DRIVING FILAMENT SLIDING:

Weak binding cross-bridge states, strong binding cross-bridge states, and the power stroke

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1. INTRODUCTION

The terms weak binding cross-bridge states and strong binding cross-bridge states by now are widely used in the literature on muscle contraction and cell motility, although the physiological significance of the actin attachment of cross-bridges in weak binding states is still questioned quite frequently. In addition, the term 'weak binding cross-bridge state' is often used when binding to actin with low affinity is described without clear distinction whether the binding occurs to the activated or inactive actin filament. Furthermore, the role of weak binding cross-bridge states (weak binding cross-bridge conformation) in relation to the power stroke is still disputed.

In this paper we therefore review our work on (i) characterizing weak and strong binding states of the actomyosin cross-bridge, (ii) possible physiological relevance of attachment of cross-bridges in weak binding states to the actin filament, and on (iii) the relation of weak and strong binding cross-bridge states to the power stroke of the actomyosin cross-bridge. All this work was performed with the structurally intact contractile system of skinned fibers of the M. psoas of the rabbit to preserve the natural steric relations within the contractile apparatus. By this approach we can exclude interactions with actin, specifically of cross-bridges in weak binding states, which are the result of lost steric relations and thus the loss of possible steric hindrance of myosin head attachment by regulatory proteins as is possible in solution studies on isolated proteins. Initial characterization of the weak binding states of the myosin head had been performed

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under relaxing conditions to avoid active cross-bridge cycling in the presence of ATP, i.e., to avoid generation of a mixture of weak and strong binding states of the myosin head. In later studies, binding of weak binding states not only to inactive but also to activated actin filaments was also studied by using suitable ATP-analogs or, more recently, by using analogs of the ADP.P_i states.

2. WHAT DEFINES WEAK BINDING STATE VS. STRONG BINDING STATE?

Weak binding vs. strong binding is a property of the myosin head that only depends on the state of the bound nucleotide or, more specifically, on the state of the γ-phosphate in the nucleotide binding pocket (cf. Fig. 1). Myosin heads with no γ-phosphate, or no analog of the γ-phosphate, are in strong binding states (strong binding conformation). Myosin heads with ATP (or ATP-analogs like ATPγS or AMPPNP) or with ADP and inorganic phosphate or phosphate analogs in the nucleotide binding pocket are considered weak binding states. The existence of the MDP_i^{II}/$AMDP_i^{II}$ pair had been postulated in earlier biochemical work (e.g., Stein et al., 1979, 1984), and on the basis of studies of release of phosphate from caged phosphate into skinned muscle fibers (Dantzig et al., 1992; Millar and Homsher, 1990). It remained, however, unclear whether it is a typical strong binding state of the actomyosin complex or a weak binding state or even a state with new properties. Recent work on phosphate analogs, reviewed below, allows to characterize and classify this intermediate.

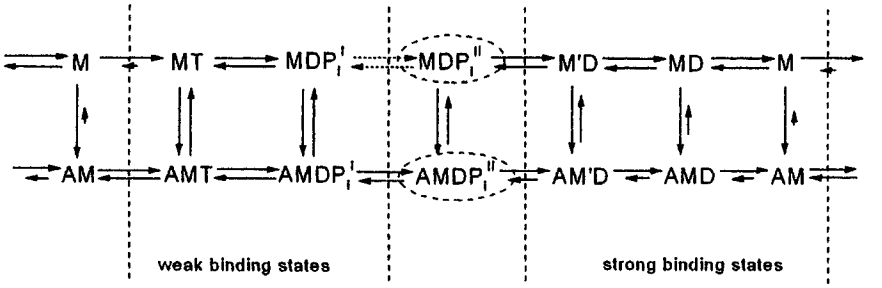

Figure 1. Kinetic Scheme of the actomyosin ATPase with the most important biochemical intermediates. M = myosin, A = actin, T = ATP, D = ADP, P_i = inorganic phosphate. Relative length of arrows qualitatively indicates equilibrium constants of the associated reaction step.

In summary, weak vs. strong binding is a property of the myosin head, and this property is independent on (i) whether the myosin head is bound to actin or not and (ii) whether the actin filament is in an activated form or not.

3. PROPERTIES THAT DISTINGUISH WEAK BINDING STATES OF THE MYOSIN HEAD FROM STRONG BINDING STATES

3.1 Actin affinity

Actin affinity is the property that originally led to the terms 'weak binding state' vs. 'strong binding state' on the basis of biochemical solution studies (e.g., White and Taylor, 1976; Stein et al., 1979).

