The Ca^{2+} sensitivity of the actin-activated ATPase of scallop heavy meromyosin

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The actin-activated scallop heavy meromyosin (HMM) ATPase was monitored turbidometrically during a limited number of turnovers. At 4 μM actin, the turnover rate in the presence of Ca^{2+} (1.2 s^{-1} per head) was 650-fold higher than in its absence (1.8×10^{-3} s^{-1}), a Ca^{2+} sensitivity which approaches that expected in vivo. The extent of Ca^{2+} activation was much larger than that observed by steady-state measurements, where the rate, in the absence of Ca^{2+}, is dominated by a small proportion of unregulated molecules. Acto-HMM formation, and its dissociation by ATP, were Ca^{2+} insensitive.

Kinetics *ATPase* *Heavy meromyosin* *Myosin-linked regulation* *Ca^{2+} activation*

1. INTRODUCTION

The contraction of scallop adductor muscle is controlled by the direct binding of Ca^{2+} to the myosin filament [1,2]. In this system, myosin regulatory light chains inhibit the actin-activated ATPase in the absence of Ca^{2+}. Thus activation by Ca^{2+} involves a deinhibition, just as in the actin-linked system of vertebrate skeletal muscle. In both systems, the interaction between myosin and actin is so strong, in the absence of ATP, that the rigor state forms, regardless of the Ca^{2+} concentration. At low ATP concentrations, the cooperative nature of the actin-linked system gives rise to a characteristic property, whereby the formation of a few rigor links appears to displace the tropomyosin from its inhibitory position. Consequently, the whole actin filament becomes activated, in the absence of Ca^{2+} [3]. This phenomenon also reveals itself in the form of a lag phase when myosin heads bind to regulated actin in the absence of Ca^{2+} [4]. While cooperativity is evident in the myosin-linked system of scallop muscle [5], there is no reason, a priori, to suppose that a limited number of rigor complexes override regulation in this species. However, experimental evidence regarding this point is conflicting [2,6].

Steady-state ATPase measurements show a Ca^{2+} activation of 10–25-fold for acto-scallop myosin, which is reduced to only about 4-fold for acto-scallop HMM. We report here on the properties of acto-scallop HMM ATPase during the transient state and demonstrate the extreme efficiency of its regulatory system – a feature not apparent from steady-state measurements.

2. MATERIALS AND METHODS

Heavy meromyosin and subfragment 1 were prepared from scallop (*Pecten maximus*) myosin as in [7]. F-Actin was prepared from an acetone-dried powder of rabbit skeletal muscle [8]. The protein concentrations quoted throughout this paper refer to the concentration of heads, in the case of HMM, and the concentration of monomer subunits for F-actin.

The dissociation and reassociation of acto-HMM, during ATPase activity, were measured

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Abbreviations: HMM, heavy meromyosin; Tes, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid

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turbidometrically. Rapid phases were followed using an Aminco-Morrow stopped-flow apparatus attached to a DW2 dual-wavelength spectrophotometer set at 320 nm (with the reference monochromator set at 420 nm). Slower time courses, when additions could be carried out manually, were recorded using a Pye-Unicam SP8-100 or a Perkin-Elmer LS5 spectrophotometer; the latter being used for multiple assays in conjunction with an autochanger attachment. Steady-state ATPase activity was measured using a Radiometer RTS822 pH-stat [7]. MgATP was the substrate utilized for all the ATPase activities measured.

3. RESULTS

Steady-state ATPase activity can be measured using a pH-stat to follow the rate of proton release during ATP hydrolysis. Fig.1A shows the pH-stat record of the actin-activated ATPase of a typical scallop HMM preparation. At 3.5 μM actin, the ATPase rate, per HMM head, was 1.18 s^{-1} in the presence of Ca^{2+} and 0.31 s^{-1} in its absence. Thus Ca^{2+} activated the ATPase by a factor of 3.8, corresponding to a Ca^{2+} sensitivity of 74%. However, the significance of this sensitivity is ambiguous. For instance, it could arise from 26% of the HMM molecules being totally insensitive and the remainder being fully regulated, or it could reflect a 26% reduction in the efficiency of the regulatory system of all the molecules present. This ambiguity was resolved by recording the time course of the turbidity of the acto-HMM preparation during a limited number of turnovers of ATP.

