Phosphorylation of chicken gizzard myosin and the Ca^{2+}-sensitivity of the actin-activated Mg^{2+}-ATPase

Heather A. Cole, Valerie B. Patchell and S. Victor Perry

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, England

Received 16 May 1983

A method is described for the preparation of partially and fully phosphorylated chicken gizzard myosin. When fully phosphorylated it possessed an actin-activated Mg^{2+}-ATPase of similar specific activity to that of mammalian skeletal muscle myosin. The Mg^{2+}-ATPase activity of these preparations was related in a non-linear fashion to increasing phosphorylation of the P light chain. When P light chain phosphorylation occurred during enzymic assay the Mg^{2+}-ATPase activity remained constant. Fully phosphorylated preparations of gizzard myosin possessed an actin-activated Mg^{2+}-ATPase that was not Ca^{2+}-sensitive, whereas the Mg^{2+}-ATPase of partially phosphorylated myosin preparations was Ca^{2+}-sensitive.

Phosphorylation *Ca^{2+}-Sensitivity* *P light chain* *Mg^{2+}-ATPase* *Smooth muscle* *Myosin light chain kinase Actin activation*

1. INTRODUCTION

With few exceptions it has been widely reported that the specific Mg^{2+}-activated ATPase of vertebrate smooth muscle actomyosin is significantly higher in those preparations in which the P light chain is phosphorylated [1–3]. Nevertheless, the precise nature of the correlation between the extent of phosphorylation and the ATPase activity is more controversial [4–7].

The situation is complicated by the fact that myosin possesses two enzymically active heads, each of which contains a phosphorylatable (P) light chain. From studies on myosin from striated muscle there is no clear evidence for assuming that either or both heads of the myosin molecule require to be phosphorylated to activate the Mg^{2+}-stimulated ATPase of actomyosin. In [8], only quantitative differences were reported, compared to the striated enzyme, in the actin-activated ATPase activity of subfragment 1 in which the P light chain was partially degraded during preparation from smooth muscle myosin. However, it is possible that in the intact molecule of smooth muscle myosin some kind of cooperativity exists between the two heads. The non-linear relationship between ATPase activity and light chain phosphorylation has been interpreted as indicating that both heads require to be phosphorylated for activation of the MgATPase and that negative cooperativity leads to preferential production of myosin with only one head phosphorylated [9].

Evidence of the complexity of the relationship between enzymic activity and phosphorylation state is given by the fact that with some preparations of smooth muscle actomyosin, changes in phosphorylation can occur without changes in ATPase activity [4,5,10]. Factors have also been described that increase the activity without increasing the level of phosphorylation [4,7,11].

Another aspect which could complicate the analysis of the system would be a dependence of the Ca^{2+} sensitivity of the actomyosin ATPase on the extent of the phosphorylation of the P light chain. The general view has been that the Ca^{2+} sensitivity of partially phosphorylated smooth muscle actomyosin ATPase has simply been a reflection of the Ca^{2+} requirement of the kinase itself (see [12]).

Published by Elsevier Science Publishers B.V.
00145793/83/$ 3.00

The regulatory light chain of molluscan adductor myosin that is homologous to the P light chain in vertebrate muscles, but which does not appear to be a substrate for myosin light chain kinase [13,14], is essential for the calcium sensitivity of the actomyosin ATPase. The possibility that the P light chain of vertebrate smooth muscle has a similar function which could be modulated by phosphorylation does not appear to have been considered. Here we present evidence that suggests P light chain phosphorylation may change the Ca^{2+} sensitivity of the Mg^{2+}-ATPase of smooth muscle actomyosin.

2. MATERIALS AND METHODS

2.1. *Preparation of myosin*

Myosin was prepared by a method similar to that in [1] as described [4]. It was further purified by gel filtration on Sepharose 4B by a method similar to that in [15]. Myosin (~40 mg) was dissolved in 0.8 M KCl, 1 mM EDTA, 1 mM EGTA, 20 mM imidazole–HCl, 1 mM DTT, 3 mM NaN_3, 0.5 mM ATP (pH 7.5, 10°C) and applied to a column (90 cm × 1.5 cm) equilibrated and eluted with the same buffer. The fractions representing the eluted peak of myosin were pooled and dialysed against 30 mM KCl, 20 mM imidazole–HCl, 0.5 mM EDTA, 0.5 mM DTT (pH 7.0, 10°C) and the myosin precipitated by the addition of $MgCl_2$ to 10 mM final conc. To obtain the most active preparations, myosin was pre-phosphorylated prior to gel filtration by incubation for 5 min at 30°C in 50 M imidazole–HCl (pH 7.0) 0.2 mM $CaCl_2$, 5 mM DTT, calmodulin (25 mg/ml), smooth muscle light chain kinase (40 μg/ml), 5 mM $MgCl_2$, 5 mM ATP (pH 7.0).

