Monitoring of intracellular calcium in *Saccharomyces cerevisiae* with an apoaequorin cDNA expression system

(bioluminescence/Ca$^{2+}$-signaling/cell cycle/mating pheromone/recombinant DNA)

JUNKO NAKAJIMA-SHIMADA*, HIDETOSHI IIDA*, FREDERICK I. TSUI†, AND YASUHIRO ANRAKU*

*Division of Cell Proliferation, National Institute for Basic Biology, 38 Nishigakou, Myodaijicho, Okazaki 444, Japan; †Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan; and ‡Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92039

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ABSTRACT A method is described for measuring cytosolic free Ca$^{2+}$ and its time-dependent changes in the yeast *Saccharomyces cerevisiae* by using the luminescent protein apoaequorin as a Ca$^{2+}$-specific indicator. This method with intact yeast cells is labeled "in vivo" to distinguish it from methods with cell extracts, labeled "in vitro." A plasmid in which the apoaequorin cDNA was joined downstream from the glyceraldehyde-3-phosphate dehydrogenase gene promoter was constructed and introduced into yeast cells. The intracellular concentration of apoaequorin expressed by the cDNA was approximately 1 μM, which was high enough to detect the cytosolic Ca$^{2+}$. Growth of the transformed cells was normal. In the *in vitro* method, apoaequorin in crude cell extracts was regenerated into apoaequorin by mixing with coelenterazine, the substrate for the luminescence reaction, whereas in the *in vivo* method, apoaequorin was regenerated by incubating intact cells with coelenterazine. Simultaneous addition of 10 mM CaCl$_2$ and 10 μM A23187, a Ca$^{2+}$-ionophore, to coelenterazinerincorporated cells generated luminescence. Coelenterazine-incorporated cells also responded to native extracellular stimuli. A mating pheromone, α-factor, added to cells of mating type a or α, generated extracellular Ca$^{2+}$-dependent luminescence specifically in a mating type cells, with maximal intensity occurring 45-50 min after addition of α-factor. Glucose added to glucose-starved G$_1$/G$_2$ cells stimulated an increase in extracellular Ca$^{2+}$-dependent luminescence with maximal intensity occurring 2 min after addition. These results show the usefulness of the apoaequorin system in monitoring [Ca$^{2+}$]$_i$ response to extracellular stimuli in yeast cells.

Aequorin, found in the jellyfish *Aequorea victoria*, is a photoprotein containing coelenterazine as a chromophore (1, 2). The binding of Ca$^{2+}$ to aequorin generates a transient bioluminescence, yielding as products light [λ$_{max}$ = 470 nm (hv$_{470}$)], CO$_2$, and a blue fluorescent protein (1, 3). Since aequorin has a high specificity for Ca$^{2+}$, it has been used as a biological indicator of Ca$^{2+}$ (4). Aequorin consists of a complex of apoaequorin (apoaprotein), coelenterazine (substrate), and molecular oxygen. In the Ca$^{2+}$-triggered bioluminescence, the coelenterazine is oxidized to coelenteramide, and the excited state of coelenteramide binds to apoaequorin (blue fluorescent protein) in the reaction (5). Aequorin can be regenerated *in vitro* from apoaequorin by incubation with coelenterazine, molecular oxygen, 2-mercaptoethanol, and EDTA (5):

Aequorin + 3 Ca$^{2+}$ → apoaequorin + coelenteramide + CO$_2$ + hv$_{470}$

+ coelenterazine + O$_2$ + 2-mercaptoethanol + EDTA

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The cDNA for apoaequorin has been cloned, and the primary structure of the protein has been deduced from the nucleotide sequence (6). Aequorin is composed of 189 amino acid residues with three EF-hand structures.

In mammalian cells, Ca$^{2+}$ plays an important role in the regulation of the cell cycle (7, 8). Growth factors induce a transient increase in the intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) (9, 10). In the yeast *Saccharomyces cerevisiae*, specific roles for Ca$^{2+}$ have been found recently during the cell cycle and mating process (11, 12). To measure [Ca$^{2+}$]$_i$ in these studies, fura-2, a Ca$^{2+}$-specific probe, was used in conjunction with digital-image processing (11). A second method is now described that overcomes some of the problems associated with the fura-2 method. Aequorin, expressed by apoaequorin cDNA, is regenerated in the intact yeast cells into apoaequorin and is used to follow changes in [Ca$^{2+}$]$_i$ in response to such external stimuli as mating pheromone and glucose; this method with intact yeast cells is labeled "in vivo" to distinguish it from methods with cell extracts, labeled "in vitro.

