

Is *Amoeba proteus* Myosin VI Immunoanalogue a Dimeric Protein?

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Summary. Myosin VI is an unusual actin-based motor protein moving, unlike other known myosins, towards minus end of actin filaments. This implies a unique role of this protein in cell migration and intracellular transport. There are serious concerns whether myosin VI heavy chains may dimerize as its amino acid sequence contains heptad repeats responsible for dimerization but both native and recombinant myosins VI were found to be monomeric proteins. Recently, we have detected 130-kDa myosin VI immunoanalogue in *Amoeba proteus* that also exhibits many features characteristic for mammalian myosins VI (Dominik *et al.* 2005). It seemed interesting to check whether it is a monomeric or dimeric protein. Using a zero-length crosslinker, N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC), we have shown that under our experimental conditions the 130-kDa band corresponding to myosin VI immunoanalogue disappeared with the concomitant accumulation of about 260-kDa protein band. Similar results have been obtained when the coiled-coil skeletal muscle myosin rod was subjected to the EDC-crosslinking. These data indicate that the heavy chains of *A. proteus* myosin VI immunoanalogue may form dimers. These results also suggest that *in vivo* myosin VI immunoanalogue may play a role of an active transporter that requires dimerization and processivity.

Key words: *Amoeba proteus*, crosslinking, dimerization, myosin VI immunoanalogue.

Abbreviations: EDC - N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide

INTRODUCTION

Myosins are actin-based molecular motors that hydrolyze ATP and convert its chemical energy into mechanical power. They are ubiquitously expressed as multiple isoforms in all eukaryotic cells, including protozoans and plants. They are composed of one or two

heavy chains, and one or more light chains non-covalently attached to the heavy chain. Myosin heavy chains follow the common organization pattern, with an N-terminal globular motor domain containing nucleotide and actin-binding sites; a neck domain containing at least one IQ motif [light chain(s) binding site]; and the most variable C-terminal tail domain (Sellers 1999). Based on the diversity of amino acid sequence of motor domains, myosins have been divided into at least 18 distinct families (classes) (Berg *et al.* 2001). The classic, two-headed myosins, also called conventional myosins, form class II, and other myosin families are referred to as

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unconventional myosins. The tail regions of conventional as well as some of the unconventional myosins (for example myosins V, VI VII and X) contain specific sequence pattern (known as the heptad repeats) that enables the molecules to form stable coiled-coil and dimerize. Nonmuscle myosins are involved in various cellular functions, e.g. cell motility (myosins I, II, VI and X), intracellular transport of particles, vesicles and organelles (myosins I, V, VI and VII), endo- and exocytosis (myosins I, V and VI), cell contacts formation (myosins II and VII), cytokinesis (myosins II and VI), and transcription (myosin I).

In this study we have focused on class VI myosin, which is the only myosin known to date that moves towards the minus (pointed) end of actin filaments (Wells *et al.* 1999, Walker *et al.* 2000). It is involved in vesicle transport, cell migration and membrane tension maintenance (Buss *et al.* 1998, Sellers 1999, Hasson 2003, Frank *et al.* 2004). It has been postulated that myosin VI may play an important role during both endo- and exocytosis, but its exact role still needs to be established. In all cell types examined, myosin VI is associated with uncoated endocytic vesicles and carries these vesicles away from actin-rich sub-plasma membrane regions (Aschenbrenner *et al.* 2003). Yoshida *et al.* (2004) showed that myosin VI may be responsible for cell migration, because inhibit myosin VI expression in high-grade ovarian carcinoma cells impeded cell spreading and migration *in vitro*. Mutations in the myosin VI gene lead to deafness in mice (for references see Rędowicz 2002) and humans (Melchionda *et al.* 2001, Ahmed *et al.* 2003, Mohidin *et al.* 2004). In the deaf mice (*Snell's waltzer* syndrome), the major phenotype is manifested in the disorganized stereocilia of inner ear hair cells, but other defects can also be found. For example, in fibroblasts of *Snell's waltzer* mice the Golgi complex size and protein secretion are significantly reduced as compared with wild-type cells (Warner *et al.* 2003). Also, it has recently been shown that severe defects are found in hippocampus of these animals: there are less synapses, abnormally short dendritic spines, symptoms of astrogliosis, and the hippocampal neurons display a significant deficit in the stimulation-induced internalization of one type of glutamate receptors (Osterweil *et al.* 2005).

