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Binding of murine monoclonal antibodies to the active and inactive configurations of aequorin

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Murine monoclonal IgG_1 antibodies (MAb), designated Aq-11 and Aq-12, were prepared against the photoprotein aequorin from jelly fish. Aequorin is a calcium-sensitive photoprotein which consists of a single polypeptide chain, apoaequorin, and a functional chromophore, coelenterazine. Native aequorin consists of two species with molecular masses of 25 and 23.5 kDa. MAb Aq-12 was found by immunoblot analysis to bind specifically to the 25 kDa species, while MAb Aq-11 reacted with the 23.5 kDa protein. Activation of apoaequorin with coelenterazine was associated with a shift of the 23.5 kDa molecule to the 25 kDa species. In contrast, treatment with calcium ions induced a shift back to the 23.5 kDa form. These changes between the active and inactive forms were identified by reactivity with MAbs Aq-11 and Aq-12. The results thus indicate that these MAbs should be useful in monitoring activation of this photoprotein.

Aequorin; Monoclonal antibody; Active form; Inactive form

1. INTRODUCTION

Aequorin is a bioluminescent protein from the jelly fish which emits blue light in the presence of calcium ions [1,2]. Aequorin has thus been used as a calcium indicator [3,4]. Aequorin is dissociated into apoaequorin and the oxidized chromophore, coelenteramide. Dissociated apoaequorin can be reconstituted by incubation with coelenterazine in 2-mercaptoethanol (2ME), EDTA and 30 mM Tris-HCl buffer (pH 7.6) for 2 h at 4°C [5]. Activation of aequorin with coelenterazine is associated with a shift in apparent molecular mass from 23.5 to 25 kDa. In contrast, calcium treatment induces conversion of the activated 25 kDa form back to the inactivated 23.5 kDa species [6,7] (fig.1). A cDNA for apoaequorin has recently been cloned and sequenced [8–11]. However, the structure and function of the aequorin emission site remain unclear. Moreover, previous studies have not identified monoclonal antibodies against aequorin, particularly against epitopes involved in the function of this photoprotein. In this presentation, we demonstrate the production of MAbs against the active and inactive configurations of aequorin.

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2. MATERIALS AND METHODS

2.1. *Animals and chemicals*

New Zealand Black-White F1 female mice aged 5 weeks were used for immunization (CLEA-Japan, Inc. Tokyo). Highly purified natural aequorin was obtained from Dr J.R. Blinks (Mayo Foundation, Rochester, MN) [12], and coelenterazine, 2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo-(1,2-alpyrazin-3-one), was synthesized by previously described techniques [13].

2.2. *Preparation of monoclonal antibodies*

The mice were immunized with purified aequorin. Spleen cells from immunized mice were fused with mouse myeloma cells, NS1/1.Ag4.1 [14]. Myeloma cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 15% horse serum (Pel-Freeze Nippon Bio-supply, Tokyo), 2 mM L-glutamine (WAKO pure chemicals, Tokyo), 1 mM sodium

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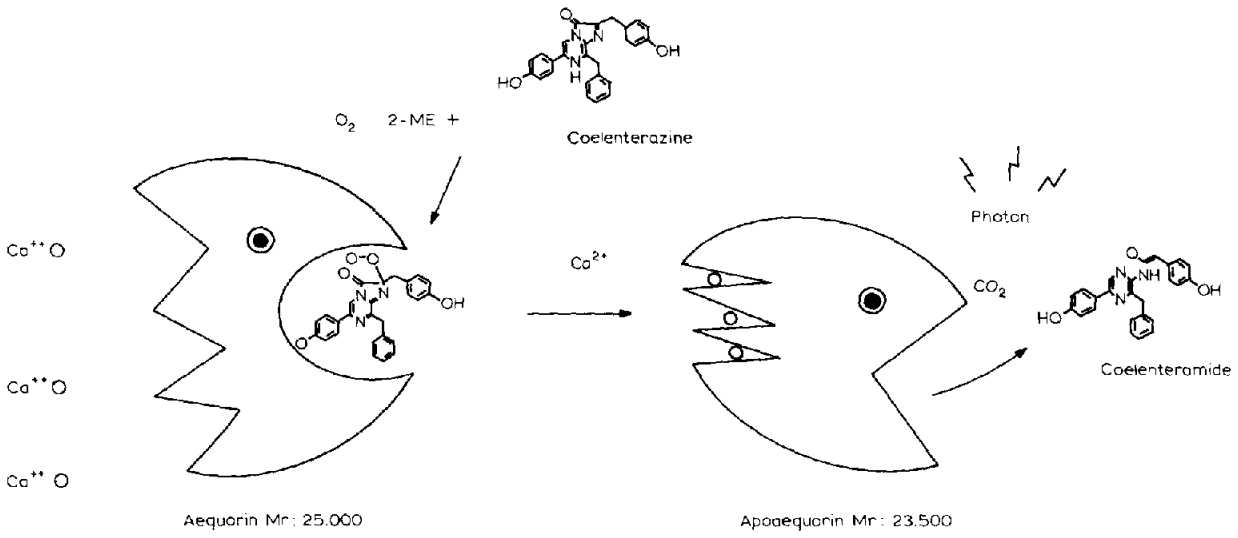

Fig.1. A model of the active and inactive configurations of an aequorin molecule.

pyruvate (Sigma) and 10 mM Hepes (Sigma). Polyethylene glycol (50%; 1540 Da from BDH chemicals Poole, England) was used for the fusion.

