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NUCLEOTIDE BINDING TO MYOSIN IN CALCIUM ACTIVATED MUSCLE

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Summary

The nucleotides bound to calcium-activated muscle fibres or myofibrils were separated and estimated. Most of the bound nucleotide was found to be ADP, as in relaxed muscle. The maximum ADP binding was not significantly altered by activation but the dissociation constant of the bound ADP was slightly reduced. These results are discussed in terms of the possibility of formation of a ternary ADP—myosin—actin complex.

In our previous paper evidence was given that the dominant steady state intermediate of myosin ATPase in relaxed glycerol extracted muscle is a myosin—ADP—P_i complex [1,2]. In this report the study is extended to activating conditions.

Single glycerol extracted rabbit psoas muscle fibres were incubated with 160 μM [^{14}C]ATP; mannitol was used as a volume marker and an ATP regenerating system was included in the medium. The free calcium concentration was maintained at 1.1 μM by a CaEGTA buffer. After 2 min incubation the fibres were transferred to trichloroacetic acid and their excess nucleotide content determined. The steps in this procedure have been described in a previous paper [2].

Under these conditions the excess ADP in the fibres, presumed to be bound to myosin on previous evidence [1,2], was large and not significantly different from the value obtained in relaxed muscle (Table I). Activated fibres could not be successfully incubated at lower ATP concentrations because of their high ATPase activity (Table I) and the consequent diffusional gradient of substrate across the fibre. In order to reduce the diffusional pathway we

TABLE I

THE EFFECT OF CALCIUM ACTIVATION ON ADP BINDING AND ATPase ACTIVITY IN GLYCEROL EXTRACTED FIBRES AND GEL-EMBEDDED MYOFIBRILS FROM RABBIT PSOAS MUSCLE

B_m and K_m are calculated by the hyperbolic fitting method of Wilkinson [12].

	Relaxed ($< 10^{-6}$ M calcium)	Activated (10^{-6} M calcium)
Bound ADP (μmoles per l of fibres)		
In fibres at 0.16 mM ATP [a]	179 ± 12	162 ± 15
Calculated B_m in fibres	227 ± 6 (ref. 2)	
Calculated B_m in myofibrils [b]	204 ± 17	206 ± 16
Dissociation constant for ADP binding (μM)		
In fibres	14 ± 1 (ref. 2)	
In myofibrils [b]	13 ± 4	8 ± 3
ATPase activity (μmoles/min per ml fibres) [c]		
In fibres	0.29	2.0
In myofibrils	0.31	2.4

[a] Mean ± S.E. of 8 assays.
[b] Mean ± S.E. of calculated B_m and K_m in 3 myofibril preparations.
[c] Mean of four experiments.

homogenised the muscle fibres and dispersed them in a 1% agarose gel; the gel was then cut into 1 mm^3 blocks which were handled in the same manner as the fibres.

Such myofibril preparations showed a comparable ATPase to the fibres (Table I). Binding experiments at different ATP concentrations in the absence of free calcium revealed a variation of bound ADP with free ATP similar to that seen previously in relaxed fibres (Fig. 1; cf. ref. 2) and the values of B_m (maximum amount bound) and K_d (dissociation constant) calculated by a hyperbolic fit to the data were not significantly different from those obtained in fibres (Table I); the experimental method is therefore probably sound.

On addition of calcium the ATPase of the homogenised fibres was strongly activated (Table I), but the shorter diffusional path across the myofibrils allowed measurement of binding down to low ATP concentrations (Fig. 1). The observation that the excess ATP was as high in activated fibres as it was in non-activated fibres shows that the creatine kinase/creatine phosphate system effectively regenerated the ATP and so it is unlikely that the values of bound ADP were greatly contaminated by excess free ADP. The creatine kinase activity was 50 $\mu moles \cdot min^{-1} \cdot ml^{-1}$ within the gel whilst the effective myofibrillar ATPase activity was approximately 0.4 $\mu moles \cdot min^{-1} \cdot ml\ gel^{-1}$ (equivalent to a catalytic centre activity of 0.15 s^{-1}). On the basis of a K_m of 100 μM for creatine kinase [3] calculation for the Michaelis—Menten relation leads to an expected ADP concentration of 0.8 μM, which would cause a 4 μM maximum overestimate in the binding of ADP.

If this is correct the excess ADP found in calcium activated myofibrils may be compared with that in relaxed myofibrils (Fig. 1) and similar calculations of B_m and K_d performed (Table I). There was no apparant change in the B_m on activation although the experimental variation might have concealed

