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The ternary complex of DNase I, actin and thymosin β4

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Abstract **We have recently described a method for identifying contact sites between actin and thymosin β4 (Tβ4) by following spectrophotometrically the extent and kinetics of distinct, thiol-specific crosslinking reactions between appropriate derivatives of the two proteins [Reichert et al. (1996) J. Biol. Chem. 271, 1301–1308]. In the present study this method was used to show that such crosslinking, which is indicative of complex formation, occurs to the same extent with the actin-DNase I complex as with pure actin, although at a somewhat lower rate. Further evidence for the formation of the ternary complex was given by gel electrophoresis. From fluorescence spectroscopy the K_D value of Tβ4 from the actin-DNase I complex was found to be identical to that from pure actin. In line with these data, the capacity of actin for inhibiting DNase I was not affected by the addition of Tβ4. In conclusion, DNase I and Tβ4 are independent of each other in their interaction with actin, suggesting that the binding sites of thymosin β4 and DNase I on actin do not overlap. A ternary complex of DNase I, actin and Tβ4, if obtained in crystalline form, could thus provide an approach for studying the interface of Tβ4 and actin by X-ray analysis.**

Key words: Actin; Thymosin β4; DNase I; Ternary complex; Thiol-specific crosslinking

1. Introduction

Thymosin β4 (Tβ4) is one of the small proteins in non-muscle cells that bind to monomeric actin and thus prevent unregulated polymerization [1–4]. From the Tβ4-complexed monomers, polymerization can be started in vitro either by decapping barbed ends of filaments [5,6], or by adding nucleus stabilizers such as myosin S1 or phalloidin [7–11]. The relatively low affinity of Tβ4 for actin (ca. 1 μM [4]) may be functionally significant in allowing the instant release of polymerizable actin into cytoplasm, if required.

Since an X-ray analysis of the actin-Tβ4 complex has so far not been achieved we have tried to identify contact sites between the two proteins by a chemical approach [12]. For this, five Tβ4 analogs were synthesized, each of them with one cysteine residue substituted for a hydrophobic amino acid in the Tβ4 chain. Using a set of thiol-specific crosslinkers of varying length, we assayed whether in the complex with actin the differently positioned thiol groups in Tβ4 come close enough to distinct thiol groups in actin to allow the formation of crosslinks. In this way two contact sites between the two proteins could be identified, one between the C-terminus of actin (Cys 374) and position 6 of Tβ4 and a second between the γ-phosphate of the actin-bound nucleotide in actin and the sequence 17–28 in Tβ4.

X-ray analysis of monomeric actin took advantage of a facilitated crystallization of actin when complexed with DNase I. Provided DNase I exerted a similar effect on Tβ4-actin as well, co-crystallization with DNase I would allow the interface of actin and Tβ4 in the ternary complex to be studied. Corresponding complexes have been reported for DNase I, actin and profilin, or ADF/cofilin, respectively [14,15]. However, the ternary complex can be expected to form only if the binding of DNase I and that of Tβ4 to actin do not strongly interfere. We therefore examined whether DNase I affects the affinity of Tβ4 for actin by studying one of the crosslinking reactions mentioned above in the presence of DNase I.

2. Materials and methods

2.1. Protein purification

Actin was prepared from rabbit muscle as described by Spudich and Watt [16] and further purified by a gel filtration step on a Fractogel TSK HW 55 column (3×120 cm) (E. Merck, Darmstadt) in buffer G (2 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.02% NaN_3, pH 7.8). Thymosin β4 was isolated from bovine lungs according to Spangelo et al. [17], the thymosin β4 analog (S-isopropylthio)-L-Cys^{6}Tβ4 was prepared according to [12]. DNase I was purchased from Sigma (München).

