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The effects of formins on the conformation of subdomain 1 in actin filaments

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Abstract

In this study we investigated the effects of formins on the conformation of actin filaments by using the method of fluorescence quenching. Actin was labelled with IAEDANS at Cys³⁷⁴ and the quencher was acrylamide. The results showed that formin binding induced structural changes in the subdomain 1 of actin protomers which were reflected by greater quenching constants (K_{SV}). Simultaneously the fraction of the fluorophore population accessible for the quencher (α) decreased. These observations suggest that the conformational distribution characteristic for the actin protomers became broader after the binding of formins, for which the structural framework was provided by a more flexible protein matrix in the microenvironment of the label. The effects of formins depended on the formin:actin molar ratio, and also on the ionic strength of the medium. These observations are in agreement with previous results and underline the importance of the intramolecular conformational changes induced by formins in the structure of actin filaments.

Keywords

Formin; Actin; fluorescence spectroscopy; fluorescence quenching; Protein conformation

1. Introduction

Actin can be found in all eukaryotes as a part of the cytoskeleton. The cytoskeleton has significant roles in different cellular processes like migration of cells, cytokinesis and endocytosis [1,2]. Actin nucleation factors have peculiar role in the development of the filamentous structure of actin. The nucleation factors described so far are the ARP 2/3 complex [3], Spire [4], Formins and Leiomodin [5-7].

In this study we focused on the interactions between actin and formins. Formins are multidomain polypeptides with a single polypeptide chain. The FH1 and FH2 (formin homology 1 and 2) domains are common in all formins [8]. The FH2 domain is responsible for the binding to actin and for alteration of the polymerization properties of filaments. The FH1 domain can modulate this effect through interactions with profilin-actin [9-11]. Different formins can have other specific properties as they can depolymerise, sever or bundle filamentous actin [9,12-16]. The effect of the formins on the structure of actin has been previously investigated in the case of FH2 domain of mouse Diaphanous-related protein 1 (mDia1-FH2) and it was found that the structure of the formin-bound filaments is significantly different from those polymerized in the absence of formins. Binding of the mDia1-FH2 to barbed ends of filaments made the structure of actin filaments more flexible,

while side-binding of formins stabilised the conformation of the filaments [17,18]. Subsequent experiments using EPR methods gave further evidences regarding the formin induced changes in actin filaments [19]. It was also shown, that binding of tropomyosin can reverse these conformational modifications [20].

In the present study the effect of the mDia1-FH2 on actin filaments was investigated by using the method of fluorescence quenching. In previous studies fluorescence quenching proved to be a powerful method to describe the conformational changes of macromolecules [21-24]. We applied acrylamide as a quencher and characterise the accessibility of the IAEDANS label attached to Cys³⁷⁴ of the actin protomers. The results showed that binding of formins to actin filaments modified the microenvironment of the Cys³⁷⁴ residue. Binding of formins substantially increased the Stern–Volmer quenching constant, while the fraction of quenchable fluorophores decreased. The formin effect depended on the formin:actin molar ratio, and also on the ionic strength of the solution. These observations provided further details regarding the formin induced conformational changes in the actin filaments and corroborated our previous results [17-19].

2. Materials and methods

2.1. Protein preparation and purification

Actin was prepared from acetone dried rabbit skeletal muscle [25] according to the method of Spudich and Watt [26]. Actin monomers were stored in buffer. A containing 4 mM Tris–HCl (pH 7.3), 0.2 mM ATP, 0.1 mM CaCl₂ and 0.5 mM DTT. The concentration of G-actin was determined with the absorption coefficient of 0.63 mg⁻¹ ml cm⁻¹ (26,650 M⁻¹ cm⁻¹) at 290 nm [27] on a Shimadzu UV-2100 type spectrophotometer. The molecular mass of 42,300 was used for G-actin [28].

The FH2 fragments of the mammalian formin mDia1 (mDia1-FH2) were prepared as described previously [29]. The concentration of FH2 was determined with the absorption coefficient of $A_{280} = 21,680 \text{ M}^{-1} \text{ cm}^{-1}$ (ProtParam, <http://us.expasy.org/tools/>). The purified protein was frozen in liquid nitrogen and stored at –80 °C. Formin concentrations are given as mDia1-FH2 monomer concentrations throughout this article.

