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Cadmium-induced luminescence of recombinant photoprotein obelin

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Abstract

It has been shown for the first time that Cd^{2+} ions induce substantial bioluminescence of a Ca^{2+}-binding photoprotein: recombinant obelin. The optimum pH for the bioluminescent reaction in the presence of Cd^{2+} ions is pH 6. The intensity, L, of the light emission for the Cd^{2+} ions is 75% of the intensity of the signal in the presence of Ca^{2+}. The quantum yields of the reactions in the presence of Cd^{2+} and Ca^{2+} are 0.18 and 0.24 respectively. The slope of the straight line (between 5 and 90% of L_{max}) in the coordinates of $\log(L/(L_{max}-L))$ vs. $\log([Cd^{2+}])$ is 1.75 ± 0.06, which indicates positive cooperative character of this reaction. At a concentration exceeding $1 \cdot 10^{-3}$ M, Cd^{2+} inhibits the bioluminescent reaction.

Keywords: Photoprotein; Obelin; Cadmium; Bioluminescence

1. Introduction

The calcium-activated photoproteins occur in many species of luminescent marine coelenterates including both the *Cnidaria* and the *Ctenophora* [1]. One of the representatives of such proteins is obelin from the hydroids. Native obelin from the hydroid *Obelia longissima* is a small single-chain protein of approximate molecular mass of 20 kDa that contains a noncovalently bound chromophoric group –coelenterazine (substituted imidazolopyrazinone) [2]. The interaction of the photoprotein with Ca^{2+} ions results in intramolecular conversion of coelenterazine to coelenteramid followed by light emission at $\lambda_{max} = 469$ nm. It was reported recently that Mn^{2+} ions also stimulate the bioluminescent reaction of obelin [3]. The gene of obelin was cloned and expressed in *Escherichia coli* (*E. coli*) cells [4]. The gene of the recombinant protein encodes 195 amino acid residues (M_r 22200). A recurring Ca-binding motif found in many calcium proteins is the helix-loop-helix site also known as EF structure [5]. Obelin has three EF-structures, which have homology to EF-structures of other Ca^{2+} binding proteins [6].

Extensive literature data indicate that different ions, especially lanthanides, besides calcium, can stimulate the bioluminescence of photoproteins. However, cadmium has never been reported as an ion stimulating this reaction with substantial yields in comparison to calcium. Cadmium and calcium are members of group II of the periodic table of the elements and the ionic radius of Cd^{2+} ions (0.95 Å) is close to that of Ca^{2+} (0.99 Å) [7].

2. Materials and methods

2.1. Chemical reagents

Casein hydrolysate (peptone No. 140), yeast extract and kanamycin sulfate were obtained from Gibco BRL (Life Technologies, UK). Ampicillin (sodium salt), bovine serum albumin (BSA) and electrophoresis reagents were obtained from Sigma (USA). Coomassie brilliant blue R-250 was from Merck (Germany).

For chromatographic purification DEAE-Sepharose fast-flow (Pharmacia/LKB, Sweden) and Bio-Gel P6 DG (Bio-Rad, USA) were used. Coelenterazine (C-2944) was purchased from Molecular Probes (USA). Parvalbumin agarose was obtained from the Institute of Biophysics (Pushchino, Russia). All solutions were prepared in water purified with the Nanopure system (Sybron-Barnstead, USA) with the output resistivity set at > 16.7 MΩ/cm.

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All solution were stored in plastics. All other chemicals were of analytical grade.

2.2. Cultivation of cells

Recombinant apoobelin was extracted from *E. coli* cells strain C600 containing the plasmid pOL110 [4]. Cells were cultivated in Luria-Bertani medium (10 l), containing 50 mg/l ampicillin and 50 mg/l kanamycin, at 30°C until the optical absorbance at 590 nm reached the value of 2 units. After thermoinduction at 42°C for 30 min, cultivation was continued for 1 h at 37°C. Before starting of thermoinduction, sucrose was added to the media to a final concentration of 50 mM. After cultivation was finished the cells were spun down by centrifugation: 6000 rpm for 9 min at 20°C. The precipitate was washed twice with 0.9% NaCl solution.

