Role of the Actin Ala-108–Pro-112 Loop in Actin Polymerization and ATPase Activities

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Actin plays fundamental roles in a variety of cell functions in eukaryotic cells. The polymerization-depolymerization cycle, between monomeric G-actin and fibrous F-actin, drives essential cell processes. Recently, we proposed the atomic model for the F-actin structure and found that actin was in the twisted form in the monomer and in the untwisted form in the filament. To understand how the polymerization process is regulated (Caspar, D. L. (1991) Curr. Biol. 1, 30–32), we need to know further details about the transition from the twisted to the untwisted form. For this purpose, we focused our attention on the Ala-108–Pro-112 loop, which must play crucial roles in the transition, and analyzed the consequences of the amino acid replacements on the polymerization process. As compared with the wild type, the polymerization of P109A was accelerated in both the nucleation and the elongation steps, and this was attributed to an increase in the frequency factor of the Arrhenius equation. The multiple conformations allowed by the substitution presumably resulted in the effective formation of the collision complex, thus accelerating polymerization. On the other hand, the A108G mutation reduced the rates of both nucleation and elongation due to an increase in the activation energy. In the cases of polymerization acceleration and deceleration, each functional aberration is attributed to a distinct elementary process. The rigidity of the loop, which mediates neither too strong nor too weak interactions between subdomains 1 and 3, might play crucial roles in actin polymerization.

Significance: This knowledge is important for understanding the polymerization mechanism.

Actin is one of the most abundant proteins in eukaryotic cells, where it plays fundamental roles in a variety of cell functions, such as locomotion and division (1, 2). In cells, actin is present in two states: a monomeric state (G-actin) and a fibrous state (F-actin). The transitions between the two states, i.e., polymerization and depolymerization, drive several essential cellular processes. To understand the processes (29), the atomic structures of G-actin and F-actin are essential. The G-actin crystal structure was solved by Kabsch et al. (3) in 1990. On the other hand, an atomic model for the F-actin structure was first proposed by Holmes et al. (4) in 1990, based on x-ray fiber diffraction analyses. In 2009, we proposed a new model (5) and found conformational changes that are associated with the G- to F-actin transition. A recent study of the F-actin structure, using high resolution electron cryomicroscopy (6), confirmed the conformational changes.

The actin molecule has two major domains enclosing an ATP-binding cleft (3). These domains rotate relative to each other upon the G- to F-actin transition, and thus the actin molecule is flattened in F-actin (5). Within each molecule, the conformational changes are associated with the sliding of subdomain 1 relative to subdomain 3. The interface between two subdomains is formed by the side chains extending from the β-sheet core of subdomain 3 (green), the central α-helix including Gln-137–Gly-146 (wine), and the loop including Ala-108–Pro-112 of subdomain 1 (Fig. 1, cyan) (7). The loop shifts substantially relative to subdomain 3 upon sliding. Moreover, the preceding part of the loop contacts the side chain of Gln-137, which participates in both the conformational transition of the actin molecule and the ATPase reaction (8, 9). The successive part of the loop contacts the diagonal subunit in F-actin. In addition, the conformation of the loop is strongly restricted by two proline residues, Pro-109 and Pro-112, and it might behave as a stable structural unit.

