Requirement of the C-terminal proline residue for stability of the Ca^{2+} -activated photoprotein aequorin

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cDNA coding for the Ca^{2+} -activated photoprotein aequorin from the jellyfish *Aequorea victoria* has been engineered to investigate the role of the C-terminal proline residue in bioluminescence. Recombinant aequorin proteins were synthesized by PCR followed by *in vitro* transcription/translation, and characterized by specific activity, stability, and affinity for coelenterazine. The C-terminal proline residue of aequorin was shown to be essential for the long-term stability of the bound coelenterazine. Aequorin minus proline had only 1% of the specific activity of the wild-type after 2 h, and was virtually inactive after 18 h. The instability of this variant was further demonstrated by re-activating with a coelenterazine analogue

(ϵ -coelenterazine), where maximum reactivation was reached in 15 min, and the luminescent activity was almost completely abolished within 3 h. Replacement of the C-terminal proline residue with histidine or glutamic acid decreased the specific activity to 10 and 19% of that of the wild-type respectively. However these variants were also unstable, having $t_{1/2}$ values of 2.4 h and 2.3 h respectively. Enhancement of the Ca^{2+} -independent light emission when proline was replaced by histidine confirmed the stabilizing role of the C-terminal proline. No significant effect of removal of the C-terminal proline was detected on the affinity for coelenterazine.

INTRODUCTION

Aequorin is the Ca^{2+} -activated photoprotein from the jellyfish *Aequorea victoria*. The protein is monomeric with an M_r of 21.4 kDa. When extracted it consists of an apo-aequorin of 189 amino acids (Charbonneau et al., 1985) and a prosthetic group, coelenterazine, a substituted imidazopyrazine (M_r 423) covalently linked to apo-aequorin through a peroxide bond (Musicki et al., 1986). However the cDNA sequence showed an extra seven amino acids at the N-terminus (Inouye et al., 1985). Within the protein there are three EF-hand Ca^{2+} binding sites (Persechini et al., 1989), and a 'hydrophobic region' postulated to be the coelenterazine-binding site (Tsuiji et al., 1986). When Ca^{2+} binds to these sites, a conformational change takes place, resulting in the oxidation of coelenterazine to coelenteramide and the emission of blue light [λ_{max} ~ 470 nm] (Shimomura and Johnson, 1978).

The three Ca^{2+} binding sites of aequorin show sequence similarity to Ca^{2+} sites I, III and IV in calmodulin (Tsuiji et al., 1986), indicating a possible common evolutionary origin. However, full apo-aequorin has some 48 more amino acids than calmodulin. In particular, the C-terminus of aequorin extends for 25 amino acids after the last Ca^{2+} -binding site, whilst in calmodulin there are only eight residues. Also, unlike all the sequence-related EF-hand Ca^{2+} -binding proteins, the aequorin gene contains no introns. Furthermore, it has been reported that removal of the C-terminal proline residue virtually abolishes light emission (Nomura et al., 1991).

Formation of the complete photoprotein requires oxygen and coelenterazine, the latter being coupled to the apoprotein as a result of the coelenterazine oxygenase activity of the protein. In this form the coelenterazine is stable. However, coelenterazine is very unstable in solution (Campbell et al., 1989); in luminous marine organisms it is stably stored in a sulphated form or bound to a binding protein (Cormier and Hori, 1964). There are therefore three possible reasons why the C-terminus of aequorin has evolved: for coelenterazine oxygenase activity, for high-quantum-yield Ca^{2+} -activated chemiluminescence or to provide an environment where coelenterazine can be maintained in a stable form for long periods.

The aim of the present paper was to engineer aequorin to distinguish between these three possibilities. PCR, followed by *in vitro* transcription/translation (Sala-Newby et al., 1991) was used to remove the C-terminal proline residue (AEQ-P) or to replace it with either histidine (AEQH) or glutamic acid (AEQE), so that the effects on formation, stability and Ca^{2+} -activated chemiluminescence could be assessed.