MATERIALS AND METHODS

Media and Chemicals. Media for yeast cells were prepared essentially as described by Sherman et al. (13). YPD contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. Synthetic medium (SD) contained 0.67% Bacto-yeast nitrogen base without amino acids and 2% (111 mM) glucose. In calcium-deficient medium (SD – Ca), CaCl$_2$ was omitted and calcium pantothenate was replaced by sodium pantothenate as described by lida et al. (11). The concentration of calcium in SD – Ca medium was 0.24 μM (11). Glucose-deficient SD medium contained 0.67% Bacto-yeast nitrogen base without amino acids, and 0.02% (1.1 mM) glucose. All minimal media were supplemented with appropriate auxotrophic requirements.

Native aequorin was purchased from Wako Pure Chemical (Osaka, Japan). Calcium ionophore A23187 (free acid), 2-deoxyglucose, 6-deoxyglucose, and α-factor were from Sigma. Coelenterazine, 2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, was chemically synthesized (14). Other chemicals were commercial products of the highest grade.

**Yeast and Escherichia coli Strains.** Yeast strains used were H207-2D-A (MATa his3-D1 leu2-3,112 ura3-52 trp1-289 sstl-2) and H207-2D-B (MATa his3-D1 leu2-3,112 ura3-52 trp1-289 sstl-2). H207-2D-B was constructed by switching the mating type of H207-2D-A with YCP50 bearing the HO gene as described by Boeke et al. (15). The two strains were thus isogenic except for the mating locus. Yeast transformations were performed by the lithium acetate method (16). E.

Abbreviation: [Ca$^{2+}$], intracellular free Ca$^{2+}$ concentration.

†Present address: Department of Parasitology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113, Japan.

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coli strains HB101, JM83, and dam 3704 (17) were used for plasmid propagation and isolation.

DNA Manipulation. Plasmid DNA was isolated from E. coli by using the alkaline lysis or SDS-lysis method (18). DNA restriction endonucleases (Boehringer Mannheim), Klenow fragment of DNA polymerase I (New England Biolabs), and T4 DNA ligase (Takara Shuzo, Kyoto, Japan) were used as recommended by the suppliers.

Construction of Aequorin cDNA Expression Plasmid. The plasmid pQi5 containing the aequorin cDNA has been described (6). Deoxyoligonucleotides, 5'-CCCGGG-GATGGTCA-3' (that contains the SacII site as noncoding sequence) and 5'-CGCTTGAACCATCCCGG-3', were synthesized by Applied Biosystems Japan (Tokyo), annealed to each other, and inserted into the ClaI/HindIII fragment of pQi5, yielding plasmid pQi5L in which the Sac II site is located just upstream from the cDNA. Cloning vector pYS1, provided by K. Tanaka (University of Tokyo), was based on a Yep vector and contained the promoter of a S. cerevisiae glyceraldehyde-3-phosphate dehydrogenase (GAPD) gene. The selection marker used for the yeast cells was TRP1. The expression plasmid pGAPAQ1 was constructed by inserting the Sac II/EcoRI 0.6-kilobase (kb) fragment of the aequorin cDNA in pQi5L into the pYS1 vector at the EcoRI site. The nucleotide sequence of the junction region in pGAPAQ1 plasmid was determined with the T7-Sequencing kit (Pharmacia). The GAPD promoter by which the cDNA is expressed constitutively was used because yeast cell growth is not affected by conditional expression of aequorin under the control of the GAL1 promoter (19).

