Identification of Contact Sites in the Actin-Thymosin β4 Complex by Distance-dependent Thiol Cross-linking*

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The physiological conditions within nonmuscle cells favor the assembly of actin monomers. Therefore, the pool of monomeric actin has to be maintained by complexation with small G-actin binding proteins. Particularly thymosin β4 (Tβ4)†, which forms a 1:1 complex with actin monomers, is believed to be involved in preventing actin polymerization (1–6). Dissociation constants of the actin-Tβ4 complex were found to be in the range of 0.4–0.7 μM for platelet actin and 0.7–2.0 μM for muscle actin (4, 7). While complexed with Tβ4, actin (12) has shown that binding of Tβ4 to actin is mainly mediated by the hexamotif LKKTET (17–22), since loss of this sequence is paralleled by an almost complete loss of inhibitory activity. Alterations in the N-terminal part (1–16) of the peptide strongly influence the inhibitory activity of Tβ4, whereas alterations in the C-terminal part (31–43) seem to be of minor importance (9). As shown by 1H NMR spectroscopy (10, 11) Tβ4 does not contain an ordered conformation in aqueous solution but tends to form an α-helical conformation between residues 5 and 16 (11). It has been proposed that Tβ4 is likely to adopt a unique conformation upon binding actin (12).

One of the binding sites of Tβ4 on the actin molecule seems to be located in subdomain 1 as suggested by cross-linking studies (13, 14). In order to gain more knowledge about contact sites in the actin-Tβ4 complex, we performed a structural analysis using bifunctional thiol-specific reagents of the type alkylene-bis-[5-dithio-(2-nitrobenzoic acid)] for intermolecular cross-linking of two cysteine residues. Such reagents were successfully used for cross-linking two distinct cysteine residues in muscle actin as well as for preparing a defined disulfide-linked actin dimer (15, 16). By varying the length of the cross-linkers (as well as using Ellman’s reagent for zero-length cross-linking), information can be obtained about the distance up to which two thiol groups in the complexed proteins can approach. In a first reaction, the cross-linkers (9.2 Å to 18.4 Å) were anchored monovalently at one of three thiols in monomeric actin. Since native Tβ4 does not contain any cysteine, Tβ4 analogs were synthesized, each containing cysteine at one of the positions 6, 17, 28, 34, and 40. The substitutions were distributed over the whole protein but were restricted to hydrophobic amino acids. After adding the Tβ4 analogs to the actin derivatives, the kinetics and extents of cross-linking were followed by spectrophotometric analysis of the 2-nitro-5-thiobenzoate released.

MATERIALS AND METHODS

Protein Purification—Actin was prepared from rabbit muscle as described by Spudich and Watt (17) 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 CaCl2, 0.02% NaN3, pH 7.8). Thymosin β4 was isolated from bovine lungs according to Spangel et al. (18) or obtained by synthesis.

Preparation of Actin Derivatives—Actin374SS-(CH2)n-SSAr was prepared by reacting G-actin (3.8 × 10−3 M) in buffer G with a 3 molar excess of the reagent ARSS-(CH2)n-SSAr (n = 3, 6 or 9) (see Fig. 1). The cross-linkers were prepared according to Refs. 15 and 16, and their corresponding lengths were 9.2, 13.8, or 18.4 Å, respectively. The mixture was kept at 4 °C until one equivalent of 2-nitro-5-thiobenzoate (ArS−) was released (εmax = 14,550 M−1 cm−1). By exhaustive dialysis in buffer G, the major part of excess reagent was removed together with ArS−, before the protein was purified on a Bio-Rad P2 column (2 × 45 cm) equilibrated with buffer G. Labeling of the actin derivative was 80–90% as determined from protein concentration of the purified derivative, and the amount of ArS− detected at 412 nm after deavage with excess of diithiethiol (DTT). For zero-length cross-linking, we prepared actin374SSAr by reacting G-actin with 3-fold excess of Ellman’s reagent under the same conditions.

[Text continues...]

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‡The abbreviations used are: Tβ4, thymosin β4; ArSS-(CH2)n-SSAr, alkylene-bis-[5-dithio-(2-nitrobenzoic acid)]; ArS−, 2-nitro-5-thiobenzoate; NEM, N-ethylmaleimide; DTT, diithiethiol; EDC/NSHS, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide; MOPS, 3(3-N-morpholinopropanesulfonic acid; HPLC, high performance liquid chromatography; ATP-S, adenosine 5'-O-(thiotriphosphate); Fmoc, N-(9-fluorenylmethoxy carbonyl).