MATERIALS AND METHODS

Materials

Oligonucleotide primers were prepared using an Applied Biosystems 381A DNA synthesizer and purified as 'trityl-off' oligonucleotides. Their sequences were as follows (the codons for position 189 are in bold with an underbar):

5: TAATACGACTCACTATAGGGAGAATGGTCAAGCTTACATCAGACTTCGAC (5' end sense including the T7 polymerase promoter)

10: CTCCTTGAGCTCGTCGACTTAGGGGACAGCTCCAC (3' end antisense with C-terminal proline)

33: CTCCTTGAGCTCGTCCGATTAGACAGCTCCACCGTAGAG [3' end antisense removing C-terminal proline (189)]

34: CTCCTTGAGCTCGTCCGATTAATGGACAGCTCCACCGTA (3' end antisense replacing C-terminal proline with a histidine)

35: CTCCTTGAGCTCGTCCGATTATTCGACAGCTCCACCGTA (3' end antisense replacing C-terminal proline with a glutamic acid)

Abbreviations used: AEQ-P, aequorin with proline at position 189 removed; AEQH, aequorin with proline at position 189 replaced with histidine; AEQE, aequorin with proline at position 189 replaced with glutamic acid; CL counts, chemiluminescent counts.

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Amplitaq DNA polymerase, T7 polymerase, RNAase inhibitor and rabbit reticulocyte lysate were from Promega, nucleotides were from Pharmacia and [α - 32 P]UTP (3000 Ci/mmol) and stabilized [35 S]methionine (> 1000 Ci/mmol) were purchased from Amersham International. All other AnalaR-grade reagents were from Sigma Chemical Co. and BDH Chemicals.

Methods

Preparation of mutant aequorin

Addition of the T7 RNA polymerase promoter (TAATACGACTCACTATAGGGAGA) (Stoflet et al., 1988) as well as all the other mutations were carried out using PCR. Aequorin (*Aequoria victoria*) genomic DNA was amplified in a solution containing Promega TAQ Buffer, 2 mM MgCl₂, 0.2 mM of each of the four deoxynucleoside triphosphates, 0.5 μ M of each primer and Amplitaq DNA polymerase (5000 units/ml). The cycling reactions were carried out in a Perkin-Elmer thermal cycler. Each of the 25 cycles consisted of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C plus a 5 s extension on each cycle. Klenow fragment of *Escherichia coli* DNA polymerase (40 units/ml) was added after the completion of the 25 cycles, and incubated for 30 min at 37 °C.

The final product was extracted twice with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and precipitated with 0.1 vol. of 3 M sodium acetate plus 2.5 vol. of 100% ethanol. The DNA concentration was assessed visually from ethidium bromide-stained agarose gels by comparison with the bands of a standard DNA (Sambrook et al., 1989).

Wild-type aequorin DNA preceded by the T7 RNA polymerase promoter was prepared using oligonucleotide primers 5 and 10 (see under 'Materials'). The C-terminal proline-deleted cDNA was prepared using primers 5 and 33. The substitution of the C-terminal proline with histidine and glutamic acid was carried out using the primers 5 and 34, and 5 and 35, respectively.

Formation of aequorin *in vitro*

PCR products were transcribed as described by Sala-Newby et al. (1990). The RNA was capped using 0.5 mM m⁷G(5')ppp(5')G and 0.1 mM GTP and precipitated twice with 0.2 vol. of 7.5 M ammonium acetate and 2.5 vol. of ethanol. The RNA (100–300 ng), in 2 μ l of a solution containing 10 mM Tris/HCl, 1 mM-EDTA (pH 7.4), 0.4 μ l of Promega 1 mM complete amino acid mixture, 0.4 μ l of Promega ribonuclease inhibitor, 3.2 μ l of diethyl pyrocarbonate-treated water and 14 μ l of Promega rabbit reticulocyte lysate, was incubated for 1.5 h at 30 °C. The aequorin was re-activated in the dark at room temperature in 50 μ l of 10 mM Tris/500 mM NaCl/1 mM EDTA/0.1% (w/v) gelatin/5 mM β -mercaptoethanol, pH 7.4, and 1 μ mol/l normal coelenterazine or ϵ -coelenterazine (Shimomura et al., 1988) was added. Formation of active photoprotein was monitored for up to 18 h (Campbell et al., 1990). Luminescent activity as chemiluminescent (CL) counts was measured in a home-built chemiluminometer (Campbell, 1988) for 10 s at room temperature with 5 μ l reconstitution mixture in 500 μ l of 200 mM Tris/0.5 mM EDTA, pH 7.5. The reaction was started by the injection of 500 μ l of 50 mM CaCl₂. The amount of protein synthesized was measured by including 15 μ Ci of [35 S]methionine/10 μ l of translation cocktail. Proteins were separated on SDS/9% (w/v) PAGE under reducing conditions (β -mercaptoethanol) (Laemmli, 1970) followed by fluorography and exposure to preflashed X-ray film. The aequorin bands were excised from the gel, radioactivity was measured in a liquid-scintillation counter and the amount of protein was estimated by taking into account

