Mapping the binding site of thymosin \( \beta_4 \) on actin by competition with G-actin binding proteins indicates negative co-operativity between binding sites located on opposite subdomains of actin

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The \( \beta \)-thymosins are small monomeric (G-)actin-binding proteins of 5 kDa that are supposed to act intracellularly as actin-sequestering factors stabilizing the cytoplasmic monomeric pool of actin. The binding region of thymosin \( \beta_4 \) was determined by analysing the binding of thymosin \( \beta_4 \) to actin complexed with DNase I, gelsolin or gelsolin segment 1. Binding was analysed by determining the increase in the critical concentration of actin polymerization from the actin–T\( \beta_4 \) fragment 1 or phalloidin induce actin repolymerization from the actin–T\( \beta_4 \) complex. It has been shown that profilin \( \beta_4 \) and a number of other G-AbPs can stimulate actin repolymerization from the actin–T\( \beta_4 \) complex. It is suggested that the dissociation of thymosin \( \beta_4 \) binding to actin is caused by negative co-operativity between their spatially separated binding sites on actin. A similar negative co-operativity was observed between DNase I and gelsolin segment 1 binding to actin. The results therefore indicate that the respective binding sites for DNase I and segment 1 on subdomains 1 and 2 of actin are linked in a negative co-operative manner.

INTRODUCTION

Thymosin \( \beta_4 \) (T\( \beta_4 \)) and related peptides (the \( \beta \)-thymosins) are small G-actin-binding proteins (AbPs) of molecular mass 5 kDa. They are supposed to be the main actin-sequestering factors in many eukaryotic non-muscle cells, where they are present in sufficiently high concentration to stabilize approx. 50% of the total actin in monomeric form. They bind monomeric actin with intermediate affinity (\( K_s \) in the region of 0.2–2 \( \mu \)M), forming a stoichiometric 1:1 complex. On cell activation, actin can be mobilized from the \( \beta \)-thymosin complex and enter the polymeric pool.

The exact mechanism by which the actin–T\( \beta_4 \) complex is separated \( \textit{in vitro} \) is still not clear, although considerable evidence from experiments \( \textit{in vitro} \) indicates that profilin [1] and a number of other F-actin-stabilizing proteins [2] can stimulate actin repolymerization from the actin–T\( \beta_4 \) complex. It has been shown that profilin is able to lower the critical concentration of actin polymerization (\( C_c \)) [1] and might thus shift the equilibrium between free G-actin and actin–\( \beta \)-thymosin, although the exact mechanism of its effect is still unclear. Besides profilin a number of other G-AbPs can influence the stability of the actin–T\( \beta_4 \) complex by direct competition. Indeed, it was recently demonstrated that F-actin-stabilizing factors such as myosin subfragment 1 or phalloidin induce actin repolymerization from the actin–T\( \beta_4 \) complex [2]. In addition, a sudden increase in the number of free actin filament ends also leads to repolymerization of actin from its complex with T\( \beta_4 \) [2]. Similar observations were reported when using extracts obtained from activated neutrophils containing an elevated concentration of free F-actin ends [3]. The repolymerizing effect of free F-actin ends could also be due to a shift of the G/F equilibrium, because the affinity of monomeric actin for filament ends seems to be higher than that for T\( \beta_4 \). Another mechanism could be the alteration of the stability of the actin–T\( \beta_4 \) complex by direct interaction of AbPs with the complexed actin. Furthermore the exact knowledge of the T\( \beta_4 \) binding site on actin will also allow conclusions to be drawn about the mechanism of \( \beta \)-thymosin function. We have therefore analysed the interaction of the actin–T\( \beta_4 \) complex with other G-AbPs by measuring the formation of ternary complexes with a number of different techniques. This analysis allowed the determination of the binding region of T\( \beta_4 \) on actin and in addition indicated the existence of negative co-operativity between binding sites located on different subdomains of actin.

MATERIALS AND METHODS

Materials

The chemical cross-linkers FFD (1,5-difluoro-2,4-dinitrobenzene) and EDL [1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide] were obtained from Serva (Heidelberg, Germany) and Pierce (Rockfford, IL, U.S.A.) respectively. Pyrenylidodeacetamide was purchased from Paesel & Lorei (Frankfurt, Germany).