2.2. Fluorescence labelling of actin

IAEDANS labelling of actin at Cys³⁷⁴ was completed according to the method of Miki and co-workers [30]. The concentration of the fluorescent dye bound to the actin monomers was determined by using the absorption coefficient of 6100 M⁻¹ cm⁻¹ at 336 nm [31]. The extent of labelling was 0.8–0.9 mol/mol of IAEDANS/actin monomers.

2.3. Steady-state fluorescence experiments

Steady-state fluorescence measurements were carried out on a PerkinElmer LS50B Luminescence Spectrometer and Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter at 22 °C with 5 μM actin. The excitation wavelength was 350 nm and the emission was monitored through a wavelength range of 466–476 nm. The optical slits were set to 5 nm in both the excitation and emission paths. The acrylamide concentration was increased up to 0.5 M in subsequent steps during the measurements. The experiments were carried out in the presence of 1 mM MgCl₂ and 50 mM KCl (higher ionic strength) or 0.5 mM MgCl₂ and 10 mM KCl (lower ionic strength).

2.4. Fluorescence lifetime measurements

Fluorescence lifetime measurements were done at 22 °C with an ISS K2 multifrequency phase fluorometer (ISS Fluorescence Instrumentation, Champaign, IL). Sinusoidally

modulated light (350 nm) from a 300 W Xe arc lamp was used for excitation and the emission was monitored through a 385FG03–25 high-pass filter. The modulation frequency was changed in 10 steps from 5 to 80 MHz. The data were analysed by ISS187 decay analysis software. All data were fitted to double exponential decay curves assuming a constant, frequency-independent error in both phase angle ($\pm 0.200^\circ$) and modulation ratio (± 0.004). The goodness of the fit was determined from the value of the reduced χ^2 [32]. Average fluorescence lifetimes were calculated assuming discrete lifetime distributions [33]:

$$\tau_{aver} = \sum_{i=1}^n \alpha_i \tau_i \quad (1)$$

where τ_{aver} is the average fluorescence lifetime and α_i and τ_i are the individual amplitudes and lifetimes, respectively. The experiments were done with 20 μ M actin in the presence of 0.5 mM $MgCl_2$ and 10 mM KCl.

2.5. Theoretical considerations

The steady-state fluorescence quenching data were first analysed by using the classical Stern–Volmer equation [34]:

$$\frac{F_0}{F} = 1 + K_{sv} [Q] \quad (2)$$

where the F_0 is the fluorescence intensity of the fluorophore in the absence of quencher and F is the fluorescence intensity at varying quencher concentrations $[Q]$. The modified form of the classical Stern–Volmer relation (the Lehrer equation) was used when the samples contained more than one fluorophore population with different accessibilities [35]:

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{\alpha K_{sv} [Q]} + \frac{1}{\alpha} \quad (3)$$

where α is the fraction of the accessible fluorophore population. Time dependent fluorescence quenching results were analysed with Eqs. (2) and (3) by replacing the intensities with the corresponding fluorescence lifetimes [34].

3. Results and discussion

The mDia1-FH2 induced changes in the conformation of actin filaments were described by using steady-state and time dependent fluorescence quenching experiments. The quencher was acrylamide (a neutral quencher) and the actin was covalently labelled with IAEDANS on Cys³⁷⁴ residue (Fig. 1.).

In the absence of acrylamide the fluorescence emission of IAEDANS was somewhat smaller in the presence of formin than in the absence of it (Fig. 2A) suggesting that the binding of the FH2 domain changed the microenvironment of the fluorophore in the subdomain 1. Acrylamide decreased the fluorescence emission of IAEDANS-labelled actin filaments in either the absence or in the presence of formin (Fig. 2A). The fluorescence emission was measured at various quencher concentrations and the data were analysed first with the classical Stern–Volmer equation (Eq. (2)). In the absence of formin the plot was linear and fit of Eq. (2) to the data gave K_{sv} value of $2.3 \pm 0.1 \text{ M}^{-1}$ (Fig. 2B).

In the presence of formins the classical Stern–Volmer plot was not linear (Fig. 2B), and the data were analysed with the modified Stern–Volmer (or Lehrer) equation (Eq. (3)). (Fig. 2C). The application of Eq. (3) provides the opportunity to evaluate the experimental data even when the quenching processes underlying the decrease of the fluorescence emission are

more complex. For control we analysed the data collected in the absence of formins with the modified Stern–Volmer equation (Fig. 2C) and obtained $2.3 \pm 0.1 \text{ M}^{-1}$ for the K_{SV} . The fraction of the quenchable fluorophore population (α) was calculated as the inverse of the y-intercept. This value was 102% indicating that all the fluorophores were accessible for the quencher. These observations made in the absence of formins are in good agreements with the results of the classical Stern–Volmer analyses (Fig. 2B) and also with the data from previous quenching studies on actin filaments [18,36,37].

