COMPARISON OF THE EQUILIBRIUM BINDING OF HEAVY MEROMYOSIN AND MYOSIN TO F-ACTIN IN THE PRESENCE AND ABSENCE OF THE TROPONIN–TROPOMYOSIN COMPLEX

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Received 25 November 1981; revision received 2 February 1982

1. Introduction

The interaction of myosin cross-bridges with actin filaments and ATP provides the driving force for muscle contraction. A large amount of quantitative biochemical information has been obtained about this interaction, principally from studies using the proteolytic fragments of myosin, the double-headed fragment, heavy meromyosin (HMM), and the single-headed fragment, subfragment-one (S-1). The great usefulness of these proteolytic fragments is that they are soluble at low ionic strength. However, extrapolation of the results obtained with these proteolytic fragments to the properties of myosin itself has always been a major assumption of the work done with HMM and S-1; i.e., it has been assumed that the light meromyosin (LMM) portion of the myosin molecule is only involved in aggregation of the myosin molecule into myosin filaments, while having no effect on the actin-binding site.

To test this assumption, the equilibrium binding of myosin and HMM to actin was compared both in the presence and absence of ADP and with and without troponin–tropomyosin on the actin. The results show that both myosin and HMM have essentially the same binding constant to actin and the same cooperative response in binding to actin in the presence of troponin–tropomyosin. The differences observed between the ATPase activity of actomyosin and acto-HMM may be due to filament formation, rather than to proteolysis or to an effect of the LMM portion of the myosin molecule on the active site. A similar conclusion was reached from steady-state kinetic experiments using myosin minifilaments [1].

2. Materials and methods

Rabbit skeletal myosin, tryptic HMM, F-actin and troponin–tropomyosin complex was made as in [2,3]. The myosin was modified with iodo[^{14}C]acetamide, 1 ± 0.1 mol label/head [4]; myosin and HMM were clarified (2 h at 100 000 × *g* prior to use). The troponin–tropomyosin–actin complex (regulated actin) was prepared by mixing troponin–tropomyosin and actin in a 2:7 ratio [3].

The binding studies were done using a preparative centrifuge as in [2], whereas the competition studies were done using an air-driven centrifuge (fig.2). In these experiments, >90% of the myosin and >97% of the HMM remained in supernatant after centrifugation in the absence of actin, while <10% of the myosin or HMM remained in the supernatant after centrifugation in the presence of actin and the absence of nucleotide.

ADP and diadenosine pentaphosphate were from PL Biochemicals and iodo[^{14}C]acetamide was from Amersham.

3. Results and discussion

The binding of myosin and HMM to actin was first examined in the presence of 3 mM ADP at high ionic strength (~0.55 M), pH 8.0, conditions which favor monomeric myosin in solution [5]. By using myosin or HMM which had its SH_1 groups blocked with iodo-[^{14}C]acetamide, the binding could be easily measured by sedimenting the myosin (or HMM) bound to actin and measuring the radioactivity in the supernatant. The data from these binding studies, plotted on a

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00145793/82/0000–0000/$02.75

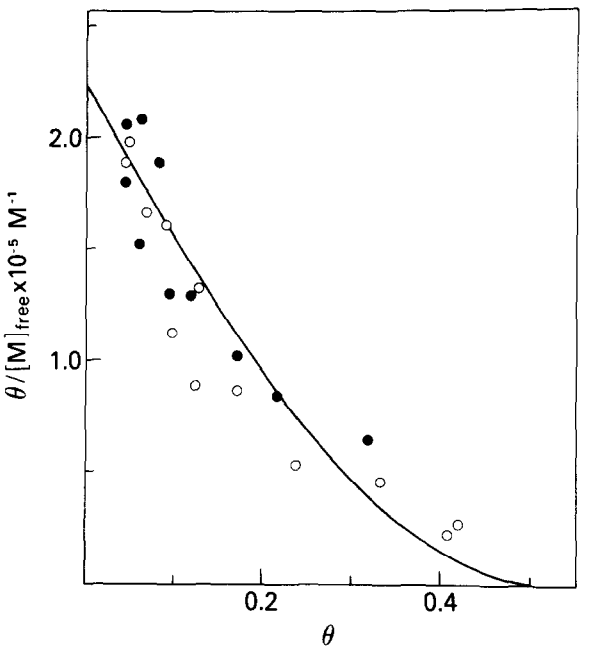

Fig.1. The binding of myosin and HMM to actin in the presence of ADP. Conditions were 0.5 M KCl, 20 mM Tris, 5.0 mM $MgCl_2$, 3 mM ADP, 5 mM KP_i, 1 mM EDTA, 0.5 mM dithiothreitol and 20 μM diadenosine pentaphosphate at pH 8.0, 25°C. The binding experiment was done using 8.4 μM actin and either 0.6–10.0 μM SH_1-blocked myosin (•) or 0.6–21.0 μM SH_1-blocked HMM (○). θ is the number of moles of HMM or myosin bound per mole of F-actin monomer. M_{free} is the concentration of unbound HMM or myosin. The solid line drawn through the data is for a binding constant equal to 2.2×10^5 M^{-1}, analyzed by assuming each molecule always occupies 2 actin sites (no one-headed binding).