As acto-HMM scatters more light than the sum of that scattered by the individual proteins, the transient dissociation of the complex, during ATP

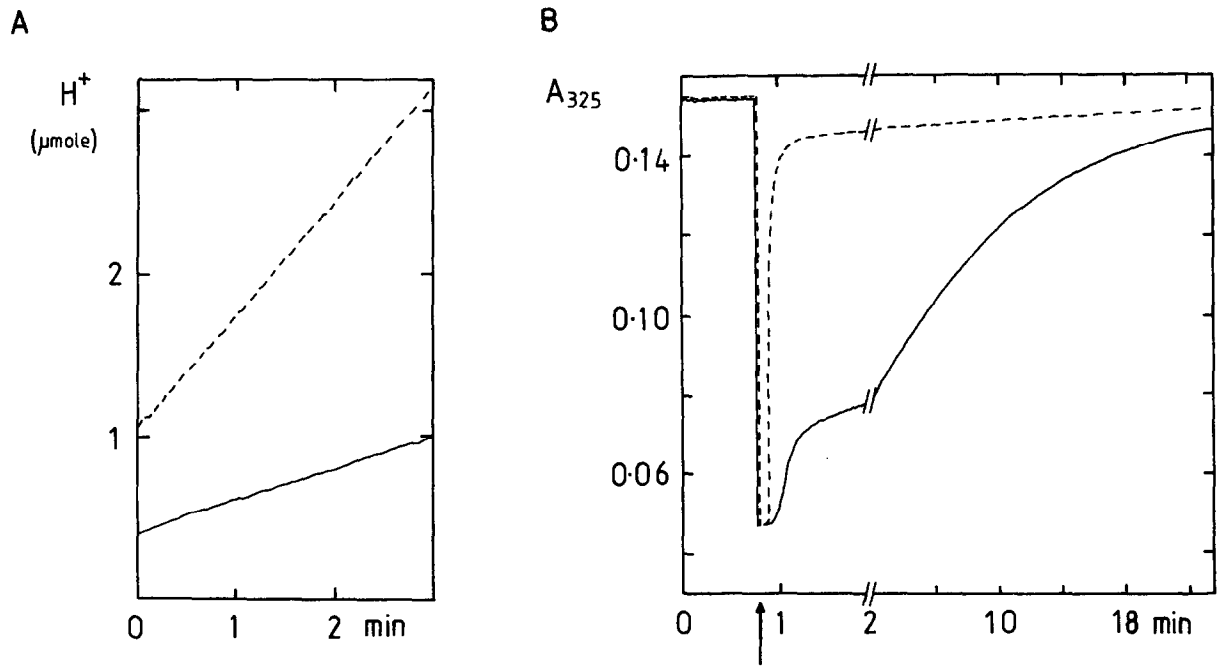

Fig.1. (A) The effect of Ca^{2+} on steady-state actin-activated scallop HMM ATPase, as measured by the pH-stat method. The solid line represents the rate of proton release (and hence ATPase activity) from an 8 ml reaction mixture containing HMM (1.2 μM heads), actin (3.5 μM) and ATP (470 μM) in 20 mM NaCl, 1 mM Mg^{2+}, 100 μM EGTA at pH 7.5 and 20°C. The broken line describes the increased rate obtained on addition of Ca^{2+} (200 μM) to this system. (B) The effect of Ca^{2+} on turbidity profiles of acto-scallop HMM, during ATPase activity. The solid line is the turbidity profile (at 325 nm) obtained from a solution of HMM (4 μM heads) and actin (4 μM) in 10 mM Tes, 20 mM NaCl, 1 mM Mg^{2+}, 100 μM EGTA, pH 7.5 (deaerated), at 20°C, to which 25 μM ATP was added at the time point indicated by the arrow. When the slow exponential phase of this profile was virtually complete (approx. 20 min after ATP addition), 200 μM Ca^{2+} was introduced, followed by a further aliquot of ATP (25 μM), to give the turbidity profile shown by the dashed line.

hydrolysis, can be followed turbidometrically [9]. At low acto-HMM concentrations, the addition of a molar excess of ATP causes a rapid and almost complete dissociation of the proteins. During hydrolysis, the proteins remain largely dissociated for a period equal to the turnover time of the ATP present. Reassociation occurs when the ATP is exhausted. On addition of ATP to acto-scallop HMM, the observed turbidity profile was markedly dependent on Ca^{2+} (fig.1B). In the presence of Ca^{2+} and a 6 molar excess of ATP, the reassociation phase occurred within 6 s of adding the nucleotide ($k_{cat} \geqslant 1\ s^{-1}$ at 4 μM actin; in agreement with the pH-stat record). In the absence of Ca^{2+}, however, acto-HMM recombination was biphasic. A partial reassociation (about 25% of the total turbidity change) occurred within 17 s, followed by a much slower phase ($t_{½}$ = 6.5 min) before full recovery was attained. We attribute the initial phase to rapid multiple turnovers of ATP by unregulated HMM molecules in the preparation, while the second, slower rate represents a single turnover of ATP by the regulated population. Thus, fig.1B may be accounted for, in quantitative terms, by proposing that 1 μM unregulated HMM (25% of the preparation) hydrolyses 22 μM ATP within 17 s, to give a k_{cat} = 1.3 s^{-1}. The 3 μM regulated HMM (75%) would turn over 3 μM ATP with a k_{cat} of $1.8 \times 10^{-3}\ s^{-1}$, deduced from the slow exponential phase. Therefore the degree of Ca^{2+} activation of regulated HMM, at 4 μM actin, is about 650-fold (Ca^{2+} sensitivity = 99.8%).

