The Effects of Severely Decreased Hydrophobicity in a Subdomain 3/4 Loop on the Dynamics and Stability of Yeast G-actin*

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Actin is a flexible molecule with its four subdomains interconnected through its bound nucleotide and metal ion (1). Allosteric interactions have been documented in the small domain between the bound nucleotide, the tightly bound metal ion, the DNase I loop (subdomain 2), and the C terminus (2–5). The subdomain coupling in the actin monomer suggests that a local structural perturbation could be transmitted to a distal part of the molecule through its bound ligand, resulting in an alteration of actin function. Normal mode analysis of the structure of G-actin† also suggests that theoretically the four subdomains of actin can move independently or collectively (6). This dynamic feature of actin structure would be important not only for actin filament dynamics (7) but also for the transformation of the G-actin monomer to the F-actin monomer during actin polymerization.

Monomer activation by added salts is the first step in actin polymerization in vitro (8). This process may involve the binding of metal ions at medium and low affinity sites of actin, presumably changing the conformation of G-actin to one that is similar to the monomer as it exists in the actin helix. Such a change would allow monomers to form nuclei or add to the ends of actin filaments more easily. The first evidence for such a conformational change was the observation that the binding of Mg$^{2+}$ to the high affinity site caused a slow fluorescence change in IAEDANS-labeled actin (9). Furthermore, studies using limited proteolysis (2) or a fluorescent probe attached to Gln41 (5) demonstrated that subdomain 2 of G-actin adopted an “F-monomer-like” structure following the substitution of Mg$^{2+}$ for the tightly bound Ca$^{2+}$.

The results described above are consistent with the difference between the atomic structure of G-actin and the conformation of the F-actin monomer in an atomic model of F-actin proposed by Holmes and co-workers (10). In this model, subdomain 2, especially the DNase I loop in F-actin, folds inward in comparison with G-actin. The central cleft of the F-actin monomer also narrows. These structural differences may lead to the decreased protease susceptibility of Mg-G-actin in comparison with that of Ca-G-actin (2).

Another major difference between the atomic structure of G-actin and the Holmes atomic model of the F-actin monomer is the conformation of the hydrophobic loop between subdomains 3 and 4. In rabbit skeletal muscle G-actin, this loop (262–274) resides against the main body of subdomain 4 with the hydrophobic residues Phe$^{266}$, Ile$^{267}$, and Met$^{269}$ near residue Tyr$^{186}$ (1). In the F-actin atomic model (10), this loop is predicted to undergo a conformational change during polymerization to form a hydrophobic plug (Phe$^{266}$–Ile$^{267}$–Gly$^{268}$–Met$^{269}$) that extends perpendicular to the filament axis and inserts into a hydrophobic pocket formed by two neighboring monomers in the opposing strand (Fig. 1, A and B). The hydrophobic plug-pocket interaction thus would become a major cross-strand contact and result in filament stabilization (11). If this hypothesized plug-pocket interaction is generally correct, the detachment of the loop from the main body of actin should be facilitated when actin monomers are activated for polymerization.

If allosteric effects play a role in actin monomer activation during polymerization, the detachment of the loop from the surface of actin might result in conformational changes in other parts of the molecule, resulting in a monomer which is more polymerization competent. Alternatively, Schutt et al. (12) argue that the hydrophobic loop (262–274) is held tightly against the main body of the molecule in the profilin–β-actin ribbon structure and is unlikely to undergo a conformational change like that proposed by Holmes since such a change would result in denaturation of the protein.

Our results with mutations in the hydrophobic plug in yeast actin, which show that the hydrophobicity of the plug is important for filament formation and stabilization, are generally consistent with the proposed hydrophobic plug-pocket cross-strand interaction (13, 14). The presumed conformational change in the monomer that accompanies movement of the loop, however, another aspect of the hypothesis, has received little attention. In this work, we took advantage of a mutant

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‡ The abbreviations used are: G- and F-actin, globular and filamentous actin; IAEDANS, N-iodoacetyl-N'-[(2-sulfopropyl)ethylidylenediamine; WT, wild-type; ε-ATP, 1,5-ethenoadenosine-5'-triphosphate; $T_m$, apparent melting temperature.
yeast actin containing a plug with markedly decreased hydrophobicity to study the effect of the conformation of the loop on other parts of the actin molecule and the structural coupling between the large and small domains of actin.