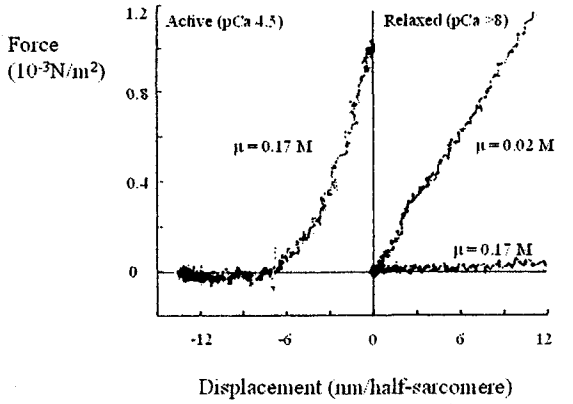

Figure 2. Fiber stiffness of a relaxed skinned fiber of rabbit psoas muscle at low (μ = 0.02M) and high (μ = 0.17M) ionic strength vs. active fiber stiffness during isometric contraction (modified from Brenner et al., 1982).

At a given ionic strength, actin affinity of weak binding states is much lower than actin affinity of strong binding states of the myosin head. In fact, actin affinity in weak binding states is so low that a large fraction of myosin heads in weak binding states is found attached to actin filaments only at low ionic strengths, e.g. at 0.02-0.05M. Thus, attachment of weak binding states to actin was initially detected and characterized by comparison of fiber stiffness at ionic strengths of 0.02M vs. 0.17M (cf. Fig. 2). The difference in stiffness of a relaxed fiber at low vs. high ionic strength was attributed to cross-bridge attachment as this component was found to scale with overlap between actin and myosin filaments (Brenner et al., 1982; Fig. 3). The stiffness seen at high ionic strength was attributed mainly to parallel elastic and viscoelastic components within the skinned fibers. Further support for cross-bridge origin of the stiffness increase seen with lowering ionic strength came later with the observation that the actin binding fragment of the protein caldesmon selectively inhibited this component of fiber stiffness (Brenner et al., 1991; Kraft et al., 1999).

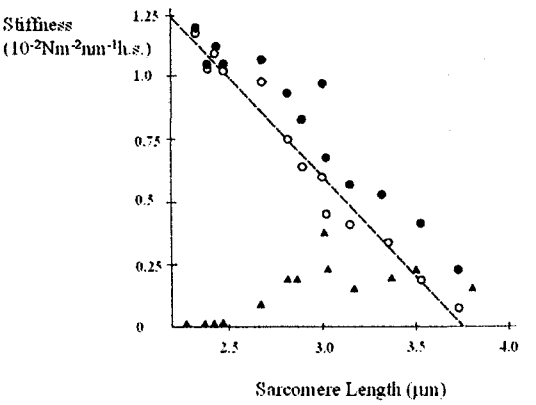

Figure 3. Fiber stiffness at different sarcomere lengths. Filled circles, stiffness at low ionic strength (μ = 0.02M); filled triangles, fiber stiffness at high ionic strength (μ = 0.17M); open circles, difference in fiber stiffness between low and high ionic strengths. Long dashed line, linear least squares fit to data represented by open circles; h.s., half sarcomere (modified from Brenner et al., 1982).

3.1.1. Fraction of myosin heads in weak binding states that is attached to actin under relaxing conditions at near-physiological ionic strength and temperature

The low actin affinity observed for weak binding states in relaxed muscle fibers raised the question of what fraction of time a cross-bridge in a weak binding state (or weak binding conformation) spends attached to an actin filament in a muscle fiber or, related to a large ensemble of myosin heads, which fraction of myosin heads in weak binding states is, at any one moment, attached to an actin filament, including physiological ionic strengths and temperature conditions. This question was addressed by making use of the already mentioned fact that an actin binding fragment of caldesmon can selectively inhibit actin attachment of myosin heads in weak binding states (Brenner et al., 1991). Thus, measuring the component of relaxed fiber stiffness that could be titrated away by addition of the actin binding caldesmon fragment we estimated the fraction of weak binding cross-bridges attached to actin under different experimental conditions. This includes higher temperatures as well as higher ionic strengths where stiffness of relaxed fibers is small. For such conditions we had initially assumed that all of the observed fibers stiffness is due to parallel elastic and viscoelastic components (Brenner et al., 1982). However, using this actin binding caldesmon fragment we found that even at physiological ionic strengths part of the observed fibers stiffness can be titrated away by the actin binding caldesmon fragment (Kraft et al., 1995). For physiological ionic strength (μ = 0.17M) and temperature of 20^{0}C we estimated that about 5% of myosin heads in weak binding states are attached to actin under relaxing conditions, i.e., that under these nearly physiological conditions a myosin head in a weak binding conformation spends about 5% of total time attached to actin (Kraft et al., 1995).

3.2. Kinetics of dissociation from actin

A second property that is distinctly different for weak and strong binding states of the myosin head are the kinetics of interaction of myosin heads with actin, specifically the kinetics for dissociation from actin.

