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Cloning and sequence analysis of cDNA for the Ca^{2+}-activated photoprotein, clytin

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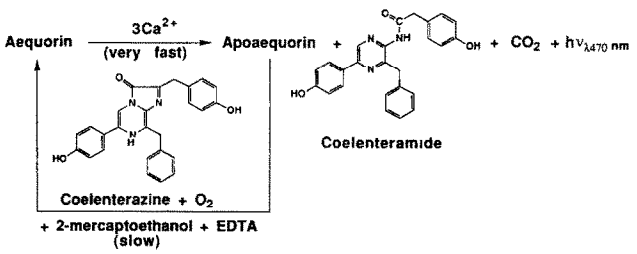
Clytin is a member of the aequorin family of photoproteins. It is made up of 189 amino acid residues, contains 3 Ca^{2+}-binding sites, and shows 62% homology in amino acid residues to those in aequorin. The cysteine, tryptophan, and histidine residues, and the C-terminal proline, that are conserved in aequorin and clytin may be involved in the Ca^{2+}-activated bioluminescence of the two proteins. Clytin may also prove useful in the determination of Ca^{2+}.

Bioluminescence; Ca^{2+}-binding protein; Coelenterazine; Calcium; Phialidin; Aequorin

1. INTRODUCTION

Up to the present time, six photoproteins have been isolated and studied from organisms in the phyla Cnidaria and Ctenophora [1]. These include aequorin [2–5], halistaurin [6,7], obelin [4,5,8], mnemiopsin [4,5,9,10], berovin [9,10], and phialidin [7,11]. All of the proteins are known to emit light on reacting with Ca^{2+}. Further, the proteins are relatively small in size (21.4–27.5 kDa) and they are thought to contain a common organic substrate (coelenterazine) and molecular oxygen bound in the form of a complex. In the case of the best studied example, aequorin, from the hydroid *Aequorea victoria*, the binding of Ca^{2+} causes a conformational change, wherein the protein is converted to an enzyme (luciferase), which catalyzes the oxidation of coelenterazine by oxygen with the emission of light (λ_{max} = 470 nm) [12]. The intramolecular light-emitting and regeneration reactions are shown below:



Scheme 1.

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While the chemical pathway leading to aequorin light emission has been studied extensively, detailed knowledge is lacking concerning the relationship between protein structure and function in aequorin. In order to study this problem, the cDNA for apoaequorin has been previously cloned and the primary structure deduced from the nucleotide sequence [13,14]. Apoaequorin consists of 189 amino acid residues (M_r 21,400) in a single polypeptide chain and contains 3 EF-hand structures characteristic of Ca^{2+}-binding sites, as well as 3 cysteine, 6 tryptophan, and 5 histidine residues. Since such a high content of cysteine, tryptophan, and histidine residues is not commonly found in a Ca^{2+}-binding protein, it has been suggested that these residues are involved in the aequorin bioluminescence reaction [15–18]. It is possible that a further understanding of aequorin and other photoprotein reactions can be gained by comparing the primary structure of aequorin with that of another Ca^{2+}-activated photoprotein. Thus, we now report the cloning and sequence analysis of the cDNA for clytin, the photoprotein also known as phialidin [7,11], from the hydroid *Clytia gregarium* (formerly *Phialidium gregarium*). The convention of using the genus name in naming clytin is adhered to in this paper.

2. MATERIALS AND METHODS

2.1. *Materials*

Specimens of *Clytia gregarium* (15–20 mm diameter) were collected using a dip net at Friday Harbor Laboratories, Friday Harbor, Washington, during the early part of October, 1991. The live specimens were frozen in liquid nitrogen and stored at −80°C until used. Bacterial strains serving as hosts were *E. coli* ER1647 [19] (New England Biolabs, Beverly, MA) and *E. coli* DH5αF′ (Bethesda Research Laboratories, Gaithersburg, MD). The sources of enzymes and chemicals were

as follows: λZap II vector [20], restriction enzymes and *E. coli* T4 DNA ligase, Stratagene, La Jolla, CA; isopropyl β-D-thiogalactopyranoside (IPTG), Calbiochem, La Jolla, CA; guanidine isothiocyanate, formamide, sodium dodecyl sulfate (SDS), dextran sulfate, and salmon sperm DNA, Sigma Chemicals, St. Louis, MO; Oligotex-dT30, Takara Shuzo, Kyoto, Japan; Sequenase (Version 2.0), United States Biochemicals, Cleveland, OH; [α-^{32}P]dCTP (3,000 Ci/mmol) and [α-^{35}S]dATP (1,000 Ci/mmol), Amersham, Arlington Heights, IL. Other chemicals were of the highest grade commercially available. Nylon membrane filters were purchased from ICN Biochemicals, Irvine, CA and XAR films were from Eastman Kodak, Rochester, NY. Coelenterazine was chemically synthesized [21].

