α -Actinin from chicken gizzard: at low temperature, the onset of actin-gelling activity correlates with actin bundling

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The effect of α -actinin from chicken gizzard on the properties of F-actin solutions at 37 °C and at 4 °C was investigated. Beside the well-known increase of the gelling activity of α -actinin, it was found that lowering temperature to 4 °C: (a) modifies the shape of the α -actinin–F-actin binding isotherm; (b) increases the light scattering of the α -actinin–F-actin mixtures; (c) induces the formation of ribbons and bundles of F-actin. It was also observed that, by warming to 37 °C, the bundles of F-actin formed at 4 °C

were dissociated into quasi-parallel actin filaments running at a distance of 25–42 nm from each other. On subsequent cooling to 4 °C, these parallel filaments were rapidly assembled into bundles. As at 37 °C, α -actinin displays a potent gelling activity on bundles but not on filaments of actin, and as the gelling activity at 4 °C is accompanied by the formation of actin bundles, it is concluded that actin bundling is a necessary condition to promote the actin-gelling activity.

INTRODUCTION

Monomeric actin is polymerized by salt to form filamentous structures, F-actin, of a size distribution up to several micrometres in length (Straub, 1943). In the presence of 15–50 mM MgCl₂, as the only salt, actin filaments undergo latero-lateral association to form bundles of parallel filaments (Hanson, 1973). Actin bundles are also formed in the presence of large concentrations, 5–7 % (w/v) of poly(ethylene glycol) 6000 (Suzuki et al., 1989). The apparent, centre to centre, interfilament distance in the bundles spans from 5.48 to 5.91 nm (Fowler and Aebi, 1982).

Polymerization increases considerably the viscosity of actin solutions. When viscosity is measured with the ‘falling-ball viscosimeter’ it is found that, beyond a given actin concentration, the ball does not fall any more because the actin network is strong enough to support it. This concentration is called the ‘critical gelling concentration’ as it is intended that actin has formed a gel (MacLean-Flechter and Pollard, 1980a). It is important to realize, however, that this is a purely operative definition. The value of the critical gelling concentration can be increased or decreased at will by increasing or decreasing the density of the ball and by increasing or decreasing the slope of the tube of the viscosimeter.

A few proteins are known: filamin (Wang et al., 1975), actin-binding protein (Hartwig and Stossel, 1975), α -actinins (Podlubnaya et al., 1975), that display an actin-gelling activity. These proteins cross-link actin filaments and, consequently, decrease the critical gelling concentration of actin. Among these proteins α -actinins possess a remarkable characteristic: their critical gelling concentration increases with temperature. At 37 °C their effect is almost undetectable when tested at 12–24 μ M actin (Goll et al., 1972; Jockusch and Isenberg, 1981; Bennett et al., 1984; Landon et al., 1985; Ohtaki et al., 1985).

Surprisingly, however, chicken gizzard α -actinin displays a significant gelling activity, even at 37 °C, when the concentration of actin is low (2–3 μ M). Furthermore, the α -actinin–F-actin binding isotherm is anomalous, as the apparent dissociation constant increases with the increase of actin concentration (Grazi et al., 1990a, 1991, 1993).

Apparently, the gelling activity at low actin concentrations

correlates with the lower apparent dissociation constant. At low concentrations actin filaments are free to diffuse and to be optimally cross-linked by α -actinin. As a consequence the gelling activity is displayed.

At higher concentrations, actin filaments are practically immobilized, the α -actinin–F-actin apparent dissociation constant increases and the α -actinin gelling activity becomes almost undetectable.

A completely different behaviour is displayed when α -actinin interacts with bundles of actin filaments, as are formed in 6.7 % (w/v) poly(ethylene glycol) 6000. Under these conditions, α -actinin is an efficient actin-gelling protein, even at 37 °C (Grazi et al., 1990b) and displays an hyperbolic binding isotherm (Grazi et al., 1992).

The dissimilar behaviour of α -actinin, when tested at 37 °C, with actin filaments and with actin bundles, raises the suspicion that the actin-gelling activity of α -actinin, at 4 °C, is associated with the conversion of actin filaments into actin bundles.

The study of the properties of the α -actinin–F-actin mixtures at 37 °C and at 4 °C shows, in fact, that the onset of actin-gelling activity of α -actinin, at 4 °C, correlates with actin bundling.

MATERIALS AND METHODS

G-actin from rabbit muscle was prepared according to Spudich and Watt (1971) and further gel-filtered through Sephadex G-150 (MacLean-Flechter and Pollard, 1980b). α -Actinin from chicken gizzard was prepared according to Feramisco and Burridge (1980). The absorption coefficients used were $A_{280}^{1\%}$ 6.2 (Gordon et al. 1976) for actin and $A_{278}^{1\%}$ 9.7 (Suzuki et al., 1987) for α -actinin. Molar concentrations were calculated on the basis of a molecular mass of 42 kDa for actin (Collins and Elzinga, 1975) and of 200 kDa for α -actinin (Suzuki et al., 1987).

