Two-site phosphorylation of the phosphorylatable light chain (20-kDa light chain) of chicken gizzard myosin

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When prepared under specified conditions chicken gizzard myosin was obtained which when incubated with ATP gave rise to a diphosphorylated as well as the monophosphorylated form of P light chain. Formation of the diphosphorylated light chain occurred more readily with these myosin preparations, but could also be obtained by prolonged incubation of the isolated whole light chain fraction with kinase preparations from rabbit skeletal and chicken gizzard muscles. Using isolated light chains as substrate the more readily formed monophosphorylated light chain contained serine phosphate while the diphosphorylated form contained serine and threonine phosphates.

Two site phosphorylation *P light chain* *Chicken gizzard myosin* *Myosin light chain kinase*

1. INTRODUCTION

It is generally assumed that when myosin light chain kinase acts on myosin or the isolated P light chain (phosphorylatable, 20-kDa, L2 or regulatory light chain) from smooth muscle that a single site, serine 19, homologous to serine 15 in the P light chain of myosin from rabbit fast skeletal muscle is phosphorylated. From time to time bands of protein in addition to the normal monophosphorylated form of the P light chain have been observed in myosin preparations from striated and smooth muscles subjected to high resolution electrophoresis under conditions in which charge determines mobility. Because of the ease with which the P light chain is modified to produce bands of different electrophoretic mobility that are not necessarily phosphorylated [1,2] it has often been difficult to decide whether these additional forms, which have been referred to as satellite forms [3], are of functional significance or merely artifacts. In the case of cardiac muscle [4,5] and slow skeletal muscle [6], it has been shown that the P light chain exists in two isoforms P1 and P2, each of which can be monophosphorylated to give derivatives, the P3 and P4 light chains respectively.

During the course of investigating the relationship between phosphorylation of smooth myosin from chicken gizzard and the actin-activated MgATPase it has been consistently observed in this laboratory that in some preparations of myosin the phosphorylated form of the P light chain can be resolved into two bands of different electrophoretic mobility in urea. Independently, Gagelmann et al. [7] have described reversible phosphorylation and dephosphorylation of two forms of the P light chain in skinned fibres from the carotid artery and Ledvora et al. [8] have reported the presence of three 20-kDa phosphorylated spots in stretched carotid artery strips.

We present evidence here that indicates that there are two sites of phosphorylation on the P light chain of chicken gizzard myosin.

2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Myosin was prepared as described [9] and either centrifuged immediately after precipitation with

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35 mM $MgCl_2$ or left in the cold overnight to settle. It was then further purified by gel filtration on Sepharose 4B [10]. Dithiothreitol (1 mM) was present in all buffers.

The myosin light chain fraction was prepared by the method of Grand and Perry [11]. Myosin light chain kinase was purified from chicken gizzard smooth muscle (2.5–10 μmol P/mg per min) and rabbit skeletal muscle (7.5–20 μmol P/mg per min) by the methods of Adelstein and Klee [12] and Nairn and Perry [13], respectively.

2.2. *Electrophoresis*

Electrophoresis of myosin was performed in prerun one-dimensional gels in 12.5% Acrylogel in 8 M urea, 20 mM Tris–125 mM glycine buffer, pH 8.6, or by the 2-dimensional method of Anderson and Anderson [14,15] with the modifications described by Westwood and Perry [3].

2.3. *Enzymic experiments*

ATPase assays and incorporation of ^{32}P into myosin were carried out as previously described [10].

The myosin light chain fraction (1.0 mg/ml) was incubated with 20 mM imidazole–HCl buffer (pH 7.5), 5 mM magnesium acetate, 1 mM $CaCl_2$, 1 mM DTT and 1 mM ATP or [γ-^{32}P]ATP (40 Ci/mol). The enzyme concentrations were chosen to ensure that 100% of the monophosphorylated form of the P light chain was obtained within 2 min on incubation at 30°C. The reaction was stopped by the addition of cold trichloroacetic acid to 5%.

2.4. *Peptide mapping*

Tryptic digestion and subsequent peptide mapping of the ^{32}P-labelled light chain samples were performed as described by Wilkinson [16] on Polygram SIL G plastic-backed thin layer nanoplates (10 × 10 cm, Camlab, Cambridge, England).