Protein preparations

Skeletal muscle actin was purified from acetone powder prepared from rabbit psoas muscle as described in [4]. Pyrene-labelled actin (pyrenyl-actin) was prepared exactly as detailed by Koujama and Mihashi [5].
Bovine pancreatic DNase I (EC 3.1.21.1) was a commercial product (Paesel & Lorei) and further purified by ion-exchange chromatography with hydroxylapatite as detailed previously [6]. Human gelsolin and the N-terminal domain of human plasmin gelsolin (segment 1) were expressed in Escherichia coli and purified as described by Way et al. [7].

TPβ1 was isolated from bovine spleen in accordance with the method described for thymosin β_s, from pig spleen [8]. Briefly, the tissue was homogenized in 5 vol. of ice-cold 0.5 M HClO₄ and kept on ice for 30 min. The precipitate was removed by centrifugation at 4 °C and the supernatant was adjusted to pH 4 with 10 M KOH. After 30 min on ice the insoluble KClO₄ was removed by centrifugation. The peptides were extracted from the supernatant solution by solid-phase extraction on LiChroprep RP-18, which was primed with methanol followed by water. To remove the salts the RP-18 material was washed several times with water and then the peptides were eluted with 30 % (v/v) n-propanol. The eluate was concentrated under reduced pressure with a grid and the gel sections were transferred into small plastic tubes containing 1 g of primed RP-18 between two frits. The columns were washed with 10 vol. of water to elute peptides and ampholines and the pH of the eluate was determined. The peptides bound to the RP-18 material were eluted with 30 % (v/v) n-propanol. In a last purification step the peptides were separated by preparative reverse-phase HPLC on a Pharmacia Super-Pac PrepS column (pore size 5 µm; 4.0 mm × 250 mm) at a flow rate of 0.75 ml/min by applying a linear gradient from 0.1 % (w/v) trifluoroacetic acid in water to 0.1 % (w/v) trifluoroacetic acid in 40 % (v/v) acetonitrile within 2 h. Detection was performed by measuring the absorbance at 215 nm.

Radioiodinated TPβ₁ was prepared after biolabelling the human lymphoblast cell line IM9 (A.T.C.C. CCL 159) with [35S]methionine. To this end the cells were initially kept in methionine-free RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum for 3 h. After the addition of 0.7 mCi of [35S]methionine they were cultured for a further 36 h. Then the cells were pelleted at 3000 g and carefully washed with PBS by repeated centrifugation. The pelleted cells were resuspended in 0.4 M HClO₄. After 30 min the pH was adjusted to 3.5 by the addition of 10 M KOH and after a further incubation of 30 min the precipitated KClO₄ was removed by centrifugation. The clear supernatant was loaded on an RP-18 column (1 g) and after being washed with distilled water to remove salts, the bound material was eluted with 40 % (v/v) n-propanol. The eluate was concentrated by vacuum centrifugation, and labelled TPβ₁ was purified by reverse-phase HPLC with an acetoni-trile gradient in 0.1 % (w/v) trifluoroacetic acid.

Vitamin D-binding protein (DbP) was purified from outdated human plasma as described previously [9] and generously provided by Dr. H. van Baelen (Liége, Belgium). Before use it was further purified with a DEAE-52 ion-exchange column (13 cm × 1.8 cm) equilibrated with 50 mM Tris/HCl (pH 8.0)/0.1 mM CaCl₂/1 mM NaN₃. The absorbed DbP was eluted at approx. 0.2 M NaCl with a linear gradient from zero to 0.5 M NaCl.

**Enzymic analysis**

DNase I activity was determined by measuring the increase in absorbance at 260 nm (hyperchromicity test) as introduced by Kunz [11] and modified by Mannherz et al. [12], with a Shimadzu UV 300 spectrophotometer.

