Yeast Actin with a Mutation in the "Hydrophobic Plug" between Subdomains 3 and 4 (L266D) Displays a Cold-sensitive Polymerization Defect

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Abstract. Holmes et al. (Holmes, K. C., D. Popp, W. Gebhard, and W. Kabsch. 1990. Nature [Lond.] 347:44-49) hypothesized that between subdomains 3 and 4 of actin is a loop of 10 amino acids including a four residue hydrophobic plug that inserts into a hydrophobic pocket formed by two adjacent monomers on the opposing strand thereby stabilizing the F-actin helix. To test this hypothesis we created a mutant yeast actin (L266D) by substituting Asp for Leu266 in the plug to disrupt this postulated hydrophobic interaction. Haploid cells expressing only this mutant actin were viable with no obvious altered phenotype at temperatures above 20°C but were moderately cold-sensitive for growth compared with wild-type cells. The critical concentration for polymerization increased 10-fold at 4°C compared with wild-type actin. The length of the nucleation phase of polymerization increased as the temperature decreased. At 4°C nucleation was barely detectable. Addition of phalloidin-stabilized F-actin nuclei and phalloidin restored L266D actin's ability to polymerize at 4°C. This mutation also affects the overall rate of elongation during polymerization. Small effects of the mutation were observed on the exchange rate of ATP from G-actin, the G-actin intrinsic ATPase activity, and the activation of myosin S1 ATPase activity. Circular dichroism measurements showed a 15°C decrease in melting temperature for the mutant actin from 57°C to 42°C. Our results are consistent with the model of Holmes et al. (Holmes, K. C., D. Popp, W. Gebhard, and W. Kabsch. 1990. Nature [Lond.]. 347:44-49) involving the role of the hydrophobic plug in actin filament stabilization.
Figure 1. Model of the proposed hydrophobic plug-pocket interaction for rabbit skeletal muscle actin. (A) Stereo drawing of the alpha carbon trace showing the conformation of the loop containing the hydrophobic plug (red) in the hook conformation as observed in the actin/DNAse I complex. The side chains of F266 and I267 are also shown. Coordinates used are those deposited by Kabsch et al. (1990) in the Protein Data Bank (Bernstein et al., 1977; Abola et al., 1987). (B) Stereo drawing of the alpha carbon trace showing the conformation of the loop containing the hydrophobic plug (red) in the extended conformation as modeled by Holmes et al. (1990). (C) Space filling model showing the interaction of the loop (red) with a hydrophobic pocket formed at the interface of two actin monomers on the opposite strand of the actin helix (blue and white). Ile267 in muscle actin which corresponds to the position of the L266D mutation in yeast actin, is shown in yellow. Coordinates used for B and C are those of Lorenz et al. (1993).
loop formation. Holmes predicted that elimination of the plug–pocket interaction would severely affect the ability of actin to form stable filaments. In this paper, we report the results of inserting into the plug a hydrophilic residue, aspartic acid, M, rabbit skeletal muscle; Y, yeast wild-type actin; L266D, substitution of Leu266 with Asp.

Materials and Methods

The site-directed mutagenesis kit, [α-35S]-Adenosine-5'-thiotriphosphate, >1,000 Ci/mmole, and [γ-32P]ATP, 6,000 Ci/mmole were purchased from Amersham Corp. (Arlington Heights, IL). Rhodamine–phallolidin was purchased from Molecular Probes (Eugene, OR). DNase I was obtained from Worthington Biochemicals Corp. (Freehold, NJ). The phagemid pGEM 3Z(−) was obtained from Promega Biotec (Madison, WI). The Sequenase 2.0 DNA sequencing kit was purchased from U.S. Biochemical Corp. (Cleveland, OH). Phallolidin was obtained from Sigma Chemical Co. (St. Louis, MO). The oligodeoxynucleotide used for site-directed mutagenesis was synthesized in the DNA Core Facility at the University of Iowa (Iowa City, IA). The SI fragment of rabbit skeletal muscle myosin was prepared by the method of Weeds and Taylor (1975). Rabbit skeletal muscle actin was prepared from acetone powder (Pardee and Spudich, 1982) by DNAse I chromatography coupled with anion exchange chromatography according to Cook et al. (1993). The SLM 4800 fluorescence spectrometer and the AVIV 62 DS spectropolarimeter used in this work are part of the Protein Structure Facility in the College of Medicine at the University of Iowa.