The heavy chain of mammalian myosins VI has a molecular weight of about 140 kDa with a motor domain containing an unique 53 amino-acid long insert followed by a short neck with one IQ motif binding calmodulin,

and a tail region. This C-terminal domain is believed to consist of a helical domain with the heptad repeats and a globular domain, responsible for a cargo binding and/or protein-protein interaction. It has been a general belief that myosin VI forms dimers *via* its coiled-coil region (Fig. 1). Surprisingly, Lister *et al.* (2004) showed that both expressed and native proteins were monomeric. However, they did not exclude the possibility that *in vivo* myosin VI may function both as a monomer and a dimer.

Recently in a highly motile free living *Amoeba proteus*, we have found a novel 130-kDa myosin VI-immunoanalogue (Dominik *et al.* 2005). Sequence comparison of several peptides derived from this protein with the other known myosins revealed a considerable homology to both human and invertebrate myosins VI. In migrating cells, myosin VI immunoanalogue was distributed in the entire cytoplasm as the punctate structures corresponding to membranous vesicular compartments where it colocalized with dynamin II; the colocalization was even more evident in pinocytotic cells. Blocking the endogenous protein with anti-human myosin VI antibody caused the inhibition of the rate of amoebae migration. To further characterize this novel ameoboid myosin VI isoform and elucidate whether its heavy chains form dimers, we performed the crosslinking experiments using the zero-length crosslinker, EDC. The obtained data indicate that under our experimental conditions *A. proteus* myosin VI immunoanalogue forms dimers.

MATERIALS AND METHODS

***Amoeba proteus* culture and preparation of high speed supernatant.** *Amoeba proteus* (strain Princeton) was cultured at room temperature in the standard Pringsheim medium [Ca(NO₃)₂·4H₂O - 0.848 mM, MgSO₄·7H₂O - 0.081 mM, Na₂HPO₄·2H₂O - 0.112 mM, KCl - 0.112 mM, FeSO₄·7H₂O - 0.007 mM, pH 6.8 - 7.2]. Amoebae were fed on *Tetrahymena pyriformis* twice a week and always used for experiments on the third day after feeding. Cells were collected by centrifugation at 2000 × g, washed three times with the ice cold TBS buffer (Tris buffer saline: 150 mM NaCl, 25 mM Tris pH 7.5), homogenized at 4°C in two volumes of a homogenization buffer containing 0.5% Triton X-100, 150mM NaCl, 10 mM KCl, 2 mM MgSO₄, 20 mM phosphate buffer pH 7.0, 1 mM EGTA, 2 mM ATP, 0.5 mM PMSF, 12% sucrose, and a set of protein inhibitors ("Complete" tablets, Roche). After centrifugation at 100 000 × g for 1 h the high-speed supernatants (HSS) were collected and subjected to cross-linking experiments.

Preparation of rabbit skeletal muscle myosin rod. Rabbit skeletal muscle myosin rod was prepared by the method described by Margossian and Lowey (1982). Briefly, myosin suspension (~10 mg/ml) in 0.2 M ammonium acetate and 2 mM EDTA, pH 7.2, was

digested at room temperature for 7 min with 0.03 mg/ml papaine dissolved in 5 mM cysteine pH 6.0 and 2mM EDTA. The reaction was stopped by 1mM iodoacetate, and the suspension was spun at 60 000 × g for 90 min. The insoluble precipitate was dispersed in solution A containing 0.6 M KCl and 0.05 M potassium phosphate pH 7.0, and three volumes of ice cold 95% ethanol were then added. The suspension was vigorously stirred for 3 h at 4°C and then spun at 20 000 × g for 30 min. The pellet was redispersed in solution A, and dialyzed overnight against the buffer in the coldroom. The supernatant was collected and further clarified by centrifugation at 70 000 × g for 90 min, and dialyzed against 30 mM KCl and 10 mM potassium phosphate, pH 7.0. The precipitated protein was collected by centrifugation at 20 000 × g for 30 min and then homogenized in two volumes of a homogenization buffer containing 0.5 M NaCl, 10 mM MOPS, pH 7.0, 0.5 mM PMSF, and a set of protein inhibitors. The homogenate was dialyzed overnight at 4°C against the same buffer, and centrifuged at 50 000 × g for 1 h. The supernatant was subjected to further experiments

The protein concentration of amoeba HSS and myosin rod preparation, measured by microbiuret method, was similar and was estimated to be about 5 mg/ml.

