F-Actin Retains a Memory of Angular Order

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ABSTRACT Modifications can be made to F-actin that do not interfere with the binding of myosin but inhibit force generation, suggesting that actin's internal dynamics are important for muscle contraction. Observations from electron microscopy and x-ray diffraction have shown that subunits in F-actin have a relatively fixed axial rise but a variable twist. One possible explanation for this is that the actin subunits randomly exist in different discrete states of "twist," with a significant energy barrier separating these states. This would result in very slow torsional transitions. Paracrystals impose increased order on F-actin filaments by reducing the variability in twist. By looking at filaments that have recently been dissociated from paracrystals, we find that F-actin retains a "memory" of its previous environment that persists for many seconds. This would be consistent with slow torsional transitions between discrete states of twist.

INTRODUCTION

It been shown by mutations (Drummond et al., 1990), chemical cross-linking (Prochniewicz and Yanagida, 1990), and proteolytic cleavage (Schwyter et al., 1990) that modifications can be made to F-actin that do not interfere with the binding of myosin, do not inhibit the activation of myosin's ATPase, but inhibit force generation. This suggests that actin's internal dynamics are important for force to result from the acto-myosin interaction. Observations from electron microscopy and x-ray diffraction have shown that subunits in the actin filament have a relatively fixed axial rise (Huxley et al., 1994) but a variable twist (Hanson, 1967; Egelman et al., 1982; McGough et al., 1997). Unexpectedly, mechanical measurements of actin filaments have not detected such a large torsional flexibility (Yasuda et al., 1996; Tsuda et al., 1996). A possible reconciliation of these seemingly conflicting observations could result if the structural disorder is static or exists on a very slow time scale. If this were the case, actin subunits within a filament might randomly exist in different discrete states of "twist," with a significant energy barrier separating these states. To test this, we have attempted to see if actin filaments undergo very slow torsional transitions.

If the energetic distribution describing torsion in F-actin is as shown in Fig. 1 *a*, where thermal motions give rise to an r.m.s. deviation of 5–6° per subunit, the filament would be quite flexible with respect to torsion. The binding of proteins such as cofilin, which changes the twist of F-actin by ~5° per subunit (McGough et al., 1997), would provide the free energy needed to shift the mean of the distribution. However, the energetic landscape shown in Fig. 1 *b* is equally compatible with the observed random disorder (Hanson, 1967; Egelman and DeRosier, 1992; Egelman et al., 1982), but would also be consistent with mechanical rigidity (Tsuda et al., 1996; Yasuda et al., 1996). To distinguish between these possibilities, we have imposed order on actin filaments and asked whether the relaxation time is slow or fast. Subunits with the ability to rotate due to thermal energy (Fig. 1 *a*) would be expected to generate filaments with a fast relaxation, while subunits locked into discrete angular orientations (Fig. 1 *b*) would be expected to show a very slow relaxation.

MATERIALS AND METHODS

Sample preparations

Actin was prepared from rabbit skeletal muscle and purified on either a Sephadex G-200 column or a Superdex column, using the AKTA-FPLC system (Pharmacia, Uppsala, Sweden). G-Ca^{2+}-actin at 0.5 mg/ml was polymerized by 0.1 M KCl, and F-actin was diluted to 3 μM by 50 mM KCl, 5 mM K-phosphate buffer (pH 7.5) (F-buffer) (Francis and DeRosier, 1990). Paracrystals were formed by adding 50 mM $MgCl_2$ to F-actin, and the mixture was incubated overnight at 4°C. One drop of the paracrystal mixture was applied to a 300-mesh copper grid (coated with carbon film) for 1 min, and for fraying filaments the grid was rinsed with one to three drops of F-buffer with 10 mM $MgCl_2$. Negatively stained samples were stained by 1% uranyl acetate. Frozen-hydrated specimens for cryoelectron microscopy were prepared on a holy carbon film, using the same method of on-the-grid washing by F-buffer with 3 mM $MgCl_2$. The samples were then rapidly frozen in a propane slush and maintained at −175°C in a Gatan 626 cryostage.

