Phosphorylation site sequence of smooth muscle myosin light chain (M_r = 20000)

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The amino terminal sequence of the myosin light chain (M_r = 20 000) isolated from chicken gizzards was found to be acetyl-Ser-Ser-Lys-Arg-Ala-Lys-Ala-Lys-Thr-Thr-Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser(P)-Asn-Val-Phe. This sequence assignment differs from that reported by Maita et al. [(1981) European J. Biochem. 117, 417] in the order of the tryptic peptides. The revised amino acid sequence exhibits greater homology with the phosphorylation site sequences of the regulatory light chains from cardiac and skeletal muscle. Moreover it is now apparent why synthetic peptides corresponding to the previously reported sequence were very poor substrates for the myosin light chain kinase.

Protein kinase *Myosin* *Specificity*

1. INTRODUCTION

The phosphorylation of the myosin regulatory light chains (M_r 20000) is believed to initiate contraction in vertebrate smooth muscles (review [1]). The enzyme responsible for catalyzing this reaction, Ca^{2+}–calmodulin-dependent myosin light chain kinase, has been purified to homogeneity and characterized [2]. This kinase phosphorylates a single serine residue near the amino-terminus of the light chain [3] which has been shown to be Ser-19 in the complete amino acid sequence of the chicken gizzard myosin regulatory light chain determined in [4]. Sequence studies on the amino-terminal region around the phosphorylation site of this light chain (Jakes and Kendrick-Jones, unpublished) however, indicated a different sequence from that in [4]. Furthermore, synthetic peptides of this region corresponding to the published sequence [4] were relatively poor substrates for myosin light chain kinase [5] whereas synthetic peptides corresponding to the alternative sequence (unpublished) were phosphorylated with apparent K_m values comparable to those of the intact light chain [5]. Since the correct amino acid sequence is vital for the design of synthetic peptide substrates and specific antigenic sites, we have re-examined the amino acid sequence on the amino-terminal side of the phosphorylatable serine in myosin light chains from chicken gizzards.

2. MATERIALS AND METHODS

Chicken gizzard myosin light chains [3,6], myosin light chain kinase [2,7] and cAMP-dependent protein kinase [8] were purified as described. Trypsin (treated with tosylphenylchloromethyl ketone) and chymotrypsin were from Boehringer. Automated sequence analysis was carried out using an Applied Biosystems gas phase sequenator [9] or manually using the Dansyl-Edman procedure [10].

3. RESULTS

3.1. *Isolation of peptic phosphopeptide*

The gizzard myosin regulatory light chain was phosphorylated stoichiometrically with myosin

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light chain kinase [7]. The reaction was stopped by adding trichloroacetic acid to 15% (w/v) and the precipitated light chains were isolated by centrifugation and oxidized with performic acid [11]. The oxidized phosphorylated myosin light chains were lyophilized, resuspended in 5% HCOOH (v/v) and digested with pepsin (92 μg/ml at 37°C for 4 h) then chromatographed on SP-Sephadex (fig.1A) with a pyridine–acetic acid gradient. The major ^{32}P radioactive peak accounted for 81% of the ^{32}P radioactivity applied to the SP-Sephadex column. The peptic phosphorylated peptide was further purified by reverse-phase HPLC on an RP-300 Aquapore column (Brownlee Labs) with a linear gradient of 12–24% CH_3CN in 0.1% CF_3COOH (v/v) over 30 min. The single ^{32}P radioactive peak was recovered with 61% yield. The amino acid composition of the peptic phosphopeptide (table 1) was in agreement with that expected from the sequence in [4]. Since the amino-terminus of the myosin light chain is blocked [4] no attempt was made to sequence the peptic phosphopeptide directly.

3.2. *Isolation of tryptic phosphopeptides*

The peptic phosphopeptide was succinylated (135-fold excess of succinic anhydride) and further purified by reverse-phase HPLC as described above except with a steeper gradient of CH_3CN (see fig.1B). The succinylated phosphopeptide was digested with trypsin [75 μg/ml in 0.1 M NH_4HCO_3 (pH 7.8) at 37°C for 2 h]. The resultant tryptic peptides were purified by reverse-phase HPLC as described for the succinylated phosphopeptide. Three major tryptic peptides were obtained (fig.1C). Only the third peptide, TsP_3, was associated with ^{32}P radioactivity. The amino acid composition of these peptides is given in table 1. Peptide TsP_1 had the composition Ser_2, Lys, Arg expected of the amino terminal tryptic peptide [4] (see below). Peptides TsP_2 and TsP_3 were subject to automated sequence analysis. The sequences were found to be: TsP_2, Ala-Lys*-Ala-Lys*-Thr-Thr-Lys*-Lys*-Arg-Pro-Gln-Arg and TsP_3, Ala-Lys*-Ala-Lys*-Thr-Thr-Lys*-Lys*-Arg-Pro-Gln-Arg-Ala-(Thr Ser Asn Val Phe).