EDC crosslinking. Amoeba HSS and rabbit myosin rod were both mixed with *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma, USA) to its final concentrations of 5 mM and 50 mM. The mixtures were incubated at room temperature for 2 h, and 10 µl aliquots were collected every 30 min and immediately mixed with the Laemmli buffer to stop the reaction. The proteins were separated using 6 % SDS-polyacrylamide gels (Laemmli 1970), and then either stained with Coomassie Brilliant Blue, or transferred to a nitrocellulose membrane (Towbin *et al.* 1979). After the transfer, the membrane was blocked for 1 h at room temperature in TBS containing 5% non-fat milk powder, 0.2% Triton X-100 and 0.05% sodium azide followed by 2-h incubation with 1:200 dilution of polyclonal anti-myosin VI antibody directed against the C-terminus of human myosin VI (M 5187, Sigma-Aldrich, USA). The primary antibody was detected using a 1:10 000 dilution of anti-rabbit antibody conjugated with alkaline phosphatase (A 3687, Sigma-Aldrich, USA). The colour reaction was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma, USA) as the substrates.

RESULTS

In order to test whether the novel *Amoeba proteus* myosin VI-immunoanalogue is a monomeric or dimeric protein, the crosslinking experiment was performed using a zero-length crosslinker, EDC (Fig. 2). This reagent activates carboxyl groups and couples them with primary amines such as lysine to generate peptide bonds thus producing a zero-angstrom-long cross-link. That ensures that only proteins directly interacting with each other are crosslinked and those that are only in close proximity are not coupled. As a positive control, rabbit skeletal muscle myosin rod that is known to almost entirely consist of heptad repeats, and form stable coiled-coil (Fig. 1) and filaments was used. It should also

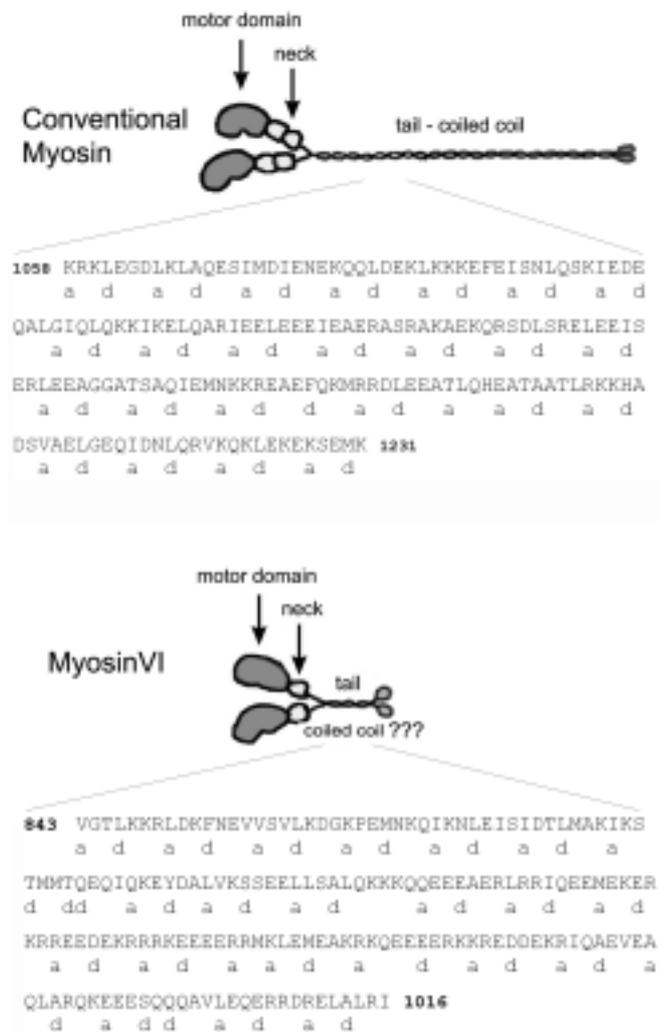


Fig. 1. Scheme of skeletal muscle myosin and myosin VI domain organization, with aminoacid sequences of the fragment (skeletal muscle myosin) or entire (myosin VI) coiled-coil region. a, d - heptarepeats hydrophobic residues, crucial for the coiled-coil formation.

be explained here that the diffusive appearance of the bands corresponding to cross-linking products resulted from the known negative effects of EDC on protein resolution.

