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Aequorin Luminescence: Relation of Light Emission to Calcium Concentration—A Calcium-Independent Component

Abstract. *Light emission from the calcium-sensitive bioluminescent protein aequorin was measured at calcium ion concentrations of 10^{-9} to 10^{-2} molar. At very low Ca^{2+} concentrations, light emission is independent of calcium ion concentration. The maximum slope of the log-log plot of light as a function of calcium ion concentration is about 2.5. The complete relation is well described by a two-state model involving three calcium-binding sites.*

Aequorin, the photoprotein extracted from the jellyfish *Aequorea* (*1*), emits light when it is exposed to calcium ions. This unusual property has generated considerable interest in the mechanism of the light-emitting reaction (*2*) and has led to the use of aequorin as an indicator of the concentration of free calcium ions $[Ca^{2+}]$ in a variety of cells (*3*). The precise nature of the relation between $[Ca^{2+}]$ and light emission is of importance to both of these subjects. Previous studies of this relation have been restricted to limited ranges of $[Ca^{2+}]$ and have generally led to the conclusion that the rate of the luminescent reaction is proportional to $[Ca^{2+}]^2$ (*4*). We report here a study of the relation between $[Ca^{2+}]$ and light emission over a range of $[Ca^{2+}]$ from less than 10^{-9} to $10^{-2}M$ which reveals two new findings: (i) at very low $[Ca^{2+}]$ there is a light emission that is independent of $[Ca^{2+}]$ and (ii) at intermediate $[Ca^{2+}]$ the relation between light emission (L) and $[Ca^{2+}]$ is not adequately described by an equation of the form $L \propto [Ca^{2+}]^2$.

Aequorin was extracted and purified (*4*) from *Aequorea* collected at Friday Harbor, Washington. For these experiments highly purified salt-free lyophilized aequorin was dissolved in distilled water in quartz. Ten microliter volumes of this solution were forcefully injected into a reaction cuvette containing 1 ml of test solution (legend to Fig. 1), giving a final aequorin concentration of about $5 \times 10^{-8}M$. The reaction cuvette was situated above a photomultiplier, and, by the combined use of variable electronic amplification and neutral density filters, light could be measured linearly over eight orders of magnitude. The peak light intensity (before significant aequorin consumption occurred) was used as the index of reaction rate.

Figure 1 shows the result of a representative experiment in which $[Ca^{2+}]$ was varied with dilutions of $CaCl_2$ and by the use of three different calcium buffers. The points for each buffer have been shifted horizontally as a group to give the best fit to the points obtained by calcium dilution. This is equivalent to a determination of the apparent association constant for the calcium buffer and has the advantage that the constant is appropriate for the conditions of pH, temperature, and ionic strength used in the experiment. The log of the apparent association constant (M^{-1}) for EGTA determined in this way at pH 7.0 is 6.45—a value between the widely accepted one of 6.65 (*5*) and others reported to be around 6.1 (*6*). It is worth noting that the conclusions which follow are equally valid if reported association constants are used, the advantage of the above procedure being only that it reduces the horizontal scatter of points in Fig. 1.

Under the conditions of our experiments, the concentration-effect curve is essentially flat at $[Ca^{2+}]$ below $10^{-8}M$. The tendency toward flattening is apparent in the calcium dilution series although the lowest points of this series must be regarded as the least reliable because they would be most influenced by calcium contamination or by EDTA contamination in the aequorin solution. The finding, however, was confirmed with all three of the calcium buffers, most convincingly with CDTA, which buffers $[Ca^{2+}]$ well over the lowest range that we explored. Furthermore, when no calcium was added, all three chelators gave the same low light level. In this case the $[Ca^{2+}]$ cannot be accurately calculated; atomic absorption spectroscopy showed that the total calcium in these solutions (no ingredients of which were passed through the Chelex column; see legend to Fig. 1) was 1×10^{-6} to $2 \times 10^{-6}M$, and on this basis the $[Ca^{2+}]$ for these points was calculated to lie between 3×10^{-11} and $6 \times 10^{-10}M$ (depending on chelator). In seven experiments with aequorin collected in five different years, the magnitude of this light (as the $\log_{10}$ of the fraction of maximal light emission) was -6.40 ± 0.16 (mean ± standard deviation, $n = 7$).

As the $[Ca^{2+}]$ is raised from low levels, the light increases so that the slope of the log-log plot increases from zero to a maximum of about 2.5 (at $[Ca^{2+}] \cong 10^{-6}M$) and then falls again to zero at saturating $[Ca^{2+}]$ levels. Between 3×10^{-7} and $3 \times 10^{-6}M$, the points lie close to a straight line with a slope of 2.52 in this experiment (determined by linear regression of the points on the log-log plot over the stated range). In eight experiments, the mean slope determined in this way from calcium dilutions was 2.46 ± 0.033 (mean ± standard error, $n = 8$). The EGTA-buffered points extend over this range and confirm this finding (slope 2.54 ± 0.039, mean ± S.E., $n = 7$). The slopes from the calcium dilution and the EGTA-buffered series are not significantly different ($P > .1$), but both are significantly greater than 2.0 ($P < .001$).

