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The accessibility of etheno-nucleotides to collisional quenchers and the nucleotide cleft in G- and F-actin

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Abstract

Recent publication of the atomic structure of G-actin (Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F., & Holmes, K.C., 1990, *Nature 347*, 37–44) raises questions about how the conformation of actin changes upon its polymerization. In this work, the effects of various quenchers of etheno-nucleotides bound to G- and F-actin were examined in order to assess polymerization-related changes in the nucleotide phosphate site. The Mg^{2+}-induced polymerization of actin quenched the fluorescence of the etheno-nucleotides by approximately 20% simultaneously with the increase in light scattering by actin. A conformational change at the nucleotide binding site was also indicated by greater accessibility of F-actin than G-actin to positively, negatively, and neutrally charged collisional quenchers. The difference in accessibility between G- and F-actin was greatest for I^-, indicating that the environment of the etheno group is more positively charged in the polymerized form of actin. Based on calculations of the change in electric potential of the environment of the etheno group, specific polymerization-related movements of charged residues in the atomic structure of G-actin are suggested. The binding of S-1 to ϵ-ATP–G-actin increased the accessibility of the etheno group to I^- even over that in Mg^{2+}-polymerized actin. The quenching of the etheno group by nitromethane was, however, unaffected by the binding of S-1 to actin. Thus, the binding of S-1 induces conformational changes in the cleft region of actin that are different from those caused by Mg^{2+} polymerization of actin. The pH dependence of collisional quenching shows that the cleft region is more accessible to collisional quenchers at pH 7 than at higher pH and suggests that changes in the environment of the cleft might contribute to the faster polymerization rates observed at lower pH.

Keywords: actin; collisional quenching; nucleotide site; polymerization; structure

The recently published atomic structure of G-actin (Kabsch et al., 1990) facilitates the search for specific conformational changes in the actin molecule at the atomic level. Because the X-ray crystallographic structure was solved for G-actin and not F-actin, a question of particular significance is how the conformation of G-actin changes upon its polymerization to F-actin. It is known that actin hydrolyzes ATP to ADP during polymerization (Straub & Feuer, 1950). Although the importance of this hydrolysis is not fully understood (see Korn et al., 1987, and references therein), it indicates a significant conformational change in the nucleotide cleft of actin. Also indicative of a conformational change in the cleft are the different exchange rates of ATP and ADP in G-actin and F-actin (Martonosi et al., 1960).

Studies of the nucleotide cleft in actin were facilitated by the development of the fluorescent etheno-labeled nucleotide analogs, ϵ-ATP and ϵ-ADP (Secrist et al., 1972; Leonard, 1984). Early work utilizing etheno-nucleotides led to a controversy over whether G- and F-actin showed different fluorescent lifetimes for bound ϵ-ATP (Mihashi & Wahl, 1975; Harvey et al., 1977). A tight correlation between the quenching of ϵ-ATP fluorescence and polymerization of actin would link conformational changes in the cleft region of actin to an important actin function.

There are still other unanswered questions about which changes on the actin molecule are significant for polymerization. Although it is well documented that actin poly-

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Abbreviations: DTT, dithiothreitol; ϵ-ATP, etheno-ATP; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol bis(*b*-aminoethyl ether)-*N*, *N*,-*N'*, *N'*-tetraacetic acid; HMM, heavy meromyosin; TCA, trichloroacetic acid; OD, optical density; S-1, myosin subfragment-1; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; TLCK, *N*-α-para-tosyl-L-lysine chloromethyl ketone.

merization is very pH dependent, a clear explanation for this phenomenon is lacking. A sharp increase in critical concentration and polymerization halftimes occurs between pH 6.6 and pH 7.4 (Zimmerle & Frieden, 1988a,b; Wang et al., 1989), which suggests that an increase in the positive charge of some region on actin is important for its polymerization. Interestingly, the pK_{a2} of ATP and ADP is in the range of 6.5–6.7 (Secrist et al., 1972). Possibly, the charge on the terminal phosphate of the nucleotide may affect the polymerization of actin.

Much evidence has accumulated on myosin-induced conformational changes in F-actin (for a review see Oosawa [1983]) and more recently in G-actin by utilizing the different effects of S-1(A-1) and S-1(A-2) isozymes of myosin on actin polymerization. S-1(A-1) binds to G-actin and induces its polymerization much like Mg^{2+} and high salt (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991). S-1(A-2) also binds to G-actin but polymerization is far slower than that of acto-S-1(A-1) (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991; Valentin-Ranc et al., 1991). It has also been reported that the cross-linking of acto-S-1 induces cooperative changes in the actin filament, which affect the actomyosin ATPase activity of uncross-linked acto-S-1 (Ando, 1989). Attempts to link the nucleotide cleft to myosin-induced changes in actin have met with mixed results. For instance, changes in the fluorescence polarization of ϵ-ADP upon the binding of HMM but not S-1 raised the question of whether actin's nucleotide site affects actomyosin interactions (Yanagida & Oosawa, 1978). In a different type of study, the fluorescence lifetime of ϵ-ADP in F-actin was reduced in a cooperative manner upon the binding of either S-1 or HMM (Harvey et al., 1977).