The determination of the role of the Ala-108–Pro-112 loop in actin function is essential to understand the mechanism of actin polymerization. To clarify its role, we studied the consequences of altering the Ala-108–Pro-112 loop in terms of actin polymerization and ATPase activities. We created the A108G and P109A substitutions and prepared the two actin mutants by...
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the use of an insect cell expression system. A108G polymerized slowly, due to an increase in the activation energy, whereas P109A polymerized rapidly, due to an increase in the frequency factor of the Arrhenius equation. P109A may affect the formation of the collision complex between a monomer and the filament end in the polymerization process, whereas A108G probably causes a conformational change of the collision complex crossing a kinetic barrier. By contrast, these substitutions have minimal effects on the actin ATPase activity. Based on these differences between the two mutants, we speculate that the rigidity of the Ala-108–Pro-112 loop is important, through maintaining a moderate interaction between subdomains 1 and 3, for the process of actin polymerization.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Actin—Skeletal muscle α-actin were prepared from chicken breast dry muscle according to the method of Spudich and Watt (10). Recombinant human cardiac muscle wild type α-actin (the WT actin) was prepared according to the method we described previously (8). The N-terminal amino acid sequence of the WT actin is MGWSHPQFEKGGIEGRDDEE; the underlined part is an additional amino acid sequence including the Strep-tag II for purification. The extra N-terminal sequence did not affect the polymerization path of the WT actin, although it modified the rate constant, and furthermore, it hardly affected the overall actin ATPase activity (8). The transfer vector was pVL1392-L21, including the enhancer sequence L21 in the pVL1392 vector (Pharmingen) (11). The transfer vectors for the expression of the actin mutants A108G and P109A were generated from pVL1392-L21, encoding the WT, by the use of a QuikChange II site-directed mutagenesis kit (Agilent Technologies). The mutagenic primers were as follows: A108G, 5’-CACCGTGTTCACAGAAGGCCCCTGAACCCCAAGG-3’, and P109A, 5’-CCTGCTCACAGAGGCCGGCTGAACCCCAAGGC-3’, where the corresponding mutation sites are underlined and only the coding sequences are described. The preparation of recombinant baculovirus encoding actin and the purification of recombinant actins were also performed according to the method we described previously (8). In the case of A108G, the method was slightly modified; at the final purification step, the actin was polymerized at 25 °C, rather than 4 °C, because A108G polymerized extremely poorly at 4 °C. The concentration of G-actin was determined from the absorbance at 290 nm, using the extinction coefficient $E_{290} = 0.63 \text{ ml g}^{-1} \text{ cm}^{-1}$ (12).

Biochemical Assays of Actin Polymerization—G-actin was prepared in G-buffer, composed of 10 mM Tris-HCl (pH 8.0), 0.2 mM CaCl$_2$, 0.5 mM ATP, and 1 mM DTT. In assays for F-actin, G-actin was polymerized by the addition of a 20-fold concentrated polymerization solution to make final concentrations of 100 mM KCl, 2 mM MgCl$_2$, and 0.5 mM ATP. Actin polymerization was detected by 90° light scattering at 660 nm (8). Ultraviolet circular dichroism (CD) spectra, time courses of scattering intensity during polymerization, critical concentrations for polymerization, and time courses of released phosphate (P$_i$) were measured according to the procedures described previously (8). F-actin was also observed by electron microscopy according to the previously reported procedures (8). The number of filaments in solution was performed according to the procedure reported by Pollard (15). Skeletal muscle α-actin was labeled by n-(1-pyrene)-iodoacetamide (pyrene actin), according to the procedure reported by Koyama and Mihashi (16). We prepared two solutions, A and B, and monitored the fluorescence at 407 nm (excitation at 365 nm) after gently mixing the two solutions at 25 °C. Solution A was prepared by mixing 15 μl of pyrene G-actin (1 mg/ml, label ratio: 5%), 79 μl of G-buffer, and 100 μl of water. Solution B was prepared by mixing 12 μl of high salt solution (2M KCl, 40 mM MgCl$_2$, 6 mM EGTA), 0–9 μl of expressed F-actin solution at steady state (0.4 mg/ml), and 27–36 μl of G-buffer. The slope is proportional to the filament concentration of F-actin in solution.

The simplest conventional nucleationelongation model was used for actin polymerization. Nucleation $nA \rightarrow N$ (A = G-actin; N = nucleation; $n = $ nucleus size) proceeds with the rate constant $k_n$. Elongation $N + A \rightarrow F$ (F = F-actin) proceeds with the rate constant $k_{+}$. The apparent maximum polymerization rate is proportional to $(k_{+}k_n)[A]^{n+1}$, and the filament concentration is proportional to $(k_{+}/k_n)[A]^{n/2}$.

The apparent rate constants for the ATPase on F-actin ($k$) were calculated by the following scheme: $G$-actin-ATP → $G$-actin-ADP + $P_i$. The reverse reactions were left out of the scheme for clarity.

$$\frac{d[F\text{-actin-ATP}]}{dt} = \text{rate (polymerization)} - k[F\text{-actin-ATP}]$$

(Eq. 1)

$$\text{rate} (P_i \text{ release}) = k[F\text{-actin-ATP}]$$

(Eq. 2)
RESULTS

Preparation of Recombinant Human Cardiac Muscle α-Ac-
tins in Insect Cells—Recombinant human cardiac muscle α-ac-
tins (the WT actin, A108G, and P109A) were expressed by the
use of a baculovirus-based expression system in insect cells.
They were purified by affinity chromatography on a
Strep-tag II
column and gel filtration on a Superdex 200 column, according
to the method we described previously (8). The actins were
quite reproducibly obtained with high purity, as shown in Fig.
2A. However, the average yields of the mutant actins, 1.4 mg for
A108G and 0.8 mg for P109A, from $8 \times 10^9$ cells/3.6 liters of
culture, were much lower than that for the WT actin (4.2 mg).

