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Polycation induced actin bundles

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ABSTRACT

Three polycations, polylysine, the polyamine spermine and the polycationic protein lysozyme were used to study the formation, structure, ionic strength sensitivity and dissociation of polycation-induced actin bundles. Bundles form fast, simultaneously with the polymerization of MgATP-G-actins, upon the addition of polycations to solutions of actins at low ionic strength conditions. This indicates that nuclei and/or nascent filaments bundle due to attractive, electrostatic effect of polycations and the neutralization of repulsive interactions of negative charges on actin. The attractive forces between the filaments are strong, as shown by the low (in nanomolar range) critical concentration of their bundling at low ionic strength. These bundles are sensitive to ionic strength and disassemble partially in 100 mM NaCl, but both the dissociation and ionic strength sensitivity can be countered by higher polycation concentrations. Cys374 residues of actin monomers residing on neighboring filaments in the bundles can be cross-linked by the short span (5.4 Å) MTS-1 (1,1-Methanedyl Bismethanethiosulfonate) cross-linker, which indicates a tight packing of filaments in the bundles. The interfilament cross-links, which connect monomers located on oppositely oriented filaments, prevent disassembly of bundles at high ionic strength. Cofilin and the polysaccharide polyanion heparin disassemble lysozyme induced actin bundles more effectively than the polylysine-induced bundles. The actin-lysozyme bundles are pathologically significant as both proteins are found in the pulmonary airways of cystic fibrosis patients. Their bundles contribute to the formation of viscous mucus, which is the main cause of breathing difficulties and eventual death in this disorder.

1. Introduction

Cells contain a number of dynamic, higher order structures, including bundles of actin filaments. These structures have indispensable role in cell physiology, in the formation of cytoskeleton, cell division, motility, adhesion and signaling, etc. Actin bundles may form via cross-linking of individual actin filaments with specific actin-bundling proteins [1] or by attracting the negatively charged filaments to the polycations which "bridge" between the filaments. The group of bivalent cross-linking proteins includes fimbrin, α-actinin, spectrin, fascin, filamin and others. These proteins [1] have two discrete F-actin binding sites and form tightly bound parallel filaments [2,3].

Polycations, including polycationic proteins or peptides and natural and synthetic polyamines, polymerize actin and induce bundle formation via non-specific electrostatic interactions, by eliminating repulsion between individual G-actin molecules or between actin filaments [4]. Calponin [5,6], lysozyme [7] MARCKS [8,9], ENA/VASP [10], fesselin [11,12] and myelin basic protein [13] are examples of such polycationic proteins. The natural polyamines spermine and spermidine [14,15] and the synthetic polyamine polylysine are known to polymerize and bundle actin [16]. The bundling effect of polycations is known to decrease with increasing ionic strength [17], but the kinetics of bundle formation, cross link formation between the filaments in the bundle, interaction of polycation-induced bundles with actin binding and severing proteins remain poorly understood or uncharacterized.

Three polycations, lysozyme, spermine and polylysine were chosen in this study to investigate the various aspects of polycation-induced actin-bundling. Lysozyme is an antibacterial polycationic protein with 9 net positive charges [7]. It is abundant in the airways of cystic fibrosis patients [18], where it forms bundles with actin [19]. These bundles contribute to the accumulation of sputum, a viscous mucus — the primary cause of bacterial infections and death in this disease [20]. The viscosity of sputum was found to be significantly decreased by the actin severing protein gelsolin [21] and by polyanions [22]. The natural polyamine spermine, which is a small tetravalent cation, is present at millimolar concentrations in proliferating cells [23], and is believed to play a significant role in cell cycle, apoptosis, and signal transduction [24,25]. The synthetic polyamine polylysine is one of the most efficient polymerizing and bundling agents and has been used as model compound in a number of studies on polycation formed actin bundles [4,26,27].

In this study, we examined the formation and structure of actin bundles induced by these three polycations using light scattering, low speed sedimentation and cross-linking methods. We found that bundles,

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which form simultaneously with actin polymerization, have a very low critical concentration at low ionic strength. The bundles are ionic strength sensitive and those induced by low millimolar concentrations of spermine or low micromolar concentrations of lysozyme or polylysine disassemble fast in the presence of 100 mM NaCl. Their ionic strength sensitivity decreases with the increase of polycation concentration. The bundles are partially disassembled by the actin filament severing protein cofilin [28], whose unbundling activity is significantly enhanced by the polysaccharide polyanion heparin. The tightly packed polycation-induced bundles contain oppositely oriented actin filaments, as indicated by the interfilament cross-links between Cys-374 residues by the short span (5.4 Å) MTS-1 cross-linking reagent.

2. Materials and methods

2.1. Materials

N-(1-pyrene)maleimide was obtained from Molecular Probes (Eugene, OR). Hen lysozyme, ATP, ADP, poly-L-lysine (MW 4000), heparin (unfractionated), dithiotreitol (DTT), N-ethylmaleimide (NEM), spermine and EGTA were purchased from Sigma Chemical Co. (St Louis, MO). 1,1-methaediyl bismethanethiosulfonate (MTS-1), was obtained from Toronto Research Chemicals Inc., North York (Ontario, Canada). Bacterial transglutaminase and N-(4-azido-2-nitrophenyl) putrescine (ANP) were generous gifts from Dr. G. Hegyi (Eotvos Lorand University, Budapest, Hungary).

