Influence of smooth muscle myosin conformation on myosin light chain kinase binding and on phosphorylation

Robert A. Cross and Apolinary Sobieszek

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

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Conventional smooth muscle myosin preparations contain a tightly bound myosin light chain kinase activity, which is incompletely removed by gel filtration at high ionic strength. We show here that by contrast, this kinase activity is released, together with calmodulin, under conditions in which myosin is in the folded configuration. The conformation-related release of kinase occurred for dephosphorylated myosin in both the presence and absence of ATP and Ca^{2+}. Binding of kinase to extended phosphorylated myosin was relatively weaker than to dephosphorylated myosin, but was nonetheless detected. The kinetic consequences of this binding behaviour were determined by measuring initial myosin phosphorylation rates as a function of KCl concentration. Rate optima occurred at 60 mM KCl and 300 mM KCl, conditions favouring respectively stable filaments and stable extended monomers. Phosphorylation of the folded monomer was uniformly slow at low KCl concentrations. The folded moysin monomer is thus a relatively poor substrate for the kinase, and is therefore unlikely to represent an analog of the relaxed crossbridge configuration in myosin filaments.

Smooth muscle myosin phosphorylation *Ca^{2+} regulation* *Myosin light chain kinase* *Calmodulin*

1. INTRODUCTION

Vertebrate smooth muscle contraction is controlled at the level of the myosin filaments by phosphorylation of the myosin regulatory light chains [1,2]. Phosphorylation is catalysed by a specific MLCK, whose activity is dependent on the binding of Ca^{2+} to its regulatory subunit, calmodulin [3]. The kinetic mechanism of the phosphorylation reaction has not as yet been unequivocally established, it being controversial whether the myosin heads are randomly or sequentially phosphorylated [4–6] and whether the resultant activation of the myosin ATPase is positively or negatively cooperative. Very recently it was proposed on the basis of some limited binding and kinetic data that the kinase and calmodulin remain tightly bound to the myosin filaments under relaxing conditions, forming therefore an inactive enzyme-substrate complex [7].

The substrate, smooth muscle myosin, has in common with non-muscle myosins the ability to adopt at physiological ionic strength either a soluble folded monomeric conformation [8–10] or an extended conformation, which self assembles into filaments [11]. It seems likely that the binding of MLCK to myosin will be affected by such gross conformational changes. Accordingly, here we have determined the influence of the folding-unfolding conformational transition of smooth-muscle myosin on MLCK binding and on the rate of phosphorylation of myosin by MLCK. We chose to work with a myosin preparation which carries a bound copurifying MLCK activity [6].

Abbreviations: MLCK, myosin light chain kinase; FPLC, fast protein liquid chromatography; HMM, heavy meromyosin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

The purified chicken gizzard preparation used here was the high MLCK content preparation described in [6] using preextracted myofibrils as a source of myosin. Aliquots were frozen in liquid N_2 and stored frozen at −70°C. FPLC was performed on a standard Pharmacia HR 10/30 Superose 6 column operated at 0.5 ml/min, corresponding to an excess pressure on the column of 0.5 MPa. Samples for the column were prepared by diluting freshly unfrozen aliquots of myosin to 10 mg/ml in 0.4 M KCl, 10 mM imidazole, 1 mM cysteine, 1 mM $MgCl_2$, either 0.2 mM $CaCl_2$ or 0.2 mM EGTA, with or without 0.2 mM ATP (Mg), pH 7.3, at 25°C; centrifuging for 5 min at 12 000 rpm in an Eppendorf table top centrifuge, and further diluting with appropriate buffer to produce various final KCl concentrations. 100 μl of 5 mg/ml sample was applied and 0.5 ml fractions collected. Phosphorylation of the sample was performed by preincubating the myosin at low ionic strength at room temperature for 1 min in the presence of 0.2 mM $CaCl_2$ and 0.2 mM ATP (Mg). Phosphorylation of the samples and of the eluted fractions was monitored using urea-glycerol gels [12]. In the case of the fractions, it was first necessary to concentrate the myosin by precipitation in ice-cold 25% trichloroacetic acid, pelleting, rinsing the pellet with water and redissolving in 8.5 M urea. MLCK and calmodulin were prepared as in [13]. Kinase assays on fractions were performed as described in [6]. Other conditions and procedures are described in the figure legends.

