Functional consequences of disulfide bond formation in gelsolin

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Abstract Gelsolin is an actin monomer binding and filament severing protein synthesized in plasma and cytoplasmic forms differing by an N-terminal amino acid extension and a disulfide bond between Cys-188 and Cys-201. To determine whether this bond altered gelsolin regulation or function, oxidized and reduced plasma gelsolins were assayed for severing, monomer binding and nucleation activity at a variety of rate-limiting calcium concentrations. The results indicate that the disulfide bond in domain 2 of gelsolin influences the transmission of information from C-terminal regulatory sites to functional sites in the N-terminus.

Key words: Gelsolin; Protein structure; Disulfide bond; Calcium binding protein; Actin binding protein

1. Introduction

Gelsolin is an actin binding protein that severs and caps actin filaments and binds actin monomers to nucleate new filaments in a calcium dependent manner [1]. Analysis of the protein sequence suggests that gelsolin consists of six similar domains with the three N-terminal domains being most similar to the equivalent domains within the C-terminus [2]. Proteolytic digestion and expression of truncated proteins indicates that gelsolin's regulatory and functional sites are differentially distributed throughout these six domains [3,4]. Truncation of the C-terminal domain, domain 6, generates a protein that can sever in the absence of calcium ions, but has lost the ability to nucleate actin polymerization [3]. Domains 1–3 also sever rapidly in the complete absence of calcium [4]. Further truncations down to domain 1 and the first 11 amino acids of domain 2 retain severing ability, but regain calcium sensitivity [3]. Domain 1 of gelsolin has only high affinity monomer binding activity [5,6]. An N-terminal truncate of gelsolin containing domains 2–6 nucleates actin polymerization normally, but has lost most of its ability to sever [4]. In total these and other studies have led to the consensus view that domain 1 contains a high affinity monomer binding domain, domain 2 has a filament binding domain, domain 4 has a monomer binding domain and the regulatory calcium binding sites are located in the C-terminus of the protein.

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Abbreviations: TRITC-phalloidin, tetramethylrhodamine isothiocyanate coupled phalloidin; DTT, dithiothreitol; F-actin, filamentous actin; G-actin, monomeric actin; PIA, pyrene iodoacetemide; DACM, 7-dimethylamino-4-methyl-(*N*-maleimidyl) coumarin; n-HPG, native human plasma gelsolin; r-HPG, bacterially expressed human plasma gelsolin lacking the disulfide bond in domain 2; ox-r-HPG, bacterially expressed human plasma gelsolin containing the disulfide bond in domain 2

Gelsolin differs from most proteins in that it is produced in intra- and extracellular forms, generated from the same gene by differential splicing [2]. These two forms are nearly identical in sequence, with plasma gelsolin having a 21 amino acid N-terminal extension not found in cytoplasmic gelsolin [2]. Many previous studies have assumed that cytoplasmic and plasma gelsolins were functionally identical, and no evidence exists to suggest this extension alters gelsolin regulation or function. However, recent analysis of the calcium sensitivity of gelsolin's severing activity suggests that plasma gelsolin would not function in the range of calcium ion concentrations reported in previous studies to activate cytoplasmic gelsolin [7–10].

Gelsolin is activated to bind actin by calcium, and Ca^{2+} binding sites have been mapped to various domains within the C-terminal of gelsolin. Truncation of intact gelsolin by 35 amino acids at the C-terminal generates a molecule that severs F-actin in the absence of Ca^{2+} [3]. The N-terminal half of gelsolin (domains 1–3) also severs in the complete absence of Ca^{2+} [4]. Domain analysis demonstrates two Ca^{2+} binding sites in domain 4 and 6, with dissociation equilibrium constants of 2 and 0.2 μM respectively [11]. There is evidence for Ca^{2+} binding to a cryptic high affinity site within domain 1 of gelsolin that is exposed by the binding to the actin monomer [12,13]. This site may be important in maintaining the high affinity interaction of domain 1 of gelsolin with actin.