The analyses with the modified Stern–Volmer equation (Eq. (3).) showed that when formin was added to the samples the value of K_{SV} increased. At 500 nM the K_{SV} was approximately three times larger ($5.8 \pm 0.1 \text{ M}^{-1}$) than in the absence of formins (Fig. 3.), while the fraction of the quenchable fluorophores (α) decreased to 71 % (Fig. 4A). When the experiments were repeated at various formin concentrations the data showed that the formin effect depended on the formin concentration, i.e. on the formin:actin concentration ratio. After reaching its highest value at around 500 nM the K_{SV} decreased at greater formin concentrations (Fig. 3.). At 3 μM formin the value of K_{SV} ($2.2 \pm 0.1 \text{ M}^{-1}$) was similar to that observed in the absence of formins ($2.3 \pm 0.1 \text{ M}^{-1}$). The formin concentration dependence of the fraction of the quenchable fluorophore population (α) followed opposite tendency, it was the lowest when the K_{SV} reached its maximum (Fig. 4B).

The experiments described above were carried out at 10 mM KCl and 0.5 mM MgCl_2 . Previously the effect of formins on the conformation of actin filaments was shown to be salt concentration dependent [17,18,20]. To test whether the quenching method was sensitive to the salt dependence of the conformational changes in actin we carried out the steady-state measurements at a higher ionic strength (50 mM KCl and 1 mM MgCl_2) as well. At this higher salt concentration the K_{SV} was similar in the absence of formins ($2.1 \pm 0.15 \text{ M}^{-1}$) to that observed at lower ionic strength ($2.3 \pm 0.1 \text{ M}^{-1}$). At 500 nM formin the K_{SV} was $2.9 \pm 0.2 \text{ M}^{-1}$ and the fraction of the quenchable fluorophores decreased to $84 \pm 3\%$. The formin concentration dependence of the quenching results was different at the two salt concentrations (Figs. 3 and 4.). Neither the value of quenching constant (K_{SV}) nor the fraction of the quenchable fluorophores (α) reached its extreme value within the applied formin concentration range, indicating that the interaction of formins with actin, and thus their effects on the actin filaments, were ionic strength dependent.

To test the magnitude of the contribution of static quenching mechanisms we measured the acrylamide concentration dependence of the fluorescence lifetimes in the absence and presence of formins. The experiments were done under the lower salt conditions (10 mM KCl and 0.5 mM MgCl_2). In the absence of formins the classical Stern–Volmer plot was linear and gave K_{SV} value of $2.6 \pm 0.3 \text{ M}^{-1}$ (Fig. 3.), close to the value obtained from the steady-state experiments ($2.3 \pm 0.1 \text{ M}^{-1}$). In the presence of formin the classical Stern–Volmer plots were not linear, and the modified Stern–Volmer equation was applied to interpret the results. When 500 nM formin was added to the sample the K_{SV} increased to $6.2 \pm 0.1 \text{ M}^{-1}$, to a similar extent that was observed in the steady-state measurements ($5.8 \pm 0.1 \text{ M}^{-1}$) under similar conditions (Fig. 3.), while the fraction of the quenchable fluorophores decreased to 53 %. The good agreement between the K_{SV} values from steady-state and time dependent fluorescence experiments indicated, that the contribution of the static quenching mechanisms to the overall quenching process was negligible.

The value of the K_{SV} is the product of the bimolecular quenching constant (k_+) and the fluorescence lifetime measured in the absence of quencher (τ_0). In the absence of acrylamide the average fluorescence lifetime of the actin bound IAEDANS was $19.6 \pm 0.6 \text{ ns}$ and $18.9 \pm 0.8 \text{ ns}$ in the absence and presence of formins (500 nM). The small formin

effect indicated that the change of the fluorescence lifetime could not be responsible for the observed formin induced differences in the value of K_{SV} .

