ELSEVIER

FEBS Letters 339 (1994) 297–301

FEBS 13682

FEBS LETTERS

The kinetics of the interaction between the actin-binding domain of α-actinin and F-actin

Philip A. Kuhlman, Jacqueline Ellis, David R. Critchley, Clive R. Bagshaw*

Department of Biochemistry, University of Leicester, University Road, Leicester, LE1 7RH, UK

Received 14 December 1993; revised version received 12 January 1994

Abstract

Measurement of the binding equilibrium for the interaction of α-actinin with F-actin is complicated by secondary reactions involving cross-linking and/or bundling of the actin filaments. To quantitate the initial binding event, we studied the interaction of the bacterially expressed actin-binding domain (ABD) of chick smooth muscle α-actinin with F-actin. Stopped-flow measurements revealed a quench in protein fluorescence and an enhancement in light scattering when ABD binds to F-actin yielding second order rate constants for association of 2×10^5, 1.8×10^6 and 4×10^6 $M^{-1} \cdot s^{-1}$ at 5°C, 15°C and 25°C, respectively. At the latter two temperatures the dissociation rate constants were 1.5 and 9.6 s^{-1}, giving equilibrium constants of 0.83 and 2.4 μM, respectively. Optical changes on mixing intact α-actinin with F-actin were dominated by secondary bundling events.

Key words: α-Actinin; Stopped-flow; Actin-bundling; Actin; Light scattering; Tryptophan fluorescence

1. Introduction

α-Actinin is a dimeric actin-binding protein originally identified as a component of the Z-line of striated muscle [1], but also found in cardiac and smooth muscle [2] and in non-muscle cells where it is located within stress fibres and adhesion plaques [3]. The protein contains an N-terminal actin-binding domain (ABD), four central spectrin-like repeats responsible for the rod shape of the molecule and the anti-parallel orientation of the two subunits, and two C-terminal EF-hand calcium-binding motifs [4]. Although attempts have been made to classify actin-binding proteins into those that cross-link and those that bundle actin filaments [5], α-actinin is capable of forming both kinds of structure depending on the conditions. Actin bundling is favoured by high actin concentrations with an α-actinin/actin molar ratio in excess of 0.05:1 [6,7].

A first step in the characterisation of the interaction between α-actinin and F-actin is an assessment of the binding affinity. Previous studies have used sedimentation of the α-actinin–actin complex as a means of quantifying the equilibrium dissociation constant. Using this approach, Meyer and Aebi [7] have estimated an apparent binding constant of 0.4 μM for the interaction between chicken gizzard smooth muscle α-actinin and F-actin at 22°C. However the binding profile was sigmoidal and the actin saturated at a stoichiometry of 0.07 mol α-actinin/mol actin monomer. This is consistent with α-actinin forming cross-links every turn of the actin helix. They also used relatively low speed sedimentation which pellets α-actinin–actin bundles but leaves the unbundled F-actin in the supernatant. On the other hand, Wachsstock et al. [6] used high speed centrifugation to pellet all the F-actin and bound α-actinin and deduced an equilibrium constant of 0.59 μM with a stoichiometry of 1 mol/mol under comparable conditions. Their data were adequately described by a hyperbola, but the error in the binding constant was relatively large in the case of the smooth muscle isoform because the total protein concentrations used were in considerable excess of the binding constant. The similarity between the values for the binding constant in these two studies [6,7] may therefore be fortuitous.

It is clear that following the initial binding of α-actinin to F-actin, subsequent bundling would favour further divalent binding in a co-operative manner [6]. The apparent binding constant may therefore appear tighter than the intrinsic interaction between α-actinin and actin. One possible approach to measure the inherent affinity between these proteins would be to study binding of the monomeric actin-binding domain (ABD) of α-actinin to F-actin. Using the ABD of chick smooth muscle α-actinin expressed in and purified from *E. coli* and a co-sedimentation assay, Way et al. [8] estimated the equilibrium binding constant to be 4.7 μM . However, we have detected a tendency for the expressed ABD to bundle F-actin, a property also displayed by myosin subfragment 1, another supposedly monovalent species [9]. To avoid the complications of secondary bundling interactions perturbing the measured equilibrium constant, we have employed a kinetic approach to quantitate the in-

*Corresponding author. Fax: (44) (533) 523369.

