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NEWS AND VIEWS

Are there two different binding sites for ATP on the myosin head, or only one that switches between two conformers?

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Introduction

Striated muscle contraction depends on the cyclic interaction of the motor domains of myosin 2 ("heads" or S1) with actin, tightly coupled to ATP hydrolysis. It is generally thought that each myosin head contains an actin and an ATP binding site whose cross-talk gives rise to the cross-bridge cycle (Geeves and Holmes 2005; Sweeney and Houdusse 2010). In the last 30 years, kinetic and structural studies greatly improved the understanding of the relation between the states of the actomyosin ATPase cycle and mechanical activity, showing highly conserved features in the myosin superfamily. However, despite this consensus, there is uncertainty as to the number of ATP sites on the myosin head (for instance Tesi et al. 1989).

In a recent study, Brenner and co-workers proposed that each of the two myosin heads has only one site for ATP switching between two conformers (Amrute-Nayak et al. 2014). They investigated the ATPase kinetics of individual myosin molecules by the use of fluorescently labelled ATP and measuring "residence times" of the labelled ATP on individual active sites from "dwell" and "waiting" times of the fluorescent signals. They found that the termination of fluorescence could originate from two different pathways as two different dwell time populations were identified in all the myosins tested (including rabbit psoas): a "long" lived time state consistent with conventional ATPase measurements from solution studies and a "short" lived time state.

From kinetic modelling studies Amrute-Nayak et al. (2014) proposed that the site for ATP on the myosin head can switch between two conformers—one allowing the complete ATPase cycle, which they labelled the M conformer, another site that binds ATP reversibly, the M′ conformer.

The intriguing proposal of Brenner and co-workers induced us to resurrect structural and transient kinetic data on the different myosin systems carried out in the 1980s from which we proposed that ATP interacts with different sites on the myosin head: an ATPase site and a site that is involved in the actomyosin dissociation.

Structural studies resurrected

From their equilibrium dialysis experiments, Schaub et al. (1983) showed that S1 has only one site for ADP. This could be because the complete triphosphate side chain of ATP is required for binding to a putative second site. Thus, Chaussepied et al. (1986b) prepared a 30 kDa fragment, comprising residues Ser562–Lys843 of S1 (shown as red cartoon in Fig. 1) that has a "polyphosphate recognition site" composed of basic residues. This fragment does not include the ATPase site of S1 and yet it binds actin in an ATP-dependent manner. It is noteworthy that whereas the actin-30 kDa complex was dissociated completely by ATP, ADP was much less effective. This does not prove that the polyphosphate site is functionally important because Trayer et al. (1991) by screening a number of peptide mimics concluded that actin docks at multiple sites on the myosin head. Furthermore, the polyphosphate recognition site is situated near the C-terminal of end of the tryptic 50 kDa domain of

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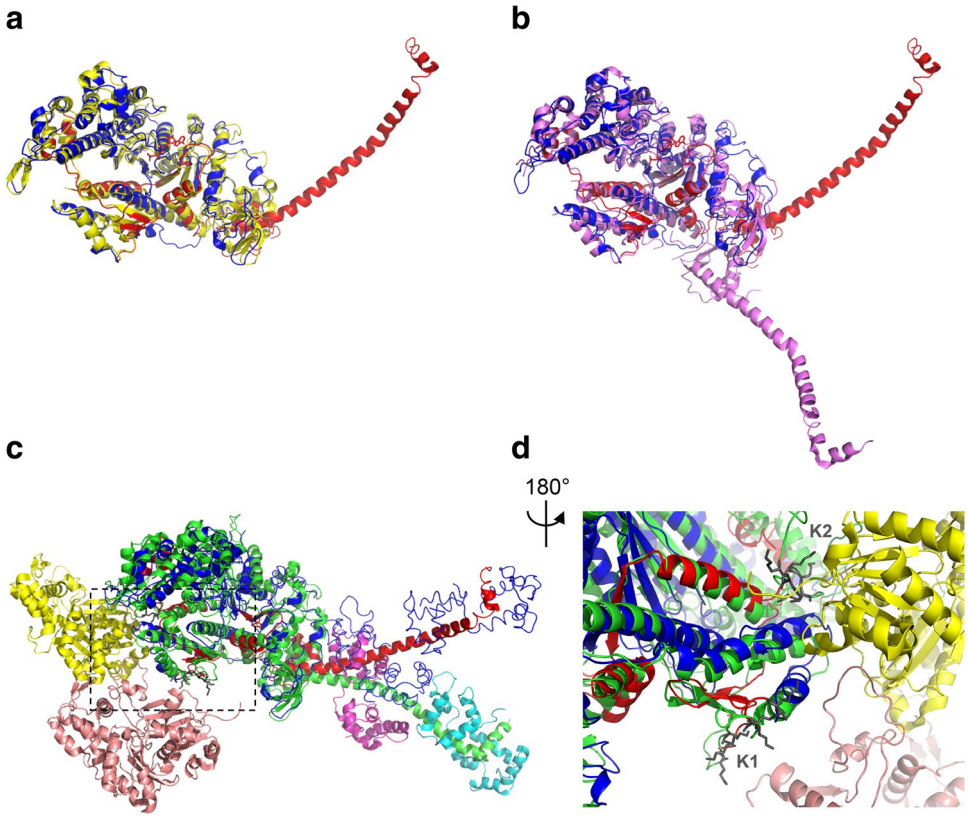

