Solubility-determining domain of smooth muscle myosin rod

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Chymotryptic digestion of chicken gizzard light meromyosin (LMM) produced a 72 kDa core fragment, which was fully soluble at 150 mM KCl, pH 6.5–7.5. The fragment showed weak self-association at 50 mM KCl. The homology of the N-terminus amino acid sequence of this fragment with the sequence of the rabbit skeletal myosin rod suggested that the N-terminus of the core fragment originated 5 kDa from the hinge common to both smooth and skeletal myosin rod. Sedimentation experiments indicated that the domain specifying the insolubility of the intact LMM was 13 kDa long. Progressive proteolytic shortening of this region produced LMM fragments of progressively increasing solubility. Electron microscopy of segments formed from full-length LMM and from LMM core suggested that this 13 kDa domain specified the 43 nm parallel and antiparallel molecular overlaps characteristic of self-assembled intact myosin.

(Smooth muscle) Myosin Myosin self-assembly Filament–monomer

1. INTRODUCTION

Smooth muscle myosin has remarkable solubility properties, being able at physiological ionic strength to adopt either a soluble, folded configuration [1,2] or an extended configuration which assembles into filaments [3]. Several recent reports describe narrow domains within the light meromyosin (LMM) domains of other myosins which are critical for filament formation [4–8]. Here we describe an apparently analogous region within chicken gizzard LMM, the removal or partial removal of which profoundly alters the solubility properties of the molecule.

2. MATERIALS AND METHODS

Purified chicken gizzard myosin was used as a source of LMMs. The 90 kDa LMM chain was prepared by digesting 12 $mg \cdot ml^{-1}$ myosin with 0.1 $mg \cdot ml^{-1}$ chymotrypsin in a buffer of 0.6 M KCl, 2 mM $MgCl_2$, 1 mM cysteine and 10 mM imidazole, pH 7.2, for 10 min at 25°C, adding phenylmethanesulphonyl fluoride (in 96% ethanol) to 1 mM final concentration, denaturing with 2 vols cold 96% ethanol, pelleting (22000 × *g*, 20 min), resuspending in the digestion buffer, clarifying (55000 × *g*, 60 min), dialysing to 20 mM KCl, 1 mM $MgCl_2$, 0.5 mM cysteine and 5 mM imidazole, pH 6.5, and pelleting the resulting aggregates. For amino acid sequence analysis, the resulting preparation was redissolved in the digestion buffer and gel filtered in the same buffer (FPLC; Superose 6, Pharmacia). The leading fractions were pooled, dialysed against 0.6 M ammonium formate and lyophilised. The 72 kDa soluble LMM was prepared by further digestion of this preparation for 120 min at 25°C using 0.05 $mg \cdot ml^{-1}$ α-chymotrypsin and the same digestion buffer. The stopped digest was dialysed to 60 mM KCl, 2 mM $MgCl_2$, 1 mM cysteine and 10 mM imidazole, pH 6.5, and centrifuged at

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150000 × g for 20 min. The supernatants were pooled and purified using FPLC as above.

For amino acid analysis, 100 μg of each fragment were loaded on a gas-phase sequinator (Applied Biosystems Inc., USA), assembled and operated as described [10]. The stepwise liberated phenylthiohydantoin (PTH)-amino acid derivatives were identified using a Cyano-HPLC analytical column (IBM) and the gradient elution described in [11]. We used a Waters HPLC system including two M6000 pumps, a WISP 710B autoinjector, an M721 system controller and an M441 fixed wavelength detector. The recovery of the PTH-amino acids was measured at each cycle using an integrative recorder (Waters data module M730).

3. RESULTS

3.1. *Solubilities of LMM fragments*

The tail or rod of smooth muscle myosin can be cleaved by chymotrypsin at or very close to a hinge region [12] generating a 40 kDa chain, subfragment 2 (SF 2), which is soluble at physiological ionic strength and a 90 kDa LMM chain, which is insoluble. Although the 90 kDa LMM is resistant in the short term to further digestion [13], prolonged incubation produced a range of truncated LMMs of decreasing molecular mass down to 72 kDa (fig.1). Samples of this digest were dialysed into buffers of two ionic strengths and various pHs, and separated into soluble and insoluble fractions by sedimentation in an airfuge (fig.1). At

