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The excimer fluorescence of *N*-(1-pyrenyl)iodoacetamide labeled to myosin and its subfragment 1

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Myosin and its subfragment 1 were labeled with the fluorescent probe *N*-(1-pyrenyl)iodoacetamide. Both of the labeled complexes exhibited the excimer band at 480 nm (pH 8.0, 25°C). SH_1 and SH_2 are labeled with this probe as judged by Ca^{2+}-ATPase of the labeled complex. Excimers arise both from the interaction of PIAAs in the two different heads within a single myosin molecule and also from the interaction of PIAAs in the same head. ATP affects these excimers depending on the concentration of Ca^{2+}.

N-*(1-Pyrenyl)iodoacetamide* *Excimer* *Myosin* *Subfragment 1* *ATP*

1. INTRODUCTION

Rabbit skeletal myosin is composed of two heads and each head (or myosin subfragment 1) has two reactive thiols, SH_1 and SH_2, of which chemical modifications are reviewed in [1]. In this study *N*-(1-pyrenyl)iodoacetamide was introduced to label these thiols. The pyrene moiety is well-known to produce excimer fluorescence at 480 nm [2]. Excimer is formed through the interaction of excited and unexcited probes [2], and this property is useful in investigating the proximity of labeled residues as well as the interaction between the labeled regions. As yet, there is no report in which the pyrene moiety has been shown to be labeled to SH groups of myosin and S1. We show here that such modification leads to excimer formation of PIAA and report on its characteristics.

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Abbreviations: S1, chymotryptic subfragment 1 of myosin; PIAA, *N*-(1-pyrenyl)iodoacetamide; DTT, dithiothreitol; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride

2. MATERIALS AND METHODS

Myosin was prepared from rabbit back and leg muscles as described by Kielley and Bradley [3]. S1 was prepared by digestion of myosin with chymotrypsin according to Weeds and Pope [4], and fractionated on AcA 34 Ultrogel column (LKB Co.). ATPase activity was measured by the method of Taussky and Shorr [5]. Protein concentrations were determined spectroscopically using the following values respectively, for myosin and S1: $A^{1\%}_{280} = 5.5\ cm^{-1}$ [6], $A^{1\%}_{280} = 7.5\ cm^{-1}$ [7]. Concentrations of labeled proteins were measured by the biuret method [8], and the amount of bound PIAA was determined from absorption at 344 nm using $A_{344} = 2.2 \times 10^4\ M^{-1}\cdot cm^{-1}$ [9]. PIAA was obtained from Molecular Probe. Myosin (15 μM) was labeled with PIAA at various ratios in 0.5 M KCl, 50 mM Tris-maleate (pH 7.0) at 0°C overnight. S1 (78 μM) was labeled with PIAA in a molar ratio of 20, in 50 mM Tris-HCl (pH 8.0) for 1 h. The labeling was terminated by adding 2-mercaptoethanol to 0.1 M and the unreacted dye was removed by exhaustive dialysis.

Fluorescence was measured with Hitachi MPF-2A spectrofluorimeter at 24.5°C. A cuvette

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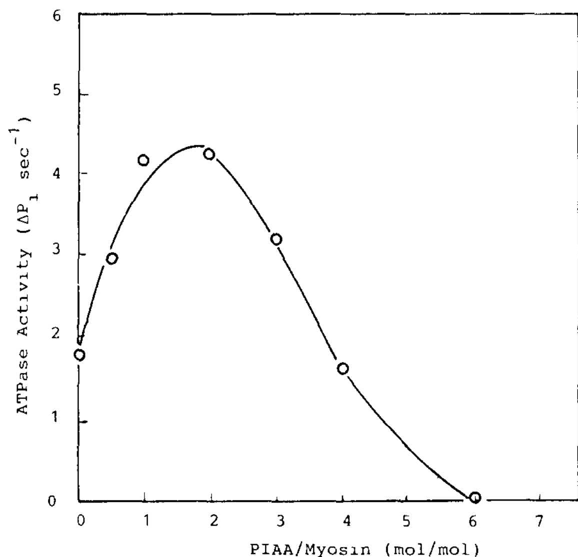

Fig.1. Ca^{2+}-ATPase of myosin as a function of the molar ratio of bound PIAA at 0.72 μM myosin in 1 mM ATP, 5 mM $CaCl_2$, 0.05 M KCl and 50 mM Tris-HCl (pH 8.0).

of size 4 × 4 mm was used to reduce the inner filtering effect at high concentration of a sample.

