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Antibodies Directed against N-Terminal Residues on Actin Do Not Block Acto-Myosin Binding†

Larry Miller,‡ Michael Kalnoski,§ Zobair Yunossi,‡ Jeannette C. Bulinski,§ and Emil Reisler*,‡

Department of Chemistry and Biochemistry, Department of Biology, and Molecular Biology Institute, University of California, Los Angeles, California 90024

Received December 9, 1986; Revised Manuscript Received March 5, 1987

ABSTRACT: Several studies using a variety of approaches have suggested a possible role for the amino-terminal residues of skeletal muscle actin in acto–myosin interaction. In order to assess the significance of acto–S-1 contacts involving the N-terminal segment of actin, we have prepared polyclonal antisera against a synthetic peptide corresponding to the seven amino-terminal residues of rabbit skeletal muscle actin (α-N-terminal peptide). Affinity-purified immunoglobulin (Ig) G (and Fab) prepared from these antisera reacts strongly and specifically with the amino-terminal segment of both G- and F-actin but not with myosin subfragment 1 (S-1). This specificity was determined by Western blot analysis of actin and its proteolytic fragments and the inhibition of the above reactivity by the α-N-terminal peptide. The α-N-terminal peptide did not interact with S-1 in solution, affect S-1 and actin-activated S-1 MgATPase, or cause dissociation of the acto–S-1 complex. In separate experiments F-actin could be cosedimented with S-1 and affinity-purified IgG or Fab by using an air-driven ultracentrifuge. Densitometric analysis of sodium dodecyl sulfate/polyacrylamide gels of pellet and supernatant fractions from such experiments demonstrated the binding of both S-1 and IgG or Fab to the same F-actin protomer. Our results suggest that, while the acidic N-terminal amino acids of actin may contact the myosin head, these residues cannot be the main determinants of acto–S-1 interaction.

A common goal in electron microscopy and solution studies of acto–subfragment 1 (acto–S-1) is to elucidate, albeit at different resolutions, the structure of the actomyosin interface and the changes in this interface during cyclical interaction of myosin cross-bridges with actin. Low-resolution electron microscopy and image reconstruction work resulted in a three-dimensional model of the acto–S-1 complex (Moore et al., 1970; Wakabayashi & Toyoshima, 1981; Taylor & Amos, 1981; Amos et al., 1982) but could not determine the regions of protein–protein contact between actin and S-1. This goal is now being vigorously pursued in solution studies.

One approach to the investigation of protein–protein contacts along the acto–S-1 interface in solution has been the chemical cross-linking of actin to S-1. Mornet et al. (1981) cross-linked actin to the 50- and 20-kDa tryptic fragments of S-1 using the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). The elegant studies of Sutoh (1982a,b, 1983) showed further that the acidic N-terminal residues of actin (Vandekerckhove & Weber, 1978a–c; Lu & Elzinga, 1977; Elzinga & Lu, 1977) can be cross-linked to either the 20- or 50-kDa fragments and that C-terminal residues of actin interact with LC-1. Consequently, it has been assumed that the N-terminal residues of actin are an important component of the myosin binding site on actin and contribute to the electrostatic attraction between these proteins. However, the assumption that the carbodiimide cross-linking sites can be equated with acto–S-1 binding sites

† This work was supported by U.S. Public Health Service Grants AR 22031 (to E.R.) and NS 19525 (to J.C.B.), National Science Foundation Grant DMB 84-08507 (to E.R.), and USPHS National Research Service Award GM 07185 (to L.M.).

‡ Department of Chemistry and Biochemistry and Molecular Biology Institute.
§ Department of Biology and Molecular Biology Institute.

0006-2960/87/0426-6064$01.50/0

was questioned by Chen et al. (1985a,b) in their detailed analysis of the acto–S-1 cross-linking reaction in both the presence and absence of nucleotides.

Since the mapping of the actomyosin interface is central to the understanding of the mechanism of cross-bridge action, it has become imperative to probe the role of the N-terminal segment of actin in acto–S-1 binding by using independent experimental approaches. To that purpose we have chosen to prepare specific antibodies directed against the seven N-terminal residues on actin. While this work was in progress, similar antibodies against actin were also obtained by Roustan et al. (1986). In their most recent preliminary paper (Mẽjean et al., 1986) these authors reported on ELISA[1] titrations of S-1 binding to actin–IgG complexes and concluded that the seven N-terminal residues on actin do not reside in acto–S-1 contact area. Solution experiments reported in this work provide an additional and a more conclusive test of the role of actin's N-terminal residues in actomyosin interaction.