2.2. Preparation of the actin derivative

Actin^{374}SS-$(CH_2)_3$-SSAr was prepared by reacting G-actin (3.8×10^{-5} M) in buffer G with a 3 molar excess of the reagent ArSS-$(CH_2)_3$-SSAr. The reagent was prepared in analogy to the nonylene reagent as described in [18]. Instead of purification by column chromatography the product was isolated by preparative TLC on Silica gel plates (Merck HF_{254} 20×20 cm) developed in chloroform-methanol-2N acetic acid (65:25:4) and detected by UV-light (254 nm). The mixture was kept at 4°C until one equivalent of 2-nitro-5-thiobenzoate (ArS^-) had been released (ε_{412}=14150 M^{-1} cm^{-1}, [19]). By exhaustive dialysis in buffer G the major part of excess reagent was removed together with ArS^- before the protein was purified on a Biorad-P2 column (2×45 cm) equilibrated with buffer G. Labelling of the actin derivative was 80–90% as determined from the protein concentration of the purified derivative and the amount of ArS^- detected at 412 nm after cleavage with excess of dithiothreitol (DTT).

2.3. Crosslinking studies

In the thymosin β4 analog, cleavage of the S-protecting isopropylthiol residue was achieved by incubating 1 mg of Cys^{6}Tβ4 in a 200-fold excess of 2-mercaptoethanol in 2 mM Tris pH 7.5 for 2 h at 4°C. Excess reagent was removed on a Biorad-P2 column (1×15 cm) equilibrated with the same buffer. The concentration of Cys^{6}Tβ4 was

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Abbreviations: Tβ4, thymosin β4; ADF, actin-depolymerizing factor; ArSS-$(CH_2)_3$-SSAr, propylene-bis-[5-dithio-(2-nitrobenzoic acid)]; ArS^-, 2-nitro-5-thiobenzoate; TLC, thin-layer chromatography; DTT, dithiothreitol; TMB, trimethylammonio-bromobimane; PMSF, phenylmethylsulfonyl fluoride

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determined by titrating an aliquot with Ellman's reagent and measuring the released ArS^- at 412 nm. The actin derivative was mixed with the Cys-Tβ4 analog at a ratio of 1:1 (concentration of the proteins was in the range of 1 to 3×10^{-5} M) and allowed to react at room temperature. During the first 30 min the amount of ArS^- released due to the crosslinking reaction was measured and the extent of crosslinking was determined.

To examine the effect of DNase I on the crosslinking reaction, the actin derivative was incubated with different amounts (0.8, 1.6, 2.4 eq.) of DNase I for 15 min at room temperature before Cys^6Tβ4 was added. The effect of salt was investigated by performing the same experiment in the presence of 100 mM KCl.

To test the specificity of the binding of Cys^6Tβ4 to the actin-DNase I complex, a competition assay was performed in which 1 eq. of native Tβ4 was incubated with the preformed actin-DNase I complex for 15 min at RT prior to the addition of Cys^6Tβ4.

All steps were performed in an argon atmosphere in order to minimize oxidation of the unprotected cysteine residue in the thymosin analog.

2.4. K_D-measurements

The dissociation constant of Tβ4 and the actin-DNase I complex was determined using the actin derivative TMB-actin, prepared according to Heintz et al. [20]. TMB-actin and DNase I were mixed at a ratio of 1:2 and fluorescence was measured in a Spex fluorolog (Spex Industries Inc., New York) between 385 and 600 nm (excitation 370 nm). Then the β-thymosins (0–12 mM) were added, and after incubation for 15 min at room temperature, the changed fluorescence spectra were recorded. The K_D values were calculated from the integrated spectra according to Pesce et al. [21].

2.5. Gel electrophoresis

Native PAGE was performed as described by Safer et al. [1] using a buffer containing 25 mM Tris, 0.194 M glycine, 0.3 mM ATP and 0.1 mM $MgCl_2$. Various mixtures of the actin derivative, DNase I and Cys^6Tβ4 were incubated for 15 min at room temperature before they were applied to gel electrophoresis.

