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The effect of troponin C removal on the Ca^{2+}-sensitive binding of Mg^{2+}AMPPNP to myofibrils

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It was previously shown that when rabbit skeletal myofibrils are titrated with Mg^{2+}AMPPNP under conditions that result in the dissociation of cross-bridges from the thin filaments (i.e. 50% ethylene glycol, 0°C), Ca^{2+}-sensitive, biphasic binding is observed. These titrations have been repeated using myofibrils from which the troponin C has been selectively removed. The disappearance of both Ca^{2+} sensitivity and biphasic binding is taken as evidence that the Ca^{2+} sensitivity is due to Ca^{2+} binding to troponin C and the biphasic binding of Mg^{2+}AMPPNP observed in intact myofibrils is not due to packing constraints or steric hindrance.

Myofibril; Troponin C; Actin-myosin interaction; AMPPNP

1. INTRODUCTION

The study of the binding of cross-bridges to thin filaments in intact muscle fibers and myofibrils has been made possible by the recent discovery of conditions that cause dissociation under equilibrium. Two sets of conditions have recently been described which weaken the cross-bridge–thin filament interaction sufficiently to allow ATP analogs to substitute for ATP. In 200–240 mM KCl, titration with pyrophosphate results in complete or nearly complete dissociation at about 4 mM $MgPP_i$ [1,2], although there is some danger of dissolving the thick filament. Tregear et al. [3,4] have found that in 50% ethylene glycol, 1 mM Mg^{2+}AMPPNP causes complete relaxation and loss of tension of rabbit psoas fibers, indicative of cross-bridge dissociation. All three groups found the equilibrium dissociation to be sensitive to the presence of Ca^{2+}, and in two of the laboratories [1,4] some evidence was obtained for cooperative interactions among the cross-bridges.

I have recently reported a study of the titration of rabbit skeletal myofibrils with Mg^{2+}AMPPNP in 50% ethylene glycol at 0°C [5], in which I observed Ca^{2+}-sensitive biphasic binding. Because the myosin cross-bridges were in rigor at the beginning of the titration and at the end point were no longer attached to thin filaments [3], the observed apparent thermodynamics of binding of Mg^{2+}AMPPNP actually included the thermodynamics of dissociation of the cross-bridges. Unfortunately, in my system, I could not directly dissect out the nucleotide-binding term, but I did attempt to use Mg^{2+}ADP binding as a model of nucleotide binding that is not coupled to dissociation to show that the biphasic binding and Ca^{2+} sensitivity that I observed with Mg^{2+}AMPPNP binding were probably due to an effect on cross-bridge dissociation rather than on nucleotide binding. Two difficulties of interpretation, however, were (i) the Ca^{2+} effect could be unrelated to the binding of Ca^{2+} to the troponin-tropomyosin complex and hence have no connection with the Ca^{2+} control of muscle contraction, and (ii) the biphasic

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Abbreviations: S-1, myosin subfragment 1; AMPPNP, adenosyl-5′-yl imidodiphosphate; Ap_5A, P^1,P^5-di(adenosine 5′)-pentaphosphate

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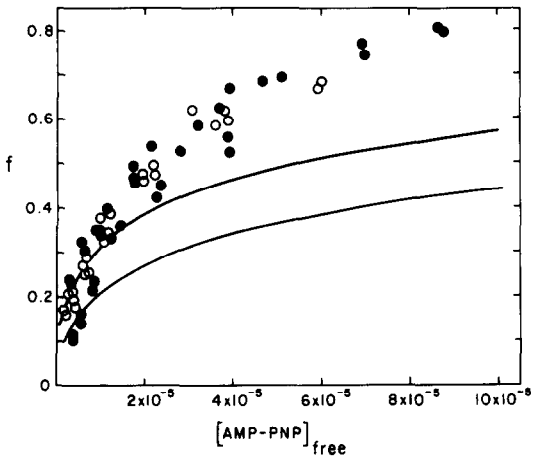

Fig.1. Fraction of myosin heads calculated to be in troponin C-extracted rabbit skeletal myofibrils containing bound Mg^{2+}AMPPNP at 0°C in 50% ethylene glycol as a function of free Mg^{2+}AMPPNP concentration. (○) With 10^{-4} M $CaCl_2$, (●) with 10^{-4} M EGTA. The upper curve represents the amount of Mg^{2+}AMPPNP that would be bound to intact myofibrils in the absence of Ca^{2+}, the lower curve corresponding to that in its presence.

binding could also be explained by the presence in rigor myofibrils of two or more classes of cross-bridge dissociation rather than on nucleotide binding. Two difficulties of interpretation, however, order to clarify the situation, I have repeated the Mg^{2+}AMPPNP-binding experiments using myofibrils from which the troponin C has been selectively removed.

2. MATERIALS AND METHODS

Myofibrils were prepared from rabbit back muscles [6], stored before use in 50% glycerol at −20°C and appeared to be fully overlapped in the phase-contrast microscope. Ap_5A was obtained from Sigma and used without purification. AMPPNP was obtained from Sigma and Boehringer Mannheim and was purified as described [5].

Nucleotide binding was measured by a centrifuge method according to [5,7] in 50% (v/v) ethylene glycol solutions using myofibril suspensions containing about 20 mg/ml total protein. The myosin content of myofibrils and nucleotide concentrations were determined as in [5].