The mechanism coupling Ca^{2+} binding to the C-terminal of gelsolin to activation of the N-terminal half of the molecule is unclear. There is a change in gelsolin's shape upon activation as indicated by a change in the diffusion constant [14]. However, this change has been localized to the C-terminus of gelsolin [15]. Recently, a disulfide bond between the two cystines in domain 2 of gelsolin was identified in plasma, but not in cytoplasmic gelsolin [16]. The presence or absence of the disulfide in gelsolin depends on the redox potential of the local environment, and changing this potential can drive the formation or dissolution of the disulfide bond [16]. Expression of plasma gelsolin in an intracellular reducing compartment such as the cytoplasm leads to plasma gelsolin lacking the disulfide bond. To address some of the apparently contradictory reports in the literature on the calcium requirements for gelsolin activation, I asked whether disulfide bond formation altered gelsolin's functional or regulatory requirements, using native and recombinant human plasma gelsolin which have or lack the disulfide bond. The absence of the disulfide bond reduces the rate at which Ca^{2+} activates gelsolin to sever F-actin, bind actin monomers and nucleate actin polymerization, but does not influence the maximal rate of severing activity. These results suggest that the disulfide bond in domain 2 of plasma gelsolin is important for the communication of regulatory information from the C-terminal Ca^{2+} binding sites to the actin monomer and filament binding sites of the N-terminus.

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2. Material and methods

Phalloidin, tetramethylrhodamine isothiocyanate coupled phalloidin (TRITC-phalloidin), 7-dimethylamino-4-methyl-(*N*-maleimidyl) coumarin (DACM), nucleotides and all other buffers and salts were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-(1-Pyrene)iodoacetamide (PIA) was purchased from Molecular Probes, Inc. (Eugene, OR).

2.1. Proteins

Monomeric (G) actin was purified from an acetone powder of rabbit skeletal muscle as previously described [17,18]. Pyrene iodoacetemide (PIA) was coupled to ATP-actin as previously described [18]. Actin concentrations were determined from the absorbance at 290 nm using an extinction coefficient of 0.62 ml mg^{-1} cm^{-1} [19]. F-actin was polymerized at concentrations of 15–30 μM in solutions containing 150 mM KCl, 20 mM HEPES, pH 7.4; 0.5 mM ATP, 0.2 mM DTT; 2 mM $MgCl_2$ and 0.2 mM $CaCl_2$.

Human plasma gelsolin was purified by ammonium sulfate fractionation followed by chromatographic separation on DE-52 ion exchange matrix as described by Kurokawa et al. [20]. Purified gelsolin was dialyzed into 75 mM NaCl, 20 mM Tris, pH 7.4 and 0.2 mM EGTA, rapidly frozen in liquid nitrogen and stored at −80°C. Gelsolin concentration was determined from the absorbance at 280 nm using the extinction coefficient 1.8 ml mg^{-1} cm^{-1}. N-terminal truncates of native human plasma gelsolin were generated as described [3,4]. The bacterially expressed N-terminal fragment of gelsolin, s1–3, was purified from *E. coli* as described [4], and was the generous gift of M. Way. Recombinant human plasma gelsolin was expressed in *E. coli* and purified by chromatography on Q-sepharose, with further purification on SP-sepharose [16]. The disulfide bond in domain two of recombinant plasma gelsolin was generated by oxidation with 2 mM oxidized glutathione [16]. Recombinant oxidized and reduced human plasma gelsolins were the generous gift of Blake Pepinksy (Biogen, Cambridge, MA).

2.1.1. Sulfhydryl analysis. The concentration of solvent accessible and total cysteines on gelsolin and gelsolin truncates was determined from the change in fluorescence intensity of sulfhydryl reactive DACM [21]. Gelsolin contains five cysteines, with four of them occurring in the N-terminal half of the molecule. A stock solution of 6.6 mM DACM in acetone was diluted to a final concentration of 3.3 μM in 150 mM NaCl, 15 mM HEPES, pH 7.4 for protein incubations. After the labeling of the native protein reached equilibrium, SDS was added to a final concentration of 0.1% to denature the protein and allow labeling of hidden sulfhydryls. Fluorescence changes were measured with a Perkin Elmer LS50-B fluorescence spectrophotometer (Newton, MA) using excitation and emission wavelengths of 380 and 470 nm, respectively.

