Myosin light chain kinase binding to plastic

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Protein kinase *Myosin* *Detergent* *Plastic* *Peptide*

1. INTRODUCTION

The myosin light chain kinase occurs in many tissues, however, it is in the regulation of smooth muscle concentration that its function is most thoroughly understood (reviewed [1]). Until recently [2] this enzyme has proved difficult to isolate in its pure, unproteolysed form. Many preparations have been reported to have low catalytic activity with V_{max} values of less than 10 μmol . min^{-1} . mg^{-1} [3]. In view of the problems with proteolysis, the low catalytic activity of these preparations was considered to have been a consequence of limited degradation. During the study of the phosphorylation of synthetic peptides by this enzyme it was found necessary to add bovine serum albumin to demonstrate peptide phosphorylation. Further investigation revealed that the myosin light chain kinase binds avidly to glass and plastic surfaces and depending on the method of dilution this may lead to a substantial underestimate of the enzyme's V_{max} (2–6-fold), even when protein substrates are employed. Inclusion of detergent, Tween-80, in the enzyme dilution and assay buffers prevented the loss of enzyme due to adsorption onto plastic surfaces and allowed an accurate measure of its specific activity.

2. MATERIALS AND METHODS

2.1. *Preparation of proteins and synthetic peptides*

The myosin light chain kinase was purified from chicken gizzard to homogeneity (M_r 128 000) by modification of the procedure of Adelstein [2] in which the gel chromatography and dialysis steps were omitted, polyethylene glycol precipitated enzyme from 25 mM $MgCl_2$ extract was chromatographed sequentially on DEAE-cellulose, calmodulin–Sepharose and concentrated on DEAE-cellulose. The myosin light chains were purified from chicken gizzard by published procedures [4,5]. Calmodulin was prepared from ovine brain as described previously [6]. Protein concentrations were determined by the procedure of Lowry et al. [7] with bovine serum albumin as standard. Synthetic peptides were prepared by the solid-phase procedure of Merrifield [8] and purified by ion-exchange and gel chromatography as described previously [9]. Purity of the synthetic peptides was assessed by quantitative amino acid analysis and high voltage electrophoresis. The synthetic heptadecapeptide used in the following experiments corresponds to part of the amino terminal 17 residues surrounding the phosphorylation site of the M_r 20 000 chicken gizzard myosin light chains, Ser-Ser-Lys-Thr-Thr-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-(P)-Asn-Val-Phe-Ser-NH_2 [10].

2.2. *Protein kinase assay*

Myosin light chain kinase was assayed in a volume of 0.08 ml of 40 mM Hepes buffer (pH 7.0), 0.1 mM EGTA, 7 mM magnesium acetate, 0.55 mM $CaCl_2$, 5 μg calmodulin, 0.5 mM [γ-^{32}P]-ATP (100–2000 cpm/pmol) myosin light chains (4 mg/ml) or synthetic heptadecapeptide substrate (0.47 mM) as indicated. Incubations were at 30°C and aliquots (0.03 ml) were taken at 3 and 6 min to obtain initial velocities. [γ-^{32}P]ATP was separated from synthetic peptide or myosin light chains as described previously [9]. Plastic polystyrene tubes

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(10 × 75 mm) were used for enzyme assays and dilutions unless otherwise stated.

3. RESULTS

3.1. *The effect of enzyme dilution*

When the concentration of the myosin light chain kinase was varied over a wide range, the activity was unexpectedly low at high dilution (fig.1A). This loss of activity at high dilution was partially mitigated by including bovine serum albumin (1 mg/ml) in the dilution buffer. At the highest concentration of enzyme (dilution 1) the reaction rate in the presence of albumin was no longer linear with respect to time. When the detergent Tween-80 was included in the enzyme dilution buffer there was a linear relationship between enzyme activity and enzyme concentration (fig.1B) over the full range. In the presence of bovine serum albumin (fig.1B) the plot of activity versus enzyme dilution was linear at the three lowest dilutions. However at the two highest dilutions in presence of albumin there was very low activity (fig.1B). These results indicate that the loss of enzyme activity at high dilution is prevented by inclusion of Tween-80 in the dilution buffer.

