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A high-throughput assay shows that DNase-I binds actin monomers and polymers with similar affinity

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Abstract

Previous conflicting reports suggest that DNase-I binds F-actin with either equal or drastically different K_D values compared to G-actin. We developed a high-throughput DNase-I inhibition assay to determine the K_D of DNase-I for F-actin. We confirmed that phalloidin-stabilized F-actin is protected from depolymerization by DNase-I and that the critical concentration at the pointed end of phalloidin–F-actin is 45.5 ± 13.9 nM. We found that DNase-I inhibition by actin follows ultrasensitive mechanics. Using varying lengths of gelsolin-capped phalloidin–F-actin, we concluded that the affinities of DNase-I for G- and the pointed end subunits of F-actin are almost indistinguishable, such that DNase-I may not distinguish between G- and F-actin conformations.

Keywords: Actin; DNase-I; Phalloidin; Gelsolin; Actin binding proteins; Ultrasensitivity; Critical concentration; Dissociation constant

DNase-I was first isolated in 1948 [1]. While actin was first characterized in 1942 [2], it was not until 1974 that the DNase-I inhibitor purified from calf spleen was identified as actin [3,4]. The ability of actin to bind tightly and inhibit DNase-I endonuclease activity has been employed to study thermal denaturation of monomeric actin (G-actin) [5], the in vitro folding of actin by eukaryotic chaperonins [6], and the basis of affinity purification of actin from cell lysates [7].

The first atomic resolution structure of monomeric actin was obtained from crystals of DNase-I bound to actin [8]. DNase-I binds the pointed end of actin, primarily to a loop in subdomain 2 that participates in an intermolecular parallel β sheet between actin and DNase-I. Subdomain 4 of actin is also involved in DNase-I interactions, forming additional hydrogen bonds.

The binding affinity of DNase-I for monomeric actin was first reported as 2 nM [9]; however, there are conflicting reports of DNase-I binding to filamentous actin (F-actin). Some sources suggest that DNase-I does not bind F-actin [6]. Cosedimentation assays including phalloidin-stabilized F-actin obtained a K_D of DNase-I for F-actin of 0.12 ± 0.09 mM [9], a 60,000-fold increase relative to G-actin binding. An investigation of the binding of DNase-I to isolated red blood cell cytoskeletons rich in short F-actin structures suggested that DNase-I binds the ends of filaments with no difference in K_D in G-actin and F-actin [10].

Recent efforts to isolate short F-actin structures for structural determination [11,12] have resulted in crystal structures that possess subunits of actin with conformations that are similar to those of G-actin. Given that these complexes have been isolated and no longer polymerize, it is possible that the subunits in these structures no longer conform to an F-actin-like state. A 60,000-fold difference in binding affinity in G-actin and F-actin would be a convenient means of determining the conformation of actin in small actin complexes prior to structural determination.

To determine the affinity of DNase-I for G- and F-actin in a biochemically pure system, we developed a high-throughput assay based on previous methods relying on

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the hyperchromicity of DNA upon cleavage [13]. Our results show that DNase-I has similar binding affinities for G-actin and the pointed end subunits of F-actin and, as such, does not distinguish between the conformations of G- and F-actin.

Materials and methods

Reagents

Unless otherwise stated, all buffer reagents and media were obtained from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Mississauga, ON). All chromatography columns were obtained from Amersham (GE Healthcare, Piscataway, NJ). Phalloidin was obtained from Molecular probes (Eugene, OR). DNase-I was from Worthington Biochemical (Lakewood, NJ).

Purification of proteins

DNase-I from Worthington was further purified by Superdex-200 gel filtration in buffer A (2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 50 mM NaCl, 0.5 mM β-mercaptoethanol). The purified DNase-I contained 95% active enzyme, compared to a commercially available DNase-I standard (Worthington).

