Identification of the trapped calcium in the gelsolin segment 1–actin complex: implications for the role of calcium in the control of gelsolin activity

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Abstract The X-ray structure of the complex of actin with gelsolin segment 1 revealed the presence of two calcium ions, one bound at an intramolecular site within segment 1 and the other bridging the segment directly to actin. Although earlier calcium binding studies at pH 8.0 revealed only a single calcium trapped in the complex (and also in the binary gelsolin–actin complex), it is here shown that two calcium ions are bound under the conditions of crystallization at physiological pH. Mutation of acidic residues in either actin or segment 1 involved in ligation of the intermolecular calcium ion resulted in loss of one of the bound calcium ions at pH <7, but not at pH 8. Thus the calcium ion trapped in the segment 1–actin complex is that located at the intramolecular site. The implications of this for gelsolin function are discussed.

Key words: Gelsolin; Actin-binding protein; Calcium binding site

1. Introduction

Gelsolin is an actin filament severing and capping protein. It is composed of six repeating segments of sequence (G1–G6) and contains 3 separate actin binding sites (reviewed in [1]). There is a high affinity G-actin binding site in G1 [2] which is essential for the severing activity of gelsolin [3]; G2 contains a filament binding site [4] and G4 a calcium-dependent monomer binding site [5].

The structure of the complex of G1 with actin was recently solved at atomic resolution [6]. X-ray analysis identified two calcium binding sites, one bound intramolecularly to G1 and the other forming a bridge between G1 and actin. The intramolecular calcium, near the N-terminus of the actin binding helix, is ligated by Asp^{66} and Glu^{97} (bidentate), together with the carbonyl oxygens of Gly^{65} and Val^{145}. The intermolecular calcium is ligated by Asp^{109} (bidentate) and the carbonyl oxygens of Gly^{114} and Ala^{116} of G1 together with the carboxylate of actin Glu^{167}.

Earlier biochemical analysis detected no calcium binding by G1 alone and only a single calcium trapped in the complex with actin [7]. This calcium probably corresponds to the trapped calcium previously identified in gelsolin–actin binary complexes which are not dissociated by EGTA [7,8]. Because the affinity of G1 for actin is markedly increased in the presence of calcium [2], and mutants of G1 truncated at their C-terminal end show an absolute requirement for calcium for actin binding [7], we predicted that the calcium bound at the intermolecular site might be the ion trapped in the G1–actin complex [6]. Here we have re-investigated the binding of calcium in the complex and confirm the presence of two bound calciums under the conditions used for crystallization at pH 6.6, but only a single calcium at pH 8.0.

To distinguish which of the two calcium sites is the one formed in the complex at pH 8, we have expressed and purified a mutant actin from yeast using the system developed to study the role of specific residues of actin in the interaction with various actin-binding proteins, including tropomyosin [9], myosin and profilin [10]. Substitution of the ligating glutamic acid residue of actin by glutamine resulted in loss of the additional calcium bound at pH 6.6 as also did the corresponding mutation of the acidic ligating residue in G1. Thus it is the calcium ion bound at the intramolecular site that is trapped in the complex at pH 8.0. Put another way, formation of the G1–actin complex generates a high affinity calcium site within the G1 moiety.

2. Materials and methods

2.1. Protein preparations

G1 and a point mutant of G1 in which aspartic acid residues 109–110 were substituted by asparagine were expressed in *E. coli* and purified as described previously [7,11]. Rabbit skeletal muscle actin was prepared as described previously [12]. Chicken β-actin containing a substitution of glutamine for glutamic acid at position 167 (E167Q) was prepared as follows: the E167Q mutation was introduced into the chicken β-actin gene by oligonucleotide-directed mutagenesis using the M13mp19/*E. coli* dut$^-$ung$^-$ system [13] and standard DNA techniques. The mutant actin was produced in *S. cerevisiae* strain K923 (HMLα, mat::*LEU2*$^+$, hmr::*TRP1*$^+$, *ura3, ade2, sir3ts*, MATa at 23°C and MATα at 34°C) using the temperature regulated expression system [14], fermenter cultivation and isolation procedures described previously [9,15], with the exception that a hollow fiber H1P10-43 (Amicon) was used to concentrate the total actin eluted from the DNase-Sepharose column. The recombinant and yeast actins were separated on a hydroxyapatite column and the mutant β-actin was dialyzed overnight against G-buffer (5 mM Tris-HCl, pH 7.6, 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol). It was then taken through a polymerization/depolymerization cycle to concentrate the actin and quick frozen as 25 μl drops in liquid nitrogen for storage. Before use, the frozen actin was rapidly thawed, centrifuged in a Beckman TLA 100 rotor at 80,000 rev/min and the resulting supernatant taken for calcium binding studies.