Plasmid Manipulations and Mutagenesis

Procedures for manipulating plasmids are those described in Ausubel (1987). Single-stranded DNA was generated from the phagemid pGEM 3Z(−) into which the yeast actin coding sequence and the yeast actin promoter had been inserted between the EcoRI and BamHI sites in the polylinker. The single stranded DNA was then used as a template for site-directed mutagenesis together with the oligodeoxynucleotide 5'-TTCCAT-CCTTCTGGTATGGTGGAATCTGCC-3' to generate the L266D mutant actin coding sequence in which Leu266 is converted to aspartic acid. Following the number system of Wertman et al. (1992), we have named this actin allele act1-157 (L266D). The resulting mutant DNA was sequenced to verify the desired mutation. An expression vector, pCEN-LD, was constructed by substituting the EcoRI–HindIII fragment containing the mutant sequence for the homologous wild-type sequence contained in the centromeric yeast vector pCEN-WN which carries the entire yeast actin coding sequence adjacent to the yeast actin promoter (Cook et al., 1991).

The plasmid was used to generate diploid cells carrying both the wild-type and mutant actin coding sequences and haploid cells containing the mutant actin coding sequence as the only active actin coding sequence in the cell (Cook et al., 1991). The plasmid was reisolated from the yeast and sequenced to verify that the transformed cells contained the desired mutation.

Yeast Strains and Manipulations

5. cerevisiae strain TDyDD was a gift of D. Shortle (Johns Hopkins University, Baltimore, MD). It is a diploid cell in which one actin allele has been partially deleted and marked with LEU2+ (Cook et al., 1991). Strains TDyLD and TDyWN are haploid derivatives of TDyDD containing the chromosome carrying the interrupted actin gene and the plasmid pCEN-LD carrying the L266D actin coding sequence or pCEN-WN carrying the wild-type actin coding region respectively. Yeast were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic defined medium (Sherman, 1990). Yeast was transformed using a modification of the method of Ito et al. (1983).

Purification of Yeast Wild-type and L266D Actins

Wild-type yeast actin was purified in an active form using the DNase I agarose procedure of Cook and Rubenstein (1992). The same procedure was used to obtain monomeric L266D actin. However, because this actin has a cold-sensitive polymerization defect, the actin was polymerized and the F-actin collected by centrifugation at 25°C instead of 4°C as previously published.

Actin Polymerization Assays

Actin polymerization was carried out in F buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl2, 0.2 mM ATP, 2 mM MgCl2, 50 mM KCl, and 0.1 mM DTT). Polymerization was followed by centrifugation or by light scattering. For the sedimentation assay at temperatures below 20°C, the actin was polymerized at the desired temperature for 15 h and centrifuged at the same temperature at 60,000 rpm in a table ultracentrifuge (TL100; Beckman Instruments, Palo Alto, CA) using a TLA 100.2 rotor. The protein concentration of the supernatant was then determined using the Pierce BCA assay. For temperatures above 20°C, polymerization was carried out for 3 h. Subsequent experiments using light scattering showed that this time was sufficient for complete polymerization.

In the light scattering assay, the sample was equilibrated in G buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl2, 0.2 mM ATP, and 0.1 mM DTT) at the appropriate temperature for 30 min in a thermostated cuvette chamber in an SLM 4800 fluorescence spectrometer with excitation and emission wavelengths both at 360 nm. The microcuvette had cross-sectional dimensions of 3 mm × 3 mm. Polymerization was initiated by adding KCl and MgCl2 to final concentrations of 50 and 2 mM, respectively, and the change in light scattering recorded as a function of time. Alternatively, polymerization was induced by the addition of phallolidin-stabilized yeast wild-type F-actin seeds at a concentration of 0.025 or 0.05 μM actin. Seeds were prepared by incubating 10 μM F-actin with 0.9 μM phallolidin followed by vigorous vortexing of the resulting filaments before the addition to the G-actin solution. After establishing the baseline, polymerization was induced as described above. For determining polymerization elongation rates, increasing amounts of wild-type or mutant G-actin were combined in G buffer with 0.05 μM phallolidin-stabilized wild-type actin seeds, and the initial rate of elongation determined at each actin concentration following introduction of 2 mM MgCl2 and 50 mM KCl to induce polymerization. In the light scattering experiments just described, we often observed one or several large sharp symmetrical peaks during the first 50 s following mixing of the samples and placing the cuvette in the fluorimeter. These mixing artifacts appeared randomly with all types of actin used and seemed more prevalent at colder temperatures. We have manually smoothed the tracings shown in Figs. 5, 6, and 9 to remove these spikes.