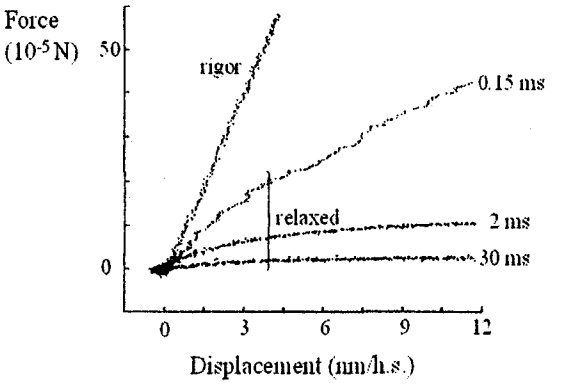

Figure 4. Original plots of force vs. length change recorded during ramp-shaped stretches of different speed imposed to one end of fibers. Number next to each trace recorded under relaxing conditions is time required for stretch by 6 nm/half sarcomere. Note that in rigor the plot of force vs. imposed length change is very insensitive to speed of stretch (c.f. Fig. 5); h.s., half sarcomere. Observed fiber stiffness was defined as the chord stiffness (force increase/imposed length change) when imposed length change had reached 2nm/half sarcomere. Modified from Brenner et al. (1982)

Characterization of the rate constant of myosin head dissociation from actin (k^-) was based on the observation that observed fiber stiffness under essentially all experimental conditions depends on the speed of the length change that is applied to one end of a fiber in order to measure fiber stiffness (Figs. 4, 5). We had shown by model simulations that the observed time course of force response during imposed stretches of different speed (Fig. 4) and the resulting dependence of apparent fiber stiffness on speed of the applied length change (stiffness-speed-profile; Fig. 5) allows to derive the rate constant (probability) of dissociation (k^-) from actin (Brenner, 1990). As a good approximation, the value of k^- can be taken as the value of speed of stretch ((nm/h.s.)/s) where stiffness has fallen to 1/e of its maximum level (Schoenberg 1985). Thus, a shift of the stiffness-speed-profile along the abscissa allows to estimate changes in k^-.

From the stiffness-speed profiles shown in Fig. 5 cross-bridges in relaxed muscle (MgATP, low Ca^{++}) dissociate with a probability of some $10^4 s^{-1}$ while in the presence of pyrophosphate dissociation occurs with some $1\text{-}100 s^{-1}$, in rigor with rate constants << $1/100\ s^{-1}$. Note that to account for the very wide speed dependence of fiber stiffness (much more than 2-3 orders of magnitude) a range of rate constants is required. This is not surprising since due to the mismatch between actin and myosin periodicities in fibers,

attached cross-bridges are strained to different extent. Different strain, however, is expected to result in different rate constants for interaction with actin, e.g. strain-dependence of the rate constant of dissociation (Hill, 1974).

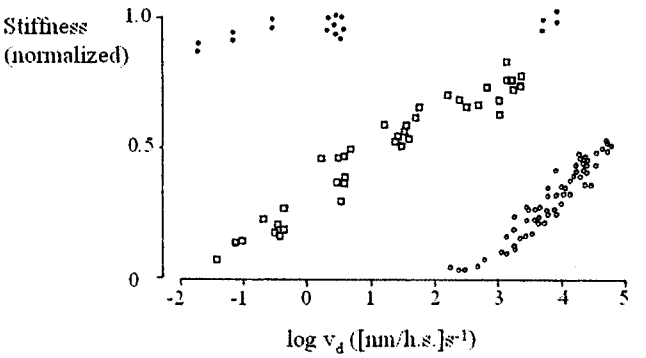

Figure 5. Observed fiber stiffness plotted vs. log of speed of stretch. Use of logarithm of speed of stretch to allow for changes in speed of stretch over several (seven) orders of magnitude. Open circles, relaxing conditions at μ = 0.02M; open squares, in the presence of 4mM Mg-pyrophosphate; filled circles, rigor conditions. (modified from Brenner et al., 1986)

3.3. Affinity for binding to activated vs. inactive actin filaments

A third characteristic difference between myosin heads in a weak vs. strong binding state (weak binding vs. strong binding conformation) is the dependence of actin affinity on interactions of the myosin head with the tropomyosin controlled sites that become accessible for binding when thin filaments are activated, e.g. at high Ca^{++}-concentrations (e.g., Greene and Eisenberg, 1980).

To address this question required an approach that allowed us to study binding of cross-bridges in weak binding states to the activated actin filament while, at the same time, active cross-bridge cycling is prevented. Otherwise, active cross-bridge cycling would result in occupancy of strong binding states by some cross-bridges, i.e., generation of a mixture of weak and strong binding states that makes definite characterization of weak binding states difficult. One approach is to use non-hydrolyzable, or slowly hydrolyzed nucleotide analogs such that strong binding states are not accessible even when thin filaments are activated. As a first example, we used the ATP-analog ATPγS that is only slowly hydrolyzed (Bagshaw et al., 1973; Goody and Hofmann,1980). To examine effects on actin affinity of weak binding cross-bridge states by (additional) interactions with the tropomyosin controlled sites, we compared attachment of myosin heads to actin in the presence of ATPγS, both at high and low Ca^{++}-concentrations by using equatorial intensities of X-ray diffraction patterns. This is based on the observation

