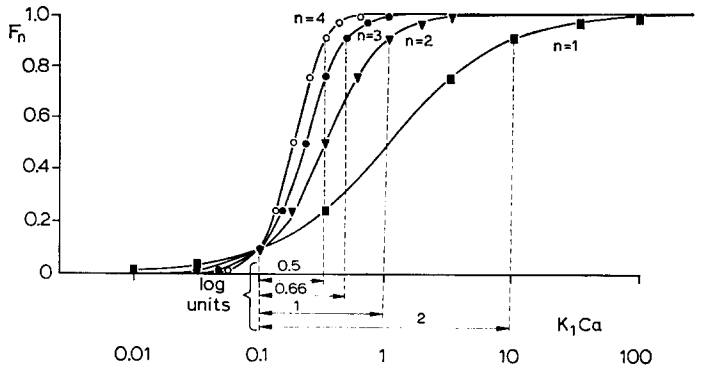

Fig. 2. A semi-logarithmic plot of the expression $F_n = K_n\,[Ca]^n/(1+K_n\,[Ca]^n)$ against $K_1\,[Ca]$. The values of K_n ($n > 1$) have been chosen such that all the curves intersect at the point where $F_n = 0.091$ ($K_n = 10^{n-1} \cdot K_1{}^n$). Thus the value of the calcium concentration at which F_n reaches a value of 0.91 is shifted by (2 log units/n) away from that corresponding to the point of intersection (see text).

which was divided by the maximal rates of utilization of the photoproteins indicated in the figure legends to give the percentage rate constant expressed in the ordinate of Figs 1 and 3. This method of mixing had a number of advantages, in addition to its relative simplicity. It enabled the photoprotein to be added in very small volumes, which did not contain a calcium-buffering system such as EDTA (ethylene diamine tetraacetic acid) or EGTA (ethanedioxy bis(ethylamine)tetraacetic acid) apart from the aequorin itself. Thus pH changes which could appear upon mixing such buffer systems were avoided and the importance of this consideration can be judged by the results presented in Figs 1a and 1b. As well as mixing the solution

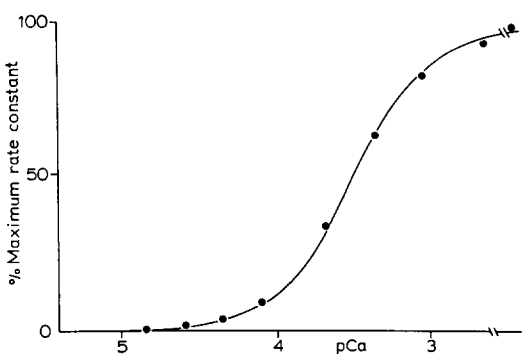

Fig. 3. Relative rate of aequorin utilization ($k_r{}^A$) as function of pCa using unbuffered Ca^{2+} in a standard solution containing 'Specpure' NaCl 0.83 M; K-TES 50 mM, pH 7.1 $\pm$0.01. The solid line is the theoretical prediction to fit the experimental points (●) with $K_1{}^A = 5 \cdot 10^2\ M^{-1}$ and $K_2{}^A = 2.5 \cdot 10^4\ M^{-1}$ (see legend Fig. 1). Here an aliquot of aequorin (30 or 60 nl) was added first into 1 ml of the standard solution and then mixed uniformly. Consequently, small volumes (1–4 μl) of $CaCl_2$ solutions of different concentrations were added onto a glass stirrer (see text). $k_r{}^A$ was calculated as described in the text. The maximal rate of utilization for aequorin was 1.2 s^{-1} at a temperature of 20 °C.

continuously throughout the experiment, the method also enabled a continuous measure to be made of the photoprotein response following either a decrease or an increase in the Ca^{2+}, as illustrated in Figs 4a and 4b.

The free calcium concentration in the Ca-buffers was calculated by the method outlined in ref. 22, employing the apparent binding constants given by ref. 24 for EGTA. The H^+ buffer TES (*N*-tris(hydroxymethyl) methyl-2-aminoethanesulphonic acid) was used throughout. The relative concentrations of total Ca and EGTA in the mixtures were accurately estimated using a titrimetric method [23].

In the experiments with unbuffered calcium, 'Specpure' (Johnson Matthey Chemical, London) NaCl was used.