Scatchard plot in fig.1, clearly show that myosin (• •) and HMM (○ ○) are not significantly different in their ability to bind actin. These data cannot be analyzed using the Scatchard equation because of the parking problem that arises because HMM and myosin bind to actin with 2 heads. Instead, equations derived in [6] were used to determine that the association constant for the binding of myosin and HMM to actin was 2.2×10^5 M^{-1} under these conditions (fig.1, ——). Therefore, at high ionic strength and in the presence of ADP, myosin and HMM bind very similarly to actin.

The binding of HMM and myosin to actin were next compared in the absence of ADP. These are conditions where both heads of HMM and myosin are expected to bind strongly of F-actin, whereas, in the presence of ADP, the second head of HMM (or myosin) does not make a strong contribution to the free energy of HMM binding to actin [2]. This binding study was conducted by having HMM and myosin compete for sites on F-actin. The competition method was used because, in the absence of nucleotide, myosin and HMM bind rather strongly to actin ($K > 10^6$ M^{-1}), making it difficult to determine directly the

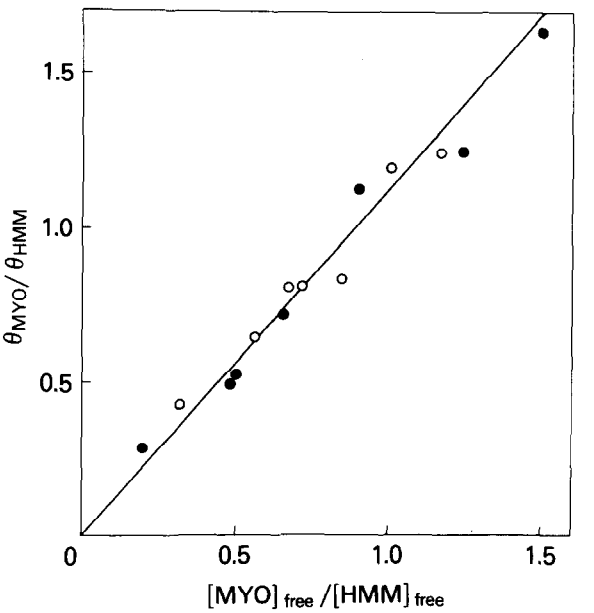

Fig.2. Competition between HMM and myosin for sites on F-actin. Conditions were: 0.5 M KCl, 20 mM Tris, 5 mM KP_i, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol at pH 8.0, 25°C. In the competition experiment between SH_1-blocked HMM and unmodified myosin (○), the conditions were 4.0–5.8 μM SH_1-blocked HMM, 2.6–7.8 μM myosin and 12 μM actin. In the competition experiment between unmodified HMM and SH_1-blocked myosin (•), the conditions were: 6.6–7.0 μM HMM, 1.5–7.5 μM SH_1-blocked myosin and 12 μM actin. θ_{HMM} and θ_{MYO} are the moles of HMM and myosin, respectively, bound per mole of F-actin monomer. $[HMM]_{free}$ and $[MYO]_{free}$ are the concentrations of unbound HMM and myosin. The line for each set of data was determined by computing for each data point a line intersecting the origin and the mean (and standard deviation) of all these lines was then determined. In these experiments, $\theta_{MYO} + \theta_{HMM} = 0.51 \pm 0.06$, indicating 1 head bound/actin monomer. All data points were corrected for the effect of SH_1 modification by iodoacetamide which caused a 25% reduction in the ability of HMM and myosin to bind to actin. The order of addition of the proteins had no effect on the results. After mixing the actin–HMM–myosin solution (2 ml) by stirring gently for several minutes, the proteins were centrifuged 30 min later in a Beckman Airfuge (1 h, 178 000 × g) at room temperature. After centrifugation, the supernatant was carefully removed and both the absorbance at 280 nm and the radioactivity were measured. From these measurements, both the concentration of unbound HMM and myosin were determined.