that attachment of cross-bridges to actin, both in weak and strong binding states, affects the intensity ratio, $I_{1,1}/I_{1,0}$, of the two innermost equatorial reflections, the 1,0 and 1,1 reflections of X-ray diffraction patterns (cf. Brenner et al., 1984, 1985). Fig. 6 shows that with increasing ionic strength, as fewer and fewer cross-bridges in weak binding states are attached to actin, the $I_{1,1}/I_{1,0}$ ratio continuously decreases, both in the absence and presence of Ca^{++}. In the presence of Ca^{++} the $I_{1,1}/I_{1,0}$ ratio is only slightly higher than in the absence of Ca^{++}. For example, at an ionic strength of 0.17M the increase in the $I_{1,1}/I_{1,0}$ ratio when Ca^{++} is raised is less than the increase in $I_{1,1}/I_{1,0}$ resulting from reduction in ionic strength from 0.017M to 0.12M in the absence of Ca^{++}. Reduction of ionic strength from 0.17M to 0.12M is expected to increase actin affinity of myosin heads about 2-fold (Greene et al., 1983). Thus, in the presence of Ca^{++}, actin affinity of weak binding myosin heads is at most 2-fold larger than in the absence of Ca^{++} (cf. Kraft et al. 1992).

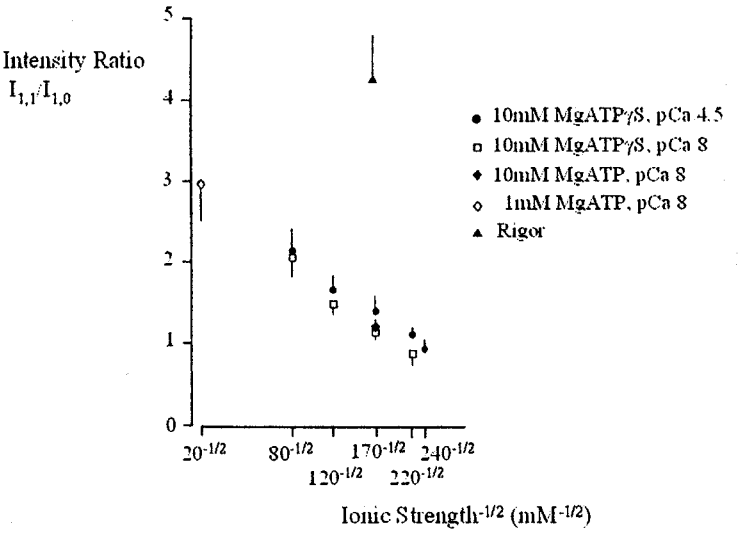

Figure 6. Intensity ratio $I_{1,1}/I_{1,0}$ of X-ray diffraction patterns recorded in the presence of ATPγS at different ionic strengths, both at high and low Ca^{++}. Modified from Kraft et al. (1992)

In conclusion, for weak binding cross-bridge states activation of thin filaments by Ca^{++} has only a rather small (≤ 2 fold) effect on actin affinity. This is quite different from strong binding cross-bridge states, e.g. in the presence of pyrophosphate, where actin binding is strongly affected by Ca^{++}-activation of the thin filaments (Brenner et al., 1986b), for solution studies cf. (Greene and Eisenberg, 1980).

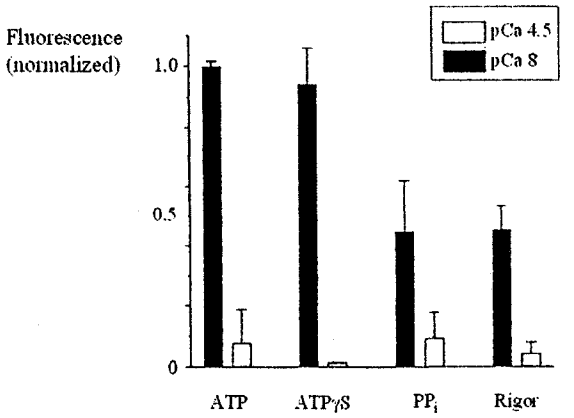

Figure 7. NBD-fluorescence with different nucleotides/nucleotide analogs at high and low Ca^{++}. Note that with ATP and ATPγS fluorescence at low [Ca^{++}] is high while in the presence of pyrophosphate (PP_i) fluorescence is quenched as it is in rigor or when Ca^{++} is raised. Quench of NBD-fluorescence is thus an indicator for activation of thin filaments which is induced by either increased [Ca^{++}] or by rigor cross-bridges or cross-bridges in presence of PP_i, i.e., by actin attachment of strong binding states of the myosin head (modified from Brenner et al., 1999).

3.4. Effect of myosin binding to actin on the state of the thin filament

A fourth characteristic property that allows to distinguish weak binding states of the myosin head from strong binding states is the ability to interfere with the state of the regulatory proteins. This results in activation of the thin filament when strong binding cross-bridges bind to regulated actin filaments at low [Ca^{++}]. This has been demonstrated by the effect of myosin head binding on the fluorescence of NBD-labeled troponin (cf. Brenner et al., 1999; Brenner and Chalovich, 1999). In contrast, attachment of weak binding states of the myosin head to actin filaments does not interfere with regulatory protein function, i.e., does not activate the thin filaments at any ionic strength studied, as is evidenced by the lack of any effect of ionic strength on the fluorescence of NBD-labeled troponin under relaxing conditions (Fig. 8). In conclusion, attachment to actin of cross-bridges in weak binding states does not interfere with regulatory protein function, i.e., does not activate thin filaments, while attachment of cross-bridges in strong binding states activates the thin filament. Note, this property is independent on ionic strength.