Fig. 1 Overlay of the crystal structures of myosin S1 from chicken (PDB 2MYS) with that of **a** *Dictyostelium discoideum* (PDB 1MMN, shown in *yellow*), **b** scallop (PDB 1KQM, shown in *pink*) or **c** with the structure of the rabbit actomyosin complex modelled from electronic microscopy images (PDB 5H53, myosin heavy chain in *green*, myosin *light* chains in cyan and *purple*, actin monomers in *yellow* and *pink*). The 30 kDa peptide generated by thrombin cut of chicken S1 (Ser562–Lys843) is shown in *red*, while the rest of the structure is in *blue*. ATP analogues from 1MMN and 1KQM structures are shown as *red sticks*. **d** Zoom on the two patches of lysine residues (depicted as *grey sticks*) seen from the opposite side compared to structure squared with *dashed line* in **c**. K1 indicates the first patch constituted of Lys 561, 567 and 568 visible in both 2MYS and 5H53; K2 the second patch including Lys 638, 639, 642, 643 and 644 only visible in 5H53. Figures were prepared with PyMOL 1.8.4.2. (Color figure online)

S1. Now, the ATP cleavage site is on the N-terminal 25 kDa region (Chaussepied et al. 1986a), so on the linear sequence of S1 the two sited are far apart. It was hypothesized that in solution, the two sites may be close enough to allow the adenine moiety of ATP to be bound to the ATPase site and the three phosphates of the same ATP to the polyphosphate recognition site. This would concord with the one site model in that with the same ATP molecule, the phosphates would bind alternatively to the two sites.

However, from recent structural studies there is no evidence for two sites for ATP per myosin head. In the crystal structures of *Dictyostelium discoideum* (Gulick et al. 1997; Fig. 1a) or scallop (Himmel et al. 2002; Fig. 1b) S1 complexed with ATP analogues, there is only one ATP binding site that corresponds to the active site. The 30 kDa peptide of Chaussepied et al. stands far away from this ATP binding site in the 3D structure.

Very recently, Fujii and Namba (2017) determined the structure of the rabbit actomyosin rigour complex by electron microscopy (Fig. 1c). They describe four interacting interfaces between actin and myosin. One of these interaction sites is particularly interesting in the context of the Chaussepied et al. experiments because it involves a patch of five lysine residues (K638, K639, K642, K643, K644) in loop 2 of the lower 50 kDa domain (K2 in Fig. 1d). This loop is part of the 30 kDa peptide but it is too flexible to be seen in the higher resolution crystal structures of S1. In the electron microscopy structure of Fujii and Namba, the loop seems to interact with acidic residues of actin, which could well compete with the polyphosphate chain of ATP, in a non-specific manner. Chaussepied et al. suggested "the involvement of the 561–641 (number +1 compared to the original paper to take into account the first methionine which was not numbered at that time) residues segment on the N-terminal part of the 30 kDa peptide in phosphoryl group recognition and intersite transmission". This segment includes three lysine residues K561, K567 and K569 (K1 in Fig. 1d) which are in loop 3. Although this loop was initially thought

to form an extensive interaction with a second monomer of actin, Fujii and Namba showed that this is not the case. Therefore, if ATP can dissociate actin from S1 by ionic competition (binding to a second ATP binding site), this should involve patch K2 rather than K1.

