Purification and Characterization of Subtilisin Cleaved Actin Lacking the Segment of Residues 43−47 in the DNase I Binding Loop

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ABSTRACT: The protease subtilisin has been reported to cleave skeletal muscle G-actin between Met 47 and Gly 48 generating a core fragment of 33 kDa and a small N-terminal peptide, which remains attached to the core fragment [Schwyter, D., Phillips, M., & Reisler, E. (1989) *Biochemistry 28*, 5889−5895]. However, amino acid sequencing and mass spectroscopy of subtilisin cleaved-actin revealed two cleavage sites, one between Met 47 and Gly 48 and a second between Gly 42 and Val 43, generating an actin core of 37 kDa and a nicked 4.4 kDa N-terminal peptide. Here we describe a procedure for purifying the actin core fragment and the attached N-terminal peptide from the linking pentapeptide comprising amino acid residues 43−47 under native conditions by anion exchange chromatography. After removal of the pentapeptide, the salt-induced polymerization of actin was abolished. However, the purified fragments could be polymerized by addition of salt plus myosin subfragment 1 or salt plus phalloidin as shown by sedimentation and fluorescence increase using *N*-(1-pyrenyl)iodoacetamide labeled actin. These results confirm earlier reports proposing that cleavage in the DNase I binding loop is affecting the ion induced polymerization of actin [Higashi-Fujime, S., et al. (1992) *J. Biochem. (Tokyo) 112*, 568−572; and Khaitlina, S., et al. (1993) *Eur. J. Biochem. 218*, 911−920]. Monomeric and filamentous subactin exhibited reduced abilities to inhibit deoxyribonuclease I (DNase I) and to stimulate the myosin subfragment 1 ATPase activity. Direct binding of subactin to DNase I was verified by gel filtration and to myosin subfragment 1 by affinity chromatography, chemical cross-linking, and electron microscopy.

Actin is one of the most widely distributed proteins in nature. It is able to interact with a vast number of actin-binding proteins (AbP).[1] Of particular interest is its interaction with myosin, which in muscle leads to the generation of contractile force. The atomic structure of rabbit skeletal muscle actin in complex with DNase I has recently been solved (Kabsch et al., 1990), thus paving the way to a better structural understanding of its interactions with other AbPs. Such an analysis would be particularly fruitful if the three-dimensional structures of other actin binding proteins were known as it is for chicken pectoralis myosin subfragment 1 (Rayment et al., 1993a). To completely understand the interaction of actin with other AbPs, it is, however, necessary to solve the high-resolution three-dimensional structure of crystalline complexes of actin and the particular actin binding protein. This has been done for the complex of actin and segment 1 of gelsolin (Mannherz et al., 1992; McLaughlin et al., 1993) and recently for the complex of actin and profilin (Schutt et al., 1993).

However, a number of AbPs preferentially bind to the filamentous form of (F-) actin or only weakly interact with monomeric (G-) actin, making it very unlikely that crystals of these complexes suitable for X-ray analysis can be obtained. Therefore, an alternative approach is the analysis of the interaction of modified or sequentially deleted actin with a particular binding protein. Cloning and site-directed mutagenesis would be one way to produce modified actins. Unfortunately, this approach has so far been hampered by difficulties in obtaining high yield expression systems allowing the actins to be purified in their native state. Therefore, in recent years experiments have been described in which actin was either chemically modified or proteolytically cleaved into defined fragments (Burtnick, 1984; Jacobson & Rosenbusch, 1976; Johnson et al., 1979; Konno, 1987; Schwyter et al., 1989; Higashi-Fujime et al., 1992; Khaitlina et al., 1993). However, these attempts have met experimental constraints, namely, the difficulties in separating the cleaved fragments and/or the need to work with mixtures of intact and cut actin.

Recently, it has been reported that subtilisin can cleave actin between Met 47 and Gly 48, thus generating a core fragment of 33 kDa and a small N-terminal peptide that remains attached to the core fragment (Schwyter et al., 1989). This nicked actin was shown to still possess polymerizing and DNase I inhibitory capacity although at a reduced extent. We discovered a second cleavage site between Gly 42 and Val 43 and developed a procedure to purify the subtilisin generated core fragment containing firmly bound the N-terminal fragment ranging from residue 1 to 42 under native conditions but devoid of the connecting pentapeptide. This allowed us to study its ability to polymerize, to inhibit and

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[1] Abbreviations: AbP, actin-binding protein, DTE, dithioerythritol; DNase I, deoxyribonuclease I (EC 3.1.21.1); EDC, 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide; 1.5 IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; S1, myosin subfragment-1; S1A1, myosin subfragment-1 containing the light chain A1; TFA, trifluoroacetic acid.

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to bind to DNase I, and to interact with myosin subfragment 1 in the absence of this pentapeptide. In native monomeric actin this region appears to be very flexible and in the actin−DNase I complex forms a hydrophobic loop interacting with DNase I. This area is also known as the DNase I binding loop (Kabsch et al., 1990) and forms part of the actin−actin interface in F-actin (Holmes et al., 1990). After addition of high salt, actin devoid of this pentapeptide is unable to polymerize, indicating that this hydrophobic pentapeptide may critically be involved in the salt-induced actin−actin association.

MATERIALS AND METHODS

Protein Preparation. Actin was purified from acetone powder of rabbit skeletal muscle as described by Spudich and Watt (1971). Myosin subfragment 1 was prepared by α-chymotryptic cleavage of rabbit skeletal muscle myosin, and the subfragment 1 isoform S1A1 was further purified on DEAE-Cellulose according to Weeds and Taylor (1975). DNase I was a commercial product of Worthington Corp. (Freehold, NJ) and further purified as outlined in Mannherz et al. (1980).

Subtilisin Digestion. G-actin (approximately 100 μM) was digested with subtilisin at a ratio of 1:1000 (w/w) in G-buffer (2 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTE) according to Schwyter et al. (1989). The reaction was stopped after 60 min by addition of 2 mM phenylmethanesulfonyl fluoride (PMSF).

Separation of Subtilisin-Cleaved Fragments by HPLC. Subtilisin-cleaved actin fragments were separated on reversed-phase HPLC using a 4.6 × 125 mm octadecyl Si 100 column (Serva) with a linear gradient of 0−100% acetonitrile in 0.1% TFA solution and monitored at 206 and 260 nm.