The present study examines the accessibility of actin-bound etheno-nucleotides to quenchers of fluorescence. The rate of fluorescence quenching of bound etheno-nucleotides during Mg^{2+}-induced polymerization was correlated with the rate of polymerization of actin. Further characterization of the conformational change in the nucleotide cleft upon polymerization was undertaken by using positively charged (thallium), negatively charged (iodide), and neutral (nitromethane) collisional quenchers. The results showed an increased accessibility of the bound etheno-nucleotide to each collisional quencher and an increase in positive charge of the environment of the etheno group upon Mg^{2+}-induced polymerization of actin. A similar analysis indicated that the binding of S-1 also increased the positive charge of the cleft. The greatest changes in the accessibility of the bound etheno-nucleotide were caused by changing the pH of the buffer. At lower pH, the accessibilities to collisional quenchers increased, but the differences in accessibilities between G- and F-actin decreased. The data from the accessibilities to differently charged collisional quenchers have been used to estimate the magnitude of a change in the charged environment of the etheno group (Ando & Asai, 1980; Ando et al., 1980) and to propose possible movements of residues that may account for the observed changes.

Results

Conformational changes upon Mg^{2+}-induced polymerization of actin

The polymerization of ϵ-ATP–G-actin by 2 mM $MgCl_2$ partially quenches the fluorescence of the bound etheno-nucleotide as shown in Figure 1A. Polymerization of actin, as monitored by light scattering, occurred simultaneously with the quenching of the etheno-nucleotide's fluorescence (Fig. 1A). Moreover, the rate of quenching of etheno-nucleotide's fluorescence (0.0053 ± 0.0002 s^{-1}) and the rate of actin polymerization (0.0051 ± 0.0002 s^{-1}) were identical (rate constants were determined by fitting curves to a single exponential). The summation of the normalized plots of the two transitions yielded a constant value (Fig. 1B) illustrating the equality of the two rates. The data suggest that the fluorescent quenching of the bound etheno-nucleotide results from conformational changes in the nucleotide cleft that accompany the conversion of G- to F-actin.

The extent of fluorescent quenching of the actin-bound etheno-nucleotide upon $MgCl_2$-induced polymerization of actin was typically 20% when using an excitation wavelength of 315 nm. The excitation wavelength of 315 nm was chosen for this experiment, as the fluorescence intensities of actin-bound and free etheno-nucleotides are approximately the same (Thames et al., 1974; data not shown). Thus, the decrease in fluorescence intensity upon polymerization of actin cannot result from the release of etheno-nucleotide from actin.

The changes in the environment of the nucleotide cleft were further characterized by using differently charged collisional quenchers. The Stern–Volmer quenching constants of bound etheno-nucleotides were expressed as a percentage of the quenching constant for free etheno-nucleotide. These values were taken to indicate the relative accessibility of the bound etheno-nucleotide to the collisional quencher. The relative accessibilities of the etheno group to both positively (Tl^+) and negatively (I^-) charged collisional quenchers increased upon $MgCl_2$-induced polymerization of actin (Fig. 2). The increase in relative accessibility of the etheno group was greater for iodide, a negatively charged quencher, which indicates that the environment around the etheno group becomes more positively charged upon polymerization of actin (Fig. 2). Harvey and Cheung (1976) previously reported low accessibilities of G-actin-bound ϵ-ATP to collisional quenchers, which is consistent with our results.

In order to selectively monitor the bound nucleotide, the excitation wavelength chosen for studies with collisional quenchers was 340 nm. At this wavelength, the fluorescence intensity of bound etheno-nucleotide is about