The fused cells were distributed into 96-well tissue culture plates (Costar, Cambridge, MA). HAT (100 μl; 0.1 mM hypoxanthine, 0.016 mM thymidine and 0.4 μM aminopterin) selection was started 24 h later. On days 2, 3, 5, 8, 11 and 14, 100 μl medium was removed from each well prior to adding 100 μl fresh HAT selection medium. Hybridoma growth was detectable by day 5 and colonies by day 10.

2.3. *Screening of monoclonal antibodies*

The hybridomas were screened by an enzyme linked immunosorbent assay (ELISA) [15]. Micro-test III flexible assay plates (Becton Dickinson, Oxnard, CA) were coated with aequorin in a 0.05 M bicarbonate-carbonate buffer (pH 9.5) by incubation overnight at 4°C. The plates were washed 7 times with 0.01 M PBS (pH 7.6) containing 0.05% Tween-20 (PBS-Tween) and blocked with 20% horse serum in PBS. Each well was washed 3 times with PBS-Tween and incubated overnight at 4°C with hybridoma supernatant diluted 1:5 in PBS. The plates were washed, reacted with 0.5 μg per ml peroxidase labeled anti-mouse IgG for 2 h at room temperature and then washed again 7 times. Enzyme substrate, 0.5 mg per ml of *o*-phenylenediamine (Sigma) in McIlvain's buffer, pH 5.5, containing 0.005% peroxide, was added to the wells and reacted for 30 min at room temperature. The reactions were stopped by adding 0.4 M sulfuric acid and the wells were then monitored for absorbance at 492 nm on a Microreader (Corona, Ibaragi, Japan).

2.4. *Immunoblot analysis*

ELISA positive wells were further selected by immunoblot analysis. Aequorin was electrophoresed in a preparative SDS/12% polyacrylamide slab gel [16] and transferred electrophoretically to nitrocellulose sheets with a Atto transfer apparatus at 70 V for 2 h [17]. The nitrocellulose sheets were blocked with 20% horse serum in 0.05 M Tris-HCl buffer (TBS) for 1 h, and then incubated with hybridoma supernatants overnight at 4°C. After washing 3 times in TBS on a shaker, the sheets were incubated with peroxidase labeled anti-mouse IgG for 90 min at room temperature and then washed again. Specifically bound antibodies were visualized by incubation with 4-chloro-1-naphthol (Bio-Rad, CA) in methanol with McIlvain's buffer (pH 5.5) and 0.01% peroxide.

2.5. *Purification of monoclonal antibody*

Antibody was purified from mouse ascites [16]. Ascites was produced by pristane (tetramethylpentadecane, Aldrich, Milwaukee, WI)-primed mice in which 1×10^7 hybridoma cells were injected intraperitoneally. The IgG fraction of the ascites was purified by precipitation with 33% ammonium sulfate followed by Sephacryl S-300 chromatography.

2.6. *Regeneration of aequorin activity*

Regeneration of aequorin activity was carried out by incubation with coelenterazine in 30 mM TBS (pH 7.6), 0.01 M EDTA and 2ME for 2 h at 4°C. Luminescence activity of regenerated aequorin was determined as described by Blinks et al. [12].

3. RESULTS

The hybridomas were screened by ELISA for reactivity with aequorin. Forty of 792 clones were selected and studied further by Western blot analysis. Two hybridomas, designated Aq-11 and Aq-12, produced high-affinity IgG_1 antibodies that reacted with purified aequorin. The reactivity of these MAbs with native aequorin is shown in fig.2. The binding of MAb Aq-11 (5 μg/ml) was approx. 10-fold greater than that of MAb Aq-12 (8 μg/ml)