**Electrophoretic techniques**

SDS/PAGE was performed by the method of Laemmli [13]. Immunoblots were performed as described previously [14]. TPβ₁ was identified by using a monoclonal antibody prepared against thymosin β₁ kindly provided by Professor B. Jockusch (Braunschweig, Germany). Native gel electrophoresis [15] was performed as detailed previously [16]. Formation of native complexes was achieved by incubation of the proteins for 30 min at room temperature. A control lane containing sample buffer with Bromophenol Blue to indicate the electrophoretic progress was replenished with dye after its complete elution. The separation was stopped when the dye had passed through the gel twice (native actin–TPβ₁ complex) or 1.5 times (mixtures of native and cross-linked actin–TPβ₁ complex). The gels were stained with Coomassie Blue. When SDS/PAGE as second dimension was to follow, the native gels were stained briefly with Coomassie Blue. Bands to be analysed by SDS/PAGE as second dimension were carefully excised with a razor blade and placed into the slots of the stacking gel.

**Fluorescence measurements of polymerized actin**

Cᵢ for actin alone or in the presence of a monomer-binding protein was determined by measuring the fluorescence increase of native actin supplemented with 4 % of pyrenyl-actin after a preincubation period of 16 h at room temperature. The excitation and emission wavelengths were set at 365 nm and 385.5 nm respectively. Relative fluorescence readings were done on a Shimadzu RF-5001-PC spectrofluorometer. The amount of un-polymerized actin in equilibrium with F-actin was derived from pyrene fluorescence measurements of serially diluted solutions of F-actin containing a constant amount of DNase I, DbP, segment 1 or TPβ₁. For these experiments the proteins were taken up in a buffer containing 5 mM Hepes/NaOH, pH 7.4, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.2 mM ATP and 0.2 mM NaN₃ (buffer A).

**Chemical cross-linking**

Chemical cross-linking was performed in buffer A with FFD, which bridges a distance of 0.3 nm. For cross-linking, defined concentrations of actin and TPβ₁ (as described in the Figure legends) were preincubated for 30 min at room temperature. The pH of the samples was raised by adding triethanolamine, pH 8.5, to 0.1 M final concentration; FFD dissolved in dimethylformamide was then added to give a final concentration of 0.1 mg/ml and further incubated at room temperature for the times indicated in the Figures. The cross-linked actin–TPβ₁ complex used for native gel electrophoresis was generated by an overnight incubation with FFD at room temperature.

**Quantitative protein determination**

Protein concentrations were determined by following the procedure given by Bradford [10], with BSA as standard. This technique leads to an underestimation of the concentration of DNase I by a factor of approx. 2.3 compared with measuring the absorbance at 280 nm [6]. However, we used the colorimetric procedure throughout this work to avoid effects of the nucleotide-containing buffers.
RESULTS

Ternary complex formation of actin, DNase I and DbP

The methods of detecting ternary complex formation were first tested with two AbPs that bind to actin with similar affinities and whose binding sites on actin are known to be spatially separated. For this reason DNase I and the DbP were used. First we used a kinetic approach to measure the increase of Cc in the absence or presence of these AbPs. Figure 1(A) gives the results of experiments in which F-actin containing co-polymerized 5% pyrene-labelled actin was incubated with constant concentrations of either DNase I, DbP or both proteins. The decrease in pyrene fluorescence was measured after equilibrium had been reached (normally after 16 h of incubation at room temperature). It can be seen that either AbP leads to an increase in Cc. When both AbPs were added no further increase in Cc was obtained, indicating that both AbPs bind simultaneously to the same actin molecule. Ternary complex formation of DNase I and DbP with actin was further verified by gel filtration (results not shown) and chemical cross-linking with FFD as described in the Materials and methods section (Figure 1B).

No stable ternary complex of actin, Tβ4 and DNase I is formed under native conditions

First we used the kinetic procedure to test for ternary complex formation between actin, Tβ4, and DNase I. An additional increase in Cc was obtained in the simultaneous presence of both Tβ4 and DNase I (Figure 2), indicating that both AbPs do not bind simultaneously to the same actin molecule. Indeed, the final increase in Cc is almost the sum of the increases in Cc produced by each AbP on its own. When radiolabelled Tβ4 was used to analyse its binding to actin by gel filtration, it was found that in the absence of DNase I the radioactivity co-eluted with actin (Figure 3A). After the addition of DNase I, however, the radiolabelled Tβ4 did not elute with the actin–DNase I complex; instead it was shifted to lower molecular mass, indicating its dissociation from actin (Figure 3B).

