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Authors: Kanzawa, Nobuyuki, Yabuta, Hiroko, Fujimi, Takahiko J., and Tsuchiya, Takahide

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[SHORT COMMUNICATION]

Solubility Properties of a 65-kDa Peptide Prepared by Restricted Digestion of Myosin with Astacin-like Squid Metalloprotease

Nobuyuki Kanzawa*, Hiroko Yabuta, Takahiko J. Fujimi and Takahide Tsuchiya

Department of Chemistry, Faculty of Science and Technology,
Sophia University, Tokyo 102-8554, Japan

ABSTRACT—Substructure of the myosin rod and its correlation to filament formation is largely based on studies of proteolytic digests and expressed proteins. However, tryptic digestion of myosin always produces polymorphous peptides. Consequently, it is difficult to determine the relation between myosin substructure and filament formation. Similarly, filament formation with recombinant myosin protein is also difficult to interpret because it is never clear whether the recombinant protein folds like the native protein. We recently reported a novel metal protease isolated from squid liver, astacin-like squid metalloprotease (ALSM), which can specifically hydrolyze *in vitro* myosin heavy chain. In the present study, we examined the solubility properties of the 65-kDa peptide and light meromyosin (LMM) prepared by ALSM isoform II and trypsin digestion, respectively. The 65-kDa peptide is much less soluble than LMM under physiological conditions, even though the length of 65-kDa peptide is shorter than that of LMM. These results suggest that a novel substructure of myosin drives filament assembly.

Key words: ALSM, light meromyosin (LMM), myosin, self assembly

INTRODUCTION

Myosin is one of the principal protein components of numerous contractile systems, comprising almost 50% of the total protein in skeletal muscle. Myosin II (referred to as conventional myosin) is composed of two globular heads joined to a rod-like tail (Cohen and Parry, 1990; McLachlan and Karn, 1982, Fig. 1). Myosin polymerizes into bipolar filaments both in muscle and *in vitro* when the solution is at or below physiological ionic strength. The essential domains for self-assembly of myosin rods have been widely studied (McLachlan and Karn, 1982; Morris and Lu, 1987; Parry, 1981), as has the effect of pH, ionic strength, and effectors such as inorganic ions, on filament formation (Chowrashi and Pepe, 1989; Ozog and Béchet, 1995; Persechini and Rowe, 1984; Pinset-Härström, 1985). The amino acid sequence in the coiled-coil region forms a heptad repeat ([*a*, *b*, *c*, *d*, *e*, *f*, *g*]n), where positions *a* and *d* are predominantly occupied by hydrophobic residues and positions *e* and *g* are frequently acidic and basic residues, respectively (Cohen and Parry, 1990).

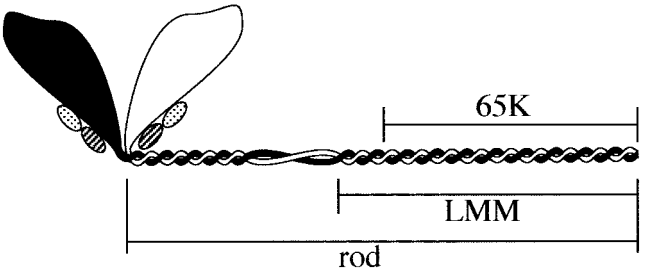

Fig. 1. Schematic diagrams of myosin molecule. Myosin II, referred to as conventional myosin is composed of two globular heads joined to a rod-like tail. Proteolytic cleavage of myosin at the hinge region where two heavy chains are more loosely bound than those in other part produces heavy meromyosin and light meromyosin (LMM). A 65-kDa peptide (65K) and LMM are generated by digestion with ALSM or trypsin, respectively.

Rod-rod interaction is essential for the self-assembly of myosin filaments. The myosin rod consists of a highly charged 28-amino acid cluster that is repeated four times as a heptad. To study myosin filament self-assembly, many investigators use proteolytic digests of myosin or recombi-

* Corresponding author: Tel. +81-3-3238-3363;
FAX. +81-3-3238-3361.
E-mail: n-kanza@sophia.ac.jp

nant myosin expressed *in vitro* under physiologically appropriate conditions (Akutagawa and Ooi, 1982; Atkinson and Stewart, 1991a; Atkinson and Stewart, 1991b; Ball *et al.*, 1987; Maeda *et al.*, 1991; Sohn *et al.*, 1997). Nonetheless, these methods have not been adequate for elucidating the exact mechanism of myosin filament assembly.

