Isolation from bovine brain of tropomyosins that bind to actin filaments with different affinities

Thomas Keiser and Albrecht Wegner

Institut für Physiologische Chemie, Ruhr-Universität, Universitätsstr. 150, D-4630 Bochum, FRG

Received 13 May 1985

Tropomyosin was isolated from bovine brain using mild conditions thereby avoiding heat precipitation. Separation by DEAE ion exchange chromatography yielded a 33 kDa tropomyosin and a mixture of 30 and 32 kDa tropomyosin. Binding of the tropomyosins to actin filaments was measured by a newly developed method. The binding was assayed by the retarding effect of tropomyosin on actin polymerization. The 33 kDa tropomyosin was found to bind to actin filaments with considerably higher affinity than the 30 and 32 kDa tropomyosin.

Tropomyosin *Actin* *Polymerization kinetics* *Fluorescence*

1. INTRODUCTION

Tropomyosin is an actin filament-associated protein that has been isolated from various types of cells [1–6]. Tropomyosin occurs in many isoforms that are specific for cells and organs [6–13]. The isoforms have been shown to bind to actin filaments with different affinities [5,14–16]. In this paper we quantified the assembly of tropomyosins isolated from bovine brain with actin filaments by a newly developed method. Binding of tropomyosin was assayed by its retarding effect on actin polymerization [17].

2. MATERIAL AND METHODS

2.1. *Preparation of proteins*

Actin was prepared according to the method of Rees and Young [18] with the modifications described in [19]. Part of the protein was covalently modified with *N*-ethylmaleimide and 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole to produce fluorescently labeled actin [20]. The actin concentration was determined photometrically at 290 nm using an extinction coefficient of 24900 $M^{-1} \cdot cm^{-1}$ [19].

Tropomyosin was prepared according to a newly developed method. Heat precipitation frequently used by others was avoided in order to obtain the tropomyosin in a native as possible state. Tropomyosin was prepared from bovine brain of freshly killed animals. The brains were freed of meningae and blood vessels and stored at −18°C. Ether ethanol powder was prepared as described by Fine et al. [5]. Powder (100g) was extracted overnight in 1.6 l buffer A (1 M KCl, 30 mM potassium phosphate, pH 7.0, 3 mM EDTA and 0.5 mM PMSF [5]). After centrifugation for 30 min at 20000 × *g* tropomyosin was precipitated isoelectrically by adjusting the supernatant to pH 4.1 using 5 M HCl. The precipitate was collected by centrifugation for 45 min at 20000 × *g* and resuspended in 400 ml buffer A which was vigorously stirred for several hours. The suspension was centrifuged for 45 min at 20000 × *g* to remove insoluble particles. The isoelectric precipitation was repeated. The pellet was resuspended in 240 ml buffer A thereby vigorously stirring overnight. After centrifugation for 45 min at 20000 × *g* ammonium sulfate was added to the

Abbreviation: PMSF, phenylmethanesulfonyl fluoride

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

supernatant to reach 40% saturation. The precipitate was centrifuged for 45 min at 20000 × g and was discarded. The supernatant was adjusted to 60% ammonium sulfate saturation. The precipitate consisting of protein and potassium sulfate crystals was collected by centrifugation for 45 min at 45000 × g and redissolved in 30 ml buffer B (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol and 0.5 mM EDTA). After exhaustive dialysis against buffer B the protein was applied to a DEAE ion exchange column (1.5 × 15cm, TSK DEAE-650(S), purchased from Merck). The column was carefully washed with 100 ml buffer B to remove unbound material. Tropomyosin was eluted by a linear NaCl gradient. The starting buffer contained 100 mM NaCl (200 ml buffer B) and the final buffer contained 500 mM NaCl (200 ml buffer B plus 400 mM NaCl). For the binding studies, tropomyosin was dialyzed against buffer C (100 mM KCl, 5 mM triethanolamine HCl, pH 7.5 and 1 mM dithiothreitol). The tropomyosin concentration was determined according to Lowry et al. [21].