DNase I is inhibited by G-actin and actin–Tβ4 complex

Next we tested the ability of actin on its own or actin–Tβ4 complex to inhibit the DNase I activity. In agreement with previous reports [17,18] it was found that the inhibitory capacity of G-actin on DNase I is not impaired even when preincubated for 60 min in the presence of a 2-fold molar excess of Tβ4. The addition of equimolar G-actin or actin–Tβ4 resulted in an identical DNase I inhibition of approx. 90%. In contrast, when F-actin was preincubated with Tβ4 for 60 min and then mixed rapidly with DNase I (within 3–5 s, to minimize F-actin depolymerization by DNase I during the test), a dependence of the DNase I inhibitory capacity on the Tβ4 concentration was obtained. The observed values were approx. 40%, 62% and 85% (n = 4) at molar actin-to-Tβ4 ratios of 13:1, 6:1 and 0.5:1 respectively. In contrast, the addition of F-actin on its own did not lead to a significant DNase I inhibition under these conditions. The inhibition of DNase I by F-actin in the presence of Tβ4 can only be attributed to an increasing shift of the G/F equilibrium after the addition of Tβ4 towards monomeric actin.
because the formation of the actin–T\(_4\) complex removes free G-actin from its equilibrium with F-actin, which subsequently depolymerizes to re-establish the G/F equilibrium. If only G-actin formed inhibitory complexes with DNase I, the inhibition should not be altered by the addition of T\(_4\). However, increasing the T\(_4\) concentration resulted in a further increase in DNase I inhibition. This result can be interpreted in two ways: (1) both G-actin and the actin–T\(_4\) complex are able to inhibit DNase I, or (2) DNase I rapidly shifts the equilibrium between G-actin and the actin–T\(_4\) complex. To decide between these possibilities, we performed the following experiments.

**Formation of a chemically cross-linked ternary actin–T\(_4\) complex**

Direct interaction of the actin–T\(_4\) complex with DNase I was demonstrated by chemical cross-linking. The results in Figure 4 (lanes 1, 2, 5, and 6) show that FFD is able to generate binary actin–T\(_4\) and actin–DNase I complexes. In the presence of both AbPs a new species of approx. 80 kDa was generated (Figure 4, lanes 5 and 6). Immunoblots with the monoclonal antibody against T\(_4\) and corresponding lanes in (B) were marked identically. Protein concentrations were determined by the method given by Bradford [10]. As detailed in the Materials and methods section this procedure leads to an underestimation of the DNase I concentration. Abbreviations: A, actin; B, cross-l., cross-linked.

**Figure 4** Demonstration of the formation of a ternary cross-linked complex consisting of actin, T\(_4\), and DNase I

Actin (A) (46.6 \(\mu\)M) and T\(_4\) (95 \(\mu\)M) were incubated for 30 min at room temperature. The cross-linking reaction was performed as described in the Materials and methods section. After 30 and 60 min, 3.2 \(\mu\)l of the reaction mixtures were treated with SDS sample buffer and analysed by SDS/PAGE (5% (w/v) gel) (lanes 1 and 2). Lanes 3 and 4 show an immunoblot of an identical gel reacted with the monoclonal antibody against T\(_4\). After 2.5 h, DNase I was added to the cross-linking reaction. The protein concentrations were changed to 18.4 \(\mu\)M actin, 36.6 \(\mu\)M T\(_4\), and 21.9 \(\mu\)M DNase I. For analyzing the progress of the cross-linking reaction, 0.2 \(\mu\)l samples were removed, mixed with SDS sample buffer and analysed by SDS/PAGE (lanes 5 and 6) and immunoblotting (lanes 7 and 8). The cross-linking pattern obtained 30 min after the addition of DNase I is shown in lanes 5 and 7 and after 60 min in lanes 6 and 8.

**Figure 5** Interaction of cross-linked actin–T\(_4\) complex with DNase I

(A) Native gel electrophoresis (7.5% (w/v) polyacrylamide) of a mixture of native and cross-linked actin–T\(_4\) complex (12.5 \(\mu\)M actin and 24.6 \(\mu\)M T\(_4\)) that reacted with 2.34 \(\mu\)M DNase I (lane 2). Lane 1 shows DNase I on its own and lane 3 the mixture of native and cross-linked actin–T\(_4\) complexes. (B) The protein spots were stained, excised, incubated for 2 h with non-boiling SDS sample buffer and analysed by SDS/PAGE (10% (w/v) gel). Protein spots in (A) and corresponding lanes in (B) were marked identically. Protein concentrations were determined by the method given by Bradford [10]. As detailed in the Materials and methods section this procedure leads to an underestimation of the DNase I concentration. Abbreviations: A, actin; B, cross-l., cross-linked.