SSDI 0014-5793(94)00066-5

teraction between the bacterially expressed ABD and F-actin. Stopped-flow experiments have allowed the association and dissociation rate constants to be measured for this process.

2. Materials and methods

2.1. Expression and purification of the ABD of chicken smooth muscle α-actinin from E.coli lysates

An *NcoI–HincII* restriction enzyme fragment encoding the ABD of chicken smooth muscle α-actinin (residues 2–269) cloned into the *NcoI–StuI* cut *E. coli* expression vector pMW172 [8] was expressed (16 h, 30°C) in the BL21(DE3) strain of *E. coli*. Purification of the ABD was achieved using DE-52 anion exchange and Sephadex G-75 gel filtration chromatography. The ABD was concentrated using a mono Q column (Pharmacia) and was approximately 98% pure as determined by SDS polyacrylamide gel electrophoresis. ABD concentrations were determined using the Bradford method standardised against BSA.

2.2. Purification of rabbit skeletal muscle actin and chicken smooth muscle α-actinin

Rabbit skeletal muscle actin was purified from an acetone powder essentially as described by Pardee and Spudich [10]. The actin concentration was determined using the extinction coefficient $A^{1\%}_{290} = 6.2$ [11]. α-Actinin was purified from frozen chicken gizzard [12]. The purified α-actinin concentration was determined by its absorbance at 278 nm using an extinction coefficient of $A^{1\%}_{278} = 9.7$ [13].

2.3. Stopped-flow fluorescence and light scattering measurements

Stopped-flow fluorescence measurements were performed using a SF-17MV stopped-flow spectrometer (Applied PhotoPhysics, Leatherhead, UK) as described in Ellis et al. [14]. Tryptophan fluorescence was excited at 295 nm with the emission selected using a 340 nm cut-off filter placed in front of the photomultiplier. For the light scattering measurements, the incident wavelength was 340 nm. The temperature of the sample handling system was maintained using a water circulating temperature control mechanism. Sample stock solutions were diluted to the desired concentrations immediately prior to loading into the syringes. Typically, 10–15 traces were collected for signal averaging and subsequent non-linear regression analysis. The sample buffer comprised 2 mM Tris-HCl, 0.2 μM ATP, 50 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol and 1 mM NaN_3 adjusted to pH 8.0 at 20°C.