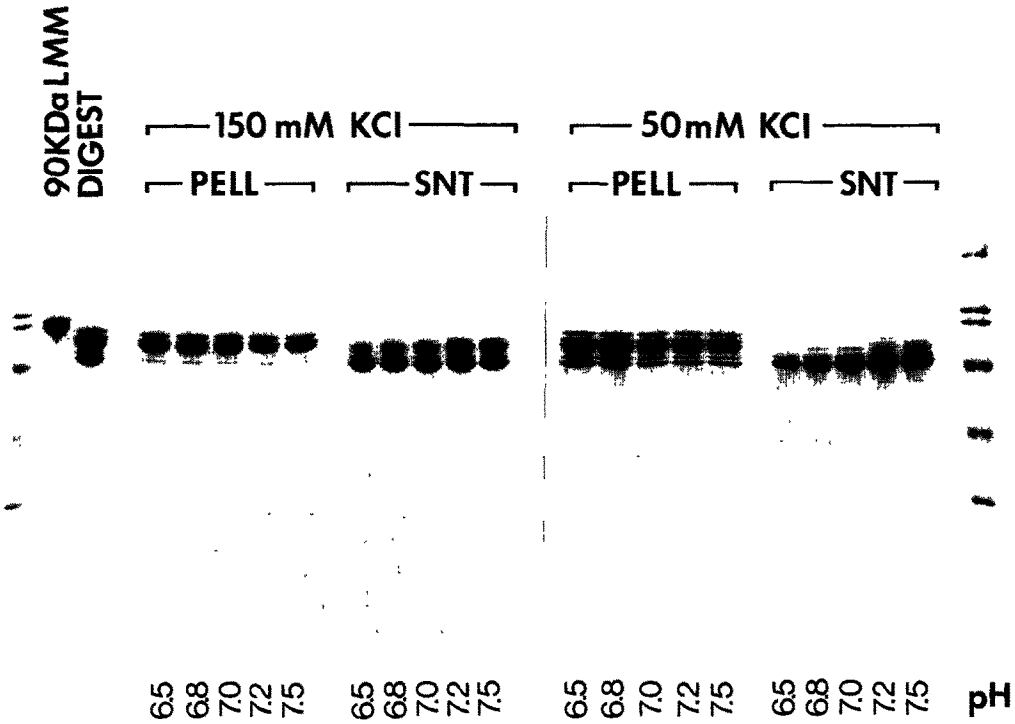

Fig.1. 11–22% microslab gradient PAGE showing sedimentation of whole digest of chicken gizzard LMM as a function of ionic strength and pH. Flanking lanes, molecular mass markers (in kDa): myosin, 205; β-galactosidase, 116; phosphorylase *b*, 94; bovine serum albumin, 67; ovalbumin, 45; carbonic anhydrase, 29. The next two lanes on the left show respectively the source LMM and a 2 h chymotryptic digest performed in 0.6 M KCl, 1 mM cysteine, 1 mM $MgCl_2$ and 10 mM imidazole, pH 7.2, at 25°C and a 1:100 enzyme/substrate ratio. The blocks labelled SNT and PELL represent the peptide compositions of the supernatants and pellets of this digest following dialysis of 200 μl samples of the digest to either 150 mM KCl or 50 mM KCl, 10 mM imidazole, 1 mM $MgCl_2$, 1 mM cysteine, pH 6.5–7.5, and centrifugation in the airfuge at 150000 × g for 20 min. Supernatants were diluted with 1 vol. SDS buffer (17.5% SDS, 7% β-mercaptoethanol, 0.437 M Tris, pH 6.8). Pellets were dissolved in this buffer and adjusted to the same volume as the supernatant samples. The gels [20,21] are thus quantitative.

150 mM KCl, the original 90 kDa LMM was completely sedimented, whereas the 72 kDa fragment remained in the supernatant over the pH range 6.5–7.5. The removal of 18 kDa from the original 90 kDa LMM chains was thus sufficient to render the molecule soluble at physiological ionic strength. Intermediate sized fragments had intermediate solubility properties. If the experiment was repeated at 50 mM KCl, most of the intermediate size material was pelleted together with some of the 72 kDa LMM. The greater part of this 72 kDa material nonetheless remained in the supernatant fraction, particularly at high pH (fig.1).