¶ The identity of the authors is illustrated by the use of the above symbols.
Identification of Contact Sites in Actin-Thymosin β4 Complex

To introduce the cross-linkers into position 10 of actin, the cysteine residue in position 374 was blocked by incubating G-actin in buffer G with a 100-fold excess of N-ethylmaleimide (NEM) at 4°C for 30 min. The reaction was quenched with excess DTT, and the protein was separated on a Bio-Rad P2 column equilibrated with buffer G. NEM-actin was polymerized by the addition of 0.2 mM EGTA, 1 mM MgCl2, and ATP, and polymerization of ATP was achieved by incubation with heokinase (5 units/ml actin solution, Sigma) and 0.4 mM glucose for 90 min at room temperature (19). After centrifugation at 100,000 × g, the pellets were allowed to soften on ice in ADP buffer (2 mM Tris, 1 mM ADP, 0.02% NaN3, pH 7.8) for 30 h. After that time, Cys39 was completely accessible (20) and could be reacted with one end of the cross-linking reagent. The 374NEM-actin/SS-(CH2)4-SSAr was purified as described above. Yield of the labeling reaction was ∼80–90%.

For labeling of ATP-S (Sigma), the nucleotide was reacted with 1.5 equivalents of reagent (n = 3 or 9) in 1 M imidazole, pH 6.5, at 4°C overnight. Excess of reagent was removed on a Sephadex LH20 column (2 × 45 cm) (Pharmacia Biotech Inc.), equilibrated with 10% Tris, pH 7.0, and 90% methanol. After removal of methanol in vacuo at 4°C, the fractions containing the labeled nucleotide were used as softening buffer (∼0.1 ml labeled ATP-S, 2 mM Tris, 0.1 mM CaCl2, 0.02% NaN3, pH 8) as described above.

Incorporation of ATP-S-S-(CH2)4-SSAr was performed only with actin blocked at Cys39 and Cys44 in order to exclude any unspecific reaction. For this, ADP-actin with both cysteines exposed was prepared as described above. Reaction with NEM and removal of excess reagent were achieved as described for 374NEM-actin. The resulting 103NEM-actin was polymerized, and ATP was removed by the heokinase reaction. The 103NEM-actin pellet was allowed to soften on ice in the preformed softening buffer in order to incorporate the labeled ATP-S. Directly before use, excess of ATP-S-S-(CH2)4-SSAr was removed on a Bio-Rad P2 column (1 × 18 cm) equilibrated with ADP buffer 2 (2 mM Tris, 0.2 mM ADP, 0.1 mM CaCl2, 0.02% NaN3, pH 7.8) yielding a fraction of actin that contained nearly one equivalent (95%) of the labeled nucleotide.

Synthesis of Thymosin β4 Analogs—The thymosin β4 analogs, (S-isopropylthiolio)-L-Cys11β4, (S-isopropylthiolio)-L-Cys34β4, (S-isopropylthiolio)-L-Cys40β4, and (S-isopropylthiolio)-L-Cys374β4 were prepared with an Econos P peptide synthesizer (Eppendorf-Biotronik, Mainz, Germany) using the Fmoc technique (21) and a 4-alkoxybenzyl alcohol resin (22). The side chains of the Fmoc amino acids were protected as tert-butyloxycarbonyl derivatives (lysine), and S-isopropylthio derivatives (cysteine) (23). After cleavage from the resin and deprotection with trifluoracetic acid/thioanisole/anisole/water (14:5:0:60:0:25 (v/v)), the peptides were precipitated with anhydrous diethyl ether and dried. Desalting of the crude peptides was performed by gel chromatography on a TSK-HW 40 S (Merck, Darmstadt, Germany) column (1.6 × 100 cm) with 5% acetic acid as eluent. The peptides were precipitated with anhydrous diethyl ether and dried. Desalting of the crude peptides was performed by gel chromatography on a TSK-HW 40 S (Merck, Darmstadt, Germany) column (1.6 × 100 cm) with 5% acetic acid as eluent. The peptides were precipitated with anhydrous diethyl ether and dried.

In SDS-PAGE, these actin derivatives were indistinguishable from normal actin apart from a slightly increased critical concentration. In SDS-PAGE, these actin derivatives were indistinguishable from normal actin apart from a slightly increased critical concentration.

RESULTS

A method was developed that allowed the investigation of contact sites between actin and thymosin β4 by assessing whether two thiol groups in the protein complex could approach sufficiently close to allow cross-linking by thiol-specific cross-linkers of different lengths.