Electron microscopy and image analysis

Specimens were examined in a JEOL 1200 EXII electron microscope at an accelerating voltage of 80 keV and a nominal magnification of 30,000×. Negatives were densitometered with a Leaf 45 scanner, using a raster of 4 Å/pixel. Fourier transforms were calculated from filament sections containing ~100 subunits, after straightening by a spline fitting procedure (Egelman, 1986). The twist, t, of each filament was calculated from the axial positions of the "first" (Z_1, $n = 2$), "sixth" (Z_6, $n = -1$), and "seventh" (Z_7, $n = 1$) layer lines, using

$$t = 2. + 0.5*[(Z_1/Z_6) + (Z_7 - Z_6)/Z_6]$$

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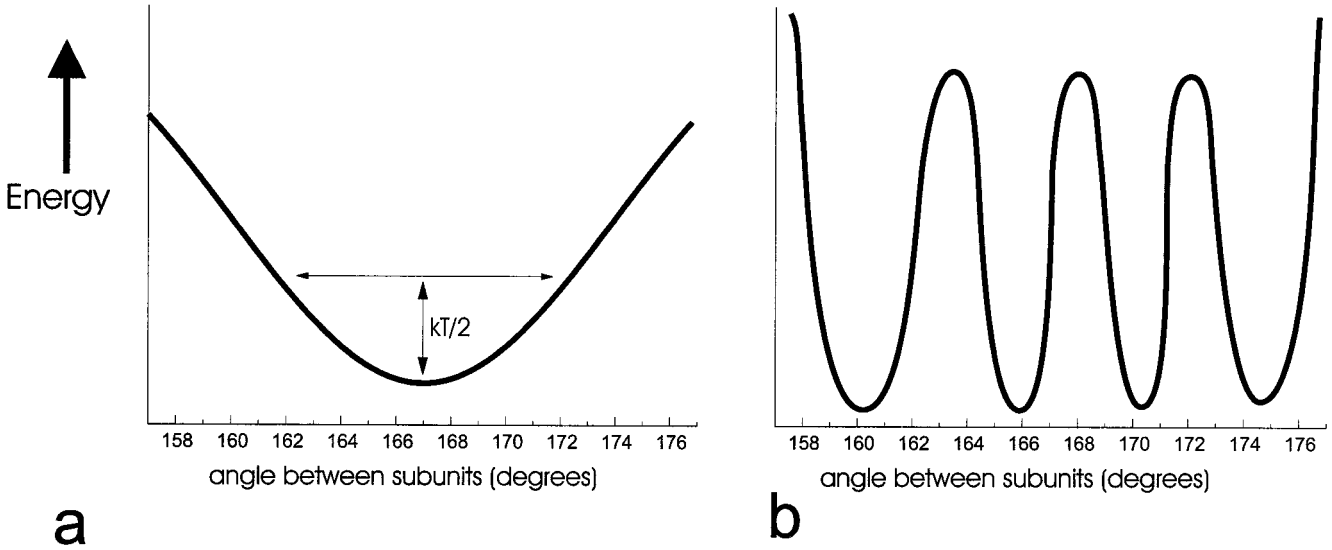

FIGURE 1 Possible energetic landscapes for the variable twist observed in F-actin. If there was a shallow distribution, with thermal energies of $kT/2$ corresponding to angular excursions of ± 5–$6°$ (*a*), one would expect to find a large torsional flexibility for F-actin, and Brownian torsional motions would occur on a rapid time scale. If, however, actin subunits existed in multiple, discrete states of twist, with a significant energy barrier separating these states (*b*), thermal torsional motions would occur on a very slow time scale.