To demonstrate the existence of the ternary complex in a gel band of the native PAGE the corresponding band was cut out of the gel. The gel piece was homogenized in 100 μl of elution buffer (100 mM $NaHCO_3$, 8 M urea, 3% SDS, 0.5% Triton X-100). After elution at 50°C overnight, the slurry was transferred into a Micropure-.22 unit, placed into a Microcon-30 (30 000 molecular weight cut off) and spun at $13\,000\times g$ for 30 min (Micropure Inserts and Microcon Microconcentrators were purchased from Amicon (Witten, Germany). The protein in solution remaining on the filter was applied to a 10% SDS-PAGE and stained by a silver-dye according to Bloom et al. [22].

2.6. DNase I inhibition assay

The DNase I inhibition assay was performed according to Blikstadt et al. [23]. DNase I (8×10^{-8} M in 50 mM Tris-HCl, 0.1 M $CaCl_2$, 0.01 mM PMSF, pH 7.5) was incubated with different amounts of actin (3.5×10^{-9} M to 1.1×10^{-7} M) for 2–5 s. After the addition of DNA (40 μg/ml calf-thymus DNA in 0.1 M Tris-HCl, 4 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.5) the activity of DNase I was determined from the increase in absorption at 260 nm as the slope in the linear parts of the curves [$\Delta OD\times s^{-1}$]. 100% activity of DNase I was detected in a control experiment without actin added. To examine the influence of Tβ4 on DNase I activity, actin solutions (3.5×10^{-9} M to 1.1×10^{-7} M) were mixed with equimolar amount, or excess (1.5–10×), of Tβ4 prior to the addition of DNase I.

In addition, we assayed the inhibitory capacity of the reaction product of the actin derivative actin^{374}SS-$(CH_2)_3$-SS-Ar with Cys^6Tβ4 (62% covalency, see above). For this, unbound Cys^6Tβ4 was removed by dialysis against buffer G before the solution was applied to the assay in concentrations of 3.5×10^{-9} M to 1.1×10^{-7} M, related to actin.

Table 1
Dependence of the extent of thiol-specific crosslinking between actin^{374}SS-$(CH_2)_3$-SSAr and Cys^6Tβ4 on the amounts of DNase I added

	10 min	30 min
Aktin^{374}SS-$(CH_2)_3$-SSAr + Cys^6Tβ4 (1 eq.)	59%	62%
Aktin^{374}SS-$(CH_2)_3$-SSAr + DNase I (0.8 eq.) + Cys^6Tβ4 (1 eq.)	48%	62%
Aktin^{374}SS-$(CH_2)_3$-SSAr + DNase I (1.6 eq.) + Cys^6Tβ4 (1 eq.)	46%	62%
Aktin^{374}SS-$(CH_2)_3$-SSAr + DNase I (2.4 eq.) + Cys^6Tβ4 (1 eq.)	40%	59%

Values are expressed as percentage of ArS^- released from the actin derivative due to the crosslinking reaction (100% corresponding to the actual amount of thiol added as determined by titration of Cys^6Tβ4 with Ellman's Reagent). ArS^- was measured by spectrophotometry at 412 nm.