To demonstrate that this slow phase was not due to a non-specific turbidity increase, perhaps due to protein ageing, the following experiment was performed. The acto-HMM ATPase reaction was initiated in the presence of EGTA, as described in fig.1B, in 6 cuvettes in a spectrophotometer fitted with an autochanger attachment. Subsequently, 200 μM Ca^{2+} was added in turn to each sample at different time points on the slow exponential phase. In all cases, addition of Ca^{2+} caused the turbidity to rise rapidly (at a rate comparable to that recorded in fig.1B in the presence of Ca^{2+}) to the value expected for the associated acto-HMM complex.

Addition of 150 μM ATP to 4 μM acto-HMM, under the conditions given in fig.1B, prolonged the lifetime of the dissociated state. In the absence of Ca^{2+} the two phases were still evident, although they were not as clearly separated. In the presence of Ca^{2+}, the observed turnover time of 32 s gave a more accurate assessment of k_{cat} = 1.2 s^{-1} than could be determined from the experiment in fig.1B. At 5 μM ATP, in the absence of Ca^{2+}, the first phase of reassociation became too fast to measure by manual addition, although the slow phase remained essentially unchanged. Stopped-flow measurements revealed that the first phase was much reduced in amplitude because of the decrease in the dissociation rate of acto-HMM at low ATP concentrations.

The effect of increasing the actin concentration on the steady-state pH-stat assay and the turbidometric assay was studied. A 2-fold increase in the actin concentration doubled the steady-state rates observed in fig.1A. An increase in the ATPase rate was also evident in the turbidometric profile recorded in the presence of Ca^{2+} (cf. fig.1B). In the absence of Ca^{2+}, however, the slow exponential phase, attributed to a single turnover by regulated HMM, was barely affected.

Scallop subfragment 1, regardless of light chain content, shows no Ca^{2+} sensitivity by the pH-stat method [1]. The turbidometric assay confirmed that the ATP was turned over rapidly in the presence or absence of Ca^{2+}. Indeed, Ca^{2+} appeared to inhibit the ATPase rate by about 20%, a phenomenon noted previously for desensitized myosin [10]. We envisage that the unregulated molecules of an HMM preparation are proteolysed in the neck region, such that they behave like subfragment 1.

Stopped-flow studies were carried out to investigate the effect of Ca^{2+} on the formation of the acto-HMM complex and its dissociation by ATP. In contrast to the results obtained with unregulated rabbit skeletal myosin heads binding to regulated actin [4], Ca^{2+} had little influence on the rate of binding of regulated scallop HMM to unregulated actin (fig.2A). The observed rate constant increased with increasing HMM concentration, to give a second-order association constant of about $10^6\ M^{-1} \cdot s^{-1}$. Thus there is no evidence for the regulatory light chain operating via a steric blocking mechanism. The observed rate of dissociation induced by ATP was also independent of Ca^{2+} (fig.2B) and yielded an apparent second-order rate constant of $3 \times 10^5\ M^{-1} \cdot s^{-1}$ for ATP binding. This result supports the assumption, made in the