This also answers a frequently asked question whether a strong binding cross-bridge state is changed to a weak binding state of the myosin head by raising ionic strength and thus weakening actin affinity or, whether a weak binding state by lowering ionic strength can be transformed into a strong binding state of the myosin head. Since the ability of a strong binding cross-bridge state to activate the thin filament still remains at high ionic strength while the inability of a weak binding cross-bridge state to change the state of activation of the thin filament remains unchanged at low ionic strength, the properties 'weak binding' and 'strong binding', i.e., ability to activate thin filaments or not, do not simply depend on the magnitude of actin affinity but is rather a property that is

independent of ionic strength but rather determined by the state of the nucleotide, specifically of the γ-phosphate.

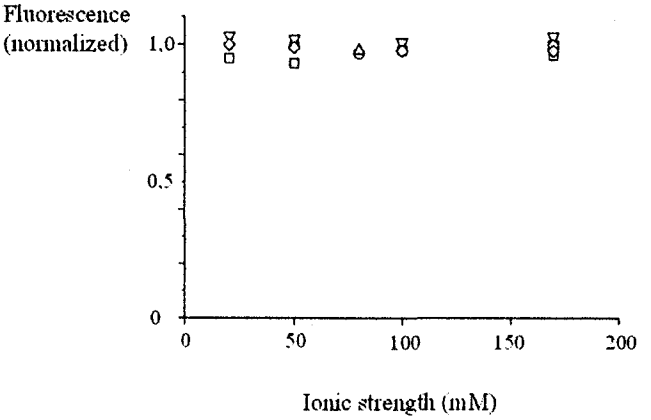

Figure 8. Fluorescence of NBD-labeled troponin when attachment of cross-bridges in weak binding states is increased by lowering ionic strength under relaxing conditions (MgATP, pCa 8). While actin attachment of cross-bridges in weak binding states increases with lowering ionic strength, fluorescence is not quenched as is expected if cross-bridges in weak binding states could interfere with regulatory proteins resulting in (partial) activation of thin filaments (modified from Brenner et al., 1999).

4. THE MODE OF CROSS-BRIDGE ATTACHMENT TO ACTIN: SPECIFIC VS. NON-SPECIFIC BINDING AND STEREOSPECIFIC VS. NON-STEREO-SPECIFIC BINDING

Another frequently raised question concerns the mode of actin attachment of weak and strong binding conformations of the myosin head, specifically, whether attachment of weak binding cross-bridge states to actin is specific or not, i.e., whether it goes to specific sites on actin. We had addressed this question by using the actin binding fragment of caldesmon. Although this is a highly elongated molecule, actin binding of cross-bridges in weak binding states, e.g., relaxing conditions (MgATP, pCa 8) or in the presence of MgATPγS (pCa 8 or pCa 4.5) could be inhibited (Brenner et al., 1991; Kraft et al., 1995). This suggested that attachment of weak binding cross-bridges involves specific sites on actin. From the pronounced ionic strength dependence and the fact that attachment occurs under relaxing conditions it is thought that attachment of cross-bridges in weak binding states involves the electrostatic sites on the actin monomers that were shown not to be controlled by the tropomyosin (e.g. Lehman et al., 1994; 1995). Modeling of 2D-Xray patterns suggested that the attachment of weak binding myosin heads under relaxing conditions mainly involves the N-terminus of actin (Gu et al., 2002). Altogether, actin

binding of myosin heads in weak binding states involves specific sites, i.e., is site-specific.

Table 1. Summary of properties of weak binding states vs. strong binding states of the myosin head

Distinct differences between weak and strong binding states of the myosin head

	weak binding state	strong binding state
actin affinity (at comparable ionic strength)	low	high
actin binding kinetics (rate constant for dissociation)	very fast	much slower
effect of activation of thin filament on actin affinity	small (≤ 2 fold)	large (> 50-100 fold)
ability to activate thin filament	no effect	activates thin filaments even in absence of Ca^{++}

A separate question, however, is whether the actin binding of cross-bridges in weak binding states is also stereospecific, as it is for strong binding cross-bridge states, i.e., that at least part of the attached myosin heads, e.g. the catalytic domain, have a defined orientation relative to the actin monomers. As a result, stereospecific attachment labels the helical features of the actin filament and thus is expected to enhance actin based layer lines in 2D-X-ray diffraction patterns. Such enhancement of actin based layer lines is characteristic for binding in rigor or in the presence of $MgPP_i$ or during isometric contraction. Associated with enhancement of the actin based layer lines is weakening or loss of the myosin based layer lines. We therefore tested the question about stereospecific vs. non-stereospecific binding of myosin heads in weak binding states by recording 2D-X-ray diffraction patterns under relaxing conditions at high and low (0.05M) ionic strengths (Xu et al., 1997; Kraft et al., 1999) where in the latter condition many more of the myosin heads in the weak binding states are attached to actin. No enhancement of actin based layer lines was found nor did we see much change in the myosin based layer lines. Lack of enhancement of actin based layer lines suggests that while the actin attachment of cross-bridges in weak binding states goes to specific sites, it is non-stereospecific, i.e., the attached heads do not have a fixed orientation relative to the actin

monomers to which the heads are attached and thus do not have a fixed orientation relative to the actin helix.