A real-space determination of cross-over spacings was made from the frozen-hydrated images by 1939 overlapping segments from 40 filaments for the control, and 1926 segments from 55 filaments for the dissociated paracrystals. Each segment was ~270 Å long and was incremented by ~55 Å along the filament. Cross-correlations were performed against a model filament (containing 12 subunits), generated with the mean twist of the observed filaments, that was rotated in 4° increments and projected onto two dimensions. The cross-correlation search was restricted to ±14 Å axially. Filament segments that yielded a polarity opposite that of adjacent segments in the cross-correlation with the model were excluded. The cross-over spacing was taken as the distance between 180° rotations.

RESULTS

At high Mg^{2+} concentrations, all actin filaments exist in paracrystals. Within these paracrystals, the actin periodicity is more fixed. This increase in order appears to occur as an annealing process, as the order present increases over time. Actin filaments were incubated in 50 mM Mg^{2+} overnight, and Fig. 2 *a* shows the resulting paracrystals imaged in negative stain. Paracrystals were then washed (Francis and DeRosier, 1990) while on the electron microscope grid with a solution of 10 mM Mg^{2+}, producing the frayed ends of paracrystals shown in Fig. 2 *b*. Because of the possibility that the 10 mM Mg^{2+} can induce structural changes in filaments, control filaments were incubated overnight in 10 mM Mg^{2+}. Fourier transforms of isolated control filaments (Fig. 3 *a*), bundles of filaments in paracrystals (Fig. 3 *b*), and filaments emerging from frayed paracrystals (Fig. 3 *c*) were analyzed for the mean number of units per turn of the 59-Å-pitch, left-handed helix by measuring ratios of layer line spacings (Egelman et al., 1983). It can be seen that the variance in this parameter is decreased between the isolated control filaments and the paracrystals. This change in variance is highly significant ($p < 0.002$, F-distribution). Strikingly, this variance remains reduced in the filaments that have recently been (<1 min) dissociated from paracrystals, suggesting that they retain a "memory" of the imposed order that existed within the paracrystals. However, the simplest model would not explain the observed change in the mean twist between the paracrystals (mean = 2.164) and the filaments dissociated from the paracrystals (mean = 2.161), which is small but significant ($p < 0.001$). We discuss below how supercoiling can account for this shift.

The possibility that the increased variance in twist seen between the paracrystals (Fig. 3 *b*) and the control filaments (Fig. 3 *a*) is due mainly to measurement error appears unlikely, as the filaments dissociated from paracrystals (Fig. 3 *c*) display the same reduction in variance. However, there are potential problems with measuring layer line spacings, as other sources of noise might account for the variation seen in these ratios. In addition, there might be artifacts associated with the negative staining of specimens. We therefore have used an entirely different approach to measuring angular disorder in frozen-hydrated F-actin filaments as an independent check on these results. Fig. 2 *c* shows unstained paracrystals imaged in ice by cryo-EM, and Fig. 2 *d* shows unstained frayed paracrystals. Images of both frozen-hydrated control filaments in 10 mM Mg^{2+} (not shown) and the filaments from the frayed paracrystals (Fig. 2 *d*) were cut into segments containing ~10 subunits, and these were cross-correlated against images of an atomic model of F-actin (Lorenz et al., 1993). This yielded an estimate of the azimuthal rotation of each segment, and the