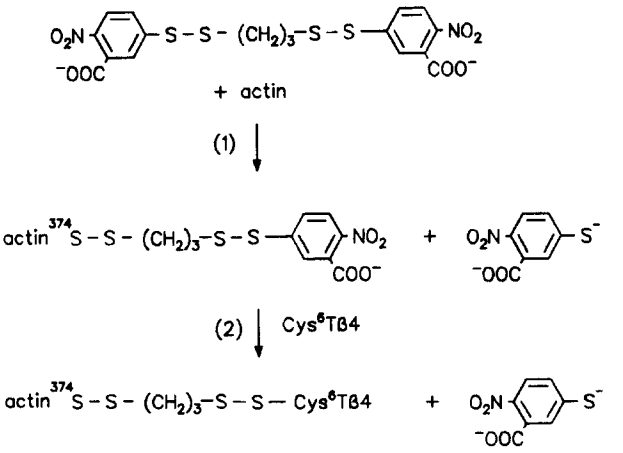

Fig. 1. Reaction of the crosslinking reagent propylene-bis-[5-dithio-(2-nitrobenzoic acid)], (ArSS-$(CH_2)_3$-SSAr), with cysteine 374 of actin (1) and subsequent reaction of the actin derivative with Cys^6Tβ4 (2). Both reactions were followed by the release of the yellow 2-nitro-5-thiobenzoate (ArS^-).

3. Results and discussion

The crosslinking reaction that provided the basis for the present study is shown in Fig. 1. The sole exposed thiol group of actin (cysteine 374) was selectively modified, via a disulfide bridge, with a 9.2 Å linker bearing a thiol-capturing moiety (reaction 1). In a previous study [12] we demonstrated that the resulting actin derivative binds Tβ4, as well as the analog Cys^6Tβ4, with an affinity comparable to that shown by actin itself [12]. On the addition of Cys^6Tβ4, complex formation leads to a high-yield crosslinking reaction, which can be followed spectrophotometrically from the release of a stoichiometric amount of 2-nitro-5-thiobenzoate (reaction 2). Actin that contains the crosslinking moiety is still an inhibitor of DNase I, exhibiting virtually the same inhibitory capacity for DNase I as pure actin (data not shown). We concluded that reaction 2, when performed in the presence of DNase I, would represent an assay system useful for monitoring the formation of the ternary complex between DNase I, the actin derivative, and Cys^6Tβ4.

In a control experiment the crosslinking reaction in the absence of DNase I was nearly complete after 10 min, with a final yield of ca. 60% after 30 min (Table 1). In the presence of increasing amounts of DNase I, yield of the crosslinking reaction remained nearly unaffected, even at an 2.4-fold excess of DNase I; however, the reaction proceeded at lower rates. The kinetics of the first 10 min of the crosslinking reaction in the absence or presence of 1.6 eq. DNase I are shown in Fig. 2a and b.

In an earlier study of this kind it was concluded that the absorbance measured at 412 nm, representing the variable of

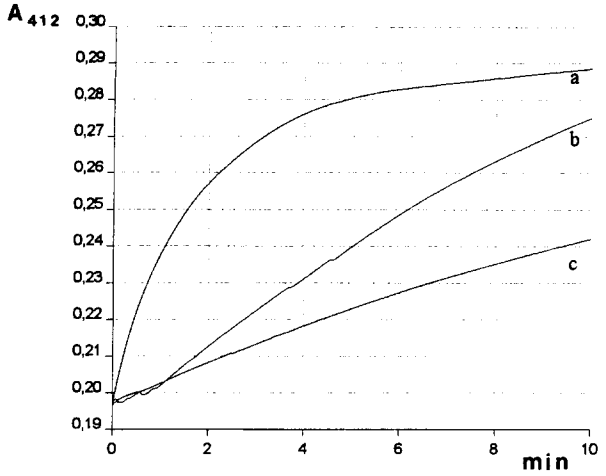

Fig. 2. Reaction kinetics of actin^{374}SS-$(CH_2)_3$-SSAr and Cys^{6}Tβ4 in the absence (a) or presence (b) of 1.6 eq. of DNase I. In the presence of DNase I the crosslinking reaction between the actin derivative and the thymosin derivative is retarded, but proceeds to the same yield. The crosslinking reaction (b) is specific, since it is competitively retarded when the actin derivative is preincubated with one equivalent of native Tβ4 (c).