Assay for Aequorin Activity in Vitro and in Vivo. The in vitro assay consisted of a modification of the methods of Blinks et al. (4) and Inouye et al. (20). Yeast cells expressing aequorin (=1 x 10^8 cells) were harvested by centrifugation at 1600 x g for 3 min at room temperature and disrupted with glass beads as described by Iida and Yahara (21). After 100 |mu|l of 30 mM Tris-HCl, pH 7.60/10 mM EDTA, and the aequorin in a 100 |mu|l aliquot which were generated into aequorin by adding 1 |mu|l of 2-mercaptoethanol (14.2 M) and 6 |mu|l of coelenterazine (1 |mu|g/|mu|l of 99.5% methyl alcohol) and allowing the mixture to stand in an ice bath for 2 hr. The aequorin was assayed by injecting 2.5 |mu|l of the incubation mixture into 500 |mu|l of 30 mM CaCl2/10 mM Tris-HCl, pH 7.60. The initial maximal light intensity was measured at 25°C with a photon-counting system in which the photomultiplier of a photometer (model CAF-100; Japan Spectroscopic, Tokyo) was connected to the preamplifier of a photon counter (model C-1230; Hamamatsu Photonics, Hamamatsu, Japan).

For measuring Ca^{2+}-dependent luminescence in vivo, exponentially growing cells carrying pGAPAQ1 (5 x 10^8 cells/ml) in 5 ml of SD medium at 25°C were harvested by centrifugation as described above and resuspended in 30 |mu|l of the same medium containing 50 |mu|M coelenterazine. The cell suspension was incubated for 10 min at room temperature, after which the cells were washed once on a filter (Millipore; type HA; pore size, 0.45 |mu|m) with 10 mM 2-(N-morpholino)ethanesulfonic acid and were resuspended in 500 |mu|l of SD – Ca medium. The suspension was transferred to a cuvette (9.7 x 2.5 x 20 mm) and magnetically stirred with a tiny stirring bar at 600 rpm. Intracellular aequorin luminescence was triggered by injecting 5 |mu|l of 1 M CaCl2 into the suspension, with or without 1 mM A23187, and the luminescence intensity was monitored with the photon-counting system.

Immunoblot (Western) Analysis. Yeast cells carrying pGAPAQ1 or pYS1 were harvested by centrifugation and disrupted with glass beads as described above; after addition of 200 |mu|l of SDS sample buffer, aliquots were subjected to SDS/12.5% polyacrylamide gel electrophoresis (22). Proteins in the gel were electrophoretically transferred to an Immobilon™ membrane (Millipore) and incubated with rabbit antiserum against aequorin, followed by incubation with peroxidase-labeled anti-rabbit IgG.

In Vivo Monitoring of [Ca^{2+}], After Addition of a-Factor. Exponentially growing cells carrying pGAPAQ1 (2.5 x 10^8 cells/ml) were preincubated with a-factor (6 |mu|M) for 15 min at 25°C in 10 ml of SD medium. The cells were harvested by centrifugation at 1600 x g, resuspended in 30 |mu|l of SD medium containing 6 |mu|M a-factor and 50 |mu|M coelenterazine, and incubated for 10 min at room temperature. The cells were then washed once with 10 ml of SD medium containing 6 |mu|M a-factor or SD – Ca medium containing 6 |mu|M a-factor by filtration and were resuspended in 500 |mu|l of SD medium containing 6 |mu|M a-factor or SD – Ca medium containing 6 |mu|M a-factor, respectively. The cell suspension was transferred to a cuvette and monitored with the photon-counting system for luminescence intensity starting 35 min after addition of a-factor at 25°C.

In Vivo Monitoring of [Ca^{2+}], After Glucose Starvation. Glucose starvation and glucose readdition were carried out essentially as described by Kaibuchi et al. (23). To determine cell number and the percentage of budding cells, yeast cells carrying pGAPAQ1 and growing exponentially (3 x 10^6 cells/ml) in 10 ml of SD medium were harvested by filtration, washed once by filtration with 10 ml of glucose-deficient SD medium, and resuspended in 10 ml of glucose-deficient SD medium. After 24 hr of incubation at 25°C, glucose or its hexose derivative was added to give a final concentration of 25 mM, and the cell number and percentage of budding cells were determined as described by Iida and Yahara (24), except that fixation of cells with formaldehyde was omitted. To determine changes in [Ca^{2+}], 10 ml of yeast cells starved for glucose for 24 hr as described above (final cell density = 6 x 10^6 cells/ml) were harvested by filtration, washed once with 10 ml of 0.1 M 2-(N-morpholino)ethanesulfonic acid adjusted with 1 M Tris (pH 10.8) to pH 6.5 (Mes/Tris buffer), resuspended in 10 ml of the same buffer, and incubated for 2 hr at 25°C. The cells were harvested by centrifugation at 1600 x g, resuspended in 30 |mu|l of Mes/Tris buffer containing 50 |mu|M coelenterazine, and incubated for 10 min at room temperature. The cells were then washed once by filtration with 10 ml of Mes/Tris buffer with or without 10 mM CaCl2, and resuspended in 1.2 ml of the same buffer with or without 10 mM CaCl2, respectively. Glucose or its hexose derivative (5 |mu|l) was added to 500 |mu|l of the cell suspension to give a final concentration of 25 mM, and luminescence intensity was measured at 25°C.