In the following we describe the results of our work. We have prepared polyclonal antisera against a synthetic peptide corresponding to the seven N-terminal amino acid residues of rabbit skeletal muscle actin. Affinity-purified IgG and Fab fragments prepared from these antisera react strongly and specifically with the N-terminal segment of both G- and F-actin, but not with S-1. Both IgG and Fab could be cosedimented with acto–S-1 by using an air-driven ultracentrifuge. Densitometric analysis of SDS/polyacrylamide gels of pellet and supernatant fractions from these experiments demonstrated the binding of both S-1 and IgG or Fab to the same F-actin promoter. Our results confirm the observation (Mẽjean et al., 1986) that although the N-terminal amino acid residues of actin may contact the myosin head, these residues cannot be the main determinants of acto–S-1 interaction.

Materials and Methods

Reagents. Distilled, millipore-filtered, and deionized water and analytical grade reagents were used in all experiments. Papain, alkaline phosphatase conjugated goat anti-rabbit IgG, cyanogen bromide activated Sepharose, subtilisin Carlsberg (subtilopeptidase A, type VIII), 4-chloro-1-naphthol, alkaline phosphatase substrate, and Freund's adjuvant were from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase conjugated goat anti-rabbit IgG was from Cappel (Cooper Biomedical, Malvern, PA). Iodoacetic acid was from Aldrich Chemical Co. (Milwaukee, WI). The synthetic peptide used for immunization was purchased from the custom peptide synthesis facility at the University of California, San Diego. Specific activity of the ^{14}C-labeled peptide was 35 cpm/nmol. Aquacide II and keyhole limpet hemocyanin (M_r 200 000) were from Calbiochem (La Jolla, CA). Protein A conjugated Sepharose CL-4B was from Pharmacia (Piscataway, NJ).

Preparation of Proteins. Rabbit skeletal muscle myosin was prepared as described by Godfrey and Harrington (1970). Actin was prepared by the procedure of Spudich and Watt (1971). Subfragment 1 (S-1) was prepared by chymotryptic digestion of myosin according to the method of Weeds and Pope (1977).

Immunological Assays. Enzyme-linked immunosorbent assays (ELISA) performed as described by Atherton and Hynes (1981) were used to test titers of antisera. Either 5 μg of skeletal muscle G-actin or 5 μg of α-N-terminal peptide conjugated to bovine serum albumin (BSA) with bis(diazobenzidine) (Bassiri et al., 1979) was used as the coating antigen. The competitive ELISA procedure was that of Raugi et al. (1982). For all ELISAs, optical density at 410 nm was read with a Dynatech MR600 microplate reader. Western blots of SDS gels were performed according to the method of Bulinski et al. (1983). Western blots of isoelectric focusing (IEF) gels were as described by Otey et al. (1986a). Dot blots were performed exactly as Western blots except that antigens (10–15 μg) were applied directly to the nitrocellulose membrane.

Preparation of Peptide Antibodies. The peptide used for immunization (α-N-terminal peptide) had the sequence Ac-Asp-Glu-Asp-Glu-Thr-Thr-[^{14}C]Ala-Tyr. The purity of this peptide was checked with reversed-phase HPLC. The first seven amino acids correspond to N-terminal residues 1–7 of skeletal muscle actin (Vandekerckhove & Weber, 1978a–c; Lu & Elzinga, 1977; Elzinga & Lu, 1977). The tyrosine residue at position 8 was included to facilitate efficient tyrosine–tyrosine coupling to a carrier protein for immunization (Bassiri et al., 1979). A conjugate of the α-N-terminal peptide with keyhole limpet hemocyanin (KLH) using bis(diazobenzidine) as the coupling reagent was prepared as described by Otey et al. (1986b). Coupling efficiencies were 75–85% as determined by specific activity of the peptide. Conjugates were dialyzed exhaustively against water and then against 0.15 M NaCl. Two female New Zealand white rabbits were immunized with the peptide–KLH conjugate as described by Otey et al. (1986b).