[³H]N-Ethylmaleimide-labelled α -actinin was prepared and radioactivity determined as previously described (Grazi et al., 1991).

The incubation mixtures contained F-actin and α -actinin at the appropriate concentrations, 0.1 M KCl, 2 mM MgCl₂, 0.2 mM ATP, 1 mM dithiothreitol and 10 mM Tris/HCl buffer, pH 7.5. Temperature is indicated in the single experiments.

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Low-shear-rate viscosity was measured with home-made 'falling ball' viscosimeters (capillary diameter 1.13 mm, capillary slope 14°) maintained in a thermostatically controlled water bath.

Centrifugation was performed at the temperature of each single experiment in a TL100 rotor of a TL100 Beckman centrifuge. The samples were centrifuged for 10 min either at 9 900 *g* to sediment actin bundles, or at 366 000 *g* to sediment actin filaments.

The apparent F-actin- α -actinin dissociation constant was defined as follows:

$$\frac{[\text{F-actin}][\text{free } \alpha\text{-actinin}]}{[\text{actin-bound } \alpha\text{-actinin}]} = K_{\text{app.}}$$

where free α -actinin is the α -actinin remaining in the supernatant solution after centrifugation and bound α -actinin is α -actinin sedimented in the presence of F-actin minus the amount of α -actinin sedimented in the absence of F-actin. The latter amount was usually less than 1% of total α -actinin.

Light scattering was determined at 540 nm with a 90° observation angle by using a Jasco FP 550 spectrofluorimeter equipped with an FP 2060 temperature controller unit.

Protein was determined by the Coomassie Blue method (Bradford, 1976) as modified by Stoscheck (1990).

For electron-microscopy analysis the samples were diluted five times with the incubation buffer, immediately applied to carbon-coated 400-mesh grids, washed with one drop of water and stained with five drops of 1% (w/v) uranyl acetate, pH 4.25. Electron microscopy was performed with a Hitachi H-800 electron microscope.

RESULTS

Effect of temperature and of α -actinin concentration on the low-shear-rate viscosity of F-actin and of actin bundles

As is shown in Figure 1, the low-shear-rate viscosity of F-actin (2.4 μM as monomer) increases from 2.1×10^{-3} to $2.75 \times 10^{-3} \text{ Pa}\cdot\text{s}$ when temperature is decreased from 35 °C to 4 °C (points on the ordinate axis). However, due to the large effect of temperature on the viscosity of the solvent, the specific viscosity of the solution increases with temperature; being 0.77 at 4 °C, 0.81 at 10 °C, 2.1 at 20 °C and 3.0 at 35 °C.

Addition of α -actinin increases the viscosity of the solution. At 20 °C and 37 °C the low-shear-rate viscosity increases two-fold in the presence of 1–2 μM α -actinin. At lower temperatures, actin gels (the ball does not fall in the tube of the viscosimeter and the value for the viscosity of the gel is not determined). At 4 °C actin is gelled by 0.2 μM α -actinin and at 10 °C by 1 μM α -actinin.

The effect, at 37 °C, of α -actinin on the low-shear-rate viscosity of actin bundles (3.4 μM as monomer) formed in 6.7% (w/v) poly(ethylene glycol) is also presented. Apparent viscosity increases from 0.015 $\text{Pa}\cdot\text{s}$, in the absence, to 0.037 $\text{Pa}\cdot\text{s}^{-1}$ in the presence of 0.16 μM α -actinin. In the presence of 0.32 μM α -actinin the sample is gelled (Figure 1).

Effect of temperature on the apparent F-actin- α -actinin dissociation constant and on the sedimentation of the F-actin- α -actinin mixtures

As is shown in Figure 2, at 37 °C, the apparent dissociation constant of α -actinin from actin filaments increases from 20 μM at 3.6 μM actin to 140 μM at 48 μM actin, while the apparent dissociation constant of α -actinin from actin bundles changes from 10 μM at 3.6 μM actin to 18 μM at 48 μM actin.

Figure 1 Effect of α -actinin on the low-shear-rate viscosity of F-actin at different temperatures

Left hand panel: the mixtures contained F-actin (2.4 μM as monomer) and α -actinin as indicated. After 60 min of incubation either at 4 °C (○), at 10 °C (△), at 20 °C (●), or at 35 °C (▲), in the tubes of the falling-ball viscosimeters, the low-shear-rate viscosity of the samples was determined. The broken lines at the bottom of the figure indicate the viscosity of water at different temperatures. Right-hand panel: the mixtures contained F-actin (3.4 μM as monomer), α -actinin as indicated and 6.7% (w/v) poly(ethylene glycol) 6000. After 60 min of incubation at 35 °C, in the tubes of the falling-ball viscosimeters, the low-shear-rate viscosity of the samples was determined. The broken line at the bottom of the figure indicates the viscosity of a 6.7% (w/v) poly(ethylene glycol) 6000–water solution.