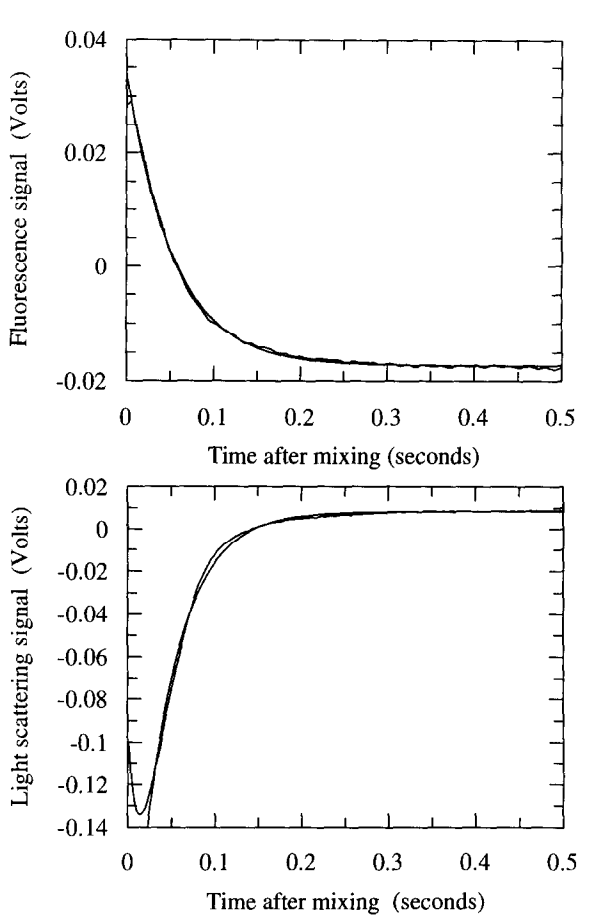

Fig. 1. Changes in tryptophan fluorescence and light scattering during the interaction of the ABD of α-actinin with F-actin. The interaction of 10 mM ABD with 2 mM F-actin (reaction chamber concentrations) at 15°C produced a fluorescence quench of 5% (A) with a concurrent increase in light scattering of 14% (B). The data were fitted to a single exponential to give a pseudo first order rate constant of 18.7 s^{-1} for the fluorescence change and 26.0 s^{-1} for the light scattering change.

3. Results

Binding of the ABD of α-actinin to F-actin was followed under pseudo first order conditions with a molar excess of ABD over F-actin (latter expressed in terms of equivalent G-monomers throughout). Following the rapid mixing in the stopped-flow instrument, the interaction between the ABD with F-actin was accompanied by a quench in tryptophan fluorescence and an increase in light scattering (Fig. 1). With increasing concentrations of ABD the relative change in fluorescence became less due to the higher background signal from unbound ABD. When mixed at stoichiometric concentrations above the binding constant (see below) a maximum quench in tryptophan fluorescence of 23% was observed.

The measurement of the initial rate of binding of the ABD of α-actinin to F-actin at a range of ABD concentrations allowed calculation of the equilibrium dissociation constant (K_d) at a number of different temperatures. A plot of the pseudo first order association rate constant (k^{app}) against ABD concentration was linear (Fig. 2), thus conforming to the equation $k^{app} = k_{+1}[ABD] + k_{-1}$, and yielded association rate constants (k_{+1}) of 0.2 $\mu M^{-1} \cdot s^{-1}$, 1.78 $\mu M^{-1} \cdot s^{-1}$ and 4.05 $\mu M^{-1} \cdot s^{-1}$ at 5°C, 15°C and 25°C, respectively. The corresponding dissociation rate constants (k_{-1}) determined from the intercept were < 0.5 s^{-1}, 1.47 s^{-1} and 9.65 s^{-1}, respectively. As $K_d = k_{-1}/k_{+1}$, these rate constants reflect equilibrium dissociation constants of 0.82 μM at 15°C and 2.38 μM at 25°C. The intercept which defines the dissociation rate constant for the data obtained at 5°C was insufficiently resolved from origin to determine an accurate K_d.

At higher ABD and F-actin concentrations than used above, a slower phase in light scattering was observed while the fluorescence emission showed little change. We have also seen F-actin bundles induced by ABD by electron microscopy (data not shown). It is possible that the

truncated region of the ABD presents an artifactual surface that interacts weakly with actin, as has been observed for the proteolytic subfragment 1 of myosin [9]. Nevertheless, the observation of a significant change in the light scattering signal without an appreciable change in fluorescence argues that the quench in fluorescence observed in Fig. 1 is not an artifact arising from the increased turbidity producing an inner filter effect, but reflects an environmental change in several tryptophan residues.

In order to support the estimates of the dissociation rate constants obtained from the intercept values (Fig. 2), we subjected the preformed ABD–actin complex (5 μM) to rapid dilution using a 1:10 syringe volume ratio in the stopped-flow apparatus. At 25°C we observed an enhancement in tryptophan fluorescence with a rate constant of 15 s^{-1}, consistent with intercept measurement. At lower temperatures the signal was insufficient to determine an accurate rate constant, in line with the higher affinity of binding which would result in less dissociation.

Binding of intact chick smooth muscle α-actinin to F-actin produced a change in tryptophan fluorescence quench which was too small to permit the interaction to be followed. A much diminished signal was expected because the tryptophan content of intact α-actinin (2×16 tryptophan residues) leads to a higher background. However, the changes in light scattering were much larger than for ABD binding, particularly when actin was in molar excess over the α-actinin (Fig. 3). It is likely that these changes reflect bundling of the F-actin. The light scattering profile clearly deviated from a mono exponential and we have not attempted a detailed analysis. At a constant F-actin concentration (4 μM), α-actinin concentrations above 250 nM produced similar light scattering profiles (Fig. 3A,B). At a concentration of 100 nM α-actinin the trace revealed a greatly reduced rate of change in light scattering, with a pronounced lag (Fig. 3C). Lower concentrations of α-actinin showed very little scattering change (data not shown).