Anchoring of the Cross-linkers to Actin—The actin derivatives of the type actinβ4SS-(CH2)4-SSAr (n = 3, 6, 9) were obtained in a nearly quantitative reaction of G-actin with a 3-fold excess of the cross-linkers (Fig. 1). Stoichiometry of the actin derivatives was proved by the release of approximately 1 equivalent of ArS− during the cross-linking reaction, as well as by analysis of the purified actin derivative, which released 0.8–0.9 equivalents of ArS− on the addition of DTT. It was shown that actin derivatives of this type polymerized similar to normal actin apart from a slightly increased critical concentration. In SDS-PAGE, these actin derivatives were indistinguishable from G-actin (Fig. 2a).

![Fig. 1. Reaction of the cross-linking reagent alkylenebis-(5-dithio-(2-nitrobenzoic acid)), (ArSS-(CH2)4-SSAr), with cysteine 374 of actin. Yield of the actin derivative (actinβ4SS-(CH2)4-SSAr) was followed by the release of the yellow 2-nitro-5-thiobenzoate (ArS−) monitored at 412 nm.](image-url)
Two potentially reactive thiol groups in actin, yielding tramolecular cross-linking of the modified nucleotide with the typical 47-kDa band (Fig. 2B). SDS-PAGE of native actin and several actin derivatives cross-linked with native Tβ4 using EDC/NHS. The presence of similar amounts of the 47-kDa band representing the covalently linked actin-Tβ4 complex indicates that none of the cross-linking reagents attached to actin inhibited binding of Tβ4. Yield of cross-linking was 25 ± 1% for G-actin and all actin derivatives as determined by densitometric measurements. 1, G-actin; 2, G-actin cross-linked with native Tβ4; 3, actin<sub>374NEM-(CH<sub>2</sub>)<sub>9</sub>-SSAr</sub> cross-linked with native Tβ4; 4, <sub>374NEM-actin</sub>(<sub>SS-(CH<sub>2</sub>)<sub>3</sub>-SSAr</sub> cross-linked with native Tβ4.

In order to prepare the actin derivative with the cross-linker anchored at the actin-bound nucleotide, the cross-linking reagent had first to be attached to ATP<sub>S</sub>. The modified ATP<sub>S</sub> was identified by its <sup>1</sup>H NMR spectrum as well as by UV spectrometry (Fig. 3a). The presence of a 1:1 adduct of ATP<sub>S</sub> and nonylene-5-dithio-2-nitrobenzoate was proved by evaluating the amount of ArS<sub>S</sub> (ε<sub>295</sub> = 14,150 M<sup>-1</sup> cm<sup>-1</sup>) released after treatment with DTT (Fig. 3b), which corresponds to the amount of cross-linker present in the modified nucleotide. (The molar extinction coefficient of the cross-linking part is ε<sub>338</sub> = 9400 ± 50 M<sup>-1</sup> cm<sup>-1</sup>, a value that agrees with the extinction coefficient previously reported for n-octyl-5-dithio-2-nitrobenzoate (ε<sub>338</sub> = 9050 M<sup>-1</sup> cm<sup>-1</sup> (26)). Considering the contribution of the cross-linking part to the absorbance at 259 nm (0.4 × E<sub>338</sub> (26)) the absorption of the adenine part at that wavelength (ε<sub>295</sub> of ATP = 16,415 M<sup>-1</sup> cm<sup>-1</sup>) (Fig. 3a) reveals a ratio of 1.097 for the ATP<sub>S</sub> part and the cross-linking part. The modified nucleotide was exchanged for ADP in 10<sup>374</sup>(NEM)<sub>2</sub>-actin, which was prepared in order to avoid intramolecular cross-linking of the modified nucleotide with the two potentially reactive thiol groups in actin, yielding 10<sup>374</sup>(NEM)<sub>2</sub>-actin-ATP<sub>S</sub>(SS-(CH<sub>2</sub>)<sub>3</sub>-SSAr) (n = 3, 9). Since the affinity of the modified ATP<sub>S</sub> for actin is lower than that of ATP (see below), loading with the labeled nucleotide was optimized by separating the excess of unbound, labeled ATP<sub>S</sub> just before use. Incorporation of the labeled nucleotide into actin at the time of the experiment was then as high as 95%.

For making sure that ATP<sub>S</sub> could indeed be used as an anchoring point in actin, the affinity of the modified nucleotide to actin was assayed by determining the exchange rate of the modified ATP<sub>S</sub> bound to 10<sup>374</sup>(NEM)<sub>2</sub>-actin for ε-ATP. This exchange rate was found to be accelerated 5-fold in comparison with normal ATP bound to 10<sup>374</sup>(NEM)<sub>2</sub>-actin (k = 2.8 × 10<sup>-3</sup> ± 0.2 × 10<sup>-3</sup> s<sup>-1</sup> in comparison with k = 5.8 × 10<sup>-4</sup> ± 0.3 × 10<sup>-4</sup> s<sup>-1</sup>).