the current assay, was not caused by an unspecific disulfide exchange reaction, but reflected the extent of crosslinking quantitatively. This was confirmed by the observation that the increase in absorbance was paralleled by a corresponding increase of a 47 kDa band in SDS-PAGE, representing the conjugate of the actin derivative (42 kDa) and the Tβ4 analog (5 kDa) [12]. In the present study a similar 47 kDa band was found (see below), indicating that even in the presence of DNase I the crosslinking reaction had proceeded specifically. Additional proof of the specificity of the reaction was obtained from the slowing observed when 1 eq. of native Tβ4 was added to the crosslinking reaction prior to the addition of Cys^{6}Tβ4 (Fig. 2c). The lower reaction rate observed, for example 27% yield of crosslinking after 10 min instead of 48% in the absence of native Tβ4, is taken to reflect competitive binding of Tβ4 and Cys^{6}Tβ4.

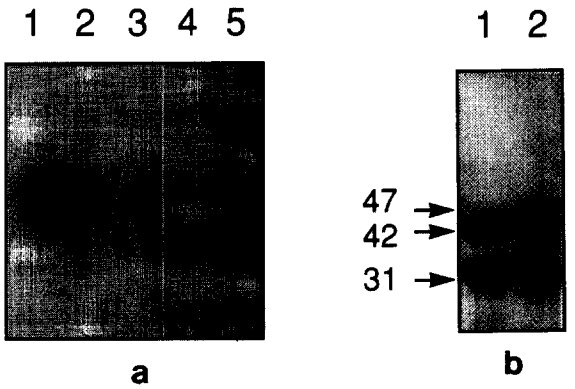

Fig. 3. Native PAGE of the ternary complex of the actin derivative, DNase I and Cys^{6}Tβ4 (a). (1) DNase I; (2) actin^{374}SS-$(CH_2)_3$-SSAr + DNase I (1.6 eq.) + Cys^{6}Tβ4 (1 eq.); (3) actin^{374}SS-$(CH_2)_3$-SSAr + DNase I (1.6 eq.); (4) actin^{374}SS-$(CH_2)_3$-SSAr + Cys^{6}Tβ4 (1 eq.); (5) actin^{374}SS-$(CH_2)_3$-SSAr. The strong band of lane 2 was extracted and applied to SDS-PAGE (b). It contained the actin derivative crosslinked to the thymosin derivative (47 kDa), the actin derivative (42 kDa) and DNase I (31 kDa) (lane 2). The 47 kDa band disappeared in the presence of DTT (lane 1).

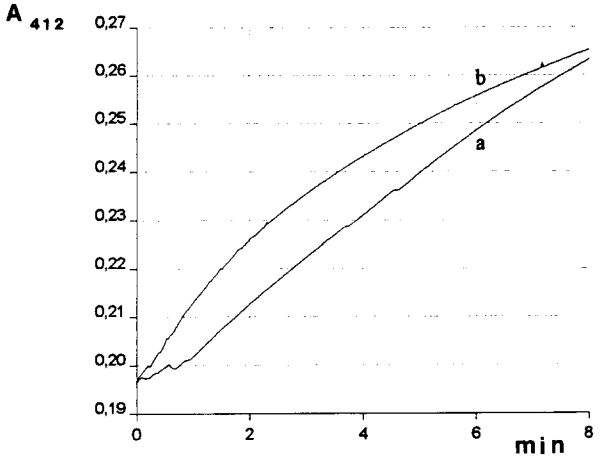

Fig. 4. Reaction kinetics of the actin^{374}SS-$(CH_2)_3$-SSAr-DNase I complex with Cys^{6}Tβ4 in the absence (a) or presence (b) of 100 mM KCl.