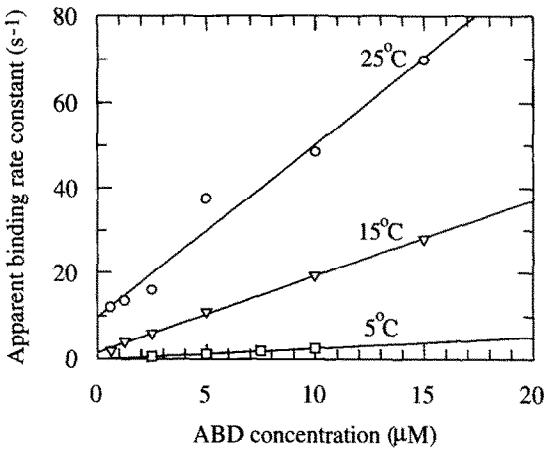

Fig. 2. Temperature dependence of the apparent binding constant for the interaction of the ABD of α-actinin with F-actin. The pseudo first order rate constant for the tryptophan fluorescence change on mixing ABD and F-actin is plotted as a function of ABD concentration at 5°C, 15°C and 25°C. The [F-actin] was at least fourfold lower than that of the [ABD]. The intercepts and gradients of the lines of best fit reflect dissociation rate constants of < 0.5 s^{-1} for 5°C, 1.47 s^{-1} at 15°C and 9.65 s^{-1} at 25°C, and association rate constants of 0.2 $\mu M^{-1} \cdot s^{-1}$ at 5°C, 1.78 $\mu M^{-1} \cdot s^{-1}$ at 15°C and 4.05 $\mu M^{-1} \cdot s^{-1}$ at 25°C. The ratio of these rate constants reflect K_d's of 0.82 μM at 15°C and 2.38 μM at 25°C.

4. Discussion

Determining the K_d for the interaction between actin cross-linking proteins such as α-actinin with F-actin is complicated by the formation of actin filament bundles. One method of circumventing this problem is to use the monovalent ABD. Nevertheless, under some conditions even the ABD has been observed to promote actin filament bundling. Such a secondary interaction may distort the estimates of binding constants as measured by centrifugation methods where the α-actinin–actin mixture is often preincubated for an hour or more. Using stopped-flow techniques described here, the initial rate of the interaction between the ABD of α-actinin and F-actin have been measured. The ratio of the derived association and dissociation constants provide equilibrium dissociation constant (K_d) for the interaction of 0.82 μM at 15°C and 2.38 μM at 25°C, and are in reasonable agreement with the value (K_d 4.7 μM at room temperature) for the same protein determined by co-sedimentation [8]. The temperature dependence of the binding equilibrium between the ABD of α-actinin and F-actin is similar to observations made with whole α-actinin [7,15]. A reduction in binding affinity with increasing temperature implicates hydrophobic interactions in the binding of α-actinin to F-actin.

The temperature dependence of the individual rate constants is also informative. The high activation energy for the association reaction (100 ± 40 kJ/mol) suggests the binding process involves some rearrangement in protein conformation following the initial collision [16,17]. The dissociation rate constant shows an even greater temperature dependence giving rise to a higher affinity for ABD binding at lower temperatures. These data do not support the assumption of Wachsstock et al. [6] that the association constant between α-actinin and actin is diffusion limited. Therefore the assumption that the association rate constants for all isoforms of α-actinin are constant is invalid.

