Role of the C-terminal extremities of the smooth muscle myosin heavy chains: implication for assembly properties

Sophie Quevillon-Chéruel[a], Georges Foucault[a], Michel Desmadril[b], Anne-Marie Lompré[a], Jean-Jacques Béchet[a,*]

[a] *Laboratoire des Gènes et Protéines Musculaires, CNRS EP 1088, Bâtiment 430, Université de Paris-Sud, 91405 Orsay Cedex, France*
[b] *Laboratoire de Modélisation et Ingénierie des Protéines, CNRS EP 1088, Université de Paris-Sud, 91405 Orsay Cedex, France*

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Abstract The two light meromyosin isoforms from rabbit smooth muscle were prepared as recombinant proteins in *Escherichia coli*. These species which differed only by their C-terminal extremity showed the same circular dichroism spectra and endotherms in measurements of differential scanning calorimetry. Their solubility properties were different at pH 7.0 in the absence of monovalent salts. Their paracrystals formed at low pH differed by their aspect and number. These data suggest a role for the C-terminal extremity of myosin heavy chains in the assembly of myosin molecules in filaments and consequently in the contractility of smooth muscles.

Key words: Myosin heavy chain; Smooth muscle; Assembly; Solubility

1. Introduction

Myosin II is a major contractile protein of skeletal, cardiac and smooth muscles. It is composed of two heavy chains (approximately 200 kDa) and two pairs of light chains (approx. 20 kDa). The myosin heavy chains form a dimer consisting of two globular amino-terminal heads and carboxy-terminal α-helical coiled coil tails. The heads bind the light chains and contain the ATP- and actin-binding sites whereas the tails are involved in the assembly of myosin molecules into filaments.

Numerous isoforms of myosin differing by their heavy and (or) light chains have been identified. In the case of striated muscles the heavy chain isoforms are expressed by different genes and have been associated with distinct mechanical properties of these muscles. In smooth muscle cells, four smooth muscle specific myosin heavy chain isoforms are produced by the alternative splicing of a single gene, and moreover two non-muscle myosin heavy chain isoforms NMA and NMB have also been detected ([1] and references therein). Their relative expression depends on the muscle type, the development stage and the tissue differentiation state. Two muscle specific isoforms (named SMB and SMA, respectively) are defined by the presence or the absence of an insert of seven amino acids in the N-terminal globular head region. This insert is in a loop which could be a modulatory element of myosin kinetic properties [2]. The two others (called SM1 and SM2) differ just at their C-termini by two different non-helical sequences with different lengths. It has been suggested that these two tailpieces which are not present in cardiac or skeletal myosin heavy chains may differentially influence filament stability or packing (for review see [3]), or even the contraction velocity in vivo [4].

To specify the role of the myosin heavy chain C-terminus extension in smooth muscle function we have prepared the two light meromyosin isoforms of rabbit (called LMM1 and LMM2 in Fig. 1) by expression of their recombinant DNAs in *Escherichia coli* and compared various physicochemical properties of these two species.

2. Materials and methods

2.1. General DNA manipulations and expression of recombinant light meromyosins (LMM)

Oligodeoxynucleotides were synthesized by Cybergene S.A. (Saint-Malo, France). Restriction endonucleases and DNA modification enzymes were from New England Biolabs and were used as recommended by the supplier. Expression vector pET-28a and expression strain BL21(DE3) were from Novagen. LB medium was from Genaxis and isopropyl β-D-thiogalactopyranoside (IPTG) was from Sigma.

The PBRUC1 clone encoding part of the myosin heavy chain type SM1 from rabbit uterus was a generous gift from Dr. Muthu Periasamy (University of Cincinnati, USA) [6]. PBRUC1 was digested with *Sph*I, blunted with Klenow fragment and digested with *Eco*RI. The insert encoding LMM1 (Met-1295 to Glu-1972 of SM1) was subcloned into the (*Nco*I)-*Eco*RI site of the expression vector pET-28a, to form pET-LMM1. The LMM2 isoform was constructed by PCR using pET-LMM1 as template, using primers 5′SMHC-*Nco*I (5′CATGCCATGGGCCGCGAG-3′) and 3′SM2-*Hin*dIII (5′-CCC-AAGCTTTCATTGAGAGGTTTCTTGCGGTGGAGGACCCCTG-AGCTTGCT-3′), containing *Nco*I and *Hin*dIII recognition sites, respectively. The PCR product was digested with *Nco*I and *Hin*dIII and subcloned into the *Nco*I and *Hin*dIII site of pET-LMM1, to form pET-LMM2. The nucleotide sequence of the PCR fragment was verified using the T7 sequencing kit from Pharmacia.