4. Conclusions

The effect of static quenching processes could be excluded based on the experimental results. We conclude that the formins induced changes in the quenching parameters by changing the conformation of the protein matrix surrounding the Cys³⁷⁴ residue, i.e. in the subdomain 1 of actin protomers. The increase of the value of K_{SV} and the simultaneous decrease of the fraction of the quenchable fluorophore population (α) indicated that the conformational distribution of actin was modified, it was broader in the presence of formins than in the absence of them. After the binding of formins a population of the actin protomers (maximum approximately 30%) established a conformation where the fluorophore was not accessible for the solvent. Another protomer population became more accessible for the quenchers, which was reflected by the increase of the K_{SV} values. These intramolecular changes suggest that the subdomain 1 of the actin protomers became more flexible after the binding of formin and this change provided the structural framework for the establishment of the broader conformational distribution. This conclusion and the observed ionic strength dependence of the formin effects are in correlation with our previous observations [17-19].

To explain the formin concentration dependence of the changes that we observed in the quenching parameters we use the model published previously [18]. At lower formin concentrations (below approximately 500 nM) the formin is binding preferably to the high affinity ($K_D = 20\text{--}50$ nM, [6,38]) binding-sites at the barbed end of filaments. Formin binding to the barbed ends induce long range allosteric conformational changes which make the actin filaments more flexible [17,18], and result in the observed changes of the quenching parameters. At higher salt concentrations the formin binding to the filament ends is weaker and thus the development of its conformational effect in actin requires more formin. At greater formin concentrations the low affinity binding sites ($K_D = 3$ μ M [39]) on the sides of the actin filaments becomes more saturated. The formins attached to the sides of the filaments stabilise the interaction between the neighbouring actin protomers and thus stabilise the structure of the actin filaments too [17,18]. The reversal of the changes of the quenching parameters described in this study were attributed to this effect of the formins bound to the sides of actin filaments.

Our present observations, together with those made in previous studies underline the importance of the intramolecular conformational changes induced by formins (and possibly by other actin nucleation factors as well) in the structure of actin filaments. These results give further support to the idea [17,18] that actin nucleation factors can regulate the formation of cellular protein complexes by tuning and controlling the conformation, and thus the affinity of actin filaments for actin-binding proteins.

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Fig. 1.

The left panel shows the schematic representation of an actin filament (created based on pdb file 3B5U) showing six protomers. The position of the Cys³⁷⁴ is indicated with a dark surface. The right panel shows a magnified view of an actin monomer (pdb file 1ATN) indicating the four subdomains and the position of the fluorescent label used in this study.

Fig. 2.

Acrylamide quenching of the steady-state fluorescence emission of IAEDANS-labelled actin filaments. (A) Fluorescence intensity as the function of the wavelength. Black curves show initial intensities for 5 μM actin filaments while gray curves show IAEDANS intensities after the addition of 0.1 mM acrylamide. 5 μM IAEDANS-labelled actin filaments were used in the absence (left panel) or in the presence (right panel) of mDia1-FH2 (500 nM). (B) Classical Stern–Volmer plot for formin free actin filaments (filled squares), and for the filaments in the presence of 500 nM mDia1-FH2 (empty circles). Linear fit to the data (Eq. (2).) obtained in the absence of formins gave K_{SV} value of $2.3 \pm 0.03 \text{ M}^{-1}$ and α value of $99 \pm 0.01\%$. (C). Modified Stern–Volmer plots for actin filaments in the absence (filled squares) and in the presence (empty circles) of formin (500 nM). Fit of Eq. (3) to the data gave K_{SV} values of $2.3 \pm 0.03 \text{ M}^{-1}$ and $6.2 \pm 0.02 \text{ M}^{-1}$ for filaments in the absence and presence of formin, respectively. The corresponding α values were $93 \pm 4\%$ and $68 \pm 4\%$.

Fig. 3.

Dependence of the K_{SV} values obtained from the modified Stern–Volmer analyses on the formin concentration. Salt concentrations were 10 mM KCl and 0.5 mM MgCl₂ (filled squares and empty triangles) or 50 mM KCl and 1 mM MgCl₂ (empty circles). The data for filled squares and empty circles were obtained from steady-state measurements while empty triangles are from fluorescence lifetime measurements.

Fig. 4. Dependence of the fraction of fluorophores accessible for the quencher (α) on the formin concentration. The figure shows results obtained at 10 mM KCl and 0.5 mM MgCl_2 (A) or at 50 mM KCl and 1 mM MgCl_2 (B).