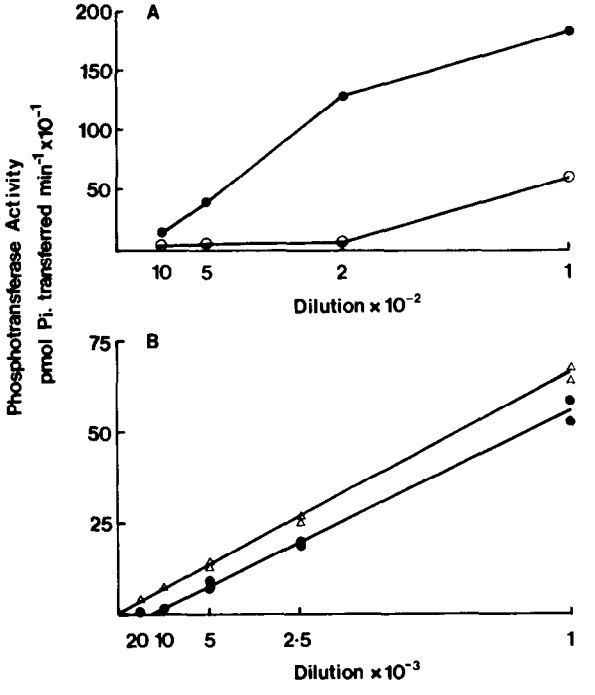

Fig.1. The effect of enzyme dilution on activity: Serial dilutions of the enzyme were made from a concentrated stock (3 mg/ml). (A) Myosin light chain kinase was diluted in 25 mM Tris · HCl buffer (pH 7.5) with (•) or without (○) addition of bovine serum albumin (1 mg/ml). The enzyme was assayed with myosin light chains as substrate without the addition of bovine serum albumin. (B) Myosin light chain kinase was diluted in 25 mM Tris · HCl buffer (pH 7.5) with bovine serum albumin (•) or 0.1% Tween-80 (△). The enzyme was assayed with myosin light chains as substrate in the presence of bovine serum albumin (1 mg/ml).

3.2. *The binding of enzyme to plastic*

When it was found that myosin light chain kinase activity was lost at high dilution and that this was partially prevented by including bovine serum albumin, it was of interest to test whether the enzyme was bound to the plastic tubes used in its dilution. Repetitive transfer of diluted enzyme in empty plastic tubes resulted in complete loss of activity after two transfers in the absence of bovine serum albumin (fig.2). At the high dilutions of

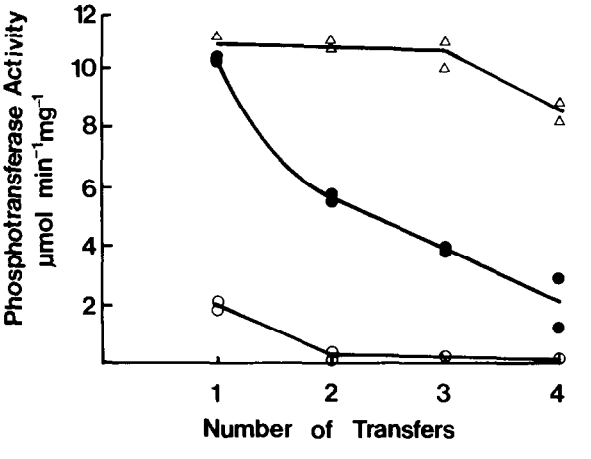

Fig.2. The effect of serial transfers on enzyme activity. Myosin light chain kinase stock (3 mg/ml) was diluted 60-fold in 25 mM Tris · HCl buffer containing bovine serum albumin (1 mg/ml). This diluted enzyme was diluted a further 200-fold into Tris · HCl buffer (○), Tris · HCl buffer with 0.1% Tween-80 (△) or Tris · HCl buffer with bovine serum albumin (1 mg/ml) (•) to give a final dilution of 12 000-fold. This was taken as the first transfer. The diluted enzyme was transferred a further 3 times by sequential transfers into empty plastic tubes. A 200 μl aliquot was left after each transfer for assessing the residual activity. All enzyme assays contained 0.1% Tween-80 and bovine serum albumin (1 mg/ml).

myosin light chain kinase required for initial rate activity determinations it was not possible to measure its binding to plastic directly using protein determinations. In the presence of serum albumin there was approximately 30% loss of activity after each transfer. Measurement of the protein concentration after each of these transfers indicated that there was no detectable loss of total protein (results not shown). There is therefore no loss of bovine serum albumin during the transfer corresponding to the loss of myosin light chain kinase. Tween-80 completely prevented the loss of enzyme activity for three repetitive transfers (fig.2).