**Figure 3** Dissociation of the actin–T\(_4\) complex after the addition of DNase I

(A) Actin (1 mg) and T\(_4\) (64 \(\mu\)g) supplemented with \(^{35}\)S-T\(_4\) were mixed in a final volume of 0.15 ml and incubated in buffer A for 2 h on ice. Then the reaction mixture was separated on an AcA-44 column (1 cm × 40 cm) at a flow rate of 10 ml/h and 30 fractions of 3 ml each were collected. The protein concentration (\(A_{280}\)) and the radioactivity (c.p.m.) were plotted against the fraction number. Portions (300 \(\mu\)l) of the fractions indicated were analysed further by SDS/PAGE (15% (w/v) gel) as shown in the inset. (B) Identical experiment to that shown in (A) except that, after a preincubation period of 2 h, DNase I (23.8 nM) was added. The mixture was incubated for a further 30 min. Gel filtration on the AcA-44 column and the evaluation were performed as detailed above. The fraction size was 2 ml. The inset gives the SDS/PAGE analysis of the fractions indicated. The 180 kDa marker did not migrate into gel.

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(A) Native gel electrophoresis (7.5% (w/v) polyacrylamide) of a mixture of native and cross-linked actin–T\(_4\) complex (12.5 \(\mu\)M actin and 24.6 \(\mu\)M T\(_4\)) that reacted with 2.34 \(\mu\)M DNase I (lane 2). Lane 1 shows DNase I on its own and lane 3 the mixture of native and cross-linked actin–T\(_4\) complexes. (B) The protein spots were stained, excised, incubated for 2 h with non-boiling SDS sample buffer and analysed by SDS/PAGE (10% (w/v) gel). Protein spots in (A) and corresponding lanes in (B) were marked identically. Protein concentrations were determined by the method given by Bradford [10]. As detailed in the Materials and methods section this procedure leads to an underestimation of the DNase I concentration. Abbreviations: A, actin; B, cross-l., cross-linked.
Binding site of thymosin \( \beta_4 \) on actin

Figure 6 Interaction of the actin–T\( \beta_4 \) (A:T\( \beta_4 \)) complex with gelsolin

(A) Native gel electrophoresis [7.5% (w/v) polyacrylamide] of an incubation mixture containing native and cross-linked actin–T\( \beta_4 \) complex (12.3 \( \mu \)M actin and 24.6 \( \mu \)M T\( \beta_4 \)) and 2.58 \( \mu \)M gelsolin (lane 2). The position of free gelsolin and actin–T\( \beta_4 \) complex are shown in lanes 1 and 3 respectively. (B) The marked spots in (A) were excised, treated as detailed above and analysed by SDS/PAGE [10% (w/v) gel]. (C) Immunoblot of an SDS/polyacrylamide gel parallel to (B) after reaction with the monoclonal T\( \beta_4 \) antibody. Abbreviation: cross-l., cross-linked.

Figure 7 Effect of gelsolin segment 1 and T\( \beta_4 \) on \( C_c \)

Determination of \( C_c \) on actin alone (line A), in the presence of 0.75 \( \mu \)M gelsolin segment 1 (seg 1) (line B), 3.1 \( \mu \)M T\( \beta_4 \) (line C) and both AbPs (line D).

actin–T\( \beta_4 \) generated a new spot; however, this exhibited the same mobility as the binary actin–DNase I complex (results not shown). To prove ternary complex formation, we used actin–T\( \beta_4 \) complex that had been treated with FFD and therefore contained a mixture of cross-linked and native complex. After incubation of this material with DNase I, a shift in mobility on native gels was observed that was identical with that obtained after the addition of DNase I to untreated actin–T\( \beta_4 \) complex (Figure 5A). The spots separated by native gel electrophoresis were then excised and loaded on SDS/polyacrylamide gel as the second dimension. The results obtained are given in Figure 5(B). They demonstrate that the product spot 2a generated by the addition of DNase I contained cross-linked actin–T\( \beta_4 \) complex together with DNase I, indicating that a ternary actin–T\( \beta_4 \)–DNase I complex can be formed or rather that DNase I can bind to actin chemically cross-linked to T\( \beta_4 \).