3. RESULTS

3.1. *Distinction of myosin conformations using FPLC*

The effect of various solution conditions on the conformation of smooth muscle myosin was followed using Superose 6 gel filtration chromatography on a Pharmacia FPLC system. In initial experiments a standard sample of dephosphorylated myosin, prepared as described in section 2, was applied to preequilibrated columns. If the column was run in 150 mM KCl, ATP-containing buffer, the myosin peak eluted at a V_e of 11.0 ml (fig.1), only slightly less than that of HMM (11.6 ml), and indicating therefore that

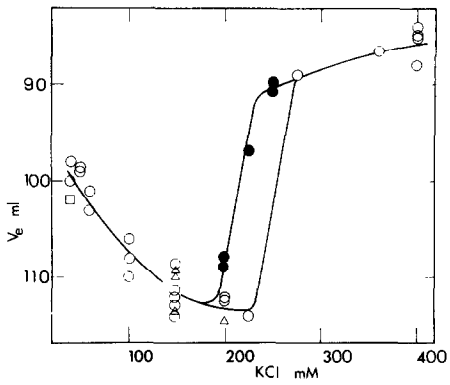

Fig.1. KCl dependence of the elution volume of smooth muscle myosin from the FPLC column. 100 μl of 5 mg/ml samples of unphosphorylated (○) myosin were applied to a Superose 6 column preequilibrated in 10 mM imidazole, 1 mM $MgCl_2$, 1 mM cysteine, 0.2 mM EGTA and from 40 to 400 mM KCl, pH 7.3, at 25°C. The sample in all cases was dissolved in this same buffer containing 180 mM KCl. (□) EGTA was replaced by 0.2 mM Ca^{2+} in the column buffer, (△) 0.2 mM ATP was present in the column buffer. Phosphorylated myosin (●) was run only in the presence of Ca^{2+} and ATP.

the myosin was in the folded configuration. If in contrast the KCl concentration of the column buffer was greater than 250 mM, the position of the myosin peak shifted to a V_e of 9.0 ml. This is close to the V_e for myosin rod, and indicated that the myosin had been unfolded during the run. The unfolding transition was apparently fast on the time scale of the run (20 min for the myosin to elute), since there was no detectable tendency for the peak to trail. At KCl concentrations below 150 mM, a similar but smaller decrease in V_e was again apparent. This was due to a tendency for the folded molecules to dimerise [10], as was confirmed by low angle platinum rotary shadowing of the present myosin preparation (fig.2). Both monomers and dimers (arrows) were present, and no extended molecules were observed. The molecular dimensions of the folded molecules can be seen in this figure to be similar to those of HMM, consistent with these 2 species having eluted at similar V_e values.

3.2. *Effects of Ca^{2+}, MgATP and phosphorylation on myosin conformation*

The plot of KCl concentration vs elution volume

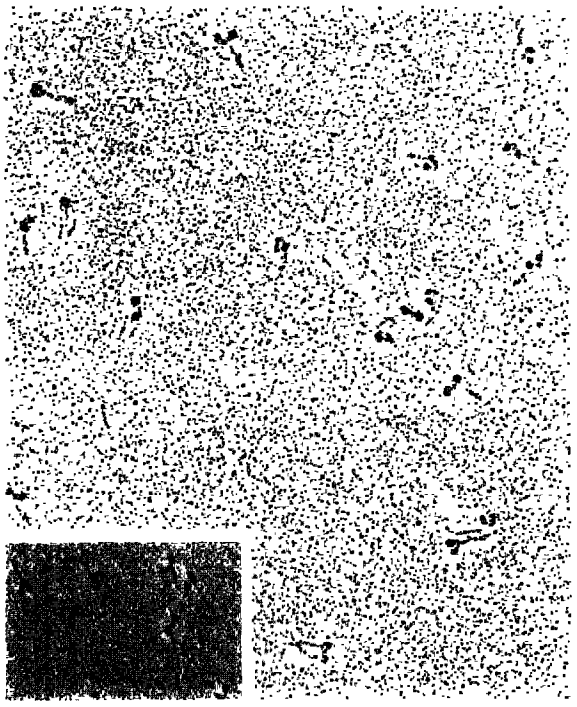

Fig.2. Rotary shadowing of myosin at 50 mM KCl. A sample of the present myosin preparation was dissolved in 50 mM KCl, 1 mM $MgCl_2$, 1 mM cysteine, 10 mM imidazole, 50% glycerol, pH 7.3, at 25°C, to a final concentration of 20 μg/ml, sprayed onto freshly cleaved mica, dried in vacuo (2×10^{-5} Torr) and rotary shadowed with Pt at 6°C. Replicas were picked up on 300 or 400 mesh Cu grids. Micrographs were recorded at a nominal 25000 × in a Zeiss EM 10 operated at 80 kV. Inset, HMM at an equal magnification.