Amino Acid Sequencing. The N-terminal peptide was dissolved in 0.1 M NH_4HCO_3 buffer, pH 8.0, containing 0.8 M guanidinium hydrochloride. Twenty-five micrograms was digested with 0.5 μg of endoproteinase ASP-N (Boehringer, Mannheim, Germany) for 18 h at 37 °C. The reaction was stopped by adding 1 μL of TFA. The resulting peptides were separated by C8 reversed-phase HPLC using a 2.1 × 100 mm Brownlee Aquapore RP-300 column with a linear 0−60% acetonitrile gradient in 0.1% TFA solution. The purified peptides were submitted to automated Edman degradation using an ABI470A gas phase sequenator [Applied Biosystems (ABI), Weiterstadt, Germany]. The actin-core protein was directly applied to the polybren-coated glass filter of the sequencer. The program cycles supplied by ABI were modified (one additional coupling step) to increase the yield during the repetitive cycles. The phenylthiohydantoin derivatives of amino acids were identified by HPLC in an ABI 120A PTH analyzer with an ABI 900 Data Analysis Module. The HPLC injection vent was equipped with a 100 μL sample loop, allowing the analysis of 83% of the phenylthiohydantoin samples.

Mass Spectroscopy. The molecular mass of subactin and HPLC separation products of subactin was determined by mass spectroscopy using the matrix assisted laser desorption mass spectrometer Finnigan MAT. Subactin at 1.3 mg/mL was dissolved in 20 mM sinapine acid and laser activated at 337 nm. As an internal standard, cytochrome *c* (M_r 12 361) was added. Mass spectrometry indicated the presence of a peptide of M_r 4373.1 in the purified subactin core whose M_r was 36 759. The peptide separated by HPLC at 10 mg/ml was dissolved in 50 mM cyanohydrocinnamic acid. Its molecular weight was determined to be 4406 when using renin (M_r 1760) as internal standard, corresponding well with the calculated M_r of 4327.78 for the first 42 amino acids of actin.

Purification of the 37 kDa Actin Core Associated with a 4.4 kDa N-Terminal Fragment ("Subactin"). Subtilisin-generated actin (subactin) was purified by a step-gradient of KCl on DEAE-cellulose. The anion-exchanger (Whatman DE 52) was saturated with nucleotide (ATP) prior to the separation and washed with 200 mL of starting buffer (10 mM imidazole-HCl, pH 7.5, 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.1 mM DTE) supplemented with 100 mM KCl. After the proteolytic digestion the sample was loaded on a DE 52 column (2.5 × 20 cm) equilibrated in starting buffer. Usually 20 fractions of 10 mL each were collected in each step. The elution profile was monitored at 290 nm. The second step (starting buffer supplemented with 500 mM KCl) eluted subactin as a sharp peak. The third step (1 M KCl final concentration) eluted a second peak containing denatured actin and actin fragments as verified by subsequent SDS−PAGE analysis.

Labeling of F-Actin with 1.5-IAEDANS and N-(1-Pyrenyl)-iodoacetamide. The labeling of Cys 374 with 1.5-IAEDANS followed the method of Takashi (1979) using a 20-fold molar excess of the fluorescent dye over actin. The labeling of F-actin by *N*-(1-pyrenyl)iodoacetamide was performed as described by Cooper et al. (1983). F-actin at 1 mg/mL was incubated with 7.5 molar excess of pyrene for 24 h at 4 °C. After labeling, the actin was precipitated by ultracentrifugation, resuspended in G-buffer, and dialyzed for 48 h to depolymerize. It was then clarified by centrifugation, and the supernatant was further purified by gel-filtration on a Sepharose S 200 column. The labeling ratio was regularly greater than 50% using an extinction coefficient of 22 000 M^{-1} cm^{-1} for pyrene (Kouyama & Mihashi, 1981).

Fluorescence Spectroscopy. Steady-excitation fluorometry was performed with an SLM Instruments 8000 photon counting spectrofluorometer operated in time resolved mode. Pyrene was excited at 365 nm and detected at 407 nm with 8 nm slit width. The concentration of pyrene−actin and myosin subfragment 1 was 15 μM each. The fluorescent signal of ε-ATP bound to actin or subactin was used to measure their binding to nucleotide (Waechter & Engel, 1975; Mannherz et al., 1980).

Determination of Enzymatic Activities. The Mg^{2+}-dependent ATPase activities of subfragment-1 or acto-S1 were determined using the coupled optical assay system as given by Mannherz et al. (1975). DNase I activity was determined with the optical hyperchromicity test (Kunitz, 1950) as described by Mannherz et al. (1980).

Cross-Linking Reactions. Phalloidin stabilized subactin filaments or F-actin at 1 mg/mL were incubated with myosin subfragment 1 at equimolar ratio and cross-linked by EDC at a final concentration of 5 mM in G-buffer at 25 °C according to Mornet et al. (1981). In a time course analysis samples were suspended in boiling sample buffer to stop the reaction and analyzed by polyacrylamide gelelectrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) according to Laemmli (1970).

Affinity Chromatography. DNase I agarose was used as an affinity matrix to immobilize subactin or intact actin.

Usually 20 mg of DNase I were coupled to about 10 mL of vinylsulfone agarose (Mini-Leak), and the actin was allowed to bind at equimolar ratio. Binding of subfragment-1 to immobilized actin−DNase I complex has been demonstrated previously (unpublished observation).

Electron Microscopy. Carbon-coated copper grids were treated with a solution of phalloidin-stabilized subactin filaments or F-actin at 20 μg/mL for 5 min at 25 °C and negatively stained with 1% uranyl acetate for 5 min. To decorate the filamentous actin samples with subfragment 1, excess nucleotide was removed by gel-filtration and F-actin was incubated with equimolar S1A1 for 15 min before the negative staining reaction. The grids were examined in a Zeiss EM 902 electron microscope operated at 80 kV.