The critical concentration (Cc) for actin association was also determined using light scattering. For measurements at 25°C, F-actin was diluted to the appropriate concentration in F buffer at the appropriate temperature for 3 h, and the change in light scattering was determined. For measurements at 10°C and 4°C, the incubation time was extended to 24 h. For every concentration tested, the light scattering of an equivalent amount of G-actin in G buffer was determined and deducted as a baseline value.

Actin–ATP and Actin–Myosin S1 Interactions

The rate of dissociation of ATP from actin was determined according to Mockrin and Korn (1980) using radiolabeled ATP as a probe. The intrinsic
rate of ATP hydrolysis by G-actin was determined by a combination of the methods of Haerter et al. (1990) and Tobacman and Korn (1982). The activation of myosin S1 ATPase activity by actin was measured according to Cook et al. (1992) based on the procedure of Spudich (1974).

**Electron Microscopy**

Actin filaments, negatively stained by 1.5% uranyl acetate, were examined by EM of samples on carbon-coated Formvar grids using an electron microscope (7000; Hitachi Instruments, Inc., San Jose, CA). This work was performed in the Central Electron Microscope Facility at the University of Iowa.

**Staining with Rhodamine Phalloidin and Calcafluor**

Yeast cells were stained with rhodamine phalloidin or Calcafluor, and fluorescence micrographs were obtained on a microscope (Carl Zeiss, Oberkochen, Germany) using TMAX 400 film according to the procedure of Pringle et al. (1991).

**Actin Filament Viscometry**

The viscosity of F-actin solutions was determined with a falling ball viscometer (Cooper, 1992) using a path length of 7.9 cm and an angle of inclination of 12°. Actin was polymerized at a given temperature for a specific period of time after which it was drawn into the microcapillary pipette and incubated for an additional 1/2 h at the same temperature. Measurements were obtained in duplicate or triplicate on a number of different actin preparations with similar results obtained from different preparations of the same actin.

**Circular Dichroism Measurements**

For these experiments, the 1.4 μM actin in a buffer containing 2 mM Hepes, pH 8.0, 0.2 mM CaCl₂, 0.2 mM ATP, and 0.5 mM β-mercaptoethanol was placed in a 3-ml stoppered quartz cuvette with a path length of 1 cm. Measurements were performed on an AVIV 62 DS spectropolarimeter equipped with a thermostatically controlled temperature and an immersible thermocouple, accurate to ±0.4°C, for direct monitoring of the temperature of the sample. The sample was heated at a constant rate of 1°C/min over a temperature range extending from 0°C to 80°C with constant stirring of the sample over the entire range tested. Changes in ellipticity were monitored at 222 nm. Buffer alone showed no change in ellipticity with temperature and was therefore ignored during data analysis. Data were analyzed using a Macintosh version of a nonlinear least-squares fitting program developed by Johnsson and Frasier (1985). Data were processed and presented using Cricket Graph (Computer Associates International, Inc., West Conshohocken, PA).

**Results**

**In Vivo Studies with the L26D Mutant Actin**

When we transformed the yeast with the plasmid containing the actin coding sequence carrying the Leu266→Asp mutation, we obtained transformants with the same frequency as when we used a plasmid containing the wild-type actin coding sequence. Dissection of tetrads following the induction of these cells to undergo meiosis allowed us to readily obtain viable haploid cells (TDyLD) in which the L266D actin, synthesized from the plasmid-borne coding sequence, was the only actin in the cell. Furthermore, these cells appeared to grow on YPD agar plates at temperatures ranging from 20°C to 37°C as well as wild-type cells. By microscopic examination, the morphology and size of the L266D cells also appeared normal. Thus, contrary to our prediction based on the Holmes model, introduction of an acidic residue in the hydrophobic plug apparently did not significantly destabilize the actin helix in vivo under the conditions tested.

It is difficult to obtain quantitative differences in growth of yeast on agar plates. We thus measured the generation times for cells expressing L266D actin versus wild-type actin at various temperatures in liquid culture. Table I shows no difference in the ratio of growth rates of the mutant and wild-type cells between 20°C and 37°C. However, as the temperature decreased below 20°C the ratio of the growth rates of these two cultures increased reaching a value of about 1.4 at 10°C. Thus, the L266D mutation causes a non-lethal cold-sensitive phenotype. L266D cells grown at 10°C were stained with rhodamine phalloidin to visualize F-actin and with Calcafluor to visualize the pattern of chitin deposition. No differences were observed between the cells carrying the wild-type and L266D actins (data not shown).