2.2. Functional assays

2.2.1. Severing. The rate of severing by gelsolin and gelsolin truncates was measured using gelsolin's ability to displace TRITC-phalloidin from F-actin, as previously described [7]. F-actin was added to buffers containing 1 μM TRITC-phalloidin and the fluorescence increase upon phalloidin binding measured in the fluorescence spectrophotometer at excitation and emission wavelengths of 540 and 575 nm. The solutions were continuously stirred using the stirrer of the LS-50b on the low setting. Buffers differing in free Ca^{2+} were generated by addition of various concentrations of EGTA, pH 7.4 to F-buffer which contained 1 mM instead of 0.2 mM $CaCl_2$. Ca^{2+} concentrations were measured as previously described [7] using Mag Fura 5 (Molecular Probes, Eugene, OR). Stopped flow fluorescence measurements were conducted using a Hi-Tech (Salisbury, UK) SF-11 rapid kinetics accessory, generously lent by Paul Matsudaira (Whitehead Institute, Cambridge, MA). Severing rates were determined from the rate of fluorescence loss of TRITC-phalloidin bound F-actin upon addition of gelsolin or gelsolin truncates [7].

2.2.2. Monomer binding assay. Rates of actin monomer binding by gelsolin were determined from the increased emission of G-pyrene iodoacetemide coupled actin (G-PIA actin) upon interaction with gelsolin [22]. G-PIA actin was prepared by dilution of labeled PIA actin to 1 mg/ml followed by centrifugation at 150 kg for 45 min. Fluorescence changes of PIA-actin were measured at excitation and emission wavelengths of 365 and 386 nm respectively. 400 nM G-PIA was incubated in buffers differing in free calcium concentrations as described above and 200 nM gelsolin added with gentle vortexing. Fluorescence changes were followed for up to 2 h, and there was no change in PIA-actin fluorescence in the absence of gelsolin during this time. Reaction rates and kinetic constants were determined as described in Ditsch and Wegner [8].

2.2.3. Nucleation. The nucleation of filament assembly from G-actin by gelsolin was assayed using the fluorescence change of PIA labeled actin [23]. The supernatant was collected and the actin concentration determined using an extinction coefficient of 0.62 mg^{-1} cm^{-1} at 290 nM. G-PIA actin was rapidly mixed with gelsolin in 2 mM Tris, pH 7.4; 0.2 mM $CaCl_2$, 0.5 mM ATP and 0.2 mM DTT. The sample was incubated for 30–45 s and then salts were added to bring the buffer conditions to 150 mM KCl, 2 mM $MgCl_2$, 2 mM Tris, pH 7.4; 0.2 mM $CaCl_2$, 0.5 mM ATP and 0.2 mM DTT. The fluorescence change of the PIA actin upon assembly into filaments was monitored over time at excitation and emission wavelengths of 365 and 386 nm.

3. Results

The difference in disulfide bond structure between bacterially expressed human plasma gelsolin and native plasma gelsolin led me to examine the functional activities of these molecules. The disulfide bond in plasma gelsolin is in domain 2 [16], which contains elements necessary for severing and nucleation. The specific severing activity of native and recombinant plasma gelsolin was determined using the phalloidin displacement assay [7]. Native plasma gelsolin displaces two molecules of phalloidin from F-actin in the presence of Ca^{2+}, while the preparations of recombinant HPG tested displaced 1.7 (±0.5 S.D., $n=4$) mol of phalloidin per mol of rHPG. This result is not significantly different from previous measurements of native gelsolin activity [7].

3.1. Severing

Gelsolin requires Ca^{2+} to interact with F-actin, and previous work demonstrated that both the rate of severing and monomer binding is highly dependent on the free Ca^{2+} concentration [7,8]. We measured the rate and calculated the apparent second order forward rate constant k_{sev} of phalloidin displacement from F-actin by native, recombinant and refolded recombinant gelsolin at different free Ca^{2+} concentrations. It should be noted that the apparent rate constant k_{sev} is a sum of many separate processes [7]. As indicated in Fig. 1, recombinant gelsolin lacking the disulfide in domain 2 (r-HPG) consistently severed more slowly than native plasma gelsolin at the Ca^{2+} concentrations tested, while recombinant gelsolin oxidized to generate the disulfide bond in domain 2 (ox-r-HPG) severed at a rate identical to that of native plasma gelsolin (Fig. 1).

