

# The actin-activated ATPase of regulated and unregulated scallop heavy meromyosin

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Received 20 December 1985

Single-turnover kinetic analysis indicates that scallop heavy meromyosin (HMM) preparations contain a small fraction of unregulated molecules which dominate the steady-state ATPase activity in the absence of  $\text{Ca}^{2+}$ . This fraction was removed by rapid centrifugation during an effective single turnover of the ATPase in the presence of actin. The maximum ATPase activity of a scallop myosin head was estimated as  $10 \text{ s}^{-1}$  by cross-linking subfragment 1 to F-actin. HMM became  $\text{Ca}^{2+}$ -insensitive during the cross-linking procedure. Attachment of spectroscopic probes to the reactive thiol group of scallop HMM resulted in the retention of ATPase activity but a loss in  $\text{Ca}^{2+}$  sensitivity.

*ATPase    Heavy meromyosin    Myosin-linked regulation     $\text{Ca}^{2+}$  activation*

## 1. INTRODUCTION

Contraction of molluscan muscle is controlled by the binding of  $\text{Ca}^{2+}$  to the regulatory domain of myosin [1,2]. In vitro this is reflected by the  $\text{Ca}^{2+}$  sensitivity of the myosin ATPase activity in both the presence and absence of actin [3,4]. Previously we proposed that such steady-state measurements give a gross under-estimate of the degree of  $\text{Ca}^{2+}$  sensitivity of the ATPase activity because the rate in the absence of  $\text{Ca}^{2+}$  is dominated by a small fraction of unregulated, permanently activated molecules [5,6]. This was deduced from a transient kinetic study of scallop heavy meromyosin (HMM). The first indication of the presence of a mixed population comprising unregulated and highly regulated molecules in HMM preparations came from a turbidity assay in the presence of actin [5]. Addition of near stoichiometric concentrations of ATP to acto-

HMM in the absence of  $\text{Ca}^{2+}$  caused a rapid drop in turbidity followed by a biphasic rise as the unregulated and regulated HMM rebound to form rigor complexes. Rapid ultracentrifugation of the incubation mixture at the end of the first phase should therefore pellet the acto-unregulated HMM selectively, leaving the regulated HMM with its bound nucleotide in the supernatant.

Measurement of the maximum actin-activated ATPase activity requires the use of very high actin concentrations or the cross-linking technique of Mornet et al. [7]. We adopted the latter approach in an attempt to evaluate the dynamic range of ATP turnover rates displayed by a highly regulated system.

The reactive thiol group of scallop myosin (probably homologous to  $\text{SH}_1$  of rabbit skeletal myosin) provides a convenient site on the heavy chain for the introduction of spectroscopic probes [8,9]. Previous steady-state measurements suggested that there was some loss in  $\text{Ca}^{2+}$  sensitivity of the ATPase on labelling but, due to the sub-stoichiometric incorporation, the severity of the effect could not be deduced unambiguously [8].

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## 2. MATERIALS AND METHODS

HMM and subfragment ( $S_1$ ) were prepared by the methods in [8] from *Pecten maximus* obtained from the Marine Biological Association, Plymouth, England. F-Actin was prepared from rabbit skeletal muscle [10]. Nucleotides, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-iodoacetyl-*N*-(1-sulpho-5-naphthyl)ethylenediamine (IAEDANS) were obtained from Sigma (Poole, England) and 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinoxyl (ISL) from Syva (Palo Alto, CA). Other reagents were obtained from BDH, Poole or Fisons, Loughborough, England.

Steady-state ATPase measurements were performed with a Radiometer RTS822 pH-stat in 20 mM NaCl, 1 mM  $MgCl_2$  at pH 7.5 and 20°C. Turbidity assays were carried out using a Pye Unicam SP8-100 spectrophotometer at 325 nm. In some cases the signal from the spectrophotometer was digitised using a Datalab DL902 transient recorder under external time-base control provided by an Apple II+ computer. Experiments were analysed using a non-linear, least-squares fitting procedure [11]. Rapid ultracentrifugation was carried out with a Beckman airfuge using the 30° rotor.