Finally, we examined the ability of these cells to grow on agar in hyperosmolar medium. Chowdhury et al. (1992) recently reported that introduction of yeast to hyperosmolar medium causes a temporary cessation of growth and a gross fragmentation and rearrangement of the actin cytoskeleton. Under these conditions, cells containing certain mutant actins could not grow. If nucleation of filaments and their subsequent assembly in vivo was affected by the L266D mutation in a manner suggested by the Holmes model, proper reassembly of the cytoskeleton might be compromised in hyperosmolar medium, especially at lower temperatures where the defect is most evident. However, relative to wild-type cells, L266D cells grew between 10°C and 30°C to the same extent on agar in hyperosmolar medium (YPD containing 1.2 M KCl or 1.8 M sorbitol) as they did on normal YPD plates.

**Polymerization of the L266D Mutant Actin In Vitro**

We then purified the actin for biochemical studies in vitro. The L266D actin bound to a DNAseI affinity column, a step in the purification procedure, indicating that the mutation produced no gross distortion of the monomeric structure of actin. Repeated attempts to polymerize the actin by incubation at 4°C overnight in F buffer after incubation at room temperature for 30 min were unsuccessful. However, polymerization of the L266D actin occurred readily when the actin was incubated in F buffer for 2 h at 25°C. Thus the hydrophobic plug mutation resulted in a cold-sensitive polymerization defect.

To further characterize the effects of temperature on the polymerization of the L266D actin, we measured the centrifugation properties of the actin as a function of temperature (Fig. 3). For these experiments, the starting actin concentration was 3 mg/ml (70 μM). Under the conditions of the assay, the amount of wild-type actin pelleted remained constant over different temperatures extending from 0°C to 80°C with constant stirring of the sample over the entire range tested. Changes in ellipticity were monitored at 222 nm. Buffer alone showed no change in ellipticity with temperature and was therefore ignored during data analysis. Data were analyzed using a Macintosh version of a nonlinear least-squares fitting program developed by Johnsson and Frasier (1985). Data were processed and presented using Cricket Graph (Computer Associates International, Inc., West Conshohocken, PA).

**Table I. Doubling Times of TDyLD and TDyWN Cells at Different Temperatures**

| Temperature (°C) | TDyLD (h) | TDyWN (h) | LD/WN |
|------------------|-----------|-----------|-------|
| 9°C              | 33        | 22.5      | 1.5   |
| 10.5°C           | 22.7      | 17.2      | 1.4   |
| 15°C             | 8.7       | 7.1       | 1.2   |
| 20°C             | 4.9       | 4.9       | 1.0   |
| 30°C             | 2.1       | 2.1       | 1.0   |
| 37°C             | 4.7       | 4.7       | 1.0   |

Shown are the doubling times and the ratios of the doubling times for the TDyLD and TDyWN yeast strains grown in YPD medium. Growth at temperatures between 9°C and 20°C were monitored with cultures in a revolving drum. Growth at higher temperatures was monitored using a shaking platform. Each doubling time represents the average of three parallel cultures grown simultaneously. For each temperature, growth of both cell types were monitored concurrently. LD/WN is the ratio of the doubling times.
Figure 3. Sedimentation of wild type and F-actin as a function of temperature. The vertical axis represents the concentration of actin remaining in the supernatant. (s) wild-type actin; (●) L266D actin.

We observed that the amount of protein remaining in the supernate at 22°C was about twice that of the wild-type protein. As the temperature decreased to 10°C, the actin remaining in the supernate increased from 0.2 mg/ml (4.6 μM) to ~0.5 mg/ml (11.6 μM). As cooling continued to 4°C, there was a marked increase in supernatant actin to a level of about 2 mg/ml (46.5 μM), suggesting the occurrence of a cooperative transition.

