Analysis of filamin and α-actinin binding to actin by the stopped flow method

W.H. Goldmann and G. Isenberg*

Technical University of Munich, Department of Biophysics, E22, D-85747 Garching, Germany

We ascertained by the stopped flow method the overall association rate constant, k_{+1}, of filamin and α-actinin to fluorescently labelled filamentous actin of ~ $1.3 \times 10^6 \, M^{-1} \cdot s^{-1}$ and ~ $1.0 \times 10^6 \, M^{-1} \cdot s^{-1}$ as well as the overall dissociation rate constant, k_{-1}, of ~ $0.6 \, s^{-1}$ and ~ $0.4 \, s^{-1}$, respectively. The overall equilibrium constant, K, for filamin and α-actinin to actin deduced from the relation $K = k_{+1}/k_{-1}$ agree well with published data.

Actin; Filamin; α-Actinin; Transient kinetics

1. INTRODUCTION

Filamin is a 160 nm elongated, highly flexible homo-dimer of ~ 500.000 molecular mass, predominantly exhibiting β-strand conformation. In its dimeric form it possesses actin-binding sites at each (N-terminal) end and self-association sites at the (C-terminal) end [1,2]. Filamin is an actin-binding protein and a major constituent of smooth muscle [3,4]. It also plays a critical role in determining the three-dimensional arrangement of actin filaments in non-muscle cells.

α-Actinin is a dumb-bell shaped homodimer of ~ 200.000 molecular mass. It has an actin-binding site at each end and is able to cross-link (like filamin) filamentous actin. α-Actinin is found in various muscle cells as well as in non-muscle cells, where it is believed to be involved in cell transformation and locomotion [5].

Although filamin and α-actinin have been known as actin cross-linking proteins, for a long time only values exist for the overall equilibrium constant, K, with actin [1,5]. We present here the association (k_{+1}) and dissociation (k_{-1}) rate constants of filamin/α-actinin actin binding. This allows us to evaluate the function of these two proteins in a more precise manner.

2. MATERIALS AND METHODS

2.1. Proteins

Filamin was isolated by the method of Shizuta et al. [6]. Low ionic strength extraction of chicken gizzard was followed by Mg^{2+} and $(NH_4)_2SO_4$ precipitations, and ion-exchange and gel-filtration column chromatographies. For all experiments, an additional hydroxylapatite column was used to further purify the protein.

α-Actinin was prepared from smooth muscle as described by Craig et al. [7] with the modification that fresh turkey gizzard was cut into cubes of ~ 1 cm side length and used as starting material for the glycerol extraction step. Actin was prepared according to Spudich and Watt

[8] from acetone powder obtained from rabbit back muscle followed by a gel filtration step as described by MacLean-Fletcher and Pollard [9]. Actin was labelled with 7-chloro-4-nitrobenzeno-2-oxa- 1,3-diazole (= NBD) for fluorescence measurements following the protocol of Detmers et al. [10]. The labelling efficiency was ~ 0.7 (NBD/actin molecule). In all transient kinetic studies the following buffer was used: 100 mM KCl, 2 mM Tris-HCl, pH 8.0; 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT (dithiothreitol) and 0.005% NaN_3 at 20°C. Under these experimental conditions actin was in a polymerized state up to ~ 95% [11]. (Note: the signal of fluorescently labelled globular (G)-actin under non-polymerizing conditions was significantly weaker when measuring filamin binding [12]).

2.2. Transient kinetics

All experiments were performed on a stopped flow fluorimeter SF 61 supplied by Hi-Tech (Salisbury, Wilts., UK). The unit consists of two 5 ml drive syringes which can be filled from reservoirs. The syringes are driven simultaneously by compressed air at 4 bar. The dead time of the apparatus was ~ 1 ms. The solutions were rapidly mixed in a quartz observation/reaction cell. Light was transmitted to the cell via a quartz fibre optic light guide. Emitted light from the reaction/observation cell was sent directly to the photomultiplier. The flow was stopped using a syringe. A micro-switch was pressed by the stopping syringe to give a trigger pulse. Light was supplied from a 75 W Xenon lamp (Hamamatsu, L 2194-02), driven by an arc lamp power supply LPS-220 from Photo Technology International and passed through a Hi-Tech M 300 monochromator with 3 nm band pass width. The light was passed through a OG 520 cut-off filter and detected from the photomultiplier at 90° to the incident light.

(NBD) fluorescence was monitored by exciting at 480 nm and measuring emission at 540 nm. Output from the photomultiplier was electronically filtered, and the time constant was never more than 5% of the half time of the reaction observed. The temperature of the stopped flow machine was maintained within ± 0.1°C at 20°C during the course of the experiments. Kinetic data were collected on a Highscreen 486/33 computer using a 10% pre-trigger and analysed by a fitting routine (IS-2) supplied by Hi-Tech.