Preparation of Affinity-Purified α-N-Terminal Peptide Antibodies. The IgG fraction from serum was prepared by adsorption to protein A–Sepharose CL-4B (Reeves et al., 1981; Kilion & Holtgrewe, 1983) and subsequent elution in 0.2 M glycine, pH 2.3. Fab was prepared by digestion of this IgG (7–10 mg/mL) with papain (1/100 w/w) for 1 h at 37 °C in a solution of 0.1 M NaH_2PO_4, 0.02 M L-cysteine, and 0.002 M EDTA, pH 6.5. Digestion was stopped by addition of 10 mM iodoacetic acid. The digest was dialyzed at 4 °C vs. phosphate-buffered saline (PBS) (0.14 M NaCl, 2 mM KH_2PO_4, 8 mM Na_2HPO_4, and 3 mM KCl, pH 7.4) to which 2 mM iodoacetic acid had been added. Undigested IgG and F_c fragments were removed by adsorption onto protein A–Sepharose CL-4B. Specific Fab directed against the N-terminal part of actin was obtained from the Fab preparation by immunoaffinity chromatography with G-actin covalently coupled to Sepharose. This resin was prepared by incubation of G-actin (10 mg/mL swelled resin) with cyanogen bromide activated Sepharose in 5 mM triethylamine for 2 h at room temperature and then overnight at 4 °C. Unreacted groups on the resin were blocked with 0.2 M glycine, pH 8.6, for 2 h at room temperature. Specific Fab was eluted from the column with 0.2 M glycine, pH 2.3. Column fractions were neutralized immediately with 1 M Tris base. Purified Fab was dialyzed vs. PBS containing 2 mM iodoacetic acid, concentrated with Aquacide II [(carboxymethyl)cellulose], and then redialyzed vs. PBS. Affinity-purified IgG was prepared from the IgG serum fraction by adsorption to G-actin–Sepharose as described for purification of Fab fragments.

Airfuge Binding Studies. G-Actin was polymerized with 2 mM $MgCl_2$ and 0.1 M NaCl for 30 min at room temperature. The F-actin (2.9 μM final) was incubated with Fab at

[1] Abbreviations: BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; S-1, myosin subfragment 1; α-N-terminal peptide, synthetic peptide containing the first seven N-terminal amino acid residues of α-actin from skeletal muscle (Ac-Asp-Glu-Asp-Glu-Thr-Thr-[^{14}C]Ala-Tyr); ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; Ig, immunoglobulin; HPLC, high-performance liquid chromatography; ATPase, adenosinetriphosphatase.

concentrations between 1.4 and 11.7 μM for 35 min at room temperature in PBS. The mixture was centrifuged at 140000g for 12.5 min in an air-driven ultracentrifuge (Beckman Instruments). Pellets were resolubilized with PBS. Experiments measuring binding of Fab to actin in the presence of a 1.2 molar excess of S-1 over actin were conducted as described above except that F-actin was first incubated with S-1 for 30 min at room temperature. Resolubilized pellet and supernatant fractions were denatured and loaded onto 12.5% SDS/polyacrylamide gels (Laemmli, 1970). Coomassie blue R stained bands were scanned with a Biomed Instruments (Fullerton, CA) Model SL-504-XY soft laser scanning densitometer equipped with an integrator and interfaced to an Apple IIe computer. Analysis of these densitometric traces determined the distribution of S-1, actin, and Fab between the pellet and supernatant fractions. The density of the actin band in each pellet fraction was used as an internal standard for variations in gel loading.

Equilibrium Dialysis. Equilibrium dialysis experiments were conducted in an apparatus consisting of two 1.5-mL chambers separated by dialysis membrane with a molecular weight cutoff of 12000–14000. Incubations of S-1 (3.5 mg/mL) and the α-N-terminal peptide at a 10-fold molar excess over S-1 were carried out in the equilibrium dialysis apparatus for 36 h at 4 °C. This was the time required in control experiments for complete equilibration of free peptide across the membrane. Peptide distribution across the membrane was determined by specific activity of the peptide. PBS was used as buffer.

Hydroxylamine Cleavage of Actin. Hydroxylamine cleavage of actin was conducted according to Sutoh (1981). The extent of cleavage was assessed by densitometry of SDS/polyacrylamide gels.

Concentration Determinations. Protein concentrations were determined spectrophotometrically by using the following extinction coefficients: myosin, $E_{280}^{1\%} = 5.55$ cm^{-1}; actin, $E_{280}^{1\%} = 11.0$ cm^{-1}; S-1, $E_{280}^{1\%} = 7.50$ cm^{-1}; IgG, $E_{280}^{1\%} = 15.0$ cm^{-1}; Fab, $E_{280}^{1\%} = 16.0$ cm^{-1}; papain, $E_{280}^{1\%} = 25.0$ cm^{-1}.

Actin-Activated ATPase Measurements. Actin-activated MgATPases were measured at 25 °C by colorimetric assay of liberated phosphate (Reisler, 1980).