2.2. *Binding of tropomyosin to actin filaments*

2.2.1. Polymerization retardation assay

Rabbit skeletal muscle tropomyosin has been reported to retard actin polymerization because tropomyosin inhibits spontaneous fragmentation of filaments [17]. We used the retardation of actin polymerization for assaying the binding of bovine brain tropomyosin to actin filaments. 1 ml of various concentrations of tropomyosin dialyzed against buffer C was mixed with 100 μl of 1 M KCl, 100 μl of 100 mM $MgCl_2$ and 800 μl of a 3.75 μM monomeric actin solution in fluorescence cells. The actin contained 10% fluorescently labeled actin and was dissolved in a buffer containing 0.5 mM ATP, 0.2 mM $CaCl_2$, 200 mg/l NaN_3 and 5 mM triethanolamine HCl, pH 7.5. The final composition of the mixtures was 1.5 μM actin, various concentrations of tropomyosin, 5 mM $MgCl_2$, 100 mM KCl, 0.08 mM $CaCl_2$, 0.2 mM ATP, 160 mg/l NaN_3, 0.5 mM dithiothreitol and 4 mM triethanolamine HCl, pH 7.5. The temperature of all solutions and samples was kept at 37°C. Polymerization was measured by the 2–2.5-fold increase of the fluorescence intensity on transition of actin from the monomeric to the polymeric state [20]. The initial fluorescence intensity F_i was somewhat higher in the presence of tropomyosin than in the absence. The increase of the fluorescence intensity ΔF on actin polymerization was independent of the tropomyosin concentration. The concentration of polymerized actin c_p was calculated from the fluorescence intensity F according to the following equation:

$$c_p = \frac{F - F_i}{\Delta F} \Delta c_p \quad (1)$$

where Δc_p is the final concentration of polymerized actin. The assay is most sensitive if traces of polymeric actin are removed from monomeric actin by centrifugation for 25 min at 50000 × g before addition of monomeric actin to tropomyosin. However, the polymerization retardation assay works also without centrifugation of monomeric actin. If binding of tropomyosin is studied under other experimental conditions the actin concentration has to be altered so that polymerization is complete within a time comfortable for fluorescence measurements (e.g. 2 h).

2.2.2. Centrifugation assay

The centrifugation assay was used as an independent method [2,14]. Actin tropomyosin filaments and unbound tropomyosin were separated by centrifugation for 40 min at 50000 × g. The supernatant and the pellet were applied to an SDS-polyacrylamide gel [22]. The gels were stained with Coomassie blue.

3. RESULTS

3.1. *Heterogeneity of tropomyosin*

Tropomyosin was eluted from the DEAE ion exchange column as a peak with a preceding shoulder at about 150 mM NaCl (fig.1). The fractions of the tropomyosin peak were analyzed by SDS-polyacrylamide gel electrophoresis. The preceding shoulder (fig.1, pool A) revealed a 33 kDa band (fig.2a). The fractions which were collected when the absorption was maximum (fig.1, pool C) contained 4 bands migrating at 30, 32, 33 and 36 kDa. The last pool (fig.1, pool B) contained bands of 30 and 32 kDa and a minor 36 kDa band (fig. 2b). The staining intensities of the 30 and 32 kDa bands of the last fractions were similar suggesting that the 30 and 32 kDa peptide form a heterodimer.

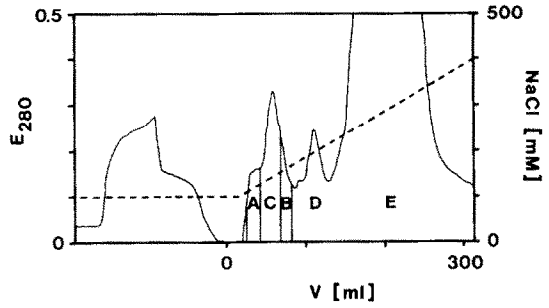

Fig.1. DEAE ion exchange chromatography (Fractogel TSK DEAE-650(S)). E_{280}, absorption at 280 nm; V, volume of effluent; ---, NaCl concentration. Tropomyosin was combined in 3 pools (A,B,C). Peak D, unidentified 48 kDa protein; peak E, RNA.