Troponin C was removed from myofibrils by extraction at 0°C in 2 mM Tris-EDTA (pH 7.8) for 24 h [8]. Disc-gel electrophoresis indicated that only the troponin C band was missing. Protein determinations indicated that about 10% of the protein was gone. The extraction was performed at 0°C to avoid extraction of myosin light chain 2 [9].

3. RESULTS

Fig.1 shows the results of titrating troponin C-depleted myofibrils with Mg^{2+}AMPPNP in the presence and absence of 10^{-4} M $CaCl_2$. Table 1 compares the fitted parameters for Mg^{2+}AMPPNP binding to troponin C-depleted myofibrils with the previously published data for intact myofibrils [5]. The intact myofibrils show a 3-fold tightening of Mg^{2+}AMPPNP binding in the

Table 1

Fitted parameters of binding of Mg^{2+}ADP and Mg^{2+}AMPPNP to rabbit skeletal myofibrils at 0°C in 50% ethylene glycol

Nucleotide	Myofibril type	Buffer[a]	$K_{1/2}$[b] (μM)	Hill coefficient	Bound nucleotide/ myosin head at saturation
Mg^{2+}ADP	intact[c]	Ca^{2+}	360 ± 50	1.0 ± 0.1	0.8
Mg^{2+}AMPPNP	intact[c]	EGTA	22 ± 1	0.60 ± 0.03	0.8
Mg^{2+}AMPPNP	intact[c]	Ca^{2+}	80 ± 14	0.6 ± 0.1	0.8
Mg^{2+}AMPPNP	no troponin C[d]	EGTA	27 ± 9	1.08 ± 0.12	0.9
Mg^{2+}AMPPNP	no troponin C[d]	Ca^{2+}	21 ± 3	1.04 ± 0.10	0.9

[a] Buffer: 80 mM KCl, 30 μM Ap_5A, 5 mM Hepes (pH 7.5 before glycol), 10^{-4} M $CaCl_2$ or EGTA, 5 mM $MgCl_2$, 2 mM NaN_3

[b] Concentration of nucleotide at which there is half-saturation

[c] From [5]

[d] This work

Values (± SD) of parameters were determined by a nonlinear least-squares computer program to give the best fit of the data to an equation of the form $f = f_0C^n/(K_d + C^n)$ where C is free AMPPNP concentration and n the Hill coefficient

absence of Ca^{2+} and exhibit biphasic binding with a Hill coefficient of 0.6 in both the presence and absence of Ca^{2+}. The binding to troponin C-depleted myofibrils, however, is neither Ca^{2+}-sensitive nor biphasic, though the binding constant itself is about the same as for intact myofibrils in the absence of Ca^{2+}.

4. DISCUSSION

The loss of Ca^{2+} sensitivity of Mg^{2+}AMPPNP binding with troponin C removal indicates that it is the binding of Ca^{2+} to troponin C which effects the binding of Mg^{2+}AMPPNP to intact myofibrils rather than to myosin light chains or to some other location in the myofibril.

Methods for making troponin C-depleted myofibrils and fibers have only recently been described [10] and the effects of troponin C removal have not been characterized in detail; however, basically, these fibers and myofibrils are frozen in the 'off' state observed with intact muscle in the absence of Ca^{2+} and have only low ATPase activity. The easy removal of troponin C from fibers and myofibrils has allowed the substitution of different troponins C, such as from cardiac or smooth muscle or a labeled troponin C. The use of labeled troponin C allows for precise measurement of Ca^{2+} binding to only the troponin C-binding sites [11]. One of the results of such experiments has been the reconfirmation of the finding of Bremel and Weber [12] that Ca^{2+} binding to troponin C is linked to the status of the cross-bridges. Guth and Potter [13] found that the K_a for Ca^{2+} changed by a factor of two for overlapped vs non-overlapped fibers and by a factor of ten between rigor and activated fibers. Since Mg^{2+}AMPPNP binding under my conditions is also linked to dissociation, it is perhaps not surprising that their mutual linkage to cross-bridge dissociation would make Mg^{2+}AMPPNP binding Ca^{2+}-sensitive.

One could argue that the effect of troponin C removal is simply to break the linkage between Mg^{2+}AMPPNP binding and cross-bridge dissociation so that the cross-bridges remain attached, even in 1 mM AMPPNP. However, Mg^{2+}AMPPNP binds only very weakly to attached cross-bridges [14] so that a drastic change in the binding constant would be expected if there were no dissociation occurring.

Biphasic binding was observed in my previous paper [5] on the binding of Mg^{2+}AMPPNP to intact myofibrils. At that time it was not possible to rule out the existence of two or more classes of binding sites caused by steric constraints as opposed to cooperativity between identical or potentially identical binding sites. The present data, however, tend to rule out the possibility of physically different classes of sites, since it is difficult to imagine how merely the removal of troponin C could eliminate the difference between the classes.

Greene and Eisenberg [15] and Geeves and Halsall [16] have studied the equilibrium binding of myosin subfragment 1 (S-1) to regulated F-actin in the presence and absence of nucleotides. They observed that under some conditions both Ca^{2+} sensitivity and positive cooperativity occurred, neither of which is seen when S-1 binds to naked F-actin. These effects are believed to be related to the mechanism by which troponin and tropomyosin regulate the contraction process, and a model explaining these data in terms of an 'off' and 'on' conformation of the actin-tropomyosin-troponin complex has been developed [17]. The Ca^{2+} sensitivity and cooperativity of cross-bridge–thin filament interactions observed by us and others in fibers and myofibrils may be related to this model and future work will attempt to test this possibility.

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