Effects of the A108G and P109A Mutations on the Overall
Structure of the Actin Molecule—To determine whether the
expressed mutants adopt the canonical structure of the actin
molecule, we measured the CD spectra, which reflect the sec-
secondary structure content, and the temperature dependence of
the CD spectra. As shown in Fig. 2B, the CD spectra of A108G
and P109A G-actin were almost identical to that of the WT
actin at 25 °C (Fig. 2B). The specific minima at 210 nm started
to disappear as the sample temperature was gradually raised
and completely disappeared at 70 °C. To clarify the melting
process, we monitored the mean residue ellipticity at 222 nm
and determined the melting temperature ($T_m$) at the inflection
point of the melting curve. The $T_m$ of P109A was 5 °C lower as
compared with those of the WT actin and A108G (Table 1).
These results indicated that these mutants adopt the canonical
structure of the actin molecule at room temperature, although
the P109A substitution reduces the thermal stability of the
actin molecule. The instability could be due to small defects in
the contacts between subdomain 1 and subdomain 3. More-
over, to confirm whether the expressed actin mutants assemble
into the canonical F-actin under the polymerizing conditions,
we examined the preparation by electron microscopy. The neg-
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Polymerization of the A108G and P109A Mutants—The critical concentration for polymerization ($C_c$) is a thermodynamic index of F-actin stability at steady state. It is defined by the ratio of the dissociation and association rate constants. The $C_c$ of P109A was almost identical to that of the WT actin at 4 °C (Table 3). By contrast, the $C_c$ of A108G was ~10 times higher than that of WT actin, irrespective of the incubation temperature (Table 3). This result indicated that the A108G substitution destabilizes F-actin and favors G-actin.

The time courses of actin polymerization, detected by light scattering, are shown in Fig. 3A. With A108G, the apparent maximum rate of polymerization was 3.2 times slower than that of the WT actin. By contrast, with P109A, the rate was 2.4 times faster than that of the WT actin. The time courses of both mutants showed an initial short lag phase, followed by a rising phase, and a stationary phase, similar to that of WT actin. We analyzed the nucleation of the actin mutants by the conventional nucleation-elongation model (17, 18). From the double logarithmic plot of the apparent maximum rate of polymerization versus the actin concentration (13, 18), the nucleus sizes for polymerization were estimated to be 3.6–4.4. These values were identical to that of the WT actin (Fig. 3B). These results suggested that the polymerization of these actin mutants occurs via processes similar to those for the WT actin, although at distinct reaction rates. To estimate the relative rate constants for nucleation and elongation, we measured the numbers of filaments in solution at the steady state. The numbers of filaments were estimated from the initial rates of muscle pyrene phosphate assay kit (19) (Fig. 4). The time courses also showed an initial short lag phase followed by a rising phase and a stationary phase, similar to the polymerization time course. In all cases, the total amount of $P_i$ released for 45 min after the initiation of polymerization corresponded to the amount of F-actin.

Activation Energy for Polymerization of A108G and P109A—The Arrhenius plots of the maximum polymerization rates of these actin mutants are shown in Fig. 3C. The slopes of the plots yield the activation energy for the polymerization reaction. For the WT actin, the activation energy was calculated as 133 kJ/mol (Table 5). This value is close to the value of 100 kJ/mol, reported previously by Kasai (14). For A108G, the activation energy was 163 kJ/mol and was higher than that for the WT actin, which indicates that the kinetic barrier for polymerization is higher (Table 5). In contrast, the plot with P109A is 1.1-fold, respectively, as compared with that from the WT actin.

Incubated at 4 °C (overnight)
WT $0.71 \pm 0.11$ ($n = 4$)
P109A $0.65 \pm 0.16$ ($n = 4$)

Incubated at room temperature (overnight)
WT $0.18 \pm 0.09$ ($n = 4$)
A108G $1.52 \pm 0.20$ ($n = 4$)

Incubated at room temperature (overnight), followed by 4 °C (overnight)
WT $0.56 \pm 0.06$ ($n = 4$)
A108G $5.82 \pm 0.34$ ($n = 4$)

**DISCUSSION**

The amino acid substitutions of Ala by Gly or of Pro by Ala increase the allowed range for the dihedral angles of the resi-
dues in the polypeptide chain, thus increasing the permissible conformations of the polypeptide chain (20). Actually, in the case of P109A, the main chain of Ala-108–Ala-109 is deformed, and Ala-109 is separated from His-161 in subdomain 3 despite an almost invariant arrangement with Ile-163, partly because of the lack of an interaction between Pro-109 and His-161 (Protein Data Bank (PDB) codes: 3A5N (21) and 1C0F (22)). Furthermore, the main chain has slightly larger B-factors in the crystal structure of Dictyostelium P109A than that of the wild type actin. Although both A108G and P109A conferred similar perturbations of the Ala-108–Pro-112 loop, the two substitutions altered the polymerization rates in opposite manners. P109A polymerized more rapidly at room temperature because of increases in both the elongation and the nucleation rates.