RESULTS AND DISCUSSION

In Figs 1a and 1b are shown the experimental curves which relate light production, expressed as a fraction of the maximal rate of consumption of the two photoproteins, versus Ca^{2+} concentration at two different pH values employing Ca-EGTA buffers to stabilize the free calcium concentration. The theoretical curves presented in Fig. 2 illustrate the relationship between the theoretical function, F_n versus p*Ca* (see legend) which provides the steepest relationship which can be achieved by the cooperative action of nCa^{2+} in a given process. Thus if the ratio, R, between the calcium concentration corresponding to approx. 90 % and that corresponding to approx. 10 % of the maximal saturated experimental value is close to 100, that is 2 log units, then only $1Ca^{2+}$ per functional unit is necessary to explain the curve; if $10 < R < 100$ ($1 < \log R < 2$) then the respective process could be explained in terms of at least $2Ca^{2+}$; if $4.6 < R < 10$ ($0.66 < \log R < 1$) then at least $3Ca^{2+}$ must be involved in the reaction scheme, while for $4Ca^{2+}$ to be required $3.16 < R < 4.6$ ($0.5 < \log R < 0.66$) must be the case. This analysis was applied to the experimental results in Figs 1a and 1b and Fig. 3 and suggested at least $2Ca^{2+}$ per functional unit for aequorin. The obelin curves were however much steeper implying the cooperative action of at least $3Ca^{2+}$ for the process of light emission. All the experimental results presented in this paper can only be fitted by theoretical curves derived from the consecutive reaction scheme [5] utilizing $2Ca^{2+}$ for aequorin and $3Ca^{2+}$ for obelin. The general form of the reaction scheme can be expressed as follows:

$$Ca^{2+} + PhP \overset{K_1}{\rightleftharpoons} (CaPhP) + Ca^{2+} \overset{K_2}{\rightleftharpoons} \cdots \overset{K_n}{\rightleftharpoons} (Ca_nPhP) \xrightarrow{k} h\nu + \text{Products} \qquad (1)$$

where K_i represents the apparent affinity constant of the photoprotein, PhP, for the ith calcium and k is the overall rate of photoprotein utilization ($1.5\,s^{-1}$ for aequorin and $3.9s^{-1}$ for obelin at 23 °C at pH 6.5–7.5). Since the reaction steps in which Ca^{2+} is involved are considered to equilibrate much faster (approx. $300–500s^{-1}$) for aequorin [25] and this has been assumed also for obelin, it follows that light intensity, L is

$$L = \gamma k[Ca_nPhP] = \gamma k_r \cdot k[PhP]_0 \exp(-k_r k t) \qquad (2)$$

where $[PhP]_0$ is the initial concentration of the photoprotein, γ is its quantum yield, t is the time and k_r is given by the following expression

$$k_r = K_1K_2 \cdots K_n[Ca]^n/(1 + K_1[Ca] + K_1K_2[Ca]^2 \cdots K_1K_2 \cdots K_n[Ca]^n) \qquad (3)$$

and has the meaning of the apparent relative rate constant of the photoprotein utilization. The apparent affinity constants, K_i, to which the experimental results were fitted are quoted in the appropriate figure legends. It is important to note that the apparent affinity constants of the two photoproteins for Ca^{2+} are significantly affected by a change in pH for the conditions indicated in Fig. 1. A drop in pH from 7.1 to 6.8 shifts the curve for aequorin by approx. 0.2 p*Ca* units towards higher Ca^{2+} concentrations, while that for obelin is shifted by about 0.37 p*Ca* units, without affecting the maximum rate constants for the consumption of either of the photoproteins. This pH effect could be explained by assuming a simple competitive binding of Ca^{2+} and H^+ to the active sites of the protein. If this is the case, then the values by which the curves (Fig. 1) are affected suggests that under these experimental conditions, aequorin should have a high apparent affinity constant ($10^{7.5}$–10^8 M^{-1}) for one H^+ for every Ca^{2+} required to activate the process of light emission. While obelin should have, in addition, a relatively high apparent affinity constant for a second H^+ ($10^{6.8}$–$10^{7.5}$ M^{-1}) for every Ca^{2+} bound. The two photoproteins are considerably less sensitive to pH changes when the Mg^{2+} and monovalent cation concentration is higher, as is the case in vivo, since these ions are also able to compete with Ca^{2+} for the active sites of the photoproteins. This monovalent cation inhibition has not been reported before for either of the photoproteins and this phenomenon must certainly be taken into account when light emission vs p*Ca* curves such as Figs 1 and 3 are being constructed.

It has been suggested by other investigators [19] that EGTA might itself interact with aequorin, therefore we have also used unbuffered Ca^{2+} concentrations to assess the relationship between Ca^{2+} and the process of light production. Since it is more convenient and reliable to work at high unbuffered Ca^{2+} concentrations, we have made use of the observation that monovalent cations can compete quite strongly for the active calcium binding sites of the photoproteins. The results presented in Fig. 3 illustrate the dependency of aequorin light emission upon unbuffered Ca^{2+} concentration (see legend for details). The experimental points are again satisfactorily predicted by a consecutive scheme for the co-operative interaction of two calciums as for Fig. 1. These observations strongly suggest that Ca-EGTA buffers have no unexpected effect upon the aequorin light emission and might imply that observation by other workers to the contrary could be attributed to the effect of monovalent cations in the supporting medium. The present results emphasise, therefore, that in order to construct an accurate calibration curve for aequorin or obelin in unbuffered Ca^{2+}, great care must be taken to ensure a constant pH and an essentially constant ionic composition throughout the whole series of individual observations at different Ca^{2+} values.