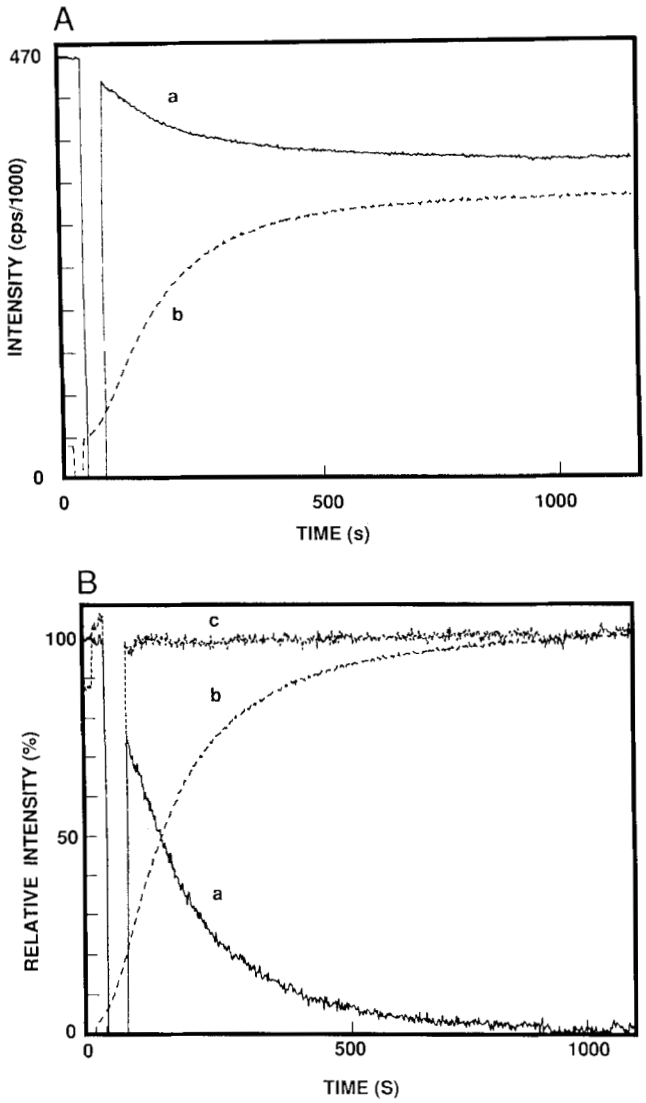

Fig. 1. A: Time dependence of the polymerization of actin and the quenching of fluorescence of the etheno-nucleotide. The polymerization of actin (3.0 μM) was induced by the addition (indicated by the early lapses in the data) of 2 mM $MgCl_2$ to G-actin in ATP-free G-buffer. Excess ϵ-ATP was removed from the actin by a Penefsky spin column. The intensity of fluorescence (a) was measured using excitation and emission wavelengths of 315 nm and 420 nm, respectively. Light scattering (b) was monitored from a 90° angle at 350 nm. Measurements were performed at 23 °C. **B**: Normalized time course of actin polymerization and fluorescence quenching. The data from Figure 1A were normalized so that the maximum intensity of each time course was taken as 100% and the minimum intensity of each curve was set to 0%. Equal rates of change in scattering and fluorescence intensities are indicated by a constant value for the sum (c) of the light scattering (b) and fluorescence quenching (a).

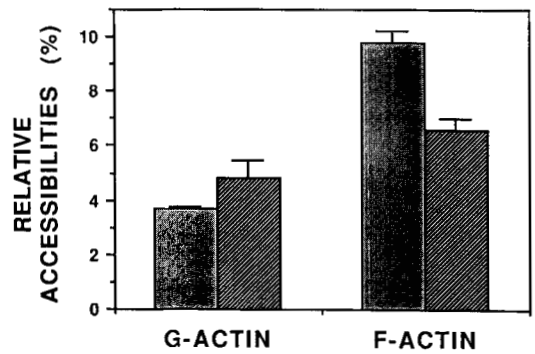

Fig. 2. Relative accessibilities of G- and F-actin to negatively and positively charged collisional quenchers of etheno-nucleotide fluorescence. Stern–Volmer quenching constants for thallium (▨) and iodide (▩) quenching of actin bound etheno-nucleotides were divided by the quenching constant for free ϵ-ATP to yield the relative accessibility. Determinations of the Stern–Volmer quenching constants were made in ATP-free G-buffer, with 3.0 μM actin at 23 °C. Excess ϵ-ATP was removed by Penefsky spin columns. F-actin was formed by polymerization of G-actin with 2 mM $MgCl_2$ for 15 min. Excitation and emission wavelengths were 340 nm and 420 nm, respectively. Bars represent standard errors ($P < 0.001$).

six times greater than that of the free etheno-nucleotide (Miki et al., 1974; data not shown). Consequently, on samples from which the free etheno-nucleotide has been removed by Penefsky spin columns, the signal from any remaining free etheno-nucleotides excited at 340 nm is very low. At an excitation wavelength of 350 nm, the fluorescence intensity from actin-bound etheno-nucleotides is about 15-fold greater than that from free etheno-nucleotides (data not shown). Nevertheless, similar relative accessibilities of etheno-nucleotides were obtained by using either 340-nm or 350-nm excitation wavelengths (data not shown). (The intensity of the fluorescent signal was much lower from the 350-nm excitation wavelength and consequently more noisy [data not shown].)

The differences between G- and F-actin in relative accessibilities of the bound nucleotide to quenching could not be attributed to differences between bound ϵ-ATP and ϵ-ADP. The ϵ-ADP–G-actin was prepared by treatment of ϵ-ATP–G-actin with hexokinase. TLC of etheno-nucleotides extracted from the hexokinase-treated actin confirmed that a large proportion of the ϵ-ATP had been hydrolyzed (data not shown). Both ϵ-ATP–G-actin and ϵ-ADP–G-actin had similar relative accessibilities to quenching by iodide (Fig. 3). As in the present study, only small differences between the Stern–Volmer quenching constants for free ϵ-ADP and ϵ-ATP have been detected previously (Ando & Asai, 1980). On the other hand, we cannot exclude the possibility that magnesium binding to ADP-actin could induce conformational changes that might contribute to the differences in accessibility between G-actin and Mg^{2+}–F-actin.

pH effects

The fluorescence intensities of bound and unbound etheno-nucleotides were elevated somewhat (data not shown) by either lowering the pH from 7.6 to 7.0 (free ϵ-ATP,