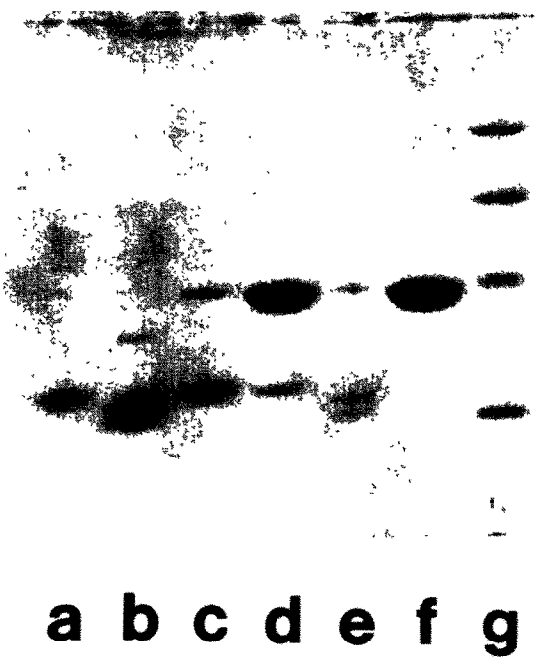

Fig.2. SDS-polyacrylamide gel electrophoresis. (a) 33 kDa tropomyosin (3 μg) (fig.1, pool A), (b) 30 and 32 kDa tropomyosin (6 μg) with a minor 36 kDa tropomyosin (fig.1, pool B), (c–f) binding of 2 μM tropomyosin to 15 μM actin analyzed by centrifugation. The pellets were dissolved in the original volume. 25 μl of each sample was applied to the gel. (c) Supernatant 33 kDa tropomyosin, (d) pellet 33 kDa tropomyosin, (e) supernatant 30 and 32 kDa tropomyosin, (f) pellet 30 and 32 kDa tropomyosin, (g) reference proteins; phosphorylase *b*, 90 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa.

The proteins of pool A and B were identified as tropomyosin by the amino acid composition [5].

3.2. *Binding of the tropomyosins to actin filaments*

We investigated the 33 kDa tropomyosin of pool A and the mixture of 30 and 32 kDa tropomyosin of pool B because these 2 tropomyosin pools were the most homogeneous fractions available. Binding of tropomyosin to actin filaments was studied by its retarding effect on actin polymerization. At salt concentrations of 1 mM $MgCl_2$ and 100 mM KCl, no polymerization retardation by tropomyosin was detectable. 5 mM $MgCl_2$ and 100 mM KCl were necessary for assembly of tropomyosin with actin. Similar observations were reported by Côté and Smillie [16] for platelet tropomyosin. The time courses of actin polymerization in the presence of various tropomyosin concentrations are depicted in fig.3 and 4. At concentrations below 0.2 μM, the 33 kDa tropomyosin has no significant effect on the rate of actin polymerization. Above 2 μM, the 33 kDa tropomyosin retards actin assembly maximally, suggesting that tropomyosin covers actin filaments completely. An intermediate retardation is found at 0.5 or 1 μM 33 kDa tropomyosin (fig.3). Considerably higher con-

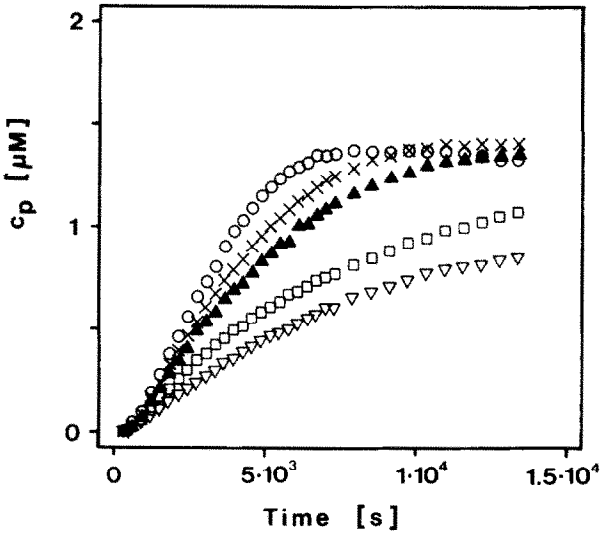

Fig.3. Polymerization retardation assay of the 33 kDa tropomyosin. Tropomyosin concentrations: ○, 0 μM; ×, 0.28 μM; ▲, 0.56 μM; □, 1.08 μM; ▽, 2.15 μM. c_p, Concentration of polymeric actin.