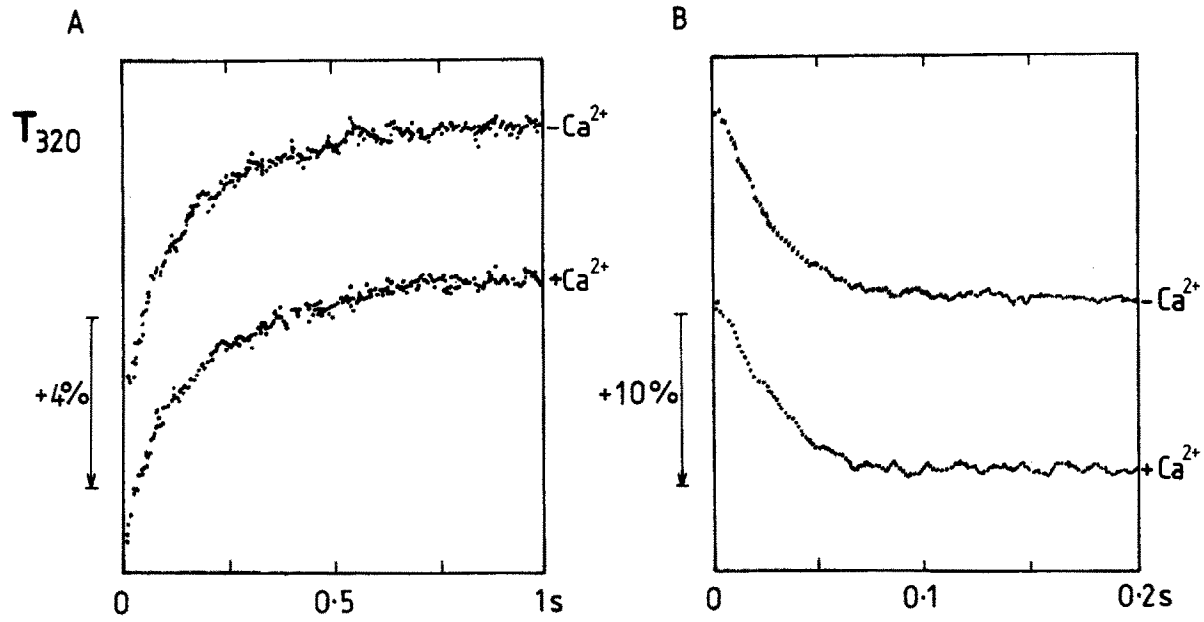

Fig.2. (A) Stopped-flow measurements showing the lack of an effect of Ca^{2+} on acto-scallop HMM formation. The rate of acto-HMM formation was measured as a decrease in transmittance at 320 nm. HMM (8 μM heads) in one syringe was mixed with 4 μM actin in the other syringe at 20°C in 10 mM Tes, 20 mM NaCl, 1 mM Mg^{2+}, 100 μM EGTA, pH 7.5 (deaerated). The experiment was repeated after the addition of 200 μM Ca^{2+} to both syringes and a similar rate was observed. The traces are displaced for presentation. (B) Stopped-flow measurements showing the lack of an effect of Ca^{2+} on ATP-induced acto-scallop HMM dissociation. Transmittance change (%) was recorded when 10 μM acto-HMM in one syringe was mixed with 200 μM ATP in the other syringe under the same conditions as in A. The rate of dissociation (recorded as an increase in transmittance) was not affected by Ca^{2+} (200 μM). The traces are displaced for presentation.

interpretation of fig.1B, that both the unregulated and regulated acto-HMM complexes bind ATP equally well. The observed rate of dissociation increased with increasing ATP concentration, until it became too fast to measure, and indicated that the dissociation of actin from the ternary complex was $>150\ s^{-1}$.

4. DISCUSSION

The degree of Ca^{2+} activation, revealed by the turbidometric assay of acto-scallop HMM, is 650-fold at 4 μM actin (99.8% Ca^{2+} sensitivity). This is much higher than that reported previously using the pH-stat assay, because, in the absence of Ca^{2+}, the activity of the small proportion of unregulated molecules present dominates the turnover of ATP during the steady state. In such an assay, the ratio of the rates, in the absence and presence of Ca^{2+}, provides a measure of the proportion of unregulated molecules (26% in fig.1A), but it does not provide a true estimate of the Ca^{2+} sensitivity of the fully regulated molecules. The degree of activation reported here, which increases further at higher actin concentrations, approaches that expected for in vivo muscle contraction. The long-standing discrepancy between the ATPase rates observed in relaxed vertebrate skeletal muscle and actomyosin preparations has been attributed to a damaged, or incomplete, regulatory system [11]. Unfortunately, single turnover analysis of such an actin-linked system is unlikely to reveal any fully regulated molecules, if present, because the rigor links formed in the initial stages of the transient would override the control proteins [12]. On the basis of fig.1B, rigor complex formation, in the myosin-linked regulation system of scallop, does not appear to influence the control of neighbouring myosin molecules [2]. Nevertheless, the steep activation profile, implicit in our results,

is consistent with the proposal that two Ca^{2+} are required to activate the HMM heads cooperatively [5].

It has previously been demonstrated that the myosin-linked regulatory system operates by effectively blocking the binding of the myosin–nucleotide complex to actin [1]. The actin binding step per se is unlikely, however, to be the exclusive point of control, because the maintenance of the thermodynamic balance would require that the equilibrium constant of acto-HMM formation shows the same high degree of Ca^{2+} sensitivity. There is no evidence for this in the amplitude or rate of the turbidity change (fig.2A). Therefore the myosin-linked regulatory system appears to modulate a transition between nucleotide-bound states of myosin (associated and/or dissociated from actin); a conclusion also reached in [13]. Our HMM preparations are Ca^{2+} sensitive in the absence of added actin (cf. [14]), as determined by the lifetime of the small fluorescence enhancement during ATPase activity, but the influence of contaminant actin (<1%, w/w) has not yet been rigorously excluded. Further studies on the kinetics, in the total absence and also at near saturating actin concentrations, are required to identify the Ca^{2+}-sensitive steps.

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