CALCIUM BINDING REGIONS OF MYOSIN 'REGULATORY' LIGHT CHAINS

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Received 18 September 1976

1. Introduction

All the vertebrate myosins so far examined contain 'regulatory' light chains, with molecular weights of about 19 000, which bind to 'desensitized' scallop myofibrils (replacing the released scallop light chain) and restore calcium regulation [1]. On the basis of their ability to restore full calcium binding to desensitized scallop myofibrils, these regulatory light chains can be divided into two groups: (1) light chains from smooth muscles, where preliminary evidence indicates myosin linked calcium regulation is present [2,3], restore full calcium binding, and (2) light chains from skeletal and cardiac muscles, where biochemical evidence indicates the absence of myosin linked regulation, have no effect on calcium binding [1]. The primary sequence around the calcium binding site of the light chain from a smooth muscle myosin was therefore determined and compared with the published sequence of the skeletal myosin light chain [4] in an attempt to understand the differences in the calcium binding affinity of these two groups of light chains.

The light chains from vertebrate smooth and skeletal myosins are phosphorylated by a highly specific calcium requiring light chain kinase, which would further imply a regulatory function [5,6]. The serine residue phosphorylated in the skeletal light chain is near the N-terminus [4] and in this study we have located and identified the phosphorylated residue in the primary sequence of the smooth muscle light chain.

2. Materials and methods

The source of vertebrate smooth muscle chosen for these studies was from chicken gizzards. Actomyosin was prepared from fresh chicken gizzards by a procedure previously described [7]. Scallop myofibrils were prepared and 'desensitized' by the method outlined by Kendrick-Jones et al. [1]. Rabbit myosin light chain kinase was prepared by the procedure described by Pires et al. [8].

2.1. *Gizzard myosin light chains*

The light chains were isolated from gizzard actomyosin by dissociation in 6 M guanidine–HCl as described by Perrie and Perry [9] and separated by chromatography on DEAE–cellulose (Whatman DE-52) (fig.2).

2.2. *Phosphorylation*

The gizzard 'regulatory' light chain (M_r = 20 000 G_I) was phosphorylated by two methods: (1) Gizzard actomyosin (10 mg/ml) was phosphorylated by endogeneous kinase by incubating in 60 mM NaCl, 25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM $CaCl_2$ and 2.5 mM [γ ^{32}P]ATP (1 μCi/μmol). (2) The isolated regulatory light chain was incubated with rabbit myosin light chain kinase under the conditions outlined by Frearson et al. [6]. The extent of ^{32}P incorporation was followed with time by precipitating aliquots of the incubation mixtures with 5% trichloroacetic acid. The protein precipitates were washed on millipore filters, dried, dispersed in toluene scintillation fluid containing 20% Biosolv (Beckman) and counted. After 10 min incubation at 30°C (0.5–0.7 mol ^{32}P incorporated per mole of regulatory light chain) the reaction was terminated: (1) In the case of the actomyosin by the addition of solid guanidine HCl and the phosphorylated light chain separated from the non phospho-

rylated light chains by chromatography on DEAE–cellulose and (2) with the isolated light chain by addition of solid urea to 2 M final concentration and desalting on G25 Sephadex in 50 mM ammonium bicarbonate, pH 8.1.

The phosphorylated light chains (300 nmol) in 50 mM ammonium bicarbonate, pH 8.1, were digested with chymotrypsin (100:1 protein/enzyme ratio w/w at 37°C for 4 h) and the [^{32}P]peptides isolated by electrophoresis, at pH 6.5, in the first dimension and by descending chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:10:12 by vol.) in the second dimension. The radioactive peptides were detected by autoradiography, eluted and sequenced by the manual Edman technique [10]. Regulatory light chain fragments were prepared by digesting gizzard actomyosin (6–8 mg/ml) in 60 mM NaCl, 2 mM EDTA, 20 mM Imid, pH 7.0, 1 mM DTT at 25°C with (a) papain (2000:1 protein/enzyme ratio w/w) for 4 min. Iodoacetic acid (final concentration 1 mM) added to terminate digestion. (b) Chymotrypsin (200:1 protein/enzyme ratio w/w) for 5 min. Digestion terminated by addition of phenylmethylsulphonyl fluoride (0.5 mM). The precipitated actomyosin was collected by centrifugation, washed to remove solubilised material and the light chains released by 6 M guanidine–HCl treatment [9] and fractionated on DEAE–cellulose (fig.2).