2.3. Isolation and purification of obelin

Washed cells (50 g) were suspended in a ratio 1:4 (w/v) in buffer 20 mM Tris-HCl (pH 7.0) and ruptured using a French press. The suspension was centrifuged (13 000 rpm for 40 min) and the supernatant was discarded. The precipitate was washed with portions (150 ml) of 20 mM Tris-HCl (pH 7.0) containing: 0.9% NaCl (twice), 1% Triton X-100 (twice) and 5 mM $CaCl_2$ (once). From this precipitate the protein was extracted with the same buffer containing 5 mM $CaCl_2$ and 6 M urea at 4°C overnight with constant stirring. Subsequently, the solution was centrifuged (13 000 rpm × 40 min) and the precipitate was discarded. The supernatant was diluted in a ratio of 1:3 (v/v) with 20 mM Tris-HCl, 5 mM $CaCl_2$ (pH 7.0). Then β-mercaptoethanol was added to this solution to a final concentration of 5 mM. As a result, protein precipitated. The precipitate was removed by centrifugation (13 000 rpm × 30 min). The supernatant was applied on DEAE-Sepharose fast-flow (5 × 2 cm), equilibrated with buffer containing 20 mM Tris-HCl, 5 mM $CaCl_2$, 2 M urea (pH 7.0); at a speed of 100 ml/h. Obelin was eluted from the column with a gradient of NaCl (0–0.5 M) in the same buffer. Fractions were tested for the presence of obelin by the activity assay and active fractions were pooled and concentrated using an Amicon concentrative cell equipped with a YM-10 membrane. To the sample obtained after concentration EDTA was added to a final concentration of 5 mM and afterwards the sample was desalted using a Bio-Gel P6 DG (0.8 × 26 cm) column equilibrated with 20 mM Tris-HCl, 500 mM NaCl (pH 7.0). Active fractions containing obelin were combined and reconcentrated using the Amicon system.

2.4. Purity of obtained obelin

The protein concentration was measured with the microbiuret method [8], using BSA as a standard. The degree of purity of obelin preparations was determined by SDS-gel electrophoresis in 12.5% polyacrylamide gel [9]. According to these data the purity of the protein was more than 98%.

2.5. Preparation of calcium-free solutions and plasticware

Polyethylene containers and other plasticware were soaked for 24 h in 1 N HCl at 70°C, then in deionized water for 24 h at 70°C and finally rinsed five times with metal-ion-free water. The trace metal ions were removed from all the solutions using Chelex 100. The decalcination of the solutions was done using parvalbumin agarose.

2.6. Reactivation of obelin and bioluminescence measurements

Obelin was reactivated overnight at 4°C in buffer containing: 20 mM Tris-HCl, 500 mM NaCl, 5 mM β-mercaptoethanol, 1 mM EDTA (pH 7.0); 2.35 μM protein and 2.35 μM coelenterazine. The bioluminescent activity of obelin was measured using a bioluminometer BLM 8801 (SCTB 'Nauka', Krasnoyarsk, Russia) calibrated with a Hastings-Weber radioactive light standard [10]. The solution to be tested contained: 500 μl of buffer 50 mM Bis-Tris propane of defined pH and 5 μl of reactivated obelin. The reaction was started by injecting 200 μl of the tested ions solutions. The final concentration of ions in the reaction was $3.3 \cdot 10^{-4}$ M. The bioluminescence reaction under calcium-free conditions was initiated by manual injection of photoprotein solution (10 μl) into the 1 ml of 10 mM Hepes containing Cd^{2+} or Ca^{2+} ions. The quantum yield was calculated according to the following equation [11]: $Q_B = \phi_{sp} \cdot M_r / A$, in which Q_B is bioluminescence quantum yield, ϕ_{sp} (specific photon yield) is the number of emitted photons per g of photoprotein, M_r is the relative molecular mass, A is Avogadro's number.

3. Results and discussion

The optimum of light emission of recombinant obelin in the presence of Ca^{2+} ions is optimal over a broad range of pH 6–10 (Fig. 1). The quantum yield of this reaction, calculated from the L_{max} and protein concentration, is 0.24. The Cd^{2+} ions initiated bioluminescent reaction of recombinant obelin exhibits a maximum at pH 6 (Fig. 1) with a yield equal to 75% of that with the Ca^{2+}-stimulated reaction. The quantum yield for Cd^{2+}-stimulated bioluminescent reaction is 0.18. The log-log plot of obelin bioluminescence against Cd^{2+} concentration (Fig. 2) shows a sigmoid curve with maximum in the range of Cd^{2+} concentration $3.3–8.1 \cdot 10^{-4}$ M. The slope of the straight line (between 5 and 90% of L_{max}) in the coordinates of $\log(L/(L_{max} - L))$ vs. $\log([Cd^{2+}])$ was 1.75 ± 0.06 (mean $\pm$ standard deviation, $n = 11$, $r = 0.995$) (Fig. 3), which