3. RESULTS

Fig.1 shows the Ca^{2+}-ATPase of the labeled myosin as a function of the amount of bound PIAA. The ATPase activity first increased with the labeling ratio and then turned to decrease at 2 PIAA/myosin, and became equal to the normal value at 4 PIAA/myosin. Concomitantly K-EDTA-ATPase was inhibited (not shown). According to previous reports [3,10], these results indicate the labeling of PIAA at the so-called SH_1 and SH_2 in myosin head.

Fig.2a shows the emission spectra of PIAA labeled to S1 (labeling ratio = 2.5 PIAA/S1). The spectra had three peaks at 386, 405 and 480 nm. The former two peaks correspond to the emission of the pyrene monomer and the last one to that of the pyrene excimer [11]. The ratio of excimer fluorescence intensity (I_E at 480 nm) to monomer fluorescence intensity (I_M at 405 nm) of S1 depends on the protein concentration. The concentration dependence of S1-excimer suggests complex formation of S1 (intermolecular excimer). We

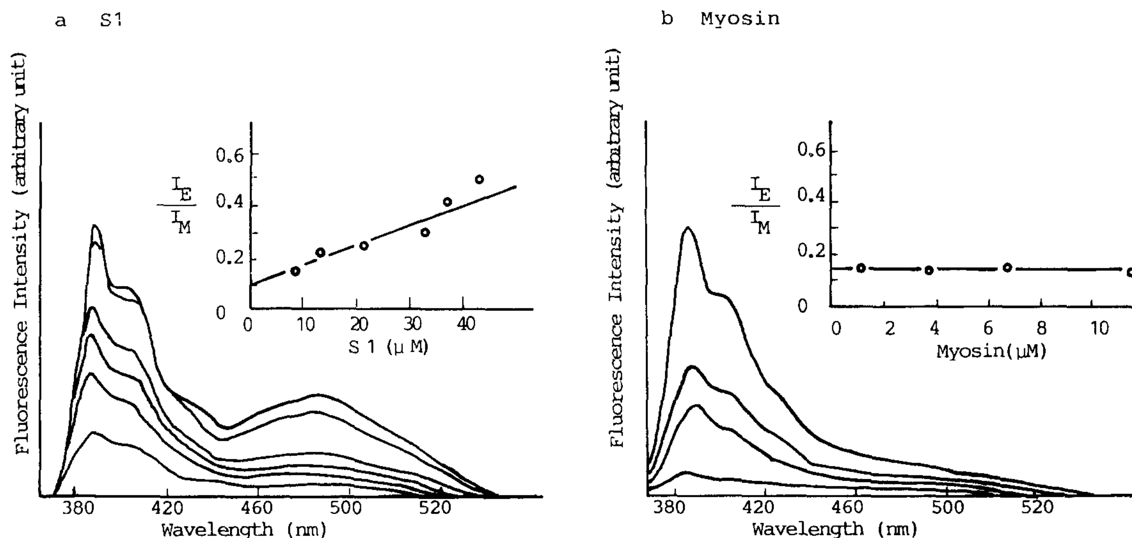

Fig.2. The fluorescence emission spectra of PIAA in myosin and S1 after excitation at 344 nm. (a) S1 in 10 mM $K\text{-}PO_4$ (pH 6.8), 1 mM DTT; the labeling ratio = 2.5 PIAA/S1. S1 curves (from the top to the bottom): 43, 37, 33, 22, 13 and 8.7 μM. Inset plot: I_E/I_M to S1. (b) Myosin in 0.5 M KCl, 50 mM Tris-HCl (pH 8.0); the labeling ratio = 3.5 PIAA/myosin. Myosin curves (from the top to the bottom): 11.5, 6.9, 3.8 and 1.3 μM. Inset plot: I_E/I_M to myosin.

note that the presence of intramolecular excimer in S1 is also suggested from the intercept of the inset plot of fig.2a which does not cross the origin.