The light chain fragment sequence data was obtained on a Beckman model 890B automated sequencer using peptide program 11 13 74 which utilises a *N,N*-Dimethyl-*N*-allylamine buffer system, and the PTH amino acids identified by thin layer chromatography [11], gas–liquid chromatography [12] and amino acid analysis following hydrolysis with HI [13].

3. Results

The gizzard 'regulatory' light chain (G_I M_r = 20 000) in common with all the other muscle proteins whose sequences are known, contains a blocked N-terminus. Therefore to produce light chain material which might be amenable to sequence analysis by an automatic sequencer, this blocked N-terminal residue must be removed. Although fragments, in the 14–16 000 mol. wt range, are formed by brief chymotryptic or tryptic digestion of the isolated G_I light chain, a far more homogeneous light chain fragment is produced if gizzard actomyosin is digested briefly with either chymotrypsin or papain. Examination of the proteolytic susceptibility of the G_I light chain when bound to the myosin reveals that it is initially cleaved to a fragment, with a molecular weight of about 16 000, which remains attached to the myosin (fig.1). This fragment can only be differentiated from the other gizzard light chain (G_{II} M_r = 17 000) when low protein concentrations are run on the gels. The G_I light chain fragment prepared by brief chymotryptic or papain digestion of actomyosin can be separated from the intact G_I and the G_{II} light chains by chromotography on DEAE–cellulose (figs.1 and 2). Characterisation of these fragments indicates a loss of about 20–24 amino acid residues, including the blocked N terminal residue. The primary sequence of the N terminal regions (47 amino acid residues) of both the papain and chymotryptic G_I fragments, together with sequence of the chymotryptic phosphoserine containing peptide, have been determined and compared with the sequence of the same region in the rabbit skeletal (DTNB) [4] and scallop (EDTA) 'regulatory' light chains [14] (fig.3). The most striking observation is the extent of homology in this region in all the light chain sequences (73% of the amino acid residues are identical in the gizzard and rabbit sequences and most of the substitutions are conservative). The serine residue specifically phosphorylated in both the rabbit and gizzard light chains has been positively identified (serine 15 in the rabbit light chain sequence). The same serine residue is phosphorylated in the gizzard light chain whether partially purified rabbit myosin light chain kinase or 'endogeneous gizzard muscle kinase' are used. It is interesting that the scallop light chain under the same conditions is not phosphorylated.

The three dimensional structure of carp calcium binding parvalbumin [15] has served as a model for identifying potential calcium binding regions in troponin C [16] and rabbit 'DTNB' light chain [4]. In parvalbumin, each calcium binding site lies in a 'pocket' surrounded by helical regions on either side and within the site, calcium is coordinated to oxygen atoms from six amino acids (the position of the amino acids in capital letters in fig.3), which form the vertices