for unphosphorylated myosin was unchanged by inclusion of 200 μM Ca^{2+} in the column buffer instead of EGTA, or by the presence or absence of 0.2 mM ATP in the column. This latter finding is of particular interest, since the column removed the ATP present in the sample and bound to myosin, yet the myosin retained its folded conformation. ATP was thus necessary to promote formation of the folded monomer [14], but not to maintain it during the run. The excess pressure on the column (0.5 MPa) may have played a role in maintaining the myosin in the folded configuration. Once eluted from the column, the folded molecules do seem in the absence of ATP slowly to relax to the extended conformation, since filaments begin to form in the eluted fractions within 5–10 min, as detected by turbidity assays [11].

In agreement with others [10,14], phosphorylation of the myosin had the effect of shifting the range of KCl concentrations over which the folded⟶extended conformational change occurred (fig.1). Under the present conditions, 1 mM Mg and pH 7.3, the elution volume vs KCl concentration curves are shifted to about 25 mM higher KCl concentration compared to those obtained by others at 10 mM Mg and pH 7.5 [10,14], but are otherwise congruent. It was not possible to examine the conformational behaviour of phosphorylated myosin in the absence of Ca^{2+} and ATP, since attempts to do this consistently led to variable levels of dephosphorylation of the samples during the run.

3.3. *Myosin conformation and kinase binding*

The myosin preparation contained sufficient MLCK activity to phosphorylate fully the myosin within 1 min of the addition of Ca^{2+} and ATP, as judged by urea-glycerol-PAGE [12]. The MLCK activity was totally Ca^{2+} sensitive. It was possible by assaying this activity in the fractions from the FPLC column to relate kinase binding to myosin conformation. Fig.3 shows the KCl dependence of the association of MLCK activity with the myosin peak. For dephosphorylated myosin, the amount of MLCK bound decreased in a step-change from ~60% of the total activity to ~5%, in the region

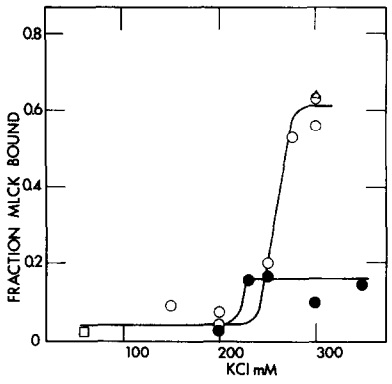

Fig.3. KCl dependence of the amount of MLCK eluting with myosin from the FPLC column. Runs were performed as described in fig.1, except that above 0.18 M KCl, samples were dissolved and applied in column buffer. Symbols as in fig.1.

240–280 mM KCl (figs 3,4), paralleling the unfolding transition of myosin as reflected in the change in the V_e for myosin. Significantly, the presence or absence of Ca^{2+} in the column buffer did not affect the elution pattern of either myosin or MLCK (fig.3). Similarly the presence or absence of ATP had no effect on separation. For phosphorylated myosin, a smaller but nonetheless readily detectable step-change in MLCK binding also accompanied myosin folding. This finding is in agreement with earlier sedimentation experiments, in which MLCK was found to bind phosphorylated myosin filaments [7], but is at variance with the data of Sellers and Pato [15] in which no binding of a purified MLCK to phosphorylated myosin was detected. The conformation-related release of MLCK thus occurred in both the presence and absence of Ca^{2+} and, to differing extents, for both phosphorylated and dephosphorylated myosin. The simplest interpretation of this finding might be that the presence of the myosin tail in the head region obscures the light chain substrate from the kinase. It is also possible, however, that a conformational change in the head region [8,16–18] induces both MLCK release and the folding of the molecule. In this case the MLCK and the tail might have a common binding site in the head region. At present we cannot distinguish between these 2 alternatives.