Preparation of the Thymosin β4 Analogs—Since thymosin β4 does not contain any cysteine residue, five different Tβ4 analogs were synthesized, each containing one cysteine in a defined position (Fig. 4). The distribution of the cysteines in the Tβ4 sequence was such as to replace hydrophobic residues only. To ensure that these substitutions did not influence binding of Tβ4 to actin, cross-linking studies using EDC/NHS were performed with all analogs. These studies showed that all analogs were still able to bind actin similar to normal Tβ4 as indicated by the occurrence of the 47-kDa band in SDS-PAGE (Fig. 5). To confirm this result, and to ensure that the substitution even in the hexamotif of Tβ4 had no significant effect on the affinity to actin, the K<sub>d</sub> value of Cys<sup>374</sup>Tβ4 was determined according to Ref. 13. It was shown to be 0.8 μM ± 0.1 μM and thus in the same range as the K<sub>d</sub> value of native Tβ4, which was reported to be 0.7–2.0 μM (4, 7).

Cross-linking Studies—Cross-linking reactions were detected by measuring changes in absorbance at 412 nm that occur when actin derivatives and thymosin β4 analogs were allowed to form a complex. In order to prove that the ΔOD really reflected cross-link formation between the two proteins, the reaction mixtures were investigated in parallel by SDS-PAGE (Fig. 6). The amounts of the 47-kDa bands, representing the covalently linked actin-thymosin β4 complex, were measured by integration of the gel bands and compared with the absorbance values detected at the same time. From the good agreement of the two sets of data, it was concluded that the release of ArS<sub>S</sub> measured by UV spectroscopy at 412 nm indeed reflected the formation of cross-links. Proof of the disulfide nature of the linkage between the two proteins was obtained by SDS-PAGE where the 47-kDa band disappeared in the presence of DTT. Since each of the two proteins in the complex was exposing only one thiol group, a positive cross-linking reaction could be taken as evidence that the two thiols had approached to a distance that could be bridged by the length of the cross-linker. In total, more than 40 kinds of cross-linking experiments were performed, the results of which are compiled in Table I. In control experiments, it was shown that native Tβ4

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2 A. Reichert, D. Heintz, H. Ehner, W. Voelter, and H. Faulstich, unpublished data.
Identification of Contact Sites in Actin-Thymosin β4 Complex

**Fig. 4.** Thymosin β4 does not contain any cysteine (a). Therefore five different Tβ4 analogs were synthesized, each containing one cysteine in distinct positions (b-f). Substitutions were distributed over the whole molecule and were restricted to hydrophobic amino acids.

**Fig. 5.** SDS-PAGE of G-actin cross-linked to the five Tβ4 analogs using EDC/NHS. Comparable amounts of the 47 kDa band representing the covalently linked actin-Tβ4 complex were formed from all thiol-protected analogs similar to native Tβ4. This indicates that none of the substitutions made in Tβ4 abolished binding to actin. 1, actin + Cys6-Tβ4; 2, actin + Cys17-Tβ4; 3, actin + Cys28-Tβ4; 4, actin + Cys34-Tβ4; 5, actin + Cys374-Tβ4; 6, actin; 7, actin + Tβ4

**Fig. 6.** Reaction mixtures of different actin derivatives cross-linked with Cys374-Tβ4 were analyzed on SDS-PAGE after 60 min. Yield of cross-linking was determined by densitometric evaluation of the gel bands and was in good agreement with the corresponding spectrophotometric values representing the amount of ArS released due to the cross-linking reactions. For lanes 1–4, calculations of the yields of cross-linking took into account that the actin derivatives were present in excess (2:1) over Tβ4, 1, actin374SS-(CH2)9-SSAr + Cys374-Tβ4 mixed at a ratio of 2.1 (yield of cross-linking, 60%); 2, actin374SS-(CH2)5-SSAr + Cys374-Tβ4 mixed at a ratio of 2.1 (yield of cross-linking, 55%); 3, actin374SS-(CH2)3-SSAr + Cys374-Tβ4 mixed at a ratio of 2.1 (yield of cross-linking, 54%); 4, actin374SSAr + Cys5-Tβ4 mixed at a ratio of 2:1 (yield of cross-linking, 29%); 5, actin; 6, NEM-actin374SS-(CH2)5-SSAr + Cys374-Tβ4 mixed at a ratio of 1:1 (yield of cross-linking, 11%); 7, NEM-actin374SS-(CH2)3-SSAr + Cys374-Tβ4 mixed at a ratio of 1:1 (yield of cross-linking, 9%); 8, NEM-actin374SS-(CH2)3-SSAr + Cys5-Tβ4 mixed at a ratio of 1:1 (yield of cross-linking, 6%).