3. RESULTS

3.1. Stopped flow studies of filamin-actin and α-actinin-actin association and dissociation

The association reaction between filamin and actin
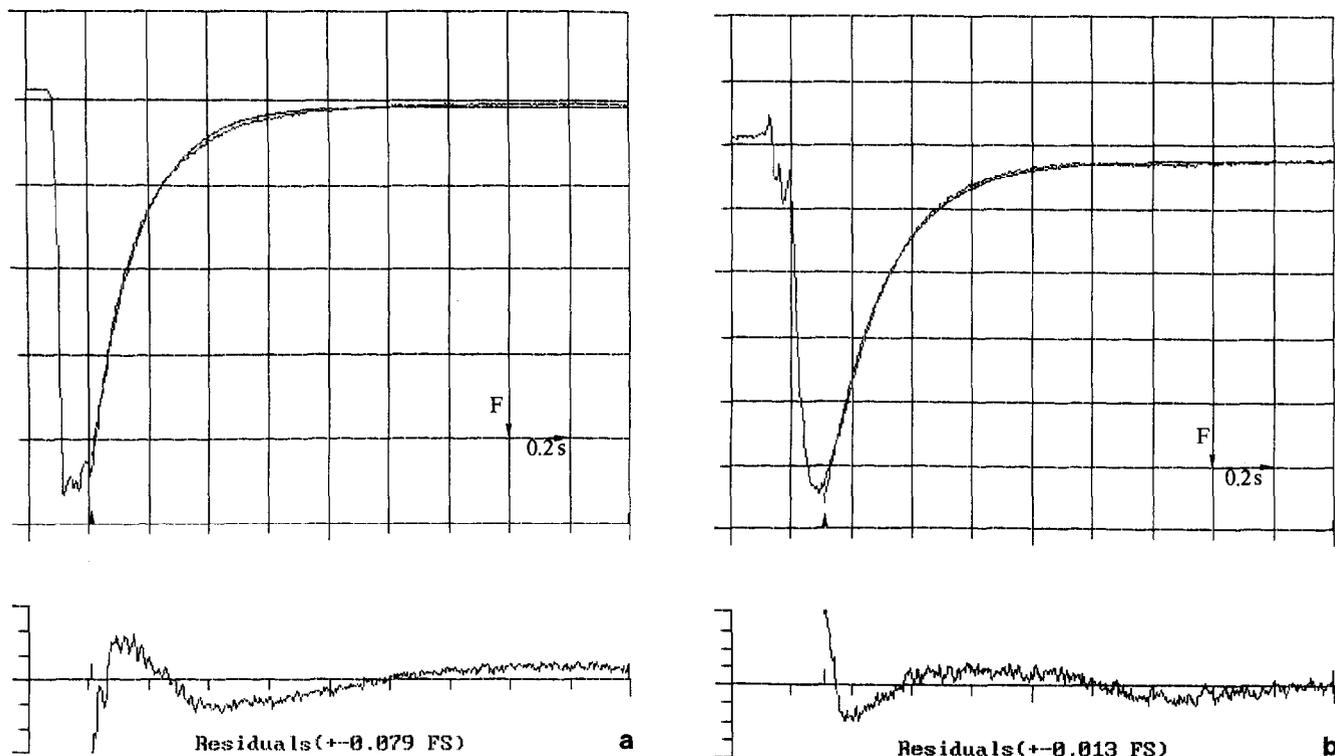
*Corresponding author.

Fig. 1. The trace represents the average of 5 consecutive measurements in the stopped flow machine. The arrow indicates the start of the reaction and fit. The best fit to a single exponential is shown superimposed with the residuals given below. Buffer conditions as stated in section 2. Protein concentration: 2 μM (NBD) labelled actin and 3 μM filamin or α-actinin. The observed rate, k_{obs} = 6.5 s^{-1} for filamin (a) and k_{obs} = 5.0 s^{-1} for α-actinin (b). One square represents F (= fluorescence) = 20%.

and between α-actinin and actin was studied using the change in (NBD) fluorescence of labelled actin mixed with filamin or α-actinin. The fluorescence decay could be described by a single exponential at the given concentration. Fig. 1a,b shows a typical result for fluorescence transients with a least squares best fit exponential superimposed. Assuming the equation

$$k_{obs} = k_{+1} \times ([A] + [B]) + k_{-1}$$

describes this reaction adequately, then the observed rate with respect to [A] + [B] determines the value of k_{+1} of the binding reaction ([A] is the concentration of actin and [B] is the concentration of filamin or α-actinin). We obtain ~ 1.3 × 10^6 M^{-1}·s^{-1} for filamin and ~ 1.0 × 10^6 M^{-1}·s^{-1} for α-actinin.