The quench in tryptophan fluorescence could reflect residues in either or both the ABD of α-actinin and actin. Structural studies indicate the α-actinin binding site on actin involves sub-domain 1 (residues 360–372 and 112–115) on the opposite face to the myosin binding site [19]. This region is the location of the four tryptophan residues of rabbit skeletal muscle actin. The ABD resi-

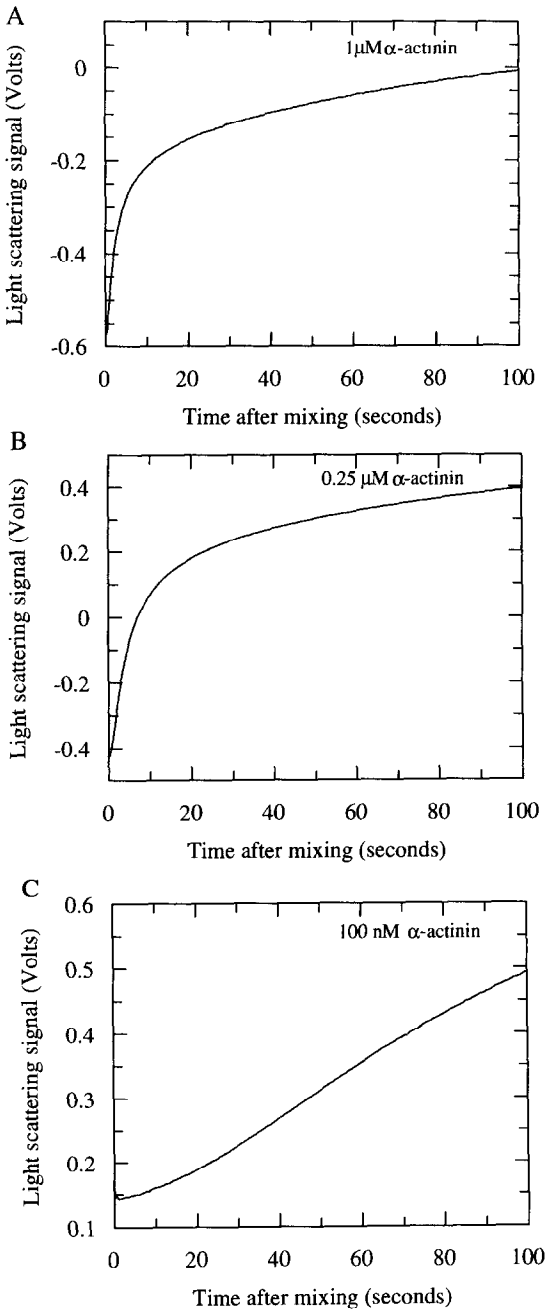

Fig. 3. The effect of α-actinin concentration on the bundling of actin filaments. Light scattering signals were monitored on mixing α-actinin with F-actin in a stopped-flow apparatus. The change in signal shown occurred when 1 μM (panel A), 0.25 μM (panel B) and 0.1 μM (panel C) α-actinin was mixed with 4 μM F-actin (reaction chamber concentrations).

dues involved in binding actin have been identified as 120–134 [18] which includes one tryptophan (residue 129) from a total of six in the expressed ABD (residues 2–269). The maximum amplitude we have observed of a 23% quench suggests more than one tryptophan is affected.

Light scattering has long been used as an empirical tool to follow the kinetics of decoration of F-actin filaments with myosin heads [17]. The binding profile of ABD to F-actin does not greatly deviate from a mono-exponential (Fig. 1B) suggesting a similar decoration process is involved. On the other hand, interaction of actin filaments with α-actinin produces a large, multiphasic change in light scattering, presumably due to the binding and subsequent bundling of the actin filaments. We have not found conditions where we can clearly separate these phases. The bundling process itself is likely to show complicated kinetics as the observed signal is a weighted sum from a heterogeneous population, with larger bundles having a stronger scattering characteristics. The light scattering signal nears completion after several minutes, in line with Meyer and Aebi's [7] limit for bundle formation of <15 min. At a constant [actin] of 4 μM, α-actinin concentrations above a threshold of 250 nM produce very similar light scattering changes. Concentrations of α-actinin lower than 250 nM show markedly reduced rates of bundle formation, while less than 50 nM α-actinin shows little or no signal. These results agree with Meyer and Aebi [6] who reported that a stoichiometry of 0.05 mol α-actinin:actin was sufficient to cause extensive bundling. Below this stoichiometry, their data and the modelling of Wachsstock *et al.* [6] suggests that α-actinin may crosslink the actin filaments to lock them into an isotropic network but actin bundles are not favoured. Such cross-linking is unlikely to result in a significant light scattering change as the bulk of the F-actin remains as single, entangled filaments.