Purification of LMM1 and LMM2 was performed according to [7] with some modifications. The recombinant proteins were prepared from 1 l of BL21(DE3)/pET-LMM1 or pET-LMM2 grown at 37°C in LB-kanamycin (50 μg/ml) broth to a cell density of $A_{600nm} = 1$. Expression was induced at 37°C for 4 h by addition of 1 mM IPTG. All subsequent steps were conducted at 4°C. Cells were collected by centrifugation (5000×*g* for 10 min), washed with ice-cold buffer A (20 mM Tris-HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl), resuspended in 4 ml of buffer B (50 mM Tris-HCl, pH 8.0; 1 mM EDTA; 20% glycerol (v/v); 1 mM DTT), rapidly frozen in liquid nitrogen and kept at −20°C overnight. Cells were incubated 10 min at 4°C with lysozyme (1 mg/ml); a solution of 2 M NaCl as added to a final concentration of 1 M, and cells lysed by sonication. After centrifugation at 10000×*g* for 30 min, the lysate was heated 5 min at 95°C and cooled on ice for 1 h. After another step of centrifugation, the refolded proteins were fractionated by ammonium sulphate precipitation at 60% saturation. The pellet was dissolved in 30 mM pyrophosphate (pH 8.0), 1 mM EDTA, 1 mM DTT and purified by anion exchange chromatography on a DEAE Sephacel column equi-

*Corresponding author. Fax: +33 1 69 85 37 15.
E-mail: jean-jacques.bechet@bpc.u-psud.fr

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LMM1  1 MLSEAE.....KSKLRRGNETSFVPTRRSGGRRVIENADGSEEEVDARDADFNGTKSSE 678

LMM2  1 ------.....-----GPPPQETSQ 644
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Fig. 1. Sequences of LMM1 and LMM2. LMM1 and LMM2 contain 678 and 644 amino acid residues and their amino acid sequences are identical with the LMM regions (sequences 1295–1972 and 1295–1938, respectively) of rabbit smooth muscle myosin heavy chains [5,6]. Dashes represent identical amino acid residues and dots represent the common sequence 1301–1924 not rewritten here.

librated with the same buffer. The LMM isoforms were eluted with a linear gradient of 0 to 500 mM NaCl and the interesting fractions collected at about 250 mM NaCl. Nucleic acids were eliminated in these conditions.

Protein concentrations were determinated by the BCA protein assay (Pierce) using skeletal myosin rod as standard or by measuring the absorbance at 295 nm of ionized tyrosine residues at alkaline pH. In this last assay the molar absorbance was taken equal to 2500 M^{-1} cm^{-1} and LMM isoforms were considered to have ten tyrosine groups per molecule as deduced from sequences.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [8], using 10% polyacrylamide slab gels containing 0.1% SDS. After staining with the Coomassie Brillant Blue the LMM isoforms showed a single band at about 75 kDa as expected (Fig. 3, insert). These bands were slightly shifted, one compared with the other due to the different expected molecular weights of isoforms.

2.2. Circular dichroism and microcalorimetry measurements

Circular dichroism spectra were recorded over 200—250 nm using a Jobin-Yvon Mark-V dichrograph connected to an Apple 2e microcomputer. The protein concentration was 0.05 mg/ml and the measurement cell was 0.2 cm wide.

Differential scanning microcalorimetry (DSC) was carried out using a MicroCal model MC2. The buffer used was 50 mM phosphate (pH 7.0) containing 0.5 M NaCl. DSC scans were performed at a rate of 90 K/h in a temperature range from 20 to 80°C. Each sample (1 mg/ml) was thoroughly degassed before measurement.

2.3. Solubility tests

Solubility of the two LMM isoforms (at the concentration of 0.2 mg/ml) was determined at variable NaCl concentration and different pH in the range of 6.0 to 8.0. The buffers (50 mM) used were sodium phosphate (pH 6.0—7.5; pH adjusted with NaOH) and Tris-HCl (pH 8.0). After 1 h incubation on ice, the suspensions were centrifuged for 30 min at $13000 \times g$. Pellets and supernatants were denaturated and analyzed by SDS-PAGE according to Laemmli [8]. Analysis of the Coomassie Brillant Blue stained gels was performed by scanning with a Personal densitometer SI (Molecular Dynamics) and data were computed using ImageQuaNT as software.

