ADP binds similarly to rigor muscle myofibrils and to actomyosin-subfragment one

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The binding of Mg^{2+} ADP to both rabbit skeletal and bovine cardiac myofibrils has been studied at two different temperatures. In each case a single class of binding sites was observed with a binding constant very close to that reported for the analogous actomyosin-subfragment one but much weaker than that seen with the analogous myosin subfragment one alone. These findings are discussed in terms of the constraints on the myosin cross-bridges imposed by the regular array of thick and thin filaments found in myofibrils.

Bovine cardiac myofibril *Rabbit skeletal myofibril* *Actomyosin ATPase* *Mg^{2+} ADP binding* *Actomyosin-subfragment one* *Cross-bridge cycle*

1. INTRODUCTION

Rabbit skeletal muscle rigor myofibrils [1,2] and glycerinated fibers [3,4] have been found to have at least 95% of their myosin heads attached to actin despite the steric constraints imposed by the mismatch in the thick and thin filament helical parameters [5,6]. It has also been shown that addition of ADP changes neither the X-ray diffraction pattern [7] nor the stiffness [8] of glycerinated rabbit muscle fibers in rigor. Therefore ADP binding can be thought of as a non-perturbing probe of the state of the myosin heads in muscle tissue or tissue fragments such as myofibrils in which the basic filament structure of muscle is intact. Actomyosin-subfragment one, or 'decorated actin' is a synthetic mixture of purified actin and subfragment one (S-1) of myosin. S-1 is made by chymotryptic digestion of solubilized myosin and consists of just the globular 'head' of myosin and contains both the nucleotide and actin binding sites but lacks the larger, rod-shaped portion which aggregates to form the thick filament.

We have developed a centrifuge method to measure the binding of Mg^{2+} ADP to both cardiac and skeletal rigor myofibrils and have found that greater than 90% of the ADP binding sites in myofibrils are of a single class and display the weaker binding characteristic of acto-myosin relative to myosin alone [9]. Moreover, the binding constant of ADP to both types of myofibrils is very close to the binding constant of ADP to the respective acto-myosin subfragments one (acto-S1s), even though the entire thick filament is missing in these proteolytic fragments of myosin, and the binding constants differ by a factor of 100 between muscle types. We conclude that not only are most of the myosin heads in cardiac and skeletal rigor myofibrils attached to actin but also their nucleotide binding sites and perhaps their overall conformation is not grossly different from that of acto-S-1.

2. MATERIALS AND METHODS

Myofibrils were prepared from either bovine ventricals or rabbit back muscles by the method of [10] and stored before use in 50% glycerol at $-20°C$. They appeared to be fully overlapped in the phase-contrast microscope. ADP (dicyclohexyl ammonium salt) and Ap_5A were obtained from Sigma.

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Binding experiments were performed either on ice or at room temperature by mixing 1 ml ADP solution with approx. 4.5 ml of myofibril suspension (approx. 30 mg/ml protein) in a centrifuge tube, spinning at 15000 rpm for 15 min, and measuring the A_{259} of the supernatant relative to a blank containing no ADP. Control experiments showed that the apparent degree of ADP binding increased 10% per h, presumably due to breakdown of the Ap_5A-myokinase inhibitor. The binding experiments were finished in 15 min or less.

Myosin content was determined by comparison of known amounts of column-purified rabbit skeletal myosin with myofibrils dissolved in 5% SDS using electrophoresis in 5% polyacrylamide gels in buffer containing 1% SDS and scanning the intensity of the myosin heavy chain band.

Myofibrils dissolved in 5% SDS and containing 1 mg/ml myosin were found to have an A_{280} (corrected for light scattering) of 1.624 for skeletal and 2.38 for cardiac.

Purified myosin was assumed to have an A_{280} = 5.3 and M_r = 470000. The concentration of ADP was calculated using ϵ_{259} = 15400.

3. RESULTS

We developed a centrifuge method for measuring the binding of nucleotides to myofibril suspensions which is essentially the same as that used in [11] to measure binding to acto-S1. Fig.1 shows the results of titrating cardiac and skeletal myofibrils with Mg^{2+} ADP.

We were able to fit the cardiac myofibril data by assuming that a single class of independent binding sites have a K_d = 14 μM at 23°C (table 1). This is considerably larger than the dissociation constant from purified cardiac myosin S-1 [14] but is the same as the constant found for cardiac acto-S1 [12]. The dissociation constant is moderately sensitive to temperature, decreasing to 3 μM at 0°C.

ADP binding to skeletal myofibrils is considerably weaker than to cardiac myofibrils and a linear term for non-specific binding was required to fit the data at high ADP concentrations in addition to a single class of independent binding sites. Because of this term, the fitted parameters have considerably more error than the parameters for cardiac myofibrils but the same basic conclusions