3.2. *N-terminal amino acid sequencing*

To establish the location of the soluble 72 kDa LMM core fragment within the parent 90 kDa LMM, we performed N-terminal amino acid sequencing of the purified fragments (fig.2). The two N-termini were nonidentical, indicating some loss of material from the 90 kDa N-terminus in generating the 72 kDa. The amino acid sequence of the gizzard myosin rod has not yet been determined, and so we searched for homology of the sequences to the known rabbit skeletal myosin sequence [14,15]. A search of the rabbit data between the hinge region and residue 484 of the rod produced a unique fit for the N-terminus of the gizzard 72 kDa LMM fragment starting at residue 379 of the rabbit sequence, about 5 kDa from the hinge common to gizzard and myosin rods (fig.2). At this position 5 out of 12 residues matched. This degree of homology is similar to that observed for the best fit of rabbit LMM fragments to the nematode data [5]. The present assignment, if correct, implies that all 5 amino acid identities are on the charged outside surface of the coiled coil, away from the hydrophobic seam, and also that 5 kDa of sequence has been lost from the N-terminus of intact LMM in generating the 72 kDa LMM core. We were unable to align unambiguously the gizzard data with the nematode sequence, or to fit the 90 kDa gizzard LMM N-terminus unambiguously against the rabbit sequence. Purely on the basis of molecular length, however, this must lie close to the hinge [12].

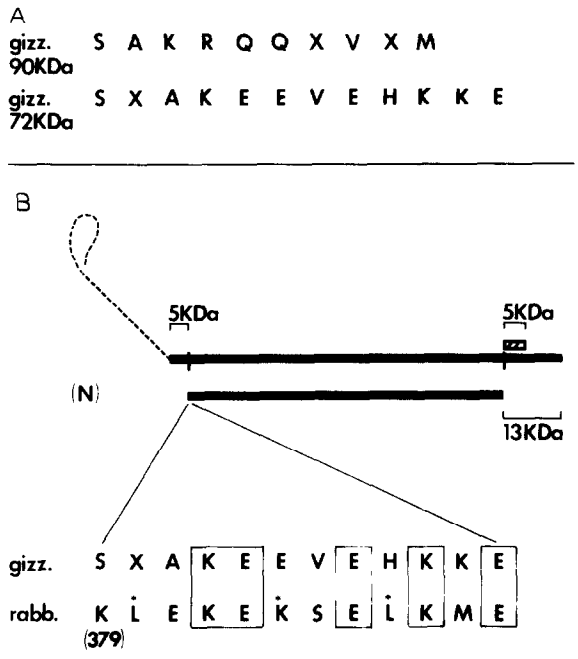

Fig.2. (A) N-terminal amino acid sequences of 90 and 72 kDa chain chicken gizzard LMMs. (B) Alignment of 72 kDa LMM N-terminus with the known rabbit skeletal myosin rod sequence. Amino acid residues are designated by the one letter notation. An X indicates an ambiguous assignment. Homologous residues are boxed. Those marked with an asterisk occur at the hydrophobic interfaces between adjacent chains. The most likely alignment indicates that the 72 kDa LMM core arises by cleavage of 5 kDa from the N-terminus and 13 kDa from the C-terminus of the parent 90 kDa LMM chain.

3.3. *Size of the solubility-determining domain*

The gels in fig.1 indicate that the 90 kDa LMM could be shortened to 85 kDa without affecting its solubility properties. Further, LMM fragments intermediate in size between 85 and 72 kDa show a progressive change in solubility, indicating that the whole of the 13 kDa difference piece is involved in modulating the ability to self-assemble. If, as seems likely, this material forms a continuous solubility-determining domain within the myosin rod, then the sequence data require that it maps to the C-terminus of the molecule, with the N-terminus of 72 kDa LMM therefore 5 kDa from the hinge (fig.2).

3.4. *Segment formation*

Gizzard myosin rod and LMM form characteristic segments [16] when precipitated with divalent metal ions from KSCN-containing buf-

fers. The segments are characterised by specific 43 nm molecular overlaps, occurring with adjacent molecules both antiparallel and parallel. To throw further light on the origin of the 13 kDa of solubility-determining material within the full-length LMM chain, the segment forming properties of the 90 kDa and 72 kDa LMMs were compared. As shown in fig.3A, the 90 kDa LMM formed compound segments as expected, exhibiting both the 43 nm parallel and antiparallel molecular staggers. Low numbers of segments showing apparently only the parallel overlap were also observed (fig.3B). For the 72 kDa LMM, the order of the segments was disrupted, a large amount of loosely-aggregated material appearing on the grids, together with larger but poorly ordered segments (fig.3C,D). These appeared to arise as a result of side by side association of molecules, followed by loose end-to-end abutment of these parallel assemblies.