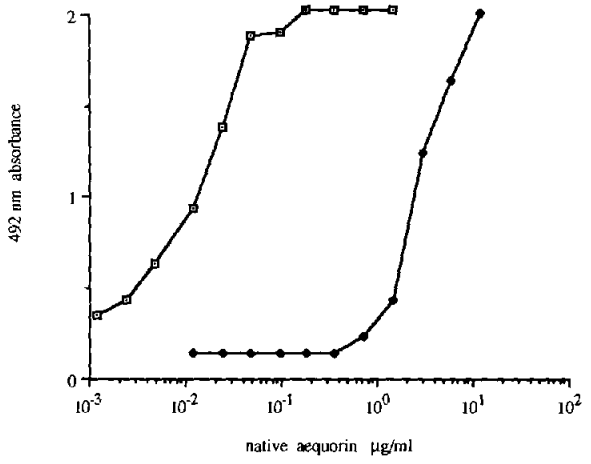

Fig.2. Reactivity to natural aequorin of Aq-11 and Aq-12 by ELISA. The reactivity of MAbs Aq-11 (—□—) and Aq-12 (—●—) to serial 2-fold dilutions of native aequorin.

against the same aequorin preparation (fig.2). Furthermore, MAb Aq-11 was found to react with recombinant apoaequorin, while there was no detectable reactivity of MAb Aq-12 with this protein (fig.3). These findings indicated that MAbs Aq-11 and Aq-12 recognize distinct epitopes on the aequorin molecule. The electrophoretic mobility of aequorin in a SDS-polyacrylamide gel is shown in fig.4A. Coomassie blue staining revealed two species with apparent molecular masses of 25 and

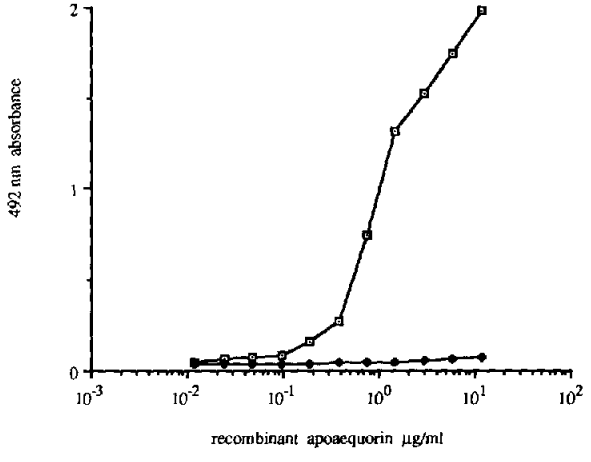

Fig.3. Reactivity of MAbs Aq-11 and Aq-12 with recombinant apoaequorin by ELISA. The reactivity of MAbs Aq-11 (—□—) and Aq-12 (—■—) to serial 2-fold dilutions of recombinant apoaequorin was determined by ELISA.

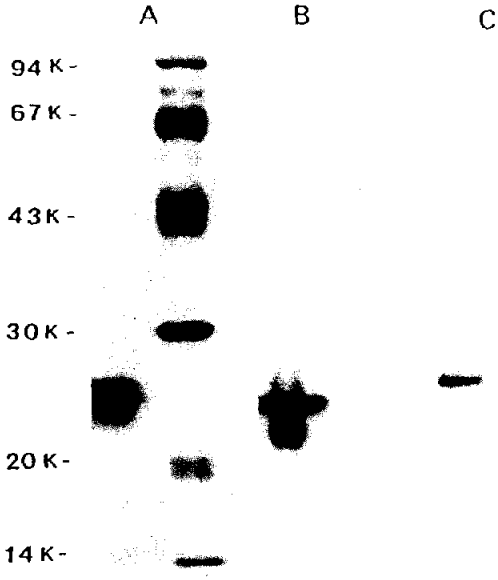

Fig.4. Reactivity of MAbs Aq-11 and Aq-12 with natural aequorin. Natural aequorin was subjected to electrophoresis in a SDS/12% polyacrylamide gel, transferred to nitrocellulose paper and reacted with MAb Aq-11 (lane B) or MAb Aq-12 (lane C). Molecular mass markers (lane A) and natural aequorin (lane A) were stained with Coomassie blue after transfer.

23.5 kDa. Of interest, MAb Aq-11 reacted only with the 23.5 kDa species (fig.4B). In contrast, MAb Aq-12 had specific reactivity with the 25 kDa aequorin molecule. The presence of relatively greater amounts of the 23.5 kDa species was in agreement with the demonstration in fig.5 that MAb Aq-11 was more reactive than MAb Aq-12 with this aequorin preparation. The electrophoretic mobility of native aequorin and calcium-inactivated aequorin is shown in fig.5A. Calcium treatment was associated with an increase in electrophoretic mobility of the apparent 25 kDa species (fig.5A). Furthermore, binding of MAb Aq-11 with the active and inactive forms of aequorin demonstrated the presence of the 23.5 kDa species in both preparations (fig.5B). In contrast, binding of MAb Aq-12 to the 25 kDa protein was detectable with the active, but not the calcium-inactivated aequorin (fig.5C). These findings suggested that MAb Aq-12 reacts specifically with the active 25 kDa species, while MAb Aq-11 detects only the inactive 23.5 kDa form. Indeed, there was increased reactivity with MAb Aq-12 and de-