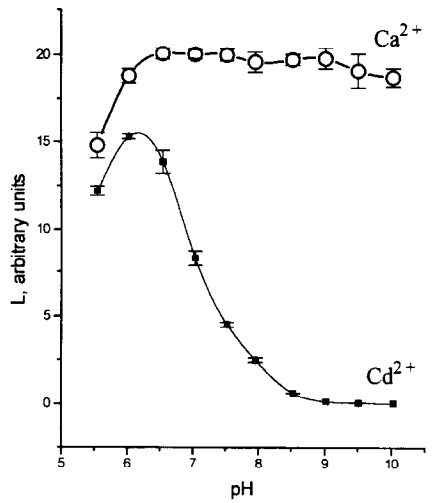

Fig. 1. The pH dependence of the bioluminescent reaction of obelin in the presence of Ca^{2+} and Cd^{2+} ions in 50 mM Bis-Tris propane solutions.

indicated positive cooperative character of this reaction [12]. For concentrations of Cd^{2+} exceeding $1 \cdot 10^{-3}$ M there is a decrease of the intensity, thus an inhibition of bioluminescent reaction.

The similar ionic radii of Cd^{2+} (0.95 Å) and Ca^{2+} (0.99 Å) and the data that Cd^{2+} can form the seven-coordinate geometry, in the same way as calcium does, in the Cd parvalbumin [13,14] suggest that the Cd^{2+} ions bind with EF-structures of obelin and thus produce light with the high quantum yield. It has not yet been determined how many ions of Cd^{2+} stimulate the bioluminescent reaction. From the value of the slope in the log-log plot,

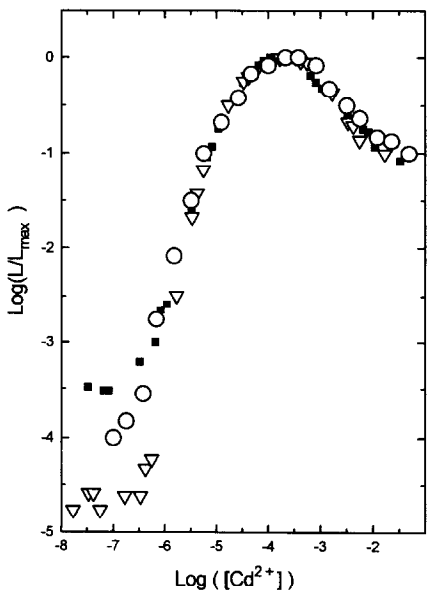

Fig. 2. Relation between Cd^{2+} concentration and obelin luminescence in the presence of (■) $7.1 \cdot 10^{-9}$ M EDTA, (▽) $7.1 \cdot 10^{-6}$ M EDTA, (○) without EDTA (calcium-free conditions). The curve (▽) is corrected for the presence of the free EDTA in the reaction system.

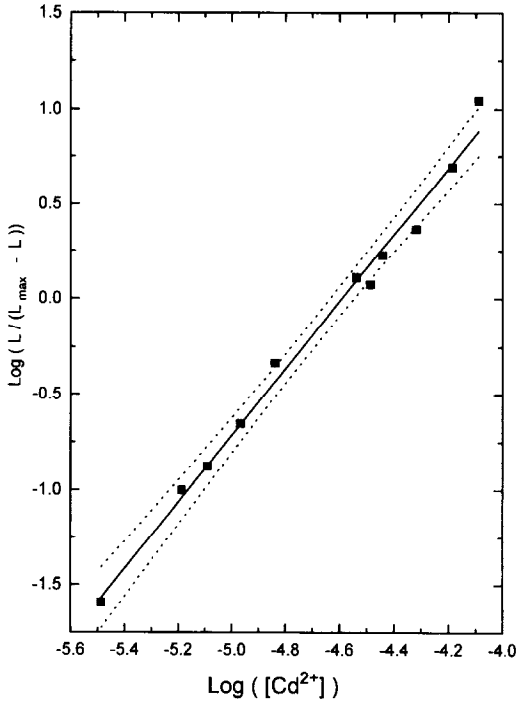

Fig. 3. The linearization of the bioluminescent signals of obelin (between 5 and 90% of L_{max}) in the presence of Cd^{2+} ions after correction on the free EDTA concentration in the reaction system.

we assume that in the stimulation of the bioluminescent reaction more than one ion of Cd^{2+} is involved. A precise stoichiometry determination might be possible with Cd-NMR monitored titrations on highly concentrated obelin preparations. The difference in pH dependence of the reaction in the presence of calcium and cadmium is not due to the formation of cadmium hydroxide, since the first pK for the hydrolysis of Cd(II) is ≈ 8 [15]. At this time we have no explanation for the observed difference. We cannot exclude the interaction of Cd^{2+} ions with SH-groups in the protein molecule, because it is known that Cd^{2+} blocks sulfhydryl groups in enzymes [16]. This phenomenon may explain the inhibition of the bioluminescent reaction of obelin in the presence of Cd^{2+} at concentrations higher than $1 \cdot 10^{-3}$ M, because the amino acid sequence data for obelin show that this molecule contains five cysteine residues and three of them are situated where substrate binding is presumed to occur [6].