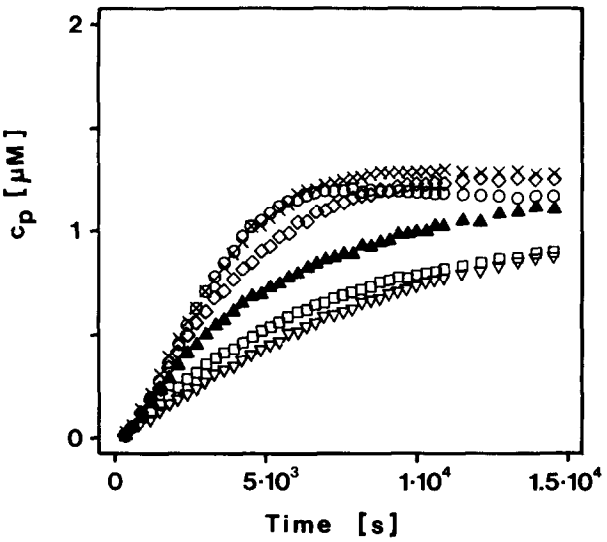

Fig.4. Polymerization retardation assay of the 30 and 32 kDa tropomyosin. Tropomyosin concentrations: ○, 0 μM; ×, 1.3 μM; ◇, 2.6 μM; ▲, 5.2 μM; □, 10.4 μM; ▽, 15.6 μM. c_p, Concentration of polymeric actin.

centrations of the 30 and 32 kDa tropomyosin are required for retardation of actin polymerization. Fig.4 shows that concentrations 1 or 2 μM 30 and 32 kDa tropomyosin do not effect actin polymerization. Above 10 μM maximal retardation of actin polymerization is reached. An intermediate effect is detected at 5 μM. About 10-fold more 30 and 32 kDa tropomyosin is necessary for partial saturation of actin filaments than 33 kDa tropomyosin.

Binding of tropomyosin was also studied by separating filaments and unbound tropomyosin by centrifugation. The sedimented filaments and the unbound tropomyosin in the supernatant were analyzed by SDS-polyacrylamide gel electrophoresis. The concentration of both the 33 kDa tropomyosin and the mixture of 30 and 32 kDa tropomyosin was 2 μM. The actin concentration was 15 μM. Fig.2 shows that the 33 kDa tropomyosin is bound to actin filaments (fig.2d) whereas the mixture of 30 and 32 kDa tropomyosin is not bound (Fig.2f). The 36 kDa tropomyosin present at low concentrations has evidently the highest affinity for actin filaments as this tropomyosin cosediments with actin filaments (fig.2d,f). The tropomyosins appeared in the supernatants (Fig.2c,e). The sedimentation studies are in agreement with the polymerization retardation assay. According to the polymerization retardation assay, 2 μM 33 kDa tropomyosin binds to actin filaments (fig.3) whereas the mixture of 30 and 32 kDa tropomyosin remains unbound (fig.4).

4. DISCUSSION

In this study, tropomyosin was isolated from bovine brain using mild conditions. Heat precipitation that has been widely used for purification of tropomyosin was avoided as tropomyosin from skeletal muscle has been shown to be unfolded at high temperatures [23].

The assembly of tropomyosin with actin filaments was investigated by the retarding effect of tropomyosin on actin polymerization and by centrifugation. The polymerization retardation assay turned out to be easy to handle and to yield semi-quantitative data. Other methods described in the literature are light scattering and flow birefringence [14,24]. Light scattering required large amounts of tropomyosin and experience in preparation of samples. The light scattering method is less suitable for routine use than the polymerization retardation assay and centrifugation. However, light scattering provides detailed information about binding of tropomyosin to actin filaments. By light scattering skeletal muscle tropomyosin could be shown to bind several 100-fold more tightly to a binding site contiguous to another tropomyosin molecule than to an isolated binding site along actin filaments. Thus, binding of tropomyosin to actin filaments is highly co-operative [14]. We have no experience with flow birefringence which is another useful method [24]. The polymerization retardation assay, centrifugation and light scattering lead to consistent results as demonstrated here and in [17].