Next we asked the question whether actin attachment of cross-bridges in weak binding states might become stereospecific when the actin filament is activated by Ca^{++} (cf. Holmes, 1995) or when the actin filament has changed to its closed form by lowering ionic strength below 0.05M, e.g. to 0.03M (Head et al., 1995; Geeves and Conibear, 1995). Lowering ionic strength to 0.03 M under relaxing conditions (MgATP, pCa 8) did not enhance actin based layer lines nor were the myosin based layer lines much weakened (Kraft et al., 1999). In the presence of MgATPγS as an ATP-analog to avoid turnover at high calcium, a small enhancement of the 5.9nm actin based layer line was found when Ca^{++} was raised to pCa 4.5. This small enhancement was somewhat larger than the enhancement of the 5.9nm actin based layer line when fibers were first stretched beyond filament overlap, i.e., when the observed enhancement can not be due to cross-bridge attachment but instead is presumably due to changes in the thin filaments by Ca^{++}-activation. The small additional enhancement on the 5.9nm actin based layer line in full overlap might indicate a small actin labeling effect, e.g. due to some restriction of the otherwise highly variable angles of attachment when myosin heads in weak binding states also interact with the tropomyosin controlled sites at high Ca^{++} (Kraft et al., 1999).

Thus, when the actin filament is activated by Ca^{++} but not by lowering ionic strength to 0.03M, a slight restriction of the non-stereospecific binding of myosin heads in weak binding states is observed.

This is very different from cross-bridges in strong binding states where attachment is stereospecific, i.e., the attached myosin heads have a quite fixed orientation relative to the actin monomers and thus to the actin helix. This results in quite prominent enhancement of actin based layer lines when such cross-bridges attach to actin, e.g. in rigor (Kraft et al., 1999, 2002), in the presence of ADP or during isometric contraction (Kraft et al., 2002).

In summary, myosin heads in weak binding states bind to specific sites on actin but non-stereospecifically when the actin filament is inactive but with some restriction of non-stereospecific binding when actin filaments are activated by Ca^{++}.

4. INTERACTION OF MYOSIN HEADS IN WEAK BINDING STATES WITH THE TROPOMYOSIN CONTROLLED BINDING SITES ON ACTIN

Since there is only a small effect of activation of thin filaments on the intensity ratio of the two innermost equatorial reflections, $I_{1,1}/I_{1,0}$, of X-ray diffraction patterns (Fig. 6) the question may be raised whether myosin heads in weak binding states (weak binding conformation) are at all able to interact with the tropomyosin controlled binding sites on actin that become accessible at high Ca^{++}-concentrations. That myosin heads in weak binding states indeed interact with these sites at high Ca^{++}-concentrations is suggested by the slight enhancement of the 5.9nm actin based layer line. Interactions with the tropomyosin controlled sites, however, are much more evident when fiber stiffness is plotted vs. speed of applied stretch for fibers incubated with MgATPγS both at low and high Ca^{++}-concentrations (Fig. 9). As a result of interactions with the tropomyosin controlled binding sites on actin at high Ca^{++}, the stiffness speed relation at high Ca^{++} is

shifted by more than 2 orders of magnitude to slower stretch velocities. This indicates more than 100-fold slower probability of dissociation from actin at high Ca^{++} compared with low Ca^{++}-concentrations (Kraft et al., 1992).

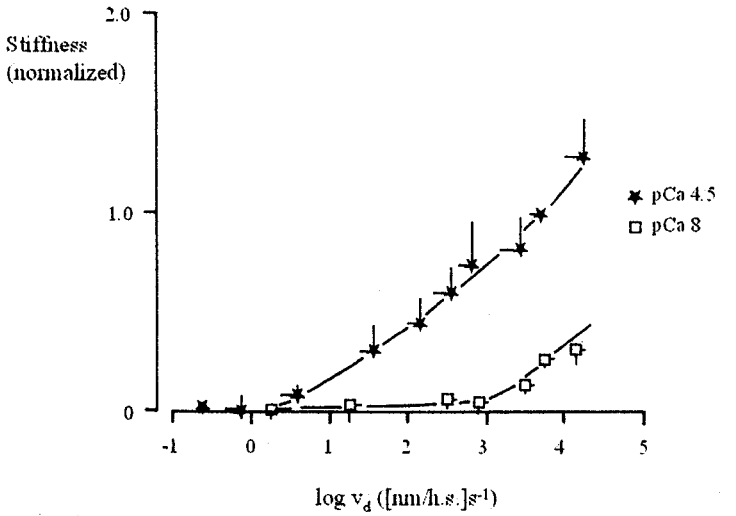

Figure 9. Stiffness speed relations obtained in the presence of 10 mM MgATPγS at low and high Ca^{++}-concentrations. Ionic strength 0.075M, temperature -3°C. Note that 10mM MgATPγS and low temperature were needed to keep myosin heads saturated with MgATPγS at high Ca^{++}- concentrations (cf. Fig. 10); modified from Kraft et al., 1992.