Results

Characterization of α-N-Terminal Antibodies. Antisera prepared against N-terminal actin peptide were characterized to determine the specificity of reaction against actin. These antisera consistently showed high reactivity in ELISA against both an α-N-terminal peptide–BSA conjugate and skeletal muscle G-actin. Serum dilutions as high as 1/100000 showed ELISA reactivity when compared to preimmune serum. These sera also showed strong reactivity in dot blot assays in which G- or F-actin (10–15 μg) applied directly to nitrocellulose was used as antigen.

The cross-reactivity of antisera against purified muscle proteins used in this study was tested by Western blot analysis. As shown in Figure 1, affinity-purified IgG reacted strongly against actin electrophoretically transferred onto nitrocellulose from SDS/PAGE (Figure 1, lanes B1 and B3). When myosin and S-1 were transferred from the same gel (Figure 1, lanes C2 and C1), we observed no reaction with the affinity-purified IgG (Figure 1, lanes C3 and C4). Amido black staining of replicate pieces of nitrocellulose verified that both myosin and S-1 transferred and bound to the nitrocellulose membrane (data not shown).

Two types of experiments were performed to determine the specificity of the actin peptide antibodies with the N-terminus

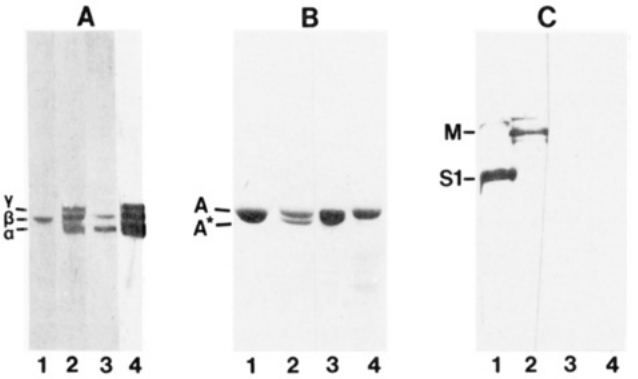

FIGURE 1: Immunoblot of IEF and SDS gels stained with α-N-terminal antibody. Panel A: 20 μg of rabbit aorta extract and 50 μg of rat heart extract were focused and transferred to nitrocellulose. Lanes 1 and 3 were stained with α-N-terminal antibody diluted 1/2000; lanes 2 and 4 were the same blots restained with C4 actin monoclonal diluted 1/1000. The positions of α-, β-, and γ-actin are labeled. Panel B: Duplicate samples were electrophoresed on 10% SDS/polyacrylamide gels stained with Coomassie brilliant blue (lanes 1 and 2) or transferred to nitrocellulose (lanes 3 and 4). Lanes 1 and 3 contain 10 μg of purified skeletal muscle actin; lanes 2 and 4 contain hydroxylamine-treated actin. Lanes 3 and 4 were stained with a 1/2000 dilution of α-N-terminal antibody. A corresponds to actin, and A* corresponds to actin$_{13-375}$. Panel C: Duplicate samples were electrophoresed on 10% SDS/polyacrylamide gels and stained with Coomassie brilliant blue (lanes 1 and 2) or transferred to nitrocellulose (lanes 3 and 4). Lanes 1 and 2 contain 10 μg of S-1; lanes 2 and 4 contain 10 μg of myosin. Lanes 3 and 4 were stained with a 1/2000 dilution of α-N-terminal antibody. M and S-1 refer to myosin and S-1, respectively.

of actin. First, reactivity against actin isoforms with different N-terminal sequences was examined. Western blots of tissue extract proteins separated by isoelectric focusing (Otey et al., 1986a) were reacted with N-terminal antisera and rereacted with monoclonal antibody C4 (Otey et al., 1986b). The C4 antibody shows reactivity against all actin isoforms and thus identifies their positions on the blots. As shown in Figure 1 (lane A1), α-N-terminal antibodies reacted with only one species in a rabbit aorta extract. By rereacting the same blot with C4 antibody (Figure 1, lane A2), we identified this protein as nonmuscle β-actin (N-terminal sequence Ac-Asp-Asp-Asp-Ile). The antibody was not reactive with the smooth muscle α- (Ac-Glu-Glu-Glu-Asp-) actin or the smooth muscle γ- (Ac-Glu-Glu-Glu-Thr-) actin present in the aorta extracts. Similar analysis of rat heart extracts (Figure 1, lanes A3 and A4) showed that α-N-terminal antisera reacted with cardiac α-actin (Ac-Asp-Asp-Glu-Glu) and the nonmuscle β isoform but not with the nonmuscle γ isoform (N-terminal sequence Ac-Glu-Glu-Glu-Ile). N-Terminal antibodies were highly specific for the N-terminus of three out of six actin isoforms, those whose N-terminal amino acid sequences closely resembled that of skeletal muscle actin (Ac-Asp-Glu-Asp-Glu). [Sequence data are from Vandekerckhove and Weber (1978a–c).]