Three isoforms of astacin-like squid metalloprotease (ALSM), which have been cloned and characterized (Okamoto *et al.*, 1993; Tamori *et al.*, 1999; Yokozawa *et al.*, 2002), hydrolyze myosin at a specific site. ALSM isoform II (ALSM-II), which is the most abundant form in the Japanese common squid, hydrolyzes rabbit skeletal muscle myosin (GenBank accession# U32574) between Glu^{1379} and Thr^{1380} to produce the 155-kDa head and a 65-kDa peptide of the rod region. In this study, we compared the dependence of pH and ionic strength on solubility of the novel 65-kDa myosin rod peptide with that of light meromyosin (LMM).

MATERIALS AND METHODS

Protein preparations

Japanese common squid (*Todarodes pacificus*) were purchased at the Tokyo Central Wholesale Fish Market. ALSM-II was purified from Japanese common squid liver as previously described (Tamori *et al.*, 1999). Rabbit skeletal muscle myosin was purified according to the method of Perry (1955). Protein concentration was determined by the method of Bradford (1976) with the use of bovine serum albumin (BSA) as a standard.

Purification of LMM

LMM was prepared as by the method of Szent-Györgyi (1960). Briefly, purified myosin was dialyzed against 10 mM sodium phosphate buffer (SPB) (pH 7.0), 0.5 M KCl. Protein concentration of the dialysate was adjusted to 4.0 mg/ml, and trypsin was added to the solution to a final concentration of 4.0 μg/ml. After incubation at 23°C for 10 min, tryptic digestion was stopped by the addition of trypsin inhibitor (Sigma, St. Louis, MO, U.S.A.). The sample was dialyzed against 10 mM potassium phosphate buffer (PPB) (pH 7.0) and was centrifuged at 60,000×*g* for 10 min. Precipitated fragments were dissolved in 0.5 M KCl and 10 mM PPB (pH 7.0). LMM was precipitated by the addition of 3 volumes of 95% ethanol and maintained at room temperature for 3 hr. LMM obtained by centrifugation at 10,000×*g* for 10 min was homogenized in 0.6 M KCl and 10 mM PPB (pH 7.0) and dialyzed against the same solution. Insoluble proteins were precipitated by centrifugation at 10,000×*g* for 10 min.

Purification of the 65-kDa myosin rod fragment (65K)

Purified myosin was dialyzed against 10 mM $ZnCl_2$ and 100 mM SPB (pH 7.0), and the protein concentration of the dialysate was adjusted to 0.6 mg/ml. Protease-inhibitor cocktail was added to the sample, resulting in final concentrations of 5 mM EDTA, 4 mM MIA, 8 mM PMSF, 20 μg/ml pepstatin A and 20 μg/ml leupeptin. Digestion of myosin was initiated by adding ALSM to a final concentration of 1 μg/ml. After incubation at 37°C for 3 hr, the reaction mixture was dialyzed against 0.5 M KCl and 10 mM PPB (pH 7.0). 65K was purified by addition of ethanol as described in the method for LMM purification.

Solubility tests of myosin fragments

LMM and 65K were dialyzed against 10 mM Tris-HCl (pH ranges of 6.5 to 8.5). Protein concentration of the dialysate was adjusted to 0.7 mg/ml. Aliquots (10 μl) of dialysate were gradually added to various concentrations of KCl (between 50 and 300 mM) and were then incubated on ice for 1.5 hr. After the incubation, samples were centrifuged at 10,000×*g* for 30 min. The supernatants were saved, and the precipitates were quickly washed with a precipitating solution and then dissolved in the same volume of precipitating solution containing 0.6 M KCl. Both supernatant and dissolved precipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% resolving gels (Laemmli, 1970). Band intensities detected in precipitated fractions were scanned and analyzed three times with NIH Image software to ensure accurate and consistent measurements. A two-factor analysis of variance (ANOVA) was used to analyze the results of solubility tests. Results were presented as the mean and standard deviation of three separate measurements.

RESULTS AND DISCUSSION

To analyze the effect of size and nature of novel myosin rod fragments on filament formation, we purified 65K and LMM from myosin. SDS-PAGE analysis showed that 65K was obtained as a single band (Fig. 2, lane c), whereas LMM generated by digestion with trypsin was composed of several fragments (Fig. 2, lane d). LMM fragments prepared by trypsin proteolysis of myosin might be generated by cleavage at Arg^{1306}–Gly^{1307} as well as at carboxy-terminal residues described previously (Nyitray *et al.*, 1983).