To begin to determine the basis for this unusual centrifugation behavior of the L266D actin, we examined preparations of the protein by EM after the addition of 2 mM MgCl₂ and 50 mM KCl. Fig. 4 C shows that the mutant actin forms normal looking filaments at room temperature. At 10°C stringy aggregates were observed, but defined filaments were not detected. When polymerization was carried out in the presence of 0.05 μM phalloidin stabilized wild-type actin seeds, we sometimes detected normal appearing filaments (Fig. 4 B). More often we observed disordered or broken filamentous-like structures in which actin subunits were clearly visible suggesting that the filaments may be breaking apart during preparation of the EM specimens. This may have been due in part to the fact that for the 10°C samples, we polymerized the actin at 0.8 mg/ml (18.6 μM) and then diluted the sample to 0.3 mg/ml (7 μM) with gentle pipetting just before application of the sample to the grid. We did not convincingly observe structures that would have indicated an unwinding of the F-actin helix into single strands. At 4°C, where little if any actin pelleted in the centrifugation assay at actin concentrations below 2 mg/ml (46.5 μM), no filaments were seen either with 11 μM pure mutant actin or with mutant actin in the presence of wild-type actin seeds (data not shown). However, if equimolar amounts of phalloidin and L266D actin were mixed in the presence of wild-type actin seeds, normal looking filaments were readily apparent (Fig. 4 A). These results suggest that the L266D mutation results in a pronounced decrease in the ability of the actin to nucleate filament assembly and a decrease in the stability of the filament once it is formed. The ability of the mutant actin to generate normal filaments in the presence of seeds and phalloidin at 4°C demonstrates that the alterations caused by the mutation do not result in structural changes that affect the addition of monomers to a filament per se.

We used light scattering to determine the effect of the L266D mutation on the kinetics of association of the actin monomers at 25°C, 10°C, and 4°C. As shown in Fig. 5 A, at 25°C there is a significant lag in the onset of the elongation phase compared with that observed with wild-type yeast actin. At 10°C, using 0.8 mg/ml (18.6 μM) of actin, the 1,500-s lag in the onset of monomer association was even more pronounced when compared with that seen with wild-type actin (Fig. 5 B). At 4°C no increase in light scattering was observed for at least 6,000 s after the addition of F-salts although wild-type actin readily polymerized under these conditions (Fig. 5 C).

If the L266D mutation affected nucleation of filaments, addition of preformed F-actin nuclei should decrease the lag phase of the observed light scattering curve. The addition of phalloidin-stabilized wild-type actin nuclei to 10°C to a concentration of 0.025 to 0.05 μM increased the rate at which the L266D actin monomers polymerized (Fig. 6 A) although nucleation and elongation were still slower than with wild-type actin. We observed a similar effect at 4°C where, in the absence of added nuclei, polymerization was virtually
Figure 5. Effects of temperature on the polymerization of L266D actin. Polymerization of yeast wild-type and L266D-actins was followed as a function of time using a light scattering assay. For each experiment, the actin concentration was 18.6 μM. (—) L266D actin; (······) wild-type actin. (A) 25°C; (B) 10°C; (C) 4°C.

nonexistent (Fig. 6 B). However, at this temperature, due to the effect of the mutation on the ability of the actin to form stable filaments, we propose that this nucleation produced amorphous aggregates instead of filaments shown in Fig. 4.

We next determined the effect of the L266D mutation on the elongation rate of actin polymerization by measuring the rate at which different concentrations of actin polymerized at 22°C and 10°C in the presence of a constant amount of stabilized F-actin seeds. Fig. 7 shows that at both temperatures, the elongation rate for the L266D actin was two to three times slower than that observed with the wild-type actin. A similar difference was observed at 4°C. The failure to observe recognizable filaments under these conditions at 4°C under the electron microscope made interpretation of this last piece of data difficult. However, the ability of the L266D actin to form filaments in the presence of phalloidin at 4°C suggests that the seed-induced increase in light scattering at 4°C reflects an actin–actin association occurring in the normal polymerization process. In all light scattering experiments, no increase in light scattering was observed with the seeds alone.

Having demonstrated an effect of the mutation on both filament nucleation and elongation, we next determined the Cc for association at temperatures ranging from 4°C to 25°C using a light scattering assay which is largely independent of filament length (Tobacman and Korn, 1982). The results with L266D actin are shown in Fig. 8, and the critical concentrations for both mutant and wild-type actins are shown in Table II. The apparent Cc at 25°C for the L266D mutant (1.2 μM) was two to three times that determined for wild-type actin (0.4 μM). At 10°C and 4°C, the value for Cc for the L266D actin increased to 6.5 and 11 μM, respectively, compared with 0.65 and 1.7 μM for the wild-type actin at the same temperatures. This behavior mirrored that observed in the centrifugation assay.