Since the intercept of an assumed plot of k_{obs} vs. [A] + [B] is small and not sufficiently accurately determined by this method, the rate of release of filamin or α-actinin from (NBD)-actin was measured directly by displacing the (NBD)-actin with an excess of native actin. Such an experiment is shown in Fig. 2a,b where 2 μM (NBD)-actin and 0.5 μM filamin or α-actinin were displaced by the addition of a fivefold excess of native actin. The observed rates are for filamin 0.61 s^{-1} and for α-actinin 0.44 s^{-1} which correspond to k_{-1}. The ob-

served rates were unaffected by increasing the actin concentration. The dissociation rate measured here is a factor of two lower than the value obtained from the relation $K = k_{+1}/k_{-1}$. In this case, the values obtained by direct displacement are preferred. (Note: dissociation rates from intercepts are inaccurate when the observed rates are much greater than the value of the intercept).

4. DISCUSSION

In the present study we have determined the binding parameters of filamin and α-actinin to filamentous actin in solution by a kinetic approach. Previously, the filamin-to-actin and α-actinin-to-actin binding have been defined in numerous co-sedimentation and centrifugation assays [1,5]. We were interested to determine the overall equilibrium constant, K, by using transient kinetics and measuring the association rate constant, k_{+1}, and the dissociation rate constant, k_{-1}, of actin-filamin and actin-α-actinin complexes. For this reason we employed the stopped flow apparatus. This technique allowed the determination of the overall association rate constant, k_{+1}, for filamin-to-actin (~ 1.3 × 10^6 M^{-1}·s^{-1}) and α-actinin-to-actin (~ 1.0 × 10^6 M^{-1}·s^{-1}) with an overall dissociation rate constant, k_{-1}, of (~ 0.6 s^{-1}) and (~0.4 s^{-1}), respectively. These rates compare well with
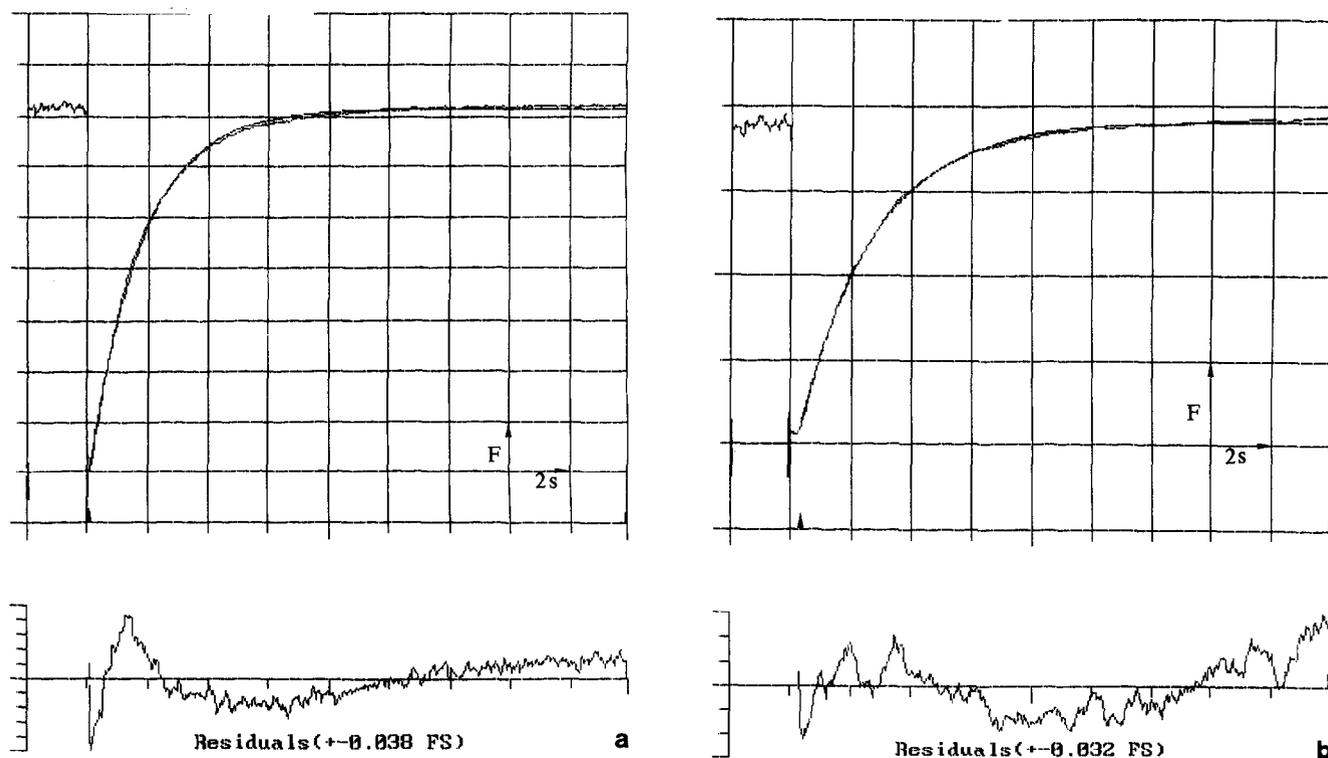
Fig. 2. Displacement of (NBD)-actin from (NBD)-actin–filamin complex by the addition of excess native actin. Change in fluorescence on mixing $2\,\mu M$ (NBD)-actin and $0.5\,\mu M$ filamin or α-actinin with $10\,\mu M$ native actin. The trace is an average of three reactions in the stopped flow apparatus and the best fit exponential is superimposed with the residuals given below, $k_{obs} = 0.61\ s^{-1}$ for filamin (a) and $k_{obs} = 0.44\ s^{-1}$ for α-actinin (b). The arrow indicates the start of the reaction and fit. Buffer conditions as stated in section 2. One square represents in (a) F (= fluorescence) = 14% and in (b) F = 25%.