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Abbreviations: G1, segment 1 of human gelsolin; PIP_2, phosphatidylinositol 4,5 bisphosphate.

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2.2. Calcium binding assays and complex formation

Calcium binding was measured by equilibrium dialysis as described previously [7]. G1 (24 μM) in 10 mM Tris-HCl pH 8.0, 10 μM EGTA was mixed with 20 μM G-actin that had been dialysed into G-buffer containing 0.08 mM $^{45}CaCl_2$ at either pH 8.0 or pH 6.6 (using 5 mM imidazole-HCl). The mixtures were left on ice for 2 h and dialysed against the same buffer for 36 h. Protein concentrations were estimated from extinction coefficients, calculated at 290 nm, because of interference from ATP at 280 nm. Values for A_{290} = 1.0 cm^{-1} were: actin = 37.4 μM, G1 = 71.8 μM, actin in the complex with G1 = 23.0 μM. Counts were measured using 150 μl samples: all samples for counting were weighed to minimise errors. Complex formation was checked using gel-electrophoresis under non-denaturing conditions as described previously [16], but using 7.5% polyacrylamide gels.

3. Results

Mutant actin E167Q was retarded behind endogenous yeast actin on hydroxyapatite as described previously for other mutant actins [17]. The β-actin mutant E167K was also prepared but did not separate from yeast actin on hydroxyapatite. DNase inhibition assays [18] indicated a recovery of 7.3 mg from 184 g of pelleted yeast cells. The recovery of actin after a polymerization and depolymerization cycle and storage in liquid nitrogen was 3.4 mg.

Fig. 1 shows the mobility of the actins and complexes with G1 on native gel-electrophoresis. With the exception of the mutant G1–actin complex shown in lane 5, there was no excess actin visible on the gels, indicating complete complex formation.

Previous experiments showed that there was no calcium binding by G1 alone [7]. This was confirmed in controls carried out in this study. Even at pH 6.6, calcium bound was only 0.27 mol/mol G1 at 0.08 mM calcium. Thus in the absence of actin, there is little binding of calcium to G1 under these conditions.

Table 1 shows calcium binding by complexes of G1 with muscle actin and mutant β-actin. Calcium bound per mol of complex with muscle actin at pH 8.0 was 1.2 mol/mol, minus the calcium bound by actin alone. At pH 6.6 this value was 2.0. The former confirms previous measurements [7], while the latter is consistent with the calcium binding predicted from the crystal structure [6]. When G1 was bound to mutant β-actin lacking the co-ordinating glutamate residue at position 167, one calcium was bound at both pH 8 and pH 6.6. This suggests that when the co-ordinating glutamate carboxylate of the actin is removed, which would be expected to disrupt the intermolecular calcium site, there is a reduction of calcium binding only at the lower pH value.

A further set of experiments was carried out using muscle actin, making complexes both with G1 and with a G1 mutant in which the two aspartic acid residues 109–110 were both substituted by asparagine. Previous experiments had shown

Table 1
Calcium binding by muscle actin and mutant actin in complex with G1

pH	Actin	Actin E167Q	G1–actin complex	G1–E167Q complex	G1–actin minus actin	G1–E167Q minus E167Q
8.0	0.54	0.38	1.67	1.74	1.13	1.36
			1.85		1.31	
6.6	0.42	0.58	2.23	1.39	1.81	0.81
	0.93		3.06		2.13	

Calcium binding measured in mol/mol actin at pH 8.0 and 6.6 for actin and mutant actin alone and in complex with G1. Results from two separate experiments are shown for muscle actin.