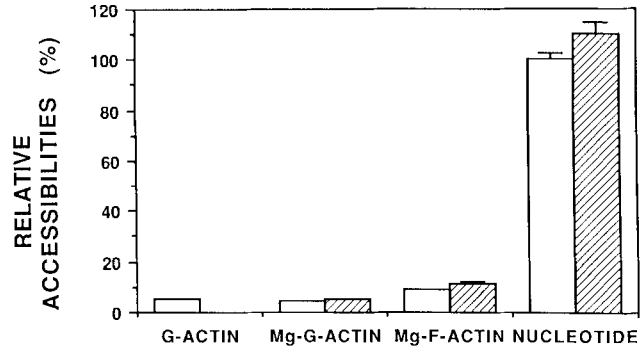

Fig. 3. Relative accessibilities of ϵ-ADP and ϵ-ATP and their actin-bound derivatives to collisional quenchers. Relative accessibilities (normalized to ϵ-ATP) were determined from iodide quenching of ϵ-ATP and actin-bound derivatives before (□) and after (▨) treatment with 70 U/mL hexokinase to hydrolyze ϵ-ATP to ϵ-ADP. G-actin refers to actin (3.0 μM) in ATP-free G-buffer. Mg-G-actin denotes actin (3.0 μM) in ATP-free G-buffer plus 20 μM $MgCl_2$ and 2 mM glucose. Mg-F-actin refers to ϵ-ADP-F-actin derived from the Mg-G-actin by addition of 2 mM $MgCl_2$. Nucleotide denotes free etheno-nucleotides in the same buffer as Mg-G-actin. Determinations were made at 23 °C with excitation and emission wavelengths of 340 nm and 420 nm, respectively. Excess nucleotide was removed from G-actin by a Penefsky spin column. Bars represent standard errors ($P < 0.005$).

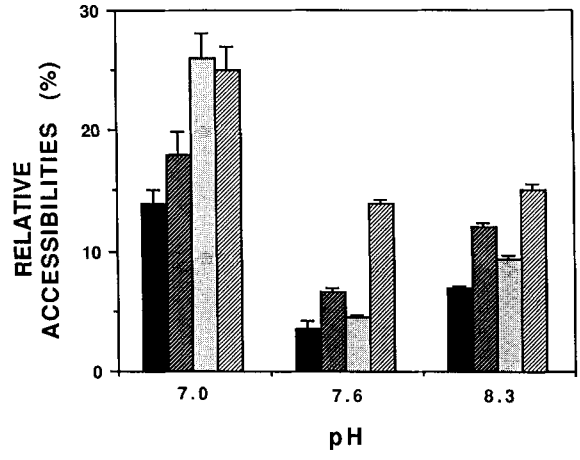

Fig. 4. Variation in relative accessibilities of G- and F-actin with pH. The relative accessibilities for G-actin to iodide (■), G-actin to nitromethane (▩), F-actin to iodide (▩), and F-actin to nitromethane (▨) were determined at three different pH values. At pH 7.6, ATP-free G-buffer was used. At pH 7.0, 15 mM imidazole was added to the ATP-free G-buffer. At pH 8.3, 15 mM Tris was added to the ATP-free G-buffer. F-actin was formed by addition of 2 mM $MgCl_2$ for 15 min. Determinations were made at 23 °C with excitation and emission wavelengths of 340 nm and 420 nm, respectively. Excess nucleotide was removed from G-actin by a Penefsky spin column. Bars represent standard errors ($P < 0.005$).

54%; ϵ-ATP-G-actin, 83%; and ϵ-ADP-F-actin, 100%, increase in fluorescence intensity) or by raising the pH from 7.6 to 8.3 (about 20–30% increase in fluorescence intensity). A similar pattern of pH dependence to the above intensity changes was observed for the relative accessibilities of bound etheno-nucleotides to collisional quenchers (Fig. 4). In contrast, the Stern–Volmer quenching constants of free ϵ-ATP were virtually unaffected by pH (data not shown). These data raise the possibility that the charge on the etheno-nucleotide may be an important factor in the pH effects. The complex pH profile suggests that more than one aspect of the actin structure is affected by pH, and the greatest change is associated with the reduction of pH from 7.6 to 7.0.

The relative accessibilities of ϵ-ATP-G-actin and ϵ-ADP-F-actin to collisional quenchers are more similar at pH 7.0 than at higher pH (Fig. 4). This result suggests that the conformations of the nucleotide cleft in G- and F-actin may also be more similar at pH 7.0 than at higher pH and may thereby promote the enhanced polymerization of actin at lower pH values (Zimmerle & Frieden, 1988a,b; Wang et al., 1989).

The effect of S-1 binding on the nucleotide cleft

The two isozymes of S-1, S-1(A-1) and S-1(A-2), which differ in the attached light chains, have different effects on the polymerization of G-actin (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991). S-1(A-1) induces rapid polymerization of actin, whereas S-1(A-2) alone is deficient at nucleating actin (but promotes fast elongation) and thus polymerizes it at a slow rate (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991). The different effects of the two isozymes on G-actin enabled a comparison between G-acto-S-1(A-2) and F-acto-S-1(A-1). Although the fluorescent intensity of ϵ-ADP-F-acto-S-1(A-1) was somewhat lower than that of ϵ-ATP-G-acto-S-1(A-2) (data not shown), the Stern–Volmer quenching constants of ϵ-ATP-G-acto-S-1 and ϵ-ADP-F-acto-S-1 were similar (Fig. 5A,B). Thus, the effects of S-1 binding on the relative accessibilities of the nucleotide cleft to collisional quenchers cannot be attributed to the polymerization of actin.