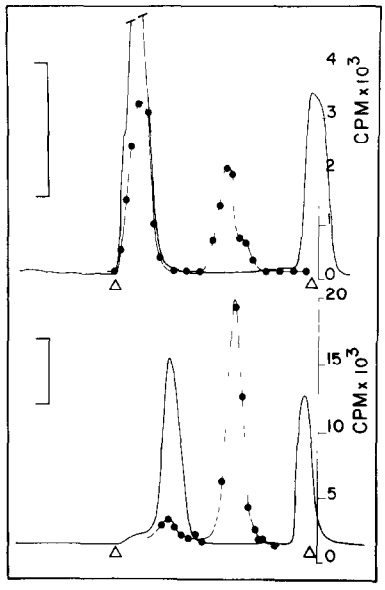

Fig.4. Representative FPLC elution profiles. Above: The sample (section 2) was applied in 0.3 M KCl, 1 mM $MgCl_2$, 1 mM cysteine, 10 mM imidazole, 0.2 mM EGTA, 0.2 mM ATP, pH 7.3 (25°C). The column was run in the same buffer lacking ATP. Below: The sample (section 2) was applied in 180 mM KCl, ATP-containing buffer, and the column run in 150 mM KCl buffer lacking ATP. (●) MLCK activity. Note that at the lower KCl concentration, the myosin peak is shifted, indicating molecular folding, and the MLCK activity is almost completely released. (△) Void and included volumes. The large peak of absorbance at the ends of the profiles is ATP. The bars at left represent 0.05 A_{280nm} (1 cm light path).

3.4. *Elution position of calmodulin*

The MLCK which copurifies with myosin has approximately stoichiometric amounts of calmodulin associated with it [6]. To obtain sufficient material to detect this in column fractions, we had to scale up to a much larger conventional Sepharose 4B gel filtration column (5 × 100 cm). Elution profiles from such runs are shown in fig.5. Selected fractions were concentrated about 100 × by dialysis in water, lyophilisation and redissolution in 8.5 M urea. Such samples were then run on SDS and urea-glycerol microslab gels [12,19]. Calmodulin and MLCK could then be visualised using Coomassie blue staining (fig.5). In spite of the relatively low resolution of these columns (the myosin peak was found to trail into the MLCK-containing fractions) calmodulin was clearly seen to elute together with the MLCK in both the presence (fig.5) and absence (not shown) of Ca^{2+}. Thus, calmodulin and MLCK formed here a Ca^{2+}-independent complex, since in the absence of binding calmodulin would be expected to elute much later than MLCK (V_e = 18.4 ml on FPLC, fig.6).

3.5. *Integrity, shape and* M_r *of MLCK*

The MLCK studied here eluted, under conditions under which it was unbound, as a single peak at a position corresponding to an apparent M_r of 160000, using globular proteins for calibration (fig.6). This is slightly higher than the value estimated from SDS-PAGE (140 kDa, fig.5), consistent with calmodulin (17.4 kDa) remaining bound. In low angle platinum rotary shadowing, purified smooth muscle MLCK presents a globular, or in some orientations a triangular profile (fig.7). Taken together therefore, the data