Calibration of the Aequorin Signal. The aequorin luminescence intensity was calibrated in a solution [100 mM KCl/10 mM 3-(N-morpholino)propanesulfonic acid (Mops), potassium salt, pH 7.20] by using Ca^{2+}-EGTA buffers (25). A calibration curve was drawn relating free Ca^{2+} concentration to the ratio of peak light intensity observed to maximum light intensity measured at saturating Ca^{2+} concentration (26). [Ca^{2+}], was estimated from the ratio of aequorin light intensity of the yeast cells to maximum light intensity calculated from total light yield when the cells were lysed at the end of the experiment with 0.5% Triton X-100 (26). The Ca^{2+}-independent luminescence of cells containing the vector pYS1 loaded with coelenterazine was subtracted as a blank value from all of the other results.

RESULTS

Constitutive Expression of Aequorin cDNA in S. cerevisiae. Yeast cells carrying pGAPAQ1 and pYS1 growing
in SD medium were monitored for cell growth and expression of apoaequorin. Fig. 1A shows that the growth curves were essentially the same for cells carrying pGAPAQ1 and pYS1. Fig. 1B gives the time course of apoaequorin production, calculated from a calibration curve relating luminescent intensity of native aequorin to apoaequorin protein concentration (data not shown). In cells carrying pYS1, no luminescence activity was detected, whereas apoaequorin was constitutively expressed in exponentially growing cells carrying pGAPAQ1. At the stationary phase, there was a decrease in the amount of apoaequorin. The expression of apoaequorin was also confirmed by Western blot analysis with anti-aequorin antibody (Fig. 1C).

The content of apoaequorin in exponentially growing cells was estimated to be \(1 \mu M\) with the above calibration curve. This concentration is high enough to monitor changes in \([Ca^{2+}]_i\) even though apoaequorin has an in vivo half life of 6.5 min (data not shown).

**Localization of Aequorin.** Cell fractionation was carried out by the method of Bandlow and Bauer (27). Assay for aequorin after regeneration showed that 93.5 ± 1.5% of the total luminescent activity was present in the 165,000 × g supernatant, indicating that apoaequorin is localized largely in the cytosol.

**Introduction of Coelenterazine into Intact Yeast Cells.** Yeast cells carrying pGAPAQ1 require coelenterazine to regenerate apoaequorin into aequorin and show intracellular luminescence. Coelenterazine, a hydrophobic compound with a molecular weight of 423, was taken up best when added directly to cell suspensions compared with other methods tested, including electroporation. To investigate in vivo localization of coelenterazine, intact yeast cells were incubated in SD medium containing 50 \(\mu M\) coelenterazine, washed three times with SD medium, and harvested by centrifugation. Extracts were prepared as described by Bandlow and Bauer (27). The extracts were centrifuged at 165,000 × g, and the supernatant and pellet were incubated for 2 hr with apoaequorin-containing extracts of cells carrying pGAPAQ1. When assayed with \(Ca^{2+}\) for luminescent activity, >70% of the coelenterazine was found present in the cytosol.