Amoeba proteus high speed supernatant (HSS) (Fig. 2A) and rabbit skeletal muscle myosin rod (Fig. 2B) were treated at room temperature with both 5 and 50 mM EDC for 30, 60, 90, and 120 min, as indicated in the figures. The formation of the cross-linking products was monitored using the SDS-PAGE gels and, in the case of amoebae proteins, Western blots. *A. proteus*

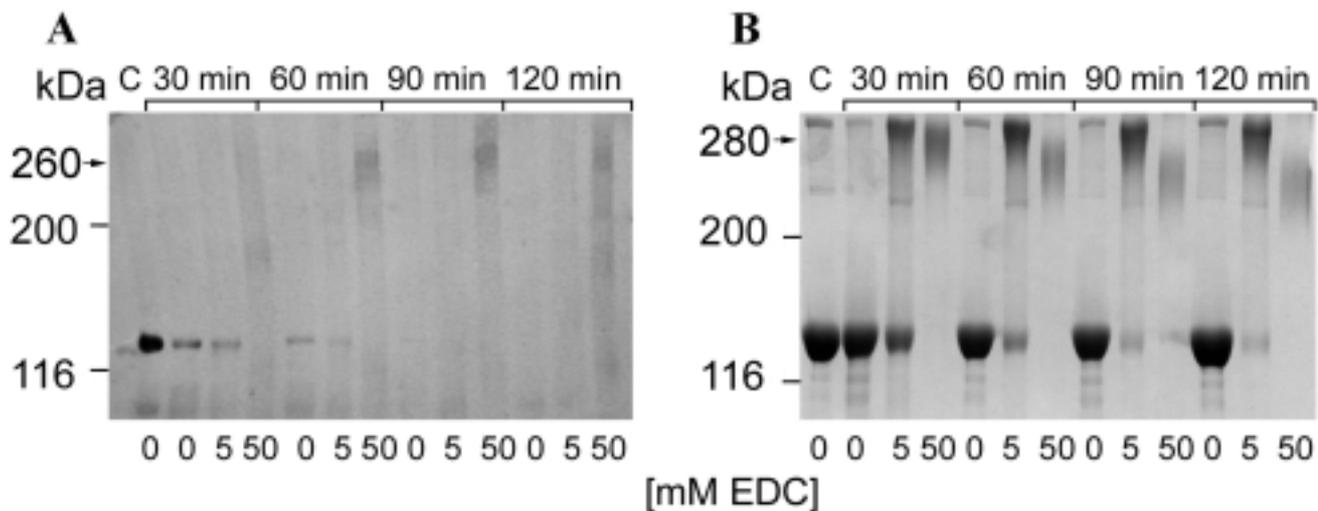


Fig. 2. Time course of EDC-crosslinking. **A** - *Amoeba proteus* high speed supernatant (HSS); **B** - rabbit skeletal muscle myosin rod; were subjected to crosslinking with no (0), 5 mM or 50 mM EDC as indicated in the figure. Myosin VI immunoanalogue and its crosslinking product were detected with polyclonal anti-human myosin VI antibody, and muscle myosin rod and its crosslinking product were visualized with Coomassie Brilliant Blue staining. C, HSS and myosin rod not treated with EDC; 30 min, 60 min, 90 min, 120 min, times of incubation with EDC. Arrows points to the crosslinking products

myosin VI immunoanalogue was visible in not-treated HSS as the about 130-kDa band (Fig. 2A, lane C). During the course of the experiment, the band corresponding to myosin VI immunoanalogue diminished and a new band, corresponding to 260 kDa, appeared (Fig. 2A, arrow). This molecular mass seems to be consistent with that of putative dimers of myosin VI immunoanalogue heavy chain. The presence of the band was much more evident when the higher EDC concentration was used (Fig. 2A). Interestingly, while the 260-kDa cross-linking product was stable after its first appearance after 60 min, the intensity of the initial 130-kDa band was gradually decreasing even when the incubation was performed without EDC (Fig. 2A, lanes 0). It can be explained in terms of higher susceptibility of non-crosslinked protein to non specific proteolytic cleavage, even in the presence of protease inhibitors in the solution; crosslinking within the coiled-coil region may protect the protein from the protease attack.

Myosin rod not treated with EDC (Fig. 2B) was visible as the one band corresponding to about 140 kDa and remained stable during the 2 h incubation at room temperature. Addition of EDC, 5 or 50 mM, to myosin rod preparation resulted in the appearance of a new protein band of about 280 kDa (which corresponded to the known molecular weight of a rod dimer) with the

concomitant and gradual disappearance of the 140-kDa myosin rod band (Fig. 2B, arrow).

These data indicate that 130-kDa myosin VI immunoanalogue heavy chains may be crosslinked with each other, similarly to muscle myosin rod.