## 3. RESULTS

### 3.1. Separation of the regulated and unregulated HMM populations

Fig.1a shows a turbidity assay of scallop acto-HMM during a turnover of a limited molar excess of ATP. As described in [5], the profile can be accounted for by postulating the existence of two populations of HMM molecules: regulated and unregulated. The relative proportions of these populations was estimated in two ways. (i) From the amplitude,  $A_x$  of the turbidity change at time  $t_x$  (when only the unregulated molecules have reassociated with the actin) relative to the total turbidity change (fig.1). By this procedure the regulated fraction was calculated to be 82% of the preparation. (ii) From the relative steady-state ATPase rates in the presence and absence of  $Ca^{2+}$ . The ATPase of the regulated fraction in the absence of  $Ca^{2+}$  is so low that it barely contributes to ATP turnover during the steady state. If it is assumed that the ATPase activity of the un-

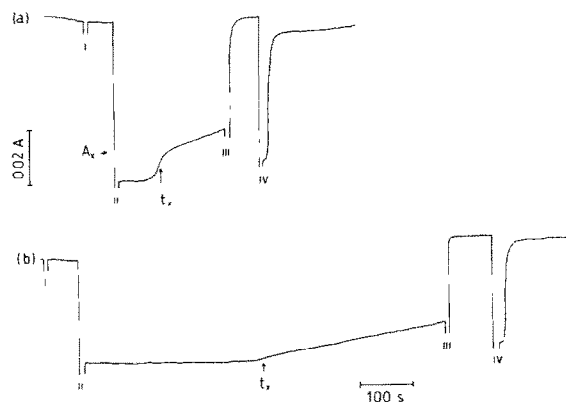


Fig.1. Enrichment of the regulated HMM ATPase population by rapid ultracentrifugation. (a) Turbidity assay of starting acto-HMM preparation by the method of Wells and Bagshaw [5]. 2  $\mu$ M acto-HMM (initial  $A_{325} = 0.08$ ) in 20 mM NaCl, 1 mM  $MgCl_2$ , 10 mM Tes at pH 7.5 and 20°C was treated with (i) 100  $\mu$ M EGTA (ii) 20  $\mu$ M ATP, (iii) 200  $\mu$ M  $Ca^{2+}$  and (iv) 20  $\mu$ M ATP. Turbidity was followed by the apparent absorbance at 325 nm in a 1 cm cell. Note the rapid recovery in the turbidity on adding  $Ca^{2+}$  to the preparation which was undergoing a slow ATP turnover in the presence of EGTA (iii). (b) Turbidity assay for the acto-HMM preparation enriched by ultracentrifugation. 200  $\mu$ l of 50  $\mu$ M acto-HMM, in the presence of 100  $\mu$ M EGTA, was treated with 500  $\mu$ M ATP and the mixture transferred rapidly to an airfuge rotor. The rotor was brought to 80000 rpm ( $90\,000 \times g$ ) within 60 s of adding the ATP and centrifugation continued for a further 3 min. The top 100  $\mu$ l of the supernatant was removed and diluted to 1 ml in the assay buffer, containing  $\sim 10 \mu$ M  $Ca^{2+}$ , and 2  $\mu$ M actin was added. The observed turbidity increase indicated that the supernatant contained about 1.3  $\mu$ M HMM and all the ATP from the centrifugation step had been hydrolysed. The turbidity profile was then recorded for additions as in (a), i.e. (i) 100  $\mu$ M EGTA, (ii) 20  $\mu$ M ATP, (iii) 200  $\mu$ M  $Ca^{2+}$  and (iv) 20  $\mu$ M ATP.

regulated fraction equals that of the  $Ca^{2+}$ -activated, regulated fraction, then the ratio of the observed rates in the presence and absence of  $Ca^{2+}$  provides a measure of the fraction of regulated molecules. The steady-state rates were estimated from the time taken to deplete the ATP as indicated by the rapid rise in turbidity. From the comparison of the depletion times observed in fig.1a(ii) vs (iv) (80 vs 16 s) the regulated fraction was calculated to be about 80% of the population.

