Volume 281, number 1,2, 23–26 FEBS 09540 April 1991

ADONIS 001457939100249R

Rabbit skeletal muscle myosin

Unfolded carboxyl-terminus and its role in molecular assembly

Kayo Maeda[1], Andrea Rösch[1], Yuichiro Maéda[2], Hans Robert Kalbitzer[1] and Alfred Wittinghofer[1]

[1]*Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, W-6900 Heidelberg, Germany* and [2]*European Molecular Biology Laboratory at DESY, Notkestraße 85, W-2000 Hamburg 52, Germany*

Received 17 December 1990; revised version received 4 February 1991

We have expressed in *E. coli* segments of the rod portion of rabbit skeletal fast muscle myosin and compared physical properties of two different species, LMM-30 and LMM-30C'. LMM-30 consists of 263 amino acids including the original C-terminus of myosin heavy chain. LMM-30C' is colinear with LMM-30, but is devoid of 17 residues at the C-terminus. ^{1}H NMR spectroscopy indicates that the C-terminus of LMM-30, but not of LMM30C' is unfolded and freely mobile. Furthermore, the present results show that the unfolded C-terminus is essential for molecular assembly of LMM-30; at pH 8.0 LMM-30, but not LMM-30C', formed aggregates upon decreasing the ionic strength.

Myosin; α-Helical coiled-coil; Self-assembly; Recombinant DNA technique; Nuclear magnetic resonance

1. INTRODUCTION

The skeletal muscle myosin molecule consists of two heavy chains and four light chains. The C-terminus of the heavy chain is responsible for assembly of myosin into thick (myosin) filaments [1,2]. Limited tryptic digestion splits a myosin molecule into the C-terminal section of the rod (light meromyosin, LMM) and the rest (heavy meromyosin, HMM).

Not much is known about the mechanism responsible for filament formation. Electrostatic interactions between superhelical coiled-coil molecules through the regular distributions of charges have been proposed to play a role, based on the amino acid sequence of nematode myosin [3,4]. To study the assembly of myosin, we have expressed 30 kDa segments of LMM in the expectation that these molecules are long enough to maintain the original molecular interactions of intact myosin and short enough to facilitate NMR structural studies. Recombinant proteins should have several advantages over LMM prepared by proteolysis of myosin. These conventional LMM preparations contain a mixture of products encoded by a gene family and they are heterogeneous due to proteolysis at the C- as well as the N-terminus [5–10].

Correspondence address: A. Wittinghofer, Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, W-6900 Heidelberg, Germany

Abbreviations: LMM, light meromyosin; NMR, nuclear magnetic resonance

2. MATERIALS AND METHODS

2.1. *Expression of recombinant proteins*

Standard DNA technology was used for construction of the vectors as outlined in Fig. 1A. For the introduction of a stop codon pLMM in M13mp9 was mutagenized with the oligonucleotide 5'-GGTC-AACTAACTGCG-3' using the thiophosphate method [11]. After overnight induction with isopropyl-β-D-thiogalactopyranosid (IPTG), the bacterial cells were broken by lysozyme and the proteins were extracted with a high salt solution as described [12]. The protein was then enriched to a purity of more than 90% by two cycles of dialysis against 100 mM KCl and extraction with 600 mM KCl, both in 10 mM phosphate buffer, pH 6.5. The protein LMM-30C' was enriched as for LMM-30, followed by further gradient purification on a DEAE-Sepharose column pre-equilibrated with 20 mM pyrophosphate, pH 8.0, 1 mM DTE, 1 mM EDTA, 30 mM KCl. LMM-30C' was eluted at about 280 mM KCl.

2.2. *NMR measurements*

^{1}H NMR spectra were recorded with a Bruker AM-500 NMR spectrometer operating at 500 MHz. The water signal was suppressed by selective presaturation. For the NMR experiments the protein was precipitated by 11-fold dilution of the sample with buffer A (10 mM potassium phosphate, 30 mM KCl in H_2O, pH 6.5). The precipitate was dissolved in a small volume of buffer B (10 mM potassium phosphate, 0.6 M KCl in D_2O, pH 6.5). After precipitation of the protein by a 10-fold dilution of the sample with D_2O, the precipitate was redissolved in 500 μl of buffer B and 5 μl of a 0.01 M solution of trimethyl-silyl-perdeuteropropionic acid (TSP) were added, providing the internal reference signal.