Some properties of the Ca^{2+}-independent light were studied. It is absent when the aequorin has been discharged by Ca^{2+}. When pH was varied, light intensity was least at pH 7 and was increased by a factor of 2 to 3 at pH 5 and 9. When the KCl concentration in the test solution was increased from 0.01 to $0.50M$, the Ca^{2+}-independent light was reduced by a factor of 2. As temperature was increased from 0° to 40°C, the Ca^{2+}-independent light increased by a factor of 67. An attempt was made to see whether the Ca^{2+}-independent light was dependent on free oxygen. We could detect no difference in the light emission of solu-

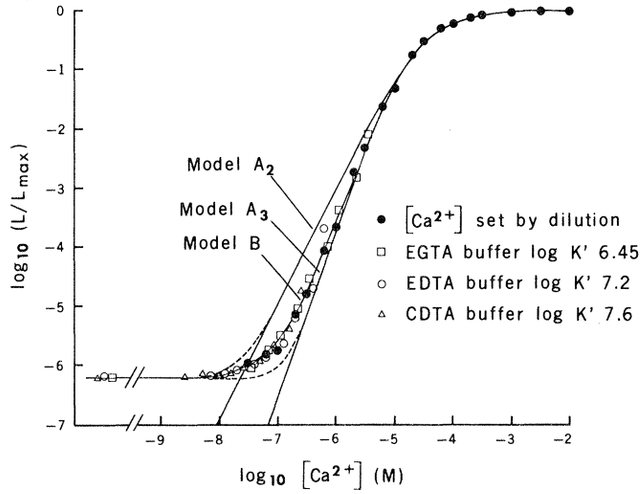

Fig. 1. The relation between light emission and $[Ca^{2+}]$ for aequorin. All solutions contained 150 m*M* KCl, 5m*M* PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], *p*H 7.0, and were at room temperature (22°C). Points are experimental data, lines are predictions from theoretical models described in text. Ordinates: $\log_{10}$ of the ratio of peak light intensity to the peak light intensity in saturating $[Ca^{2+}]$. Abscissae: $\log_{10}$ of the free calcium concentration (*M*). ●, $[Ca^{2+}]$ determined by dilutions of 1*M* $CaCl_2$. Before addition of $CaCl_2$, these solutions were passed through a Chelex 100 (Bio-Rad Laboratories) column which reduced $[Ca^{2+}]$ to less than $10^{-8}M$. □, ○, Δ, $[Ca^{2+}]$ set by EGTA [ethylenebis(oxyethylenenitrilo)tetraacetic acid], EDTA (ethylenediaminetetraacetic acid), and CDTA [(1,2-cyclohexylenedinitrilo)tetraacetic acid] buffers, respectively. Buffer solutions were made by mixing two solutions containing: (i) 1 m*M* chelator, and (ii) 1 m*M* Ca chelator, in addition to the other salts. Solutions (i) and (ii) were mixed in proportions 0.1 to 10. The logs of the apparent association constants (log *K*′) determined by the method described in the text are given on the graph. For comparison, the reported log apparent association constants (M^{-1}) for EDTA and CDTA (*11*) are 7.3 and 7.8, respectively. The three points on the left (undefined abscissae) were obtained with solutions (i) alone. Lines were calculated from theoretical models, with equations derived in the text: For model A_2, $K = 3.5 \times 10^4 M^{-1}$, $n = 2$. Addition of Ca^{2+}-independent light = −6.22 (units of ordinate) gave dashed line. For model A_3, $K = 6.2 \times 10^4 M^{-1}$, $n = 3$, Ca^{2+}-independent light = −6.22. For model B, $K_R = 7 \times 10^6 M^{-1}$, $K_{TR} = 1.18 \times 10^2$, $n = 3$.

tions equilibrated with 100 percent O_2 and with argon containing O_2 at 0.5 part per million. Silver ions increase the Ca^{2+}-independent light substantially when as little as $10^{-9}M$ $AgNO_3$ is added. Addition of 5 m*M* $MgCl_2$ was found to reduce Ca^{2+}-independent light by a factor of 2.