The same sequences were obtained on tryptic peptides derived from two independent isolations of the peptic phosphopeptide from the myosin light chains. PTH-lysine residues were not

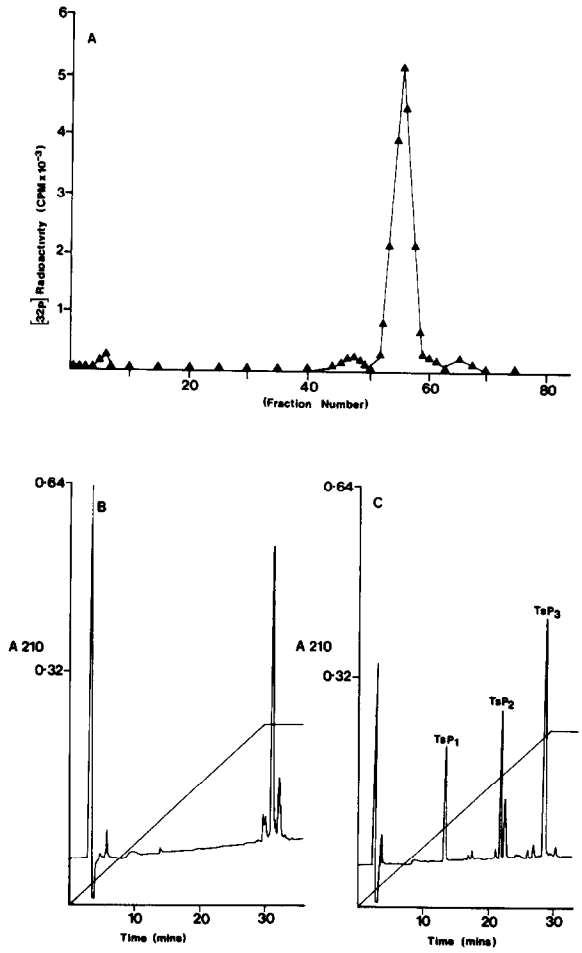

Fig.1. Peptide purification. (A) SP-Sephadex ion-exchange chromatography following peptic digestion of phosphorylated gizzard myosin light chains. Phosphorylated peptide was eluted with a linear gradient of 0.05–2.0 M pyridine in acetic acid. Fractions (7.5 ml) were collected and an aliquot (20 μl) subjected to liquid scintillation counting. The major ^{32}P radioactive peak was collected. (B) Purified phosphorylated peptic peptide was treated with succinic anhydride and the succinylated phosphopeptide purified by reverse-phase HPLC on an RP-300 column (Brownlee Labs). Peptides were eluted with a linear gradient of 0–24% CH_3CN in 0.1% CF_3COOH (v/v) over 30 min at a flow rate of 1.0 ml/min and detected by monitoring absorbance at 210 nm. The major absorbance peak was collected. (C) The purified succinylated phosphopeptide was digested with trypsin (see text) and the digest chromatographed on an RP-300 reverse-phase HPLC column (Brownlee Labs). Peptides were eluted as in B and major absorbance peaks collected.

Table 1

Amino acid analysis of phosphopeptides from myosin light chain

Amino acid	P	TsP_1	TsP_2	TsP_3
Asp/Asn	1.10 (1)			1.29 (1)
Thr	2.47 (3)		2.26 (2)	2.58 (3)
Ser	2.89 (3)	2.03 (2)		0.74 (1)
Glu/Gln	1.05 (1)		1.11 (1)	1.41 (1)
Pro	0.95 (1)		1.11 (1)	1.04 (1)
Ala	3.03 (3)		2.08 (2)	3.26 (3)
Val	0.93 (1)			2.21 (1)
Phe	0.74 (1)			0.74 (1)
Lys	5.20 (5)	0.91 (1)	3.49 (4)	2.64 (4)
Arg	3.12 (3)	1.05 (1)	1.95 (2)	2.09 (2)

Serine and threonine were corrected for 10 and 5% destruction, respectively. Hydrolyses were for 24 h in 5.6 M HCl at 110°C. The figures in parentheses are expected values based on the sequence reported here. P, peptide phosphopeptide. TsP_1, TsP_2 and TsP_3 are the 3 tryptic peptides isolated following tryptic digestion of the succinylated peptic phosphopeptide

positively identified since the lysine was succinylated. Blanks were obtained after Edman degradation at 4 steps corresponding to the 4 lysine residues expected from the amino acid composition. The carboxyl terminal pentapeptide of TsP_3 was lost from the sequencer on both occasions. From the tryptic digestion results we would have expected a fourth tryptic peptide, Ala-Thr-Ser-Asn-Val-Phe, however, this peptide was not recovered from HPLC chromatography of the tryptic digest (fig.1C).

3.3. *Chymotryptic digestion of phosphorylated light chain*

Chymotryptic digestion of the phosphorylated light chains [3] yielded 3 ^{32}P-labelled peptides on two-dimensional chromatography and electrophoresis as in [3]. The sequences of two of these peptides were obtained by subtractive Edman degradation. The first, Arg-Ala-Thr-Ser(P)-Asn-Val-Phe was reported in [3], the second being Lys-Arg-Pro-Glx-Arg-Ala-Thr-Ser(P)-Asn-Val-Phe. The sequence of the chymotryptic phosphopeptides agrees with that obtained with the tryptic peptides (see above).