3.3. *The activity of bound enzyme*

Tween-80 prevented the loss of enzyme activity but was ineffective in extracting bound enzyme from the plastic (results not shown). The activity of the bound enzyme was assessed by adding the myosin light chain kinase assay components direct to plastic tubes into which diluted enzyme had been added, removed and then the tube rinsed with dilution buffer containing Tween-80. Approximately 66% of the diluted enzyme was bound since the residual unbound activity was equivalent to 34% of the total. Myosin light chain kinase activity (2.97% of the bound fraction) was present in the tubes in which the enzyme had been diluted. Since the enzyme activity associated with the plastic was only 3% of that bound, the results indicate that the major fraction of the bound enzyme (97%) is catalytically inactive under the conditions employed.

3.4. *The binding of enzyme to glass*

When it was found that the myosin light chain kinase bound tightly to plastic it was of interest to test if it was bound by glass and siliconized glass. Binding to glass was tested in a transfer experiment analogous to that used in fig.2. After four transfers greater than 87% of the myosin light chain kinase activity was lost. Inclusion of either albumin or Tween-80 did not prevent the loss of activity (results not shown). Siliconizing the glassware did not reduce the loss of enzyme activity.

3.5. *The effect of Tween-80 on calmodulin dependence*

Previously Sharma et al. [11] reported that the detergent Triton X-100 inhibited the binding of calmodulin to the calmodulin dependent phosphodiesterase. In the present study Tween-80 had

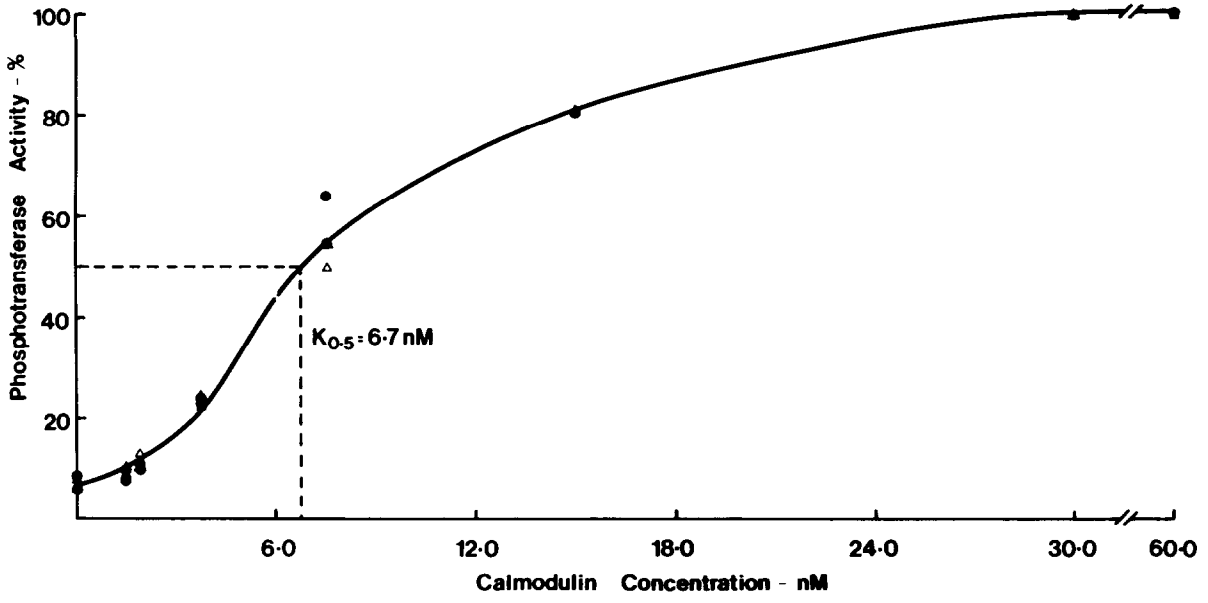

Fig.3. Effect of Tween-80 on calmodulin dose dependence. The myosin light chain kinase was assayed with increasing concentration of calmodulin without (•) or with 1% Tween-80 (△). The enzyme was diluted 2000-fold in presence of serum albumin (1 mg/ml) and assayed with synthetic peptide as substrate.

no effect on the calmodulin dependence of the myosin light chain kinase activity when assayed with synthetic heptadecapeptide substrate (fig.3). The synthetic substrate overcomes the need to prepare myosin light chains free of calmodulin. These results indicate that inclusion of Tween-80 to inhibit enzyme binding to plastic does not have any adverse effect on the capacity of calmodulin to stimulate activity.