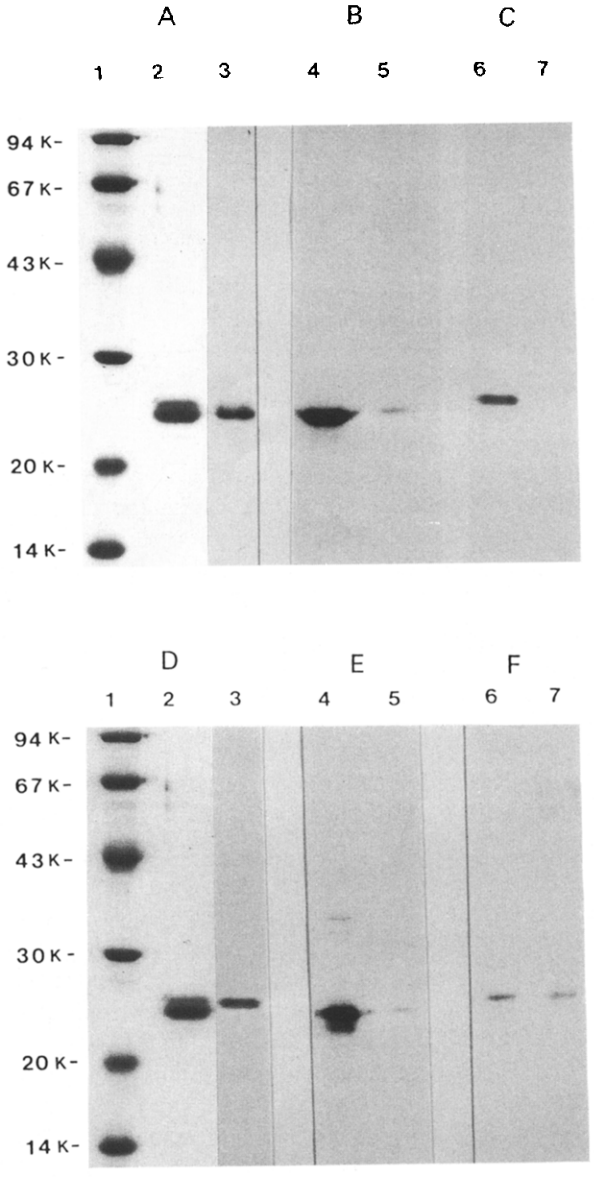

Fig.5. Reactivity of MAbs Aq-11 and Aq-12 with the active and inactive forms of aequorin. Natural aequorin (lanes A2, B4, C6, D2, E4, F6), calcium-inactivated aequorin (A3, B5, C7) and coelenterazine-activated aequorin (D3, E5, F7) were subjected to electrophoresis in a SDS/12% polyacrylamide gel, transferred to nitrocellulose paper and stained with Coomassie blue (A and D), reacted with MAb Aq-11 (B and E) or reacted with MAb Aq-12 (C and F). Lanes A1 and D1 represent molecular mass markers stained with Coomassie blue.

creased binding of MAb Aq-11 when the aequorin preparation was activated with coelenterazine (fig.5D–F). Taken together, these results demonstrate the selectivity of these MAbs for the active and inactive conformations of aequorin.

4. DISCUSSION

Electrophoresis of native aequorin revealed two species with apparent molecular masses at 25 and 23.5 kDa. MAb Aq-12 was found to react with the 25 kDa molecule, while MAb Aq-11 was specifically reactive with the 23.5 kDa protein. Calcium induced a shift in electrophoretic mobility of the 25 kDa species to the 23.5 kDa form. In contrast, regeneration with coelenterazine shifted the 23.5 kDa inactive form to the active 25 kDa conformation. Purified recombinant apoaequorin was prepared from *E. coli* expressing a plasmid, the apoaequorin cDNA (Inouye, in preparation). The electrophoretic mobility of apoaequorin revealed an apparent molecular mass at approx. 23.5 kDa. MAb Aq-11 reacted with the recombinant apoaequorin and the 23.5 kDa species of native aequorin. In contrast, MAb Aq-12 had no detectable reactivity with apoaequorin. Furthermore, while MAb Aq-11 reacted with the inactive form of aequorin, MAb Aq-12 had no detectable reactivity after calcium inactivation of native aequorin. These findings suggest that the apparent molecular mass of aequorin shifts with a change in configuration between the active and inactive forms. Furthermore, the results suggest that the 25 kDa species of aequorin represents the active form, while the 23.5 kDa species is the inactive form. Finally, the present findings represent the first demonstration of the identification of activated inactive configuration of a protein by specific monoclonal antibodies.

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