2.2. *Myosin light chain kinase*

A crude preparation of myosin light chain kinase was extracted from partially washed chicken gizzard myofilaments [1] with 3 vol. wash buffer containing 25 mM $MgCl_2$. The precipitate obtained between 40% and 60% ammonium sulphate saturation, was separated by gel filtration on an 'Ultrogel' AcA 44 column (90 cm × 2 cm) in 25 mM imidazole–HCl, 0.3 NaCl, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 1 mM DTT, 3 mM NaN_3^-, streptomycin (0.1 g/l) pH 7.5 and the peak of enzyme activity pooled.

2.3. *ATPase assays*

These were carried out under conditions described in the text by incubation for 5 min at 30°C in 1 ml final vol. After an initial preincubation for 5 min the reaction was started by the addition of the ATP. Electrophoresis and determination of P light chain phosphorylation was done as in [4].

3. RESULTS AND DISCUSSION

When fully activated by pre-phosphorylation the column-purified chicken gizzard myosin usually possessed Mg^{2+}-ATPase activities over 200–600 nmol $P \cdot min^{-1} \cdot mg\ myosin^{-1}$ at 30°C in the presence of excess actin and tropomyosin; i.e., similar to actomyosin from skeletal muscle assayed under similar conditions (fig.1). Less active preparations have been reported in the literature (e.g., [9,15,16]) but we believe studies on preparations of high activity are of more significance than those on preparations which, even when measured

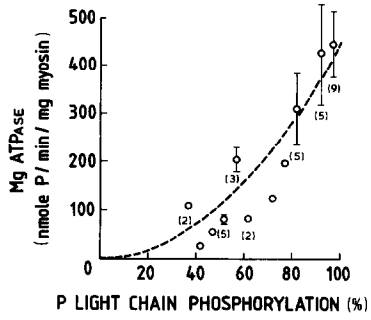

Fig.1. Phosphorylation of the P light chain of chicken gizzard myosin and the actin-activated Mg^{2+}-ATPase. Chicken gizzard myosin (0.5–1.0 mg/ml) incubated with 50 mM imidazole–HCl buffer (pH 7.0), 6 mM $MgCl_2$, 5 mM Na^+K^+-ATP, 0.2 mM $CaCl_2$, 2 mM dithiothreitol, chicken gizzard tropomyosin (0.2 mg/ml), bovine brain calmodulin (25 μg/ml), rabbit skeletal actin (0.4–0.7 mg/ml) incubated in 1 ml total vol. for 5 min at 30°C. Light chain phosphorylation determined on original myosin after 2.5 min and after 5 min incubation. Values plotted are those at 2.5 min when phosphorylation was usually complete. Collected results from 14 different myosin preparations (total number of assays 36) obtained from 5 different batches of washed myofilaments. Dotted line indicates ATPase activity expected if only myosin with two heads phosphorylated was active and if phosphorylation of the heads occurred in a random fashion.

under comparable conditions have spec. act. 30–50 nmol . min^{-1} . mg $myosin^{-1}$.

After the gel filtration step the standard myosin preparations had Mg^{2+}-ATPase activities in the presence of rabbit skeletal actin of 50–150 nmol P . min^{-1} . mg $myosin^{-1}$ and the P light chains were normally 25–40% phosphorylated. The endogenous myosin light chain kinase activity of the preparations was low, and little further phosphorylation occurred under the conditions of ATPase assay. The Mg^{2+}-ATPase activity was increased by preincubation with ATP and added smooth muscle light chain kinase before the final gel filtration step. This step usually increased the phosphorylation of the P light chain to 90–100%.

When the activities of all the different preparations, including those that were pre-phosphorylated, were plotted against the percentage phosphorylation of the P light chain, the pattern in fig.1 was obtained. If the light chains were dephosphorylated completely by preincubation with light chain phosphatase, ATPase activity was reduced to a very low level. The most active preparations were those that were fully phosphorylated or close to it. It was also noted that even with myosins that were 90–100% phosphorylated, the actin-activated ATPase of different preparations could vary by a factor of 6.