All known bioluminescent reactions are thought to involve the oxidation of an organic substrate (luciferin or chromophore). However, the Ca^{2+}-dependent light emission of photoproteins such as aequorin does not require exogenous O_2. Thus it seems likely that the active photoprotein molecule contains some form of bound oxygen and that, when the protein combines with Ca^{2+}, a conformational change occurs which allows the bound oxygen to oxidize the chromophore with the emission of light (*7*). Two general sorts of mechanisms might be responsible for the Ca^{2+}-independent light: either the luminescent reaction proceeds at a low rate in a manner unrelated to the Ca^{2+}-dependent luminescence (model A) or, alternatively, the same mechanisms that lead to Ca^{2+}-dependent luminescence occur at a low rate in the absence of Ca^{2+} (model B). An example of the first alternative would be the oxidation of the chromophore by exogenous oxygen. The absence of a detectable effect of O_2 depletion argues against this mechanism, although the possibility remains that oxidation of the chromophore might require an activated oxygen species rather than O_2 (*7*). The finding that the Ca^{2+}-independent light is increased by Ag^+ raises the possibility that this component might be due entirely to trace concentrations of ions (such as Ag^+) that are not strongly bound by the Ca^{2+} chelators used. We have eliminated this possibility, at least for Ag^+, by showing that addition of 1 m*M* CN^-, which binds Ag^+ very strongly, generally has little or no effect on the Ca^{2+}-independent light. (It is worth noting that the exceedingly low concentrations of Ag^+ released by some *p*H reference electrodes or an Ag wire in the Ca^{2+}-chelated solution can increase Ca^{2+}-independent light considerably; the increase is eliminated by addition of CN^-.) An example of the second alternative would be that a conformational change leading to oxidation of the chromophore might occur spontaneously in the absence of Ca^{2+} but be greatly accelerated by the binding of Ca^{2+}.

A way of trying to decide between these alternatives is to construct models of the proposed mechanisms for comparison with the experimental data. It has been proposed (*8, 9*) that two calcium ions must be bound to aequorin simultaneously to produce light emission and that light intensity is proportional to the concentration of Ca_2 aequorin (model A_2). More generally, for each binding site

$$Ca^{2+} + A \underset{}{\overset{K}{\rightleftharpoons}} CaA$$

where K is the equilibrium association constant and A represents aequorin. If n identical sites on each aequorin molecule must be occupied for luminescence to occur, then:

$$\frac{L}{L_{max}} = \left(\frac{[CaA]}{[A] + [CaA]}\right)^n = \left(\frac{K[Ca^{2+}]}{1 + K[Ca^{2+}]}\right)^n \quad \text{Model A}$$

where L/L_{max} = (peak light intensity)/(peak light intensity at saturating $[Ca^{2+}]$). Curves calculated from model A when $n = 2$ and 3 are shown in Fig. 1. In each case, K has been chosen to give the optimum fit to the upper experimental points. The dashed lines show how the numerical addition of a fixed amount of light equal to the Ca^{2+}-independent light affects these curves. The maximum possible slope of these curves on a log-log plot is n, so that model A_2, with a maximum slope of 2, is inconsistent with our data.

Model B is based on the assumptions that each of the sites to which Ca^{2+} binds has two states, T and R, which are in equilibrium and that light is emitted by the molecule only when n sites on the molecule are in the R state. Calcium binds much more avidly to the R state, and hence at high $[Ca^{2+}]$ most of the sites will be in the R state. For each site

$$\begin{array}{ccc} & & K_R \\ & R + Ca^{2+} & \rightleftharpoons RCa \\ K_{TR} & \uparrow\downarrow & \\ & T & \end{array}$$

where K_R is the equilibrium association constant and $K_{TR} = [T]/[R]$. In the usual two-state model (*10*) Ca^{2+} also binds to T with a binding constant, K_T. We find the curves fit our data well, provided that $K_T \ll K_R$; hence we have made $K_T = 0$ to reduce the number of arbitrary constants in the model. The proportion of sites in the R state is the expression within the large parentheses shown below. If we assume that n sites on one molecule must simultaneously be in the R state and that the fractional light intensity is equal to the proportion of molecules in that form, then

$$\frac{L}{L_{max}} = \left(\frac{[R] + [RCa]}{[R] + [T] + [RCa]}\right)^n = \left(\frac{1 + K_R[Ca^{2+}]}{1 + K_{TR} + K_R[Ca^{2+}]}\right)^n \quad \text{Model B}$$

A curve calculated from model B ($n = 3$) is shown in Fig. 1. This curve fits the experimental points substantially better than those from models A_2 and A_3 (all have three arbitrary constants). An almost equally good fit can be obtained with model B when $n = 4$ (with adjustment of the other constants), but values of $n = 2$ or 5 will not give a satisfactory fit. The fit of model A_3 can be slightly improved (at the expense of additional arbitrary constants) by giving the three sites different microscopic binding constants, but the fit of model B remains substantially better. Model B is also the most attractive in the sense that the Ca^{2+}-independent light is an integral part of the model rather than simply an added constant. More detailed structural and chemical information about the aequorin molecule will be required to finally decide among these and other possible models.