the concentration of methionine in the lysate. Rate constants were measured by recording 100 time points over a 2 s period in a home-built luminometer, connected to an IBM LSI-11 computer. The data were then analysed using BIOSOFT FIG.P software and plotted as ln CL counts versus time (s), the rate constant being the gradient of the plot.

For re-activation time courses, Ca²⁺-activated luminescence activity was measured at 30 s, 5 min, 15 min, 30 min, 60 min, 90 min, 120 min, 180 min, 210 min, 240 min and 18 h (times varied depending on which variant was being investigated). For Ca²⁺-independent light-emission measurements, the re-activated protein was drop-dialysed by placing the reconstitution mixture on to Millipore VS (0.025 μ m pore size) filters and dialysing against 12.5 mM Hepes/2.5 mM EGTA, pH 7.0, for 1 h. This was carried out to remove excess coelenterazine and the high salt, as high salt lowers the Ca²⁺-independent light emission (Allen et al., 1976). The Ca²⁺-activated light emission was then measured by placing the dialysed protein in 12.5 mM Hepes/2.5 mM EGTA, pH 7.0, and the decay was monitored over time. At every time point 5 μ l aliquots were taken in order to monitor the decay of the Ca²⁺ activated CL counts. The coelenterazine dose-response experiment was carried out with the wild-type and AEQ-P aequorin. A separate translation was set up for each concentration; the concentrations were as follows: 0 nM, 10 nM, 100 nM, 1000 nM, 2000 nM, 3000 nM, 4000 nM and 5000 nM [final coelenterazine concentration in re-activation mixture (100 μ l)]. For every concentration the coelenterazine was added to the re-activation mixture in the same volume of methanol (1 μ l). After the coelenterazine had been added, the samples were re-activated in the dark for 2 h at room temperature. For Ca²⁺-activated luminescence, 10 μ l aliquots were taken and measured as described previously.

RESULTS

In vitro synthesis of engineered aequorin variants

The PCR products were characterized using three criteria: size on agarose-gel electrophoresis, ³²P-labelled mRNA and translation *in vitro* of the mRNA to form light-emitting protein. The PCR generated a single DNA band of the correct predicted size (620 = 567 + T7 + stop and restriction sites at 5' and 3' ends) for all the recombinant DNA, and this was confirmed by using agarose-gel electrophoresis (Figure 1). The ability of the PCR

Figure 1 Agarose-gel electrophoresis of cDNAs prepared by PCR

Wild-type aequorin cDNA was amplified with oligonucleotides 5–10 (lane 1). In lanes 2, 3 and 4 are the 3' mutants: AEQ-P prepared using 5–33, AEQH using 5–34 and AEQE using 5–35. Size markers were *Hind*III-digested λ -phage DNA and *Hae*III-digested ϕ X-174-phage DNA (lane 5).

Figure 2 Autoradiograph of *in vitro* translation products

mRNAs were translated in a rabbit reticulocyte-lysate system containing [35 S]methionine. The proteins were separated by SDS/PAGE under reducing conditions and exposed to X-ray film to produce a band of the correct molecular mass. Wild-type (lane 1), AEQ-P (lane 2), AEQH (lane 3) and AEQE (lane 4) proteins are shown (molecular mass 21.5 kDa). The following molecular-mass (*M*) markers were run prestained: phosphorylase (106 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa).

products to form mRNA which could be translated to protein of the correct molecular mass was confirmed using 35 S labelling and SDS/PAGE (Figure 2). Furthermore, incorporation of [32 P]UTP into RNA and [35 S]methionine into recombinant protein allowed specific activities to be expressed as CL counts/ μ g of RNA or CL counts/ng of protein (Table 1).