The effect of ionic strength on solubility properties of 65K and LMM were examined. In the present experiments, KCl was gradually added to the samples according to the method of Pinset-Härström (1985). At pH 6.5, both fragments were detected in precipitates at low ionic strength,

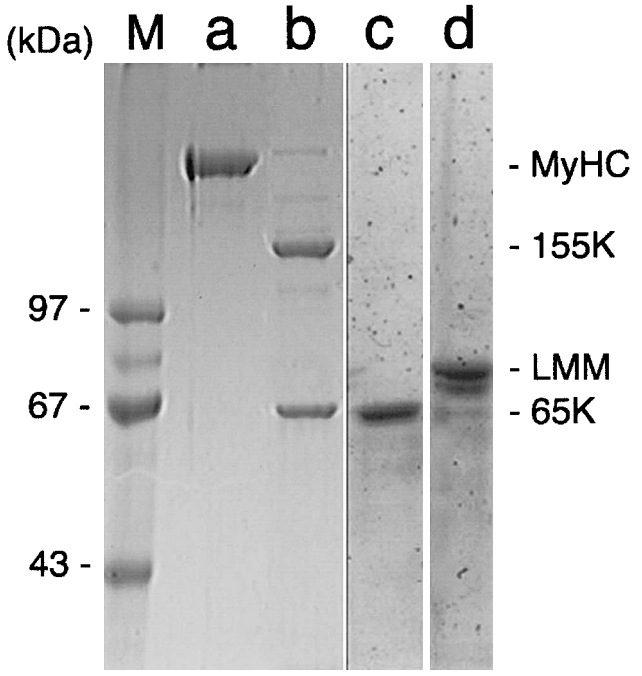

Fig. 2. Purification of 65K and LMM from myosin. A 65-kDa fragment was prepared by digestion of rabbit skeletal muscle myosin (MyHC) (lane a) with ALSM-II. After incubation at 37°C for 3 hr, myosin heavy chain was hydrolyzed to 155 kDa (155K) and 65-kDa peptides (65K) (lane b). LMM and 65K were purified as described in the Materials and Methods. M, molecular weight marker.

and in supernatants at high ionic strength. Thus, at pH 6.5, ionic strength appeared to have no effect on 65K or LMM solubility. At pH 7.5, however, ionic strength did affect solubility (Fig. 3A). Fig. 4 represents the solubility properties of 65K and LMM. Near physiological conditions (150 mM KCl, pH 7.5), approximately 70% of 65K was precipitated, whereas less than 30% of LMM was precipitated (Fig. 4B). No significant difference in 65K or LMM solubility was observed over any of the various ionic strengths tested at pH 6.5 (Fig. 4A). Thus, we compared the solubility of 65K and LMM at a single ionic strength (150 mM KCl) and at pHs of 6.5, 7.0, 7.5, 8.0, and 8.5 (Fig. 3B). In the presence of 150 mM KCl, both fragments were detected in the precipitate at pH 6.5 and in the supernatant at pH 8.5. However, at pHs of 7.0, 7.5, and 8.0, the precipitate found in the 65K sample was greater than that found in the LMM sample, suggesting that 65K is less soluble than LMM under physiological conditions.

Hydrophobic interactions form the basis for the interaction of the two heavy chains of the molecule. The outermost positions, *b*, *c*, and *f*, are highly charged with repeating negative and positive patches spaced 14 residues apart. This pattern suggests that if myosin molecules were staggered by an odd multiple of 14 residues, electrostatic interactions could provide the energy for myosin assembly (McLachlan and Karn, 1982). Because the charge periodicity is present in both 65K and LMM, both molecules would be expected to have similar solubility properties. However, if the strength of the interaction between myosin rods was determined by complementation of the charge repeat, then the interaction energy between molecules would be expected to be proportional to their length. Our result shows that 65K solubility is rather less than LMM solubility under physiological conditions, even though the length of 65K is shorter than that of LMM, putting in question the role of the 28-residue charge

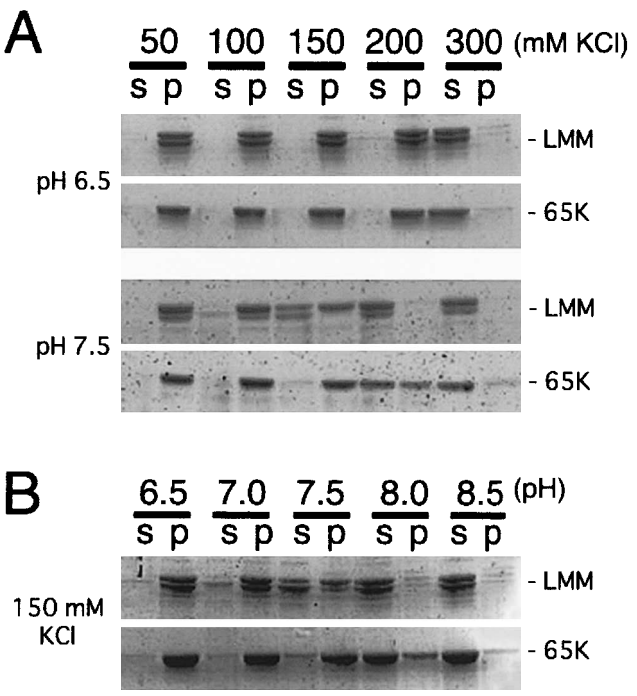

Fig. 3. Solubility of 65K and LMM. A: LMM and 65K were incubated in various concentrations of KCl (50, 100, 150, 200 and 300 mM) at pH 6.5 (upper panels) or at pH 7.5 (lower panels). B: A LMM and 65K were incubated in solutions at various pH (6.5, 7.0, 7.5, 8.0 and 8.5) in the presence of 150 mM KCl. After the incubation, samples were centrifuged, and supernatant (s) and precipitates (p) were resolved by SDS-PAGE.