In yeast actin, the hydrophobic plug is comprised of Val266–Leu267–Gly268–Leu269. For studying the postulated hydrophobic plug-pocket interaction in actin filament stability, we previously made a yeast actin double mutant V266G/L267G (GG) in which the hydrophobic side chains of the first two residues of the plug were eliminated (14). We have shown that the GG mutation did not grossly change the conformation of actin but prevented its polymerization unless near stoichiometric amounts of WT actin or actin filament stabilizing agents such as beryllium fluoride and phalloidin were present. However, it is not clear what effect the GG mutation would have on the conformation of G-actin. The elimination of most of the hydrophobicity of the plug should make the loop more easily detachable from the main body of actin, because the decreased hydrophobicity of the plug and the increased flexibility of the Gly residues would result in a lower energy barrier for making the hydrophobic plug totally accessible to solvent. The “parked” position of the loop in G-actin appears to restrict the movement of subdomains 3 and 4 relative to one another, and the adenine ring of the bound nucleotide is located near this domain interface. A structural perturbation of this loop might alter the conformation of the central cleft and/or other structural elements in G-actin through the bound adenine nucleotide. There are allosteric interactions between the large and the small domains of G-actin. Alternatively, the perturbation caused by the GG mutation might destabilize the actin molecule and result in a local or more extensive denaturation of G-actin thereby preventing polymerization (12). Here, we report the results of experiments designed to differentiate between these alternatives.

**MATERIALS AND METHODS**

\[^{32}P\]ATP (3000 Ci/mmol) was purchased from the Amersham Corp. Phalloidin, 1-N6-ethenoadenosine 5'-triphosphate (e-ATP), \(\alpha\)-chymotrypsin, trypsin, subtilisin, ATPase (w/luciferase) assay mix, and the ATP disodium salt were purchased from Sigma. The bicinchoninic acid assay reagent was purchased from Pierce. AG 1-X8 anion exchange resin was purchased from Bio-Rad.

**Purification of Yeast Wild-type and GG Actin**—Purification of actins was done as described previously using a DNase I affinity chromatography-based protocol (14). Ca-G-actin was stored at 4°C in Ca-G-buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl\(_2\), 0.2 mM ATP, and 0.1 mM dithiothreitol). Mg-G-actin was obtained by converting actin from the Ca-G form into the Mg-G form as described by Strzelecka-Golaszewska et al. (2). The buffer conditions for Mg-actin are 10 mM Tris-HCl, pH 7.5, 0.1 mM MgCl\(_2\), 0.05 mM CaCl\(_2\), 0.2 mM EGTA, 0.2 mM ATP, 0.1 mM dithiothreitol.

**Limited Proteolysis of Actin**—F-actin was prepared by adding 2 mM MgCl\(_2\) and 50 mM KCl to 5 \(\mu\)M G-actin in the presence of equimolar phalloidin at room temperature for 2 h. The digestion experiments were carried out according to Strzelecka-Golaszewska et al. (2). G-actin or F-actin (5 \(\mu\)M) was incubated with trypsin (5 \(\mu\)g/ml), subtilisin (1 \(\mu\)g/ml), or \(\alpha\)-chymotrypsin (22.5 \(\mu\)g/ml) at 37°C for the desired time. The digestion with trypsin was stopped with 10 mM trypsin inhibitor, and digestion with the other two proteases was stopped with 1 mM protease.
inhibitor phenylmethylsulfonyl fluoride. The samples were then separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and visualized by staining with Coomassie Blue.

ATP Exchange Rate Assay—Nucleotide exchange experiments were done as described by Cresbie et al. (4). G-actin was pre-equilibrated with α-ATP and the excess nucleotide removed with a PenEFsky column (15). This G-actin (3 μM) was mixed with 0.5 mM ATP, and the decay of fluorescence was monitored in a SLM4800 fluorescence spectrometer with the excitation and emission wavelengths set at 340 and 410 nm, respectively. Nucleotide exchanges were derived by fitting the data to a single exponential expression using a nonlinear least squares program (16).