3.2. Monomer binding and filament nucleation

Gelsolin binds actin monomers, and the gelsolin-two actin monomer complex acts as a pointed end nucleus. Generation of nuclei by gelsolin is more rapid than the de novo nucleation of micromolar concentrations of actin monomers, so that addition of gelsolin in the presence of calcium to actin monomers strongly enhances the polymerization rate. The rate of monomer binding to recombinant human plasma gelsolins containing and lacking the disulfide bond was determined at various concentrations of free calcium as described in Section 2. Similar to the studies of severing illustrated in Fig. 1, r-HPG bound monomers several fold more slowly than ox-r-HPG at two different free calcium levels (Fig. 2). In agreement

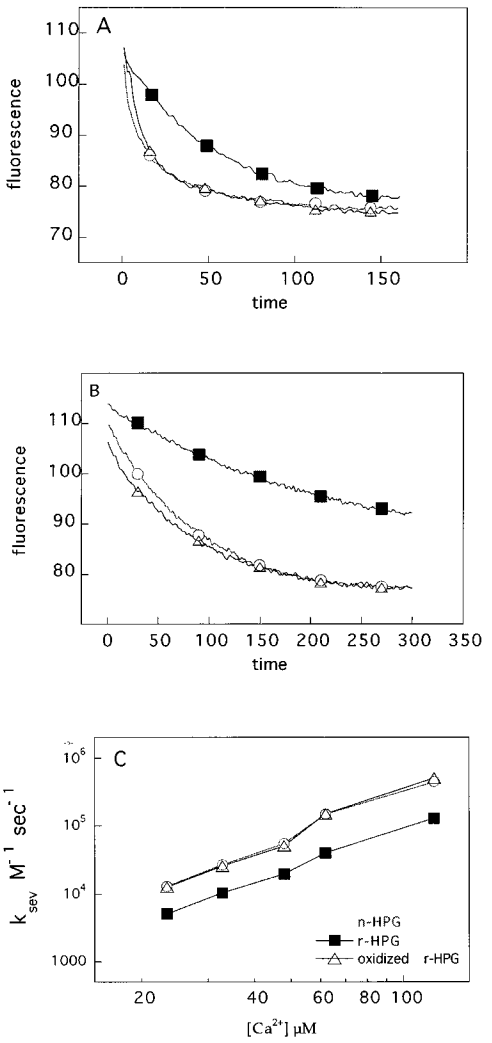


Fig. 1. Oxidized and recombinant gelsolins sever at different rates. Actin severing by native HPG (open circles), r-HPG (closed squares) and ox-r-HPG (open triangles), assayed by the displacement of TRITC-phalloidin, at 32 μM (A) and 64 μM (B) free Ca^{2+} is illustrated. The TRITC-phalloidin concentration was 500 nM; the actin and gelsolin concentrations were 400 nM and 200 nM respectively. C: Second order rate constant for severing by the various gelsolins at several free Ca^{2+} concentrations.

with the work of Ditsch and Wegner [8], who reported that two calcium binding sites with K_d of 25 and 200 μM regulated gelsolin actin-monomer interactions, we observed no significant increase in PIA actin fluorescence in the presence of either form of gelsolin at 14 μM free calcium (data not shown). In contrast, previous studies have demonstrated there was detectable severing activity at 14 μM free calcium [7,10,18].

These differences in rates of interaction with actin are also apparent in nucleation experiments. As indicated in Fig. 3, both gelsolins greatly enhanced the polymerization rate of actin monomers compared to actin alone. Yet, at the low fixed calcium concentrations analyzed (14–46 μM), the gelsolins diminished but did not completely remove the lag phase. Furthermore, there was a significant increase in the polymerization rate at 14 μM free calcium in the presence of gelsolin, conditions where monomer binding was not detected. Apparent nucleation at concentrations of free calcium where monomer binding was not observed was also reported by Ditsch and Wegner [8].