The binding of S-1 to actin had no detectable effect on the Stern–Volmer quenching constant by nitromethane (Fig. 5A). However, the actin-bound etheno-nucleotide was much more accessible to collisional quenching by iodide when S-1 was bound to actin (Fig. 5B). This result indicates that the binding of S-1 to actin increases the positive charge of the environment of the etheno group. Because a similar increase in positive charge occurs during Mg^{2+}-induced actin polymerization, this change might be related to the elongation of actin filaments.

The accessibility to collisional quenchers of actin-bound etheno-nucleotides in acto-S-1 increased as the pH was lowered from 7.6 to 7.0 (Fig. 6). This result is similar to the pH dependence of G- and F-actin illustrated in Figure 4. Another important result shown in Figure 6 is

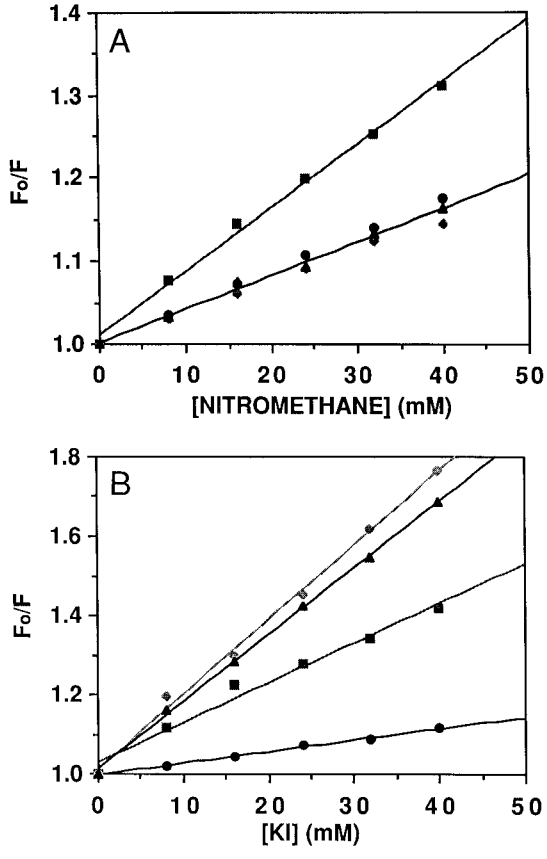

Fig. 5. **A**: Stern–Volmer plot of etheno-nucleotide quenching by nitromethane. The fluorescence of actin-bound etheno-nucleotide was quenched by the addition of nitromethane (0–40 mM). The unquenched fluorescence intensity, F_0, was divided by the quenched fluorescence intensity, F, and plotted versus the concentration of nitromethane. G-actin (●), 2 mM $MgCl_2$-polymerized F-actin (■), F-acto-S-1(A-1) (▲), and G-acto-S-1(A-2) (◆) were incubated for 10 min in ATP-free G-buffer prior to quenching by nitromethane; 3.0 μM actin and 6.0 μM S-1 concentrations were used in ATP-free G-buffer, pH 7.6. Determinations were made at 23 °C with excitation and emission wavelengths of 340 nm and 420 nm, respectively. Excess nucleotide was removed from G-actin by a Penefsky spin column. The standard errors in determining the Stern–Volmer constants were less than 13% ($P < 0.005$). **B**: Stern–Volmer plot of etheno-nucleotide quenching by iodide. G-actin (●), 2 mM $MgCl_2$-polymerized F-actin (■), F-acto-S-1(A-1) (▲), and G-acto-S-1(A-2) (◆) were quenched by iodide (0-40 mM). Experimental conditions are the same as in Figure 5A. The standard errors in determining the Stern–Volmer constants were less than 8% ($P < 0.005$).

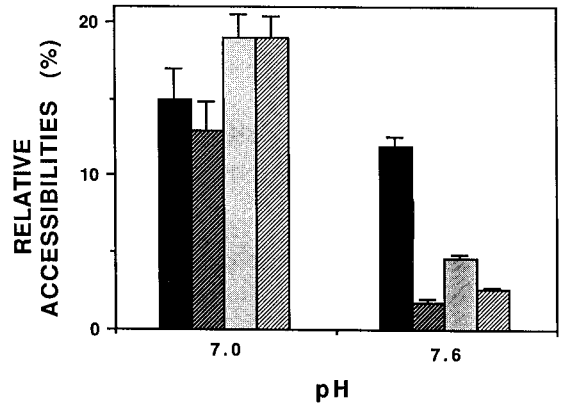

Fig. 6. The pH dependence of relative accessibilities of acto-S-1 (A-1 + A-2) in the presence and absence of $MgCl_2$. The relative accessibilities for acto-S-1 in the absence of $MgCl_2$ to iodide (■) and to nitromethane (▨), and for acto-S-1 in the presence of 2 mM $MgCl_2$ to iodide (▨) and to nitromethane (▨) were determined at two different pHs. Actin (3.0 μM) and S-1 (6.0 μM) were incubated for 15 min before quenching. Experimental conditions were the same as for Figure 4. Bars represent standard errors ($P < 0.005$).