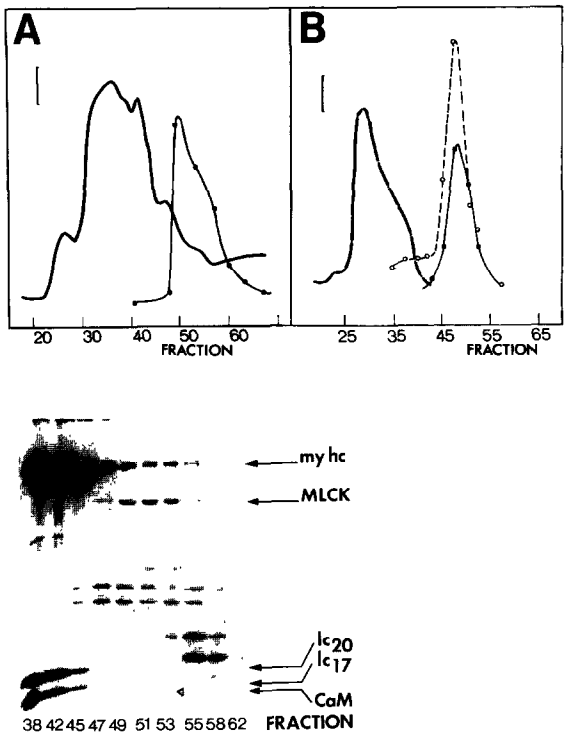

Fig.5. Elution of calmodulin from Sepharose 4B gel filtration columns of the present myosin preparation. Above: A 5 × 100 cm column of Sepharose CL-4B was run at 60 ml/h, taking 30 ml fractions. (A) Phosphorylated myosin, 290 mM KCl, 1 mM $MgCl_2$, 1 mM cysteine, 10 mM imidazole, 0.05 mM $CaCl_2$, 0.5 mM ATP, pH 7.3 (4°C). (B) Unphosphorylated myosin, 210 mM KCl, 1 mM $MgCl_2$, 1 mM cysteine, 10 mM imidazole, 0.2 mM EGTA, pH 7.3 (4°C). (●—●) MLCK activity with no added calmodulin, (○---○) MLCK activity with added excess calmodulin. The bars at left represent 0.1 A_{280nm} (1 cm light path). Below: SDS 11–22% gradient microslab gel electrophoresis of selected fractions from (A) above. Bands corresponding to MLCK (M_r 140000) and calmodulin (M_r 17400) are marked. Calmodulin was identified according to its Ca^{2+}-dependent mobility on urea-glycerol gels (not shown).

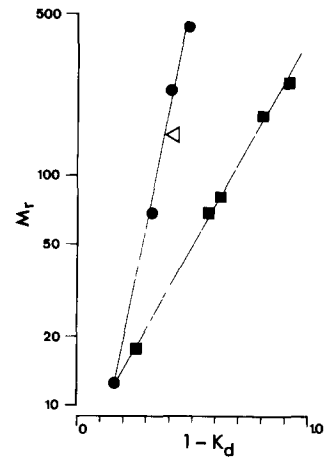

Fig.6. Calibration plot for M_r determinations using the FPLC column. The column was calibrated at a flow rate of 0.5 ml/min using the following standards: (globular standards) ferritin, 440 kDa; catalase, 232 kDa; bovine serum albumin, 68 kDa; cytochrome *c*, 14.5 kDa; (rodlike standards) myosin rod, 254 kDa; smooth muscle light meromyosin, 180 kDa; smooth muscle subfragment 2, 80 kDa; rabbit skeletal tropomyosin, 68 kDa; calmodulin, 17.4 kDa. The void volume was 7.4 ml, determined with blue dextran, and the included volume 22.0 ml, determined with acetone.

argue strongly that the MLCK studied here was a single monomeric species of native size. We feel it important to emphasise the structural integrity of this MLCK, since much of the considerable variation in published estimates of the kinetic parameters and binding properties of MLCK may have arisen from inhomogeneity in the kinase preparations. Kinase fragments of ~105 and ~80 kDa have been shown to retain respectively Ca^{2+}-sensitive and Ca^{2+}-insensitive activities [3,20].

3.6. *Myosin conformation and phosphorylation rates*

Unphosphorylated myosin diluted to low ionic strength (60 mM KCl) at neutral or slightly acid pH forms filaments which are resistant to the dissolving action of ATP [21], and which contain high levels of MLCK [6]. Such filaments were prepared and made to dissolve in a stepwise manner by varying the KCl concentration (fig.8). Initial rates of myosin phosphorylation were then measured as a function of KCl concentration. Superimposed upon an apparently strong KCl inhibition of MLCK activity (determined using purified MLCK and light chain), 2 rate optima were apparent. The first at ~60 mM KCl corresponds to conditions for stable filaments and the second at ~300 mM to conditions for the folded⟶extended monomer transition. This suggested that the folded conformation, which is ex-