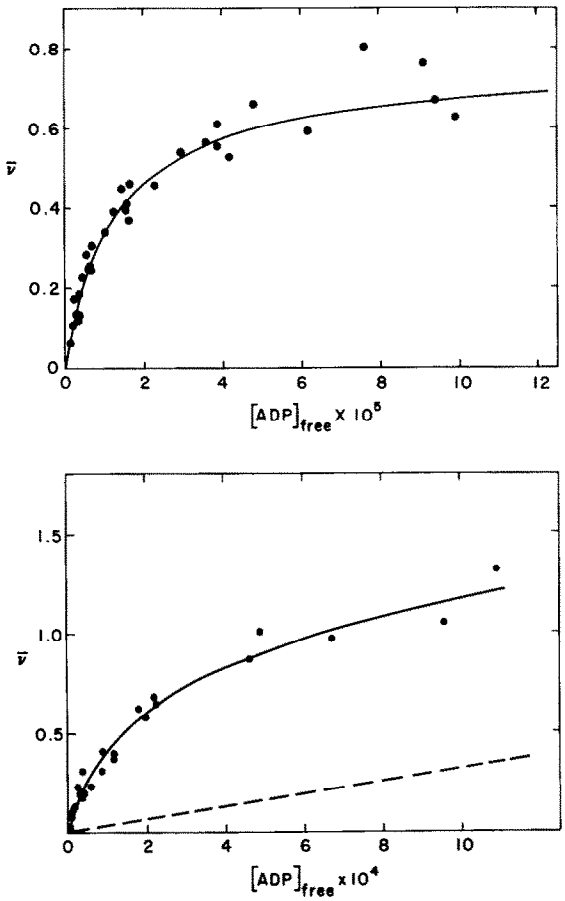

Fig.1. Fraction of myosin heads containing bound ADP at 23°C as a function of free Mg^{2+} ADP in A, beef cardiac rigor myofibrils, and B, rabbit skeletal rigor myofibrils. Solid lines are the calculated fit assuming a single class of non-interacting binding sites, using the parameters listed in table 1 which were determined by a non-linear least-squares fitting program. The dashed line is the linear term representing non-specific binding which was needed to fit the data at high ADP concentrations. Conditions: 80 mM KCl, 30 μM Ap5A, 5 mM Hepes (pH 7.5), 10^{-4} M $CaCl_2$, 5 mM $MgCl_2$, 2 mM NaN_3.

can be drawn from the data: (i) greater than 90% of the myosin heads appear to bind ADP with a K_d = 170 μM at 23°C. (ii) This dissociation constant is over two orders of magnitude weaker than that seen with free skeletal S-1 [13]. (iii) The constant is essentially the same as that seen with skeletal acto-S1 [11]. Unlike cardiac myofibrils, however, there appears to be almost no temperature dependence of this binding constant.

Table 1

Binding affinity of Mg^{2+} ADP for myofibrils, acto-S1 and S-1

	K_d (μM)	Temperature (°C)	mol bound ADP/ mol myosin head	Reference
Myofibril				
Skeletal	170 ± 50	23	1.0 ± 0.3	Here
	120 ± 40	0	1.0 ± 0.3	Here
Cardiac	14 ± 3	23	0.76 ± 0.07[a]	Here
	2.8 ± 0.4	0	0.77 ± 0.02[a]	Here
Acto S-1				
Skeletal	140	22	–	11
	100	4	–	11
Cardiac	5 ± 1	15	–	12
S-1				
Skeletal	1.6	23	–	13
	0.4	0	–	13
Cardiac	0.7	20	–	14

[a] With cardiac myofibrils there is a well-defined end point at 77% of the calculated amounts of myosin heads. This could be interpreted to mean that some of the myosin is inactive or inaccessible to ADP, but it could also reflect any error in determining the myosin content of myofibrils, such as the assumption that cardiac and skeletal myosin heavy chains bind equal amounts of dye in polyacrylamide gels. Titrations of purified myosin usually yield a stoichiometry around 0.8 rather than 1.0 bound nucleotide per myosin head [9,15,16]

4. DISCUSSION

Although we are unaware of any previous measurements of ADP binding to myofibrils, ADP binding to rabbit skeletal fibers has been measured by the author in [8] to be about 40 μM. However, the presence of myokinase activity, which he recognized as a possible source of error, could easily have lowered the apparent binding constant by a factor of 4. We have found it necessary here to use a myokinase inhibitor, Ap5A, to prevent contraction. In a control experiment, the inhibitor was found not to bind significantly to myofibrils. Authors in [17] measured the dissociation constant of ϵ ADP (a fluorescent analog of ADP) from glycerinated rabbit skeletal muscle fibers to be 350 μM in the presence of 100 μM Ap5A.

If some myosin heads in fully overlapped rigor myofibrils were unattached to actin for steric reasons, one might expect to observe two classes of ADP binding sites, one stronger, reflecting the unattached heads, and one weaker, representing the attached heads. The fact that greater than 90% of the ADP binding is of only one class to both types of myofibrils is further confirmation of reports [1–4] that essentially all myosin heads are attached to actin in the rigor state.

Since nearly all the cross-bridges in myofibrils are attached to actin thin filaments despite the mismatch of helical parameters and steric constraints, it has been postulated [4] that some flexible element in the thick filament or the cross-bridge may allow the preferred orientation of the cross-bridge to be achieved with perhaps some strain being generated by bending or stretching. This 'strain' would be related to the position *vs* free energy diagram that has been developed in [18] as a thermodynamic model for the cross-bridge cycle. The close similarity of the ADP binding constants of myofibrils and acto-S1, shown in table 1, is surprising because in acto-S1 the entire thick filament has been removed with no apparent effect of nucleotide binding. The primary effect of this removal would be to relieve any strain that the

cross-bridges are experiencing. Therefore, our myofibril data can be interpreted in two ways: (i) the cross-bridges are not under enough strain to change significantly the ADP binding site, or (ii) the ADP binding site remains unchanged as long as the cross-bridge remains attached to the thin filament, regardless of the presence or absence of strain. In support of the second possibility, it has been shown [19] that the conformation of a portion of the cross-bridge near the actin binding site does not change when stress is applied to a rabbit rigor muscle fiber. More recently, authors in [20] have presented preliminary evidence that the orientation of a spin-labeled ADP analogue at the nucleotide-binding site of a rabbit muscle fiber does not change with tension.

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