

# Binding of phosphorylated and dephosphorylated heavy meromyosin to F-actin

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The effect of myosin light chain phosphorylation in skeletal muscle was investigated with respect to the binding affinity of phosphorylated and dephosphorylated heavy meromyosin (HMM) for F-actin in the absence of ATP. For phosphorylated HMM the affinity was 2.5-times weaker in the presence of  $\text{Ca}^{2+}$  as in its absence (HMM divalent binding sites saturated only with Mg). For dephosphorylated HMM the reverse was true, the binding being 2.4-times higher in the presence of  $\text{Ca}^{2+}$ .

Meromyosin; Phosphorylation; Dephosphorylation; Actin binding; (Skeletal muscle)

## 1. INTRODUCTION

Since the discovery of myosin phosphorylation in skeletal muscle [1,2] many attempts have been made to demonstrate its effects at the level of the actin-activated ATPase activity of myosin. In contrast to smooth muscle (reviews [3-7]) however, this phosphorylation appears not to be essential for the ATPase activity [8,9] and only under certain conditions [10,11] or in fresh preparations [12] could pronounced effects be demonstrated. There are two reasons, however, that suggest that the phosphorylation of the myosin regulatory light chain must be of significance. Firstly, the enzyme responsible for this phosphorylation, myosin light chain kinase (MLCKase), must be active during stimulation, *in vivo*. Secondly, numerous *in vivo* studies in intact muscle, have demonstrated that the level of myosin phosphorylation increases from a relatively high resting level of 50% [13-19] to virtually 100% after tetanic stimulation [18,20,21].

Very recently we have shown some differences in the flexibility of F-actin complexed with either

phosphorylated or dephosphorylated heavy meromyosin (HMM). These differences were diametrically opposite in the presence and absence of  $\text{Ca}^{2+}$  [22-26]. Similarly, HMM displayed two different forms of arrowheads depending on whether its regulatory light chains were fully phosphorylated, or dephosphorylated [27]. Thus, it would appear that phosphorylation of skeletal muscle myosin may affect its binding to F-actin rather than its  $\text{Mg}^{2+}$ -ATPase activity. To test this conclusion we have undertaken the present study and report here supporting evidence showing that there are differences in the affinity of phosphorylated and dephosphorylated HMM for F-actin.

## 2. MATERIALS AND METHODS

Rabbit skeletal muscle myosin was extracted and purified, in the fully phosphorylated or dephosphorylated state, as described in [11]. HMM was made as before by chymotrypsin digestion [27]. F-Actin was prepared by the method of Spudich and Watt [28]. Protein concentrations were determined by the biuret method [29] or from the absorbance at 280 nm with extinction coefficients ( $E_{280}^{1\%}$ ) equal to 5.6, 6.49 and 11 for myosin, HMM and F-actin,

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respectively. The  $M_r$  values were taken as 480 000, 350 000 and 42 000 for myosin, HMM and actin, respectively.

The binding of HMM to F-actin in the presence (0.1 mM  $\text{CaCl}_2$ ) and absence (1 mM EGTA) of  $\text{Ca}^{2+}$  was always performed in parallel experiments. The binding medium contained 60 mM KCl, 2 mM  $\text{MgCl}_2$ , 0.3 mM DTT and 10 mM imidazole at pH 7.6. In the experiments successive dilutions of phosphorylated and dephosphorylated HMM were made and then added into airfuge tubes containing fixed amounts of F-actin. After mixing with a 100  $\mu\text{l}$  pipette and 15 min incubation at 25°C, the actin-HMM complexes were pelleted using a Beckman airfuge (top speed 15 min).