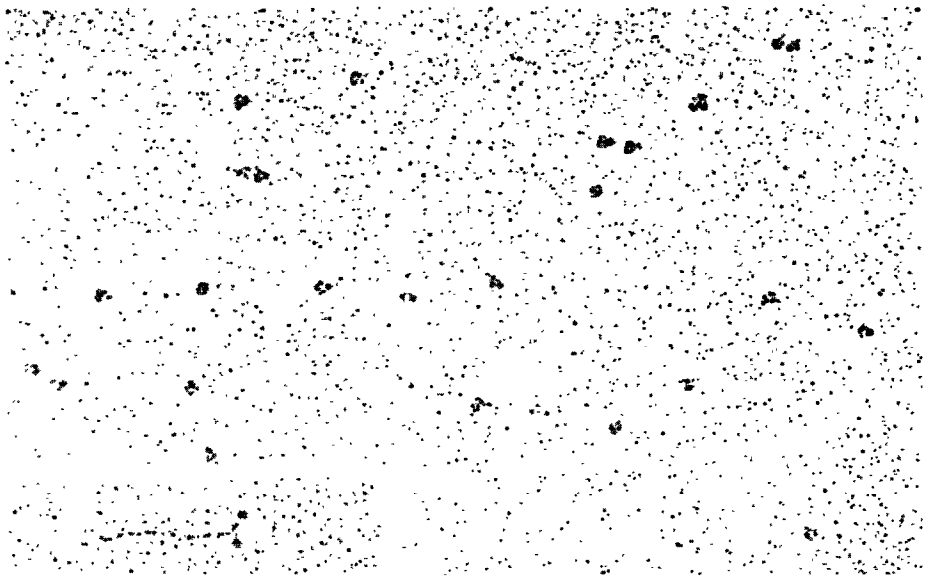

Fig.7. Rotary shadowing of purified smooth muscle MLCK. Pig stomach MLCK prepared as in [13] was rotary shadowed with Pt as described in fig.2. Inset, chicken gizzard myosin from the same shadowing run, at the same magnification as for MLCK. The myosin was dissolved in 0.6 M ammonium formate, 30% glycerol. Other conditions were identical to fig.2.

pected to predominate in the intervening range of KCl concentration, was phosphorylated only slowly. That this was the case was confirmed in experiments on myosin preparations containing uniquely the folded conformation. Such samples could be generated by dilution of myosin dissolved at high KCl concentration and pH 7.6 in the presence of ATP, to low ionic strength. No filament formation was detectable in turbidity measurements [11,21] on the resulting solutions. Fig.8 shows that phosphorylation of these samples was much more weakly dependent on KCl concentration in the region 50–150 mM KCl than was either the phosphorylation of filamentous myosin by the intrinsic MLCK, or of isolated light chain

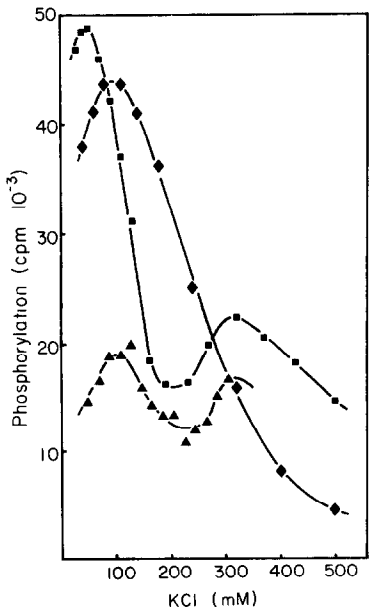

Fig.8. KCl dependence of initial phosphorylation rates. (■—■) Myosin filaments in 30 mM KCl, 1 mM cysteine, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM imidazole, pH 7.0, were adjusted to higher KCl concentration. Phosphorylation was initiated by addition of [γ-^{32}P]ATP and stopped after 10 s as in [6]. (◆—◆) Isolated myosin regulatory light chain was phosphorylated by purified chicken gizzard MLCK. Conditions as for the myosin filaments except that the assay time was 10 min. (▲—▲) Myosin in 300 mM KCl, 1 mM cysteine, 1 mM $MgCl_2$, 10 mM imidazole, 0.2 mM EGTA, 0.5 mM ATP, 20 mM Hepes, pH 7.55, was diluted to various KCl concentrations, Ca^{2+} and [γ-^{32}P]ATP added, and the reaction stopped after 20 s.

by purified MLCK. At high (~300 mM) KCl concentration, the 'folded' and 'filamentous' samples showed comparable-sized rate optima due to unfolding of the myosin.