Intrinsic ATPase Activity Assay—The intrinsic ATPase activity of G-actin was measured as described by Chen et al. (13). Ca- or Mg-G-actin, pre-equilibrated with γ-[32P]ATP at 4 °C, was raised to the desired temperature, and aliquots of the reaction solution were taken at 20-min intervals. The amount of inorganic phosphate released was determined by the procedure of Spudich (17).

Circular Dichroism and Intrinsic Trypsin Fluorescence—The melting temperatures of G-actin were determined by CD according to Chen et al. (18). The ellipticity of a G-actin solution (1.4 μM or 0.7 μM) was monitored at 222 nm using an AVIV 62 DS instrument. Data were fitted to a two-state model with a single transition between the native and denatured form of the protein (19, 20) using a nonlinear least squares program (16). Since the denaturation of actin is irreversible, only a pseudo-melting temperature was derived.

The secondary structures of 6 μM WT or GG actin solutions were monitored by far UV CD spectroscopy between 200 and 260 nm. The buffer conditions were the same as that of G-buffer except that the concentration of Tris-HCl was 2 mM instead of 10 mM. Intrinsic trypsin fluorospectra of WT or GG actin were recorded with the excitation and emission wavelengths set at 290 and 335 nm, respectively.

ATP Binding Affinity Assay—The apparent association constants for the G-actin-ATP complex were determined according to Chen et al. (18) using a procedure based on the determination of bound and free nucleotide with firefly luciferase.

RESULTS

The GG Mutation Changes the Conformation of Subdomain 2 of Actin—Subdomain 2 (DNase I loop) of G-actin is a rather mobile structure, and the conformation of subdomain 2 and its structural transitions are important for actin function (7). Conformational changes in subdomain 2 can be probed by limited proteolysis with trypsin, subtilisin, and α-chymotrypsin (21, 22). Trypsin cleaves subdomain 2 of Ca-G-actin at Arg62–Gly63 and Lys64–Tyr65, yielding 33-kDa C-terminal fragments. Subtilisin cleaves subdomain 2 of Ca-G-actin at Met44–Gly45 and Leu67–Lys68, producing 35- and 33-kDa fragments. These muscle actin digestion sites are conserved in yeast actin, and the protease susceptibility of subdomain 2 in GG actin is similar to that of rabbit skeletal muscle actin (18).

To determine the effect of the GG mutation on the conformation of subdomain 2 of G-actin, we carried out limited proteolysis of GG and WT actins. For both WT actin and GG actin, Mg-G-actin is less susceptible than Ca-G-actin, and F-actin is less susceptible than G-actin to protease digestion (Fig. 2, A and B), consistent with previous results (18). However, the susceptibility of GG actin is different from that of WT actin. Although controlled digestion of G-WT and G-GG actin by trypsin, subtilisin, and α-chymotrypsin yielded the same digestion pattern, the proteolytic fragments of Ca2+– or Mg2+–GG actins were always less abundant than those of its WT counterpart. These results indicate that the digestion sites of these proteases on subdomain 2 of GG actin are more protected than those of WT actin. The conformational change associated with Ca2+-GG actin mimics that caused by substituting the tightly bound Ca2+ of WT actin with Mg2+, which protects subdomain 2 of Mg-actin from digestion. The results suggest that the GG mutation may induce G-actin to change its conformation to a more F-monomer like structure.

GG actin cannot polymerize by itself in vitro but can polymerize in the presence of filament stabilizing agents (14). To evaluate how the GG mutation would affect the topology of F-actin, we tested the protease susceptibility of phallodin-stabilized actin filaments. Fig. 2, A and B, shows that subdomain 2 of phallodin-stabilized F-GG actin appeared to be more susceptible to protease digestion by subtilisin and α-chymotrypsin but not trypsin. These results suggest that the GG mutation alters the conformation of subdomain 2 in phallodin-stabilized actin filaments by allowing subdomain 2 to be more accessible to protease digestion.