2.3. *Solubility tests*

Solubility of LMM-30 and LMM-30C' were studied by measuring percent protein precipitated. The concentrated protein dissolved in 0.6 M KCl, 1 mM EDTA, 1 mM DTE, 10 mM phosphate buffer was diluted into low-salt buffer. After overnight incubation at 4°C, each mixture was centrifuged for 30 min at 100 000 × *g* with a Beckmann TL-100 ultracentrifuge. The pellet thus obtained was dissolved in 100 μl of 0.6 M KCl. The protein concentration was measured by the

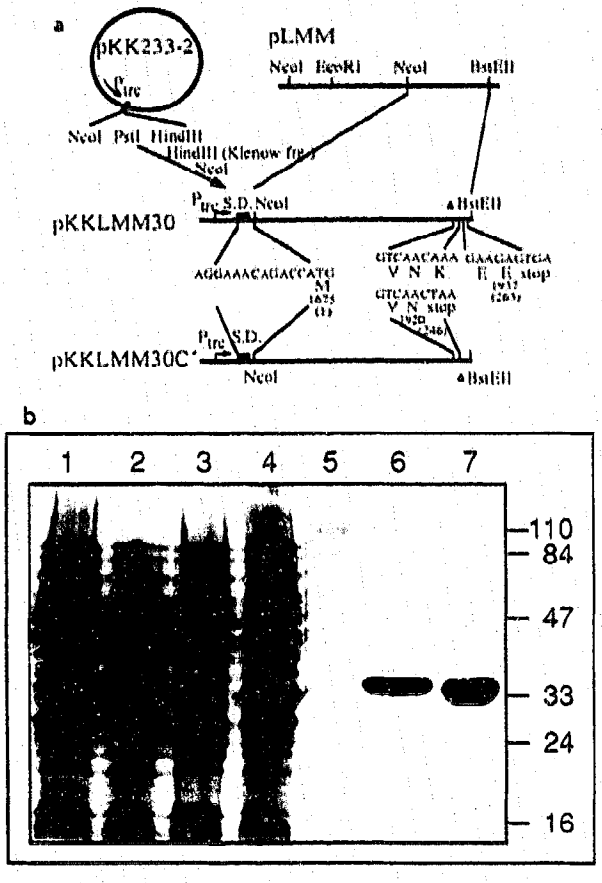

Fig. 1. Expression of LMM fragments. (A) LMM cDNA from pLMM [12] was cloned into pKK233-2 containing the P_{trc}-promoter [14] as outlined. The Lys-1923 codon AAA was mutagenized to a stop codon to produce pKKLMM30C′ that expresses LMM30C′ which has 246 amino acids instead of 263 for LMM30. (B) SDS-PAGE analysis of *E. coli* lysates with pKKLMM30 (lanes 1,2) and pKKLMM30C′ (3,4) minus (1,3) and plus (2,4) 1 mM IPTG, respectively. Purified LMM30 (6) and LMM30C′ (7) are also shown, with molecular weight markers in lane 5 having the indicated molecular weights.

method of Bradford [13]. The buffers (10 mM) used were potassium phosphate (pH 6.5, 7.5) and Tris-HCl (pH 7.5, 8.0), 1 mM EDTA, 1 mM DTE with variable concentration of KCl.

3. RESULTS AND DISCUSSION

3.1. *Expression of LMM-fragments in* E. coli

We have shown earlier that recombinant rabbit short light meromyosin (LMM74) and a fragment thereof could conveniently be expressed in *E. coli*. These proteins were shown to have properties resembling those of proteolytic rabbit LMM [12]. Since we wanted to find the minimum size of LMM protein that could form paracrystals and have solubility properties resembling native LMM we expressed LMM30 comprising amino acids 1675–1939 and LMM20 from 1768–1939 (not shown). Fig. 1 shows the construction of an expression vector in pKK233-2 [14] and the high-level expression of recombinant proteins after induction with IPTG. The proteins could be purified as described for full-length LMM before [12], see section 2.