4. DISCUSSION

In contrast to authors in [15], who employed a purified MLCK preparation in binding studies, we have used here a native myosin preparation which carries an intact copurifying MLCK. The value of this approach lies in its potential to preserve the native arrangement of the myosin-MLCK complex.

We have shown here that both MLCK and calmodulin bind to extended myosin monomers in both the presence and absence of Ca^{2+}. This was true for both unphosphorylated and phosphorylated myosin monomers, although in the latter case the binding was weaker. The folding of the myosin monomers to produce the 10 S conformation was accompanied by a dramatic reduction in the affinity of myosin for MLCK and calmodulin, and a corresponding reduction in the rate at which myosin was phosphorylated. MLCK and calmodulin which were released by the folding of myosin remained bound together in both the presence and absence of Ca^{2+}.

The finding that MLCK and calmodulin remain bound to extended myosin in both the presence and absence of Ca^{2+} suggests that these 3 components are already tightly associated in relaxed muscle, and that therefore Ca^{2+} activation of smooth muscle occurs via a Ca^{2+}-induced conformational change in this preexisting regulatory complex. The role of ATP binding to MLCK or to myosin in this process is not yet clear.

It has been suggested that the folded conformation of myosin might represent an analog of a particular crossbridge conformation in the thick filament [14]. From the present data, we conclude that this could not correspond to the relaxed state, since levels of MLCK binding and rates of phosphorylation were both low for the folded monomer.

In this and previous work [7] MLCK was shown to bind detectably to phosphorylated myosin in the extended conformation. This finding indirectly implicates some other factor in releasing MLCK from phosphorylated myosin. Possibilities include release via binding of MLCK to the thin filaments [15], perhaps involving a further myosin conformational change.

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REFERENCES

[1] Sobieszek, A. (1977) in: The Biochemistry of Smooth Muscle (Stephens, N.L. ed.) pp.413–443, University Park Press, Baltimore, MD.
[2] Sobieszek, A. (1977) Eur. J. Biochem. 73, 477–483.
[3] Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K. and Hartshorne, D.J. (1978) Biochemistry 17, 253–258.
[4] Persechini, A. and Hartshorne, D.J. (1981) Science 213, 1383–1385.
[5] Sellers, J.R., Chock, P.B. and Adelstein, R.S. (1983) J. Biol. Chem. 258, 14181–14188.
[6] Sobieszek, A. (1985) Biochemistry 24, 1266–1274.
[7] Sobieszek, A. (1985) J. Muscle Res. Cell Mot. 6, 122.
[8] Suzuki, H., Kamata, T., Onishi, H. and Watanabe, S. (1982) J. Biochem. (Tokyo) 91, 1699–1705.
[9] Trybus, K.M., Huiatt, T.W. and Lowey, S. (1982) Proc. Natl. Acad. Sci. USA 79, 6151–6155.
[10] Trybus, K.M. and Lowey, S. (1984) J. Biol. Chem. 259, 8564–8571.
[11] Craig, R., Smith, R. and Kendrick-Jones, J. (1983) Nature 302, 436–439.
[12] Perrie, W.T. and Perry, S.V. (1970) Biochem. J. 119, 31–38.
[13] Sobieszek, A. and Barylko, B. (1985) in: Smooth Muscle Contraction (Stephens, N.L. ed.) pp.283–316, Marcel Dekker, New York.
[14] Ikebe, M., Hinkins, S. and Hartshorne, D.J. (1983) Biochemistry 22, 4580–4587.
[15] Sellers, J.R. and Pato, M.D. (1984) J. Biol. Chem. 259, 7740–7746.
[16] Ikebe, M. and Hartshorne, D.J. (1984) J. Biol. Chem. 259, 11639–11642.
[17] Onishi, H. and Watanabe, S. (1984) J. Biochem. (Tokyo) 95, 899–902.

[18] Kumon, A., Yasuda, S., Murakami, N., Tashiro, Y. and Matsumura, S. (1984) Eur. J. Biochem. 144, 629–635.
[19] Laemmli, U.K. (1970) Nature 277, 680–685.
[20] Walsh, M.P., Dabrowska, R., Hinkins, S. and Hartshorne, D.J. (1982) Biochemistry 21, 1919–1925.
[21] Kendrick-Jones, J., Cande, W.Z., Tooth, P.J., Smith, R.C. and Scholey, J.M. (1983) J. Mol. Biol. 165, 139–162.