Effect of the L266D Mutation on Filament Viscosity

We next examined the viscosity of the L266D actin under various conditions using falling ball viscometry. The results are shown in Table III. At room temperature, the viscosities observed for both wild-type and L266D actin were similar, although we consistently observed that the viscosity of L266D actin was actually slightly higher than that of the wild-type actin. At 10°C, the viscosity of the mutant actin was about one-fourth that of the wild type. Addition of seeds produced no increase in the viscosity of the mutant. However, when polymerization of both mutant and wild-type ac-
Figure 7. Elongation rates of L266D and wild-type actins as a function of temperature. The initial rates of polymerization of wild-type (○) and L266D (●) actins in the presence of 0.05 μM wild-type actin seeds were measured and the elongation rates were plotted as a function of actin concentration. (lsu) light scattering units. (A) 23°C; (B) 10°C; (C) 4°C.

Figure 8. The Cc of L266D actin as a function of temperature. Values were determined by light scattering. (○) 25°C; (○) 10°C; (●) 4°C.

Table II. Critical Concentration for Polymerization of Wild-type and L266D Actins

| Temperature | Wild-type actins (μM) | L266D actins (μM) |
|-------------|-----------------------|-------------------|
| 23°C        | 0.4                   | 1.2               |
| 10°C        | 0.7                   | 6.5               |
| 4°C         | 1.7                   | 11                |

Table III. Viscosity Measurements of Wild-type and L266D Actins at Different Temperatures

| Temperature | 4°C | 10°C | 23°C |
|-------------|-----|------|------|
| Wild type   | 0.8 ± 0.1* | 3.0 ± 0 | 1.7 ± 0 |
| L266D       | 0.9 ± 0.1† | 0.7 ± 0.1 | 2.3 ± 0.1 |
| L266D plus seeds | 1.4 ± 0.1‡ | 0.8 ± 0.1 | n.d. |
| Wild-type plus phalloidin | 45 ± 8 | 39 ± 4 | n.d. |
| L266D plus phalloidin | 38 ± 5 | 45 ± 5 | n.d. |

Values shown for the L266D actin are derived from data shown in Fig. 8. Values for wild-type actin were obtained by carrying out a similar set of experiments with this actin. Values are expressed in s/cm. All concentrations are 11.6 μM (0.5 mg/ml) except as noted.
* 4.7 μM (0.2 mg/ml)
† 18.6 μM (0.8 mg/ml)
‡ n.d., not done

Effect of the L266D Mutation on the DNAse I-induced Depolymerization of Actin

To investigate further whether the L266D mutation destabilizes the actin filament, we measured the effect of DNAse I on these filaments. Since DNAse I binds to monomers rapidly, its presence causes a gradual depolymerization of the actin filaments. If the L266D F-actin is less stable than wild-type F-actin, it should depolymerize more rapidly in the presence of DNAse I. Fig. 9 demonstrates a faster depolymerization of the L266D actin than wild-type actin in the presence of DNAse I. In a control experiment in which buffer
Figure 9. Effect of DNAse I on the stability of yeast wild type and L266D F-actin filaments. F-actin (23 μM) and DNAse I were combined in equimolar amounts in F buffer and the light scattering of the solution monitored at 25°C as a function of time after addition of the DNAse I. The square on the vertical axis denotes the starting light scattering value for both actins. (——--) L266D actin; (———) wild-type actin.

only was added instead of DNAse I, no decrease in light scattering was observed with either actin. The observed filament instability may be caused by a faster off rate of the L266D monomer from the filament ends compared with a wild-type actin filament. Alternatively, the gentle mixing of the actin and DNAse solutions with a Pasteur pipette at the beginning of the experiment may have resulted in a larger number of filament ends causing the more rapid depolymerization observed. Although we cannot presently distinguish between these possibilities, the result supports our assertion that the L266D actin filament is less stable in solution compared with its wild-type counterpart.

Effect of the L266D Mutation on the Activation of Myosin S1 ATPase Activity

Although at temperatures >20°C, L266D filaments appeared normal by EM, the mutation may have caused a change in the surface topology of the filament leading to an altered ability to interact with various F-actin binding proteins. We therefore assessed the relative abilities of the L266D and wild-type yeast actins to activate the skeletal muscle myosin S1 ATPase activity. If an alteration in surface topology interfered with the normal actin-myosin interaction, curves of myosin ATPase activity versus actin concentration should have different slopes. As shown in Fig. 10, the slopes were the same although the curve for L266D actin was laterally displaced indicating that the amount of functional L266D F-actin per total actin added was less than when a comparable amount of wild-type actin was added. This is the result expected if the mutation affected Cc for the actin but did not significantly affect its filamentous structure.