When added to the actin derivatives did not induce the release of ArS·.

Based on extent and kinetics of the ArS· release, three types of reactions could be distinguished. In the first type, the reaction proceeded rapidly reaching its end point (>50%) within less than 10 min (Fig. 7, a–c). For one of these reactions, a complete kinetic analysis was performed showing that the half-maximal value was actually reached after about 1 min (data not shown). Reaction kinetics of this type were taken as indicating the close proximity of the two thiols in the protein complex. Based on this type of kinetics it was possible to identify three sites of very close contact (~9.2 Å) between the two proteins. One of these contacts is between Cys374-actin and Cys5-Tβ4. Cross-linking at this site was almost independent of the length of the cross-linker as yields and kinetics of actin374SS-(CH2)n-SSAr were similar when n was 3, 6, or 9. The proximity of Cys374-actin and Cys5-Tβ4 was even close enough to allow for zero-length cross-linking as shown for actin374SSAr when allowed to complex with Cys5-Tβ4. However, zero-length cross-linking was distinctly slower than the cross-linking reactions described first, and thus belongs to the second type of kinetics described below. The two other sites of close contact were identified from the rapid reactions of the cross-linkers attached to the actin-bound ATPγS with Cys17-Tβ4 and, to a lower extent, with Cys374-Tβ4.

In the second type of kinetics, yield of cross-linking was low at the beginning (~10% after 10 min) but became extensive with time (Fig. 7d). It appears that in this type of cross-linking reaction, the two thiols are not in close proximity but can come close to each other due to the mobility of one, or both, of the partners. Examples of this second type of kinetics are, besides the reaction already mentioned, the cross-links between actin374SS-(CH2)n-SSAr (n = 3, 6, 9) and the cysteines located in the central part of thymosin β4. Particularly Cys17-Tβ4, and to a much lower extent also Cys374-Tβ4 showed considerable extents of cross-linking with cysteine 374 of actin, although with low reaction rates. Cross-linking reactions of this type were not regarded as identifying sites of strong contact.

The third type of cross-linking reactions comprises those with very low amounts (~10%) of ArS· released during the first 10 min followed by an only slight increase within 90 min (Fig. 7e). This reaction pattern was the most frequent one and, in contrast to the other two types of kinetics, was taken as an indication that the two thiol groups were remote from each other. This type of kinetics was found e.g. in all experiments involving Cys40-Tβ4, suggesting that this position in thymosin β4 must be located distant from both cysteine residues in subdomain 1 of actin as well as from the actin-bound nucleotide. This type of kinetics was likewise found in all cross-linking experiments involving 374NEM-actin374SS-(CH2)n-SSAr (n = 3, 6, 9).

Functional Interactions between the Thymosin β4 Analogs and Actin—All five thymosin β4 analogs obtained by peptide synthesis were able to bind actin as shown from the cross-linking studies illustrated above (Fig. 5). In order to assay the influence of the substitutions in Tβ4 on the polymerization-inhibiting capacity, polymerization of G-actin was monitored in the presence of each of the analogs. Generally, we found that all Tβ4 analogs, which proved positive in one of the cross-linking reactions, also showed a reduced inhibitory capacity on actin polymerization (Fig. 8). Lowest inhibitory capacities were found for those two analogs that showed the highest yields in the cross-linking reactions with the actin-bound nucleotide (Cys17-Tβ4 and Cys374-Tβ4). In line with this, even Cys34-Tβ4, which reacted with the actin-bound nucleotide only to a small amount, had likewise lost part of its inhibitory capacity. The substitution in position 6 of Tβ4 resulted in an only slight alteration in the polymerization-inhibiting capacity, whereas the substitution in position 40 of Tβ4 had no influence at all. In accordance with this, the capacity of the latter to inhibit actin polymerization was virtually indistinguishable from that of native Tβ4.