If the acto-HMM present at time  $t_x$  in fig.1a could be rapidly removed, then the remaining

HMM preparation should comprise 100% regulated molecules. In practice, acto-HMM could be sedimented on a small scale within about 4 min using an airfuge. When this procedure was adopted about 65% of the initial HMM remained in the supernatant and this had a substantially increased proportion of regulated molecules (fig.1b). The amplitude of the transient recovery phase at time  $t_x$  was barely measurable and indicated that the fraction of regulated molecule was now  $> 96\%$ . From the relative ATP depletion times, the fraction of regulated molecules was estimated as 93–94%. Thus ultracentrifugation allows the physical separation of the two populations of HMM and substantiates the interpretation of our kinetic data.

### 3.2. Cross-linked acto-HMM

Scallop  $S_1$  and HMM were cross-linked to actin, under a number of conditions, both by the method of Sutoh [12] and by that of Rouayrenc et al. [13]. In all cases a considerable activation of the ATPase was noted as cross-linking occurred but the  $\text{Ca}^{2+}$  sensitivity of the HMM ATPase was lost. Prolonged incubation of the proteins with EDC resulted in over cross-linked material which failed to enter a 7–20% gradient gel and the ATPase activity decreased. There was no indication that the light chain subunits became cross-linked, even on prolonged incubation. In order to have sufficient time to monitor and optimise the cross-linking reaction, experiments were normally performed at  $0^\circ\text{C}$ .

Under these conditions the peak ATPase activity was reached after 6–12 h. Typical values for the maximum observed ATPase rates were about  $10 \text{ s}^{-1}$  with  $S_1$  and  $4\text{--}6 \text{ s}^{-1}$  with HMM (fig.2). It is not clear whether only one head of HMM can interact with actin on cross-linking, or whether HMM is more prone to over cross-linking (e.g. by the formation of head-head links). The loss in  $\text{Ca}^{2+}$  sensitivity appeared to occur concomitantly with the cross-linking reaction regardless of whether this was performed in the presence or absence of  $\text{Ca}^{2+}$ . The ATPase values for the cross-linked preparations are comparable to the  $V_{\text{max}}$  of the actin-activated ATPase estimated from assays performed with a related species in the absence of NaCl [14].

### 3.3 Effect of labelling the reactive thiol group

Incubation of scallop myosin with a 1–2-fold molar excess (over the head concentration) of an iodoacetamide-based spin label (ISL) or fluorescent label (IAEDANS) results in the selective labelling of the 24 kDa tryptic peptide region of subfragment 1 [8,9]. The ATPase activity of the labelled scallop myosin was still actin-activated, although there appeared to be some loss in  $\text{Ca}^{2+}$  sensitivity [8]. To explore this further, the effect of labelling scallop HMM was investigated using the turbidity assay. Fig.3 shows the effect of preincubation of HMM with IAEDANS, on the turnover of ATP in the presence of actin and absence of  $\text{Ca}^{2+}$ . The major effect of labelling was to alter the relative amplitudes of the fast and slow phases, without changing the rate of the slow phase significantly. Thus it appears that labelling the reactive thiol group rendered HMM completely  $\text{Ca}^{2+}$ -insensitive, causing a switch in the proportions of the regulated and unregulated popula-