Transient kinetic studies resurrected

From early steady state measurements, it was suggested that each myosin head has more than one site for ATP (references cited in Tesi et al. 1989). Eccleston (1980) extended these studies to S1 with ribose 5-phosphate (RTP) as substrate. With RTP, $k_{cat} = 0.016\ s^{-1}$ compared with $0.038\ s^{-1}$ for ATP. As with ATP, upon mixing RTP with S1, there was an increase in the intrinsic fluorescence of S1. The kinetics of this increase were studied by stopped-flow. The results did not fit to a "single site for ATP" situation on S1. He concluded that his data are compatible with two sites for RTP on S1 but that "if this second site also binds ATP there is at present no evidence for this".

Etheno-ATP gives a fluorescent signal upon interaction with S1. Rosenfeld and Taylor (1984) carried out a fluorescence stopped-flow study on this interaction. The time courses that they obtained were biphasic and they proposed a branched pathway for etheno-ATP binding although they did not exclude a "two-sites for ATP" situation. This work was confirmed by Tesi et al. (1988) who proposed that the fast fluorescent transient is due to the tight binding and hydrolysis of etheno-ATP of S1 and the slow phase to a site that does not hydrolyse etheno-ATP.

The experiments reported below are based on the "cold ATP chase method", a method that measures specifically the kinetics of the essentially irreversible binding of ATP to the ATPase site of S1 and is a way to titrate these sites. In particular, the ATP chase method is sensitive to the kinetics the binding of ATP to secondary sites (Barman and Travers 1985, and references cited therein).

In the experiments outlined below, myosin and its subfragment refer to rabbit psoas muscle. The data obtained with S1 were interpreted by Scheme 1, where steps 2 and 4 are irreversible on the time scale of the experiments. Thus, once the ATP is bound in M*.ATP, it can only come off by being hydrolysed. We illustrate the cold ATP chase method in Fig. 2.

Most of the experiments were carried out in the presence of 40% ethylene glycol. This cryosolvent has two effects. First, it allows for extensive Arrhenius and van't Hoff plots, and second, it may affect the kinetics of the individual rate constants of the steps of the reaction pathway of an enzyme in different ways which can lead to mechanistic information (Douzou 1977a, b). With S1, the kinetic data obtained in 40% ethylene glycol fit well to the Bagshaw–Trentham reaction Scheme 1 that was obtained in the absence of the glycol.