3.2. *Low salt solubility*

Upon decreasing ionic strength, LMM-30 and LMM-30C′ form aggregates (Fig. 2), a property which they share with conventionally prepared LMM. At pH 7.5 or lower, both species precipitated LMM-30C′ being more soluble than LMM-30. At pH 8.0, however, LMM-30C′ was completely soluble, while LMM-30 precipitated. The solubility curves are dependent on pH, but independent of the kind of buffer used. The simplest interpretation is that at pH 8.0 the C-terminus is essential for the assembly. This implies that the molecular assembly of LMM is caused by two types of interactions, the presumably electrostatic interactions between the helical parts of LMM and a still undefined C-terminus-mediated interaction. At pH 7.5 or lower, the electrostatic interactions would be strong enough to induce aggregation even without substantial contribution of the C-terminus-mediated interaction. The present observation may be related to the well-known differences between myosin filaments formed at pH 7 and pH 8 [15–17]. The pH 8 filaments resemble the native thick filaments in length distribution and in thickness, while the pH 7 filaments are thicker and longer.

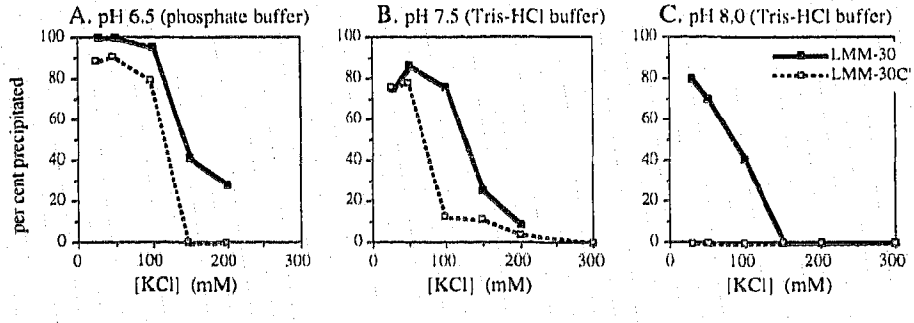

Fig. 2. Low salt solubilities of LMM-30 and LMM-30C′. They were measured as % precipitation at various pH (see section 2). The protein concentrations were 0.093 mg/ml for LMM-30 and 0.121 mg/ml for LMM-30C′.

3.3. *Structure of the C-terminus of LMM-30*

The ^{1}H NMR spectrum of LMM-30 exhibits a series of rather narrow resonances with line widths in a range of 4-6 Hz typical for peptides (Fig. 3). These resonances are superimposed on a background of broad resonances which are expected for a protein with the size of LMM-30. The narrow resonances were assigned by two-dimensional NMR methods to the spin systems of one histidine, two valines, one isoleucine, two serines, two glutamates and one lysine per protomer, as indicated (details of the assignment procedure will be published elsewhere). The amino acid composition derived from NMR matches exactly the composition of the C-terminus of LMM-30 (-Val-His-Ser-Lys-Val-Ile-Ser-Glu-Glu). This suggests that the sharp lines arise only from the nine residues at the C-terminus. The spectrum of LMM-30C', which is devoid of 17 amino acids from the C-terminus, shows the same broad background and a few sharp lines. The resonances of the spin systems identified in LMM-30 are no longer observed (Fig. 3), which proves their suggested assignment to the C-terminus of the protein.