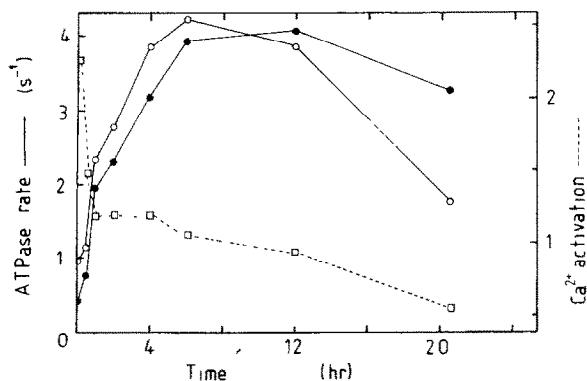


Fig.2. Effect of cross-linking time on steady-state ATPase rate of HMM. Actin was cross-linked to scallop HMM by the method of Sutoh [12]. 5 mM EDC was added at time zero, to a mixture of actin ( $4 \mu\text{M}$ ) and HMM ( $2 \mu\text{M}$  heads) in 20 mM NaCl, 1 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$   $\text{CaCl}_2$ , 10 mM Tes at pH 7.5 and  $0^\circ\text{C}$ . Aliquots were removed at the times indicated for steady-state ATPase assays using a pH-stat in the presence of 100  $\mu\text{M}$  EGTA (●) or 100  $\mu\text{M}$  excess  $\text{Ca}^{2+}$  (○). The actin concentration was maintained at  $4 \mu\text{M}$  in all assays, while the amount of the cross-linked preparation was varied to give a measurable rate. The  $\text{Ca}^{2+}$  activation (□) is expressed as the ratio of the ATPase rate in the presence of  $\text{Ca}^{2+}$  to that in its absence, and falls below 1 on prolonged cross-linking.

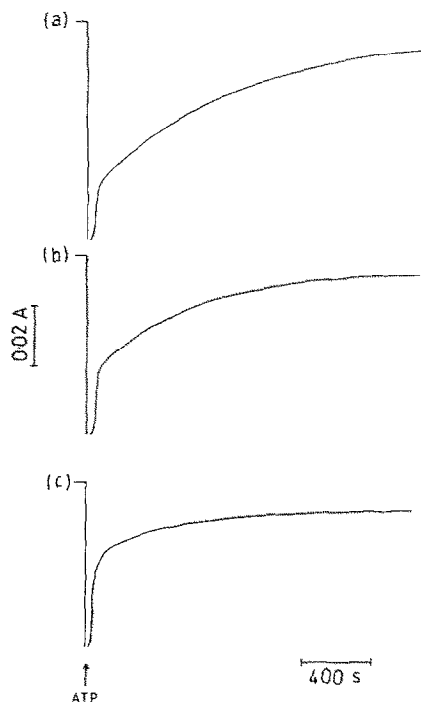


Fig.3. Loss of the regulated HMM population on labelling with IAEDANS. (a) Turbidity assay of HMM before labelling.  $17 \mu\text{M}$  ATP was added to  $2 \mu\text{M}$  acto-HMM in  $20 \text{ mM}$  NaCl,  $1 \text{ mM}$   $\text{MgCl}_2$ ,  $100 \mu\text{M}$  EGTA,  $10 \text{ mM}$  Tes at pH 7.5 and  $20^\circ\text{C}$ . (b)  $2 \mu\text{M}$  HMM was preincubated with  $4 \mu\text{M}$  IAEDANS for 15 min at  $0^\circ\text{C}$ . It was then brought to  $20^\circ\text{C}$ ,  $2 \mu\text{M}$  actin added and a turbidity assay carried out as above. (c) As above but HMM was incubated with IAEDANS for 85 min. In addition to the chart recorder of the spectrophotometer, each time course was digitised and stored using a transient recorder as described in section 2. Exponential fits to the slow phase of the reaction yielded rate constants of (a)  $1.4 \times 10^{-3} \text{ s}^{-1}$ , (b)  $1.8 \times 10^{-3} \text{ s}^{-1}$  and (c)  $2.2 \times 10^{-3} \text{ s}^{-1}$ . The relative amplitudes of the slow phase, with respect to the total change in turbidity, were (a) 0.69, (b) 0.53 and (c) 0.29.

tions. The residual  $\text{Ca}^{2+}$  sensitivity measured in conventional steady-state assays [8] therefore probably reflects the remaining unlabelled molecules. In any event it is clear that labelling the thiol group is detrimental to the regulatory mechanism. A similar result was observed when HMM was incubated with a spin label (ISL), but iodoacetate itself had little effect on the turbidity profile.