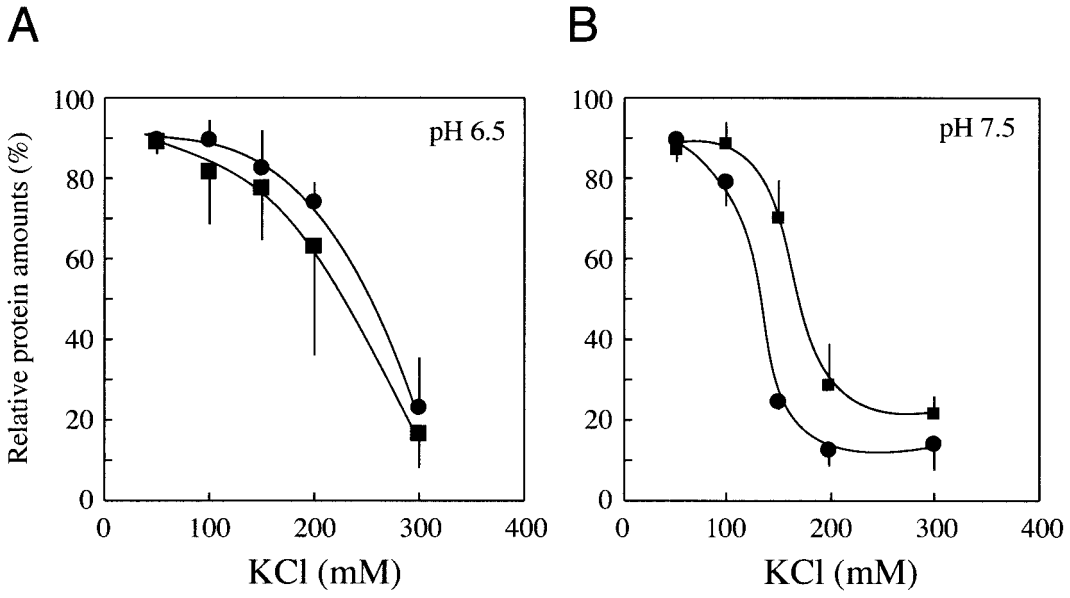

Fig. 4. Ability of 65K or LMM to form filaments. LMM (closed circle) and 65K (closed box) were incubated with KCl ranging between 50 and 300 mM at pH 6.5 (A) or pH 7.5 (B) as described in the legend for Fig. 3A. Relative filament formation ability was estimated as described in the Materials and Methods. Static analysis reveals that solubility for 65K is significantly different from that for LMM ($P < 0.001$). The error bar indicates the mean value ± standard deviation of three determinations.

repeat in the myosin rod assembly. Our results indicate that another domain located where 65K and LMM do not overlap is negatively driving myosin filament assembly. In vertebrate striated muscle myosins, a short region (29 residues) near the carboxyl terminus is thought to contribute to filament assembly (Atkinson and Stewart, 1991a; Nyitray *et al*., 1983; Shoffner and De Lozanne, 1996; Sohn *et al*., 1997). Our preparations of LMM contained polymorphous peptides, and we found that the solubility of the shorter LMM peptides was greater than that of the longer LMM peptides at pH7.5 and 150 mM KCl (Fig. 3A). However, at pH 7.5 and 200 mM KCl, the amount of precipitate was greater for 65K than for LMM, suggesting that a domain that negatively drives filament assembly lies in the region (Gly^{1307}–Glu^{1379}) where 65K and LMM do not overlap.

We have reported three isoforms of ALSM; ALSM-I hydrolyzes rabbit myosin Ala^{1150}–Thr^{1160} to produce peptides of 130 and 90 kDa. ALSMs-II and –III hydrolyze rabbit myosin Glu^{1379}–Thr^{1380} and Glu^{1098}–Asp^{1099} to produce peptides of 155 kDa plus 65 kDa and 120 kDa plus 100 kDa, respectively (Okamoto *et al*., 1993; Tamori *et al*., 1999). These novel sizes of peptides prepared by digestion of myosin with ALSM may be useful tools to examine functional domains involved in myosin filament assembly.

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