The chemical shift values of the C-terminal amino acids are rather close to the values found in random-coil model peptides [18,19]. Together with the long transversal relaxation times (small line widths), this means that the C-terminus is extremely mobile and unfolded. This conclusion is also supported by the observation of

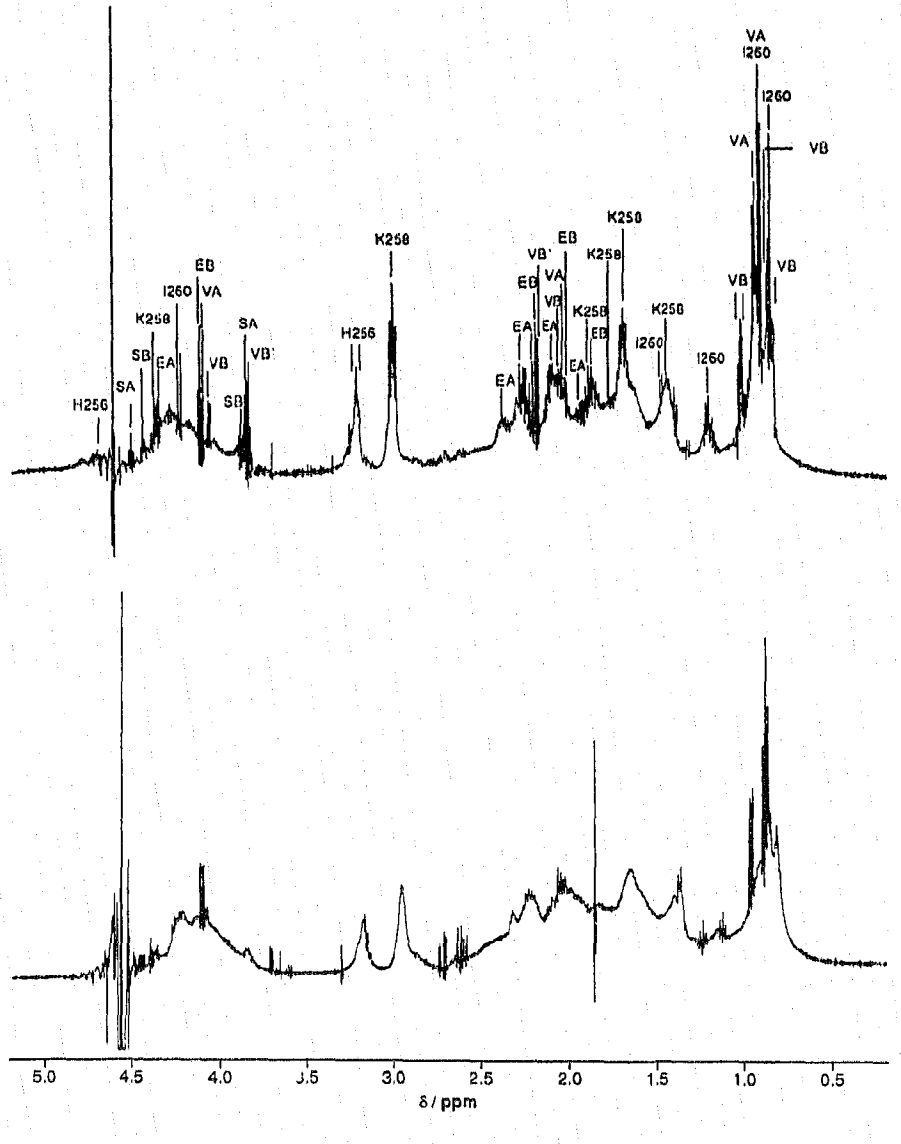

Fig. 3. 500 MHz ^{1}H NMR spectra of LMM30 (above) and LMM30C' (below). Protein concentration was 1 mM in 0.6 M KCl, 10 mM potassium phosphate pH 6.6 in 99.75% D_2O, at 308°K. Resolution enhanced spectrum (Lorentzian-to-Gaussian transformation). Assignments were obtained by two-dimensional NMR spectroscopy (not shown).

only one set of resonance lines for the C-termini in the molecule; the two C-termini of the coiled-coil molecule must have the same time-averaged conformations and the same mutual interactions. The only exceptions were the resonances of Val B and B', where two separate sets of resonance lines with half the intensity were observed. This indicates that these valine residues experience two different microenvironments likely due to the interaction between the two C-terminal peptides at position 255, the border between the helical and the random conformations. The high mobility of the C-terminus is not simply due to its position at the end of the polypeptide chain. The N-terminus shows no sign of high mobility (e.g. the N-terminal methionine would give rise to a sharp singlet methyl resonance at 2.1 ppm which was not observed). Moreover, the spectrum of the C-terminus of LMM-30C' shows no indication of such a high mobility.