#### 4. DISCUSSION

The ability to increase the  $\text{Ca}^{2+}$  sensitivity of scallop HMM preparations by ultracentrifugation substantiates the existence of two populations of molecules and supports the interpretation of the turbidity profile offered previously [5]. The method is, however, an analytical one which would be difficult to scale up because of the requirement to sediment the unregulated acto-HMM before the regulated HMM has turned over its bound nucleotide to a significant degree. Even using a rapidly accelerating airfuge we experienced some variability in the success of this method. The efficiency of mixing the ATP with the acto-HMM and the timing of the centrifugation, with respect to the profile of ATP turnover, are crucial factors.

The  $\text{Ca}^{2+}$  sensitivity of the HMM freshly prepared by the airfuge method was  $> 93\%$  (fig.1b). In order that the true  $\text{Ca}^{2+}$  sensitivity of the regulated fraction would be expressed in steady-state ATP assays, the regulated fraction would need to comprise  $> 99.9\%$  of the population. There are two reasons why this is unlikely ever to be achieved in practice, apart from the difficulties in rapid sedimentation alluded to above. Firstly, the regulatory light chains tend to dissociate to a small extent under the conditions of the ATPase assay. It is estimated that up to 17% of the light chain would dissociate from  $4 \mu\text{M}$  HMM in  $1 \text{ mM}$   $\text{MgCl}_2$  at  $20^\circ\text{C}$  over a period of several hours [15]. Secondly, it is possible that there is a third, minor population of HMM molecules which possess ATPase activity but which does not interact with actin. Szentkiralyi [16,17] reported that if the  $\text{S}_1$  heavy chain was nicked then the preparation was no longer activated by actin. Such a population might remain in the supernatant together with the regulated fraction.

The ATPase rate of regulated HMM in the absence of  $\text{Ca}^{2+}$  is estimated to be about  $0.002 \text{ s}^{-1}$  from the time course of a single turnover of this population (fig.3 and [5]). The ATPase rate of an  $\text{S}_1$  head maximally activated by cross-linking to actin is around  $10 \text{ s}^{-1}$ . Thus the ATPase can be potentially activated by a factor of 5000. Unfortunately, it has not been possible to demonstrate this dynamic range in the same assay because the  $\text{Ca}^{2+}$  sensitivity of HMM is lost on cross-linking.

Three possible explanations for the loss are:

(i) Regulation by  $\text{Ca}^{2+}$  is achieved by controlling the binding of actin rather than a chemical step of the mechanism. This is unlikely in the view of the binding measurements of Chalovich et al. [14].

(ii) Cross-linking is performed under rigor conditions which may lock the system in an activated state.

(iii) EDC is a highly reactive species which may modify other amino-acid residues of the HMM and interfere with the regulatory mechanism (cf. the effect of modifying the reactive thiol group). This is at least a contributory factor. When EDC was incubated with HMM, in the absence of actin, there was a partial loss of intrinsic  $\text{Ca}^{2+}$  sensitivity of the ATPase.

Labelling of the reactive thiol group destroys the  $\text{Ca}^{2+}$  sensitivity of the HMM ATPase. Our previous observation that  $\text{Ca}^{2+}$  had no effect on the head mobility of spin-labelled myosin [8,18] cannot be interpreted unambiguously in the light of this finding. It does not, however, alter the conclusion concerning the large reduction in head mobility on removal of regulatory light chains [8].

#### ACKNOWLEDGEMENT

We thank the Science and Engineering Research Council for financial support.

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