Fluorescence polarization study of the Ca^{2+}-sensitive photoprotein aequorin

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1. INTRODUCTION

The photoprotein aequorin (M_r 20 000) obtained from the jellyfish *Aequorea*, emits a bluish light when a trace of Ca^{2+} is added, independently of the presence of O_2 [1,2]. In the light-emitting reaction, aequorin having no visible fluorescence is converted into a blue-fluorescent protein product (BFP), which is a complex of apoaequorin, coelenteramide (the light-emitting chromophore) and 2 Ca^{2+} [3]. It has been thought that the light-emitting intramolecular reaction is triggered by a conformational change that is caused by the binding of Ca^{2+}, although no firm evidence supporting it had been submitted.

These data on the intensity and depolarization of intrinsic fluorescence of aequorin and BFP suggest that the conformation of aequorin is considerably more rigid than that of BFP. This finding is consistent with the results of the papain digestion of these proteins which showed that aequorin is far more resistant than BFP to papain.

2. MATERIALS AND METHODS

Aequorin was extracted and purified as in [4,5]. The concentration of aequorin was determined by absorbance at 280 nm ($A\,]^{\%}_{cm} = 27.0$) or by light-emitting capacity when Ca^{2+} is added (4.5×10^{15} photons/mg at 25°C [4]. Aequorin solution freshly prepared by dissolving $(NH_4)_2SO_4$-precipitated aequorin or freeze-dried aequorin was left standing for $\geq$ 30 min before using it in the fluorescence study, because the fluorescence emission intensity at 334 nm gradually increases in this period before reaching a constant value of > 2-times the initial value. BFP was obtained by adding 15 μl 1 M calcium-acetate into 3 ml aequorin solution containing 2 mM EDTA.

Fluorescence anisotropy was measured on an instrument built essentially as in [6]. Two Schoeffel 250 monochromators in tandem were used to obtain the excitation beam of 280 nm from a 150 W xenon lamp. For the two emission beams, narrow-band 334 nm interference filters (Corion Corp.) were inserted to separate fluorescence emission from scattered excitation light. The excitation light was plane polarized by an air-gap Glen-Taylor prism while emission beams were polarized with UV-transmitting polarizing films (Polaroid HNB'P). The intensities of emitted light polarized parallel and perpendicular to the polarization of exciting light are denoted by I_V and I_H, respectively. Outputs from two lock-in amplifiers (Ithaco 391 and PAR 128A) were fed into an analog divider to obtain digital readout of I_V/I_H. Anisotropy is defined as $(I_V - I_H)/(I_V + 2I_H)$, where $I_V + 2I_H$ is proportional to the emission intensity of the sample [7]. Fluorescence emission spectra were measured with a Perkin-Elmer fluorescence spectrophotometer model MPF-44B. All measurements were carried out in a 1 cm square cell with 3 ml sample solution at room temperature.

Papain digestion of aequorin (20 μg) was done by incubating the sample in 2 ml 10 mM Tris–HCl buffer (pH 7.2) containing 0.5 M NaCl, 1 mM EDTA, 0.5 mg cystein, 0.4 mg 2-mercaptoethanol, and 25 μg papain (Sigma, type III), at 22°C for 30 min. BFP (20 μg) was digested under the same conditions as used for aequorin, except that 1 mM EDTA was replaced with 2 mM $CaCl_2$. The

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amounts of aequorin and BFP that remained unaltered were assayed by luminescence produced by adding, respectively, 1 ml 10 mM calcium-acetate for aequorin, and 10 μl methanolic coelenterazine (250 μg/ml) for BFP [8] and compared with the results of control samples that lacked papain.

3. RESULTS

Aequorin solution excited at 280 nm shows a fluorescence emission maximum at 334 nm (fig.1A) due to the presence of tryptophan residues in the molecule [4,9]. When aequorin in the solution was converted to BFP by adding Ca^{2+}, the fluorescence intensity of the solution at 334 nm increased 4-fold, accompanied by the appearance of a new blue fluorescence band (λ_{max} 468 nm) due to the formation of coelenteramide (fig.1B). Acidification of this sample (pH 2) resulted in the loss of the 468 nm emission band and also a slight increase of the 334 nm emission peak (fig.1C). Acidification of the original aequorin solution resulted in the same fluorescence spectrum as fig.1C.

Fluorescence depolarization of aequorin and BFP when excited at 280 nm and measured at 334 nm indicated two distinct values of anisotropy covering a wide range of pH: a high value of 0.098 for aequorin over pH 2–9, and low value of 0.074 for BFP over pH 5–12 (fig.2). The high value of anisotropy of aequorin can be brought down irreversibly to the level of the low value by raising pH to ~ 12, causing decomposition of the coelenterazine moiety in aequorin molecule. However, the low value for BFP can be reversibly changed to the level of the high value simply by lowering the pH to < 2. Stepwise addition of EDTA, up to 30 mM, to BFP solution containing 3 mM Ca^{2+} did not alter the value of anisotropy, indicating that the anisotropy of BFP is independent of $[Ca^{2+}]$.

