A Fluorescent Probe for Conformational Changes in Skeletal Muscle G-Actin*

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Carl Frieden, Deborah Lieberman, and Helen R. Gilbert

From the Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Actin from rabbit skeletal muscle has been modified with the fluorescent label N-iodoacetyl-N'-[(5-sulfo-1-naphthyl)ethylenediamine (1,5-I-AEDANS). Under conditions where the actin is in the unpolymerized form (G-actin), the addition of Mg"+ or KCl results in enhancement of the fluorescence. Titration of the labeled G-actin with Mg"+ at varying concentrations of CaCl2 gives, by extrapolation, a value for the dissociation constant for Mg"+ of 35 nM in the absence of Ca"+ and a calculated value of 10 nM for Ca"+ in the absence of Mg"+. The two metal ions compete with each other. The fluorescence enhancement induced by Mg"+ is reversed by the addition of Ca"+ and both processes are time-dependent, indicating a reversible conformational change of G-actin as a consequence of addition of divalent metal. KCl also enhances the fluorescence of the labeled G-actin but does not appear to compete with the divalent metal ion. The enhancement of the fluorescence is very rapid and any conformational change induced by KCl is probably different from that induced by divalent metal ions. Finally, it is shown that loss of fluorescence of the labeled G-actin may be associated with inactivation of the actin.

At concentrations greater than the so-called critical concentration, the addition of MgCl2 and/or KCl to G-actin induces a polymerization to fibrous or filamentous F-actin. The polymerization process is usually regarded as involving at least two steps: a nucleation step in which several G-actin monomers aggregate, followed by a highly cooperative elongation reaction (1, 2). There have been a number of studies which indicate that actin changes conformation during the G to F transformation (3-5), but little information has been presented to indicate whether conformational changes occur within the G-actin itself or as a consequence of the formation of the double-stranded polymer. In one study of this question, Rich and Estes (6) showed that the addition of KCl to actin at concentrations below those required to form a polymer decreased the rate at which the actin was proteolytically degraded. Since the rate of proteolysis induced by KCl was similar to that of F-actin, these authors postulated a new form of actin which they termed F-actin monomer.

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In this paper, we have labeled G-actin with N-iodoacetyl-N'-[(5-sulfo-1-naphthyl)ethylenediamine (1,5-I-AEDANS) and have observed that the addition of Mg"+ or KCl will enhance the fluorescence at sufficiently low concentrations of actin where no polymerization occurs. Under these conditions, it is possible to obtain the dissociation constants of Mg"+, Ca"+, and KCl for G-actin. Further, the Mg"+-induced fluorescence enhancement is, in part, time-dependent and therefore presumably represents a conformational change after binding of this cation. Finally, we find that the fluorescence of 1,5-I-AEDANS-G-actin is decreased when the actin is inactivated and this decrease can be used as a monitor of inactivation.

MATERIALS AND METHODS

G-actin was extracted from rabbit skeletal muscle acetone powder as described by Spudich and Watt (7). However, 30 min after adding 100 mM KCl and 2 mM MgCl2 to induce polymerization, KCl was added to 0.8 M (rather than 0.6 M) and the solution was stirred at 4°C for 30 min prior to centrifugation. Actin was labeled with 1,5-I-AEDANS by a procedure similar to that of Tawada et al. (8). G-actin (20 mg/ml) in 2 mM Tris/Cl, pH 8, containing 0.2 mM ATP, 0.2 mM CaCl2, and 0.01% NaN3, was treated with an equimolar amount of the dye (solved in dimethylformamide and serially diluted with buffer to give a final concentration of dimethylformamide of less than 0.5%). The reaction was allowed to proceed for 50 h at 4°C in the dark. The labeled F-actin was centrifuged at 160,000 × g for 2 h and suspended in 2 mM Tris/Cl, pH 8, containing 0.2 mM ATP, 0.2 mM CaCl2, and 0.01% NaN3 and dialyzed extensively against this buffer to depolymerize the actin. Before use, the labeled G-actin was clarified by centrifugation at 160,000 × g for 90 min. The concentration of label was determined from the absorbance at 337 nm using an extinction coefficient of 6,000 M-1 cm-1 (8). Actin concentration was measured by the absorbance at 290 nm using an A290/280 = 0.63 and corrected for absorbance due to the dye by subtracting 0.21 A337 from A290 (5, 9). The molar ratio of dye to actin in several preparations was 0.8 ± 0.1.

Fluorescence studies were performed on a Spex Fluorolog spectrophuorometer using excitation and emission wavelengths of 340 and 480 nm, respectively, for maximal fluorescence enhancement. The fluorometer was used in the E/R mode which corrects for any changes in light intensity during the experiment. Fluorometer titrations were performed by the addition of small volumes of ligand with correction for any dilution (total dilution was maintained at less than 10%). Titrations were performed by the addition of small volumes of ligand with correction for any dilution (total dilution was maintained at less than 10%). Titrations were performed as described in Fig. 1, for example) were completed within 40 min of dilution of labeled G-actin into the cuvette. All titrations were performed at 20°C.