Materials. Subtilisin was obtained from Sigma, Munich, Germany. DNase I was a commercial product of Worthington Corp., Freehold, NJ. EDC and 1.5-IAEDANS were obtained from Pierce, Rockford, IL, and Serva, Heidelberg, Germany, respectively. The fluorescent ATP analogue 1:N^6-etheno-ATP (εATP) was synthesized according to Secrist et al. (1972) and a generous gift from Mrs. M. Matuska (MPI für medizinische Forschung, Heidelberg, Germany). Agarose activated with vinylsufone (Mini-Leak) was obtained from KEM EN TEC, Copenhagen, Denmark. The pentapeptide V-G-V-G-M was synthesized by Dr. R. Pipcorn, Deutsches Krebsforschungszentrum, Heidelberg, Germany. Phalloidin and endoproteinase ASP-N were from Boehringer-Mannheim, Mannheim, Germany. All other reagents were of analytical grade.

RESULTS

Detection of a Second Cleavage Site between Gly 42 and Val 43. Actin was treated with subtilisin exactly as given by Schwyter et al. (1989) at a 1:1000 (w/w) ratio. In order to characterize actin fragments generated by subtilisin treatment, the cleaved actin was analyzed on reversed-phase HPLC. Two major peaks and one very small peak were detected (Figure 1b). Mass spectrometry analysis revealed for the first peak a M_r value of 37 107 and for the second one a M_r value of 4374.3 obviously representing the actin core and a N-terminal fragment, respectively. The M_r value of the presumed N-terminal peptide was in good agreement with the calculated molecular weight of residues 1−42, namely, 4327.78. Amino acid sequence analysis of the two major fragments isolated on HPLC confirmed that the core fragment starts at Gly 48, in agreement with Schwyter et al. (1989). Total sequencing of the acetylated N-terminal fragment using endoproteinase ASP-N revealed that it ranged from residues 1 to 42. The pentapeptide comprising amino acid residues 43−47 was missing. By comparison with the synthetic pentapeptide V-G-V-G-M we identified the small peak as the pentapeptide on reversed phase HPLC (Figure 1b).

Purification of the Subtilisin-Generated Actin Fragments. Figure 2a shows a time-dependent SDS−PAGE analysis of the action of subtilisin on rabbit skeletal muscle actin. In this particular experiment the actin had been fluorescently labeled at Cys 374 by 1,5-IAEDANS (Takashi, 1979). It can be seen that after 10 min the actin is almost completely cleaved into the 37 kDa fragment. In contrast to the actin fragments generated by trypsin or chymotrypsin (Jacobson & Rosenbusch, 1976; Konno, 1987), subactin retains the

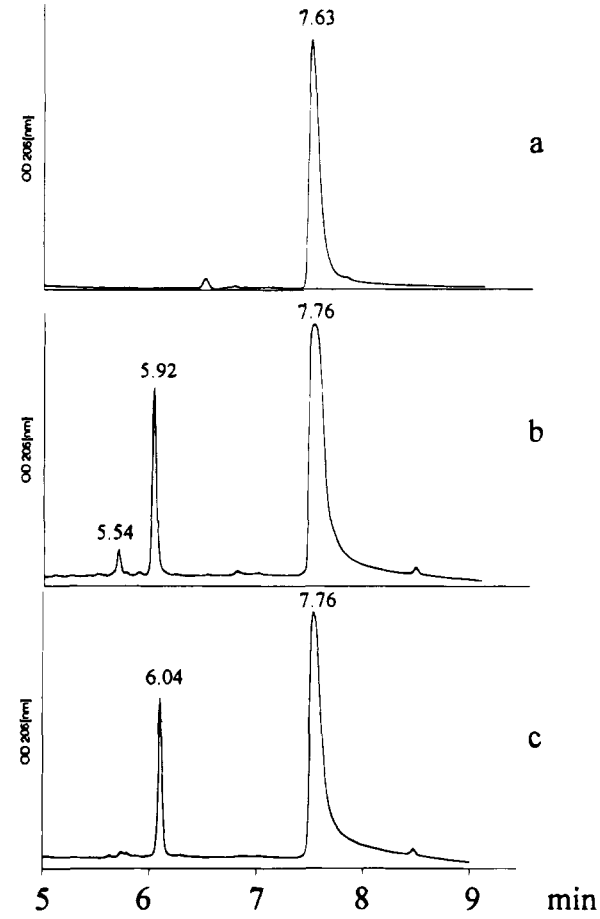

FIGURE 1: Analysis of subtilisin-cleaved actin on reversed-phase HPLC. G-actin at 2 μM was loaded on reversed-phase HPLC before (a) and after 30 min of subtilisin digestion (b) as outlined in Materials and methods. Subactin at 2 μM purified of the pentapeptide after DEAE-cellulose (c). The retention time for G-actin (a) and the 37 kDa corefragment (b and c) was at 7.63 and 7.76 min, respectively, too close to separate the peaks if a mixture was loaded on the column (data not shown). The N-terminal peptide (aminoacid residues 1−42) was detected after a retention time of 5.92 min and the pentapeptide (amino acid residues 42−47) after 5.54 min (b). After purification on DEAE-cellulose, only the 37 kDa corefragment (7.76 min) and the N-terminal peptide (6.04 min) were detected (b).

fluorescent label indicating that its C-terminus is not cleaved by the action of subtilisin.

Subtilisin-treated actin was subjected to anion exchange chromatography on DEAE-cellulose which had first been saturated with ATP and eluted in the presence of 0.5 mM ATP as described for the purification of cytoplasmic actin (Gordon et al., 1976). After loading the column with subtilisin-treated actin, it was eluted with stepwise increments of KCl (Figure 3). Two protein peaks of different size were obtained at 0.5 and 1 M KCl. SDS−PAGE of the fractions collected showed that the first peak contained pure "subactin", subtilisin-cleaved actin missing the pentapeptide 43−47 (see inset of Figure 3, lane 3). Rerversed-phase HPLC revealed the lack of the pentapeptide in subactin after separation on DEAE-cellulose (Figure 1b, c). The second peak was shown by SDS−PAGE to contain uncleaved actin and a number of low molecular weight bands (data not shown). The absence of uncleaved actin in the first peak containing subactin was verified by lack of a 42 kDa band in the SDS−PAGE after Coomassie staining.