Effect of the L266D Mutation on Properties of G Actin

According to the Holmes model, a change in the hydrophobic plug such as the L266D mutation should affect filament assembly much more than the properties of the actin monomer. The ability to purify L266D actin using DNAseI agarose affinity chromatography shows that the mutation does not cause a large-scale alteration of G-actin structure. To further investigate the effect of the L266D mutation on G-actin structure, we studied the rate at which bound radiolabeled ATP dissociated from wild-type and L266D G-actins in the presence of added unlabeled ATP. ATP binds to actin within a cleft separating subdomains 1 and 2 from 3 and 4 using binding sites on both sides of the cleft (Kabsch et al., 1990). An alteration which affects the spatial relationship of these domains should affect ATP binding. Although the mutation affected polymerization at room temperature, Table IV shows that at room temperature the ATP exchange rates for wild type and L266D actins were identical. However, at 4°C, the exchange rate for the L266D actin was only half as fast as for the wild-type actin indicating that the mutation causes a small but significant conformational change of the monomer at this lower temperature resulting in perhaps a more rigid structure.

As another probe of the conformation of the actin monomer, we compared the intrinsic ATPase activities of wild-type and L266D G-actin. Fig. 11 shows that the wild-type actin hydrolyzed ATP at twice the rate observed for the mutant protein, indicating in this case that at 30°C, the mutation induces a small conformational change in the monomer.

Table IV. The ATP Exchange Rates of Yeast Wild-type and L266D Actins at 22°C and 4°C

| °C | WT (s⁻¹ × 10⁶) | L266D (s⁻¹ × 10⁶) |
|----|----------------|-----------------|
| 22 | 5.8 ± 1.1 (n = 8) | 5.7 ± 1.0 (n = 4) |
| 4  | 2.1 ± 0.4 (n = 3) | 1.2 ± 0.2 (n = 4) |

The data is presented as the rate ± SD, n, the number of repetitions used in each determination.
type and mutant actins by monitoring their ellipticity at 222 nm as a function of temperature. Fig. 12 shows that the mutation causes a 15°C decrease in the melting temperature of the mutant actin (42°C) in comparison to wild-type (57°C) yeast or skeletal muscle (57°C) actins indicating perhaps a more flexible structure that is more susceptible to thermal denaturation. Our value for rabbit skeletal muscle actin is identical to that reported previously under the same buffer conditions (Strzelecka-Golaszewska et al., 1985; Bertazzon et al., 1990).

Discussion

The model of Holmes et al. for F-actin (1990) predicts cross-strand stabilization resulting from the insertion of a hydrophobic plug consisting of residues 265–268 of yeast actin into a hydrophobic pocket formed by the interface of two subunits on the opposing strand. Hydrophobic interactions are cold sensitive (Baldwin, 1986; Privalov and Gill, 1988); that is, they weaken as the temperature decreases. In the case of the mutant actin, insertion of Asp266 in place of leucine might destabilize this interaction although, at room temperature, not enough to drastically affect F-actin stability. As the temperature decreases, the disruption in the remaining hydrophobic interactions would increase until the disruption prevented formation of a stable polymer.

Our results are generally consistent with the predictions of this model. As judged by kinetic and viscosity measurements, both the rate and extent of the polymerization of the L266D mutant actin are drastically affected, especially at lower temperatures. Compared with wild-type actin, more mutant actin is needed to activate myosin S1 ATPase at 25°C although the catalytic efficiency of this actin is the same as that of wild-type actin once a threshold level of actin has been exceeded. The mutation does affect properties of the monomer in comparison to wild-type actin. This is evidenced by a lower intrinsic ATPase activity at 30°C, a slower rate of ATP exchange at 4°C, and a lower melting temperature. Structural alterations resulting in the lowered thermal stability may not be relevant to the effects of the mutation on polymerization since the melting temperature (Tm) is outside the temperature range used in the other studies reported here. Although these effects seem much smaller than would be needed to explain the severe effects of the mutation on the polymerization properties of actin, it is still possible that an alteration in the alignment of the four subdomains relative to one another might be partly responsible for the abnormal polymerization we observed.

Nucleation of filament formation is thought to occur by the slow association of two monomers followed by stabilization of the complex by a faster addition of a third monomer (Frieden, 1982; Zimmerle and Frieden, 1988). The filament then elongates from this complex. Partial disruption of the plug–pocket interaction in this complex would have a drastic effect on the stability of the nucleus since this hydrophobic interaction would constitute a major component of the intersubunit interactions. Our results demonstrate a large effect of the mutation on nucleation in agreement with this prediction.