The product of the crosslinking reaction between the actin derivative (1 eq.), Cys^{6}Tβ4 (1 eq.) and DNase I (1.6 eq.) was examined by native gel electrophoresis (Fig. 3a, lane 2). It shows a strong band with a mobility very similar to both DNase I alone (lane 1) and the actin-DNase I (1:1.6) complex (lane 3). While in the latter, due to the extremely high affinity of DNase I for actin (K_D ca. 1 nM [24]), no free actin was visible, the crosslinking product shows an additional faint band, which from the mobility may represent a small amount of Tβ4-actin (lane 4) or free actin (lane 5). The dominant band of lane 2 corresponds to the presence of by far the largest part of actin as a complex of the actin derivative, DNase I and Cys^{6}Tβ4. This was proven by extraction of this band and subsequent SDS-PAGE, which allowed identification of not only DNase I (31 kDa) but also the actin derivative (42 kDa) and actin^{374}SS-$(CH_2)_3$-SS-Cys^{6}Tβ4 (47 kDa) (Fig. 3b, lane 2). As expected from the disulfide nature of the linkage between the actin derivative and Cys^{6}Tβ4, the 47 kDa band disappeared when the gel was run in the presence of DTT (Fig. 3b, lane 1).

In a previous study the K_D value of the actin-Tβ4 complex was measured by fluorescence changes of TMB-actin [20]. After having shown that complexation with DNase I had no influence on the fluorescence of TMB-actin, the same assay was used to determine the K_D between Tβ4 and the actin-DNase I complex. In the presence of 2 eq. of DNase I, changes in fluorescence caused by the additon of Tβ4 to TMB-actin were virtually the same as in the absence of DNase I (data not shown). Therefore, the K_D value of 0.8 μM as determined for the actin-Tβ4 complex seems to be valid for the dissociation of Tβ4 from the ternary complex as well. Our data conflict with those of Huff et al. [25], who measured, using an ultracentrifugation assay, a 57-fold decreased affinity of Tβ4 to actin in the presence of DNase I. The inconsistency of our data with those presented in [25] may be explained by the very low Tβ4 concentration used by Huff et al., which corresponded to less than 10% of the concentration at which, given the drastically increased K_D value, 50% dissociation of the complex can be expected. The strongly reduced affinity of Tβ4 in the presence of DNase I as suggested by Huff et al. seems unlikely, since it would result in a strongly decreased rate of the crosslinking reaction between Cys^{6}Tβ4 and the