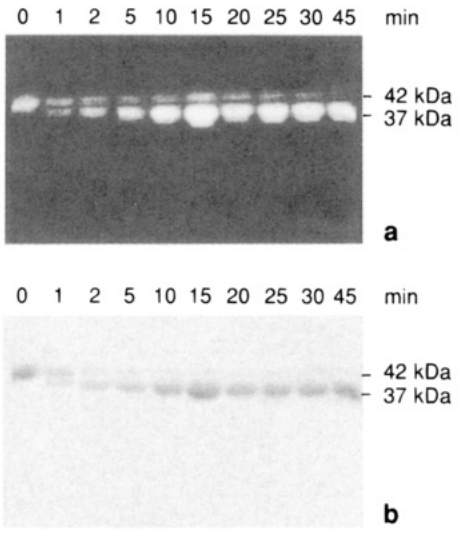

FIGURE 2: Digestion of fluorescently labeled actins by subtilisin. G-actin (42 kDa) was labeled with 1.5-IAEDANS at Cys 374 as described in Materials and Methods. (a) Fluorograph of the unstained SDS gel visualized by an ultraviolet lamp; (b) Coomassie blue stained gel. Digestion times are given for each lane. The 37 kDa fragment remains labeled during the period of digestion. Note the absence of a low molecular mass band in panels a and b; the low molecular mass fragment is not stained by Coomassie.

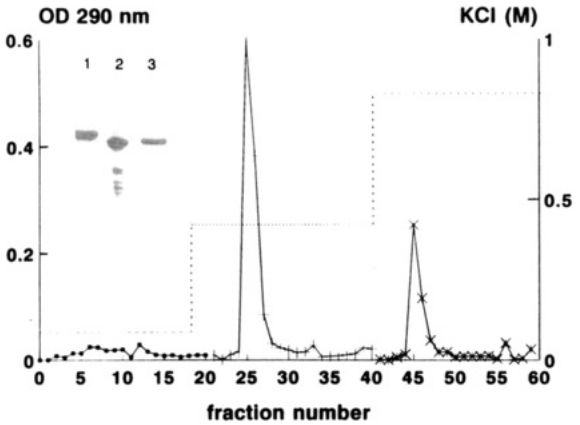

FIGURE 3: Chromatographic elution profile of subtilisin treated actin. The actin hydrolysate applied on DEAE-cellulose was eluted by stepwise increments of KCl-concentration in 10 mM imidazole, pH 7.5, as outlined in Materials and Methods. The inset gives SDS−PAGE (gradient gel of 10−15% acrylamide) of G-actin (lane 1) and subtilisin-cleaved actin before (lane 2) and after the purification on DEAE-cellulose (lane 3).

Polymerizability of Subactin. Purified subactin devoid of the pentapeptide could not be polymerized in the presence of mono- and divalent cations when tested by viscometry, sedimentation, or electron microscopy after negative staining. This was further verified by using pyrenyl-actin and pyrenyl-subactin before and after removal of the pentapeptide. In comparison to G-actin, the salt-induced polymerization of subtilisin "cleaved actin" (containing the pentapeptide) appeared to be delayed (Figure 4). No polymerization of purified subactin (after removal of the pentapeptide) was observed (Figure 4). However, in the additional presence of equimolar amounts of phalloidin or myosin subfragment 1 (S1) subactin was able to polymerize, although at a slower rate than cleaved actin without phalloidin (Figure 4). After incubation for 30 min with either S1 or phalloidin, subactin could also be sedimentated using a Beckman airfuge (data not shown). Furthermore, it was not possible to restore salt inducable polymerizability of subactin after readdition of the pentapeptide (data not shown). We attribute this to the high mobility of the DNase I binding loop (McLaughlin et al., 1993) which may exclude a correct and stable association of the highly hydrophobic pentapeptide.

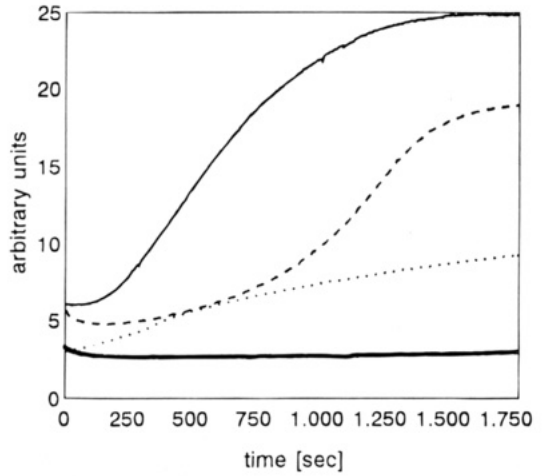

FIGURE 4: Polymerization of pyrenyl-labeled actins. Intact pyrenyl-actin (solid line) and subtilisin-cleaved pyrenyl-actin before (broken line) and after removal of the pentapeptide (thick solid line) at 15 μM were incubated in G-buffer supplemented with 2 mM $MgCl_2$ and 30 mM KCl. The purified subtilisin-cleaved actin was polymerized by addition of 2 mM $MgCl_2$ and 30 mM KCl and phalloidin at equimolar ratio (dotted line). The increase in fluorescence intensity was measured for 30 min at 20 °C with excitation and emission wavelengths at 365 and 407 nm, respectively.

Negative staining revealed the formation of actin filaments from purified subactin in the presence of equimolar phalloidin (Figure 5c) or myosin subfragment 1 (S1A1) (Figure 5d). The subactin filaments obtained in the presence of phalloidin were not obviously different from untreated F-actin (Figure 5a). In contrast to S1-decorated F-actin (Figure 5b), the decorated subactin filaments did not exhibit a well ordered arrowhead orientation (Figure 5d).

Interaction of Subactin with DNase I. We tested the ability of the purified subactin to interact with DNase I. First, its ability to inhibit the DNA degrading activity was compared with intact actin. As can be seen from Figure 6, subactin inhibits DNase I activity although higher concentrations are needed to achieve equal inhibition. Binding of subactin to DNase I could also be demonstrated by affinity adsorption to immobilized DNase I. Immobilized DNase I retained subactin effectively. It was not eluted from this column by increasing the ionic strength up to 100 mM KCl or inclusion of 1 mM EDTA into the washing buffer (data not shown). Furthermore, complex formation of subactin and DNase I was demonstrated directly by gel filtration over an AcA 44 column. A single peak containing both subactin and DNase I was eluted at a position corresponding to a molecular mass of about 68 kDa (Figure 7). In a separate experiment DNase I alone (31 kDa) was shown to elute at position 27 (data not shown).