The above anisotropy data indicate that the local rigidity around the tryptophan residues in the molecule of aequorin is greater than that in the molecule of BFP, consequently implying that the molecular conformation of aequorin is probably more rigid and compact than that of BFP. The cause for the greater rigidity of aequorin molecule may stem largely from the fact, in addition to probable difference in secondary and tertiary structure, that it contains a moiety of coelenterazine tightly bound to the protein part [3,10]; the coelenterazine moiety is possibly constituting the core of the molecule that helps to increase the conformational rigidity of aequorin. Such a structure of aequorin may also explain its weak fluorescence emission at 334 nm, because intramolecular energy transfer from tryptophan residues (fluorescence maximum at 334 nm)

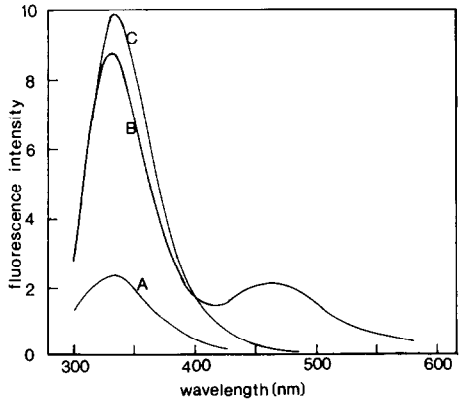

Fig.1. Fluorescence emission spectra of: (A) aequorin (33 μg/ml) in 10 mM Tris–HCl (pH 7.5) containing 2 mM EDTA; (B) BFP prepared by adding 15 μl of 1 M calcium-acetate into 3 ml solution (A); (C) after acidification of solution A or solution B to pH 2 with HCl; excited at 280 nm, at room temperature.

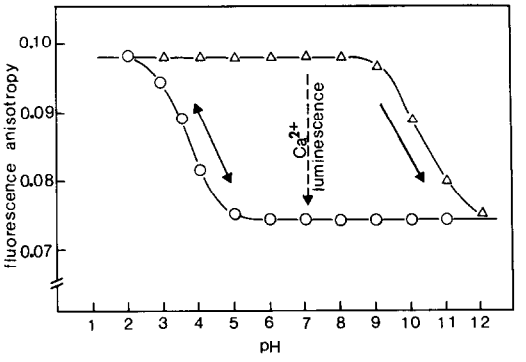

Fig.2. Relationship of pH and the fluorescence anisotropy of aequorin (△) and BFP (○), excited at 280 nm and measured at 334 nm. Solutions of aequorin and BFP (both 33 μg/ml) prepared in 10 mM Tris–HCl containing 2 mM EDTA were adjusted to the indicated pH-values with HCl or NaOH immediately before the measurements. Arrows indicate reversible and irreversible nature of anisotropy changes.

to the non-fluorescent coelenterazine moiety (absorption maximum at 300 nm) [10] is likely to occur.

In the molecule of BFP, coelenteramide is reversibly bound to apoaequorin. The molecular conformation of BFP appears to be more flexible than that of aequorin and it is constituted probably in such a manner that prevents a significant extent of energy transfer from tryptophan residues to coelenteramide. In acidic and alkaline solutions, BFP completely dissociates into apoaequorin plus coelenteramide whereas aequorin decomposes into apoaequorin plus coelenteramine [5], both producing the same protein product. Because aequorin is an acidic protein (isoelectric point: pH 4.2–4.9) [11], apoaequorin in an acidic solution of pH $\leq$ 2 may acquire a certain extent of α-helical structure which is substantially different from the structures of aequorin and BFP in neutral solution. Thus, the same value of anisotropy found for aequorin in neutral solution and in acidic solution could be incidental.

In [12] it was reported that BFP has a rigid conformation and that coelenteramide in this protein is rigidly bound to the protein part, mainly based on the studies of the blue fluorescence of BFP (λ_{max} 468 nm). We have obtained evidence showing that BFP is easily dissociable to coelenteramide plus apoaequorin even in the presence of 1 mM Ca^{2+} (dissociation constant 0.6×10^{-6} M) in the study of the relationship between the concentration of BFP and the intensity of the blue fluorescence (fig.3).

These data on the anisotropy of the fluorescence of tryptophan residues, which suggest a higher degree of conformational rigidity for aequorin over BFP, are supported by the high resistivity of aequorin to papain digestion. Thus, when incubated with papain at room temperature for 30 min under the conditions in section 2, 80% of BFP was inactivated whereas the activity of aequorin was not affected at all.

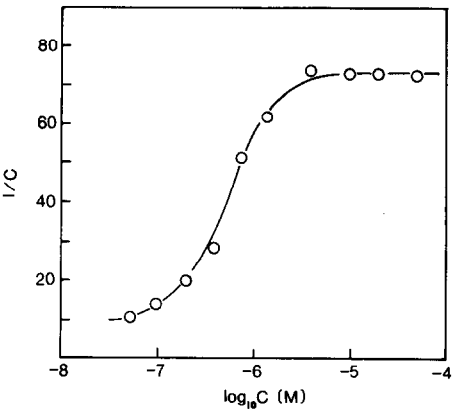

Fig.3. Relationship between the concentration of BFP (C) and the fluorescence intensity at 468 nm (I, in arbitrary units) when excited at 340 nm, in 10 mM Tris–HCl (pH 7.3) containing 1 mM $CaCl_2$, at room temperature.

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