ATP Exchange Rate of GG Actin—The decreased protease susceptibility of subdomain 2 in GG versus WT G-actin results from a conformational change due to intramolecular communication between the mutated hydrophobic plug and subdomain 2 via the nucleotide bridge. This proposition suggests that the conformation of the central cleft may be altered, too. Because the protease digestion sites are close to the top of the central cleft, the central cleft may be tighter in GG actin than in WT actin. We thus examined the ATP exchange rates of GG and WT actins. The α-ATP exchange rates for both Ca2+ and Mg2+–GG actins are greatly reduced compared with those of WT actins (Fig. 3A). The observed first-order off-rate of α-ATP for Ca2+-GG actin (1.4 ± 0.5 × 10−2 s−1) is between 2 to 3 times slower than that of Ca2+-WT actin (3.8 ± 0.6 × 10−2 s−1). The nucleotide exchange rate of muscle G-actin is affected by the tightly bound divalent cation with Mg-actin, having a higher nucleotide dissociation rate than Ca-actin (23). Here, the first-order off-rates of α-ATP of yeast Mg-actin are 3 times faster than those of Ca-actin for both GG and WT actin, and the exchange rate for Mg-2G (4.1 ±
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Equilibrium binding constants ($\times 10^7$ M$^{-1}$) of ATP to WT and GG actins

| Temperature | Ca$^{2+}$-WT | Ca$^{2+}$-GG | Mg$^{2+}$-WT | Mg$^{2+}$-GG |
|-------------|-------------|-------------|-------------|-------------|
| $^\circ$C   | $\times 10^7$ M$^{-1}$ | $\times 10^7$ s$^{-1}$ | $\times 10^7$ M$^{-1}$ | $\times 10^7$ s$^{-1}$ |
| 0           | 1.8 $\pm$ 0.4 | 1.2 $\pm$ 0.3 | 2.3 $\pm$ 0.7 | 1.3 $\pm$ 0.4 |
| 23          | 2.5 $\pm$ 0.6 | 1.6 $\pm$ 0.5 | ND$^a$ | ND$^a$ |

$^a$ ND, not determined.

with the Holmes F-actin model, which predicts that there is no significant change in the nucleotide and Ca$^{2+}$ binding site between the G-actin and F-actin monomers (10).

The similar ATP binding affinity and the 3-fold slower ATP exchange rate of GG actin suggest that the nucleotide association rate for GG actin is also slower than that for WT actin. These results are consistent with a conformational change in the central cleft of GG actin as proposed earlier. Furthermore, the similar nucleotide binding affinities for GG actin and WT actin indicate that the polymerization defect of GG actin and the conformational changes caused by the GG mutation do not result from a decreased affinity of GG actin for its tightly bound ligands or as a result of a denaturation of the lower region of the interdomain cleft.

Intrinsic ATPase Activity of GG Actin—The conformational change of the central cleft of actin caused by the GG mutation may affect the ability of G-actin to hydrolyze its bound ATP. Exchange of magnesium for calcium in the central cleft of G-actin apparently causes a conformational change in the cleft that accelerates actin’s intrinsic ATPase activity (25). The ATPase activities of Ca-WT (0.5 mmol·mol$^{-1}$·min$^{-1}$) and that of Ca-GG actin (0.4 mmol·mol$^{-1}$·min$^{-1}$) are very low and almost the same (Fig. 4A). However, Mg-G-WT (5.0 mmol·mol$^{-1}$·min$^{-1}$) actin hydrolyzes ATP about 10 times faster than Mg-G-GG actin (0.5 mmol·mol$^{-1}$·min$^{-1}$) at 17°C (Fig. 4B). Because the apparent melting temperature of monomeric Mg-GG actin is 33°C (next section), we measured the intrinsic ATPase activity of Mg-actin at room temperature (data not shown) and 17°C. All show similar differences in the rate of phosphate hydrolysis between WT and GG actins, indicating that the low ATPase activity of GG-actin is not due to denaturation of Mg-GG actin. The lower ATPase activity of GG-actin seems inconsistent with the proposal that this mutation confers an F-actin-like state on the protein. However, as discussed more fully later, the ATPase activity of the monomer may depend on its ability to form transient oligomers, which the mutation may inhibit.