that the addition of 2 mM $MgCl_2$ decreases the accessibility of ε-ADP in acto-S-1 to collisional quenchers, particularly iodide. That this inhibition is the result of a slow conformational change in F-actin was indicated by a time-dependent (on the order of seconds) increase in the fluorescence intensity of ε-ADP in acto-S-1 partially quenched with iodide upon the addition of 2 mM $MgCl_2$ (data not shown). By comparison with Figure 4, Figure 6 shows that the binding of S-1 to Mg^{2+}-polymerized F-actin also reduces the accessibility of ε-ADP to collisional quenchers. If rigor-bound S-1 partially covers the nucleotide cleft, the accessibility of the cleft to collisional quenchers could be reduced.

Discussion

Conformational changes upon Mg^{2+}-induced polymerization of actin

The present study shows that the rates of actin polymerization and quenching of etheno-nucleotide fluorescence during actin polymerization are identical (Fig. 1B). This result indicates a close relationship between conformational changes in the nucleotide cleft of actin and the polymerization of this protein.

The structure of the nucleotide cleft in actin (Kabsch et al., 1990) is similar to that of a number of ATP-hydrolyzing enzymes such as phosphoglycerate kinase (Yon et al., 1990; Flaherty et al., 1991). There is strong evidence that the cleft in phosphoglycerate kinase can assume an open and a closed conformation, which may be related to its function (Yon et al., 1990). The possibility that similar conformational changes occur in actin is consistent with the increase in the accessibility of the etheno-nucleotide to collisional quenchers during Mg^{2+}-induced polymerization of actin. In the absence of such conformational change, the contacts between actin molecules in F-actin might be expected to sterically decrease rather than increase the accessibility of the cleft. Also consistent with the opening of the nucleotide cleft upon actin polymerization is the increased reactivity to reductive methylation of Lys-336 (which is located near the adenosine moiety) in F-actin over that in G-actin (Lu & Szilagyi, 1981). From these results, it would appear that the region

around the adenosine moiety becomes more open upon actin polymerization. The decrease in nucleotide exchange rates upon actin polymerization (Martonosi et al., 1960) may be due to tighter interactions between amino acids and other sites on the nucleotide such as the phosphate groups. Possibly, a local conformational change or a rotation between the domains of actin upon polymerization might cause actin to grip the phosphates while increasing the accessibility of the adenosine group.

The accessibility of the nucleotide cleft to collisional quenchers was very sensitive to pH. The accessibility increased several-fold upon lowering the pH from 7.6 to 7.0 (Figs. 4, 6) and increased to a lesser extent upon elevating the pH from 7.6 to 8.3 (Fig. 4). The fluorescence intensity of both free and bound etheno-nucleotides shows a similar pattern of sensitivity to pH, although to a much lesser degree. This apparent correlation suggests that the pH effects on the nucleotide itself may be related to the changes in relative accessibilities of the bound nucleotide. Alternatively, conformational changes in actin may arise from pH-sensitive amino acids such as histidine, or the sensitivity of metal binding to pH may contribute to the observed effects. The reported pH sensitivity of actin polymerization (Zimmerle & Frieden, 1988a,b; Wang et al., 1989) may also be related to these changes in actin structure.

The accessibility of the nucleotide cleft to collisional quenchers upon polymerization of actin was increased to varying degrees for differently charged quenchers (iodide, thallium, and nitromethane). The increase was greater for the negatively than the positively charged quencher (Fig. 2). This difference indicates that the environment surrounding the etheno group became more positively charged in F-actin. Equations for analyzing the magnitude of such changes have been developed and utilized in proteins (Ando et al., 1980). The change in the electric potential ($\Delta\Phi$) can be expressed as

$$\Delta\Phi = \Phi_f - \Phi_g = \frac{kT}{z_q e} \ln(K_g/K_f), \tag{1}$$

in which k is Boltzman's constant, T is the absolute temperature, z_q is the charge number of the quencher, e is the magnitude of the charge of an electron, Φ_f and Φ_g are the electric potentials of F- and G-actin, respectively, and K_g and K_f are the Stern-Volmer quenching constants multiplied by the fluorescence lifetime of G- and F-actin, respectively (Ando & Asai, 1980; Ando et al., 1980). If it is assumed that the increase in positive charge in the environment of the etheno group repels quenching by Tl^+ to the same extent as it enhances quenching by I^-, then the above equation can be used with the data[2] in Figure 2 to calculate the change in electric potential to be about +0.0084 V. The fit based on this assumption takes into account the differences in both the fluorescence lifetime and accessibility to neutrally charged collisional quenchers between G- and F-actin.