2.2. *Construction of cDNA library*

Total *Clytia* RNA was prepared by the guanidine isothiocyanate method [22]. Twenty frozen specimens (5.2 g) were added to 12 ml of guanidine isothiocyanate solution (4 M guanidine isothiocyanate/2.5 mM sodium citrate/0.5% sodium *N*-laurylsarcosine/0.1 M β-mercaptoethanol) and homogenized for 1 min with a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The homogenate was stirred with 1.2 ml of 2 M sodium acetate (pH 4.0), extracted with 12 ml of water-saturated phenol, and mixed with 2.4 ml of chloroform-isoamyl alcohol (49:1). The mixture was centrifuged at 10,000 × *g* for 20 min at 4°C and the aqueous layer recovered. The RNA in the solution was precipitated with isopropyl alcohol and collected by centrifugation. The RNA was again dissolved in the guanidine isothiocyanate solution and the isopropyl alcohol precipitation repeated. The yield of total RNA was approximately 900 μg based on absorbance at 260 nm. Poly(A)$^+$ RNA was isolated using Oligotex-dT30. Two micrograms of poly(A)$^+$ RNA was used for cDNA synthesis by the method of Kakizuka et al. [23] using a Pharmacia-LKB (Piscataway, NJ) cDNA cloning kit. Twenty nanograms of cDNA possessing the *Eco*RI–*Not*I (adaptor) was ligated with 1 μg of λZap II vector [20] digested with *Eco*RI and then packaged using a Stratagene Gigapack Gold II packaging kit. The host strain was *E. coli* ER1647 [19]. The titer of the cDNA library was 6×10^6 pfu per ml and the average insert length was 0.6 kb estimated by a PCR method [24], using T7 and T3 primers present in λZap II. Amplification was carried out for 35 cycles in an Eppendorf MicroCycler (1.4 min denaturation at 94°C, 2 min annealing at 40°C, 3 min elongation at 72°C).

2.3. *Isolation and sequence analysis of clytin cDNA clone*

The clytin cDNA library was screened by the phage plaque hybridization method [25]. The probe used for screening was a *Hin*dIII–*Bam*HI fragment (521 bp) of the aequorin cDNA clone pAQ440 [13], labeled with [α-^{32}P]dCTP using a Stratagene Prime-it random primer kit. Twenty thousand independent plaques (5×10^3 plaques per 15 cm LB-broth plate) were lifted onto nylon membrane filters and then cross-linked with a Stratagene UV cross-linker. The filters were hybridized with labeled probe in a solution of 30% deionized formamide, 1 M NaCl, 0.5% SDS, 10% dextran sulfate, and 0.2 mg/ml of salmon sperm DNA at 37°C for 16 h. The filters were washed with vigorous shaking in a solution of 300 mM NaCl/30 mM sodium citrate/0.1% SDS for 30 min at room temperature and incubated in a solution of 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS for 30 min at 37°C. The washed filters were exposed to XAR film using an intensifying screen at −80°C for 16 h. Positive phage clones were isolated, the cDNA inserts excised from the λZap II as the pBluescript phagemid [20], and the nucleotide sequence determined by a modified dideoxy sequencing method [26]. The oligonucleotide primers used in the sequencing were synthesized without a 5′ dimethoxytrityl protecting group employing a Cyclone Plus DNA synthesizer (MilliGen/Biosearch, Novato, CA). The primers used were: #632, 5′-GCGGTGGCGGCCGCTCT; #633, 5′-CCCTCGAGGTCGACGGT; CL1, 5′-CAACACCAGAACAGACC; CL2,5′AAGTGGCTCAATCAGTT (Fig. 1).