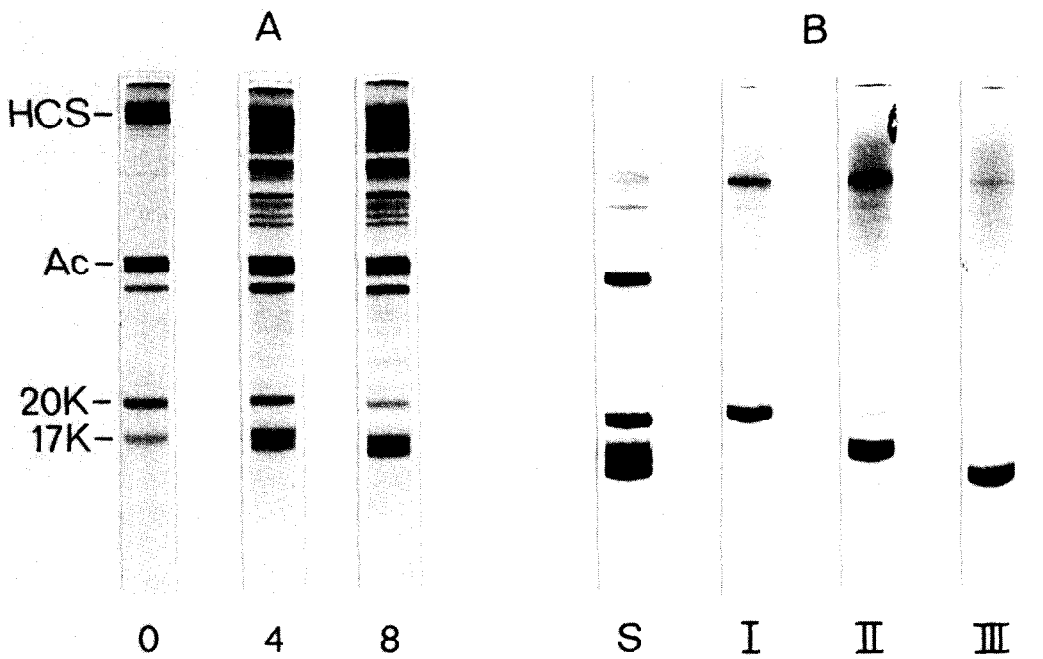

Fig.1. 10% acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of: (A) gizzard actomyosin digested with chymotrypsin under conditions outlined in the method section. Control (0), 4 and 8 min digests. (B) Pooled fractions from DEAE–cellulose chromatography of total light chain fraction isolated from gizzard actomyosin digested with chymotrypsin (fig.2). Original total light chains (S), fractions I, II and III (see fig.2).

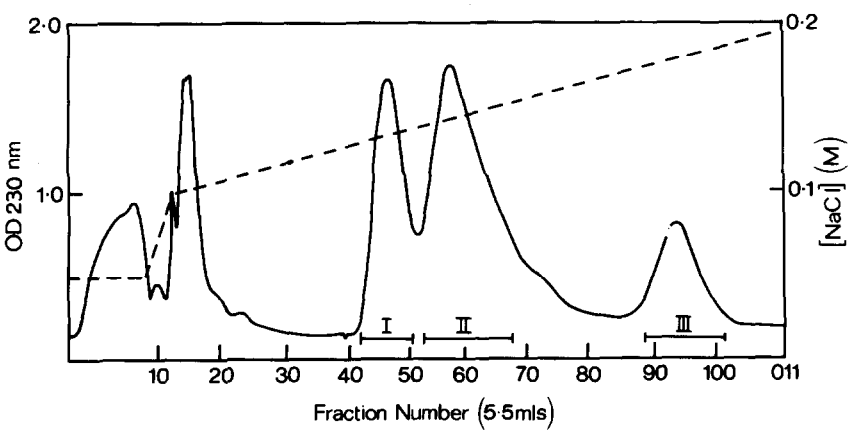

Fig.2. DEAE–cellulose chromatography of total light chains isolated from gizzard actomyosin after brief chymotryptic digestion (for conditions of digestion see Materials and methods section). Light chains (25 mg) chromatographed on DEAE–cellulose (12.5 × 1.3 cm) in 2 M urea, 25 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM DTT, 25 mM Tris–HCl buffer, pH 7.5, at 20°C and eluted with a linear gradient (total volume 600 ml) from 100–200 mM NaCl. The composition of the original light chain sample applied to the column, and the pooled fractions I, II and III are shown in fig.1.