Actin–T\( \beta_4 \) complex does not bind to gelsolin

Gelsolin contains three actin-binding sites. Ternary complex formation between actin, T\( \beta_4 \) and gelsolin (82 kDa) was analysed by native gel electrophoresis followed by SDS/PAGE. The experiments shown were performed in the presence of 0.1 mM CaCl\(_2\), which normally induces the formation of the gelsolin–2actin complex [7,16]. It was found that the addition of gelsolin to a mixture of native and cross-linked actin–T\( \beta_4 \) did not induce the formation of a new species (Figure 6A). After excision, analysis of these bands by SDS/PAGE indicated that the complex formed in spot 2b does not contain cross-linked actin–T\( \beta_4 \) complex (Figure 6B). This was again verified by immunoblotting.
an identical gel and reacting with the monoclonal antibody against Tβ4 (Figure 6C).

**Actin–Tβ4 complex does not bind to gelsolin segment 1**

Actin binds to the N-terminal 14 kDa domain of gelsolin (segment 1) independently of Ca^2+ ions with high affinity. Furthermore the three-dimensional structure of the complex of segment 1 and actin has been solved [19]. Therefore binding of actin–Tβ4 to segment 1 was tested. With the kinetic approach no indication of ternary complex formation was obtained; instead we observed an additional increase in C% in the presence of both AbPs (Figure 7). Analysis by native gel electrophoresis followed by SDS/PAGE as second dimension clearly demonstrated that cross-linked actin–Tβ4 does not bind to segment 1 (Figure 8). In contrast, the cross-linked actin–Tβ4 complex remained at its original position in the native gel. Furthermore, when gelsolin segment 1 was added to actin complexed with radiolabelled Tβ4 and analysed by gel filtration, we observed, as with DNase I, a complete dissociation of the radiolabelled Tβ4 (results not shown).

**Ternary complex formation of actin, DNase I and gelsolin segment 1**

The experiments conducted to determine ternary complex formation of actin with DNase I and Tβ4 indicated a negative allosteric coupling of the DNase I- and Tβ4-binding sites. Because Tβ4 binding to actin is completely suppressed by segment 1, we assume that segment 1 and Tβ4 bind to the same or closely spaced sites on actin. Therefore we analysed whether the binding of segment 1 to actin, like that of Tβ4, is also negatively influenced by DNase I. Figure 9 demonstrates an additional increase in C% in the presence of both AbPs, although it is well known, from the structure analysis of actin–DNase I and actin–segment 1 complexes [19,20], that their binding sites are spatially separated. This increase is, however, not completely additive like that of the pairing of Tβ4 and gelsolin segment 1 (Figure 8). Instead, the formation of a ternary actin–DNase I–segment 1 complex can be demonstrated by gel filtration, indicating that the affinity of these proteins is still sufficiently high for ternary complex formation (results not shown). The kinetic results, however, suggest that the binding sites for both proteins are also linked in a negative co-operative manner.

**DISCUSSION**

The initial aim of this investigation was to map the binding site for Tβ4 on actin. Because there is no direct structural information available about the binding site for Tβ4 on actin, its identification relied mainly on chemical cross-linking studies. From such experiments a number of studies [21–23] concluded that Tβ4 interacts with the C-terminus, whereas Safer and Nachmias [24] presented data indicating cross-linking to the N-terminus of actin. These conflicting results might be explained by the fact that the N- and C-termini of actin are both located on subdomain 1 and are highly mobile, or they might be due to the use of different chemical cross-linkers. Here we report a different approach: measuring ternary complex formation or competition of Tβ4 with DNase I or gelsolin segment 1, whose actin-binding sites are known by structure determination [19,20]. The results obtained demonstrate the complete abolition of binding of actin–Tβ4 to segment 1. Therefore we deduce that the actin-binding site of Tβ4 is within or close to the contact region of segment 1 on actin, namely the cleft between subdomains 1 and 3 [19]. Recent results obtained by chemical cross-linking support the assumption that the Tβ4-binding site on actin is close to that of segment 1. Safer and Elzinga [25] identified Asp-154, Asp-155 and Glu-167, located in subdomain 3 of actin, as residues forming part of the Tβ4-binding site. Glu-167 directly contacts the actin-binding helix of segment 1 via the shared Ca^{2+} atom [19]. A definitive answer, however, about the actin–Tβ4 interface will be possible only by direct structure determination.