Conclusive proof that the affinity-purified IgG and Fab bound specifically to the N-terminal region of actin was obtained in a second set of experiments in which actin was selectively cleaved with hydroxylamine between Asn-12 and Gly-13 (Sutoh, 1981; Sutoh & Mabuchi, 1986). Both control and hydroxylamine-cleaved actins were electrophoresed on SDS/polyacrylamide gels. Figure 1 (lanes B1 and B2) shows Coomassie blue R stained bands from these gels. As shown by Sutoh (1981) and Sutoh and Mabuchi (1986), the hydroxylamine cleavage produced only two major fragments corresponding to amino acid residues 1–12 and 13–375. The smaller fragment was not withheld by our gel system. The

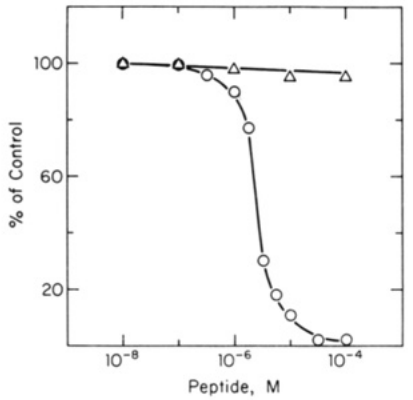

FIGURE 2: ELISA competition between α-N-terminal peptide and G-actin. α-N-Terminal antisera (1/2500 dilution) were reacted with 5 μg of G-actin adsorbed to each ELISA well. Between 10^{-8} and 10^{-4} M α-N-terminal peptide (○) was added along with the antisera. Plotted data represent values of OD_{410nm} for each experimental point divided by the value for serum in the absence of peptide. A heterologous peptide (△) [tyrosinated α-tubulin peptide; Gunderson et al. (1984)] was also used as a control in competition against immobilized actin.

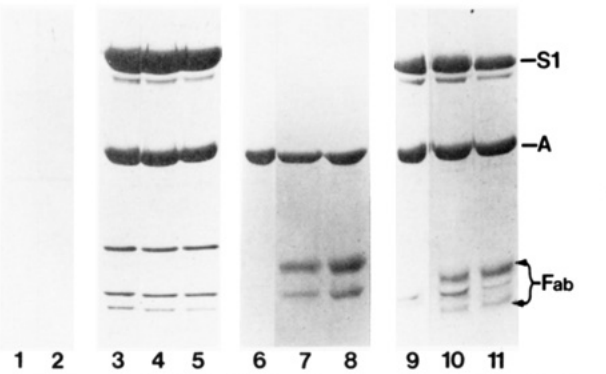

FIGURE 3: Representative electrophoretic patterns from airfuge binding experiments. Incubations, centrifugations, and electrophoresis were as described under Materials and Methods. Lanes 1 and 2: Supernatant fractions of incubations of F-actin (3.3 μM) and S-1 (3.0 μM) and α-N-terminal peptide at 0 (lane 1) and 50 μM (lane 2). Lanes 3–5: Resolubilized pellet fractions from this experiment. α-N-Terminal peptide concentrations were 0 (lane 3), 33 (lane 4), and 50 μM (lane 5). Lanes 6–8: Resolubilized pellet fractions from incubations of F-actin (2.9 μM) with Fab at 0 (lane 6), 7.3 (lane 7), and 11.7 μM (lane 8) concentrations. Lanes 9–11: Incubations of F-actin (2.9 μM) with S-1 (3.5 μM) and Fab at 0 (lane 9), 7.3 (lane 10), and 11.7 μM (lane 11) concentrations.

yield for these cleavages was approximately 40% as judged by SDS/PAGE such as shown in Figure 1 (lane B2). The native and cleaved actins were reacted in Western blots with affinity-purified IgG. As can be seen from Figure 1, the IgG reacted strongly with native actin (lane B3). However, with similar treatment of the cleaved actin sample, the IgG reacted only with the band of lower mobility (lane B4) corresponding to uncleaved actin in the hydroxylamine-treated sample. No reaction was observed with residues 13–375. This procedure verified that the antibody prepared against the first seven N-terminal residues on actin does not recognize any additional epitopes between residues 13 and 375. Thus, the cross-reactivity of this antibody is indeed confined to the N-terminal residues on actin.