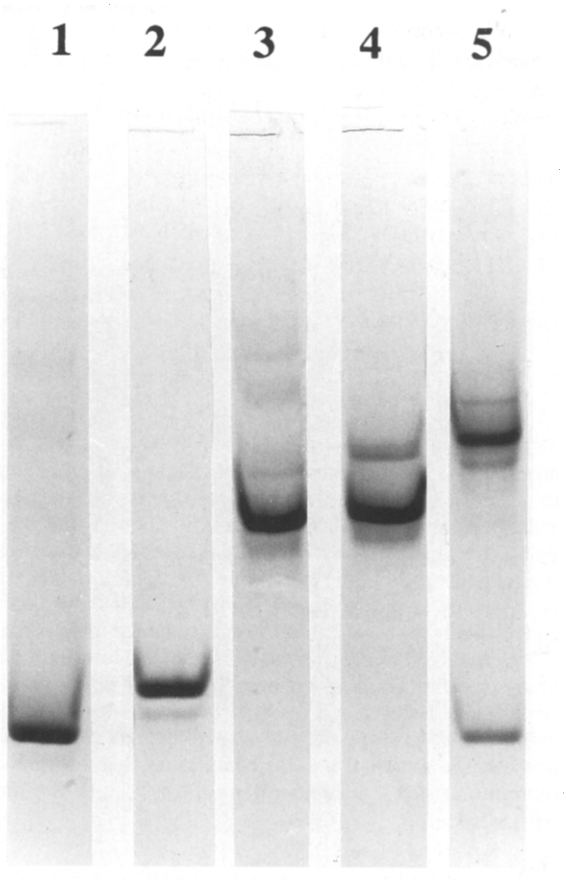

Fig. 1. Native gels demonstrating complex formation between actin and G1. Lane 1, muscle actin; lane 2, mutant actin E167Q; lane 3, G1–actin complex; lane 4, complex of G1 with mutant actin; lane 5, complex between mutant G1 and muscle actin. Neither G1 nor mutant G1 entered the gels under these conditions. Lane 5 shows incomplete complex formation with excess actin migrating as in lane 1. Complex formation in this case was about 70% and the corresponding value for calcium bound was 0.68 mol/mol.

that actin binding by this double mutant was still very tight [11]. In these experiments 0.25 mM $MgCl_2$ was added to the buffer, to approximate more closely to the conditions used in the crystallization of the complex and to minimise the possibility that calcium binding was non-specific. Gel-electrophoresis under non-denaturing conditions showed that complex formation was complete for G1 but that the mutant contained about 20% uncomplexed actin (slightly less than that in Fig. 1 lane 5). Results of calcium binding are shown in Table 2. The calcium bound per mol of complex at pH 8 was again 1 for both G1 and mutant G1. Calcium bound at pH 6.6 was 1.9 per mol complex for G1 and 1.0 for the complex with mutant G1.

4. Discussion

The mean values for calcium bound to G1–actin complexes were 1.2 (±0.1) at pH 8.0 and 1.9 (±0.2) at pH 6.6, confirming the presence of an additional bound calcium at the lower pH value. Using mutant forms of either actin or G1, in which the acidic residue contributing to the intermolecular calcium site was substituted by a neutral one, the extent of calcium binding

was 1 mol/mol complex at both pH values. The most reasonable interpretation is that the bound calcium detected at both pH values corresponds to that at the intramolecular site, while the intermolecular calcium site identified in the structure is occupied only at pH 6.6.

Because there is negligible calcium binding by G1 alone, it appears that actin binding to G1 enhances calcium affinity at this site. This site is near the N-terminus of the long actin binding helix, but on the opposite side of the helix to the actin binding site. Part of the hydrophobic contact with actin is formed by Phe^{149}, the C-terminal phenylalanine of G1. This contact imposes a constraint on the flexibility of the C-terminal region of G1, and one of the carbony groups involved in ligating this calcium ions is that of Val^{145}.

The importance of this part of the structure of G1, where the N-terminus of the long helix and the beginning of the second β-strand are brought close together, may be inferred from the conservation of the two acidic residues, Asp^{66} and Glu^{97} in all segments of gelsolin and virtually all segments of related members of the gelsolin family [19]. Asp^{66} is also involved in a buried salt bridge, the significance of which is evident from mutation of the equivalent residue in G2 in patients with familial amyloidosis (Finnish type) [6,20]. Destruction of this salt bridge in the plasma protein leads to proteolysis, loss of gelsolin activity [21] and amyloid formation.

In earlier experiments we reported that truncation at the C-terminus of G1 to Leu^{131} (termed mutant N131) resulted in loss of actin binding in the absence of calcium [7]. This deletion removes not only the calcium ligating residue Val^{145}, but the short β-strand and two conserved aromatic residues $Y^{134}F^{135}$ that form part of the hydrophobic core of G1 [6]. These two residues stabilise the position of the terminal helix and β-sheet but are at the edge of the core. Further truncation to Glu^{126} (mutant N126), involving loss of the short helix, resulted in an additional 6-fold decrease in actin binding affinity [7]. Calcium binding studies at 14 μM free calcium showed 0.54 mol calcium/mol complex for N131 and 0.18 for N126. Thus binding affinity for calcium and actin decreased together. These results suggest co-operativity between calcium binding to G1 and complex formation: interaction of G1 with actin enhances calcium binding and vice versa. This interaction probably explains the ~1000 fold increase in affinity of G1 for actin in the presence of calcium [2].