After taking 200  $\mu\text{l}$  of each of the supernatants for estimation of the free (unbound) HMM, the pellet-containing tubes were rinsed carefully with water. The pellets were then dissolved in 0.5 M KI solution during a gentle overnight mixing on a vortex-mixer in a cold room. When non-radioactive HMM was used its concentration in the supernatants and dissolved pellets was estimated by measuring the  $(\text{NH}_4)^+$ -EDTA ATPase activity as originally described by Greene and Eisenberg [30]. We established that for optimal conditions the ATPase measurements were 0.3 M  $\text{HN}_4\text{Cl}$ , 1 mM ATP (Na), 10 mM EDTA at pH 9.0, the EDTA serving as a pH buffer and divalent-cation-chelating reagent.

The radiolabelled phosphorylated HMM was obtained by phosphorylation of our dephosphorylated HMM in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , Ca and crude skeletal muscle myosin light chain kinase. The phosphorylation levels before and after the binding experiments were checked using urea-glycerol gel electrophoresis [31]. The dephosphorylated radioactive HMM was labelled by  $\text{Na}^{125}\text{I}$  in the presence of lactoperoxidase and 0.03%  $\text{H}_2\text{O}_2$  [32]. The excess radioactivity from both HMMs was removed by a gel-filtration step on an AcA 34 (LKB) column.

The data were plotted according to the Scatchard equation [33]:

$$\bar{v}/c = K_a(n - \bar{v})$$

where  $\bar{v}$  is the number of moles of HMM bound per mole of F-actin monomer,  $c$  is the concentration of free HMM,  $K_a$  is the association constant

and  $n$  is obtained by extrapolation of  $\bar{v}/c$  to infinite HMM concentration. The  $K_a$  values were obtained from the slope of the plots.

### 3. RESULTS AND DISCUSSION

Here, two experimental procedures were used for the evaluation of HMM concentration. As expected, the direct procedure, involving radiolabelling of phosphorylated HMM with  $^{32}\text{P}$  and dephosphorylated HMM with  $^{125}\text{I}$ , was more accurate than the more common measurement of  $(\text{NH}_4)^+$ -stimulated ATPase activity (also called EDTA-dependent). Nevertheless, the apparent association constants obtained using the latter method also showed similar differences for phosphorylated and dephosphorylated HMM (table 1). Only in the former case, however, was an accurate evaluation of the bound HMM possible by incorporating measurements from the redissolved pellets. The information 'contained' in such pellets is normally not used and the amount of bound HMM is estimated from the difference between the total and free ligand concentration [34,35].

As shown in table I and figs 1,2, the apparent association constant of skeletal muscle HMM for actin, in the presence of  $\text{Ca}^{2+}$ , decreases almost 4-fold following phosphorylation of its regulatory light chain. In the absence of  $\text{Ca}^{2+}$  the difference was considerably lower and, by contrast, phosphorylation resulted in an increase in the apparent affinity (table 1, fig.1). Similar counter effects on the interaction between HMM and myosin-free ghost fibers were demonstrated by polarized UV microscopy [25,26] and reflected in this case different configurations of phosphorylated and dephosphorylated HMM on actin in the presence and absence of  $\text{Ca}^{2+}$ .

These opposite changes in the affinity of HMM for F-actin imply that with  $\text{Ca}^{2+}$  present, there should be an increase in the steepness of activation of the ATPase for dephosphorylated actomyosin containing MLCKase and calmodulin. By analogy, the relaxation (e.g. dissociation of actomyosin), following  $\text{Ca}^{2+}$  removal, should be 'faster' provided that myosin is first dephosphorylated by myosin light chain phosphatase (MLCKase). From the published data [36,37] it is clear that during activation in vivo only the high-affinity and  $\text{Ca}^{2+}$ -specific site on myosin is occupied and that the same site

Table 1

Rigor-type apparent association constants<sup>a</sup> for the binding of phosphorylated and dephosphorylated HMM to F-actin in the presence and absence of  $\text{Ca}^{2+}$

HMM	Phosphorylated		Dephosphorylated	
	Ca	EGTA	Ca	EGTA
<sup>32</sup> P or <sup>125</sup> I labelled	0.6 ± 0.15	1.5 ± 0.12	2.2 ± 0.2	0.9 ± 0.15
Unlabelled <sup>b</sup>	0.5	1.2	2.2	0.8

<sup>a</sup> Units:  $10^6 \text{ M}^{-1}$

<sup>b</sup> Values obtained from measurements of  $(\text{NH}_4^+)$ -stimulated ATPase activity (see text)

is probably occupied by  $\text{Mg}^{2+}$  during relaxation. Our experimental conditions may be considered to approximate these two physiological situations.