The results are important to the use of aequorin as an intracellular calcium indicator. Clearly in the range of $[Ca^{2+}]$ where the Ca^{2+}-independent light becomes a significant fraction of the Ca^{2+}-dependent light, the utility of aequorin as a $[Ca^{2+}]$ indicator will be correspondingly reduced. Under the conditions of the experiment in Fig. 1, $[Ca^{2+}]$ below about $3 \times 10^{-8} M$ could not be determined with aequorin. However, this limiting concentration is crucially dependent on the conditions; for instance, we have found that a change in KCl concentration from 0.01 to 0.50*M* produces an approximately parallel shift of the curve to the right by 1.5 log units. In addition, there is a change in the level of Ca^{2+}-independent light (noted above). As an overall consequence the minimum detectable $[Ca^{2+}]$ at 0.50*M* KCl might be about $10^{-7} M$, but at 0.01*M* it might be $10^{-9} M$. The $[Mg^{2+}]$ and perhaps other aspects of the intracellular environment will also affect the minimum detectable $[Ca^{2+}]$.

The fact that the slope of the relation between light and $[Ca^{2+}]$ varies continuously with $[Ca^{2+}]$ introduces further difficulties into the use of aequorin as a quantitative $[Ca^{2+}]$ indicator. Previously we (*4*) and others (*8*) felt that one of the most practicable approaches to calibrating the aequorin response in vivo was to determine the light emission appropriate to some known $[Ca^{2+}]$—preferably the resting $[Ca^{2+}]$—and to assume that a square-law relation held between light and $[Ca^{2+}]$ above and below this level. It now appears that not only must the light emission from an aequorin-injected cell be determined for some known $[Ca^{2+}]$ but the shape of the relation between light and $[Ca^{2+}]$ must be determined under conditions appropriate to the intracellular milieu and over the range through which the intracellular $[Ca^{2+}]$ varies.

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Conjugal Transfer of the Gonococcal Penicillinase Plasmid

Abstract. *Certain gonococci, which heretofore have lacked a conjugal mating system, can sexually transfer a small plasmid (4.5 × 10⁶ daltons) which carries the gene for β-lactamase production. Frequencies of conjugal transfer were similar into diverse recipients (other gonococci,* Neisseria flava, *and* Escherichia coli*), which suggests that gonococci may transfer the plasmid promiscuously in nature.*

Recently, strains of penicillinase-producing *Neisseria gonorrhoeae* (PPNG) have been identified (*1*). The potential social and medical impact of these strains is considerable (*2*).

We have performed experiments demonstrating that penicillinase-producing gonococci can sexually transfer the penicillinase gene to other gonococci and to other species of bacteria. Up to the present, the only known mechanism of genetic transfer in the gonococcus has been by transformation (*3*). Past experiments that attempted to demonstrate conjugal transfer of drug resistance by gonococci were unsuccessful (*4*).

Table 1. Host range and transfer frequency of the gene for penicillinase production in penicillinase-producing gonococcal strain FA288. Strain FA306 is a spontaneous penicillinase-negative segregant of FA288. Strain FA305 is an unrelated strain derived from F62.

Penicillin-sensitive recipient	Frequency of penicillinase-producing transconjugants*
N. gonorrhoeae FA306	2×10^{-4}
N. gonorrhoeae FA305	6×10^{-6}
E. coli C600.5	2×10^{-4}
N. flava	1×10^{-5}

*Matings were generally for 16 hours in broth or on filters. Frequencies are expressed per number of donor CFU at onset of mating.

Two different naturally occurring PPNG isolates (FA288 and FA290) were tested for ability to act as conjugal donors of the penicillinase gene (*5*). Recipient strains included a spontaneous penicillinase-negative derivative of FA288, as well as other unrelated gonococci, and one isolate each of *Escherichia coli* (C600.5) and *Neisseria flava* (ATCC 14221).

Two different mating conditions were used. In the first, about 10^7 donor colony-forming units (CFU) per milliliter were mixed with the recipient (about 10^8 CFU/ml) in 10 ml of gonococcal base (GCB) broth, followed by incubation for 2 to 16 hours at 37°C with moderate agitation. Alternatively, equal amounts (about 10^8 CFU of each strain) were applied to sterile membrane filters (type HA; pore size, 0.45 μm, Millipore) and incubated overnight on GCB agar plates (*6, 7*). Deoxyribonuclease was included in the mating mixtures to inactivate any spontaneously released transforming DNA; concentrations were 100 μg on the filter and 50 μg/ml in broth. In all cases, the donor was a penicillinase-producing (or pencillin-resistant) strain, and the recipient was penicillin-sensitive. Selection for penicillinase-producing transconjugants was made on plates containing penicillin or ampicillin, and other antibiotics to inhibit the donor strain (*8*).