2.5. *Detection of phospho-amino acid*

^{32}P-labelled light chains (1–5 μg, 6 × 10^3 Cerenkov cpm) were hydrolysed in 6 M HCl for 1.5 h at 110°C and then analysed by electrophoresis at pH 3.5 or 2.0 on thin layer plates as described above. For autoradiography the plates were exposed to X-ray film for 10 h at −80°C with an intensifying screen.

3. RESULTS AND DISCUSSION

It was noted that when gizzard myosin was incubated with ATP, the pattern of phosphorylation of the P light chain by the small amount of endogenous kinase depended on the conditions of the myosin preparation. Nevertheless, the fully activated actomyosin MgATPase of all preparations was similar (170–200 nmol P/mg per min). If the myosin was recovered by centrifugation within 30 min after the first Mg^{2+}-precipitation step (see section 2), the amount of the normal phosphorylated form of the P light chain, which we designate the monophosphorylated P light chain, in the final preparation did not exceed 20–30% of the total P light chain fraction and was usually less (fig.1A(i)). On incubation with gizzard kinase and ATP the monophosphorylated form increased up to 80% or greater of the total P light chain fraction, and only a trace of a more acidic band sometimes appeared after long incubation (fig.1A(ii),(iii)). If the myosin was left to settle overnight before collection, up to 40–50% of the P light chain was present in a monophosphorylated form (fig.1B(i)). After incubation with ATP a more acidic band of phosphorylated protein which was present in low amounts in the original preparation, increased until it represented up to 30–40% of the total (fig.1A(ii),(ii),2). Careful study using 2-dimensional electrophoretic analysis of a number of preparations indicated that the new band was an additional phosphorylated form of the original P light chain and not the phosphorylated form of an isoform of the P light chain, as is the case in cardiac and slow skeletal muscles. Comparison of the specific radioactivities of the two spots obtained on electrophoresis indicated that both were phosphorylated on incubation with [γ-^{32}P]ATP although the fraction of the total P light chain represented by the monophosphorylated form showed little change. The more acidic phosphorylated light chain had a higher specific radioactivity, in some cases the values approaching twice that of the monophosphorylated P light chain.

The conclusion was confirmed by studies on the isolated whole light chain fraction of gizzard myosin. On incubation of this light chain fraction with myosin light chain kinase preparations from rabbit fast skeletal or chicken gizzard muscle the