Figure 2 Effect of increasing concentrations of F-actin on the apparent F-actin- α -actinin dissociation constant

The mixtures contained 0.21 μM [^3H]N-ethylmaleimide-labelled α -actinin (specific radioactivity 83 000 d.p.m./nmol) and F-actin as indicated. After 60 min of incubation either at 4 °C (○) or at 37 °C (●), the samples were centrifuged for 10 min at 366 000 *g* either at 4 °C or at 37 °C, and free and actin-bound α -actinin concentrations were determined as described in the Materials and methods section. Filled triangles represent the apparent F-actin- α -actinin dissociation constant determined at 37 °C and pH 7.5, in the presence of 6.7% poly(ethylene glycol) 6000. The data of the experiment in poly(ethylene glycol) are taken from Grazi et al. (1992).

Figure 3 Effect of temperature on the sedimentation of the F-actin- α -actinin mixtures

The mixtures contained F-actin ($9 \mu\text{M}$), α -actinin as indicated, with ($\blacktriangle$) or without ($\circ$, $\bullet$) 6.7% (w/v) poly (ethylene glycol) 6000. After 60 min of incubation either at 4°C ($\circ$) or at 37°C ($\bullet$, $\blacktriangle$), the samples were centrifuged for 10 min at $9900 g$, either at 4°C or at 37°C , and the supernatant solutions were assayed for total protein as described in the Materials and methods section.

Figure 4 Effect of temperature on the light scattering of the F-actin- α -actinin mixtures

The mixtures contained F-actin ($8 \mu\text{M}$ as monomer) and $0.21 \mu\text{M}$ α -actinin. Light scattering was followed as described in the Materials and methods section. First arrow, addition of F-actin; second arrow, addition of α -actinin. Third and fourth arrows, samples were taken for electron-microscopy observations.

At 4°C , the apparent dissociation constant is less influenced by the increase of actin concentration than at 37°C , being $30 \mu\text{M}$ at $3.6 \mu\text{M}$ actin and $78 \mu\text{M}$ at $48 \mu\text{M}$ actin.

The sedimentation of the F-actin- α -actinin mixtures is also influenced by the change of temperature. At 37°C , independently of the concentration of α -actinin, 90–95% of total protein is

Figure 5 Electron micrographs of the F-actin- α -actinin mixtures at 3°C and at 37°C

Samples were taken from the experiment described in Figure 4. Temperature was 3°C (a and b) and 37°C (c). The same bar represents 100 nm (b) and 333 nm (a and c).

found in the supernatant solution after 10 min of centrifugation at $9900 g$, a condition that is known to sediment actin bundles but not actin filaments. On the contrary, actin bundles, formed in 6.7% (w/v) poly(ethylene glycol), are sedimented; 90–95% of total protein is found in the pellet, after centrifugation, both in the presence and in the absence of α -actinin.

At 4°C , 91% of total protein is found in the supernatant solution when centrifugation is performed with actin alone (point on the ordinate axis). However, the amount of total protein in the supernatant solution decreases with the increase of the concentration of α -actinin, reaching 28% at $0.32 \mu\text{M}$ α -actinin (Figure 3).

Effect of temperature on the light scattering of the F-actin- α -actinin mixtures

The results of the experiments described in Figures 2 and 3 suggest that, at 4°C , the behaviour of F-actin approaches that expected for actin bundles. This view is confirmed by the effect of temperature on the light scattering of F-actin- α -actinin mixtures.

Addition of $0.21 \mu\text{M}$ α -actinin to F-actin ($8 \mu\text{M}$ as monomer)

increases the light scattering of the solution from 1.5 to 3.6 arbitrary units in about 12 min. Warming the solution to 37 °C decreases the light scattering to 1.8 arbitrary units. If the solution is then cooled again, light scattering increases more rapidly than the first time and reaches a stationary state at 6.6 arbitrary units. A second cycle of warming decreases light scattering to 6 arbitrary units at 7 °C, to 4 arbitrary units at 11 °C, and to 2.2 arbitrary units at 15 °C. A third cycle of cooling to 3 °C further increases the level of light scattering to 8 arbitrary units. Light scattering drops to 1.9 arbitrary units after the third cycle of warming to 37 °C (Figure 4).