**TABLE I**

| Complex                      | Cys<sup>8</sup> · Tβ4 | Cys<sup>17</sup> · Tβ4 | Cys<sup>36</sup> · Tβ4 | Cys<sup>36</sup> · Tβ4 | Cys<sup>36</sup> · Tβ4 |
|------------------------------|-----------------------|------------------------|------------------------|------------------------|------------------------|
| Actin<sup>74</sup>SS-(CH<sub>2</sub>)<sub>3</sub>-SSAr | 54 ± 3                | 7 ± 1                  | 1 ± 1                  | 2 ± 1                  | 4 ± 1                  |
| 10 min                       | 55 ± 4                | 13 ± 3                 | 13 ± 2                 | 2 ± 4                  | 3 ± 1                  |
| 30 min                       | 57 ± 3                | 25 ± 2                 | 22 ± 3                 | 3 ± 1                  | 4 ± 1                  |
| 90 min                       | 57 ± 3                | 43 ± 3                 | 4 ± 1                  | 4 ± 1                  | 1 ± 1                  |
| Actin<sup>74</sup>SS-(CH<sub>2</sub>)<sub>4</sub>-SSAr | 46 ± 3                | 4 ± 2                  | 2 ± 1                  | 6 ± 2                  | 2 ± 1                  |
| 10 min                       | 48 ± 4                | 17 ± 3                 | 2 ± 2                  | 1 ± 1                  | 3 ± 1                  |
| 30 min                       | 52 ± 2                | 31 ± 2                 | 2 ± 1                  | 3 ± 1                  | 5 ± 2                  |
| 90 min                       | 52 ± 2                | 13 ± 3                 | 2 ± 1                  | 5 ± 2                  | 2 ± 1                  |
| Actin<sup>74</sup>SS-(CH<sub>2</sub>)<sub>4</sub>-SSAr | 47 ± 3                | 6 ± 1                  | 2 ± 1                  | 3 ± 1                  | 6 ± 2                  |
| 10 min                       | 52 ± 4                | 12 ± 2                 | 2 ± 2                  | 3 ± 1                  | 6 ± 2                  |
| 30 min                       | 54 ± 3                | 23 ± 2                 | 2 ± 2                  | 3 ± 1                  | 6 ± 2                  |
| 90 min                       | 54 ± 3                | 41 ± 2                 | 2 ± 2                  | 3 ± 1                  | 6 ± 2                  |
| Actin<sup>74</sup>SS-(CH<sub>2</sub>)<sub>3</sub>-SSAr | 11 ± 1                | 5 ± 2                  | 2 ± 1                  | 6 ± 1                  | 2 ± 1                  |
| 10 min                       | 16 ± 3                | 15 ± 2                 | 2 ± 1                  | 3 ± 1                  | 6 ± 2                  |
| 30 min                       | 25 ± 3                | 26 ± 3                 | 2 ± 1                  | 3 ± 1                  | 6 ± 2                  |
| 90 min                       | 38 ± 4                | 26 ± 3                 | 2 ± 1                  | 3 ± 1                  | 6 ± 2                  |
| Actin<sup>74</sup>SS-(CH<sub>2</sub>)<sub>4</sub>-SSAr | 9 ± 2                 | 3 ± 1                  | 6 ± 2                  | 1 ± 1                  | 3 ± 1                  |
| 10 min                       | 12 ± 2                | 12 ± 2                 | 2 ± 1                  | 6 ± 2                  | 2 ± 1                  |
| 30 min                       | 16 ± 3                | 13 ± 2                 | 2 ± 1                  | 6 ± 2                  | 2 ± 1                  |
| 90 min                       | 21 ± 2                | 17 ± 3                 | 2 ± 1                  | 6 ± 2                  | 2 ± 1                  |
| Actin<sup>74</sup>SS-(CH<sub>2</sub>)<sub>3</sub>-SSAr | 7 ± 1                 | 7 ± 2                  | 2 ± 1                  | 5 ± 2                  | 4 ± 2                  |
| 10 min                       | 11 ± 2                | 12 ± 2                 | 2 ± 1                  | 6 ± 2                  | 2 ± 1                  |
| 30 min                       | 16 ± 3                | 13 ± 2                 | 2 ± 1                  | 6 ± 2                  | 2 ± 1                  |
| 90 min                       | 21 ± 2                | 17 ± 3                 | 2 ± 1                  | 6 ± 2                  | 2 ± 1                  |
| Actin<sup>10</sup>NEM · ATP<sub>γ</sub>SS-(CH<sub>2</sub>)<sub>3</sub>-SSAr | 5 ± 1                 | 48 ± 4                 | 27 ± 3                 | 4 ± 2                  | 13 ± 4                 |
| 10 min                       | 5 ± 2                 | 58 ± 4                 | 30 ± 4                 | 4 ± 2                  | 13 ± 4                 |
| 30 min                       | 19 ± 4                | 49 ± 5                 | 13 ± 4                 | 4 ± 2                  | 13 ± 4                 |
| Actin<sup>10</sup>NEM · ATP<sub>γ</sub>SS-(CH<sub>2</sub>)<sub>3</sub>-SSAr | 76 ± 3                | 40 ± 4                 | 23 ± 3                 | 4 ± 2                  | 24 ± 4                 |
| 10 min                       | 76 ± 4                | 41 ± 4                 | 24 ± 4                 | 4 ± 2                  | 24 ± 4                 |