Time courses of re-activation of aequorin and aequorin minus proline

To determine whether the C-terminal proline was required for coelenterazine oxygenase activity or stabilization of the eventual photoprotein, a time course of re-activation was carried out using native coelenterazine and an analogue, ϵ -coelenterazine (Figures 3 and 4). With native coelenterazine the rate of formation of AEQ-P was higher than that for the wild-type, reaching its maximum within 2 h. The Ca^{2+} -activated luminescence of AEQ-P then decayed exponentially with a $t_{1/2}$ of 3.7 h. By 18 h the activity of AEQ-P was less than 1% of that at 2 h, whereas the loss in activity of the wild-type was less than 20%. Furthermore, measurement of the specific activities of AEQ-P and wild-type recombinant aequorin at 2 h showed that AEQ-P had only 1% of the Ca^{2+} -activated light emission of the wild-type.

A similar difference in the time courses between wild-type and AEQ-P was seen when using ϵ -coelenterazine (Figure 4). In this case the AEQ-P variant reached maximum re-activation after only 15 min, and within 3 h the activity had decayed to less than 1% of maximum. This contrasted sharply with the wild-type,

Table 1 Specific activities of the aequorin variants

RNA concentrations were calculated from the amount of [32 P]UTP label incorporated into trichloroacetic acid-precipitated material. Protein concentration was assessed from the radioactivity contained in [35 S]methionine-labelled protein eluted from SDS/PAGE. CL counts were the total chemiluminescent counts recorded over a 10 s period when the proteins were discharged in saturating Ca^{2+} . Furthermore, total luminescent counts were measured at maximum re-activation for the C-terminal variants. Each value is a mean $\pm$ S.E.M. of three.

Variant	Specific activity	
	(CL counts/ng of protein) (% of wild-type)	(CL counts/ μ g of RNA)
Wild-type	$3.6 \times 10^6 \pm 0.5 \times 10^6$ (100)	$2.0 \times 10^8 \pm 0.7 \times 10^8$
AEQ-P	$5.2 \times 10^4 \pm 1.5 \times 10^4$ (1.4)	$1.8 \times 10^5 \pm 5.2 \times 10^4$
AEQH	$3.6 \times 10^5 \pm 4.7 \times 10^4$ (10)	$2.1 \times 10^7 \pm 2.7 \times 10^6$
AEQE	$7.0 \times 10^5 \pm 2.4 \times 10^4$ (19)	$1.4 \times 10^7 \pm 4.9 \times 10^5$

Figure 3 Comparison of the re-activation of AEQ-P with wild-type aequorin

Apo-aequorin was prepared by *in vitro* translation; coelenterazine was added to the translated protein in the reconstitution medium and re-activation was then monitored over time by measuring activated luminescence as described under 'Methods'. ■, AEQ-P; ▲, wild-type aequorin. Results are presented as percentages of maximum re-activation. Each point is a mean of two observations.

Figure 4 Re-activation of AEQ-P and wild-type aequorin with ϵ -coelenterazine

Re-activation was carried out as described under 'Methods' with no AEQ-P (■) and wild-type aequorin (▲).

which still had activity after 18 h. Again AEQ-P had a much faster rate of re-activation. These results suggested that the C-terminal proline was required to stabilize the photoprotein in the absence of Ca^{2+} and not for coelenterazine oxygenase activity.

Comparison of the effect of replacing the C-terminal proline residue with histidine or glutamic acid

To confirm the importance of the C-terminus in stabilizing aequorin, the proline was replaced by histidine or glutamic acid at position 189. Histidine was chosen to see whether a ring structure would stabilize the protein, and glutamic acid was chosen to place a carboxy group in a different conformation. As with AEQ-P, the AEQH and AEQE also reached maximum re-activation within 2 h and then lost activity (Figure 5). However the decays of AEQH and AEQE were slightly more rapid than that of AEQ-P, the $t_{1/2}$ values being 2.4 h and 2.3 h for AEQH and AEQE respectively, compared with 3.7 h for AEQ-P.