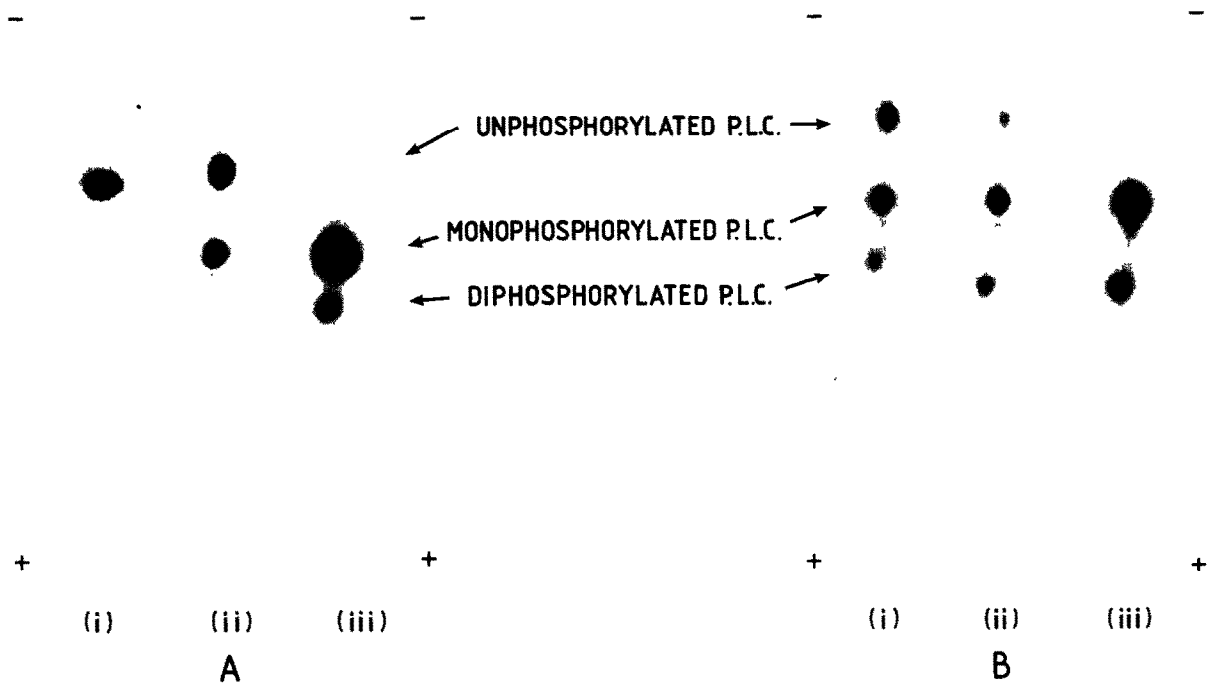

Fig.1. Two-dimensional gels of gizzard myosin preparations incubated with myosin light chain kinase and [γ-^{32}P]ATP. The regions illustrated are from 2-dimensional electropherograms corresponding to an M_r of 20000 and isoelectric point of pH 4.5–5.0 and containing only the P light chain of myosin. Incubation was carried out as described in section 2. Tracks A(i),(ii) and B(i),(ii) are stained with Coomassie blue. P.L.C., P light chain. (A) Myosin precipitated $\leqslant$30 min: (i) before incubation, (ii) after 5 min incubation, (iii) autoradiograph of (ii). It should be noted that although there is only a trace of the diphosphorylated P light chain this is strongly radioactive. (B) Myosin precipitated overnight: (i) before incubation, (ii) after 5 min incubation, (iii) autoradiograph of (ii).

normal monophosphorylated P light chain was rapidly produced. On prolonged incubation in the presence of excess enzyme the presumptive diphosphorylated P light chain was formed in addition. Under these conditions, up to 1.6 mol P per mol of P light chain could be incorporated. Comparison of tryptic digests of whole light chain fractions of myosin that contained only the monophosphorylated P light chain with those containing both the mono and diphosphorylated P light chains, indicated that an additional phosphorylated peptide was present in the latter digests (fig.3a,b). If light chains were phosphorylated to the monophosphorylated form with [γ-^{31}P]ATP and subsequently further phosphorylated with [γ-^{32}P]ATP to the diphosphorylated form, radioactivity in tryptic digests was almost entirely confined to the additional phosphorylated peptide (fig.3c). Thin layer chromatography of partial acid hydrolysates of the P light chain phosphorylated in the isolated light chain fraction indicated that phosphoserine was present when the monophosphorylated P light chain was present. When both mono- and diphosphorylated forms were present phosphoserine and phosphothreonine were detected.

Thus, we conclude that two phosphorylation sites are present in chicken gizzard P light chain; namely site a (serine) which may correspond to serine 19 and site b (threonine), the location of which is now under investigation. The more acidic phosphorylated band observed on electrophoresis would have sites a and b phosphorylated and the less acidic band has either site a or b phosphorylated.

The nature of the enzyme phosphorylating the second site is as yet uncertain, although in the isolated light chain fraction of smooth muscle myosin this site was phosphorylated, albeit slowly, by myosin light chain kinase preparations. There

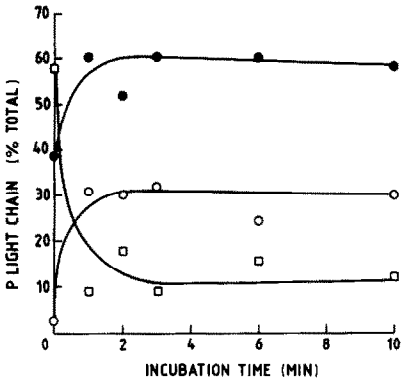

Fig.2. Myosin (1 mg/ml) incubated at 30°C with 50 mM imidazole–HCl buffer (pH 7.0), 6 mM $MgCl_2$, 5 mM ATP, 0.2 mM $CaCl_2$, 2.5 mM dithiothreitol, calmodulin (25 μg/ml), chicken gizzard tropomyosin (0.2 mg/ml), rabbit skeletal muscle actin (0.5 mg/ml) and gizzard myosin light chain kinase (~40 μg/ml). At times indicated the reaction was stopped by the addition of urea to 6.7 M, 5 mM dithiothreitol and the incubate electrophoresed in 8 M urea, pH 8.6. Bands estimated by densitometric scanning of stained gels. (□) Unphosphorylated P light chain; (●) monophosphorylated P light chain; (○) diphosphorylated P light chain.

are suggestions from studies with the isolated light chains that site a is preferentially phosphorylated, but a similar conclusion cannot yet be made about intact myosin. Nevertheless, without isolation of the phosphorylated amino acids or peptides it cannot be detected whether the a or b sites are phosphorylated in the monophosphorylated form observed on electrophoresis of intact myosin. The probability of different enzymes being involved in phosphorylation of the two sites is suggested by the fact that the more highly phosphorylated myosin preparation obtained after overnight precipitation is produced in the presence of EGTA, conditions in which myosin light chain kinase would be presumed to be inactive. The possibility of transfer of the phosphate group between the a and b sites must also be considered.