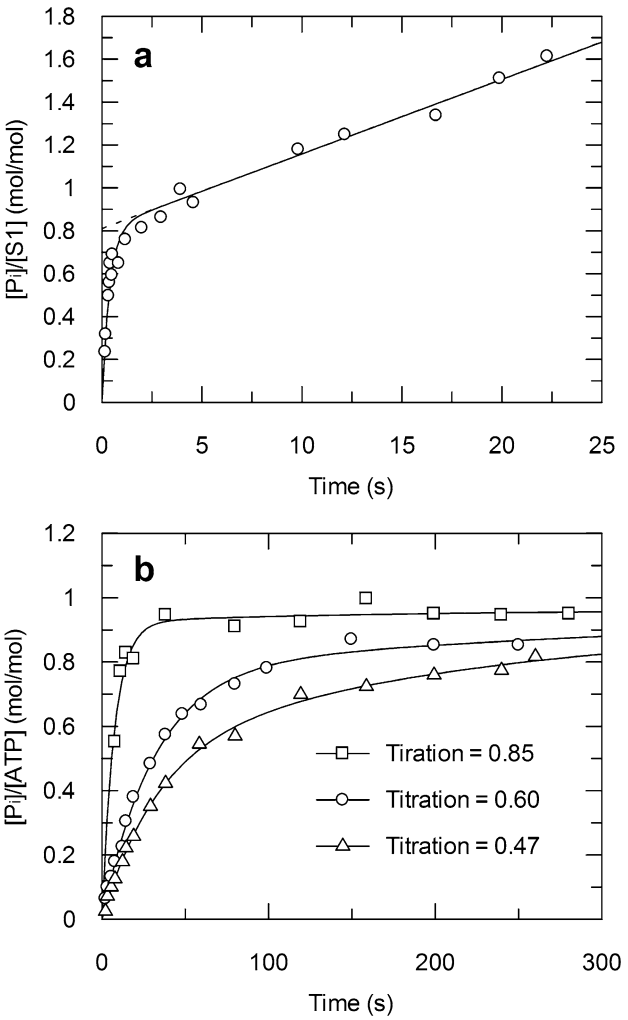

Fig. 2 **a** Time course for a cold ATP chase experiment under multiturnover conditions with S1 at 15 °C. The reaction mixtures (0.8 μM S1 plus 4 μM [γ-^{32}P]ATP) were quenched at the times indicated in 20 mM cold ATP, incubated for 3 min, quenched in acid, and the [^{32}P]P_i determined. The buffer was 2 mM Mg-acetate, 50 mM Tris–acetate pH 8 and 40% ethylene glycol. From Barman et al. (1983) in which further details are given. **b** Cold ATP chase experiments under single turnover conditions with S1 of different active site titrations at −7.5 °C. The reaction mixtures, 1 μM [γ-^{32}P]ATP plus15 μM S1 of titration amplitudes 0.85 (*open circle*), 0.60 (*open square*) and 0.47 (*open triangle*), were quenched in 20 mM cold ATP and analysed as in **a**. From Tesi et al. (1989)

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M{\cdot}ATP \xrightarrow{k_{+2}} M^*{\cdot}ATP \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} M^{**}{\cdot}ADP{\cdot}P_i \xrightarrow{k_{+4}} M + ADP + P_i$$

Scheme 1 Simplified version of the Bagshaw-Trentham scheme for myosin ATPase where M represents myosin head and the asterisks different conformations of the myosin

Experiments with S1

A typical multi turnover cold ATP chase experiment with S1 at 15 °C is illustrated in Fig. 2a (Barman et al. 1983). The time course of radioactive P_i formation fit to a "burst" of P_i of amplitude 0.81 mol P_i per S1 protein and kinetics k_{obs} 5.2 s^{-1}, followed by the steady state phase of rate constant 0.042 s^{-1}. As a first approximation, we proposed that the amplitude represents the concentration of the ATPase site (the titration value), that is to say, the site that binds tightly ATP followed by hydrolysis. The titration value of S1 from different rabbits varied in the range 0.5–0.85 and was directly proportional to the kinetics of the steady state phase (Tesi et al. 1989).

The dependence of k_{obs} is hyperbolic, with $k_{obs} = k_2 \times ATP/(ATP + K_1)$ (Fig. 3). Thus, the cold ATP chase method provides estimates for the kinetic constants of the very first steps of the S1 reaction pathway—the formation of the collision complex M·ATP, defined by the rapid equilibrium $K_1 = 11.5$ μM, and the following conformational change of S1 leading to M*·ATP of kinetics $k_2 = 15.5$ s^{-1}.

From Fig. 2a, the S1 preparation used contains a significant amount of material that did not bind tightly and hydrolyse ATP. A sensitive way to determine if this interacts with ATP is to carry out chase experiments under single turnover conditions. Thus, in a "one site only situation", the time courses of P_i production should fit to a single exponential: any additional site for ATP could manifest itself by an additional phase.

Three cold ATP single turnover chase experiments with S1 of different titration values (0.85, 0.60 and 0.47 mol site/mol S1 protein) are illustrated in Fig. 2b. For the sake of clarity only the first 280 s are shown. With the three preparations, the ATP was completely hydrolysed at incubation times >1200 s (Tesi et al. 1989).

The time courses are biphasic. The fast phases fit well to a site that binds tightly and hydrolyses ATP. The slow phases fit to a site that binds ATP loosely without hydrolysis. The rate constants for the fast phases were identical to those found under multi turnover conditions (constant as a function of the ATP concentration rather than S1 concentration as in the single turnovers).

Heavy meromyosin (HMM) and filamentous myosin also gave biphasic single turnover ATP chase progress curves (not illustrated, Tesi et al. 1989).