The specific activities of the variants were compared with the

Figure 5 Comparison of the re-activation of the AEQ-P with that of AEQH and AEQE

Re-activation was carried out as described under 'Methods' with no AEQ-P (■) and wild-type aequorin (▲).

wild-type protein at 2 h (Table 1). It was seen that removal of the C-terminal proline residue reduced the specific activity to 1.4 % of the wild-type, whereas replacement by histidine or glutamic acid resulted in much higher Ca^{2+} -activated luminescence the specific activities being 10 and 19 % respectively.

All the variants had lower rate constants than the wild-type (Table 2). The rate constant at saturating Ca^{2+} of the recombinant single clone wild-type aequorin was lower than that of the aequorin extracted and purified from *Aequorea*.

Effect of removal of the C-terminus on Ca^{2+} -independent light emission

Another way of quantifying the instability of photoproteins is to determine the Ca^{2+} -independent light emission (Allen et al., 1976). Figure 6 shows that there was a close correlation between the Ca^{2+} -independent light emission and the decay of the light emission when stimulated by Ca^{2+} , both in terms of time course and the rate constants estimated by the two methods. The rate constant of the Ca^{2+} -activated decay was $13.1 \times 10^{-5} \text{ s}^{-1}$ compared with $8.0 \times 10^{-5} \text{ s}^{-1}$ for the Ca^{2+} -independent light emission. These data suggest that the decay of the C-terminal variants resulted in light emission with a quantum yield very similar to that for the Ca^{2+} -activated luminescence. However, it was not possible to rule out some effect of the C-terminus on the absolute quantum yield.

Table 2 Rate constants of the aequorin variants

In order to calculate the rate constant, 5 μl of re-activated protein was placed in 200 mM Tris/0.5 mM EDTA, pH 7.4; 50 mM CaCl_2 was then injected into the mixture in a luminometer and 100 time points of 0.02 s were recorded over a 2 s period. The data was analysed as described under 'Methods' section. Results are expressed as means $\pm$ S.E.M. of three.

Variant	Ca^{2+} -activated rate constants (s^{-1})
Native aequorin*	1.20 ± 0.06
Wild-type	0.92 ± 0.02
AEQ-P	0.61 ± 0.02
AEQH	0.70 ± 0.02
AEQE	0.65 ± 0.02

* Extracted from jellyfish.

Figure 6 Comparison of the decay of the Ca^{2+} -independent light emission with Ca^{2+} -activated luminescence for AEQH

An *in vitro* translation system of 100 μl volume was set up for wild-type and AEQH aequorins. The apo-aequorin was re-activated, and the excess coelenterazine and high salt were removed as described in the text. The decay of the resting glow and Ca^{2+} activated luminescence were measured as described under 'Methods'. The Ca^{2+} -independent light emission (■) and Ca^{2+} -activated luminescence (▲) were compared over time. For the Ca^{2+} -independent light emission each point is the mean for six observations, while for the Ca^{2+} -activated counts each point is the mean for three observations.

Effect of modification of the C-terminus on the affinity of apo-aequorin for coelenterazine

In view of the increased rate of formation of photoprotein in the C-terminal variants, the possibility existed that the C-terminus might affect coelenterazine binding. A dose-response curve of re-activation was therefore carried out at different coelenterazine concentrations (Figure 7). AEQ-P had the same apparent affinity for coelenterazine ($K_{1/2} = 1 \mu\text{M}$) as the wild-type. It was not possible to determine a true K_m because the Lineweaver-Burk plot was not linear. The decrease in activity at the highest concentrations appeared to be due to the presence of an inhibitor in the coelenterazine, possibly coelenteramide. Inhibition was not due to methanol, as the same volume of methanol was used for each coelenterazine concentration. These results suggested that the C-terminus was not required for coelenterazine binding, as the affinities for coelenterazine of the two proteins were the same.

Figure 7 Comparison of the re-activation of wild-type aequorin and AEQ-P with different coelenterazine concentrations

The wild-type aequorin (▲) and AEQ-P (■) were translated and re-activated with a range of coelenterazine concentrations as described under 'Methods'. The CL counts were measured after 2 h re-activation as described in the text. Each point is the mean for three observations.