The most important experimental finding is that the C-terminus of LMM30 and most probably of the skeletal myosin rod is unfoled and mobile and may play a crucial role in myosin assembly. The special role of the C-terminus for assembly has also been confirmed by electron microscopy and light scattering studies (Maeda et al., unpublished). With conventionally prepared LMM such an observation might not have been possible due to proteolytic digestion of the C-terminus [5]. That the C-terminus of myosin heavy chain is non-helical has been deduced from the appearance of proline in the C-terminal amino acid sequence of myosin heavy chains from various sources, such as the nematode body wall muscle [3,4], the vertebrate smooth muscle [20] and non-muscle tissues [21,22], but it is not found in the vertebrate skeletal muscle myosin C-terminus. Experimental evidence for such a structure in myosin heavy chain has never been presented. The unfolded C-terminus has been inferred also for tropomyosin [23], another protein forming a coiled-coil structure, where it plays a crucial role in the head-to-tail interaction between molecules.

Acknowledgements: We thank K.C. Holmes for continuous support of this work and helpful discussions. One of us (A.W.) was supported by the Deutsche Forschungsgemeinschaft (Wi 371/3-2). Y.M. thanks Muscular Dystrophy Association USA for support.

REFERENCES

[1] Huxley, H.E. (1963), J. Mol. Biol. 7, 281–308.
[2] Lowey, S. (1979) in: Fibrous proteins: Scientific, Industrial and Medical Aspects, vol. 1 (Parry, D.A.D. and Creamer, L.K. eds) Academic Press, London.
[3] McLachlan, A.D. and Karn, J. (1982) Nature 299, 226–231.
[4] McLachlan, A.D. and Karn, J. (1983) J. Mol. Biol. 164, 605–626.
[5] Nyitray, L., Moez, G., Szilagyi, L., Balint, M., Lu, R.C., Wong, A. and Gergely, J. (1983) J. Biol. Chem. 258, 13213–13220.
[6] Elzinga, M. and Trus, B. (1980) in: Methods in Peptide and Protein Sequence Analysis (Birr, Chr. ed.) pp. 213–224, Elsevier, Amsterdam.
[7] Hoh, J.F.Y. and Yeoh, G.P.S. (1979) Nature 280, 321–323.
[8] Maeda, K., Szakiel, G. and Wittinghofer, A. (1987) Eur. J. Biochem. 167, 97–102.
[9] Emerson, C.P. and Bernstein, S.I. (1987) Annu. Rev. Biochem. 56, 695–726.
[10] Tong, S.W. and Elzinga, M. (1990) J. Biol. Chem. 265, 4893–4901.
[11] Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8765–8758.
[12] Maeda, K., Szakiel, G., Hofmann, W., Menetret, J.-F. and Wittinghofer, A. (1989) J. Mol. Biol. 205, 269–273.
[13] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[14] Ausman, E. and Brosius, J. (1985) Gene 40, 183–190.
[15] Kaminer, B. and Bell, A.L. 91966) J. Mol. Biol. 20, 391–401.
[16] Burke, M. and Harrington, W.F. (1972) Biochem. 11, 1456–1462.
[17] Davis, J.S. (1988) Annu. Rev. Biophys. Chem. 17, 217–239.
[18] Bundi, A. and Wüthrich, K. (1979) Biopolymers 18, 285–297.
[19] Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids John Wiley & Sons, New York.
[20] Yanagisawa, M., Mamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T. and Masaki, T. (1987) J. Mol. Biol. 198, 143–157.
[21] Warrick, H.M., De Lozanne, A., Leinwand, L.A. and Spudich, J.A. (1986) Proc. Natl. Acad. Sci. USA 83, 9433–9437.
[22] Hammer III, J.A., Bowers, B., Paterson, B.M. and Korn, E.D. (1987) J. Cell Biol. 105, 913–925.
[23] Stewart, M. and Roberts, G.C.K. (1983) J. Mol. Biol. 166, 219–225.