**Generation of Luminescence After Addition of CaCl\(_2\) to a Suspension of Intact Yeast Cells.** Fig. 2 shows the effect of adding CaCl\(_2\) or a calcium ionophore (A23187), or both, to intact yeast cells carrying pGAPAQ1 that were preincubated with 50 \(\mu M\) coelenterazine, washed, and resuspended in SD – Ca medium. When 10 \(\mu M\) A23187 was added to the cell suspension, no luminescence activity was detected (Fig. 2A), but when 10 \(\mu M\) CaCl\(_2\) was added, some light emission occurred (Fig. 2B). Much higher luminescence intensity was generated when 10 nM CaCl\(_2\) mixed with 10 \(\mu M\) A23187 was added (Fig. 2C). Addition of MgCl\(_2\), MnCl\(_2\), CuCl\(_2\), and ZnCl\(_2\) at the same concentration with 10 \(\mu M\) A23187 did not produce luminescent activity. To rule out the possibility that the observed luminescence transients (as well as those shown in Figs. 3 and 5; see below) might result from leakage or secretion of aequorin into the medium, cells carrying pGAPAQ1 loaded with coelenterazine as described above were incubated in SD – Ca medium for 10 min, and then the supernatants were separated from the cells both before and after addition of stimulants and were assayed for \(Ca^{2+}\)-dependent luminescence. The results demonstrated no detectable luminescence in the supernatants (data not shown). In addition, Western blot analysis using anti-aequorin antibody showed no detectable aequorin protein in the supernatants (data not shown).

**In Vivo Monitoring of the \([Ca^{2+}]_i\) Response to α-Factor.** It has been shown (11) with fura-2 that in single yeast cells, \([Ca^{2+}]_i\) increases with a lag time of \(40\) min in response to the mating pheromone, α-factor, and that this increase is essential for maintaining cell viability late in the mating process (11). Aequorin-bearing and control MATa cells showed the same increase in the percentage of unbudded cells and the efficiency of projection formation on the cell surface after exposure to α-factor, indicating that aequorin does not interfere with the α-factor response (data not shown). Fig. 3 shows that \([Ca^{2+}]_i\) rose in response to α-factor only in MATa cells growing in SD medium and not in cells growing in SD – Ca and in isogenic MATa cells growing in SD and SD – Ca. These results are consistent with the fact that MATa cells lack α-factor receptor for α-factor. \([Ca^{2+}]_i\) started to increase gradually in MATa cells in SD medium 35–40 min after addition of α-factor, peaking at 45–50 min and subsequently remaining above the basal level. By using a calibration method for aequorin signal, the level

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**Fig. 1.** Growth curve of *S. cerevisiae* cells carrying pYS1 (c) or pGAPAQ1 (●) and expression of apoaequorin cDNA. (A) Growth of H207-2D-A carrying pYS1 or pGAPAQ1 in SD medium at 25°C. (B) Expression of apoaequorin cDNA. At the times indicated, cells were harvested, and aequorin activity was assayed in vitro as described. The ordinate shows the amount of apoaequorin calculated from a calibration curve based on luminescence intensity of native aequorin. (C) Western blot analysis. Cell extracts were run on SDS/12.5% polyacrylamide gels and subjected to immunoblot analysis with rabbit antiserum against aequorin. Lanes: a, pYS1; b, pGAPAQ1.

**Fig. 2.** Effects of CaCl\(_2\) and A23187 on the luminescence activity of intact yeast cells. Exponentially growing cells carrying pGAPAQ1 in SD medium were harvested by centrifugation at 1600 × g and incubated with 50 \(\mu M\) coelenterazine for 10 min at room temperature. Cells were washed with SD – Ca medium and resuspended in 500 \(\mu l\) of the same medium. Luminescence intensity was measured at 25°C with the photon counting system as described. The arrow indicates the point of addition of CaCl\(_2\) or A23187, or both. (A) 10 \(\mu M\) A23187. (B) 10 mM CaCl\(_2\). (C) 10 \(\mu M\) A23187 and 10 mM CaCl\(_2\).
of the \([\text{Ca}^{2+}]\) increase in the \(\text{MATa}\) cells induced by \(\alpha\)-factor was estimated to be 590 ± 200 nM \((n = 3)\), which was about 4 times the basal level of 140 ± 40 nM \((n = 3)\). These results are in good agreement with those reported previously by the fura-2 and the \(\text{Ca}^{2+}\)-AEQ incorporation methods [11, 28].