Somewhat surprisingly, however, we found that to keep myosin heads fully saturated with MgATPγS also at the high Ca^{++}-concentrations we had to (i) increase the concentration of MgATPγS to 10-20mM and (ii) lower temperature had to –3°C, the lowest possible temperature without freezing of the solution. In contrast, in the absence of Ca^{++} about 50μM of MgATPγS were sufficient to keep myosin heads saturated at otherwise identical ionic and temperature conditions (Fig. 10).

5. PHYSIOLOGICAL SIGNIFICANCE OF ACTIN ATTACHMENT OF MYOSIN HEADS IN WEAK BINDING STATES

All these data imply that although the myosin heads in weak binding states can make additional interactions with tropomyosin controlled sites at high Ca^{++}-concentrations, actin affinity is increased at most 2-fold, while at the same time the nucleotide affinity of the weak binding myosin heads when interacting with these additional sites is lowered about 1000-fold. We have interpreted this (Brenner et al., 1998, 1999) as an indication that a myosin head in a weak binding state becomes distorted when also interacting with

the tropomyosin controlled sites, thus (i) the increase in actin affinity is almost absent despite the additional interactions between myosin head

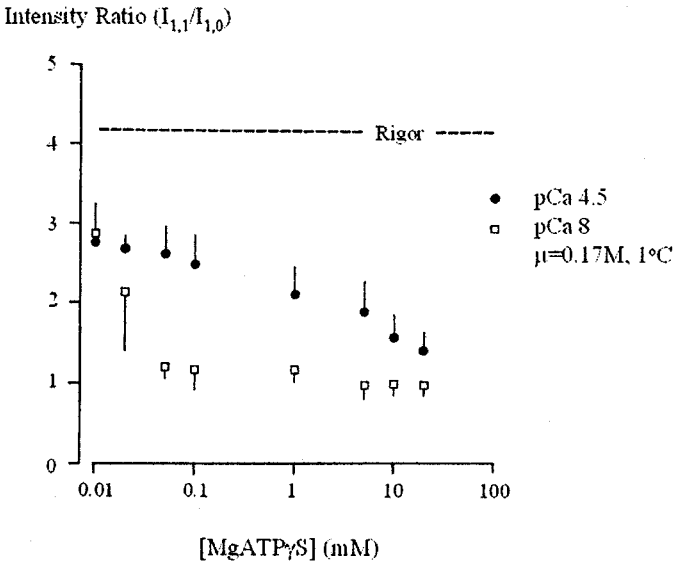

Figure 10. Intensity ratio $I_{1,1}/I_{1,0}$ of X-ray diffraction patterns recorded in the presence of different concentrations of ATPγS both at high and low Ca^{++}-concentrations. Note that in the presence of Ca^{++} the $I_{1,1}/I_{1,0}$-ratio as a measure of nucleotide-free cross-bridges decreases up to the highest nucleotide concentrations, just about approaching the value in the absence of Ca^{++} at 20mM of ATPγS, while in the absence of Ca^{++} a constant $I_{1,1}/I_{1,0}$-ratio is reached already at 50μM ATPγS (modified from Kraft et al. 1992).

and actin in the presence of Ca^{++}, and (ii) the nucleotide affinity is very much weakened. This behavior was also found for MgGTP and MgAMPPNP (Frisbie, et al., 1997), i.e., for molecules with a covalently bound γ-P_i. Moreover, we also found a weakening of AlF_4 trapped in the active site during active contraction forming an MgADP.AlF_4 complex (Chase et al., 1993), i.e., AlF_4 could be untrapped simply by activating the thin filaments by high Ca^{++} but in the presence of ATPγS which prevents active cross-bridge cycling and force generation that had previously been postulated to be required to untrap AlF_4 from the active site (Chase et al., 1993). Since we have identified the myosin head with the MgADP.AlF_4 complex formed during active cross-bridge cycling to be an analog of the weak binding conformation, the weakening of the AlF_4 simply by binding of the myosin head to the activated thin filament implies that interaction with the tropomyosin controlled sites on actin has a profound effect on the weak binding conformation of the myosin head. This results in weakening of (i) the nucleotide/nucleotide analog when the γ-phosphate (or γ-phosphate analog) is still covalently bound to the ADP moiety or (ii) of the AlF_4 trapped in the active site during active turnover. If the weakening effect seen with the AlF_4 also applies for P_i in the corresponding MgADP.P_i-complex, attachment of the weak binding states of the myosin head to the activated thin filament destabilizes the

γ-phosphate, i.e., initiates phosphate release. In other words, attachment of weak binding myosin heads to activated actin and its profound effects on the weak binding conformation of the myosin head appears to be responsible for the actin activation of the myosin ATPase as actin activation is the result of faster release of inorganic phosphate from the active site in the presence of activated actin.