Thermal Stability of GG Actin—Although the GG mutation causes small conformational changes in actin’s subdomain 2 and central cleft, structural coupling may not be limited to these parts of the actin molecule. We therefore examined the thermal stability of GG actin by circular dichroism, although the thermal unfolding of G-actin is irreversible. The molar ellipticity at 222 nm, which reflects the content of a-helical structure in G-actin, was monitored as a function of temperature. The mid-point of the thermal transition curve is defined as the apparent melting temperature for the protein ($T_m$). The GG mutation produces a thermally labile actin molecule (Fig. 5), lowering the melting temperature for Ca-WT actin from 57°C to 45°C. Even more strikingly, the GG mutation lowered the melting temperature of Mg-GG actin to 33°C compared with 51°C for Mg-WT actin. Thus, elimination of a major portion of the hydrophobicity of the hydrophobic plug causes thermal instability for the actin molecule. The effect is in the same direction caused by substitution of the tightly bound Ca$^{2+}$ with Mg$^{2+}$ but is much larger. Interestingly, the apparent melting temperature for Mg-GG actin is only 33°C, while GG cells still survive at 37°C (14), although the growth of GG cells is more compromised than that of WT cells at this temperature.
Effect of the GG Mutation on the Secondary Structure of Actin—GG-actin’s inability to polymerize by itself and its low melting temperature suggest that the GG mutation may partially denature the actin even at ambient or lower temperatures. We thus compared the secondary structures of WT and GG actin at 23 and 4°C by measuring their far UV CD spectra.

There is a subtle difference between the CD spectra of Ca\(^{2+}\)-WT and Ca\(^{2+}\)-GG actin, but the CD spectra of Mg\(^{2+}\)-WT and GG actin are identical (Fig. 6). The similar shapes of the spectra suggest that there is no significant change in the secondary structure of the mutant GG-actin. We also measured the intrinsic tryptophan fluorescence of Mg\(^{2+}\)-WT (not shown) and Mg\(^{2+}\)-GG actin. The emission spectra (Fig. 7) of WT and GG actin are again identical, and the emission maxima of WT and GG actin are the same at 325 nm whether at 23°C or 4°C. Thus, the GG mutation did not alter the tertiary structure around the G-actin tryptophan residues. Our results suggest that this mutation does not significantly disorder the local structure of actin, consistent with the similar ATP binding affinities of WT and GG G-actin.

**DISCUSSION**

Interactions between structural elements in an actin monomer may play a role in generating tension when the thin filament interacts with the thick filament during muscle contraction (7). Such interactions may be equally important in the conversion of G-actin to the F-monomer form prior to its incorporation in the growing filament. To understand these functional relationships, it is necessary to define these interactions within the actin molecule.

Our studies indicate that the hydrophobic loop between subdomain 3 and 4 may influence the conformation of subdomain 2 of GG actin via the nucleotide bridge. The elimination of the
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FIG. 7. Intrinsic fluorescence spectra of Ca\(^{2+}\)-WT and GG actin at 23 °C (A) or 4 °C (B). The excitation and emission wavelength is 290 and 335 nm, respectively. The emission maxima are around 325 nm for either WT or GG actin. ○, WT actin; □, GG actin.

hydrophobicity of the hydrophobic plug caused a closure of the central cleft, a rotation of subdomain 2 that protected the digestion sites of trypsin, subtilisin, α-chymotrypsin, and slowed the ATP exchange rate. The mutation also produced a more labile actin molecule as shown by its lower thermostability, although the mutation seems to affect the secondary structure of the actin molecule minimally at ambient or lower temperatures. These results demonstrate for the first time that there is structural coupling between the large domain, the nucleotide-binding cleft, and the small domain of actin.

The results are particularly interesting because they concern the hypothetical role of the hydrophobic plug in actin filament formation. The Holmes F-actin model (10, 11) proposes that the hydrophobic loop swings out from a parked conformation on subdomain 4 to form a major contact between two helical strands. If elements of the model are correct, the conformational change would be promoted by monomer activation through the binding of Mg\(^{2+}\) at the high affinity site and/or the binding of divergent and monovalent cations at the medium affinity sites.