2.2. Preparation of actin

CaATP-G-actin was prepared from the back and leg muscles of rabbit by the method of Spudich and Watt [29] and stored in G-buffer containing 5.0 mM Tris–HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM β-mercaptoethanol, pH 8.0 (CaATP-G-buffer). MgATP-G-actin was obtained by incubating CaATP-G-actin with 0.2 mM EGTA and 0.1 mM $MgCl_2$ at room temperature for 5 min. MgATP-G-actin ($\leq$10 μM) was used within 2 hours from the preparation. MgATP-G-actin was diluted for further treatments in MgATP-G-buffer containing 5 mM Tris–HCl, 0.1 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM ATP and 0.5 mM DTT, pH 8.0. Mg-F-actin was polymerized from Mg-ATP-G-actin by addition of 2 mM $MgCl_2$. The concentration of unlabeled skeletal muscle α-G-actins was determined spectrophotometrically using the extinction coefficients $E^{1\%}_{290} = 11.5\ cm^{-1}$. (The optical density of actin was measured in the presence of 0.5 M NaOH, which shifts the maximum of absorbance from 280 nm to 290 nm). Molecular masses of skeletal actin, yeast cofilin and hen lysozyme were assumed to be 42 kDa, 15.9 kDa and 14.3 kDa, respectively.

2.3. Cofilin preparation

Yeast cofilin was prepared as previously described [30] with minor modifications. Briefly, wild-type yeast cofilin was expressed in *Escherichia coli* BL21(DE3) cells under the T7-promoter (pBAT4 plasmid). Cells were grown to a density of 0.6 OD units, induced with 0.4 mM IPTG, harvested by centrifugation, resuspended in 20 mM Tris–HCl, pH 7.5, and lysed by sonication. The cell lysate was clarified by centrifugation, the supernatant applied to a QAE-52 column (Pharmacia Biotech) equilibrated with 20 mM Tris–HCl, pH 7.5 and the column developed with a linear 0–0.5 M NaCl gradient. Peak fractions containing cofilin were pooled, concentrated, and applied to a Sephacryl S300 gel-filtration column (Pharmacia Biotech), equilibrated with 10 mM Tris–HCl, 50 mM NaCl, pH 7.5. The peak fractions were pooled and polished by MonoQ ion-exchange chromatography using a buffer system similar to that described for the QAE-52 chromatography.

2.4. Chemical modification

Labeling of Mg-F-actin at Cys-374 with pyrene maleimide was carried out according to Kouyama and Mihashi [31] with some modifications. CaATP-G-actin was filtered through a PD-10 column equilibrated with β-mercaptoethanol free Ca-ATP-G-buffer. After filtration, actin (1.0 mg/ml) was polymerized by 2.0 mM $MgCl_2$ and 100 mM KCl at room temperature for 30 min, and reacted with pyrene maleimide (16 μg/ml) on ice, for 1 hour. The reaction was terminated with 1.0 mM DTT. The labeled F-actin was centrifuged at 38 K rpm for 2 hours, then the pellet was resuspended in Ca-ATP-G-buffer and depolymerized for over 36 h at 4 °C. Finally, actin was centrifuged again at 38 K rpm for 2 hours. The supernatant contained the purified pyrene-labeled CaATP-G-actin. The concentration of modified actin was determined by the procedure of Bradford [32] using unmodified actin as a standard. The extent of labeling, which was measured by using pyrene extinction coefficient $E_{344\ nm} = 22000\ cm^{-1}\ M^{-1}$, was ~100%. G-actin was labeled by ANP, polymerized by 2 mM $MgCl_2$ and photo cross-linked according to Hegyi et al. [33]. The extent of labeling was calculated by extinction coefficient $E_{470} = 5400\ cm^{-1}\ M^{-1}$ of ANP.

2.5. Covalent cross-linking

Cross-linking of two actin monomers between Cys-374 residues at the C-terminus was carried out with the MTS-1 disulfide reagent. This reagent has a cross-linking span of 5.4 Å and has been used before for intramolecular cross-linking of actin cysteine residues [34]. Prior to the cross-linking reaction, dithiothreitol was removed from CaATP-G-actin over a PD-10 column equilibrated with β-mercaptoethanol free Ca-ATP-G-buffer. Then CaATP-G-actin was transformed to MgATP-G-actin, polymerized by 2 mM $MgCl_2$ and finally bundled by polycations. MTS-1 was added to bundles of Mg-F-actin at 0.75:1.0 molar ratios to actin monomers. The cross-linking reaction was stopped 30–60 s after MTS-1 addition by blocking the free SH groups with 1.0 mM NEM. The cross-linked samples were analyzed by 12% SDS-PAGE and quantitatively evaluated by densitometry.

2.6. Fluorescence and light scattering measurements

The time course of pyrene-labeled actin polymerization was monitored by measuring fluorescence increase (with 365 nm excitation and 386 nm emission wavelengths) in a PTI spectrofluorometer (Photon Technology Industries, South Brunswick, NJ). The time course of light scattering changes was also measured in a PTI spectrofluorometer or in an Applied Photophysics stopped-flow apparatus (Leatherhead, Surrey, UK), with both excitation and emission wavelengths adjusted to 450 nm. All fluorescence and light scattering measurements were carried out at 22 °C.

2.7. Monitoring bundling by low speed sedimentation

After the addition of polycations, actin samples were centrifuged at 20800 rcf for 8 min in an Eppendorf centrifuge. The supernatants were analyzed by SDS-PAGE. The measurements were carried out at 22 °C.

2.8. Analysis of the kinetics of assembly and disassembly of bundles

GraphPad Prism software was used for the analysis. The explicit forms of the equations used are the following: Two phase exponential association: $Y + Ymax_1\ (1 - \exp(-K_1\ X)) + Ymax_2\ (1 - \exp(-K_2\ X))$; one phase exponential decay: $Y = Span\ (\exp(-K\ X) + Plateau$.