previously reported data for other actin binding proteins [13]. When trying to evaluate the function of filamin and α-actinin in vivo, it is important to present accurate numbers of the affinity with respect to actin binding. The rate constants reported here allow filamin and α-actinin to cross-link actin filaments within a similar time range and with similar high affinity rendering filamin to compete with α-actinin in a comparatively efficient cross-linking process and vice versa. This would be consistent with the notion that in mutant systems where α-actinin is lacking no changes in phenotype could be observed [14]. We have also used these kinetic data in our calculations and experiments to determine the viscoelastic properties of filamin and α-actinin cross-linked actin networks. In micro-rheological measurements it was found that the actin to cross-linker molar ratio needed to change a filamin–actin or an α-actinin–actin solution from sol to gel state was by a factor of two lower [15,16] than reported. The results would imply that filamin and α-actinin could regulate cytoskeletal arrangement and cellular shape changes on a more subtle basis than previously thought.

REFERENCES

[1] Hartwig, J.H. and Stossel, T.P. (1981) J. Mol. Biol. 145, 563–581.
[2] Hartwig, J.H. and Kwiatkowski, D.J. (1991) Curr. Opinion Cell Biol. 3, 87–97.
[3] Hock, R.S., Davis, G. and Speicher, D.W. (1990) Biochemistry 29, 9441–9451.
[4] Small, J.V., Fürst, D.O. and De May, J. (1986) J. Cell Biol. 102, 210–220.
[5] Meyer, R.K. and Aebi, U. (1989) In: Series in Biophysics Vol. III (Engel, A. and Aebi, U., Eds.) Springer, Heidelberg, pp. 57–59.
[6] Shizuta, Y., Shizuta, H., Gallo, M., Davies, P., Pastan, I. and Lewis, M.C. (1976) J. Biol. Chem. 251, 6562–6567.
[7] Craig, S.W., Lancashire, C.L. and Cooper, J.A. (1982) Methods Enzymol. 85, 316–321.
[8] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[9] MacLean-Fletcher, S. and Pollard, T.D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27.
[10] Detmers, P., Weber, A., Elzinga, M. and Stephens, R.E. (1981) J. Biol. Chem. 256, 99–105.
[11] Korn, E.D., Carlier, M.F. and Pantaloni, D. (1987) Science 238, 638–644.
[12] Goldmann, W.H. and Isenberg, G. (1994) J. Muscle Res. Cell Mot. 15, in press.
[13] Goldmann, W.H., Niggli, V., Kaufmann, S. and Isenberg, G. (1992) Biochemistry 31, 7665–7671.
[14] Schleicher, M., Wallraff, E., Gerisch, G. and Isenberg, G. (1989) Protoplasma Suppl. 2, 22–26.
[15] Ruddies, R., Goldmann, W.H., Isenberg, G. and Sackmann, E. (1993) Biochem. Soc. Trans. 21, 37S.
[16] Müller, O., Gaub, H.E., Bärmann, M. and Sackmann, E. (1991) Macromolecules 24, 3111–3120.