This disruption would, to a lesser degree, also affect filament elongation. The addition of each monomer to the growing filament would be compromised. However, once the filament formed, the sum of all interstrand interactions along the filament would be great enough to partially stabilize the actin helix. Although our studies show that at every temperature examined, the elongation rate for the mutant actin was slower than for the wild-type actin, the ratio of elongation rates of these actins was identical. This lack of cold sensitivity suggests that the effect of the mutation on elongation may be due more to a small conformation change in the entire actin structure rather than a disruption of the hydrophobic plug–pocket interaction.

Holmes' model predicts that the residues making up the hydrophobic loop can exist in two conformations. In the actin–DNase I complex (Kabsch et al., 1990), the loop at its base extends outward from the surface of the protein. It then hooks back in toward the surface placing the four residues...
of the hydrophobic plug in an orientation pointed away from
the solvent towards Tyr188. In this conformation, the plug
residues would not be oriented properly to interact with actin
subunits on the adjacent strand. In the model F-actin struc-
ture, the top half of the loop has rotated so that the entire
loop points away from the surface of the protein allowing the
plug–pocket interaction to occur. If this model is correct, sub-
stitution of aspartic acid for leucine at position 266 might
increase the hydrophilic character of the plug enough to al-
low the loop in G-actin to extend into the solvent instead of
bending back toward the protein surface. Although our work
suggests the L266D mutation causes small conformational
changes in the monomer, we have no explicit evidence that
these changes represent movement of the loop.

The ratio of the monomers in each of these conformations
may affect the rates at which nucleation and elongation oc-
cur. Since nucleation is the slower of the two processes, one
would predict that the favored conformation for the loop in
G-actin is the hook form. We hypothesize that once a nucleus
has formed, the interaction of a new monomer with the nu-
cleus causes a change in the loop, in effect shifting the equi-
librium position for its conformation toward the extended
position thereby facilitating monomer addition to the grow-
ing filament.

The relatively minimal effects observed with the L266D
actin in vivo do not reflect the magnitude of the effects on
nucleation and polymer formation observed in vitro. Two
possible explanations can be offered for this apparent para-
dox. First, at 10°C, when only about a 40% increase in
generation time is observed compared with wild-type cells,
other processes within the cell may become more rate limit-
ing for growth than the disruption of actin polymerization
thus masking the effect of the mutation. Second, although
spontaneous nucleation of actin filament formation and fila-
ment elongation in vitro are severely affected at 10°C, addi-
tion of pre-formed nuclei resulted in a greatly enhanced rate
of polymer formation. Furthermore, at 4°C, where not even
the addition of seeds induced the formation of stable fila-
ments, such filaments were observed in the presence of phal-
loidin which is thought to provide cross-strand stability. It
is unlikely that spontaneous nucleation of actin polymeriza-
tion in the cell is required to any great extent. A number of
known actin binding proteins can nucleate filament forma-
tion, and proteins such as tropomyosin can stabilize fila-
ments once they have formed (Stossel et al., 1985). Proteins
similar to these probably direct the deposition of actin fila-
ments in vivo, thereby overcoming the most drastic effects of
the L266D mutation.

Shutt et al. (1989), based on their work with the crystal
structure of the actin/profilin complex, have suggested a pos-
sible alternative model for the actin filament. In this ribbon
structure, the small domain consisting of subdomains 1 and
2 is in the interior of the filament where subdomain 2 con-
tacts the small domain of one neighboring monomer and the
top of the large domain (subdomain 4) of another monomer.
The interface between subdomains 3 and 4 where the hydro-
phobic loop is located is on the exterior of the filament, ap-
parently not involved directly in intersubunit contacts. For
our results to be consistent with this model, one would have
to postulate that alteration of the loop by the L266D muta-
tion produced conformational changes in subdomains 2 and/or
4 large enough to cause filament disruption. Al-
though this mutation does produce conformation changes in
the monomer, the effects on the monomer observed suggest
that these changes are minimal. Thus, although our results
do not contradict the Shutt model, we believe that the be-
havior of the L266D mutant actin is more in agreement with
the prediction of Holmes' model for F-actin.

Our work represents the first experimental test of the part
of the Holmes model involving the hydrophobic plug. Al-
though our results are consistent with the predictions of this
model, they do not prove it. Proof must await a further char-
acterization of the switch in loop conformations and an ac-
tual demonstration of an interaction between the hydropho-
bic plug and the pocket in the actin filament.

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