The formation of actin bundles and isotropic networks by α-actinin alters the equilibrium position of the initial binding interaction, tending to stabilise the bound complex. The kinetic stability of these structures results in a metastable system that is not at true equilibrium. Hence, techniques to determine the K_d of binding that involve the formation of higher order actin structures reflect both binding affinity and the stabilising effect of the actin structures. The stabilising effect of actin bundle formation has been discussed by Grazi et al. [20] who proposed that at low actin concentrations (< 5 μM) actin bundling is rapid, but at higher concentrations it is limited owing to filament entanglement. The K_d for the binding interaction when bundling is permitted is lower, 0.4 μM [7], compared to when bundle formation is inhibited by high actin concentrations, 26 μM [21]. The formation of isotropic networks may stabilise the binding of cross-linking proteins in a similar manner but to a lesser extent. Further transient kinetics measurements using intact α-actinin may be a fruitful approach for testing these ideas. In this respect perturbation methods [17] are an important complement to flow methods because in the latter, the F-actin filaments undergo partial alignment which may favour bundle formation.

Acknowledgements. We are grateful to the SERC (Molecular Recognition Initiative) and the MRC for financial support. PAK was supported by an MRC post-graduate training award.

References

[1] Ebashi, S., Ebashi, F. and Maruyama, K. (1964) Nature 203, 645–646.
[2] Small, V., Furst, D.O. and Thornell, L.-E. (1992) J. Biochem. 208, 559–572.
[3] Hemmings, L., Kuhlman, P.A. and Critchley, D.R. (1992) J. Cell Biol. 116, 1369–1380.
[4] Baron, M.D., Davison, M.D., Jones, P. and Critchley, D.R. (1987) J. Biol. Chem. 262, 17623–17629.
[5] Matsudaira, P. (1991) Trends Biol. Sci. 16, 87–92.
[6] Wachsstock, D.H., Schwarz, W.H. and Pollard, T.D. (1993) Biophys. J. 65, 205–214.
[7] Meyer, R.K. and Aebi, U. (1990) J. Cell Biol. 110, 2013–2024.
[8] Way, M., Pope, B. and Weeds, A.G. (1992) J. Cell Biol. 119, 835–842.
[9] Ando, T. and Scales, D. (1985) J. Biol. Chem. 260, 2321–2327.
[10] Pardee, J.D. and Spudich, J.A. (1982) Methods Enzymol. 85, 164–181.
[11] Gordon, D.J., Yang, Y.Z. and Korn, E.D. (1976) J. Biol. Chem. 251, 7474–7479.
[12] Feramisco, J.R. and Burridge, K. (1980) J. Biol. Chem. 255, 1194–1199.
[13] Suzuki, A., Goll, D.E., Singh, I., Allen, R.E., Robson, R.M. and Stromer, M.H. (1976) J. Biol. Chem. 251, 6860–6870.
[14] Ellis, J., Bagshaw, C.R. and Shaw, W.V. (1991) Biochemistry 30, 10806–10813.
[15] Holmes, G.R., Goll, D.E. and Suzuki, A. (1971) Biochem. Biophys. acta 253, 240–253.
[16] Gutfreund, H. (1972) Enzymes: Physical Principles, Wiley, New York.
[17] Geeves, M.A. and Gutfreund, H. (1982) FEBS. Lett. 140, 11–15.
[18] Kuhlman, P.A., Hemmings, L. and Critchley, D.R. (1992) FEBS Letts. 304, 201–204.
[19] Lebart, M.-C., Mejean, C., Roustan, C. and Benyamin, Y. (1993) J. Biol. Chem. 268, 5642–5648.
[20] Grazi, E., Cuneo, P., Magri, E., Schwienbacher, C. and Trombetta, G. (1993) Biochemistry 32, 8896–8901.
[21] Sato, M., Schwarz, W.H. and Pollard, T.D. (1987) Nature 325, 828–830.