2.4. *Expression of clytin cDNA in* E. coli *and assay for activity*

E. coli strain DH5αF′, transformed with the pBluescript phagemid, was grown with shaking in 1.5 ml of LB medium containing 50 μg of ampicillin/ml at 37°C. When the absorbance (λ = 600 nm) of the culture medium reached 0.2 (about 3 h), IPTG was added to a final concentration of 0.5 mM and the incubation continued for 3 h. The cells were harvested by centrifugation, washed with TE buffer (30 mM Tris-HCl, pH 7.2, containing 10 mM EDTA) and resuspended in 1 ml of TE buffer. The cells were disrupted (10 × 10 s, in an ice bath) with a Branson (Danbury, CT) Model 250 sonifier. After centrifugation at 12,000 × *g*, the supernatant was assayed for luminescence activity by regenerating the expressed protein with coelenterazine, as previously described for aequorin [27]. For this, 1 ml of supernatant was incubated for 2 h in an ice-bath with 5 μl of 2-mercaptoethanol and 2 μg of coelenterazine (1 μg/μl in absolute methyl alcohol). The mixture was placed in a Mitchell-Hastings photometer [28] and injected with 1.5 ml of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.6. The initial maximal light intensity was recorded with a Soltec Model S-4201 strip-chart recorder. The initial light intensity was calculated as quanta per sec using a carbon-14 light standard [29].

3. RESULTS AND DISCUSSION

Screening of the clytin cDNA library with a *Hin*dIII–*Bam*HI fragment of aequorin cDNA, carried out under low-stringency conditions, yielded 8 positive clones from 2×10^4 independent clones. Restriction map analysis, accompanied by Southern blot analysis (data not shown), gave a restriction map of the major clones shown in Fig. 1. The 8 positive clones were also analyzed for expression of apoclytin (apoprotein of clytin) using coelenterazine as substrate. Clones having the *lac* promoter under the control of the *lac* operator in the pBluescript phagemid vector would be expected to express active apoclytin. Regeneration of apoclytin with colenterazine, O_2, 2-mercaptoethanol, and EDTA would lead to clytin, as in the regeneration of apoaequorin into aequorin [27]. Subsequent addition of Ca^{2+} should then produce light emission. The advantage of the method is that the luminescence reaction is highly specific and light can be detected with high sensitivity employing a photomultiplier photometer. Using this approach, 4 clones expressing apoclytin were found among the 8 positive clones. One of the apoclytin clones, designated pCL41, was subjected to DNA sequencing. The complete DNA sequence and the deduced amino acid sequence are shown in Fig. 2. When the cDNA was expressed in *E. coli* DH5αF′ and an extract of the cells was regenerated with colenterazine,

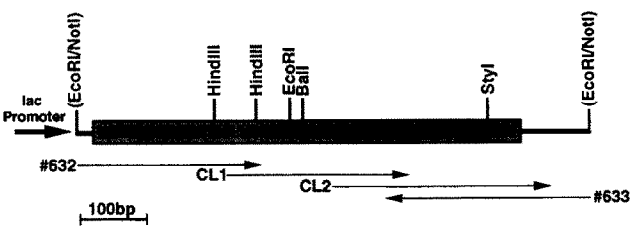

Fig. 1. Restriction map and sequencing strategy of clytin cDNA clone pCL41. Sequenced regions are shown by horizontal arrows and the synthetic primers for sequencing (#632, #633, CL1 and CL2) are given in section 2. The shaded box shows the coding region and bold arrow indicates direction of *lac* promoter.