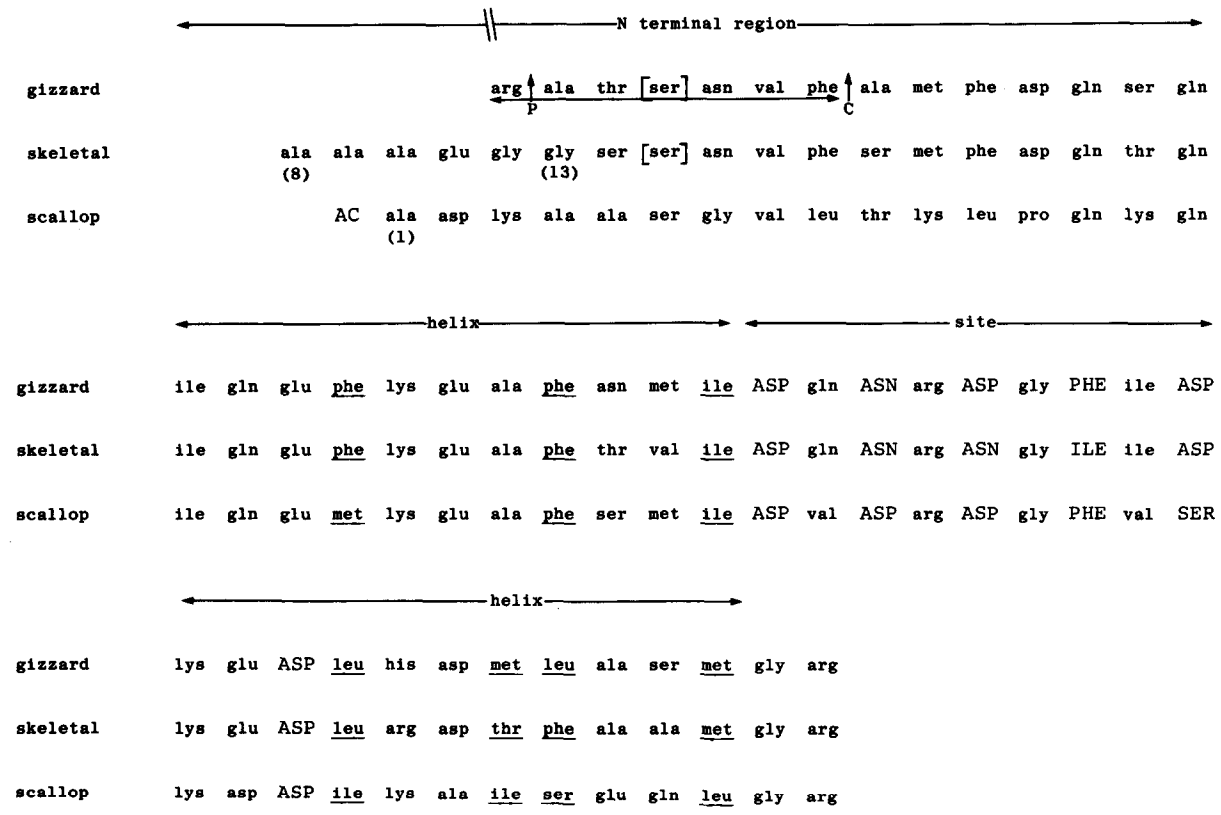

Fig.3. Primary sequence of the gizzard 'regulatory' light chain containing the phosphoserine residue and calcium binding region, compared with the same region of the sequence of the rabbit [4] and scallop regulatory light chains [14]. With the exception of the N-terminal region, the sequences have been drawn out to correspond to the main structural features of carp parvalbumin [20]. In the N terminal region, P and C (arrowed) denote the positions in the sequence of the gizzard light chain where papain and chymotrypsin respectively cleave to produce the light chain fragments and the underlined sequence that of the [^{32}P]phosphoserine containing peptide isolated from a total chymotryptic digest of phosphorylated light chain. The position of the phosphoserine residue is indicated by the square brackets. Note the position of the single phosphoserine residue in the rabbit light chain has similarly been positively identified. Preliminary findings indicate that the only difference observed between the sequences of the rabbit and chicken breast skeletal light chains in this region is the deletion of alanine at position 8 and glycine at position 13 in the chicken light chain sequence. In the calcium binding site, the positions of the residues which by analogy with parvalbumin, are believed to be involved in coordinating calcium are indicated by capital letters. In the 'helical' regions which surround each calcium binding site, the residues underlined correspond to the positions of hydrophobic amino acids which are buried and form the core of parvalbumin. Hydrophobic residues are present in each of the light chain sequences in the required positions, which would further suggest that the three dimensional structure of the light chains in this region is related to parvalbumin.

of an octahedron and within these six coordinating positions four acidic residues are present. Using these criteria and by comparison with the rabbit and scallop light chain sequences, the calcium binding site in the gizzard light chain can be recognised. Although the sequence of the calcium binding sites in all three light chains are almost identical, there is one distinct difference which may explain their different calcium binding capabilities [1]. Four acidic residues are present within the six coordinating positions in the sites of gizzard and scallop light chains and since both these light chains restore the high affinity calcium binding site ($K \sim 10^6$ M^{-1}) when they combine with desensitized scallop myofibrils (table 1), it is reasonable to assume that the binding constant for these sites is about 10^6 M^{-1}.