All nucleotides used were obtained from Sigma Chemical Co. 1,5-I-AEDANS was obtained from Aldrich and ultrapure metals were obtained from Ventron Corp. (Alpha Division).

RESULTS

1,5-I-AEDANS is believed to specifically label cysteine-373 (9) of actin and the labeled G-actin is fully capable of undergoing polymerization. The experiments reported below, however, were performed under conditions where no polymerization occurs since the concentration of actin used (10 μg/ml) was below the critical concentration. Thus, at the end of the experiments, the labeled actin remained in the supernatant fluid after centrifugation at 160,000 × g for 2 h. Also, as shown below, the G-actin was not inactivated during the time course of the experiments.

1 The abbreviation used is: 1,5-I-AEDANS, N-iodoacetyl-N'-[(5-sulfo-1-naphthyl)ethylenediamine.

2 Similar labeling was observed by reacting G-actin with a 40-fold excess of 1,5-I-AEDANS for 25 h at 4°C.
Curves measuring the enhancement of fluorescence as a function of Mg\(^{2+}\) concentration at varying levels of Ca\(^{2+}\) give information regarding the Mg\(^{2+}\) dissociation constant under these conditions. The total Ca\(^{2+}\) concentration was assumed to be about 2-fold larger and pH 8, in ATP concentration was adjusted to give a free concentration of Mg\(^{2+}\) plotted as a function of the total Ca\(^{2+}\) concentration. The total ATP concentration was adjusted to give a free ATP level of 4 \(\mu\)M prior to the addition of Mg\(^{2+}\). From the data in the inset, the Mg\(^{2+}\) dissociation constant in the absence of Ca is calculated to be 35 \(\pm\) 5 \(\mu\)M, while that for Ca\(^{2+}\) is calculated to be about 10 \(\mu\)M.

**Titration of G-Actin with Mg\(^{2+}\)**—Fig. 1 shows titration curves measuring the enhancement of fluorescence as a function of Mg\(^{2+}\) concentration at varying levels of CaCl\(_2\) at 20°C and pH 8, in 2 mM Tris/Cl buffer. In each experiment, the ATP concentration was adjusted to give a free concentration of ATP\(_2\), prior to the titration, of 4 \(\mu\)M. For these determinations, the MgATP dissociation constant under these conditions was assumed to be 3 \(\mu\)M (10) and that for CaATP was assumed to be about 2-fold larger (6 \(\mu\)M) (11). The ATP level in these experiments was sufficiently low that differences between total and free concentrations of Mg\(^{2+}\) were small even at the lower concentration of Mg\(^{2+}\) used. Scatchard plots of the raw data shown in Fig. 1 are linear when plotted as the change in fluorescence versus the ratio of change in fluorescence/free Mg\(^{2+}\) concentration. The inset in Fig. 1 shows a plot of the Mg\(^{2+}\) dissociation constant as a function of the total Ca\(^{2+}\) concentration.

The linearity of this plot demonstrates that Ca\(^{2+}\) and Mg\(^{2+}\) compete directly for a binding site of the G-actin. Extrapolation of the plot to zero Ca\(^{2+}\) indicates a Mg\(^{2+}\) dissociation constant of 35 \(\pm\) 5 \(\mu\)M in the absence of Ca\(^{2+}\) and, from the slope of the plot, a dissociation constant of about 10 \(\mu\)M for Ca\(^{2+}\) binding to G-actin under these conditions.

Fig. 2 shows that the fluorescence enhancement induced by the addition of Mg\(^{2+}\) is time-dependent. In this experiment, the concentration of labeled G-actin was 50 \(\mu\)g/ml, although the rate of enhancement is independent of the actin concentration (data not shown). Experiments at high levels of G-actin show that there are no further fluorescence changes as a consequence of polymerization. The enhancement appearing on addition of 50 \(\mu\)M Mg\(^{2+}\) to the actin solution (containing approximately 1 \(\mu\)M Ca\(^{2+}\) and 1 \(\mu\)M ATP) may be biphasic, but the major portion of the change has a t\(_{1/2}\) of about 15 to 20 s.

It may also be seen from Fig. 2 that the addition of Ca\(^{2+}\) reverses the fluorescence enhancement induced by Mg\(^{2+}\). Return of the fluorescence to its original value is also a time-dependent process and is also independent of actin concentration (data not shown). As discussed below, both of these changes probably reflect metal-induced conformational changes.

**Titration of G-Actin with KCl**—The addition of KCl to 1,5-AEDANS-labeled G-actin also results in an enhancement of the fluorescence either in the presence or absence of Mg\(^{2+}\). Fig. 3 shows a Scatchard plot of such data reflecting a titration curve of G-actin. As above, the G-actin does not polymerize under the conditions of the experiment. The Scatchard plot appears to be nonlinear at low KCl concentrations and it is not clear whether there is a limiting value of the fluorescence change reached at high levels of KCl. Furthermore, the fluorescence enhancement induced by KCl is not time-dependent as far as can be detected by our methods (i.e. the change occurs in less than 5 s). Titration experiments in the presence of Mg\(^{2+}\), Ca\(^{2+}\), or both indicate that KCl does not influence the binding of either Ca\(^{2+}\) or Mg\(^{2+}\). However, in the presence of Mg\(^{2+}\), there is a smaller fluorescence change induced by a given concentration of KCl. The change induced by KCl therefore appears to be quite different from that induced by Mg\(^{2+}\).