Recently the solution structure of the equivalent segment of villin (V1) was solved by NMR methods [22]. V1 binds with high affinity to G-actin, but differs from G1 in that binding is calcium-dependent [23]. The folding topology of V1 is similar to that of G1, but the C-terminus is highly disordered. Nevertheless, complex formation between villin and actin was found to protect Met^{125} from oxidation by *N*-chlorosuccinimide [24]. This is consistent with the structure of G1, in which the side chain of the equivalent residue, Phe^{149}, is buried at the interface with actin. In the G1–actin structure, Phe^{149} is a major component of the apolar contact site. Taken together these results suggest that the C-terminus of V1, which is flexible in the absence of actin, becomes immobilised in the complex. The V1 structure revealed two calcium binding sites with K_d values of ~1.8 and 11 mM. Measurements of the chemical shifts when calcium was added suggested that the stronger site is located near Val^{121}, which corresponds to Val^{145} of G1. The two highly conserved acidic residues Asp^{43} and Glu^{73}, located close to this calcium site, correspond to Asp^{66} and Glu^{97} of G1, i.e. the intramolecular calcium site. The weaker site is located near Asp^{85} and Glu^{86}, the former corresponding to Asp^{109} of G1, i.e. the position of the intermolecular site. Thus the two weak calcium binding sites in V1 correspond to those identified in the G1–actin complex.

There is a further parallel in the recent study of CapG [25] (formerly called macrophage capping protein [26]). Cap G contains a single calcium site of K_d ~37 μM, located in segments 2–3 of this 3-segment protein. In addition a very weak site was detected in segment 1, produced by chymotryptic digestion, with a K_d ~1.9 mM, similar to the higher affinity site in V1. Sequence comparisons with gelsolin show that the acidic residues involved in the intramolecular calcium site are conserved in segment 1 of Cap G and there is complete conservation of the two oxygen ligating residues Gly^{45} and Val^{125} as well as Phe^{129}, which corresponds to the C-terminal Phe of G1 [27]. By contrast the aspartic acid residue corresponding to that in the intermolecular site of G1 is substituted by asparagine. Based on our analysis of the G1 mutation at this position, substitution of the acidic residue by the corresponding amide results in loss of calcium binding. Although no calcium trapping was detected in functional studies of Cap G, because complexes readily dissociated in EGTA, this does not mean that there is no calcium bound at the intramolecular site in the complex [26]. Segment 1 of Cap G has a very high affinity for actin subunits based on the finding that a chimaeric protein containing this segment coupled to G2–3 of gelsolin severed actin filaments in a calcium-independent manner [28].

The C-terminal region of G1 is also implicated in polyphosphoinositide binding [29]. PIP_2 micelles bound to G1 in a gel-filtration assay, but there was little binding to N134 (G1 truncated by 15 residues at it's C-terminus). Furthermore, PIP_2 inhibited the binding of G1 to actin but not that of N134. Earlier experiments showed that the EGTA-stable complex between gelsolin and actin is dissociated by PIP_2 micelles, suggesting a direct effect of PIP_2 on the G1–actin complex [30]. The intramolecular calcium site in G1 has an incomplete co-ordination sphere with a missing ligand roughly perpendicular to the protein surface. This configuration is similar to that observed in the structure of annexin V, which is a calcium controlled phospholipid binding protein [31]. It is noteworthy that millimolar concentations of calcium are required to saturate the binding sites in annexin II in the absence of phospholipid, but affinity for calcium is increased by more than 100-fold in its presence [32]. If the annexin model provides a valid comparator for the gelsolin system, the intramolecular calcium may play a pivotal role in regulating both actin and PIP_2 interaction. Thus if PIP_2 alters the conformation of G1 in a manner that reduces

Table 2
Calcium binding by muscle actin in complex with G1 and the G1 mutant DD109NN

pH	Actin	G1–actin complex	DD109NN–actin complex	G1–actin minus actin	DD109NN–actin minus actin
8.0	0.93	1.96	1.86	1.03	0.93
6.6	0.73	2.59	1.76	1.86	1.03

Calcium binding measured in mol/mol actin in the presence of 0.25 mM $MgCl_2$ at pH 8.0 and 6.6 for actin alone and in complex with G1 or G1 mutant.

the affinity of the calcium trapped in the G1–actin complex, this will be expected to weaken actin binding. Further experiments will be needed to determine any possible role of calcium in PIP_2 binding by G1.

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