DISCUSSION

Myosins form a diverse superfamily, both structurally and functionally. They are involved in panoply of cellular processes that are vital for cell and organism survival such as - among others - cell migration, muscle contraction, cytokinesis, endo- and exocytosis, hearing and vision (see Rędownicz 2002). It is a general belief that the multiplicity of functions fulfilled by these actin-based molecular motors is realized *via* their structural diversity, and in particular *via* variability of the C-terminal tail domains (Sellers 1999, Berg *et al.* 2001). For example, in the amino acid sequence of the heavy chains of several myosins' (namely myosins II, V, VI, VII, VIII, X, XI, XII and XVIII) tails the heptad repeats responsible for coiled-coil formation were found, indicating that these heavy chains may dimerize (Berg *et al.* 2001). While it has been definitely confirmed that myosins II and V are dimeric both *in vitro* and *in vivo*, the

dimerization of other myosins' heavy chains still remains an open question.

And so is the case of myosin VI, which heavy chain contains approximately 100-amino acid-long heptad repeats region that was predicted, using COILS software (Lupas *et al.* 1991), to form coiled-coil (Fig. 1). Based on this, the assumption has been made that myosin VI is a dimeric protein (Kellerman and Miller 1992, Sellers 1999). This supposition was taken into consideration whilst creating recombinant fragments of myosin VI for structure-function relationship studies (De La Cruz *et al.* 2001, Morris *et al.* 2003). Surprisingly, Lister *et al.* (2004) showed, using size exclusion chromatography and EDC crosslinking that both expressed and native myosin VI preparations contained only one 140-kDa heavy chain. However, it should be emphasized that the cross-linking experiments were carried out in high salt and Tris buffer, pH 8, conditions not suitable for EDC coupling (see Pierce catalog and handbook). Since then no other experimental data supporting this observation have been presented, however, the detailed analysis of myosin VI amino acid sequence made by the authors revealed that the putative coiled-coil sequence contained some proline residues probably breaking the helix and a central charged region that would probably favour formation of intramolecular salt bridges rather than a coiled-coil (Lister *et al.* 2004). Our results showed that the 130-kDa heavy chain of *Amoeba proteus* myosin VI immunoanalogue, a novel protein that might be the first amoeboid myosin VI isoform (Dominik *et al.* 2005), could be crosslinked by EDC, even in a phosphate buffer known to reduce the efficiency of EDC reaction, to an about 260-kDa product, most probably corresponding to the heavy chain dimer. This seems to indicate that myosin VI immunoanalogue may form dimers, what excludes the possibility that it belongs to the monomeric myosin I or other (III, IV or IX) families. However, it cannot be excluded that the heavy chains of myosin VI immunoanalogue are not only crosslinked with each other but also with other protein with the similar molecular weight, possibly its binding partner.

Kinetic properties of mammalian myosins VI seem to depend on the state of its dimerization. It was showed in studies performed on fragments or full-length dimeric myosin VI that the protein had a high duty ratio (De La Cruz *et al.* 2001) and moved processively along actin filaments with a large step size of 30-36 nm (Rock *et al.* 2001). On the other hand, Lister *et al.* (2004) demonstrated that monomeric myosin VI was a nonprocessive

motor with a 18-nm step size undergoing a large conformational change upon addition of ATP.

The idea that both monomers and dimers of myosin VI may function *in vivo* is intriguing and may, in fact, explain the mechanisms of its functioning within the cell. Such functions as intracellular transport of vesicles and organelles require a processive dimeric motor, while processes such as maintaining tension, clustering transmembrane receptors or tethering membranes and vesicles to actin filaments would benefit more from a nonprocessive monomer (Buss *et al.* 2004). Our data indicate that under experimental conditions applied here, *A. proteus* myosin VI immunoanalogue may exist as a dimer, but most probably *in vivo* there is an equilibrium between monomers and dimers. Dimerization of myosin VI immunoanalogue may favour its role of an active transporter, translocating cargo to other compartments within these giant cells. This idea seems to be additionally strengthened by the assumption that myosin VI immunoanalogue may be the only one minus-end driven molecular motor in this microtubule-lacking environment.

Up to the date, there are no reports addressing when and where myosin VI exists as a dimer or monomer, and what factors control its dimerization. It has been proposed that during endocytosis monomers could be recruited to plasma membrane "hot spots" and/or clathrin-coated pits to achieve the concentration sufficient to initiate a spontaneous dimerization through its helical tail domain (Buss *et al.* 2004). This suggestion is based on the report showing that dimerization of kinesin Unc104/KIF1A on the lipid vesicles surface caused its processivity, and that motor dimerization could be used to control intracellular transport by this class of kinesins (Tomishige *et al.* 2002). However, it is also quite possible that association with the binding partners (e.g. Dab2, SAP95 or other yet unknown proteins) or phosphorylation/dephosphorylation of the heavy chain could contribute to dimerization process (Buss *et al.* 2004).

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