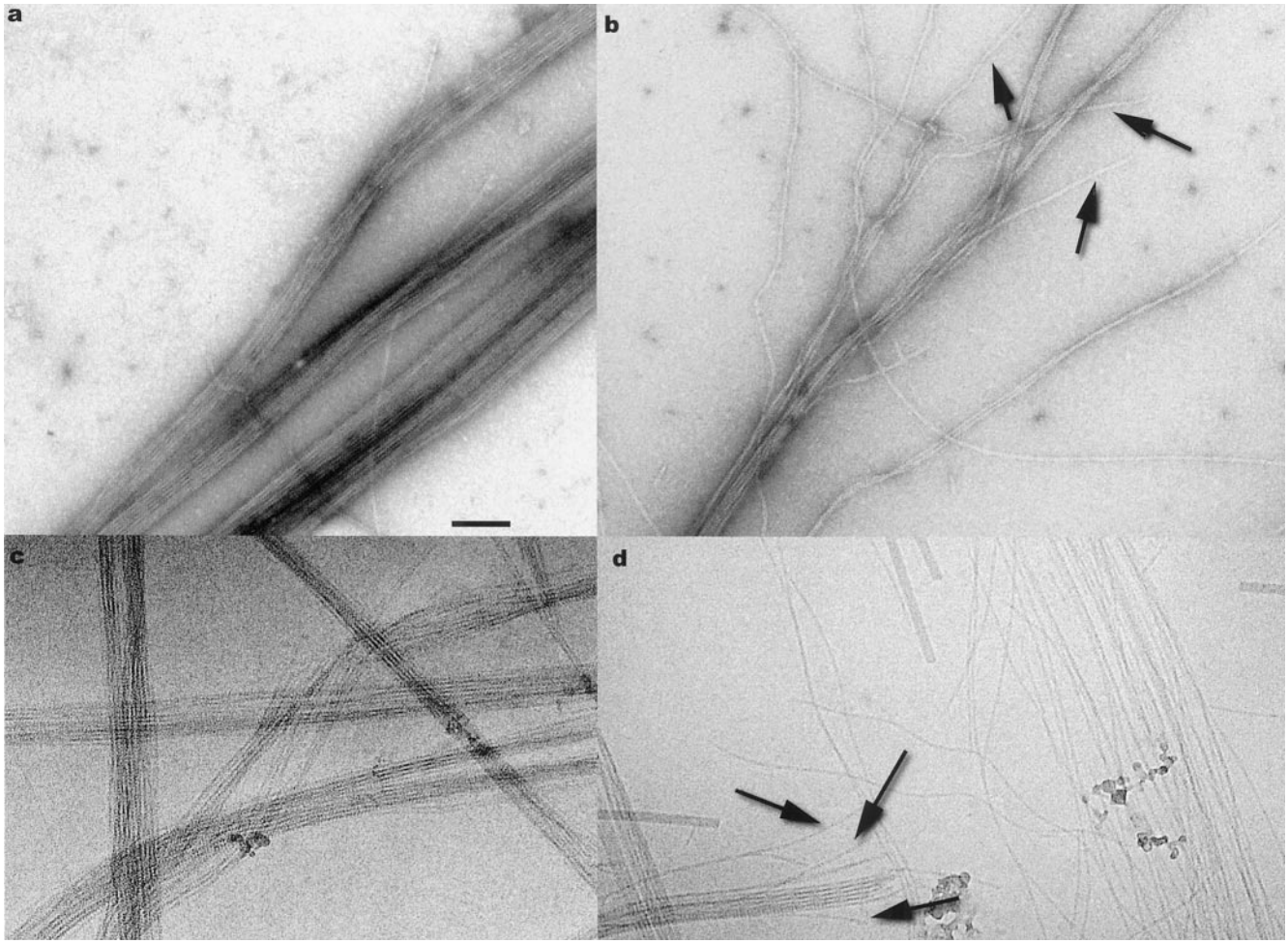

FIGURE 2 Electron micrographs of actin paracrystals (*a, c*) and partially dissociated paracrystals (*b, d*). The specimens in *a* and *b* have been prepared with negative stain, while those in *c* and *d* are unstained, frozen-hydrated samples imaged by cryo-EM. The paracrystals have been allowed to anneal during an overnight incubation of actin filaments in 50 mM Mg^{2+}. With washing of paracrystals adsorbed to an EM grid with a low-Mg^{2+} buffer, free filaments are observed (*arrows* in *b* and *d*) coming out of the paracrystal bundles. The space bar in *a* is 1000 Å.

distances between 180° rotations were taken as a measure of the cross-over distance.