3.3. Severing by N-terminal fragments

The difference in rate of severing and monomer binding between ox-r-HPG and r-HPG at equivalent calcium concentrations suggests that the disulfide in domain 2 is involved in gelsolin's activation by calcium. However, there is no evidence to suggest that domain 2 of gelsolin binds Ca^{2+} directly. N-terminal truncates of gelsolin containing domains 1–3 sever actin filaments and bind actin monomers [4,24] rapidly in the complete absence of divalent cations. I assayed N-terminal truncates containing domains 1–3 of recombinant and plasma gelsolin to determine whether the Ca^{2+} insensitive rate of severing was altered by the presence or absence of the disulfide in domain 2. Fig. 4 illustrates some representative stopped flow measurements of the displacement of TRITC-phalloidin from F-actin in the presence of bacterially expressed gelsolin s1–3, and thermolysin generated N-terminal native gelsolin. Both bacterially expressed N-terminal gelsolin and N-terminal truncates of plasma gelsolin generated by thermolysin digestion displaced phalloidin with a k_{sev} of 1.7×10^6 M^{-1} s^{-1} (S.D. $=0.9\times10^6$, $n=12$). This rate of severing is very similar

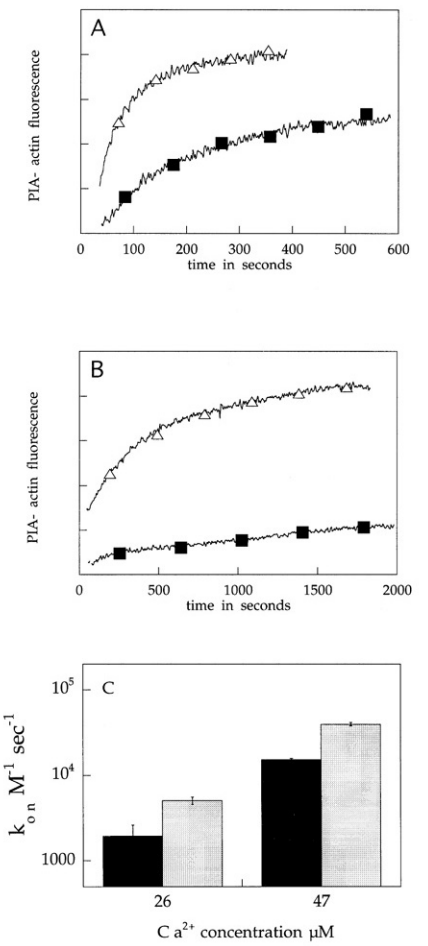
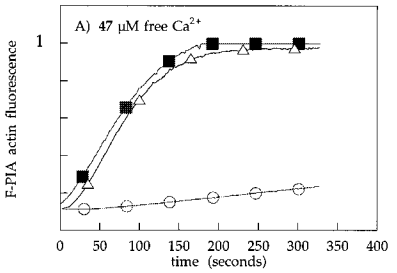


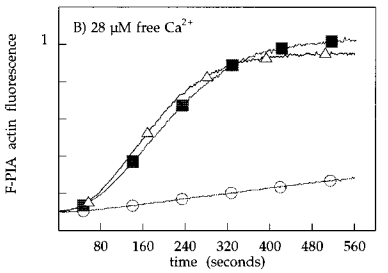
Fig. 2. Monomer binding by r- and ox-r-gelsolins. The fluorescence enhancement of 400 nM G-PIA actin upon addition of 200 nM oxidized (open triangles) or reduced (closed triangles) recombinant human plasma gelsolin is illustrated at 47 μM (A) and 26 μM (B) free calcium. C: Rate constants for the association reactions for the above reactions, solved as described in Section 2.

to the rate of severing by intact gelsolin derived either from plasma or recombinant sources in the presence of several millimolar Ca^{2+}, where k_{sev} is approximately 2.5×10^6 M^{-1} s^{-1}.

3.4. Disulfide analysis

Both recombinant s1–3 and thermolysin generated N-terminal gelsolin truncate sever at similar rates. However, these molecules maintain the differences in disulfide bond consistent with their source. Thermolysin generated N-terminal gelsolin and bacterially expressed gelsolin s1–3 have the same number of cysteines (4) and approximately the same sequence, varying by only a few amino acids at their C-terminus. DACM reacts equivalently with both thermolysin generated human plasma gelsolin N-terminal truncate and with bacterially expressed gelsolin domains 1–3 (Fig. 5). Addition of SDS to N-terminal gelsolin in the presence of DACM leads to a doubling of the fluorescence intensity observed in the absence of SDS, and suggests that there are equivalent numbers of exposed and buried sulfhydryls available for labeling in the thermolysin generated gelsolin N-terminal fragment. Recombinant gelsolin domains 1–3, in contrast, showed a 3-fold increase in labeling upon exposure to SDS, consistent with one exposed and three hidden sulfhydryls. The lack of disulfide bond in s1–3 is consistent with its expression in the cytoplasmic compartment of bacteria, and also suggests that other bacterially expressed gelsolin molecules containing domain 2 may be missing a disulfide bond.