Several studies on the binding of tropomyosin to actin filaments have been reported. Differences of the affinities of tropomyosin from different cells or organs for actin filaments have been detected. Skeletal muscle tropomyosin binds stronger to actin filaments than tropomyosin isolated from brain or platelets [5,14–16]. The two isoforms occurring in skeletal muscle have almost identical affinities for actin filaments [25]. In constrast, the isoforms from chicken embryo fibroblasts have been reported to bind to actin filaments with quite different affinities [13].

Tropomyosin has been shown to stabilize actin filaments against spontaneous fragmentation and against fragmentation by actin filament-breaking proteins [17,26–29]. Assembly and disassembly of the actin tropomyosin complex may be reactions by which the organization of actin filaments is regulated. Changes of the concentration of single tropomyosin isoforms have been suggested to regulate binding of tropomyosin to actin filaments [13,30]. Owing to the highly co-operative binding of tropomyosin to actin filaments, relatively small changes of the concentration of tropomyosin may induce transitions between actin filaments completely covered with tropomyosin and actin filaments completely free of tropomyosin [14].

ACKNOWLEDGEMENTS

We thank Mrs E. Werres for excellent technical assistance and Mrs E. Hoffmann-Posorske and Mr H. Korte for helping with the amino acid analysis. This study was supported by the Deutsche Forschungsgemeinschaft (grant We 808/5 and Sonderforschungsbereich 168).

REFERENCES

[1] Bailey, K. (1948) Biochemistry J. 43, 271–278.
[2] Laki, K., Maruyama, K. and Kominz, D.R. (1962) Arch. Biochem. Biophys. 98, 323–330.
[3] Cohen, I. and Cohen, C. (1972) J. Mol. Biol. 68, 383–387.
[4] Tanaka, H. and Hatano, S. (1972) Biochim. Biophys. Acta 257, 445–451.
[5] Fine, R.E., Blitz, A.L., Hitchcock, S.E. and Kaminer, B. (1973) Nat. New Biol. 245, 182–186.
[6] Côté, G.P. (1983) Mol. Cell. Biochem. 57, 127–146.
[7] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[8] Fine, R.E. and Blitz, A.L. (1975) J. Mol. Biol. 96, 447–454.
[9] Côté, G., Lewis, W.G. and Smillie, L.B. (1978) FEBS Lett. 91, 237–241.
[10] Bretscher, A. and Weber, K. (1978) FEBS Lett. 85, 145–148.
[11] Der Terrossian, E., Fuller, S.D., Stewart, M. and Weeds, A.G. (1981) J. Mol. Biol. 153, 147–167.
[12] Giometti, C.S. and Anderson, N.L. (1984) J. Biol. Chem. 259, 14113–14120.
[13] Lin, J.J., Helfman, D.M., Hughes, S.H. and Chou, C. (1985) J. Cell Biol. 100, 692–703.
[14] Wegner, A. (1979) J. Mol. Biol. 131, 839–853.
[15] Yang, Y.Z., Korn, E.D. and Eisenberg, E. (1979) J. Biol. Chem. 254, 7137–7140.
[16] Côté, G.P. and Smillie, L.B. (1981) J. Biol. Chem. 256, 7257–7261.
[17] Wegner, A. (1982) J. Mol. Biol. 161, 217–227.
[18] Rees, M.K. and Young, M. (1967) J. Biol. Chem. 242, 4449–4458.
[19] Wanger, M. and Wegner, A. (1985) Biochemistry 24, 1035–1039.
[20] Detmers, P., Weber, A., Elzinga, M. and Stephens, R.E. (1981) J. Biol. Chem. 256, 99–105.
[21] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Lehrer, S.S. (1978) J. Mol. Biol. 118, 209–226.
[24] Maruyama, K. (1964) Arch. Biochem. Biophys. 105, 142–150.
[25] Wegner, A. (1980) FEBS Lett. 119, 245–247.
[26] Hinssen, H. (1981) Eur. J. Cell Biol. 23, 234–240.
[27] Bernstein, B.W. and Bamburg, J.R. (1982) Cell Motil. 2, 1–8.
[28] Fattoum, A., Hartwig, J.H. and Stossel, T.P. (1983) Biochemistry 22, 1187–1193.
[29] Bonder, E.M. and Mooseker, M.S. (1983) J. Cell. Biol. 96, 1097–1107.
[30] Hendricks, M. and Weintraub, H. (1981) Proc. Natl. Acad. Sci. USA 78, 5633–5637.