Previous reports have indicated that Tβ4 binding to actin is inhibited by DNase I [26]. Because we demonstrate that DNase I binds to the cross-linked actin–Tβ4 complex, the binding site for Tβ4 must be different from the DNase I-binding site. Both sites seem to be allosterically linked and to control each other negatively. We assume that the binding of DNase I decreases the affinity of Tβ4 for actin, most probably by increasing the rate of Tβ4 dissociation. Indeed, Huff et al. [27] have recently shown by equilibrium ultracentrifugation that the Kd of Tβ4 for actin is increased approx. 57-fold when complexed with DNase I. Our
gel-filtration experiments with radiolabelled Tβ1 also demonstrate that binding of DNase I to actin–Tβ1 complex leads to the dissociation of Tβ1.

Our results are in contrast with recently published results indicating the independent interaction of DNase I and Tβ1 with actin [18]. In these experiments cross-linked actin–DNase I complex was employed, to which a modified Tβ1 was chemically cross-linked. As shown in Figure 4, we were also able to generate a cross-linked actin–DNase I–Tβ1 complex. However, chemical cross-linking perturbs the true equilibrium and might produce complexes that normally are only short-lived and/or of low affinity.

Negative co-operativity between different binding sites of actin was also indicated by the kinetic procedure when analysing ternary complex formation between DNase I and segment 1, which bind separately to actin with high affinity ($K_i$ approx. 1 nM). The negative co-operativity does not seem to decrease the affinity of the second binding protein so much as to prevent the formation of a stable ternary actin–DNase I–segment 1 complex. In contrast, the affinity of Tβ1 for actin ($K_i$ approx. 1 μM) is decreased by DNase I binding to such an extent that it apparently excludes the formation of a stable ternary complex.

DNase I and segment 1 bind to two oppositely spaced subdomains of actin bridging an interdomainal cleft (reviewed in [28]). Their binding sites are part of the actin/actin interfaces along the long-pitch F-actin helix [29]. DNase I has been shown to bind to the pointed end of F-actin [30]. In addition, it has been demonstrated that DNase I also increases the rate of monomer dissociation from the pointed end of F-actin [31]. In view of our results, this effect might also be due to negative co-operativity between the DNase I-binding site and the opposite actin/actin interface. The underlying mechanism of the observed negative co-operativity is unclear. The structure analysis of both complexes had not indicated drastic differences in the three-dimensional structure of actin when complexed with either protein [19,20]. However, it was not possible to trace the binding loop for DNase I on subdomain 2 in the segment 1 complex, most probably owing to high mobility in this region [19]. Indeed, this loop has been shown to adopt a different orientation in F-actin than in the DNase I complex [32]. In the actin–segment 1 complex only Tyr-169 in subdomain 3, a residue involved in segment 1 binding, adopted a rotamer orientation different from the actin–DNase I complex [19]. Otherwise no conformational alterations of loop regions or side chains in subdomain 1 were noted between the two binary complexes.

DNase I, segment 1 and Tβ1 are actin-sequestering proteins and their binding sites overlap with the actin/actin interfaces along the long-pitch F-actin helix [29]. Each sequestering protein can bind to only one side of actin. For effective sequestration it will be necessary to inactivate the opposite actin-binding site so as not to act as a capping protein. This might be achieved by negative allosteric coupling of the oppositely spaced interface as described here. It will be interesting to analyse other actin-sequestering or actin-capping proteins for an allosteric coupling of their binding sites to DNase I, segment 1 or Tβ1.

The function of Tβ1 in vivo is the intracellular sequestration of monomeric actin. The intermediate affinity of Tβ1 to actin ($K_i$ approx. 1 μM) allows the rapid dissociation of this complex and the repolymerization of actin. Its dissociation could also be accelerated by proteins that bind to spatially separated sites on actin and decrease the affinity of Tβ1 for actin by negative co-operativity.

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REFERENCES


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