Encouraged by the good correlation found between polymerization inhibiting capacities and cross-linking data, we assayed the retardation of the nucleotide exchange rate of actin as another functional parameter of Tβ4. The influence of Cys<sup>17</sup> · Tβ4 on the nucleotide exchange was examined in comparison with native Tβ4 and Cys<sup>36</sup> · Tβ4, the latter as an example of a Tβ4 analog, which is ineffective in the thiol-specific cross-linking reactions as well as in the polymerization-inhibiting assay. The retardation effect of Cys<sup>17</sup> · Tβ4 was found to be indeed partly abolished. While the k value of Cys<sup>36</sup> · Tβ4 (k = 2.8 × 10<sup>-4</sup> ± 0.2 × 10<sup>-4</sup> s<sup>-1</sup>) was almost indistinguishable from that of native Tβ4 (k = 2.9 × 10<sup>-4</sup> ± 0.2 × 10<sup>-4</sup> s<sup>-1</sup>), the nucleotide exchange rate of Cys<sup>17</sup> · Tβ4 was found to be accelerated to a value of k = 4.7 × 10<sup>-4</sup> ± 0.2 × 10<sup>-4</sup> s<sup>-1</sup>, a value that approaches the k value of pure actin (k = 6.2 × 10<sup>-4</sup> ± 0.3 × 10<sup>-4</sup> s<sup>-1</sup>) under these conditions (Fig. 9).

**DISCUSSION**

In order to identify contact sites between actin and Tβ4 we successfully used a method of selective cross-linking between thiols that is able to measure the closest approach of two cysteines in the protein complex. By using a set of cross-linking reagents of different lengths, or the procedure of direct activation of one of the thiols with Ellman’s reagent, we were able to assay distances between two thiols in the range from 0 to ~18 Å.

In obtaining reliable results from this kind of study it was essential that the cross-linking reaction was absolutely thiol-specific and that each of the two proteins exposed only one thiol group. The first condition was assured by the fact that the...
disulfide-exchange reaction runs with thiols only (28). The second requirement was met for Tβ4 in that the synthetic Tβ4 analogs used contained only one cysteine each. As for actin, we made use of the fact that actin in buffer G exposes only cysteine 374 (29), which could either be reacted with the cross-linking reagents or be blocked with NEM. By exchanging ATP for ADP in 374NEM-actin, cysteine 10 could be selectively uncovered (29), which could either be reacted with the cross-linking reagents or be blocked with NEM. By exchanging ATP for ADP in 374NEM-actin, cysteine 10 could be selectively uncovered (29), thus providing another distinct thiol group to be reacted or even identically, to G-actin with respect to polymerization, all actinderivatives, it was shown that they behaved similarly, providing the third point of attachment for the cross-linkers. For components was used in excess (2:1) and never exceeded 75%. The concentration of 100 mM. Values represented by the addition of KCl to a final polymerization conditions were established by the addition of KCl to a final concentration of 100 mM. Values represent the average of four measurements. Pure actin, ○: actin + Tβ4, ■: actin + Cys17Tβ4, ▽: actin + Cys34Tβ4, ●: actin + Cys6Tβ4, ●: actin + Cys50Tβ4, △: actin + Cys40Tβ4, Δ.

As in titrations using Ellman's reagent, formation of a cross-link between an actin derivative and a Tβ4 analog was accompanied by the release of ArS- detectable at 412 nm, which allowed easy determination of the extent and kinetics of cross-link formation by UV spectrometry. Since these data were in very good agreement with those obtained by integrating the corresponding gel bands in SDS-PAGE, it was concluded that the release of ArS- reflected the cross-link formation quantitatively. The extent of cross-linking as determined by spectrophotometry was independent on whether one of the components was used in excess (2:1) and never exceeded 75%. The incompleteness of the reaction may be explained by the Ki value of the actin-Tβ4 complex (−1 μM) limiting complex formation. In addition, the extent of cross-linking may be lowered by the fact that all actin derivatives were labeled only up to 80–90%. Finally, it cannot be excluded that the unprotected cysteine in the Tβ4 analogs was partially oxidized during the cross-linking reaction. On the other hand, in all reactions classified as negative, the release of ArS- was never zero. We suppose that the small amounts of ArS- (<10%) detected in these experiments were released by unspecific reactions in which the small Tβ4 reacted to some extent in a way similar to a low molecular weight thiol.