In the absence of the 13 kDa solubility-determining material segment assembly is thus disrupted, and the ability to make the 43 nm molecular staggers lost. This domain seems therefore to be responsible both for self assembly per se, and for the definition of the molecular staggers exhibited by self-assembled intact myosin molecules.

4. DISCUSSION

The above results demonstrate that a 13 kDa section of gizzard LMM plays a crucial role in determining the ability of the molecule to self-assemble at physiological ionic strength, and in defining the specific parallel and antiparallel

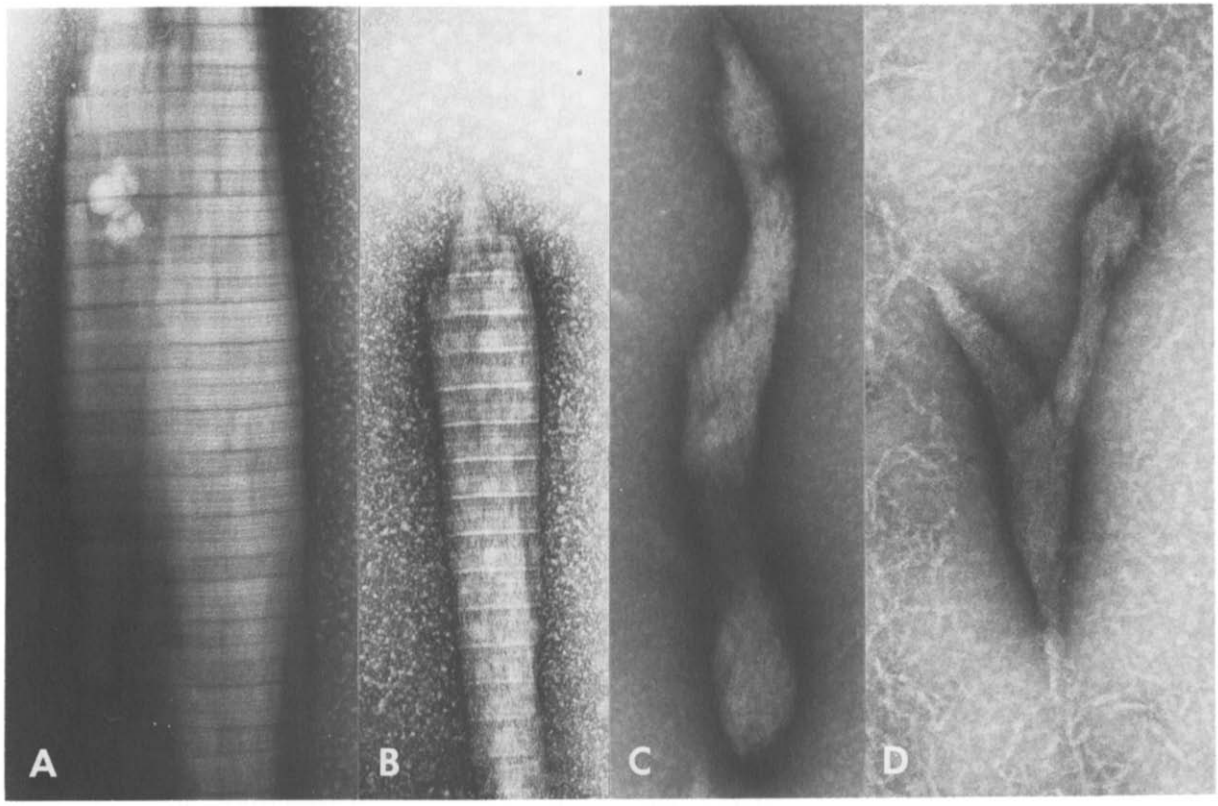

Fig.3. LMM segments. Segments were formed from 90 kDa (A,B) and 72 kDa (C,D) LMMs by dialysis of a 1 $mg \cdot ml^{-1}$ solution of the proteins in 0.6 M KCl to 85 mM KSCN, 50 mM $CaCl_2$, 50 mM Tris, pH 8.2 [16]. Negative staining was done at room temperature on 400 mesh carbon-coated Cu grids, using 1% aqueous uranyl acetate. The micrographs were all obtained at a nominal magnification of 36000 × using a Zeiss EM 10 operated at 80 kV. The period of the segment in (B) is 43 nm, using the 40 nm repeat of tropomyosin paracrystals as a calibration standard.