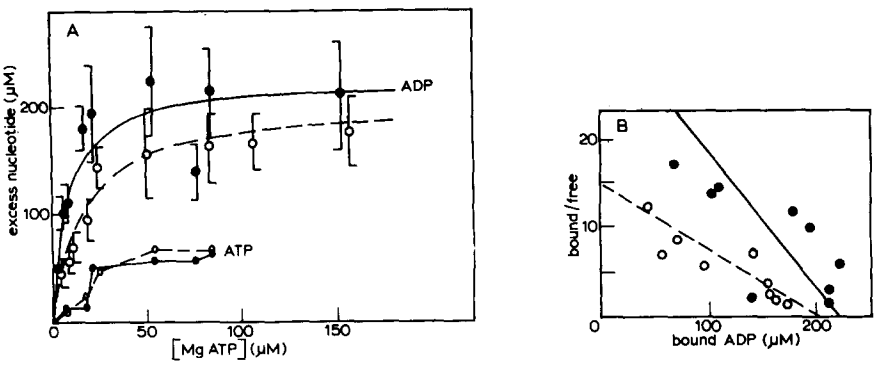

Fig.1. Excess ADP and ATP within gel-embedded myofibrils incubated in the presence of Mg-ATP. A. Direct plot. B. Scatchard plot. ○, relaxing conditions; 5 mM PIPES, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM KCl, 3 mM sodium azide, 4 mM creatine phosphate, 1—150 μM [^{14}C]ATP, 1 mM [^{14}C]mannitol. pH 7.1, ionic strength 0.09, temperature 1.5° C. ●, activating conditions. Incubating medium was the same as for relaxing conditions except for the addition of 4 mM $CaCl_2$. Gel-embedded myofibrils were prepared by mixing equal volumes of washed, homogenised fibre suspension, containing 28% fibres by volume, and melted 2% agarose at 40° C, followed by rapid cooling.
Results are expressed as μmoles nucleotide bound per litre of muscle fibres. Each point is the mean of 8 assays and the vertical bars are 5% fiducial limits. The lines for bound ADP are the calculated best fit of the 72 individual assays to the equation for simple binding calculated by the method of Wilkinson [12].

a 20 μM (10%) change. The dissociation constant decreased about 30% on activation. Maruyama and Weber [4] have reported a 5-fold increase of the dissociation constant on activation at 25 °C, but when we repeated their experiment at 1 °C we found the dissociation constant decreased by about 30%.

It therefore appears that the decrease of the dissociation constant is a property of the actomyosin system at low temperature and not an artefact of the assay procedure.

Excess ATP was found in all the experiments; the quantity and concentration dependance was similar to that previously found in whole fibres [2]. The amount of excess ATP was independant of calcium activation.

Evidence has been given that the ADP bound by relaxed glycerol extracted muscle fibres is attached to the enzymic site on myosin [1,2]. Since the quantity bound to calcium activated fibres or myofibrils is the same as that in relaxation and the affinity is only slightly changed, it is probable that the ADP bound in activated fibres is also attached to the enzymic site. It follows that all, or nearly all of the myosin molecules continued to bind ADP when the muscle was activated.

Two other observations support this conclusion. Maruyama and Weber [4] found that the total bound nucleotide (ATP plus ADP)in myofibrils is unaltered when the muscle is activated, while Schaub [5] showed by NEM inactivation that the majority of myosin active sites were occupied by nucleotide in activated myofibrils.

The significance of these observations depends on the kinetic mechanism by which myosin causes contraction. In the scheme originated by Lymm

and Taylor [6] it is proposed that a myosin—ADP—P_i complex exists in relaxed muscle which then on activation combines with actin to cause contraction. Our observations on relaxed glycerol extracted muscle [1,2] support this concept, but it has been criticised both on the grounds that no bound ADP could be detected in relaxed live muscle [7] and on the grounds that the myosin—ADP—P_i complex reacts too slowly with actin to support the actomyosin enzymic cycle [8].

If Taylor's scheme is correct the present data allow of only two possibilities. Either very few myosin molecules attach to actin on activation or those that do retain ADP. The former cannot be excluded as no estimates of the number of myosin molecules attached during contraction of rabbit psoas muscle are yet available, but in activated frog sartorius muscle X-ray diffraction experiments indicate that more myosin molecules are attached than the 10% allowed by the limits of the present experiments [9,10]. Thus the present data indicate, but do not prove, that during contraction a ternary ADP—myosin—actin complex is formed. Such a ternary complex has been shown to exist when ADP is added directly to muscle fibres [2,11]. On this basis the dissociation of ADP—myosin—actin to ADP + actomyosin would be a relatively slow process since the subsequent reaction of actomyosin with ATP is known to be very fast [6]. Positive characterisation of the proposed intermediate complex will probably require the use of transient kinetic techniques.

References

1 Marston, S.B. and Tregear, R.T. (1972) Nat. (New Biol.) 235, 23—24
2 Marston, S.B. (1973) Biochim. Biophys. Acta 305, 397—412
3 Morrison, J.F. and James, E. (1965) Biochem. J. 97, 37—52
4 Maruyama, K. and Weber, A. (1972) Biochemistry 11, 2990—2998
5 Schaub, M.C. and Watterson, J.G. (1973) FEBS Lett. 30, 305—308
6 Lymm, R.W. and Taylor, E.W. (1971) Biochemistry 10, 4627—4624
7 Barany, M. and Barany, K. (1972) Cold Spring Harbour Symp. 37, 157—167
8 Inoue, A., Shigekawa, M. and Tonomura, Y. (1973) J. Biochem. 74, 923—934
9 Huxley, H.E. (1972) Cold Spring Harbour Symp. 37, 361—376
10 Haselgrove, J.C. and Huxley, H.E. (1973) J. Mol. Biol. 77, 549—568
11 Rodger, C.R. and Tregear, R.T. (1974) J. Mql. Biol., in the press
12 Wilkinson, G.N. (1961) Biochem. J. 80, 324—332

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