Table 1
Effect of 'regulatory' light chains and their fragments on ATPase activity and calcium binding of scallop myofibrils

	ATPase activity (μmol/min per mg)		Ratio of ATPases	Calcium binding
	0.1 mM EGTA	0.1 mM Ca^{2+}	(EGTA/Ca^{2+})	Number of sites
Myofibrils	0.002	0.283	0.01	1.88
Desensitized myofibrils	0.180	0.224	0.80	1.16
Desensitized myofibrils + rabbit LC (DTNB)	0.010	0.210	0.05	1.08
Desensitized myofibrils + gizzard LC	0.014	0.239	0.05	1.93
Desensitized myofibrils + gizzard LC (papain fragment)	0.028	0.352	0.08	2.28
Desensitized myofibrils + gizzard LC (chymotryptic fragment)	0.037	0.302	0.12	1.82

Desensitized scallop myofibrils (3–4 mg/ml) were incubated with the light chain fractions (at a 1:1, light chain/myosin, molar ratio, assuming that the myosin content of the myofibrils is 65%) overnight with gentle stirring in 40 mM NaCl, 1 mM $MgCl_2$, 5 mM phosphate (pH 7.0), centrifuged in a bench centrifuge and washed three times with the same solution. Actin-activated, Mg^{2+}-dependent myosin ATPase activities and calcium binding measurements performed by the procedures previously outlined [19]. The ratio of the ATPases (EGTA/Ca^{2+}) indicates the 'calcium sensitivity' of the preparations, i.e. low values indicates calcium regulation of actin–myosin interaction. The number of calcium binding sites, with affinity constants in the range $3–7 \times 10^6$, were obtained from Scatchard plots.

The calcium binding site in the rabbit light chain however contains only three negatively charged residues within the coordination positions which may explain the lower calcium affinity of this site ($K \sim 10^5\ M^{-1}$) [17] and the failure of this rabbit light chain to restore the high affinity calcium binding site when it binds to 'desensitized' scallop myofibrils (table 1) [1].

The chymotryptic and papain fragments of the gizzard light chain are still capable of binding to desensitized scallop myofibrils and restoring both calcium regulation of myosin–actin interaction and the lost calcium binding site, i.e. are completely functional (table 1). Similarly prepared 'regulatory' light chain fragments isolated from rabbit and chicken breast skeletal myosins and the fragments produced by digestion of the isolated gizzard light chain, are, however, unable to bind to 'desensitised' scallop myofibrils. Obviously digestion of the gizzard light chain while it remains attached to the myosin preserves the 'native conformation' of the light chain which is essential for binding to scallop myosin. The papain gizzard light chain fragment, which still retains the necessary serine residue however is not phosphorylated under standard conditions which may imply that an intact N-terminus is required to promote phosphorylation.

4. Conclusions

The high degree of homology between the N-terminal sequences of the vertebrate smooth, vertebrate skeletal and molluscan 'regulatory' light chains would imply a very similar three dimensional structure and common function. Since the sequences examined are basically the calcium binding unit, composed of two helical regions surrounding the calcium binding site, calcium must play a role in the function of all these light chains. The available evidence indicates that the gizzard [2,3,18] and scallop regulatory light chains [19] are involved in calcium regulation, whereas the exact function of the rabbit skeletal light chain remains obscure. Phosphorylation appears to be a recent evolutionary acquisition, although its precise function, whether the proximity of the phosphoserine residue to the calcium binding region could possibly alter the calcium binding affinity of the light chain or whether a phosphorylation–dephosphorylation cycle exists in vivo, remains to be established.

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