It can be observed from Eqn 2 that for conditions far from calcium saturation of the photoprotein, the intensity of light emission is practically proportional to k_r which in turn is proportional to $[Ca]^n$. Therefore measurements of light intensity under these conditions can lead to an independent estimate of n, the number of Ca^{2+} involved in the process of light emission. This has been investigated for the two photoproteins using both low unbuffered (Fig. 4a) and low buffered (Fig. 4b) Ca^{2+} concentrations. The results presented here, where the intensities of the light emission from the two photoproteins is compared, confirms the results obtained at higher free calcium concentration where the light emission falls more rapidly. Namely

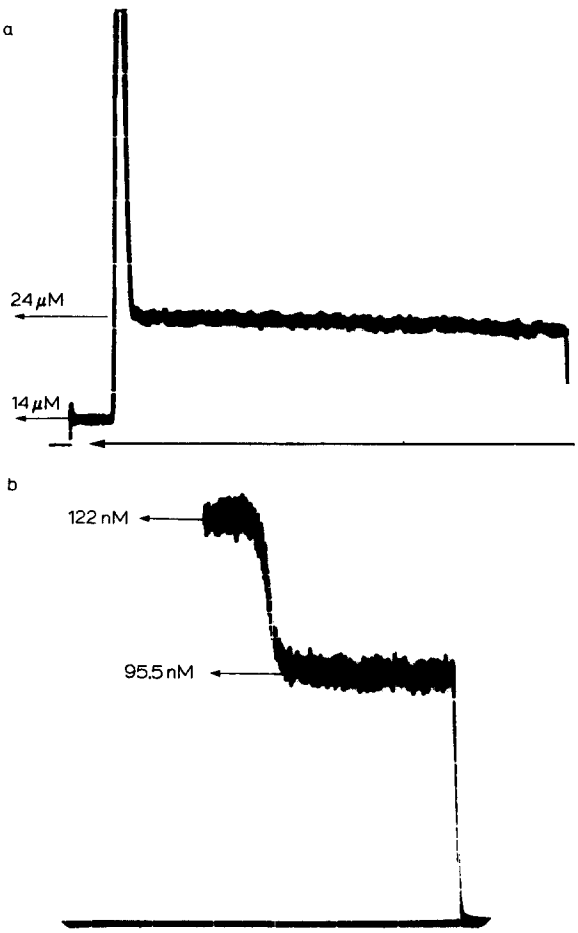

Fig. 4. Records showing the change in the intensity of light emitted by aequorin and obelin following a sudden change in Ca^{2+} concentration in conditions far from calcium saturation of the photoproteins. (a) Obelin light emission when the unbuffered Ca^{2+} concentration was changed from 14 μM to 24 μM in the standard solution mentioned in legend Fig. 3 by the same procedure as described in Fig. 3. Calibration bars: horizontal 5 s; vertical 100 nA. Note that light is proportional to the cube of Ca^{2+} concentration. (b) Aequorin light emission when the buffered Ca^{2+} concentration is decreased from $1.22 \cdot 10^{-7}$ M to $9.55 \cdot 10^{-8}$ M in 1 ml of a solution containing (mM): KCl 200, K_2EGTA + K_2Ca-K_2EGTA 9, TES 50, pH 7.1 by addition of 2 μl of a 0.65 M K_2EGTA solution, pH 7.1 following the procedure described in legend of Fig. 3. Note that here the light intensity is proportional to the square of Ca^{2+} concentration. Calibration bars: vertical 100 nA; horizontal 2 s. The base lines represent essentially the dark current from the photomultiplier tube.

that for aqeuorin the process of light emission is dependent upon $(Ca^{2+})^2$ and $(Ca^{2+})^3$ for obelin [3, 20].

The difference between the number of Ca^{2+} required to initiate the light emission process for the two proteins is only apparent, since aequorin is also likely to require three calciums for emitting light [21]. The theoretical predictions presented in Figs 1 and 2 for the light production vs p*Ca* curves, indicate the first calcium

with aequorin should be bound with a very much higher affinity than the square root of the product of affinities of the other two sites, so that usually the first calcium remains unobserved. This does not appear to be the case for the photoprotein obelin in these experiments. In an earlier study it appeared, at least over a narrow range of p*Ca* values, that the obelin light emission vs p*Ca* relationship was closer to a square-law than a cubic function [16, 17]. This present analysis, performed over a much wider range of p*Ca* values, is likely to provide a more precise stoichiometry, since a double logarithmic plot of light emission vs p*Ca* [16, 17] will only give the correct calcium stoichiometry if the photoprotein is far from saturation with calcium. In addition, as the inhibitory effect of monovalent and divalent cations upon the process of light emission by obelin has not been exhaustively investigated, this phenomenon could produce a spuriously low stoichiometry, particularly when a double logarithmic plot is employed, as opposed to the more complete curve used in the present study.

It can be concluded that obelin can also be used succesfully to follow calcium changes both in vivo and in vitro. Although less sensitive than aequorin for lower Ca^{2+} concentrations, obelin can be used to detect calcium occurring at higher values, as indicated by the shift of the light emission vs p*Ca* curve to the right (Fig. 1). The difference in the relationships between aequorin and obelin for different Ca^{2+} concentrations make them very useful complementary techniques for studying calcium-dependent processes.

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