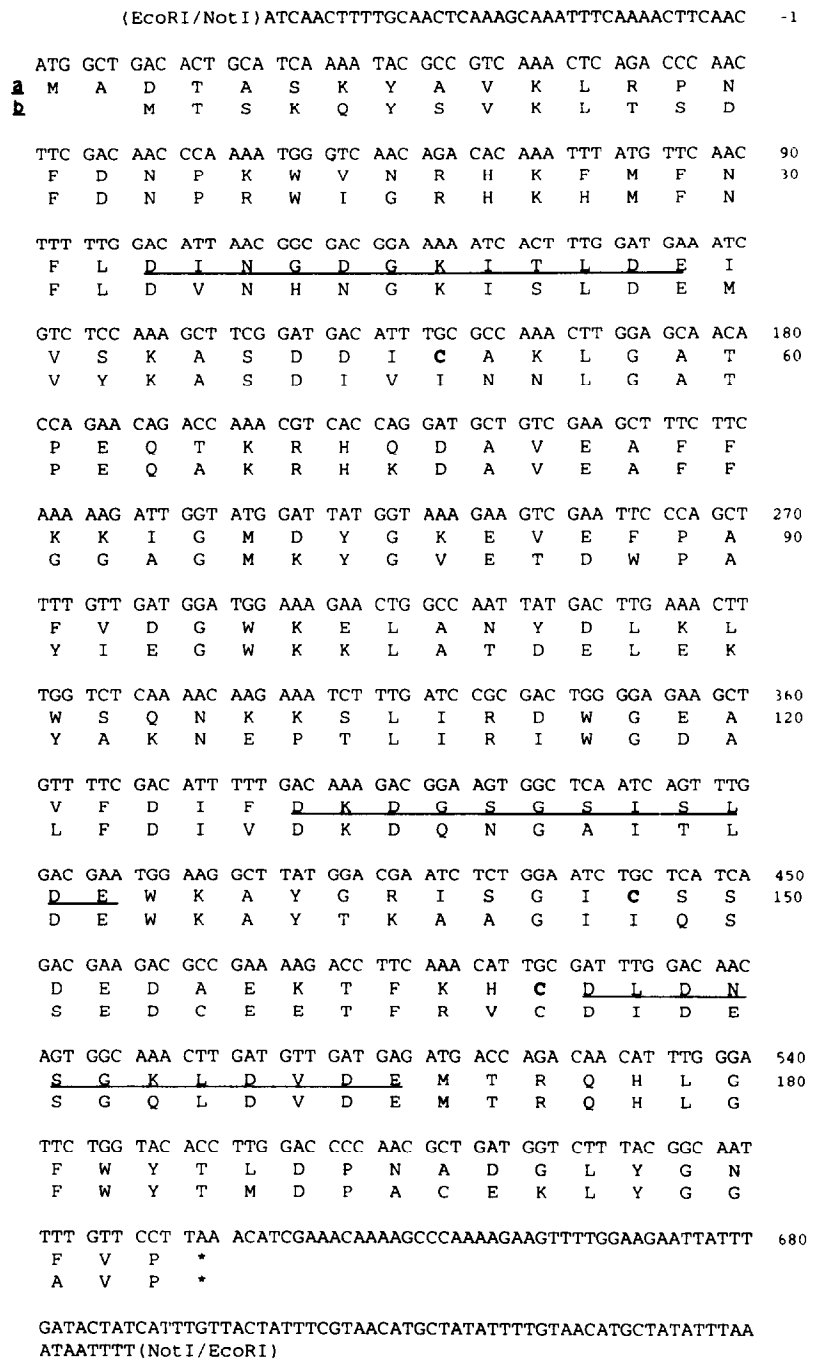

Fig. 2. Nucleotide sequence of the clytin cDNA clone pCL41, the deduced amino acid sequence of clytin (a), and amino acid sequence of aequorin (b) [13]. The putative Ca^{2+}-binding sites are underlined.

light was emitted upon the addition of Ca^{2+} (Fig. 3). The flash pattern of the luminescence was typically that of aequorin [16]. Thus, the clytin luminescence reaction appears to be similar, if not identical, to that of aequorin.

An analysis of the primary structure of clytin shows that it is made up of 189 amino acid residues and belongs to the aequorin family of photoproteins (Fig. 2). The N-terminal amino acid residue of the mature protein is assumed to be valine, since previously it was found from sequencing native aequorin that the latter has a valine at the N-terminus [13]. No role for the leader peptide (MTSKQYS) of aequorin has thus far been found. Alignment of the amino acid sequence of clytin and aequorin shows 62% homology in amino acid residues. Clytin also has 3 EF-hand structures (Ca^{2+}-binding sites) at identical locations as aequorin. Like aequorin, clytin has a proline residue at the C-terminus.

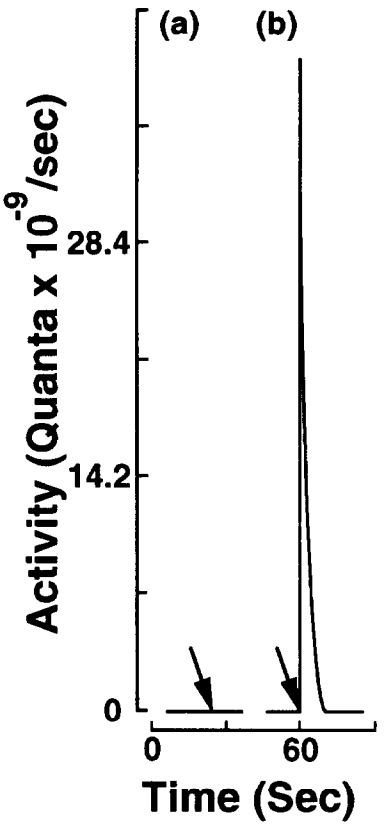

Fig. 3. Expression of clytin cDNA clone pCL41 in *E. coli*. (a) DH5αF′ with pBluescript [20] as control and (b) DH5αF′ with pCL41. Arrows indicate the point of injection of calcium solution. Experimental conditions are described in section 2.