**In Vivo Monitoring of the \(\text{Ca}^{2+}\) Response to Glucose Addition in Glucose-Starved \(\text{G}_{0}/\text{G}_{1}\) Cells.** Although it has been reported that glucose stimulates both influx and efflux of \(\text{Ca}^{2+}\) in glucose-starved cells [23], changes in \([\text{Ca}^{2+}]\) have not been demonstrated upon glucose addition. Fig. 4 shows that cell growth stopped, and the percentage of budded cells decreased when exponentially growing cells in complete SD medium (containing 111 mM glucose) were shifted to glucose-deficient SD medium (containing 1.1 mM glucose), suggesting that cells were arrested in \(\text{G}_{0}/\text{G}_{1}\). After a 24-hr starvation, >98% of the cells were viable, and the cells contained one-third of the amount of apoaequorin as cells growing in SD medium, but the level was still high enough to detect changes in \([\text{Ca}^{2+}]\) (Fig. 4C). Readdition of glucose induced bud emergence with a time lag of ~1 hr, followed by cell division (Fig. 4 A and B). Similar results were obtained when fructose or mannose was added to the glucose-starved cells. However, nonmetabolic sugars, such as 2-deoxyglucose and 6-deoxyglucose, did not induce bud emergence and cell division.

Fig. 5A shows that glucose and 2-deoxyglucose did not produce changes in \([\text{Ca}^{2+}]\) in glucose-starved cells suspended in Mes/Tris buffer lacking CaCl\(_2\). In contrast, \([\text{Ca}^{2+}]\) increased in cells suspended in the buffer containing 10 mM CaCl\(_2\), reaching a peak around 2 min after glucose addition (Fig. 5B, trace a). This increase corresponded to a change in \([\text{Ca}^{2+}]\) from the basal level of 180 ± 10 nM to 340 ± 40 nM \((n = 4)\). Similar results were obtained when fructose or mannose, but not 2-deoxyglucose or 6-deoxyglucose, was added (Fig. 5B). Luminescent activity gradually decreased about 2 min after glucose addition. When 10 \(\mu\)M A23187 was added to a cell suspension that had received glucose and in which luminescence intensity had decreased to a basal level, luminescence was again generated (Fig. 5C), indicating that the decrease was not due to an decrease in apoaequorin present in the cells but to a decrease in \([\text{Ca}^{2+}]\). These results show that glucose does elicit a \([\text{Ca}^{2+}]\) response in glucose-starved \(\text{G}_{0}/\text{G}_{1}\) cells, depending upon extracellular \(\text{Ca}^{2+}\).

**DISCUSSION**

The expression of cDNA for apoaequorin in yeast cells and subsequent regeneration of apoaequorin into aequorin provide a noninvasive and nontoxic method for measuring \([\text{Ca}^{2+}]\). Even though the amount of aequorin regenerated could not be quantified, it was high enough to detect changes in \([\text{Ca}^{2+}]\). Compared with fura-2, which is sequestered into vacuoles, and which requires excitation at 340 and 380 nm, the aequorin method seems to have the following advantages: (i) changes in \([\text{Ca}^{2+}]\) can be monitored for a long period because radiation damage to cells is not a factor; (ii) auto-fluorescence, which is a problem with the fura-2 method, is eliminated; and (iii) aequorin is not sequestered into organelar compartments. However, unlike the fura-2 method, the aequorin method has a drawback: changes in \([\text{Ca}^{2+}]\) in a single yeast cell cannot yet be monitored. Thus, the method of choice will depend on the experiments being carried out.

It is interesting that aequorin can be regenerated by using coelenterazine in intact yeast cells. For the \(\text{in vitro}\) regeneration of aequorin in a reaction mixture of native aequorin or recombinant apoaequorin, the addition of 2-mercaptoethanol to the regeneration mixture is essential to give maximum light yield [5, 20]. Other reducing reagents including dithiothreitol, cysteine, and glutathione, were also found to bring about regeneration in yeast cell extracts (data not shown). Thus, the reducing nature of the cytosol, probably due to the high content of glutathione in yeast cells, may be responsible for the regeneration [29]. It is noteworthy that obelin, another \(\text{Ca}^{2+}\)-activated photoprotein, also can be regenerated inside human neutrophils when these cells loaded with the apoprotein or mRNA purified from the hydroid Obelia are incubated with coelenterazine [30].
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