In summary, our data suggest that interaction of myosin heads in weak binding states with the activated thin filament (tropomyosin controlled sites) has a profound effect on the weak binding conformation of the myosin head leading to destabilization of the γ-P_i in the active site, i.e., speeding up P_i-release and thus resulting in actin activation of the myosin ATPase.

6. DISTORTION OF THE WEAK BINDING CONFORMATION OF THE MYOSIN HEAD UPON ATTACHMENT TO ACTIVATED THIN FILAMENTS DOES NOT RESULT IN A GLOBAL STRUCTURAL CHANGE.

As already pointed out, 2D-Xray diffraction patterns recorded when myosin heads in their weak binding conformation (weak binding states) attach to actin both at high and low Ca^{++} show no large change in the myosin based layer lines and only a slight enhancement of the 5.9nm actin based layer line, much less than seen with stereospecific attachment of myosin heads in strong binding states (Kraft et al., 1999). This implies that although generating some distortion of the catalytic domain of the attached myosin head in the weak binding conformation (weakening of nucleotide affinity, untrapping of AlF_4, almost no enhancement of actin affinity despite additional interactions with actin), interaction with the tropomyosin controlled sites does not induce a global structural change in the weak binding conformation of the myosin head domain. One might speculate that the distortion in the weak binding conformation of the myosin head generated by the interactions with the tropomyosin controlled sites may be a kind of 'forced' closure of the cleft between upper and lower 50kDa domain with destabilization of the γ-phosphate.

In contrast, binding of myosin heads in strong binding states to the activated thin filament results in very little difference in nucleotide affinity but in large increase in actin affinity (Brenner et al., 1986b) consistent with little if any distortion of the myosin head domain upon additional binding to the tropomyosin controlled sites. This might be accounted for by the cleft between upper and lower 50kDa domain being easily closed upon interaction with the tropomyosin controlled sites at high Ca^{++} when the γ-phosphate or γ-phosphate analogue is released or at least displaced (strong binding conformation of myosin heads) while such cleft closure meets large resistance when the γ-phosphate or γ-phosphate analogue is still strongly coordinated in the nucleotide binding pocket (weak binding conformation of myosin head).

Aside from the detailed structural properties, myosin heads in a weak vs. strong binding conformation apparently have quite different conformation when attached to activated thin filaments. This implies that upon binding to activated thin filaments, myosin heads in these two conformations will pass through different intermediates, i.e., will not even transiently pass through identical intermediates as postulated by the concept of Geeves et al. (1984; McKillop and Geeves, 1993).

7. RELATION OF WEAK AND STRONG BINDING STATES OF THE MYOSIN HEAD TO THE POWER STROKE

Based on the effects of release of phosphate from caged phosphate on force generation under isometric conditions it had previously been postulated that phosphate is released in a two step process. It was further postulated that the second M.ADP.P_i-intermediate contributes to fiber stiffness and possibly to active tension (Dantzig et al., 1992). During steady state force generation this state, however, is at most only a transient intermediate with little occupancy. Thus, it had so far not been possible to clearly characterize and classify this intermediate in terms of weak vs. strong binding properties.

In a recent attempt we succeeded to generate an analog state of this second M.ADP.P_i-intermediate (cf. Fig. 1) by trapping AlF_4 in the active sites of myosin heads that were bound to actin in the presence of MgADP (Mattei et al., 2004; Kraft et al., 2004). In contrast to the analog state in which AlF_4 had been trapped in the active site during active cross-bridge cycling (Chase et al., 1993) and which shows all properties of weak binding states of the myosin head summarized above, cross-bridges with the ADP.AlF_4-complex trapped in the active site by the second approach show all the features characteristic for strong binding states of the myosin head. Most characteristically, this second ADP.AlF_4 analogue state shows (i) a much larger actin affinity when binding to the activated thin filament (high Ca^{++}) compared to binding to the inactive thin filament (low Ca^{++}), (ii) much slower dissociation from activated thin filaments, and (iii) stereospecific binding to the activated thin filament as evidenced by enhanced actin based layer lines. Thus, assuming analogy for the second M.ADP.P_i-intermediate, these data suggest that the second ADP.P_i-analog is the first strong binding state of the myosin head. It has neither intermediate nor new properties other than those already characterized for the weak vs. strong binding conformation of the myosin head.

Very interestingly, trapping essentially all myosin heads in this second ADP.AlF_4 state results in an 2D-Xray pattern very reminiscent of the pattern observed under isometric conditions for which we had provided evidence that a large fraction of myosin heads are in a strong binding conformation (Kraft et al., 2002). The similarity of these 2D-patterns supports this point and is also consistent with our conclusion that during isometric force generation a large fraction of cross-bridges occupy states early in the power stroke (Brenner et al., 1995).

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