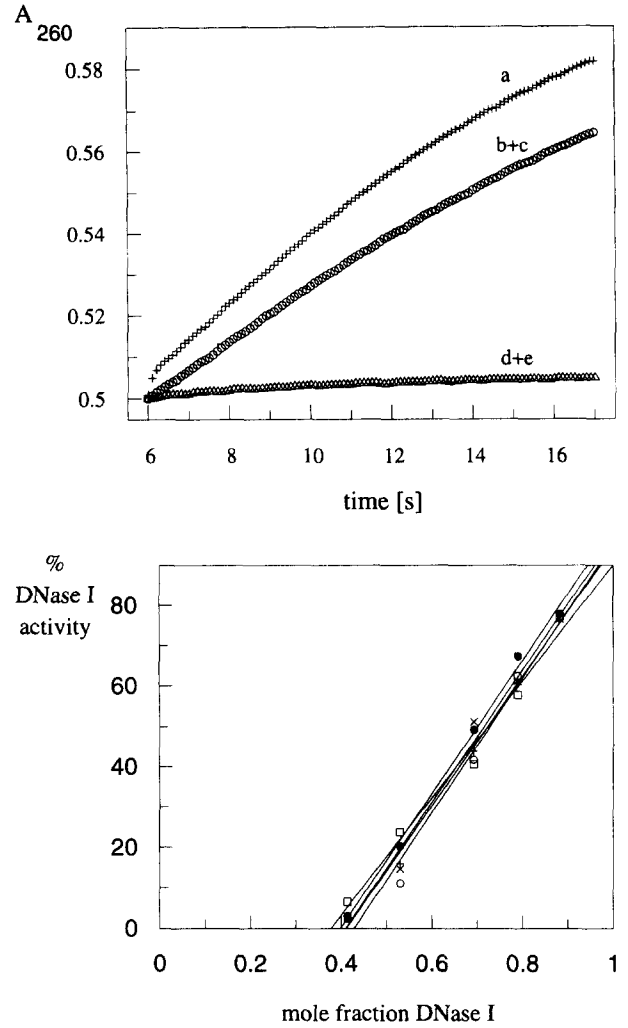

Fig. 5. (Top) Dependence of DNase I activity on the amount of actin or actin-Tβ4 (1:1.5) added, as demonstrated by $\Delta OD_{260} \times s^{-1}$: (a) without actin; (b) 2×10^{-8} M actin; (c) 2×10^{-8} M actin-Tβ4 (1:1.5); (d) 1.1×10^{-7} M actin; (e) 1.1×10^{-7} M actin-Tβ4 (1:1.5). (Bottom) Dependence of DNase I activity on the amount of actin or various actin-Tβ4 solutions added: (+) pure actin; (×): actin-Tβ4 (1:1.5); (○) actin-Tβ4 (1:3); (b) actin-Tβ4 (1:10); (□) actin^{374}SS-$(CH_2)_3$-SSAr + Cys^{6}Tβ4, complexed to 62% by covalency.

actin derivative, when complexed with DNase I. However, as outlined above, the presence of DNase I caused an only slight retardation in the reaction kinetics.

In one particular case the affinity of a β-thymosin for actin was found to be greatly decreased under polymerization conditions [26]. We therefore examined whether salt would affect the formation of the ternary complex. As shown in Fig. 4 the crosslinking reaction in the presence 0.1 M KCl was somewhat accelerated, but gave a yield similar to that in the absence of salt. Since the kinetics of the crosslinking reaction can be expected to be mainly determined by the disulfide-exchange reaction, it appears that formation of the ternary complex of DNase I, actin, and Tβ4 does not depend on ionic conditions.

In a preliminary study [20] we had claimed that thymosin β9, which essentially has the same activity as Tβ4, would reduce the inhibitory capacity of actin on DNase I. At that time this finding appeared surprizing because of a reported contact of Tβ4 with subdomain 1 of actin, which is distant from the DNase I binding region. We carefully repeated these experiments and found, in contrast to the preliminary results, that the inhibition of DNase I by, for example, 2×10^{-8} M actin was the same in the absence and the presence of 1.5 eq. of Tβ4 (Fig. 5, top). Fig. 5, below, shows that even a greater excess of Tβ4 had no influence on the inhibitory effect of actin. Likewise, full DNase I-inhibiting capacity was found for the conjugate actin^{374}SS-$(CH_2)_3$-SS-Cys^{6}Tβ4, which represented the reaction product of the crosslinking experiment between the actin derivative and Cys^{6}Tβ4. This actin species, in which ca. 60% of the actin is covalently linked to Tβ4, still exhibited the same inhibitory effect on DNase I as pure actin. These findings confirm the results of Hannappel and Wartenberg [27], who showed, with a different assay system, that Tβ4 has no influence on the capacity of actin for inhibiting DNase I.

In conclusion, we have demonstrated that there are no significant differences between actin and the DNase I complex of actin with respect to Tβ4 association. Under non-polymerizing conditions the K_D values are the same, and seem also to be identical in the presence of salt. On the other hand, Tβ4 neither enhances nor decreases the capacity of actin for inhibiting DNase I activity, a result which disproves the suggestion of Hall [28], that Tβ4, via actin, might regulate DNase I activity in cells and, in consequence, apoptosis. Although lacking any obvious physiological significance, the existence of the ternary complex of DNase I, actin and thymosin β4 may be useful for crystallization and X-ray analysis.

In the case of profilin the existence of a ternary complex with actin and DNase I was taken as an indication of the well-separated binding sites of profilin and DNase I on actin [14], a suggestion that was proven several years later by the X-ray data of the complexes of actin with DNase I [13] and profilin [29]. The absence of interference between Tβ4 and DNase I shown in the current study suggests that the binding sites of these two proteins on actin are likewise located far apart.

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