The values we obtained for the apparent association constant of HMM for actin are approximately the same as those reported in other indirect studies in which analytical ultracentrifugation [34] or fluorescence depolarization [35] was used. From experiments in which radiolabelled myosin subfragment 1 (SF1) competed with unlabelled HMM for the binding sites on actin, Greene et al. [38] concluded that HMM binds to actin about 1000-fold stronger, with an association constant of about  $10^9 \text{ M}^{-1}$ . Since our association constants are

approx. 1000-fold lower we conclude, on the contrary, that HMM and SF1 bind to actin with similar affinities.

It has to be emphasised, however, that our binding experiments were performed in the absence of ATP. Therefore, they relate to a physiologically less relevant situation in which the binding of myosin heads to F-actin is of very high affinity (also called rigor-type binding). Nevertheless, the significant effects of phosphorylation, reported here, indicate the level at which modulation by phosphorylation may be operating. Clearly, with ATP present, the affinity of myosin for actin will be dramatically reduced and the changes in the affinity may correspondingly increase. Even more pronounced effects might then be expected if

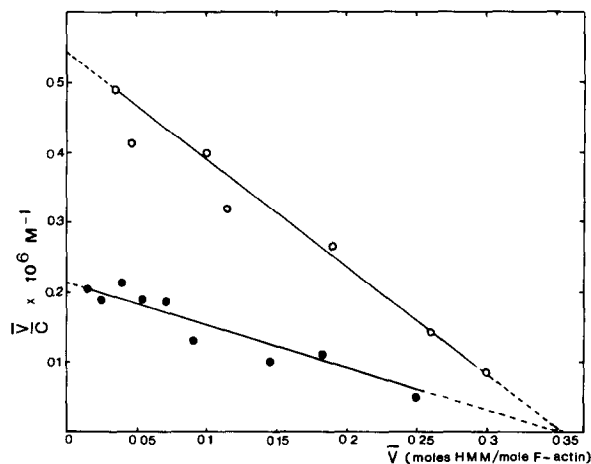


Fig. 1. Binding of phosphorylated HMM to F-actin in the presence (●—●) and absence (○—○) of  $\text{Ca}^{2+}$ . Rabbit skeletal muscle preparations were used. Actin concentration was  $15.5 \mu\text{M}$  while that of HMM was varied from  $0.3$  to  $10.3 \mu\text{M}$ .

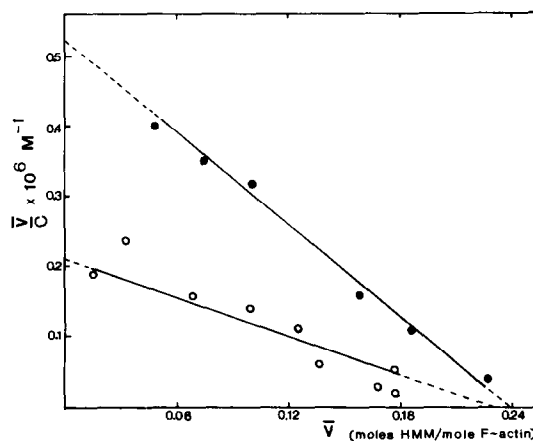


Fig. 2. Binding of dephosphorylated HMM to F-actin. All conditions were as in fig. 1 except that the HMM concentration range was from  $0.6$  to  $16.3 \mu\text{M}$ .

phosphorylation acts at a distinct step of the cross-bridge cycle, for example by affecting a particular conformation of the myosin head.

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