This terminal proline has been found essential for luminescence activity of aequorin [17]. The calculated molecular weight is 21,605 kDa compared to 21,462 kDa for aequorin. This value is consistent with the finding of 23,000 kDa reported for native clytin [11]. The hydropathy plots of the two proteins (data not shown) show a somewhat greater hydrophilicity for clytin than aequorin. Clytin also has a relatively high content of cysteine (3), tryptophan (6), and histidine (4) residues, of which Cys^{152}, Trp^{12}, Trp^{86}, Trp^{108}, Trp^{129}, Trp^{173}, His^{16}, His^{58}, and His^{169} are conserved in both proteins. Thus, these residues may have significance in the Ca^{2+}-activated light emission of aequorin and clytin.

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REFERENCES

[1] Shimomura, O. (1985) Symp. Soc. Exp. Biol. 39, 351–372.
[2] Shimomura, O., Johnson, F.H. and Saiga, Y. (1962) J. Cell. Comp. Physiol. 59, 223–239.
[3] Shimomura, O., Johnson, F.H. and Saiga, Y. (1963) J. Cell. Comp. Physiol. 62, 1–8.
[4] Morin, J.G. and Hastings, J.W. (1971) J. Cell. Physiol. 77, 305–312.
[5] Morin, J.G. and Hastings, J.W. (1971) J. Cell. Physiol. 77, 313–318.
[6] Shimomura, O., Johnson, F.H. and Saiga, Y. (1963) J. Cell. Comp. Physiol. 62, 9–15.
[7] Shimomura, O. and Shimomura, A. (1985) Biochem. J. 228, 745–749.
[8] Campbell, A.K. (1974) Biochem. J. 143, 411–418.
[9] Ward, W.W. and Seliger, H.H. (1974) Biochemistry 13, 1491–1499.
[10] Ward, W.W. and Seliger, H.H. (1974) Biochemistry 13, 1500–1510.
[11] Levine, L.D. and Ward, W.W. (1982) Comp. Biochem. Physiol. 72B, 77–85.
[12] Johnson, F.H. and Shimomura, O. (1978) Methods Enzymol. 57, 271–291.
[13] Inouye, S., Noguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T. and Tsuji, F.I. (1985) Proc. Natl. Acad. Sci. USA 82, 3154–3158.
[14] Prasher, D.C., McCann, R.O., Longiaru, M. and Cormier, M.J. (1987) Biochemistry 26, 1326–1332.
[15] Tsuji, F.I., Inouye, S., Goto, T. and Sakaki, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 8107–8111.
[16] Kurose, K., Inouye, S., Sakaki, Y. and Tsuji, F.I. (1989) Proc. Natl. Acad. Sci. USA 86, 80–84.
[17] Nomura, M., Inouye, S., Ohmiya, Y. and Tsuji, F.I. (1991) FEBS Lett. 295, 63–66.
[18] Ohmiya, Y., Ohashi, M. and Tsuji, F.I. (1992) FEBS Lett. 301, 197–201.
[19] Raleigh, E.A., Trimarchi, R. and Revel, H. (1989) Genetics 122, 279–296.
[20] Short, J.M., Fernandez, J.M., Sorge, J.A. and Huse, W.D. (1988) Nucleic Acids Res. 16, 7583–7600.
[21] Inoue, S., Sugiura, S., Kakoi, H., Hasizume, K., Goto, T. and Iio, H. (1975) Chemistry Lett. 141–144.
[22] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[23] Kakizuka, A., Yu, R., Evans, R.M. and Umesono, K., in: Developmental Molecular Biology (C.D. Stern, ed.), IRL Press, Oxford, UK, in press.
[24] Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Science 230, 1350–1354.
[25] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[26] Hattori, M. and Sakaki, Y. (1986) Anal. Biochem. 152, 232–238.
[27] Inouye, S., Sakaki, Y., Goto, T. and Tsuji, F.I. (1986) Biochemistry 25, 8425–8429.
[28] Mitchell, G.W. and Hastings, J.W. (1971) Anal. Biochem. 39, 243–250.
[29] Hastings, J.W. and Weber, G. (1965) Photochem. Photobiol. 4, 1049–1050.