Three major reaction types could be distinguished on the basis of kinetics and the extent of cross-linking. Fast reactions with high extents of cross-linking (50–75%) within a few minutes were taken as indicating close proximity of the two cysteines in the protein complex. According to this classification, one major contact was identified between the C terminus of actin and the N terminus of Tβ4. In particular, there is evidence that the thiols of Cys374 in actin and Cys6 in Tβ4 approach to within 9.2 Å. This finding confirms previous data that identified Cys374 as a part of a short distance cross-link with Tβ4 (13). Contact in this region must indeed be very close since it was even possible to form a zero-length cross-link between Cys374 of actin and Cys6 of Tβ4, although at a low rate. As a second major contact site the hexamotif of Tβ4 (position 17–22) was identified as located near the actin-bound nucleotide, since the distance of Cys17Tβ4 and the sulfur atom of ATP-γS could be bridged by a cross-linker of 9.2 Å in length. Lower, but still significant yields of cross-linking were found also between Cys50Tβ4 and the modified ATP-γS, suggesting that the whole central part of Tβ4 is in proximity to the γ-phosphate of the nucleotide. Considering the different yields of these two cross-linking reactions, Cys17 may be located closer to the nucleotide.

**Fig. 8.** Polymerization kinetics of actin (10 μM) as followed by capillary viscometry in the absence or presence of Tβ4 or the Tβ4 analogs. The β-thymosin analogs (1.5 eq) were mixed with native actin (1 eq) 30 min before the polymerization conditions were established by the addition of KCl to a final concentration of 100 mM. Values represent the average of four measurements. Pure actin, ○: actin + Tβ4, ■: actin + Cys17Tβ4, ▽: actin + Cys34Tβ4, ●: actin + Cys6Tβ4, ●: actin + Cys50Tβ4, △: actin + Cys40Tβ4, Δ.

**Fig. 9.** Time course of the exchange of actin-bound ATP (0.3 μM) in the absence of Tβ4 (+, upper curve), in the presence of Cys374Tβ4 (×, middle curve), or in the presence of Cys50Tβ4 (●, lower curve), showing the effects of the two thymosin β4 analogs on the nucleotide exchange rate.
Identification of Contact Sites in Actin-Thymosin β4 Complex

The yield of cross-linking with the actin-bound nucleotide decreased when the position of the cysteine residue in the Tβ4 analog approached the C terminus. This correlation indicates that the highly flexible C terminus of Tβ4 is most probably directed away from the actin-bound nucleotide. Since no thiol-specific cross-link formation was found in any experiment involving Cys$_{374}$Tβ4, this part of Tβ4 appears to be located in a domain of actin that is different from subdomain 1 and remote from the nucleotide region.

Reactions of cysteine 10 of actin with all Tβ4 analogs followed the third type of reaction kinetics. According to our classification, we believe that Tβ4 is not in direct contact with that side of actin bearing Cys$_{374}$, the latter known to be part of a β-sheet (30). Nevertheless, low yield cross-linking reactions with Cys$_{374}$ were measured by spectrophotometry, and confirmed by SDS-PAGE. The existence of these reactions between the cross-linker attached to Cys$_{374}$ and Tβ4 may be understood on the basis of the maximal possible reaction range of the cross-linking reagent (≈9.2 Å) that reaches far beyond the thiol of Cys$_{374}$. The reaction range of the cross-linker may be comparable with that of the first four amino acids of actin, which are believed to form a mobile structure (30) and have been reported to be involved in EDC cross-links with Tβ4 (14, 31).

Finally, we assayed whether the replacement of five hydrophobic amino acids in Tβ4 had caused any functional deficits. For all Tβ4 analogs, a clear correlation was found between the extents of cross-linking with the actin-bound nucleotide and the decrease in the inhibitory capacities on actin polymerization. The strongest reduction of inhibitory capacities was found for substitutions by cysteine in or near by the hexamotif. This observation is in line with the results of Vancompernolle et al. (9) who showed the hexamotif to be most important for binding and function. Interestingly, in the case of Cys$_{374}$-Tβ4, the greatest extent of cross-linking was paralleled not only by the most strongly reduced inhibitory capacity, but also by a significant decrease of the retardation effect on the nucleotide exchange rate in comparison to native Tβ4.

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