molecular overlaps characteristic of segments. The 13 kDa might produce its effects on solubility either directly, by binding itself to neighbouring molecules, or indirectly, by straining the conformation of the remainder of the LMM so as to induce the property of self-association. In this latter case, however, one might expect cleavage within the 13 kDa region to produce a step change in solubility as the critical strain-inducing region was cleaved. Instead, we observed a gradual increase in solubility according to the degree of truncation of the solubility-determining 13 kDa region. This indicates that the 13 kDa region itself makes specific binding interactions with other molecules. In this case the 13 kDa solubility-determining domain must be located at the C-terminus of the molecule, since it cannot otherwise specify the 43 nm antiparallel molecular overlap of the segments. This argument leads to the same conclusion as reached on the basis of amino acid sequence homology, that the solubility-determining 13 kDa domain is located at the C-terminus of the smooth muscle myosin molecule. For skeletal myosin, a similar solubility-determining region of only 5 kDa has been reported [5], and assigned to a site close to the C-terminus of the molecule. The site of this domain aligns with the forward part of that deduced here for the smooth muscle myosin molecule (fig.2).

In vitro at physiological ionic strength (150 mM KCl, pH 7.0), unphosphorylated smooth muscle myosin filaments are unstable. Addition under these conditions of stoichiometric amounts of ATP is sufficient to disassemble these filaments completely to form folded monomers, in which the tail of the molecule folds back on itself to contact the heads. By contrast at lower pH and ionic strength (50 mM KCl, pH 6.5) little filament disassembly occurs [17]. The present results suggest a partial explanation for this solubility behaviour. The pelleting experiments show that at 150 mM KCl, significant intermolecular binding occurs only between molecules containing all or part of the 13 kDa solubility-determining domain. In contrast, at 50 mM KCl we detected self-association along the entire length of the LMM. Extending these findings to the thick filament, we imagine myosin filaments at physiological ionic strength to be stabilized purely by binding interactions involving the C-terminal sticky patch, the remainder of the molecule being essentially unrestrained. Under such conditions, filament disassembly to form 10 S (folded) monomers is favoured, particularly in the presence of ATP [1,2]. In contrast, at lower ionic strengths we detected limited self-association along the entire length of LMM (fig.1). The filaments would then be expected to have a tighter structure, with disassembly being disfavoured. Filament disassembly is indeed inhibited under these conditions, even in the presence of ATP [17]. It is further possible that ionic strength-dependent binding occurs between different sections of LMM within single folded myosin monomers. It was recently shown that under low ionic strength conditions, the tail loop of the folded molecules closes, the sections of the tail appearing to bind to one another, and the molecules tending to dimerize [18]. A scheme incorporating these points is shown in fig.4.

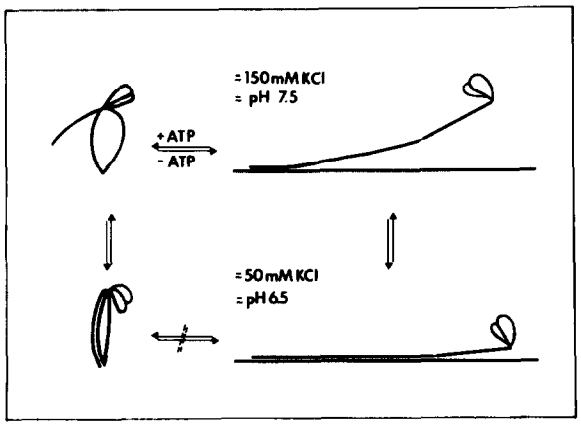

Fig.4. Outline model for the filament-to-10 S monomer transition of smooth muscle myosin. The scheme shown represents an attempt to relate the present findings to the known solution behaviour of smooth muscle myosin. At intermediate KCl concentrations (upper), the myosin filaments are supposed to be stabilised purely by binding interactions involving the C-terminal tailpiece domains of the molecules. These conditions favour filament dissociation, particularly in the presence of ATP [17]. The folded monomers so formed [1,2] have an open tail loop [18]. At lower ionic strength, the filaments are postulated to have a tighter structure, the full length of the LMM being held against the filament surface, and the filaments are resistant to dissolution [17]. Similarly, the tail loop of the folded monomers closes at low ionic strength [18], suggesting that salt-dependent binding occurs between different sections of the LMM within one molecule.

Whilst this work was in progress, Tashiro et al. [19] reported a soluble 74 kDa gizzard LMM, but were unable to localize it within the myosin rod.

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