Divalent-cation-dependent conformational changes have been implicated as the structural basis for the different properties of Mg-actin compared with Ca-actin, such as the faster nucleation rate of Mg-actin. In terms of the Holmes model, the hydrophobic loop may detach from the main body of actin more easily in Mg-actin than in Ca-actin. Molecular dynamics simulations (26) suggest that there are two populations of Mg-actin, one like the Ca-G-actin crystal structure (1) and another like the conformation of the F-monomer with the hydrophobic loop extending into solvent. Their work suggests that structural coupling may exist between the hydrophobic loop and the central cleft. Elimination of most of the hydrophobicity of the hydrophobic plug might promote detachment of the loop from the surface of subdomain 4. Such a conformational change would spread its effects to other parts of the molecule and produce a Ca-GG actin with a more “Mg-WT actin-like” or “F-monomer-like” conformation.

The change in protease susceptibility of subdomain 2 caused by the GG mutation resembles the change caused by substitution of Mg\(^{2+}\) for tightly bound Ca\(^{2+}\) in WT actin. This change may result from the “inward” folding of subdomain 2 (2), which has been proposed in the refined F-actin model (10). This movement may bring the tops of the central cleft closer to each other, which in turn might impose steric hindrance on the exchange of bound nucleotide with free nucleotides in the medium. As shown earlier, the nucleotide exchange rates of Ca- or Mg-WT actin are in fact three times faster than those of the corresponding GG actin.

While the GG mutation led to a less protease-susceptible G-actin conformation, it actually increased the protease susceptibility of phallolidin-stabilized F-actin. The DNase I loop (38–52) may form part of the hydrophobic pocket needed for cross-strand stabilization in the F-actin model (11). A decreased hydrophobic interaction between plug and pocket due to the GG mutation may allow the DNase I loop to swing away from the filament axis more easily in the phallolidin-stabilized F-actin helix, as suggested by its greater susceptibility to digestion by subtilisin and α-chymotrypsin, whose digestion sites are mainly in the DNase I loop. Thus, the conformational changes of subdomain 2 caused by the GG mutation in G-actin and F-actin, when compared with WT actin, are consistent with the predictions of Holmes’ F-actin model.

The low ATPase activity of GG actin seems to contradict the prediction that GG-actin assumes a more Mg-actin or F-monomer-like conformation. The ATPase activity that occurs following actin polymerization is much higher than that of the intrinsic ATPase of the monomer, presumably because the F-monomer conformation stabilized by the filament greatly facilitates the hydrolysis reaction. Mg-actin assumes a F-monomer-like conformation so that it has higher ATPase activity than Ca-actin. However, there are oligomers in Mg-G-actin solutions even at subcritical concentrations, although no filaments are detectable (27, 28). Thus, the higher ATPase activity of Mg-G-actin may result from transient oligomer formation from Mg-G-actin rather than from a more active monomer. Although monomeric GG actin may assume a Mg-G-actin like conformation, it may not be able to form oligomers, which are stable enough to catalyze its ATPase activity, because of its weakened cross-strand interaction. The intrinsic ATPase activity of GG actin would thus remain very low even when bound with Mg\(^{2+}\).

The presumed F-monomer-like conformation of GG actin is also consistent with a study (29) that showed that disulfide bridge formation between Cys\(^{374}\) and Cys\(^{10}\) produced an unpolymerizable actin with an F-monomer-like conformation. This actin has a slower ATP exchange rate and a lower intrinsic ATPase activity similar to that shown here for GG actin.

The GG mutation lowers the melting temperature of G-actin the way the substitution of tightly bound Ca\(^{2+}\) with Mg\(^{2+}\) does, but to a much greater degree. The drastic decrease of the apparent melting temperatures of Ca- and Mg-GG actin compared with those of WT actin suggests that the parked conformation of the hydrophobic loop on the surface of subdomain 4 greatly stabilizes the structure of G-actin possibly by immobilizing subdomain 4 and the subdomain 3/4 interface. The loop is at the intersection of subdomains 3 and 4 near the residues.
that constitute the binding site for the adenine ring of the nucleotide. The detachment of the loop may loosen the structure of G-actin by weakening the nucleotide bridge and also allowing the top of subdomain 4 to move closer to that of subdomain 2. Such a change may make it easier for G-actin to transform into a F-monomer conformation. These results suggest that the loop containing the hydrophobic plug may be involved not only in filament stabilization but also in conformational changes in the actin monomer through the nucleotide bridge.