FIGURE 3. Actin polymerization kinetics. A, time courses of the light scattering of A108G, P109A, and the WT actins were followed after the initiation of polymerization. The solution for the polymerization measurements contained 25 μM actin, 100 mM KCl, 2 mM MgCl₂, and 0.5 mM ATP in G-buffer, at 25 °C. The light scattering to 90° was measured at 660 nm. For each actin species, four independent preparations were used. B, relationship between the maximal rates of apparent elongation and the actin concentration. The solution conditions for polymerization were the same as those in the experiments described in the legend for Fig. 3. Each plot was obtained by averaging at least three measurements.

FIGURE 4. Actin ATPase rate during polymerization. Time courses of Pi release from A108G (△), P109A (■), and the WT (○) actins during polymerization are shown. The data for the WT actin are identical to those published in our previous work (8). The amounts of Pi released from actin solutions at 25 μM were measured with an EnzChek phosphate assay kit. The solution conditions for polymerization were the same as those in the experiments described in the legend for Fig. 3. Each plot was obtained by averaging at least three measurements.

TABLE 4

| Actin |  \( k_+ \) |  \( k_- \) |
|-------|----------|----------|
| A108G |  0.43    |  0.21    |
| P109A |  2.5     |  2.8     |

TABLE 5

| Actin | Temperature |  \( E_a \) (kJ/mol) |
|-------|-------------|------------------|
| WT    | 133         |                  |
| A108G | 163         |                  |
| P109A | 239         |                  |

P109A polymerized more rapidly at room temperature because of increases in both the elongation and the nucleation rates of the actin P109A species, four independent preparations were used.
rate constants. The acceleration is attributed to a slight increase in the frequency factor of the Arrhenius equation because the activation energy for the polymerization of P109A is identical to that of the WT actin (Fig. 3C). The frequency factor is related to the number of events in which G-actin molecules collide with the ends of F-actin in the proper orientation and conformation necessary to cause polymerization among the thermally fluctuating G-actin molecules; thus, under normal conditions, ~2% of the collisions of G-actin molecules with the F-actin end are capable of binding (23). The collision complexes become the newly revised F-actin end by crossing the kinetic barrier of the activation energy. In the case of P109A, the increase in the frequency factor is probably accounted for by the slight accumulation of monomers with a binding-capable conformation. The flattening is a molecular conformational transition mainly in the direction of the propeller-like thermal motion of G-actin. It is possible that similar kinds of motion are enhanced by the weakening of the interaction between the two major domains as suggested by the lower melting temperature. Another explanation is that the conformation with shifts of Ala-108–Ala-109 in the crystal is close to a binding-capable conformation. Furthermore, if the fraction of monomers with a binding-capable conformation declines with decreasing temperature, then the Arrhenius, if the fraction of monomers with a binding-capable conformation. The flattening is a molecular conformational transition mainly in the direction of the propeller-like thermal motion of G-actin. It is possible that similar kinds of motion are enhanced by the weakening of the interaction between the two major domains as suggested by the lower melting temperature. Another explanation is that the conformation with shifts of Ala-108–Ala-109 in the crystal is close to a binding-capable conformation. Furthermore, if the fraction of monomers with a binding-capable conformation declines with decreasing temperature, then the Arrhenius, if the fraction of monomers with a binding-capable conformation.

The similar role of proline has been reported for a floppy loop in arylalkylamine N-acetyltransferase (27).

Generally, polymerization occurs by the manner in which a monomer collides with the filament end, and then the collision complex adjusts its conformation to form the newly revised end. Our results experimentally demonstrated the two separate conformational changes relevant to the actin polymerization process. One is the thermal fluctuation of the conformation without activation, which probably affects the polymerization rate via the effective formation of the collision complex. The other is the conformational change crossing a kinetic barrier by extra energy. The conformational change may represent the main part of the flattening transition.

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