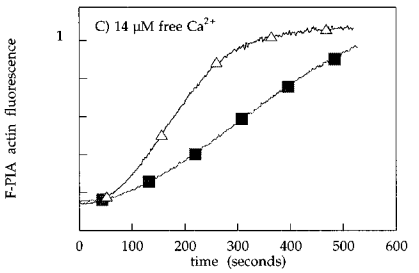


Fig. 3. Nucleation of actin polymerization by ox-r- and r-gelsolins. 1.6 μM G-PIA actin was induced to polymerize by the addition of salts, as described in Section 2, approximately 50 s after incubation in the absence (open circles) or presence of 20 nM ox-r- (open triangles) or r- (closed squares) recombinant human plasma gelsolin. Actin polymerizing in the absence of gelsolin is indicated by open circles. In A, the free calcium level was 47 μM, in B, 28 μM and in C, 14 μM.

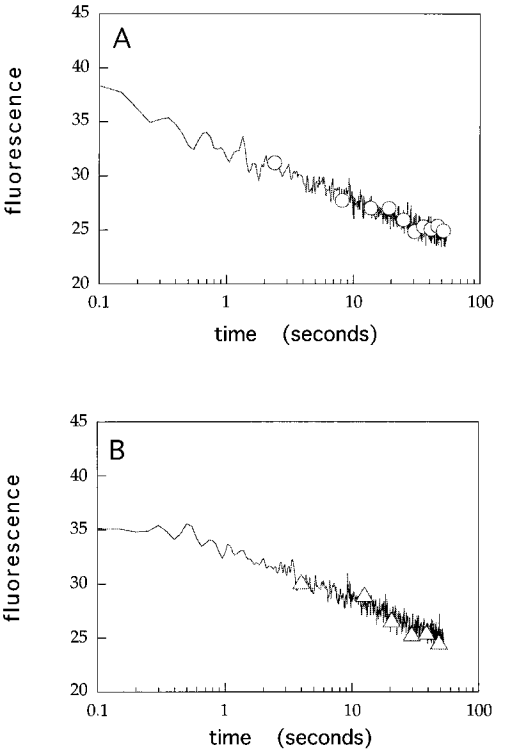


Fig. 4. N-terminal truncates of native and recombinant gelsolin sever at equivalent rates. The displacement of TRITC-phalloidin from F-actin by recombinant gelsolin s1–3 is illustrated in A. 200 nM gelsolin s1–3 was mixed with 400 nM TRITC-phalloidin bound F-actin and the loss of fluorescence measured. B: Similar experiment using 200 nM thermolysin generated N-terminal native gelsolin.

4. Discussion

Gelsolin is unusual among actin binding proteins in that it functions in two very different environments [1]. Plasma gelsolin, part of the actin scavenger system of blood, functions in a Ca^{2+} rich, oxidizing environment. Cytoplasmic gelsolin, in contrast, functions in the Ca^{2+} poor, reducing environment of the cytoplasm. Consistent with the redox state of their environment, plasma and cytoplasmic gelsolin have been demonstrated to differ by the presence of a disulfide bond between cysteines 215 and 228 of plasma gelsolin [16]. These two forms, which differ in sequence only by a 21 amino acid extension at the N-terminus of plasma gelsolin, are synthesized through differential exon usage from the same gene [2]. The results presented here suggest that the presence or absence of the disulfide bond between the two cysteines within domain 2 of gelsolin alters its sensitivity to calcium. The reduced rate of severing observed in gelsolin lacking the disulfide is lost at saturating calcium concentrations (data not shown), similar to the lack of differences observed in the severing rates (Fig. 4), and monomer binding rates [24] of calcium insensitive N-terminal truncates. These results suggest that the disulfide