2.4. Electron microscopy

Paracrystal formation and negative staining were performed essentially as described by Kakinuma et al. [9] with slight modifications adapted for the properties of smooth muscle LMM isoforms. Formation of paracrystals was started by dialysis of purified LMMs (0.5–1 mg/ml) at 4°C against a buffer containing 0.35 M NaCl and 10 mM sodium phosphate (pH 6.5, 6.0 or 5.8). The NaCl concentration of the dialysate was lowered by 0.05 M each day to a final concentration of 0.01 or 0.05 M.

Negative staining was carried out with 10 μl samples (possibly diluted in the corresponding buffer) deposited onto carbon collodion-coated grids for 20 s and rinsed for 20 s with the buffer. After removal of the excess solution, grids were stained with 1% (w/v) uranyl acetate and air-dried. Negatively stained samples were observed with a Philips model EM208 electron microscope.

3. Results and discussion

3.1. Physicochemical characterization of the recombinant polypeptides LMM1 and LMM2

The myosin tails (or myosin rod) consist of two α-helical polypeptide chains wound around one another in a left-handed coiled coil [10,11]. The recombinant polypeptides LMM1 and LMM2 expressed in *E. coli* are also predicted to have a coiled coil structure except in a few 'weak spots' (possibly coincident with some bends in myosin tails [12]) and their C-terminal segment (sequence 1934–1972 in LMM1; sequence 1931–1938 in LMM2) as shown in Fig. 2. This structural feature is confirmed by circular dichroism measurements (Fig. 3). The CD spectra of the two proteins have ellipticity peaks at 222 and 208 nm characteristic of α-helices and the values of their mean residue ellipticities at 222 nm (−33000) are identical and only a little lower than those reported for tropomyosin and light meromyosin fraction I which possess more than 90% helix [14].

Differential scanning calorimetry was performed to investigate thermodynamic properties of LMM1 and LMM2. The observed endotherms are symmetrical and have similar transition temperatures at 51.5 ± 0.5°C with a ΔH_{cal} of 275 ± 15 Kcal/mol (Fig. 4). The melting transition is reversible as observed after cooling and rescan. This result is comparable to that obtained with conventionally prepared LMM [15,16] and implies that LMM1 and LMM2 have rather uniform structural stability along their length.

Thus the C-terminal extension does not seem to change the coiled coil structure of LMM1 or LMM2 nor to confer particular physicochemical features on the isolated molecules.

3.2. Solubility properties of LMM1 and LMM2

At low pH and saline concentration of medium, LMM1 and LMM2 form aggregates. At pH 6.5 or lower, both species equally precipitate when the NaCl concentration falls lower than 0.2 M (Fig. 5A). At pH 7.0, the same result is found for [NaCl] < 0.1 M (Fig. 5B) but LMM1 (and not LMM2) remains unexpectedly soluble in the absence of NaCl (result of four independent experiments performed with two different

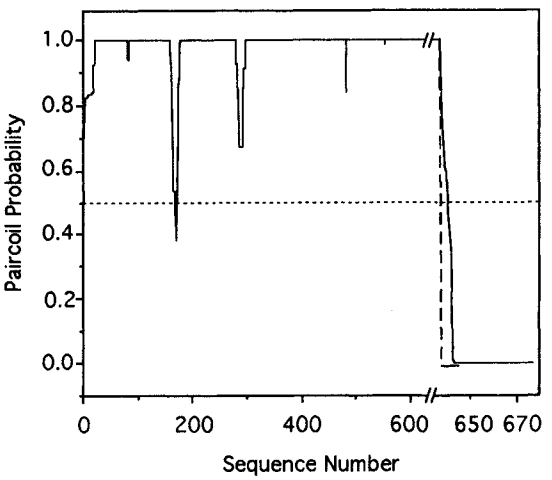

Fig. 2. Probability of forming coiled coil structures for LMM1 (solid line) and LMM2 (dashed line) using a paircoil probability program [13].