Similar results were obtained by inducing a proteolytic cleavage of actin by subtilisin (Mornet & Ue, 1984). By conducting the reaction with subtilisin (Carlsberg, subtilopeptidase A, type VIII) under suitably mild conditions, we obtained a selective cleavage of actin into 9-kDa N-terminal and 35-kDa C-terminal peptides. On Western blots, the α-N-terminal antibody reacted only with control actin and the 9-kDa N-terminal fragment (data not shown).

Further evidence for specificity of α-N-terminal antibodies for the N-terminal segment of actin was obtained from competitive ELISA experiments. In these procedures, 5 μg of G-actin was absorbed to each well of ELISA plates. Antisera were reacted with the immobilized actin in the presence of up to 10^{-4} M free α-N-terminal peptide. As can be seen from Figure 2, the peptide competed with immobilized actin for IgG binding. A heterologous peptide of similar acidic character (sequence Gly-Glu-Glu-Glu-Gly-Glu-Glu-Tyr), corresponding to the C-terminal segment of tyrosinated α-tubulin (Gundersen et al., 1984), had virtually no effect on IgG binding to actin adsorbed to the plate.

Effect of the α-N-Terminal Peptide on S-1. Equilibrium dialysis experiments were carried out to detect possible binding of the α-N-terminal peptide to S-1. No binding could be detected when up to 3.5 mg/mL S-1 and a 10-fold molar excess of peptide over the protein were used. This result implies the absence of any strong S-1–peptide binding but cannot exclude the presence of weak interactions with a binding constant $\leq 4 \times 10^3$ M^{-1}.

In a second set of experiments addressing the possible peptide–S-1 interactions, we tested the effect of N-terminal peptide on acto–S-1 complex formation and stability. F-Actin was incubated with S-1 for 40 min at 25 °C (buffer conditions 40 mM NaCl and 10 mM imidazole, pH 7.0) in the presence of up to a 15-fold molar excess peptide over actin. After incubation, these mixtures were centrifuged in the air-driven ultracentrifuge. Supernatant (Figure 3, lanes 1 and 2) and resolubilized pellet (Figure 3, lanes 3–5) fractions were denatured and run on SDS/polyacrylamide gels. Densitometric analysis of such gels revealed that S-1 bound to and sedimented with the F-actin polymer even in the presence of a 15-fold molar excess of peptide–actin. In other words, S-1 could not be displaced from the acto–S-1 complex by the α-N-terminal peptide.

The more complex schemes of S-1–peptide interaction, assuming dependence of such binding on the presence of nucleotide or actin, were assessed by measurements of ATPase activity. The α-N-terminal peptide did not appear to have any effect on the catalytic properties of S-1. The MgATPase activity of S-1 remained unchanged even in the presence of up to a 100-fold molar excess of the peptide over S-1. Similarly, the presence of the α-N-terminal peptide did not alter the ability of actin to activate S-1 MgATPase in assays containing up to a 20-fold molar excess of peptide over actin. Taken together, the results of ATPase activity and binding measurements exclude the possibility of any strong S-1–peptide or acto–S-1–peptide interaction.

Binding of Affinity-Purified Fab to the Acto–S-1 Complex. Our main goal in the preparation of antibodies directed against the N-terminal portion of actin was to test the involvement of that segment of actin in acto–S-1 binding. This region of actin was implicated in the binding of S-1 through carbodiimide cross-linking of acto–S-1 complexes (Sutoh, 1982a,b, 1983). The rationale for these experiments was that if the N-terminal segment of actin is of primary importance to the formation and stability of the acto–S-1 complex, then antibodies directed against this segment should block or inhibit acto–S-1 binding. To address this issue, we measured the binding of Fab to F-actin and to F-actin in the presence of a 1.2 molar excess of S-1.

In the first set of experiments, affinity-purified Fab was incubated with F-actin and centrifuged, and the supernatant and resolubilized pellet fractions were analyzed by densito-

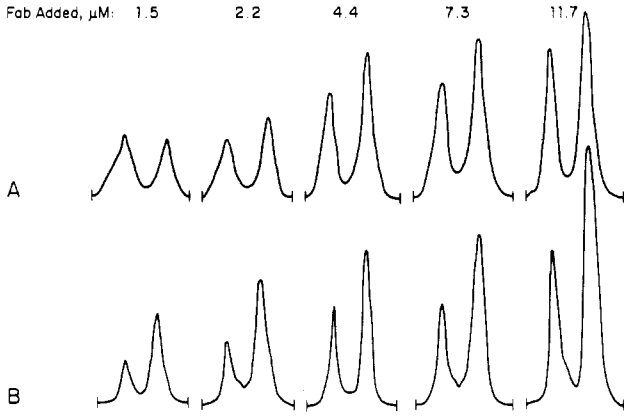

FIGURE 4: Representative densitometric traces of Fab bound to F-actin and acto–S-1. Curves represent traces of densitometric scans of Coomassie blue stained bands from SDS/PAGE (Figure 3). Densities measured were for Fab sedimenting with F-actin (B) or acto–S-1 (A) in airfuge binding studies. Fab runs as a doublet in our gel system.