3.6. *The effect of dilution buffer composition*

A variety of different enzyme dilution buffer compositions were tested. Complete activity was retained with Tween-80, Brij-35 or Triton X-100 (table 1). Deoxycholate was the least effective detergent. Elevation of the ionic strength which potassium phosphate and KCl had only a minor effect on the loss of enzyme activity following dilution. The addition of calmodulin to the enzyme prior to dilution had a small protective effect, reducing the loss of enzyme activity from 92% to 75%. These results indicate that the calmodulin binding site on the enzyme is probably not the principal region responsible for binding to plastic.

Table 1

Comparison of enzyme dilution buffers

Dilution buffer additions	Enzyme activity retained (%)
None	8
Albumin (1 mg/ml)	74
Calmodulin (3.7 μM)	22
Glycerol (25%)	25
Ethylene glycol (10%)	14
Deoxycholate (0.1%)	43
Triton X-100 (1%)	100
Brij-35 (0.1%)	100
Tween-80 (0.1%)	100
KCl (250 mM) in phosphate buffer (10 mM)	12

The myosin light chain kinase was diluted 2000-fold (2 serial dilutions) into the 25 mM Tris · HCl dilution buffer with additions as indicated and assayed in presence of bovine serum albumin (1 mg/ml) as described under Materials and Methods. Enzyme activity in the presence of Tween-80 was taken as 100%.

3.7. *The effect of Tween-80 on myosin light chain dependence*

The myosin light chain kinase velocity dependence on myosin light chain concentration was tested in the presence and absence of detergent. In addition to increasing the V_{max}, Tween-80 had a modest effect on the apparent K_m for the myosin light chains, decreasing it from 13.1 ± 1.7 μM to 8.6 ± 1.5 μM. It is not possible from these experiments to distinguish between a small direct effect of Tween-80 on the enzyme's K_m or an indirect effect resulting from preventing binding of the protein substrate to plastic. The principal effect of Tween-80 is on the V_{max}, consistent with the idea of detergent inhibiting the binding of the enzyme to plastic and consequent loss of activity.

4. DISCUSSION

The results reported in this paper demonstrate that the myosin light chain kinase binds tightly to glass and plastic surfaces. Once bound approximately 97% of the enzymes catalytic activity is lost. The binding of the myosin light chain kinase to plastic occurs even in the presence of bovine serum albumin. It is important to note that linear rates with respect to time and enzyme concentrations do not necessarily ensure that an accurate measure of the enzyme's specific activity will be obtained since significant losses of enzyme due to adsorption may occur in the initial serial dilutions.

The detergent Tween-80 was found to prevent the binding of the enzyme to plastic but was ineffective in preventing binding to glass. Once bound to plastic the enzyme could not be recovered with Tween-80. The findings reported herein indicate that previous estimates of the V_{max} for the myosin light chain kinase [2,12] are likely to be underestimates, even in cases where albumin has been included in the dilution buffer [2]. In this laboratory stored enzyme (−70°C) exhibiting a V_{max} of 18 μmol · min^{-1} · mg^{-1} when enzyme dilutions were made in albumin, had a V_{max} of 36 μmol · min^{-1} · mg^{-1} when diluted in 0.1% Tween-80. Since V_{max} values of 30–40 μmol · min^{-1} · mg^{-1} have been reported for myosin light chain kinase preparations [12] it is possible that the theoretical value is in excess of 60 μmol · min^{-1} · mg^{-1}. Since the details of the enzyme dilutions, in particular the number of serial dilutions and the material of the

tubes employed in these studies, is not usually reported it is difficult to make firm estimates. The magnitude of the myosin light chain kinases V_{max} is of significance in assessing the quality of isolated enzyme as well as in kinetic considerations of the role of this enzyme in the regulation of the speed of contraction in smooth muscle [13].

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