The histograms in Fig. 4 show that while the control filaments have a standard deviation in the cross-over distance of 54 Å, the filaments from the dissociated paracrystals have a reduced standard deviation of 40 Å. This difference in variances is quite significant ($p < 0.002$, F-distribution), given the large number of measurements from each population (135 cross-overs for the control filaments and 115 for the postparacrystal filaments). While it is likely that there is some component of measurement error in both distributions (giving rise to an additive variance with the intrinsic variability in cross-overs), it is expected that this error will be the same for the two distributions. The measurement of local distances between cross-overs shows that we observe the same effect as we did when using layer line ratios from entire filaments, suggesting that artifacts due to filament bending, other forms of disorder, etc., may be excluded.

It can also be seen in Fig. 4 that there is a small shift of the mean cross-over spacing, from 378 Å in the control filaments to 367 Å in the postparacrystal filaments. This shift is not significant ($p > 0.05$, Student's *t*-test) but would correspond to a change from 2.156 units/turn in the control filaments to 2.161 units/turn after release from the paracrystals.

DISCUSSION

Using two different methods, we have observed that actin filaments recently dissociated from paracrystals appear to maintain a reduction in the variation in twist imposed by the paracrystals. This is consistent with slow transitions between discrete states of twist (Fig. 1 *b*). It is important to consider artifacts that could influence the present results. Images (e.g., Fig. 2, *a* and *c*) show that some free filaments exist even after extended incubations with high concentrations of $MgCl_2$. While every attempt was made to include

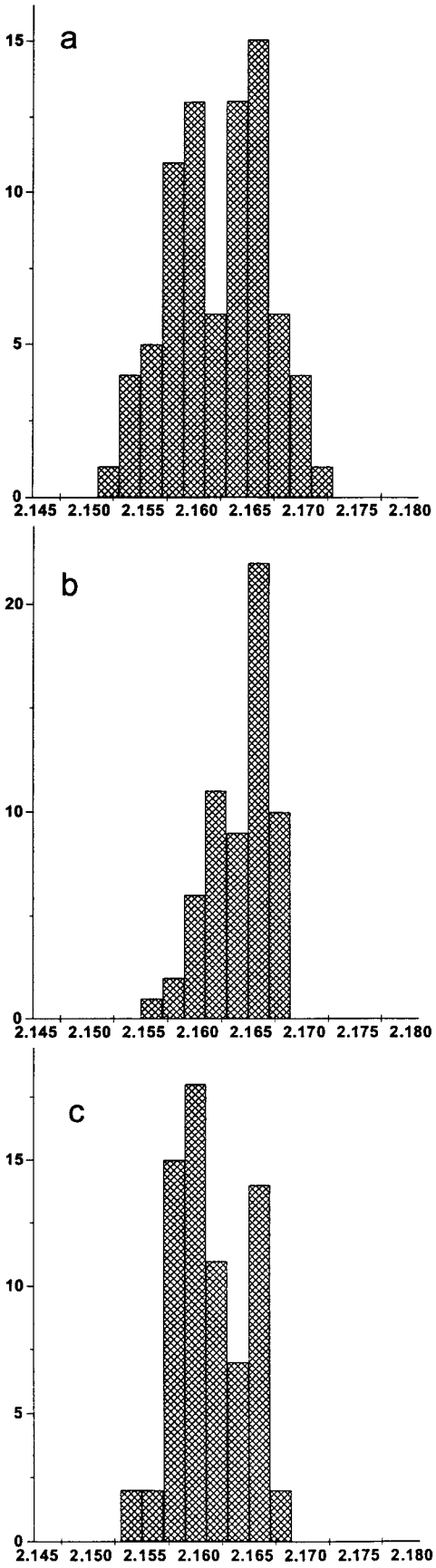

FIGURE 3 The distribution of twist (in actin subunits per turn of the 59-Å-pitch left-handed helix) for free actin filaments (*a*), actin paracrystals (*b*), and filaments observed shortly after dissociation from paracrystals (*c*). It can be seen that the variance in the twist of the filaments dissociated from paracrystals (*c*) is smaller than that of the free filaments (*a*). The means and standard deviations for these distributions are (*a*) 2.1622±.0044, (*b*) 2.1641±.0030, and (*c*) 2.1613±.0030.