Subactin Contains Bound Nucleotide. Subactin equilibrated with [^{3}H]ATP or the fluorescent ATP analogue etheno-ATP (ϵ-ATP) was gel-filtrated over a Sephadex G 100 column in order to demonstrate the firm association of nucleotide. The protein-containing fractions were identified by SDS−PAGE. By scintillation counting they were found to contain radioactivity. In addition, the comigration of

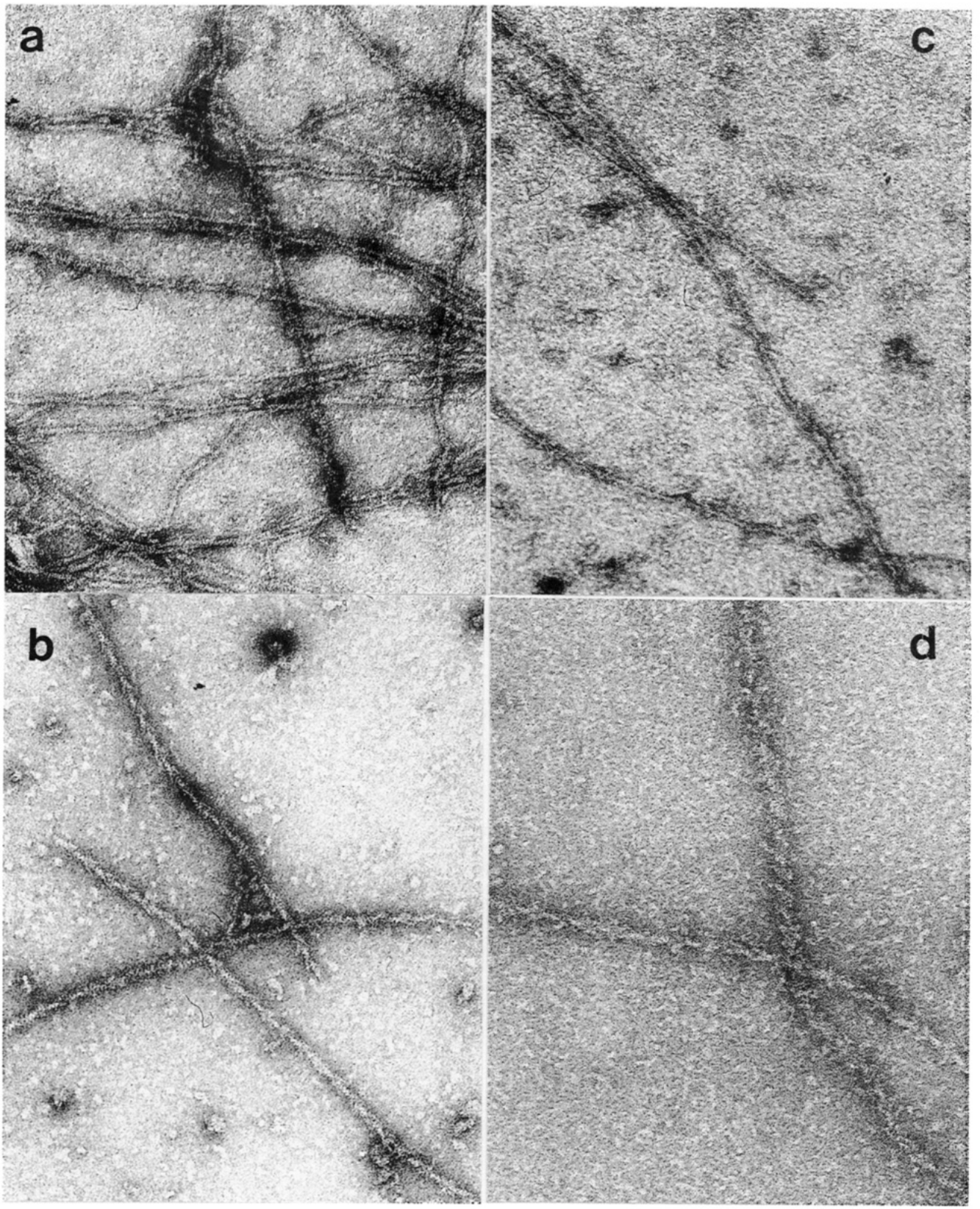

FIGURE 5: Electron micrographs of actin filaments and acto-S1 complexes. F-actin and acto-S1 concentrations were 20 μg/mL. (a) F-actin control; (b) F-actin decorated with S1; (c) filaments of purified subactin polymerized in the presence of phalloidin; (d) subactin filaments decorated with S1A1. Magnification: (a−c) 30 000- and (d) 40 000-fold.

ϵ-ATP bound to subactin was verified by analyzing the fluorescence spectra of the protein containing fractions (data not shown).

In a second experiment we absorbed G-actin or subactin containing [^{3}H]ATP firmly bound to immobilized DNase I (Figure 8a,b). Both G-actin and subactin adsorbed to immobilized DNase I were not dissociated and eluted after inclusion of 5 mM EGTA or EDTA into the washing buffer as verified by SDS−PAGE of the fractions collected (data not shown) in agreement with previous results demonstrating that nucleotide free actin remains attached to immobilized DNase I (Polzar et al., 1989). After washing the columns

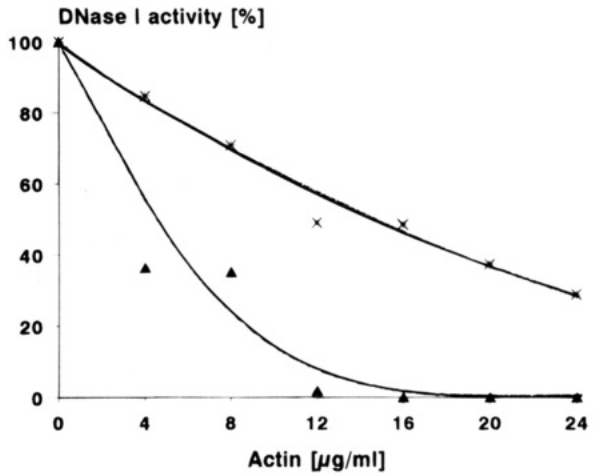

FIGURE 6: Inhibition of DNase I activity by G-actin and purified subactin. Bovine pancreatic DNase I (1 μg) was preincubated with increasing concentrations of G-actin or subactin for 30 min. The DNase I activity was determined using the optical hyperchromicity assay (Kunitz, 1950) and is expressed as relative DNase I activity of the uninhibited DNase I. G-actin (▲); subactin (×).