3.4. *Tryptic peptides of succinylated light chain*

Myosin light chain (M_r 20000) was succinylated (100-fold excess of succinic anhydride) in 6 M guanidine hydrochloride at pH 9.0. The succinylated protein was digested with trypsin (100:1, w/w) and the tryptic peptides isolated by reverse-phase HPLC (μBondapak C_{18} column, Waters Associates) in 10 mM ammonium acetate buffer (pH 4.5) and a gradient of 0–60% CH_3CN over 45 min. The resolved peptides were freeze-dried, redissolved in H_2O (100 μl) and aliquots analysed for amino acid content and dansyl amino-terminal acid identification. One of the peptides obtained (peak 6, not shown) had the composition Ser_2, Lys, Arg and when subjected to mass spectrometry [10] revealed the primary sequence acetyl-Ser-Ser-Lys. This peptide is the amino-terminus of the myosin light chain and is the same as peptide TsP_1 (table 1). Another peptide (peak 8, not shown) had the composition Thr_2, Glx, Pro, Ala_2, Lys_4, Arg_2 and following subtractive Edman degradation revealed the sequence Ala-Lys-Ala-Lys-Thr-Thr-Lys-Lys-Arg-Pro-Glx. The sequence of this peptide corresponds with that of the tryptic peptide TsP_2 (table 1) obtained by automatic sequencing of the peptic phosphopeptide following succinylation and tryptic digestion (see section 3.2).

4. DISCUSSION

The amino-terminal sequence of the myosin light chain (M_r 20000) isolated from chicken gizzards was found to be acetyl-Ser-Ser-Lys-Arg-Ala-Lys-Ala-Lys-Thr-Thr-Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe. This sequence assignment gives rise to precisely the same amino acid composition as the sequence in [4] but differs in the order of the tryptic peptides. Our sequence was determined from both tryptic and chymotryptic peptides derived from phosphorylated myosin light chains and was also supported by the sequence analysis of tryptic peptides derived from non-phosphorylated, succinylated myosin light chains.

The sequence reported herein is consistent with the phosphorylation pattern and substrate specificity properties of both myosin light chain kinase and cAMP-dependent protein kinase. We [5] have shown that the synthetic peptide corresponding to this sequence is a much better substrate for myosin light chain kinase (table 2)

Table 2

Kinetics of peptide phosphorylation[a]

Peptide sequence	cAMP-dependent protein kinase		Myosin light chain kinase	
	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})
KAKTTKKRPQRATSNVFS NH_2[b]	120	0.29	6.9	3.4
RPQRAKAKTTKATSNVFS NH_2[c]	165	0.016	193	0.39

[a] From [15]
[b] Peptide analog of sequence reported here with serine at residue 23
[c] Peptide analog of sequence reported in [4] with serine at residue 23

than the peptide corresponding to the sequence in [4]. Furthermore, it has been shown in [13] that cAMP-dependent protein kinase phosphorylates the gizzard myosin light chain at the same site as myosin light chain kinase. The cAMP-dependent protein kinase usually requires arginine residues within one or two residues of the phosphorylatable serine [14]. In our sequence there is an arginine within two residues of Ser-19 whereas in the published sequence [4] the nearest arginine is 10 residues away (table 3). The substrate specificity of cAMP-dependent protein kinase is further demonstrated when the kinetics of phosphorylation of the peptides corresponding to both sequences are compared (table 2), i.e., the peptide with our sequence is a better substrate for the enzyme.

It is of interest to compare the amino-terminal sequence of the smooth muscle light chain with those of other regulatory light chains (table 3). One of the striking features is a tribasic residue sequence located 4 or 5 residues from the phosphorylated serine in all the vertebrate regulatory light chains. This gains further significance since we have recently shown that these 3 basic residues are essential specificity determinants for smooth muscle myosin light chain kinase [5]. Whether the tribasic residues are also important determinants for the cardiac and skeletal muscle enzyme remains to be determined. The absence of these residues in the molluscan (scallop) regulatory light chain may explain our inability to phosphorylate this light chain [18] and would further exclude the possibility that light chain phosphorylation is involved in the regulation of contractile activity in molluscs.

Table 3

Comparison of the primary structures of the regulatory light chains

Tissue	Sequence	Ref.
Skeletal muscle	P [K] K A [K R R] A A E G S (S) N V F S	16
Cardiac B	P [K] K A [K K K] V E G G – (S) N V F S	17
Cardiac A	P [K] K A [K K R] I E G A N (S) N V F S	17
Gizzard	Ac S S K R A K A [K] T T [K K R] P Q R* A T (S) N V F	Here
Gizzard	X S S K R R K R P Q R A K A K T T K A T (S) N V F A	4
Molluscan (scallop)	Ac A D K A A S G V L T	19

Basic residues common to the 4 vertebrate sequences are boxed. The phosphorylatable serine residues are encircled and the arginine residue important for the cAMP-dependent protein kinase is marked with an asterisk. The acetylated NH_2-termini are marked Ac

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