From the data on the bioluminescence of other photoproteins, namely aequorin [17] and obelin from hydroids *Obelia geniculata*, *Obelia australis* [18] and *Obelia longissima* [2], it is easy to determine that the linear segment of the sigmoid curve in the log-log plot is in the range of Ca^{2+} concentration between $1–5 \cdot 10^{-7}$ and $0.5–1 \cdot 10^{-4}$ M. In the present work the total concentration of Ca^{2+} ions in the reaction mixtures, including the calcium contaminations in the solutions of the protein and cadmium, did not exceed $8.4 \cdot 10^{-7}$ M and the concentration of EDTA was $7.1 \cdot 10^{-6}$ M. Before starting the reaction by

tested ion, we did not observe any luminescence reaction. This implies that we did not have free Ca^{2+} ions in the system. Even if all the EDTA-bound calcium were substituted by cadmium (pK: Ca^{2+}, 10.58; Cd^{2+}, 16.48, where K is the effective stability constant for EDTA-metal complex [19]), then based on the data from Refs. [2,17,18] we would observe a signal of about 0.001–0.01% from L_{max} –the luminescence for saturated concentration of Ca^{2+} ($1 \cdot 10^{-4}$–10^{-3} M). In this work we measured the maximum signal for bioluminescence of obelin (L_{max}) in the presence of the saturating concentration of Ca^{2+} ions $-3.3 \cdot 10^{-4}$ M. Even if we had all EDTA ($7.1 \cdot 10^{-6}$ M) saturated by calcium and if Cd^{2+} ions substituted all calcium from this complex, we would expect to observe a signal in the range of 0.2–2% from our L_{max}. For checking the real concentration of the calcium in our experiments, we used Cu^{2+} ions because copper ions, like Cd^{2+} ions, displace the calcium from the EDTA-calcium complex (pK for Cu^{2+} is 18.80 [19]). The magnitude of light impulse in the presence of copper ions ($3.3 \cdot 10^{-4}$ M) within the range of pH 5–11 did not exceed about 0.002% of L_{max} in the presence of Ca^{2+}. Cu^{2+} ions at concentrations $1.4–3.3 \cdot 10^{-4}$ M did not inhibit Ca^{2+}-stimulated bioluminescence of obelin.

To eliminate the role of EDTA and calcium contaminations, we calculated the slope of the linear part of the curves in the coordinates of $\log(L/(L_{max} - L))$ vs. $\log([Cd^{2+}])$ in the range of cadmium concentrations that gave signals between 5 and 90% of L_{max}.

We also obtained data for the influence of the Cd^{2+} ions on the bioluminescence of obelin under Ca^{2+}-free conditions. In these experiments the value of the slope was 1.5 (calculated using the same method of calculations as in the case of the presence of EDTA). The pH optimum was around 7.0 and the quantum yield was 0.16. All these experiments were done in 10 mM Hepes and the presence of Hepes could explain the slight difference between these results and the results obtained in 50 mM Bis-Tris propane. But it is obvious that the results which we obtained under controlled concentration of calcium and EDTA could not result from calcium contamination. Also, we observed the same phenomena in the system free of calcium and in the system with the controlled calcium and EDTA concentrations.

Therefore, Cd^{2+} ions stimulated the bioluminescent reaction of recombinant obelin with high efficiency. We are not aware of specific effects of Cd^{2+} on other calcium proteins. In an early paper, Izutsu et al. reported on strong effects of many ions including Cd^{2+} (but also Cu^{2+}) on the luminescence of aequorin [20]; however, their results were subsequently suggested to be in error due to a contamination with Ca^{2+}-EDTA [21]. Thus, it appears that the present article is the first report about the role of Cd^{2+} ions in the stimulation of the high quantum yield bioluminescent reaction of Ca^{2+}-activated photoproteins. The system may have potential as a sensitive sensor for cadmium ions.

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