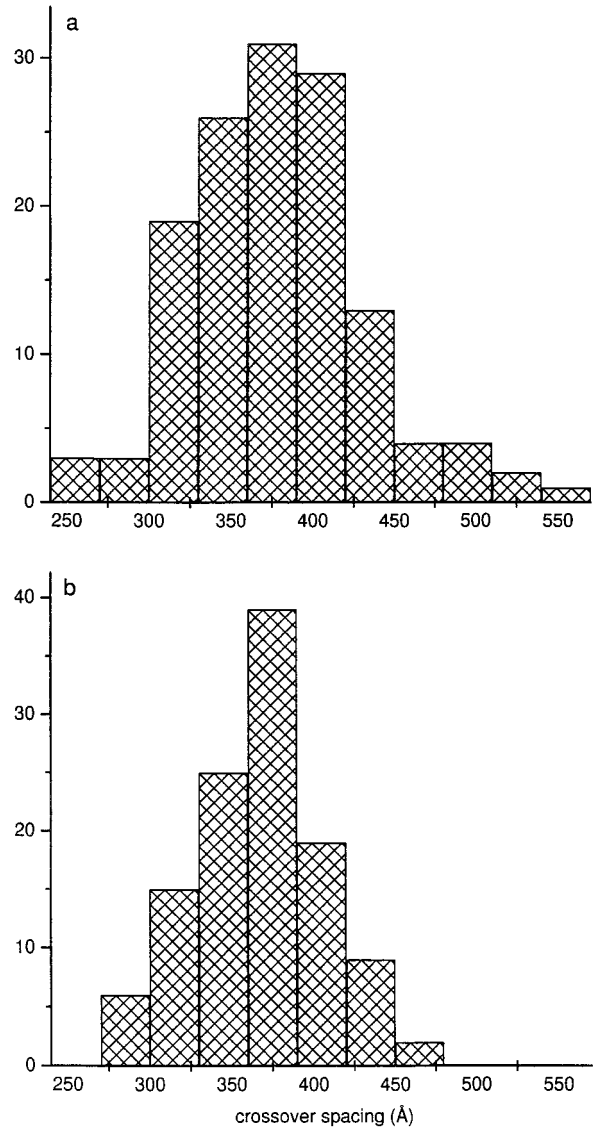

FIGURE 4 The distribution of cross-over spacings determined from free filaments (incubated in 10 mM Mg^{2+}) in ice (*a*, mean = 378 Å, σ = 54 Å) and from filaments dissociated from paracrystals (*b*, mean = 367 Å, σ = 40 Å). The cross-over spacings were determined by the method of cross-correlation against reference projections (Zhu et al., 1997) and provide an independent measure of the variability of twist from that used in Fig. 3. A similar reduction in this variability is seen (*b*) after filaments have been released from paracrystals.

only filaments that could be seen emerging from a paracrystal (Fig. 2, *b* and *d*) for the postparacrystal filaments, it is likely that some filaments were included that had not pre-

viously been part of a paracrystal. This would only serve to weaken the differences observed between the control filaments and the postparacrystal filaments and could not explain the actual differences observed.

Is it possible that the reduction in variability of twist after dissociation from a paracrystal results from an influence of the paracrystals that is propagated along the filament to a segment that is no longer within a paracrystal? We cannot exclude this possibility, but it appears unlikely, because the binding of cofilin along an actin filament does not propagate a change in twist to a segment of the same filament that is undecorated (McGough et al., 1997).