The electric potential is related to the ensemble average distance (r) between a charge (Z_f) in the environment of a fluorophore and the fluorophore's complex with a collisional quencher by the equation:

$$\Phi = \frac{Z_f e}{D} \frac{e^{a/\lambda}}{1 + a/\lambda} \frac{e^{-r/\lambda}}{r}, \tag{2}$$

in which D is the dielectric constant of the solvent, a is the mean distance of closest approach of ions, and λ is the Debye radius[3] (Ando & Asai, 1980; Ando et al., 1980). The second term is a correction factor for high ionic strength (greater than 100 mM). It is assumed that the dielectric constant and ionic strength near the nucleotide site of actin is the same as in the external solution. For ionic strengths below 40 mM and $r < \lambda$, it is easy to show that Equation 2 can be simplified to

$$\Delta\Phi = \frac{Z_f e}{D}\left(\frac{1}{r_f} - \frac{1}{r_g}\right) \quad \text{or} \quad \Delta r = r_f - r_g = \frac{r_g^2}{r_g + \dfrac{Z_f e}{D\Delta\Phi}}, \tag{3}$$

in which r_f and r_g are the ensemble average distances in F- and G-actin, respectively, and Δr is their difference (for derivation, see Ando & Asai [1980]). Because r_g can be determined from the X-ray crystallographic structure of G-actin, and all other variables from Equations 1 and 3 are known or can be estimated, the magnitude of the shift in the position of a charge near the etheno group from the Mg^{2+}-induced polymerization of actin can be calculated.

The distances from the etheno group to the nearest charged atoms in the atomic structure for G-actin were determined using the FRODO program (PS300 FRODO version 6.5, by John S. Sack, 1988). The position of the etheno group was estimated by using the average of the coordinates for N6 and N1 on ADP in the structure of G-actin. The nearest charged amino acid side chains were Glu-214 at 0.58 nm, Lys-213 at 0.73 nm, Lys-336 at 0.73 nm, and Lys-215 at 0.93 nm (Kabsch et al., 1990). Clearly, the environment around the etheno group is positively charged even in G-actin, as was suggested by the relative accessibility measurements shown in Figure 2. Based on calculations from Equation 3, the increase in positive charge in the etheno group's environment could be explained by moving the Glu-214 0.21 nm farther away from the etheno group or by shifting either Lys-213

[2] The Stern-Volmer quenching constants are 0.0037 mM^{-1} (of iodide) and 0.0048 mM^{-1} (of thallium) for G-actin and 0.0098 mM^{-1} (of iodide) and 0.0066 mM^{-1} (of thallium) for F-actin.

[3] λ is greater than 1.5 nm under the conditions in these experiments.

or Lys-336 0.18 nm closer to the etheno group. A combination of such alterations in the structure of the cleft is quite possible. It has been shown that the reactivity of 8-azido-ATP with Lys-336 increased upon polymerization of G-actin (Hegyi et al., 1986). This result indicates that Lys-336 moves closer to the adenosine moiety upon polymerization and is consistent with the increase in electric potential observed here. On the other side of the nucleotide cleft, Lys-213, Glu-214, and Lys-215 are part of the same α-helix (Kabsch et al., 1990), and a portion of this α-helix, 202–204, is a proposed actin-actin contact site (Holmes et al., 1990). It would be easy to imagine that the polymerization of actin might twist this α-helix bringing the lysines closer to the etheno group and the glutamate farther away. Another possible scenario is that the binding of Mg^{2+} to the nucleotide or actin might cause a shift in the nucleotide's position within the cleft.

The effect of S-1 binding on the nucleotide cleft

By a similar analysis, Δr can also be estimated for the binding of S-1 to actin in the absence of $MgCl_2$; if the relative accessibility of the etheno group to uncharged collisional quenchers is unaffected by the binding of S-1 to actin. This assumption is supported by the results of Figure 5A. The decrease in the fluorescence lifetime of the etheno-nucleotide upon the binding of S-1 to actin has been estimated at 12% (Harvey et al., 1977). This value is consistent with the small decrease in fluorescence intensity upon the binding of S-1 to actin observed in the present work. Using this measured decrease in fluorescence lifetime and the Stern–Volmer quenching constants from Figures 4 and 6 (0.0036 mM^{-1} and 0.012 mM^{-1} for G-actin and acto-S-1, respectively), $\Delta\Phi$ is calculated from Equation 1 to be 0.0275 V. If this change in an electric potential reflects the movement of an individual lysine, either Lys-213 or Lys-336, the lysine would move 0.38 nm closer to the etheno group. Glutamate would have to be removed from the immediate vicinity of the etheno group to cause a similar change in the electric potential. The change in electric potential of the nucleotide cleft of actin caused by the binding of S-1 to actin in the absence of $MgCl_2$ is much larger than that from Mg^{2+}-induced polymerization of actin and might possibly involve charged residues from the S-1 molecule itself.

The changes in accessibility of the actin-bound etheno-nucleotide to collisional quenchers primarily reflect the binding of S-1 to actin rather than S-1-induced polymerization of actin. The binding of S-1(A-2), which did not polymerize actin well, had similar effects to S-1(A-1), which polymerized actin rapidly (Fig. 5A,B). There are substantial differences in the effects of S-1 binding on actin in the presence and absence of $MgCl_2$. The binding of S-1 to Mg^{2+}-polymerized actin reduces the cleft's accessibility to collisional quenchers, which might occur if S-1 partially blocked the cleft. This result is consistent with the finding that the reactivity of Lys-336 to reductive methylation is decreased upon binding of S-1 to F-actin (Szilagyi & Lu, 1982). The absence of a large increase in positive charge in the nucleotide cleft upon the binding of S-1 to Mg^{2+}-polymerized actin, as opposed to Ca^{2+}-G-actin, indicates that $MgCl_2$ either induces a conformational change in the F-actin structure, or it may modify the interaction between S-1 and actin.