Full-length gelsolin was purified from *Escherichia coli* by expression in the presence of isopropyl β-D-thiogalactaside employing pGS.31 (a generous gift of B. Pope and A.G. Weeds, MRC Laboratory of Molecular Biology, Cambridge). Cells were lysed in lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride) by two passes through a French press at 16,000 psi. The cell lysate was cleared by centrifugation at 31,360*g* for 30 min. The supernatant was then applied to an 80-ml Affi-Gel Blue column (Bio-Rad, Hercules, CA) equilibrated in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). This was then developed with a linear gradient of NaCl to 1 M NaCl in TE buffer. Gelsolin-containing fractions were pooled and dialyzed overnight against TE buffer containing 1 mM β-mercaptoethanol, applied to an 8-ml Mono Q column equilibrated with TE buffer, and eluted with a linear gradient to 1 M NaCl in TE buffer. Gelsolin-containing fractions were pooled and dialyzed overnight in TE buffer containing 1 mM β-mercaptoethanol. This preparation can be stored at −80 °C for several months.

Actin nucleotide exchange

Actin was purified according to the method of Spudich and Watt [14], yielding Ca-ATP-actin. Mg-ATP-actin was prepared by incubating Ca-ATP-actin with 0.2 mM EGTA and 0.2 mM $MgCl_2$ in G buffer (2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol) for 10 min and then buffer exchanged into 2 mM Tris, pH 8, 0.2 mM $MgCl_2$, 0.2 mM ATP, and 0.5 mM β-mercaptoethanol using a HiTrap desalting column [15]. Mg-ADP-actin was prepared from Mg-ATP-actin, which had been buffer exchanged into a buffer containing 2 mM Tris, pH 8, 0.2 mM $MgCl_2$, 0.2 mM ADP, and 0.5 mM β-mercaptoethanol. The protein was then incubated with 20 U/ml hexokinase and 0.3 mM glucose for 1 h at room temperature [16]. Ca-ADP-actin was prepared by polymerizing Ca-ATP-actin that was buffer exchanged into 2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ADP, and 0.5 mM β-mercaptoethanol by adjusting the salt concentration to 50 mM KCl, 2 mM $CaCl_2$, and 2 mM ADP. During polymerization (2.5 h) the sample was sonicated three times for 10 s to promote monomer cycling. The actin was then extensively dialyzed against 2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ADP, and 0.5 mM β-mercaptoethanol. Before use the Ca-ADP-actin was centrifuged at 324,000*g* for 20 min to remove any remaining filaments or aggregates [15].

F-actin preparation

F-actin of determined length was prepared by incubating G-actin with gelsolin at fixed molar ratios (1:25, 1:50, 1:75, and 1:100). Polymerization reactions were initiated by adjusting salt concentrations to 50 mM KCl, 2 mM $MgCl_2$, 2 mM ATP and incubated for 1 h at room temperature to ensure nucleation from gelsolin. Phalloidin was then added to achieve a 1:1.5 molar ratio with actin and incubated for an additional 30 min at room temperature to complete polymerization.

DNase-I inhibition assays

The DNase-I inhibition assay used to determine the K_D of the actin:DNase-I complex was a modified version of the original assay described by Blikstad et al. [13]. For G-actin determinations, a 50-μl incubation reaction containing 0.94 nM to 47.56 μM actin and 15.4 nM DNase-I was incubated for 30 min at room temperature in the wells of a UV transparent 96-well microplate. All dilutions and reagents were prepared in G buffer containing the appropriate nucleotide and cation. The assay was initiated by adding 200 μl of 240 μg/ml salmon sperm DNA prepared in DNA buffer (100 mM Tris, pH 8, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$). The absorbance was immediately monitored at 260 nM over a period of 3 min using a UV plate reader (BioTek, Winooski, VT). The percentage of DNase-I inhibition was determined by comparing the initial linear rates of DNase-I activity to control reactions containing no actin.

F-actin determinations were performed by diluting gelsolin-capped and phalloidin-stabilized F-actin with F buffer (50 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP) in 50-μl samples containing 15.4 nM DNase-I. After incubation at room temperature for 30 min, the DNase-I activity was measured as described above.