We have observed that there is a small but significant shift in the mean twist between filaments in paracrystals (2.164, Fig. 3 *b*) and filaments dissociated from paracrystals (2.161, Fig. 3 *c*). We think that the explanation for this most likely resides in the supercoiling of actin filaments that can frequently be seen to occur in paracrystal bundles (Fig. 2, *a* and *c*). An x-ray diffraction study of actin gels had previously described the indications for the existence of supercoiling (Lednev and Popp, 1990). The supercoiling appeared when filaments in the gels made regular contacts, as they do in paracrystals. The features associated with supercoiling were observed simultaneously with a change in the F-actin cross-overs, from 370 Å in the free filaments to 355 Å in the tightly packed bundles. This would correspond to a change in the twist from ~2.160 (free filaments) to ~2.167 (tight bundles). The change that we see from the paracrystals (2.164) to the dissociated filaments (2.161) is smaller but is in the same direction. Thus there could be two determinants of the observed twist in paracrystals: the discrete distribution of states populated by the actin subunits (Fig. 1 *b*) and an additional continuous deformation compensated for by supercoiling. Rapid release of the supercoiling would then lead to a change in the mean observed twist, without changing the discrete distribution of states.

For example, consider 520 actin subunits (a 1.4-μm-long filament) having 241 turns of the left-handed 59-Å-pitch helix. This filament would have a mean twist of 2.158 units/turn. If this filament were forced to undergo a change of symmetry to 2.167 units/turn to maximize packing interactions with neighboring filaments, it would have a symmetry described by 520 actin subunits in 240 turns of the left-handed 59-Å-pitch helix, or one less left-handed turn than in the free filament. It could compensate for this change in twist by undergoing one left-handed supercoil turn in 1.4 μm. If these packing interactions were suddenly removed, the supercoil turn would rapidly disappear as the local symmetry reverted to 2.158 units/turn. This would occur on a time scale different from that of the discrete local changes in twist between subunits.

An interesting question involves determination of the time scale of the slow component of torsional motions in F-actin. We have shown in this paper that the time scale must be longer than seconds, as otherwise filaments would relax into more disordered states between their dissociation from paracrystals and the time at which they are either stained or frozen. It has not been possible to look at very long time scales (minutes or hours), as filaments have been prepared on a grid and will become dehydrated over such times. Furthermore, at long time points paracrystals would be completely dissociated, and it would be impossible to select filaments that are seen to be emerging from a paracrystal. One would thus be weakening any effect by including filaments that were never part of a paracrystal before the reduction of Mg^{2+} concentration. Other methods will need to be used to answer this question. However, our present results suggest that the spectroscopically observed submillisecond motions within F-actin (Mihashi et al., 1983; Yoshimura et al., 1984; Prochniewicz et al., 1996; Rebello and Ludescher, 1998) probably represent domain motions rather than cumulative random angular disorder in subunit positions (Egelman et al., 1982).

While a simple helical polymer like polytetrafluoroethylene (Teflon) has been shown to have a continuously variable distribution of twist (McMahon and McCullough, 1965; Egelman and DeRosier, 1982) (such as that shown in Fig. 1 *a*), it is much more likely that the complex subunit-subunit protein interface in F-actin would involve discrete states. While we cannot directly observe such discrete states, the long-lived changes in angular order that we observe are most consistent with such a situation. The present findings have implications for many cellular systems that involve F-actin, from striated muscle to the cytoskeleton of nonmuscle cells, because the ability of actin to interact with myosin cross-bridges on an incommensurate lattice, as well as to form polymorphic bundles, will likely depend upon the torsional properties of F-actin (DeRosier and Tilney, 1982). The observed rotations of actin subunits within actin bundles (Sherman et al., 1999) and muscle (Taylor et al., 1993) may be due to discrete switching between subunit-subunit contacts within the filament, rather than continuous torsional elasticity. Changes in actin's mean twist have been observed within actively contracting muscle, but these correspond to only ~0.2°/subunit when averaged over entire filaments (Takezawa et al., 1999). It is clear, however, that individual subunits have the ability to rotate by at least 5°. Because the torsional variability in F-actin is the largest internal freedom observed for actin, it is possible that the absence of force production in modified F-actin results from an inhibition of this freedom (Drummond et al., 1990; Prochniewicz and Yanagida, 1990; Schwyter et al., 1990).

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