**Fluorescence as a Measure of G-Actin Inactivation**—Dilution of 1,5-AEDANS-labeled G-actin to very low concentrations of Ca\(^{2+}\) and ATP (<1 \(\mu\)M each) results in a slow loss of...
fluorescence to about 70% of its initial value. At concentrations of G-actin of 10 μg/ml (0.23 μM) and Ca⁺⁺ and ATP concentrations of about 1 μM, the t_{1/2} for the loss of fluorescence at 20°C is about 20 min. After the loss of fluorescence, addition of about 1 pM, the G-actin of the same extent of loss of fluorescence occurs in less than 5 min in the presence of EDTA, in agreement with observations that EDTA enhances inactivation (5). Low concentrations (10 μM) of either Ca⁺⁺ or ATP and higher concentrations of Mg⁺⁺ (i.e. 1 mM) stabilize the G-actin against the loss of fluorescence and presumably, therefore, the inactivation, so that no inactivation occurs in the time course of the experiments. It should be noted that the fluorescence change observed here is in the opposite direction from that induced by Mg⁺⁺ or KCl. The titration experiments described in Figs. 1 and 3 were performed under conditions where no loss of fluorescence occurs, since inactivation is prevented by ATP or Ca⁺⁺.

**Discussion**

There have been several reports related to the use of 1,5-1-AEDANS-labeled actin (8, 9, 12). Tao and Cho (9) concluded, on the basis of fluorescence lifetime studies, that the major labeling site was cysteine-373 with labeling of other sites being considerably less. Studies which have utilized this label have not associated with polymerization, since the concentration of G-actin used is below the critical concentration and the changes which occur are rapid. Furthermore, no further change in fluorescence is observed when higher concentrations of labeled G-actin undergo polymerization. The observed fluorescence changes allow one to determine the absolute dissociation constant of G-actin for Mg⁺⁺ and Ca⁺⁺ and perhaps other metals as well and to show that these metals directly compete for a particular binding site. This site may be the so-called tight-binding site for metal. A value of ~10 μM for the dissociation constant of Ca⁺⁺ to G-actin is in agreement with estimates of 1 to 10 μM presented in the literature (13-15). In addition, inactivation of the actin may be measured as a loss in fluorescence. Thus, 1,5-AEDANS-labeled actin may be a convenient probe for several properties of G-actin.

Evidence for a KCl-induced conformational change has been reported by Rich and Estes (6). Their conclusion was based on the observation that in the presence of KCl, G-actin was degraded by proteolytic enzymes at a slower rate than in the absence of KCl. These authors termed this form the F-ATP-actin monomer. However, it is quite possible that there are a number of different conformational forms of G-actin. Our results, for example, suggest that the change induced by KCl is different from that induced by divalent metals, since 1) the former is rapid and the latter is slow; 2) the addition of KCl enhances the fluorescence even in the presence of Mg⁺⁺ (although to a smaller extent); and 3) KCl and Mg⁺⁺ do not appear to compete with each other.

Of particular interest is the time dependence of the Mg⁺⁺-induced fluorescence change and its time-dependent reversal on addition of Ca⁺⁺ (Fig. 2). Since the dissociation constants for Ca⁺⁺ and Mg⁺⁺ are 10 and 35 μM, respectively, it would be expected that dissociation of either metal from the metal-binding site should be very rapid. For example, assuming a diffusion-controlled on-rate constant of 10⁷ s⁻¹ mol⁻¹, the off-rate may be calculated as greater than 100 s⁻¹ (t_{1/2} < 0.007 s). Thus, the slow changes observed in Fig. 2 are not the consequence of a slow off-rate of the metal ion, but rather a reflection of conformational changes which occur on replacing one metal by the other. That the rate of the fluorescence change is independent of the actin concentration reinforces this view (data not shown). Furthermore, this conformational change is reversible, since the addition of Ca⁺⁺ returns the Mg⁺⁺-induced fluorescence enhancement to the original value. The data suggest the following basic scheme:

\[
\text{Mg-G-ATP} \rightleftharpoons \text{Mg-G’-ATP} \\
+ \text{Mg} \uparrow + \text{Ca} \rightleftharpoons \text{Mg} \uparrow + \text{Ca}
\]

\[
\text{Ca-G-ATP} \rightleftharpoons \text{Ca-G’-ATP}
\]

where G-ATP (G-actin with ATP bound) may exist in at least two conformational forms, G-ATP and G’-ATP, and the conversion between them is reversible. To our knowledge, the results presented here represent the first direct evidence for a metal ion-induced conformational change of G-actin. We are unable to say, at present, whether the conformational change induced by Mg⁺⁺ is an essential one for a given mode of actin polymerization.

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