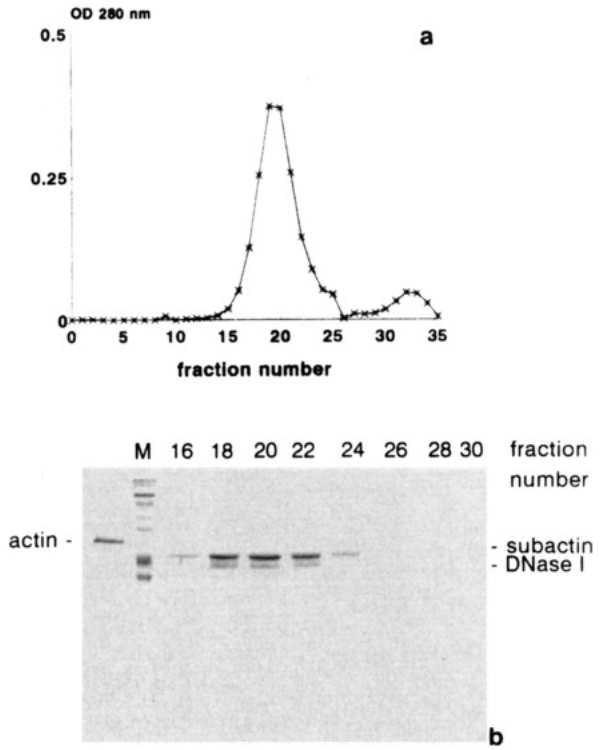

FIGURE 7: Gel filtration of subactin:DNase I complex. Subactin−DNase I complex was formed 30 min prior to the experiment by mixing both proteins at equimolar ratio and loaded on an AcA 44 (LKB, Bromma, Sweden) column equilibrated with 2 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$. Fractions of 2.5 mL were collected at 4 °C. (a) Elution profile recorded at 280 nm; (b) analysis of the protein containing fractions on 12% SDS−PAGE; (M) relative molecular mass marker proteins (prestained, Sigma, Munich, Germany) from top to bottom: α2-macroglobulin (180 kDa); β-galactosidase (116 kDa); fructose-6-phosphate kinase (84 kDa); pyruvate kinase (58 kDa); fumarase (48.5 kDa); lactic dehydrogenase (36.5 kDa); triosephosphate isomerase (26.5 kDa).

with Hepes-buffer sequentially suplemented with either 5 mM EGTA or EDTA, we observed for both actins a release of the actin-bound [^{3}H]ATP. Most of the [^{3}H]ATP was released during the final washing step containing 5 mM EDTA. Thus we detected no difference in the ion dependence of nucleotide release between G-actin and subactin in agreement with Kasprzak (1993), who reported a 2-fold decrease of ϵ-ATP affinity to subactin.

Interaction of Subactin with Myosin Subfragment 1. Since subfragment 1 was found to induce the polymerization of subactin, we further analyzed its interaction with subactin. First, we tested its ability to stimulate the Mg^{2+}-dependent ATPase activity of subfragment 1 (S1). As can be seen, subactin polymerized in the presence of phalloidin is able to stimulate the ATPase activity of S1 containing the light chain 1 (S1A1), although with lower efficiency than native F-actin in the absence or presence of phalloidin (Figure 9). This might reflect an impaired ability of S1 to interact with polymerized subactin. As mentioned previously, it was indeed observed that although polymerized subactin can be decorated by S1A1, the attached S1 molecules did not attain a clear arrow-head orientation as seen for native F-actin (Figure 5).

We also tested the ability of monomeric subactin to bind S1A1 after its adsorption to DNase I-agarose. As can be seen from Figure 10, S1 was retained by immobilized subactin−DNase I. From this affinity matrix the retained S1 was eluted after washing with Hepes buffer supplemented with 1 mM ATP (Figure 10). S1 has previously been shown not to bind to immobilized DNase I on its own (data not shown). Binding of S1 to polymerized and unpolymerized subactin could also be demonstrated in solution by chemical cross-linking using EDC. Following the reaction by time, the formation of two major cross-linked products of 110 and 170 kDa was observed after 10 min (Figure 11). Using subactin fluorescently labeled by IAEDANS at position Cys 374, the fluorescence was detected only in the 170 kDa cross-linked adduct. When we employed subfragment 1 labeled at SH 1 (residue 701), the fluorescence was detected in the 110 and 170 kDa band, indicative that both products are cross-linked acto−S1 complexes (data not shown).

DISCUSSION

The production of proteolytic actin core fragments of 33−35 kDa has been reported repeatedly in the literature. Jacobson and Rosenbusch (1976) first treated actin with trypsin or α-chymotrypsin and obtained a core fragment of actin that was shown to comprise residues 69−372. Actin treated in this way neither polymerized nor enhanced the myosin ATPase (Jacobson & Rosenbusch, 1976). Analyzing the time dependence of chymotryptic digestion, Konno (1987) demonstrated that a 35 kDa intermediate product of actin was able to polymerize. He therefore concluded that the 23 residues between the first cleavage site at Met 44 and the second cleavage site at Tyr 67 were essential for actin polymerization. Subtilisin treatment selectively cleaves native actin generating a 33 kDa actin core plus a 9 kDa N-terminal peptide. The cleavage site was reported to be between Met 47 and Gly 48 (Schwyter et al., 1989). The subtilisin-digested actin was shown to be able to polymerize, to enhance the myosin ATPase, and to bind to DNase I (Schwyter et al., 1989). Subsequently, it was shown that proteinase K also cleaves actin at this site. However, the proteinase K treated actin cannot be polymerized by mere addition of salt. Only in the additional presence of phalloidin, it was able to polymerize, to stimulate the myosin ATPase, and to exhibit motility in the vitro assay (Higashi-