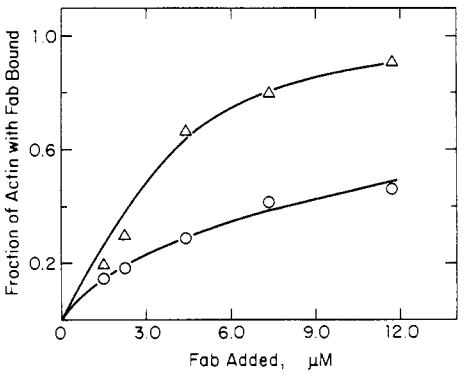

FIGURE 5: Binding of Fab to F-actin (△) and acto–S-1 (○). The fraction of actin with Fab bound was calculated from densitometric analysis of SDS/PAGE as described under Materials and Methods. When present, S-1 was at a 1.2 molar excess over actin. The maximum error margin in these experiments was ±10%.

metry of SDS/polyacrylamide gels as described under Materials and Methods. The Fab bound well to F-actin. Figure 3 (lanes 6–8) shows representative electrophoretic patterns of material sedimented with the F-actin polymer. The amount of Fab bound to actin increased with the increase in Fab concentration (up to a 4-fold molar excess over actin), as evident from the representative densitometric traces presented in Figure 4B. These traces correspond to Fab sedimenting with, and hence bound to, the F-actin polymer (Fab runs as a doublet in our gel system). By comparing the areas under curves such as shown in Figure 4 to those for standard amounts of Fab electrophoresed in the same manner, we determined the amount of Fab bound to actin. Figure 5 shows the binding of Fab to actin as a function of Fab concentration. At Fab concentration equivalent to a 4-fold molar excess over actin, we calculate that 90% of F-actin protomers contain bound Fab (i.e., Fab/actin = 0.90). The binding results shown in Figure 5 could be best fit to a theoretical curve corresponding to a binding constant of Fab to F-actin of 7×10^5 M^{-1}.

Affinity-purified Fab also bound to F-actin in the presence of a 1.2 molar excess of S-1 over actin. In these experiments, S-1 was incubated with F-actin for 30 min at room temperature. The acto–S-1 complexes were subsequently incubated with Fab, centrifuged, and electrophoresed as described under Materials and Methods. Traces of densitometric scans shown in Figure 4A demonstrate that as the concentration of added Fab increased, so did the amount of Fab bound to the sedimentable polymer. Visual examination of representative

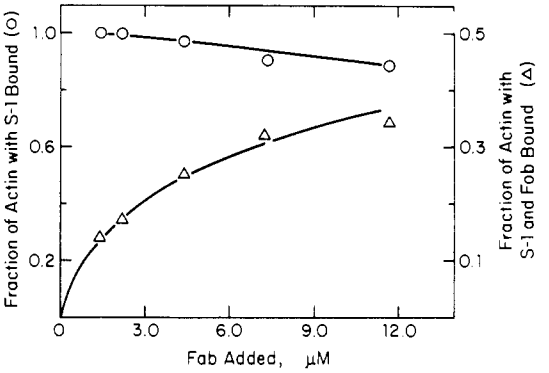

FIGURE 6: Binding of Fab and S-1 to F-actin. The fraction of actin with S-1 (○) and both S-1 and Fab (△) bound was calculated from densitometric analysis of SDS/PAGE as described under Materials and Methods. The maximum error margin in these experiments was ±10%.

electrophoretic patterns from these experiments (Figure 3, lanes 9–11) reveals that both S-1 and Fab sediment with F-actin. Figure 5 shows that at the highest concentration of added Fab (4-fold molar excess over actin) 46% of F-actin protomers contain bound Fab. Fitting of theoretical binding curves to these data yielded a binding constant of Fab to F-actin in the presence of S-1 of 1.2×10^5 M^{-1}.

The above results indicated that the presence of S-1 led to an apparent 5- or 6-fold drop in the binding constant of Fab to F-actin. In order to investigate the nature of this effect and to test for possible displacement of S-1 from acto–S-1 by Fab, we analyzed densitometric scans of S-1 bands from SDS/PAGE similar to those described in Figure 3 (lanes 9–11). This analysis showed that the binding of S-1 to F-actin was only slightly decreased by Fab binding to the N-terminal part of actin: 12% of S-1 was displaced from the acto–S-1 complex at the highest Fab concentration (Figure 6). The binding constant for S-1 and F-actin under these conditions was calculated to be on the order of 10^6 M^{-1}.