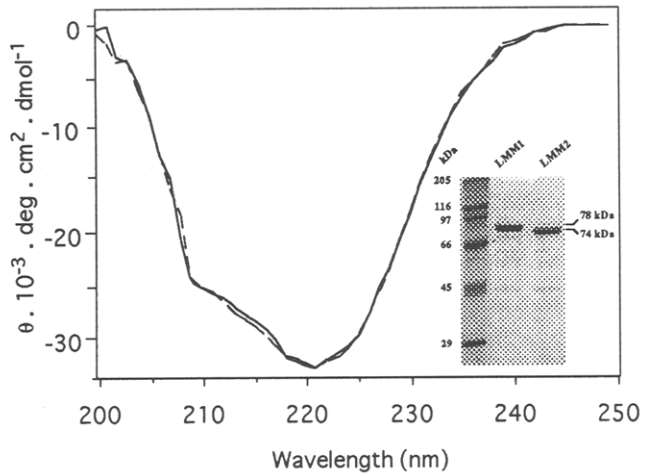

Fig. 3. Circular dichroism spectra of LMM1 (solid line) and LMM2 (dashed line) at 20°C. In insert, SDS-PAGE patterns of the two LMM isoforms.

preparations of each species). At pH higher than 7.0, LMM1 and LMM2 are soluble at all NaCl concentrations (Fig. 5C). It is well known that the smooth muscle LMM has a high solubility at low ionic strength [17], and behavior comparable to that of LMM1 at pH 7.0 has been observed for Dictyostelium non-muscle myosin [18,19]. Thus the solubility features of LMM1 and LMM2 are similar except at pH 7.0 in the absence of monovalent salt (NaCl or LiCl or KCl as experimentally verified). In these conditions the C-terminus of LMM1 which contains numerous charged amino acid residues appears to hinder the assembly of LMM1 molecules.

3.3. Ability of LMM1 and LMM2 to form paracrystals

The ability of LMM constructs to assemble was compared by electron microscopy after negative staining. LMM1 formed numerous spindle-shaped paracrystals in 0.1 M NaCl-0.01 M phosphate at pH 6.5 or lower but not at pH 7.0 (Fig. 6A). It was also sometimes observed organized in bundles of striated structures (Fig. 6B). These paracrystals showed usually a 44 nm axial repeat consisting of alternating light and dark bands with equivalent widths (Fig. 6C). In the same experimental conditions LMM2 formed rare paracrystals with the same aspect. It was only in 0.05 M NaCl and at pH 5.8 that LMM2 formed small paracrystals with axially repeated bands at 14 nm intervals (Fig. 6D).

The axial repeat patterns most commonly observed in paracrystals of LMM are 14.3 nm or 43 nm [20,21] and result from a parallel axial displacement between molecules or a combination of two displacements [22]. Thus McLachlan and Karn [10] have suggested that direct interactions between complementary charges on parallel rods staggered by 98 or 294 residues (the same theoretical staggers are found for LMM1 and LMM2 as verified) may account for these 14.3 and 43 nm axial spacings. Therefore the presence of an unstructured tail may favor the setting up of such ionic interactions and the intermolecular stagger, possibly because of steric hindrance between the tailpieces of neighboring molecules [23]. The longer the tail (as in LMM1), the more favored the larger stagger would be (43 nm instead of 14.3 nm).

SM1 and SM2 isoforms in rat and rabbit smooth muscles are differentially expressed during development [24]. The SM2 isoform usually appears only after birth and its expression is associated with an enhancement in the amount of intracellular

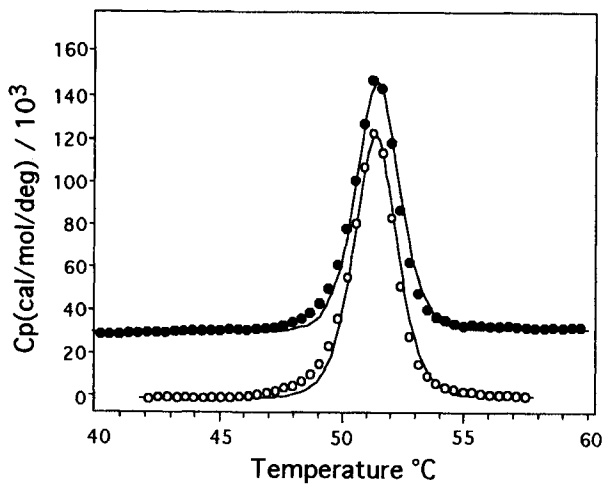

Fig. 4. DSC scans of LMM1 (●) and LMM2 (○). Solid lines result from smoothing treatment. They are shifted by 30 C_p units for convenience. C_p represents molar excess heat capacity.