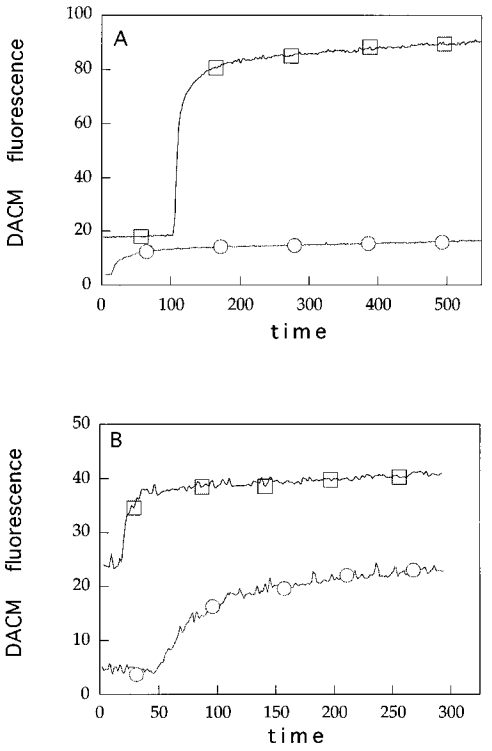


Fig. 5. DACM labeling of sulfhydryls in native and recombinant N-terminal truncates of gelsolin. The change in fluorescence of DACM upon reaction with gelsolin's free and hidden sulfhydryls was measured as described in Section 2. A: Fluorescence change upon addition of 120 nM recombinant gelsolin s1–3 to 1.2 μM DACM. The trace with open circles illustrates the fluorescence change upon addition of protein. The open square trace is a continuation of the same sample with the addition of SDS to 0.1% at 120 s. B: Fluorescence change upon addition of 120 nM thermolysin generated N-terminal of native gelsolin to 1.2 μM DACM. As in A, the open circles indicates the fluorescence change upon addition of protein, with the open squares showing the fluorescence change after addition of 0.1% SDS.

bond in domain 2 of plasma gelsolin serves to facilitate communication between Ca^{2+} binding sites in the C-terminus and active sites in the N-terminus.

4.1. Physiologic consequences of disulfide formation

In vitro, gelsolin has been shown to be activated by elevated calcium and inhibited by phosphoinositides [1]. Intracellularly, gelsolin has been shown to associate and subsequently dissociate from actin after stimulation of platelets [25] with binding and unbinding following changes in intracellular calcium and phosphoinositides. Yet in vitro studies of gelsolin activation have demonstrated that gelsolin requires tens to hundreds of micromolar Ca^{2+} to sever actin filaments or bind actin monomers rapidly [7,8]. The speculation on initiation of this work was that the lack of disulfide bond in cytoplasmic gelsolin would lower its requirement for calcium. In contrast to these expectations, the lack of the disulfide bond induces a conformation where the severing rate is several fold slower than that of oxidized gelsolin. This suggests that factors other than the oxidation state of gelsolin are important in its activation at low calcium concentrations. These results do lead to the speculation that the regulation of cytoplasmic gelsolin could be modulated both by free Ca^{2+} levels and by the redox potential of the cytoplasm.

4.2. Localization of Ca^{2+} binding sites on gelsolin

Proteolytic digestion of native plasma gelsolins, as well as expression of truncated recombinant gelsolin cDNAs, demonstrates that important Ca^{2+} regulatory sites are located in the C-terminal half of the molecule [5,11]. Calcium binding studies of gelsolin truncates containing domains 4, 5, 4–5 and 5–6 indicate that there are at least two Ca^{2+} binding sites within the C-terminal half of gelsolin [11]. To date, no evidence exists for the binding of Ca^{2+} to the N-terminal domains of gelsolin in the absence of other proteins. There is, however, strong evidence for actin stimulated binding of calcium to domain 1 of gelsolin. This calcium is tightly bound in the gelsolin-actin complex with binding of actin to domain 1 of gelsolin activating a cryptic binding site on gelsolin [12]. The importance of the actin stimulated calcium binding site in the functional activity of full length gelsolin is unclear, as gelsolin truncates containing domains 1–2 or 1–3 of gelsolin sever rapidly in the absence of calcium [3,4]. However, the minimal severing unit of gelsolin containing amino acids 1–160, which contains domain 1 and only the first 11 amino acids of domain 2 (and lacks the cysteines required to form the disulfide bond), requires Ca^{2+} in order to sever [5].

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