On the contrary, the ratio I_E/I_M of myosin (labeling ratio = 3.5 PIAA/myosin) does not show any concentration dependence (fig.2b). However, the presence of head-head interaction giving rise to interhead excimer is evident in the digestion experiment of the labeled myosin (table 1); the excimer of PIAA in myosin (labeling ratio = 1 PIAA/myosin) decreased to 1/3 after chymotryptic digestion to give singly labeled S1.

Table 1

Comparison of the excimer of PIAA in myosin (labeling ratio of 1 PIAA/myosin) with the excimer of PIAA in S1

	I_E/I_M
Myosin	0.147
S1	0.049

S1 was obtained by chymotryptic digestion of myosin labeled at a ratio of 1 PIAA/myosin. 12.8 μM myosin in 0.5 M KCl, 50 mM Tris-HCl (pH 8.0) and 0.72 μM S1 in 50 mM Tris-HCl (pH 8.0)

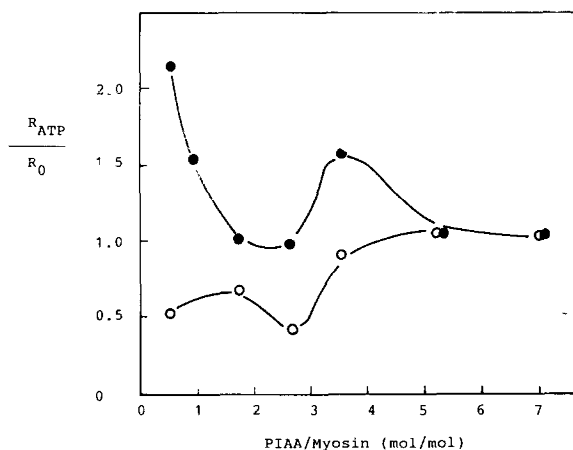

Fig.3. The effect of ATP on I_E/I_M in the presence and absence of Ca^{2+} at 0.72 μM myosin. The ratio of R_{ATP}/R_0 was plotted against the amount of labeled PIAA, here R_{ATP} denotes I_E/I_M in the presence of 1 mM ATP and R_0 denotes I_E/I_M in the absence of ATP. (○) In the presence of 5 mM $CaCl_2$, 50 mM KCl and 50 mM Tris-HCl (pH 8.0); (●) in the presence of 5 mM EDTA, 0.5 M KCl and 50 mM Tris-HCl (pH 8.0). The fluorescences were measured immediately after the addition of ATP.

It has already been suggested that ATP binding to the active site of myosin is accompanied by a conformational change in myosin [12,13]. We therefore investigated the effect of ATP binding on the excimer formation of the labeled myosin. Fig.3 shows that at a labeling ratio of less than 4 PIAA/myosin, where most of SH_1 and SH_2 are labeled with PIAA, the excimer formation is affected by ATP depending on the concentration of Ca^{2+}. In the presence of Ca^{2+} (5 mM), the excimer formation is suppressed by addition of ATP, while it is enhanced by ATP if Ca^{2+} is removed from the solution.

4. DISCUSSION

The most important finding of the present study is the excimer formation of PIAA labeled to myosin head (mostly at SH_1 and SH_2). At a labeling ratio lower than 2 PIAA/myosin, where the labeling occurs most probably at SH_1 [1], excimer formation occurs between two heads of myosin. As the labeling ratio is increased, SH_2 is labeled and intrahead excimer formation will occur. The present study showed that binding of ATP to the active site of myosin affects the excimer formation in a characteristic way depending on the concentration of Ca^{2+}, suggesting the change in head-head interaction of myosin as well as intrahead conformation change.

These structural changes may be important in the active interaction of myosin-ATP complex with actin filament.

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