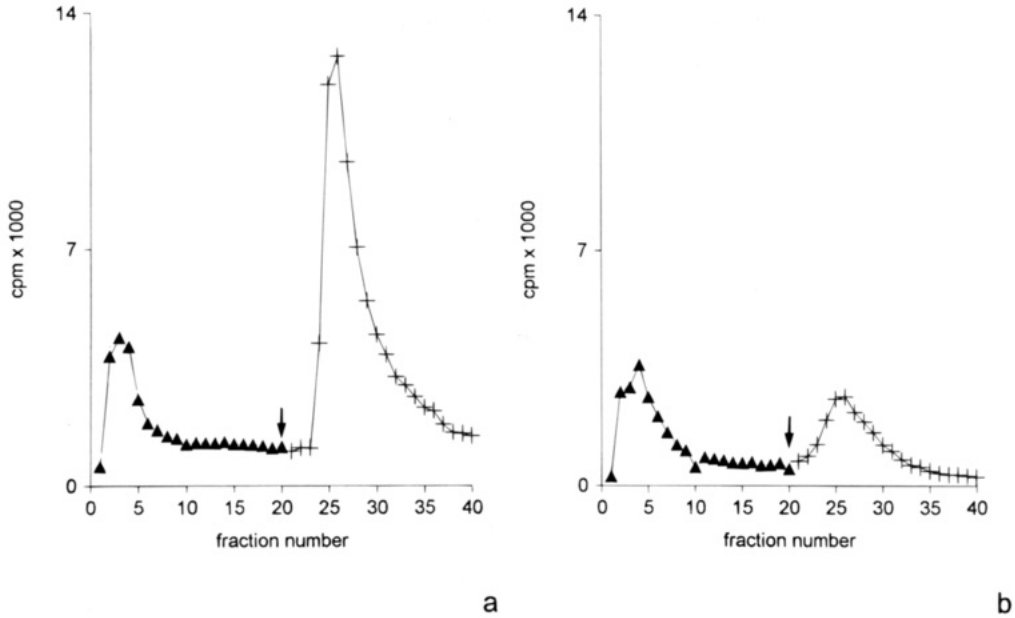

FIGURE 8: Nucleotide release of G-actin and subactin immobilized onto DNase I agarose. Prior to the experiment actin or subactin (1 mg each) were treated with 1/10 (v/v) of Dowex 50 Wx2 to remove unbound nucleotide. Subsequently they were preincubated with 10 μCi of [^{3}H]ATP for 14 h at 4 °C. Excess nucleotide was removed after loading [^{3}H]ATP-actin on a DNase I agarose column followed by washing the column with Hepes buffer (5 mM Hepes-OH, pH 7.4, 0.1 mM $CaCl_2$, 0.5 mM NaN_3, not shown). Then the columns were washed with Hepes buffer supplemented with 5 mM EGTA (▲) and subsequently with Hepes buffer plus 5 mM EDTA (arrow, +). Fractions of 2 mL were collected and analyzed for radioactivity by liquid scintillation counting. (a) G-actin and (b) identical experiment using purified subactin.

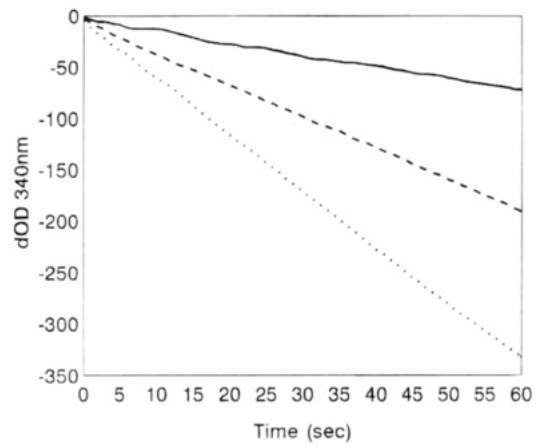

FIGURE 9: Stimulation of the Mg^{2+}-dependent myosin subfragment 1 ATPase by F-actin (dotted line) and phalloidin stabilized subactin filaments (broken line) using the coupled optical assay system as outlined in Materials and Methods. The activity of subfragment 1 on its own is given by a solid line. Subfragment 1 concentration was 20 μg; actin was 100 μg. The rate of decrease of the optical density (dOD) at 340 nm is proportional to the relative S1-ATPase activity.

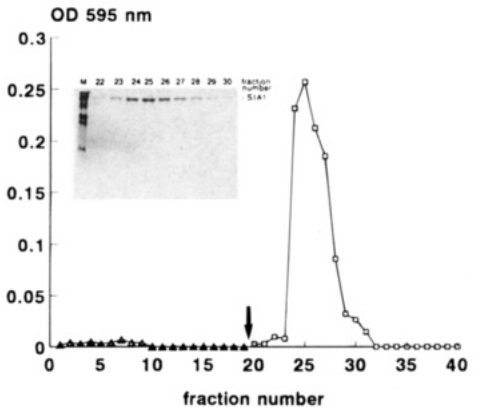

FIGURE 10: Retention of subfragment 1 (S1A1) on immobilized subactin:DNase I complex. After loading S1A1 (1 mg) in 2 mM Tris-HCl, pH 8.0, and 0.1 mM $CaCl_2$ on a Mini-leak column containing immobilized subactin−DNase I complex, the column was washed with 20 mL of the Tris buffer. Subsequently, the column was further eluted with Tris buffer supplemented with 2 mM ATP. The fractions (1 mL) were analyzed for protein by using the Bradford procedure. Arrow indicates start of elution with Tris buffer containing 2 mM ATP. The inset gives the analysis of the protein containing fractions by 12% SDS−PAGE; (M) molecular mass markers as in Figure 7.

Fujime et al., 1992). These authors therefore concluded that unspecific cleavage within the 9 kDa fragment may explain the inability of proteinase K-digested actin to polymerize. An additional explanation was proposed by Kaithlina et al. (1993), who reported that actin cleaved between residues 42 and 43 by *Escherichia coli* A2 protease did not polymerize unless tightly bound Ca^{2+} ions were replaced by Mg^{2+} ions. In these investigations the actin core fragments employed contained a mixture of digested and undigested actin, i.e., no attempt was made to separate the modified from remaining native actin, nor was it realized that the N-terminal peptides contained an additional N-terminal cleavage site. Thus in all previous investigations actin core fragments were used that contained the N-terminal peptides or a mixture of digested and undigested actin.