DISCUSSION

Engineering aequorin using PCR, in conjunction with transcription-translation *in vitro*, showed that the C-terminal proline was required for stabilizing coelenterazine and high quantum yield when bound to aequorin. At room temperature the $t_{1/2}$ of decay for aequorin without proline at the C-terminus and in the absence of Ca^{2+} (2.5 mM EGTA) was 3.7 h, so that, within 24 h, less than 1% of the Ca^{2+} -activated luminescent activity remained. In contrast, under the same conditions, native aequorin, in the absence of Ca^{2+} , decayed with a half time of about 30 days. Replacement of the C-terminal proline residue by either histidine or glutamate resulted in higher luminescent activity initially. However, these variants were also highly unstable, the loss of Ca^{2+} -activated luminescence being nearly 50% faster. The $t_{1/2}$ values for the loss in Ca^{2+} -activated photoprotein appeared to correlate with the light emission measured as Ca^{2+} -independent luminescence. This implied that the quantum yields of the Ca^{2+} -activated and Ca^{2+} -independent luminescence were the same.

It has previously been reported that the C-terminal proline residue was essential for aequorin luminescence (Nomura et al., 1991), and that various modifications, including replacement of proline by glutamic acid and addition of peptides, also virtually abolished Ca^{2+} -activated luminescence. These workers therefore suggested that the C-terminal proline residue might have a functional role in the catalytic site, such as formation of the full photoprotein from coelenterazine plus oxygen. However, these conclusions were based on data where re-activation of aequorin was assessed after only one time point of several hours. Our results show that this interpretation was not correct.

Although the absolute maximum photon yield of all our aequorin variants was considerably less than native aequorin (Table 1), the initial rate of formation of photoprotein was faster in AEQ-P, AEQH and AEQE than for the wild-type. This showed that the C-terminal proline residue does not appear to be necessary for the coelenterazine oxygenase. Furthermore, the similar affinity for coelenterazine of the AEQ-P and wild-type (Figure 7) confirmed that the proline was not necessary for coelenterazine binding.

It is well established that coelenterazine is very unstable both in aqueous and organic solvents such as methanol (Campbell et al., 1989) and dimethyl sulphoxide. Dimethyl sulphoxide actually triggers luminescence of coelenterazine and aequorin, where the quantum yield is only 0.1% of the Ca^{2+} -activated luminescence (N. J. Watkins and A. K. Campbell, unpublished work). Coelenterazine is the most common chemistry responsible for bioluminescence and is found in six phyla as varied as Protozoa and Chordata (Campbell and Herring, 1990). Nature has combated the problem of coelenterazine instability by either storing it in a sulphated form or in the presence of a binding protein (Cormier and Hori, 1964). What, therefore, is the role of proline in coelenterazine stabilization?

Proline is the only amino acid found in Nature to contain an imino group. Peptides involving an imino residue have limited conformation because of the pyrroline ring across the N-C α bond, and the peptide bond is essentially one large, relatively rigid, unit which dictates the conformation (Ramachandran and Reddi, 1976). Therefore the proline may provide a rigid region at the end of the protein which secures coelenterazine to the apoprotein. This also explains the ineffectiveness of histidine and glutamic acid in improving stability (Figure 4). These residues were chosen because histidine has a ring structure and glutamic

acid places a carboxy group in a different conformation. From the results it can be seen that stability was not enhanced. The peculiar structure of the proline residue therefore results in it being of crucial importance at the C-terminus.

These studies have important implications for the evolutionary origin of Ca^{2+} -activated photoproteins found in cnidarians, ctenophores and radiolarians (Campbell and Herring, 1990). If calmodulin were the precursor, then our studies show that 17 amino acids, including a proline residue, would have to be added to the C-terminus. It will therefore be interesting to see if proline is required for the stabilization of the photoproteins in these organisms. Four residues would also have to be added at the N-terminus, since we have also found that removal of the first 14 amino acids of aequorin abolishes activity (results not shown). Our results also have important implications when modification of the C-terminus is required, for example, for targeting to intracellular organelles. It has been shown that placing the sequence KDEL (single-letter code) after the proline gives an active stable protein but one with a reduced specific activity and it can be used to target aequorin to the endoplasmic reticulum (Kendall et al., 1992). Therefore it will be possible to place targeting sequences and binding sites at this point and use the protein as an indicator for intracellular Ca^{2+} and other cellular factors.

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