The actin-bound nucleotide joins the two domains of the protein and, therefore, confers structural stability on the monomer. Previous work from our laboratory (18) found that an ATP phosphate binding loop (residues 13–15) is important for actin's function and stability. The S14A mutation in this loop not only resulted in a decreased affinity of the protein for ATP, but also greatly destabilized the actin monomer, lowering its \( T_m \) in the Mg\(^{2+}\)-form from 52 to 40 °C. These results coupled with reexamination of the G-actin atomic structure led to the suggestion that the hydroxyl group of Ser14 not only interacts with the \( \gamma \)-phosphate of the nucleotide but also may form a contact with the amide nitrogen of Gly\(^{74}\) in a length of polypeptide leading to the top of subdomain 2. The interaction involving residue Ser\(^{14}\) may thus play a role in stabilization of the actin monomer. Thus, actin-bound ATP may not only stabilize the actin monomer but also may mediate the allosteric interactions between different structural elements necessary for polymerization, especially between actin's large and small domains.

The effect of the GG mutation on the overall stability of G-actin is quite specific. Except for the L267D mutation (13), other mutations made in this plug did not affect actin's melting temperatures (data not shown). Actin mutations V266F, V266G, L267G, L269K, and L269D, which caused no appreciable defects in actin polymerization (14), did not lower the melting temperatures of the proteins. The V266D mutation which caused a somewhat slower rate of actin polymerization and a slightly lower critical concentration at 4 °C in comparison with wild-type actin did not affect its melting temperature either. However, the L267D mutation which produced a marked cold-sensitive polymerization defect in vitro also caused a 15 °C decrease in its melting temperature. The progressive effects of the mutations on the stability of G-actin are consistent with the predictions of Holmes' model and also suggest that the hydrophobicity of the plug is important not only for filament stabilization but also for monomer stability.

The lowered thermostability of GG actin is also consistent with Schutt's argument concerning the role of the hydrophobic loop between subdomain 3 and 4. However, the far UV CD results and intrinsic tryptophan fluorescence spectra indicate at most a minimal local structural perturbation is caused by the GG mutation at 23 or 4 °C. The results also suggest that at these temperatures, detachment of the subdomain 3/4 loop from the main body does not affect monomer integrity, more in agreement with Holmes' model. The observation further supports our previous suggestion that the severe cold-sensitive polymerization defect of GG actin is not due to monomer denaturation but rather due to decreased monomer- monomer contact in the filament (14), since the presence of GG actin can stimulate the polymerization rate of WT actin even though GG actin cannot incorporate into actin filaments at 4 °C.

Yeast cells carrying only GG-actin survive at 37 °C despite the fact that Mg-GG actin denatures at 33 °C and cannot form filaments at lower temperatures unless stabilized by reagents such as beryllium fluoride or phalloidin (14). Karpova et al. (30) demonstrated that there is only a small pool of unpolymerized actin in yeast and the rest exists in a form stainable with fluorescent phalloidin. These results suggest that, in GG cells, actin-binding proteins promote the polymerization of the mutant actin, thereby stabilizing it sufficiently against denaturation so that it is compatible with yeast viability.

Thus, based on our studies, the subdomain 3/4 loop containing the hydrophobic plug at its tip may actually serve two important additional roles besides stabilizing the filament via the proposed plug-pocket interaction. First, in the parked position, the loop imposes rigidity on the monomer, which helps stabilize it against denaturation. Second, detachment of the loop away from the parked position would lead to a conformational change via the nucleotide bridge allowing the monomer to form the intersubunit contacts needed for its insertion into the growing filament. The stability lost by this detachment would then be more than compensated for by the locking of the monomer into the filament.

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