In this study we present a simple and reproducible method for the purification of the 37 kDa actin core fragment generated by subtilisin cleavage and its attached 4.4 kDa N-terminal peptide devoid of a pentapeptide located in the DNase I-binding loop. In contrast to the "nicked" actin used in previous studies (Schwyter et al., 1989, 1990), we found that polymerization of the purified subactin cannot be induced by mere addition of divalent cations. This actin core fragment polymerized only in the additional presence of phalloidin or myosin subfragment 1. We therefore conclude that the loop comprising residues 43−47 is essential for the ion-induced polymerization of actin. Since this pentapeptide is highly hydrophobic, it is unlikely to represent itself an

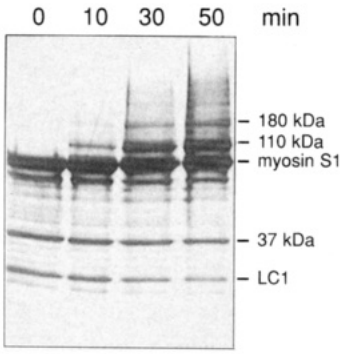

FIGURE 11: Chemical cross-linking of S1 to subactin filaments stabilized by phalloidin. S1 and subactin filaments each at 1 mg/ml were cross-linked by 5 mM EDC in 100 mM Mes, pH 6.0, and 0.2 mM $CaCl_2$. For the time-dependent analysis of the formation of cross-linked products, aliquots were withdrawn at the intervals indicated, mixed with boiling sample buffer, and loaded on a gradient SDS−PAGE gel (10−15% acrylamide).

ion-binding site. We therefore propose that the "DNase I-binding" loop comprising this pentapeptide becomes exposed after salt addition and thus induced or enabled to interact with another actin in the region of the gelsolin segment 1 binding site (Holmes et al., 1990; McLaughlin et al., 1993). This actin−actin interaction appears to be the prerequisite of the ion-induced polymerization reaction, since it completely depends on the correct alignment of the pentapeptide.

Since subactin is still able to interact with both DNase I and S1, it obviously represents native actin. This assumption is supported by the fact that it still retains ATP firmly bound [this report and Kasprzak (1993)]. Like Schwyter et al. (1989), we observed a reduced ability of subactin to stimulate the S1-ATPase or to inhibit DNase I even in the presence of the pentapeptide (data not shown). The major interaction site for DNase I on actin located within amino acid residues 41−50 [DNase I binding loop (Kabsch et al., 1990)] is removed after subtilisin cleavage. The residual presumed "minor" DNase I binding sites of actin are residues Lys 61, Lys 62, and Gly 63 on subdomain 2 and Thr 203 and Ser 204 on subdomain 4 (Kabsch et al., 1990). These sites are apparently sufficient to cause inhibition of DNase I activity.

Tetrahymena actin differs in its N-terminal region completely from mammalian α-actin and has been reported not to bind to DNase I (Hirono et al., 1989). Recently, a chimeric actin construct carrying the N-terminal amino acid residues 1−83 of Tetrahymena actin and the core fragment of Dictyostelium actin was shown to be unable to bind to DNase I (Hirono et al., 1992). Thus the contribution of subdomain 4 to DNase I binding and/or inhibition appears to be very limited. Therefore, by exclusion it follows that the DNase I interacting site on actin present on subactin must be localized on subdomain 2 between residues Lys 61 to Gly 63. This site appears to be sufficient for binding and inhibition of DNase I.

Recently, the crystal structure of the chicken pectoralis myosin subfragment 1 molecule was solved to atomic resolution (Rayment et al., 1993a). The interaction sites of subfragment 1 and actin have been identified from reconstructions of electron microscopical image analyses by Rayment et al. (1993b) and Schröder et al. (1993). In agreement with Milligan et al. (1990) these authors proposed that every subfragment 1 interacts with two actin monomers along the long-pitch actin helix. At least two different interaction modes were recognized: Ionic interactions comprise residues 1−4, 24 and 25, and Glu 99 to Glu 100 of actin and stereospecific interactions involve the hydrophobic residues Ile 341 to Gln 354, Ala 144 to Thr 148, and His 40 to Gly 42 of actin. The ionic interactions are supposed to allow the initial docking of S1 to actin (weak binding).

On the basis of this analysis it follows that all of the S1-interaction sites of actin remain after removal of the pentapeptide, although the region around the deleted pentapeptide might be distorted. Indeed, our data indicate that residues 42−47 of actin are not essential for S1 binding. Like intact G-actin, subactin bound to immobilized DNase I was found to be able to specifically retain S1 in an ATP-dependent fashion. Polymerized subactin was still able to stimulate the Mg^{2+}-dependent S1-ATPase activity, although to a reduced extent. Furthermore, direct interaction was demonstrated by chemical cross-linking. By cross-linking of subactin to S1 two products are generated: a 170 and 110 kDa product. The 170 kDa product most likely represents the 95 kDa S1 heavy chain and the 37 kDa actin core fragment in agreement to Bertrand et al. (1988), Labbé et al. (1990), and Sutoh (1982), who already noticed a lower relative mobility of the actin−S1 heavy chain adduct. In addition, we detected the formation of a 110 kDa product that is not observed when using intact actin. We assume that the 110 kDa band represents the 4.4 kDa N-terminal peptide cross-linked to the S1 heavy chain. A similar cross-linking pattern was found by Konno (1987), who used α-chymotryptic cleaved actin and observed a 105 kDa band that was identified as the adduct of the N-terminal actin peptide and the 95 kDa heavy chain. Cross-linking of S1 to different sites on actin is in accordance with the multitude of interacting sites as revealed by the structural analysis. Indeed, from the reconstruction data it appears probable that the 110 and 170 kDa cross-linked products arose from adjacent actin molecules along the long-pitch helix.

The less well ordered S1-orientation on decorated subactin filaments can be due to alterations of either the actin:actin contacts involving the DNase I binding loop (residues 41−50) or to distortions of a region close to the actin−S1 interface. The recent reconstruction of the actin:myosin interface indicated that the S1 heavy chain also contacts residues His 40 to Gly 42 and Tyr 91 to Glu 100 of the next actin below. Thus the S1 arrow-head orientation might be impaired when part of the contact area between the actin subunits is deleted as in subactin.

The region of the pentapeptide has also been implicated in interactions of actin with a number of other actin binding proteins. Purified subactin may therefore represent a useful tool to investigate their interaction with actin. Furthermore, the purification scheme of subactin might also be applicable to actins cleaved by other proteases.

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