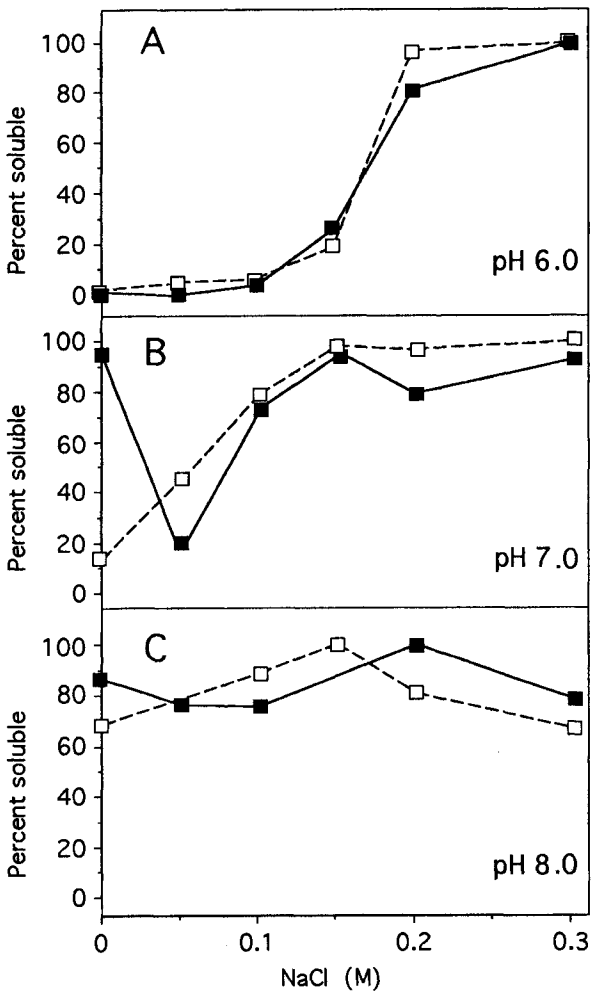

Fig. 5. Solubility of LMM1 (■) and LMM2 (□) at different pH and salt concentrations. A: pH 6.0; B: pH 7.0; C: pH 8.0. The protein concentration is 0.2 mg/ml.

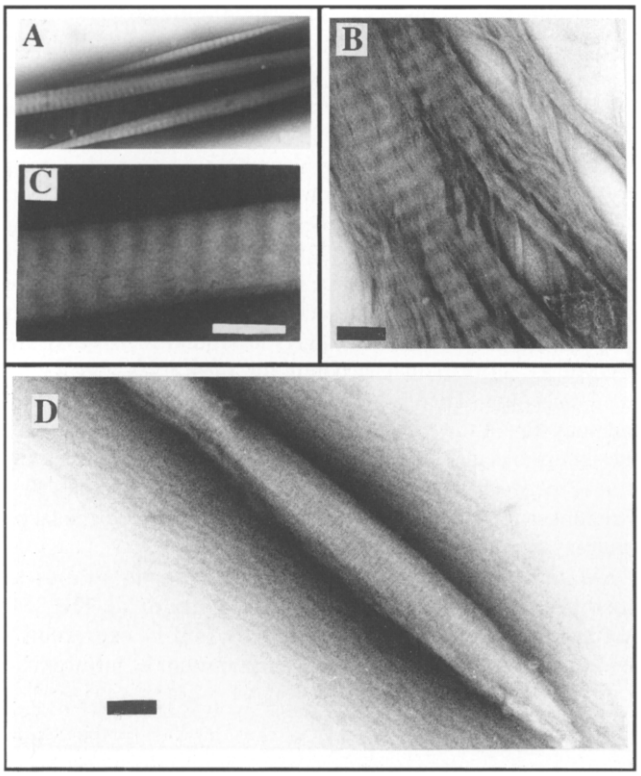

Fig. 6. Electron micrographs of paracrystals formed by LMM1 (A, B, C) and LMM2 (D). Bar, 100 nm. Paracrystals are either in spindles (A) or less often in bundles (B). They show a 44 (C) or 14 nm (D) axial repeat according to the experimental conditions or the studied species.

myofilaments and the contractile activity [25]. Myosin filaments likely have a side-polar structure with cross-bridges arranged with 14.3 nm repeat; this structure would allow smooth muscles to contract by larger amounts than skeletal muscles [26]. It may be suggested that the absence of a long tail as in SM2 isoform favors this arrangement and therefore the assembly of myosin molecules.

In conclusion LMM1 and LMM2 constructs fully retain the dimeric α-helical conformation of the native molecule as proved by the banding patterns seen on their paracrystals, their circular dichroism spectra and endotherms. The identical polypeptide chains in LMM1 and LMM2 are very likely packed parallel and in register, such as the LMM part of intact myosin [27]. These constructs have close physicochemical properties but they show nevertheless different features in their solubility and the formation of paracrystals. These differences may play a role in vivo in the assembly of smooth muscle myosin molecules in filaments.

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