In summary, $MgCl_2$ decreases the fluorescence intensity of actin-bound etheno-nucleotides and polymerizes actin at the same rates. The polymerization of actin increases the positive charge in the environment of the etheno group and increases the accessibility of the nucleotide cleft to collisional quenchers. The degree to which these changes occur is influenced by pH. The accessibility of the cleft to collisional quenchers is substantially increased upon lowering the pH from 7.6 to 7.0. These pH-dependent changes in the conformation of actin may be related to its polymerizability. The effects of S-1 binding to actin are also sensitive to both pH and the presence of $MgCl_2$. The binding of S-1 to actin in the absence of $MgCl_2$ induces a large increase in the positive charge of the environment of the etheno group. The addition of $MgCl_2$ reduces this effect, which indicates that $MgCl_2$ alters either the conformation of F-actin or the interaction between S-1 and actin.

Materials and methods

Reagents

TLCK-treated α-chymotrypsin, phenylmethylsulfonyl fluoride, bis-Tris, ATP, ϵ-ATP, and β-mercaptoethanol were procured from Sigma Chemical Co. (St. Louis, Missouri). EDTA was purchased from Fisher (Fairlawn, New Jersey). The Bradford protein assay was purchased from Bio-Rad (Richmond, California). Millipore-filtered distilled water and analytical grade reagents were used in all experiments.

Proteins

Purified rabbit skeletal myosin was prepared as described before (Godfrey & Harrington, 1970). S-1 was obtained from the myosin with α-chymotrypsin and separated according to Weeds and Pope (1977). The concentration of S-1 was measured spectrophotometrically by using $E^{1\%}_{280\,nm} = 7.5\ cm^{-1}$ (Wagner & Weeds, 1977). Rabbit skeletal muscle actin was extracted from acetone powder (Spudich & Watt, 1971). Its concentration was determined spectrophotometrically by using $E^{1\%}_{280\,nm} = 11.0\ cm^{-1}$ (West et al., 1967).

Preparation of ϵ-ATP–G-actin

G-actin, in G-buffer (0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.2 mM ATP, 2 mM Tris, pH 7.6), was dia-

lyzed for 4 h versus 20 μM ATP G-buffer. This actin was then polymerized by adding 1 mM $MgCl_2$ and then diluted to 2 mg/mL with a 10-fold molar excess of ϵ-ATP. The actin was subsequently dialyzed against two changes of 20-mL volumes of ϵ-ATP G-buffer. Actin that was not depolymerized was removed by centrifugation for 2 h at 40,000 RPM in a Beckman Ultracentrifuge. Unbound ϵ-ATP was removed immediately prior to measurements by Penefsky columns (G-50 Sephadex) equilibrated with ATP-free G-buffer. (Control experiments using radioactive ATP have shown that free ATP is efficiently removed by this method.) The concentration of the protein was estimated by Bradford protein assay as modified by Bio-Rad (Bradford, 1976). The amount of ϵ-ATP present was estimated by comparison of the absorbance of the supernatant of TCA-precipitated protein with known amounts of ϵ-ATP dissolved in similar buffer. The extent of incorporation varied between 0.5 and 1.0 mole of ϵ-ATP per mole of actin, which is similar to the results obtained by other methods (Mihashi & Wahl, 1975).

Preparation of ϵ-ADP-G-actin

ϵ-ATP-G-actin was treated with 20 μM $MgCl_2$, 70 U/mL hexokinase (in G-buffer), and 2 mM glucose for 3 h as described (Pollard, 1984). The hydrolysis of ϵ-ATP to ϵ-ADP was confirmed by TCA precipitation of the treated actin and TLC analysis of the supernatant (Schafer et al., 1978).

Fluorescence measurements and quenching

Fluorescence measurements were made in a Spex Fluorolog Spectrofluorimeter (Spex Industries, Inc., Edison, New Jersey) at 23 °C. Excitation and emission wavelengths are specified in the legends to the figures. Light scattering measurements were made at 90° to the incident beam.

Collisional quenchers, KI, TlCl, and nitromethane, were dissolved in G-buffer. The quenchers did not alter the pH of the buffer by more than 0.1 pH units at the concentrations employed. Neither nitromethane nor TlCl induced polymerization of G-actin as monitored by light scattering. KI induced polymerization only at concentrations of 40 mM or greater. Thus, only concentrations of KI below 40 mM were used. Concentrated stock solutions of the collisional quenchers were prepared fresh each day. The dilution of the actin sample by addition of the quencher was kept to a minimum. Quenching data were analyzed by Stern-Volmer plots (Ando & Asai, 1980). The Stern-Volmer constants had standard deviations of 10–30% for separate determinations. The accessibilities of samples were expressed relative to the accessibility of free ϵ-ATP to collisional quenchers measured under identical conditions.

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