The results in fig.1 suggest that both heads of the myosin molecule need to be phosphorylated for full activity. The points show some scatter and it cannot be concluded with certainty that they do not fall on the theoretical curve obtained if phosphorylation occurred randomly on the two heads and if only fully phosphorylated myosin was enzymically active. Nevertheless, quite significant levels of activity were obtained with some preparations of myosin that were 30–40% phosphorylated and in some cases different preparations were obtained with similar activities at 50% and 90–100% phosphorylation. The observation that occasionally preparations could be converted from 50–100% phosphorylation without change in ATPase activity also indicated that the relationship between phosphorylation actin-activation of the ATPase was not simple. Further evidence of the complexity of the correlation between phosphorylation and ATPase activity was obtained by carefully following the ATPase activity during progressive phosphorylation. In all cases, the ATPase rate was constant, even though the extent of P light chain phosphorylation was changing from 25–40% to 70–80% over the incubation period in which the rate was measured (e.g., fig.2). Over this range a significant proportion of fully phosphorylated myosin mulecules would be formed. These observations confirm our studies with preparations of smooth actomyosin with lower specific ATPase activities [4].

The pattern of the relation between actin-activated ATPase activity to P light chain phosphorylation shown in fig.1 shows some similarities to that reported in [9], although the maximuum activities reported by these workers are very low. It would appear that their results correspond more to those obtained by us after preincubation with ATP and kinase rather than to those illustrated in fig.2.

It was also noted that the sensitivity of the Mg^{2+}-ATPase to EGTA was related to the extent of phosphorylation (fig.3). In general, the higher the degree of phosphorylation, the lower the sensitivity of the ATPase to EGTA. It is unlikely that this is the consequence of the requirement of the kinase for Ca^{2+} as the observations were made on preparations of myosin in which the kinase activity was low and little change in phosphorylation occurred during the enzymic assay. In that event, the data cannot be explained on the assumption that only fully phosphorylated myosin is active as an ATPase which is Ca^{2+}-insensitive. They suggest that at <100% phosphorylation of myosin, in addition to the latter enzymic species, there is another which is Ca^{2+}-sensitive. This we suggest is

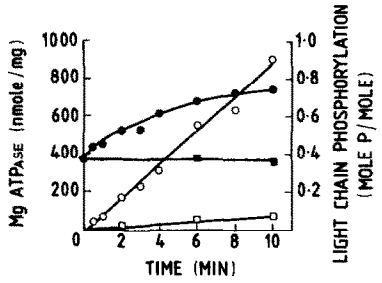

Fig.2. Effect of changing the state of light chain phosphorylation of chicken gizzard myosin during assay of the actin-activated Mg^{2+}-ATPase. Incubation conditions as in fig.1 with partially purified chicken gizzard kinase (10 μg/ml) and 2 mM EGTA added where indicated: (●) phosphorylation; (○) ATPase; (■) phosphorylation + 2 mM EGTA; (□) ATPase + 2 mM EGTA.

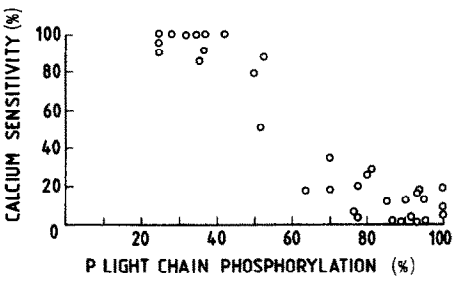

Fig.3. Ca^{2+} sensitivity of the actin-activated Mg^{2+}-ATPase of gizzard myosin and P light chain phosphorylation. Determinations were carried out on the preparations of myosin the activities of which are presented in fig.1. Assay conditions as in fig.1 ±2 mM EGTA.

$$\text{Calcium sensitivity} = 100\left[1 - \frac{\text{ATPase in EGTA}}{\text{ATPase in } Ca^{2+}}\right]$$

myosin with one head phosphorylated which possibly has a lower V_{max} than the phosphorylated form.

Thus phosphorylation of the P light chain in addition to increasing the V_{max} of the system also modulates the Ca^{2+} requirement. In effect, as the ATPase rises, the system becomes activated at lower $[Ca^{2+}]$. When the myosin is fully phosphorylated, and in the absence of other factors [4], Ca^{2+} is not essential for the Mg^{2+}-ATPase. Thus the role of the P light chain is in some ways similar to that of the regulatory light chain of molluscan adductor myosin in controlling the actomysin ATPase. Unlike the latter system, however, which has not yet been shown to involve phosphorylation of the regulatory light chain, vertebrate smooth muscle is able to modify the role of the P light chain by phosphorylation.

The Ca^{2+}-dependence of the ATPase of partially phosphorylated actomyosin could be of special importance for smooth muscle in accounting for the Ca^{2+}-dependent maintenance of tension, when the level of phosphorylation of the P light chain falls from the peak associated with the initial stimulation of carotid artery preparations [17,18].

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