Protein kinase C has been shown to phosphorylate turkey gizzard myosin at several sites in addition to that phosphorylated by myosin light chain kinase with a consequent reduction in the actin-activated ATPase [17]. In our system the relation between phosphorylation of sites a and b and activation of the MgATPase of chicken giz-

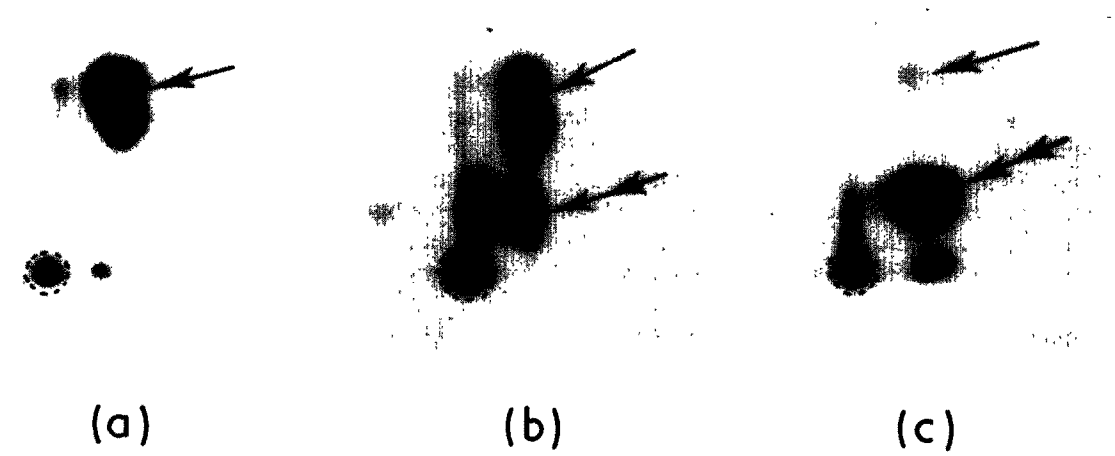

Fig.3. Autoradiographs of peptide maps of tryptic digests of the ^{32}P-labelled P light chains of gizzard myosin. Whole isolated light chain fraction incubated for 2–40 min with excess gizzard muscle light chain kinase and [γ-^{32}P]ATP under the conditions described in section 2. The incubate was digested with trypsin (1 to 50) for 4 h at 37°C and the sample applied to thin layer silica gel plates that were developed under the conditions described by Wilkinson [16]. Figures are autoradiographs of the plates. Origins indicated by dotted circle. Arrows with single head indicate site b peptide(s), with double heads site a peptide(s). (a) Monophosphorylated P light chain; (b) diphosphorylated P light chain; (c) site a phosphorylated with [γ-^{31}P]ATP and site b phosphorylated with [γ-^{32}P]ATP.

zard actomyosin is far from clear. Overnight phosphorylation in EGTA during the precipitation step did not activate the enzyme. Ca^{2+}-activated phosphorylation of myosin resulting in the conversion of 50% of the P light chain to the monophosphorylated form led to activation. Thereafter, phosphorylation leading to the appearance or an increase in amount of the diphosphorylated P light chain took place without rise in MgATPase activity. These findings explain the reports [9,10] that phosphorylation of the P light chain of gizzard myosin can occur without increase in the MgATPase of actomyosin. It is now apparent from studies on a variety of smooth muscle preparations that additional phosphorylated forms are produced both on activation of the intact fibre and when isolated myosin is incubated with kinase preparations. We do not believe that the findings we have described are a consequence of 'pseudophosphorylation' [2] as the preparation of myosin and electrophoresis were carried out in 2-mercaptoethanol or dithiothreitol to reduce the possibility of P light chain modification. Without careful analysis of the forms of the P light chain present and precise knowledge of which phosphorylated forms of smooth muscle myosin are enzymically active, meaningful conclusions cannot yet be made about the cooperative nature of myosin P light chain phosphorylation and its relation to MgATPase activation (cf. [18,19]).

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