We concluded in our previous work that ATP appears to interact at two sites in myosin preparations whether myosin itself or its sub-fragments HMM or S1. One site hydrolyses ATP by the Bagshaw–Trentham scheme (tight binding followed by hydrolysis, Bagshaw et al. 1974) and a second site to which ATP binds without detectable hydrolysis. We proposed that the slow phase in the single turnovers is a

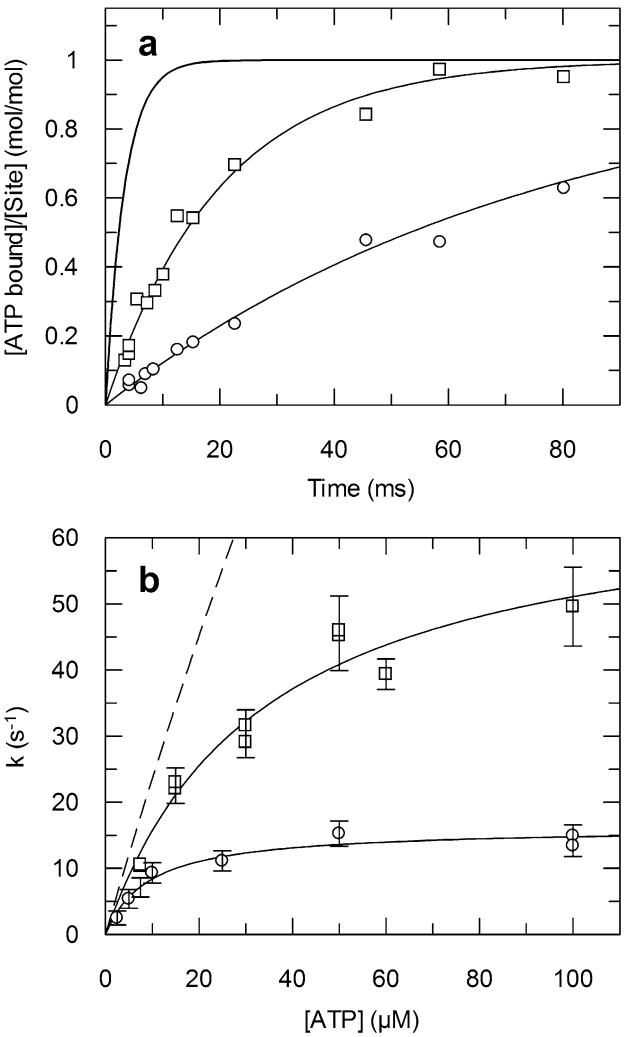

Fig. 3 **a** Time courses for cold ATP chase experiments with S1 (*open circle*) and actoS1 (*open square*) and the ATP-induced dissociation of actoS1 (*dashed line*) at 15 °C. The reaction mixtures were as follows: cold ATP chases: 100 μM [γ-^{32}P]ATP plus 10 μM S1 and 10 μM actin (actoS1) and 10 μM S1 (S1 alone). ActoS1 dissociation: 100 μM ATP plus 6 μM S1 and 6 μM actin. **b** Dependences of the binding kinetics of ATP to S1 (*open circle*) and actoS1 (*open square*) on the ATP concentration at 15 °C. The actoS1 dissociation time course dependence is indicated by a *broken line*. From Biosca et al. (1984)

reflection of the slow kinetics of the release of ATP from the second site, which is then hydrolysed by the ATPase site.

We then addressed the question concerning the origin of the slow phase in the experiments with ATP and etheno-ATP. A possibility is that the phase is due to a competitive inhibitor at the ATPase site of the S1. The phase would then be the reflection of the slow release of the inhibitor from the site which implies that the inhibitor binds tightly to S1. There is at present no evidence for such an inhibitor. Furthermore, the S1 used in our experiments had been dialysed. Pyrophosphate is a potential inhibitor but with its

low affinity for S1 this seems unlikely. Nevertheless, we confirmed its absence by treating an S1 preparation with pyrophosphatase before titration—as expected this treatment did not increase the active site concentration (unpublished).

Is the slow phase merely due to S1 with an impaired active site or is it involved in the ATP-induced dissociation of actoS1? A way to put the latter possibility to the test is to carry out cold ATP chase experiments on actoS1. Thus, we wanted to determine whether or not ATP binds as tightly to actoS1 as to S1, and if so, to investigate any connection of this process with the ability of ATP to dissociate the actoS1 complex.