Temperature effects are much less evident on the light scattering of F-actin alone. Light scattering of F-actin (8 μ M as monomer) decreases from 1.5 arbitrary units, at 4 °C, to 1.35 arbitrary units at 37 °C. The phenomenon being reversed by cooling the solution again (results not shown).

Effect of temperature on the organization of the F-actin- α -actinin mixtures

The light scattering of a rod suspension, at constant rod mass, increases significantly with the increase of the diameter of the rods. It is thus possible that the increase, at 3 °C, of the light scattering of F-actin- α -actinin mixtures is due to actin bundling. Electron-microscopy observation shows, in fact, that, at 3 °C, ribbons and compact bundles of actin filaments, up to 40 nm thick, are formed (Figure 5a). A ribbon of actin filaments is also shown at larger magnification in Figure 5(b). The same sample, after warming to 37 °C, displays very loose actin bundles, i.e. large zones of long actin filaments, running parallel to each other at the approximate distance of 25–42 nm. α -Actinin molecules (the additional masses along the actin filaments) decorate and, in some cases, cross-link actin filaments (Figure 5c).

DISCUSSION

It is well known that the actin-gelling activity of α -actinin fades with an increase in temperature (Goll et al., 1972; Jockusch and Isenberg, 1981; Bennett et al., 1984; Landon et al., 1985; Ohtaki et al., 1985), a behaviour that, in spite of its intrinsic interest, has so far received no explanation.

When deformed rapidly, α -actinin-F-actinin mixtures are more rigid than actin filaments without α -actinin, but when deformed slowly these mixtures are indistinguishable from filaments alone. These time-dependent mechanical properties are explained by multiple, rapidly rearranging α -actinin cross-links between actin filaments (Sato et al., 1987) in agreement with the mechanism proposed earlier by Frey-Wissling (1948).

Similarly, it is proposed that the increase of temperature decreases the rigidity of the α -actinin-F-actin gels because of the concomitant increase of the dissociation rate constant of α -actinin from F-actin (Sato et al. 1987). This analysis overlooks an important phenomenon: the effect of temperature on the structure of the α -actinin-F-actin gels.

Even though α -actinin is classified by Stossel et al. (1985) as an 'anisotropic filament-bundling protein', there is no doubt that it displays a more complex effect on the actin network.

At 37 °C and at low α -actinin to actin molar ratios, the interaction between α -actinin and actin filaments is accounted for by diffusion hindrance and geometry of actin filaments crossings (Grazi et al., 1993). Under these conditions only very loose bundles co-exist with the actin meshwork. Compact actin bundles, however, are formed at α -actinin to actin molar ratios larger than 1:20 (Meyer and Aebi, 1990) or 1:6 (Grazi et al., 1991), depending on the ionic conditions of the medium.

At 4 °C, the formation of compact actin bundles occurs at an α -actinin to actin molar ratio as low as 1:38 (this paper). This is in keeping with the reported effects of low temperature on the bundling of α -actinin-F-actinin mixtures (Endo and Masaki, 1982), as well as on the latero-lateral association of actin filaments (Grazi and Trombetta, 1985).

We present converging evidence that, at low temperature, actin bundling correlates with the onset of the actin-gelling activity of α -actinin. The binding isotherm, the sedimentability, the light scattering and the supramolecular organization of the α -actinin-F-actin mixtures are influenced by temperature.

In the first cycle of cooling light scattering increases slowly and moderately. Apparently, actin filaments are slowly organized into actin bundles by overcoming diffusion hindrance. Warming to 37 °C dissociates actin bundles which, nevertheless, keep, at least partially, the 'memory' of their previous state of bundles. Most of the actin filaments are now parallel and distanced 25–42 nm from each other: an arrangement that favours bundling on subsequent cooling. This is the reason why, in the second and in the third cycles of cooling, the increase of light scattering is, initially, faster than in the first cycle of cooling. The subsequent, slower increase of light scattering is due to the recruitment of new actin filaments, that, by overcoming diffusion hindrance, are organized into bundles.

We have previously shown that, at 37 °C, the conversion of actin filaments into actin bundles evokes the actin-gelling activity of α -actinin (Grazi et al., 1990b, 1992).

We show here that, at 3 °C also, the actin-gelling activity of α -actinin is linked to the formation of actin bundles. It seems therefore that actin bundling is a necessary condition for α -actinin to promote an efficient actin-gelling activity. This is in keeping with the observation that α -actinin, in the cell, is mostly co-localized with actin bundles (Lazarides, 1976; Bretscher and Weber, 1978).

This work was supported by grants 91/40/05/016 and 91 60/05/033 of the Italian Ministero della Ricerca Scientifica, and by grant 93.04459.CT04 from the CNR.

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Received 17 May 1993/9 September 1993; accepted 15 September 1993