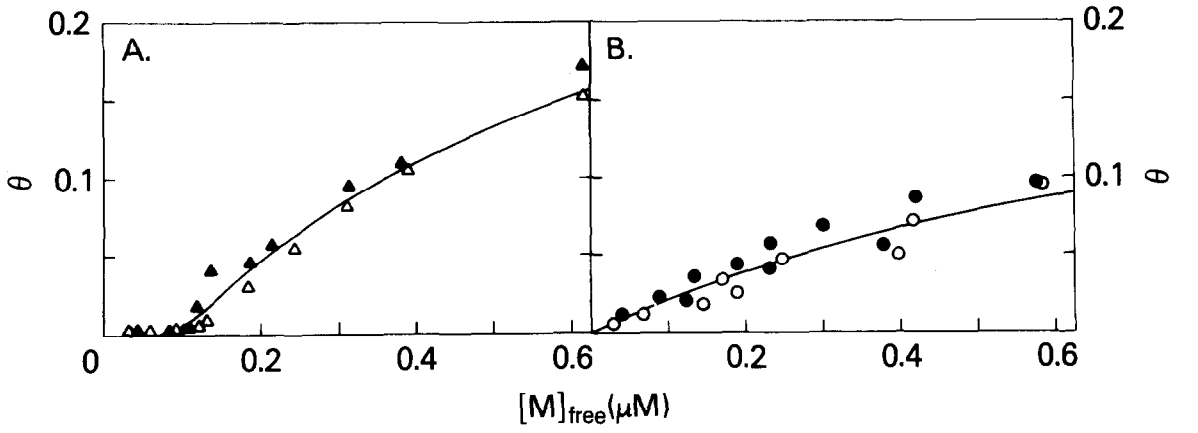

Fig.3. The binding of HMM and myosin to regulated and unregulated actin in the absence of calcium and presence of ADP. Conditions were as in fig.1 except that 1 mM EGTA was added. (A) The binding experiment was performed using 8.4 μM regulated actin, and either 0.04–2.0 μM SH_1-blocked HMM (○) or 0.05–2.0 μM SH_1-blocked myosin (●). (B) The binding experiment was performed using 8.4 μM unregulated actin and either 0.06–1.4 μM SH_1-blocked HMM (○) or 0.14–1.4 μM SH_1-blocked myosin (●).

binding constant of these proteins to actin. In the competition experiments, either myosin or HMM had its SH_1 groups blocked with iodo[^{14}C]acetamide, thereby these proteins could be easily distinguished. The results obtained from the competition experiment between SH_1-blocked HMM and unmodified myosin (○ ○) and between unmodified HMM and SH_1-blocked myosin (● ●) are plotted in fig.2 according to:

$$\frac{\theta_{MYO} = K_{MYO}\,[MYO]_{free}}{\theta_{HMM} = K_{HMM}\,[HMM]_{free}}$$

This enables the ratio of the actin–myosin association constant (K_{MYO}) to the actin–HMM association constant (K_{HMM}) to be determined from the slope of the line fitting the data. The line through the data has a slope of 1.1 ± 0.1, showing that there is no significant difference between the binding constant of HMM and myosin to actin in the absence of nucleotide.

The binding of HMM and myosin to actin was next compared in the presence of the troponin–tropomyosin complex and the absence of Ca^{2+}. Equilibrium binding studies in the presence of the troponin–tropomyosin complex had been conducted using S-1 only [3,7,8]. As shown in fig.3, both myosin (▲▲) and HMM (△△) bind nearly identically to regulated actin. Furthermore, they both bind cooperatively to regulated actin, as shown by the sigmoidal shape of the plots. There is no indication of this cooperative binding occurring when these studies were conducted over the same concentration range of myosin or HMM with unregulated actin (fig.3B). Comparing the results obtained with regulated and unregulated actin (fig.3A,B) shows that the troponin–tropomyosin complex initially inhibits the binding of HMM and myosin to actin. With increasing concentrations of unbound HMM and myosin, this inhibition no longer occurs; rather, the troponin–tropomyosin complex strengthens the binding of both HMM and myosin to actin. A similar cooperative response has also been observed for the binding of S-1 to regulated actin. The troponin–tropomyosin causes about the same inhibition of the binding of HMM and myosin to actin; this inhibition may be important in the mechanism of regulation of muscle contraction.

HMM is an excellent model for myosin in binding studies; myosin and HMM not only bind similarly to actin both in the presence and absence of ADP, but in the presence of the troponin–tropomyosin complex. Therefore, it is now possible to determine how filaments affect the ability of myosin to bind to actin without concern that differences between HMM and myosin filaments are due either to proteolysis or to the effect of the LMM portion of the molecule on the actin binding site.

Acknowledgements

I would like to thank Dr Evan Eisenberg for many helpful discussions during the course of this work and Mr Lou Dobkin for assistance in preparing the proteins.

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