Experiments with actoS1

We determined the kinetics of the ATP-induced dissociation of actoS1 by turbidimetry in a stopped-flow apparatus (Biosca et al. 1984). As illustrated in Fig. 3, we carried out three experiments at 100 μM ATP: cold ATP chases with actoS1 and S1, and the ATP-induced dissociation of actoS1.

There are three noteworthy features of the experiments in Fig. 3a. First, the ATP-induced actoS1 dissociation kinetics (300 s^{-1}) are considerably faster than those of the ATP binding kinetics in the chase experiments (49 s^{-1} with actoS1 and 13 s^{-1} with S1) from which we conclude that the ATP-induced dissociation of actoS1 is not linked directly to the tight binding of ATP to the ATPase site. Second, in the three types of experiment transient lag phases were not observed, even at concentrations of ATP at which the kinetics were similar (Gutfreund 1995), which confirms that the kinetics of the binding of ATP to the ATPase site and actomyosin dissociation process are not linked directly. Third, the ATP binding kinetics were faster with the S1 freshly released from the actoS1 than with S1 on its own. This implies that on the time scale of the experiment, the released S1 apparently "remembers" an actin-induced conformation in the actoS1 complex and reacts accordingly.

The ATP dependences of the rates of binding of ATP to actoS1 and S1 are illustrated in Fig. 3b. From the cold ATP experiments and with reference to Scheme 1, for actoS1 K_1 is 360 μM^{-1} and k_2 62.7 s^{-1}, with S1 K_1 is 11.5 μM^{-1} and k_2 15.6 s^{-1}.

Experiments with myofibrils

The myofibrils are the functional units of muscle, and with them both mechanical and kinetic experiments have been carried out (Lionne et al. 2003, and references cited therein). As with acto-S1, ATP appears to bind to two sites. Furthermore, the ATP-induced dissociation of the cross-bridges seems to occur independently of the ATP binding (Houadjeto et al. 1992; Herrmann et al. 1993; Stehle et al. 2000).

Conclusions

The one site model of Brenner and co-workers seems the more likely to explain our transient kinetics. The weakness of the two-site proposition is that there is no hard structural evidence for an extra-ATP binding site. Even a situation involving two mutually exclusive sites for ATP seems unlikely. Furthermore, the two-site proposition is based upon the Bagshaw-Trentham scheme of the ATPase cycle of myosin—a model that may be an oversimplification of the real situation. Finally, the second ATP binding site assumption was originally based on actomyosin dissociation experiments using a non-functional proteolytic fragment of S1, the folding of which is certainly different from the structure it adopts in the whole myosin head. Nevertheless, we suggest that the two-site proposition could be put to the test by further experiments such as isothermal titration calorimetry of the 30 kDa fragment or the whole S1 with a non-hydrolysable ATP analogue. Such experiments would give information on the thermodynamics of interaction (free energy, enthalpy and entropy changes), binding stoichiometry (1 or 2 ATP binding sites per myosin) and affinity constant(s) (see for example, Neves et al. 2017).

In the one site model of Brenner and colleagues (Amrute-Nayak et al. 2014), based on experiments on single molecules of the two-headed myosin, ATP interacts with one single site that switches between two conformers M and M′. Conformer M binds ATP irreversibly with hydrolysis and conformer M′ binds ATP reversibly. Can our transient kinetics be fitted to this model? It is difficult to come to a firm conclusion but we note that for equilibrium of the two conformers M and M′ to be reached 1–10 s is needed, that is on the same time range as that of slow phase in the transient kinetics. Furthermore, Brenner and colleagues pointed out that our kinetic transient kinetic data are in agreement with their single site for ATP model.

The presence and potential functional relevance in vivo of the "two types of head-pathway" of Brenner and coworkers for actomyosin hydrolysis is, of course, difficult to detect when motors are working in large arrays as in intact sarcomeres of striated muscle. Few possible insights come from recent studies of the relaxed state which report, also in skeletal muscle, a significant presence of myosin heads in an "off" state, unable to bind actin and cleave ATP (Wray et al. 1975; Wray and Holmes 1981; Woodhead et al. 2013; Fusi et al. 2015; Linari et al. 2015). These heads that loosely bind ATP without hydrolysis would promote actomyosin dissociation and contribute to keep the system relaxed in the absence of calcium. Whether or not the second site also manifests itself in the super-relaxed state of striated muscle (Stewart et al. 2010) remains an open question.

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