Taken together, the analysis of Fab and S-1 binding to F-actin demonstrates the binding of both these proteins to the same F-actin protomers. Figure 6 shows the fraction of actin bound to both S-1 and Fab as a function of added Fab concentration. At the highest Fab concentration tested, 34% of F-actin protomers bound both S-1 and Fab. In some preparations (data not shown), up to 45% of actin promoters had both S-1 and Fab bound to them. The sequential order of S-1 and Fab incubation with F-actin did not affect this value.

Similar experiments carried out with affinity-purified IgG reveal that, in analogy to Fab, both IgG and S-1 can bind to the same F-actin protomer. However, the fraction of actin protomers with both species bound is smaller (25%, at molar ratio IgG/actin = 1.5) than in experiments employing Fab. Addition of IgG to the acto–S-1 polymer led also to greater dissociation of S-1 than that induced by Fab. However, we did not attempt to determine binding constants for the formation of F-actin–IgG and F-acto–S-1–IgG complexes.

Discussion

The goal of this work was to examine the role of the N-terminal segment of actin in actomyosin interaction. The cross-linking of this part of actin to S-1 (Sutoh, 1983), fragmin (Sutoh & Hatano, 1986), depactin (Sutoh & Mabuchi, 1984), and cofilin (Muneyuki et al., 1985) suggested an important function for the N-terminal acidic residues on actin in its binding to these proteins. The assumption implicit in this suggestion, which we have tested by an immunochemical approach for the case of acto–myosin, is that the cross-linked

sites constitute an important component of the binding interface.

We have shown in this paper that antibodies to N-terminal residues 1–7 of actin will bind specifically to both monomeric and polymeric actin. This binding confirms, as predicted by EDC cross-linking studies, that the N-terminal residues are accessible on the surface of the actin filament. We are currently conducting experiments to determine the effect of Fab binding to the N-terminal part of actin on the polymerization of this protein.

We have further shown that IgG or Fab directed against the N-terminus will bind to F-actin in the presence of saturating amounts of S-1 and that this binding does not prevent the attachment of S-1 to the same F-actin protomers. It is clear, however, that binding of antibody does cause some dissociation of the acto–S-1 complex. This dissociation was particularly evident in experiments using affinity-purified IgG, which may cross-link adjacent F-actin molecules and cause further steric constraints. Therefore, the dissociating effect of N-terminal antibodies most likely arises from steric effects and partial overlap between the Fab and the main S-1 binding sites on actin. Alternatively, the dissociation may originate from direct, albeit weak, competition between S-1 and Fab for the N-terminal segment of actin. Our calculated binding constant of 10^6 M^{-1} for S-1 bound to actin in the presence of Fab is very similar to the reported range of values for acto–S-1 interaction under similar ionic strength conditions (Greene, 1980). Obviously, then, Fab does not greatly weaken, if at all, the binding of S-1 to actin. In this context, it is hardly surprising that we did not detect any significant interaction between the α-N-terminal peptide and S-1 in the presence and absence of actin and ATP.

Our results confirm the work of Mèjean et al. (1986). By immunization with trinitrophenylated actin, these investigators were able to produce two populations of antibodies. The epitope for the first antibody population was mapped to the N-terminal residues 1–7 of actin and for the second to residues 18–28. Using ELISA tests, Mèjean et al. (1986) found that the IgG recognizing residues 1–7 could still bind to acto–S-1 on the plate. Given the inherent shortcomings of ELISA experiments, as discussed below, their agreement with our solution work is gratifying.

Other experiments suggest that additional sites on actin cannot assume major myosin binding function. For example, it is unlikely that the extreme C-terminal residues of actin are essential to acto–S-1 interaction. This conclusion stems from the fact that chemical modifications of Cys-374 on actin have little effect on acto–S-1 binding (Criddle et al., 1985). Similarly, modification of Lys-61 with fluorescein isothiocyanate, which greatly inhibits actin polymerization (Burtnick, 1984), does not prevent its interaction with S-1 (Miller & Reisler, 1986). Positive identification of the binding interface is inherently more difficult. It has been attempted in studies employing peptides and renatured fragments of actin, although this approach suffers from the lack of convincing evidence for proper folding of the fragments. The immunoassay results of Mèjean et al. (1986) showing the inhibition of acto–S-1 binding by IgG directed against residues 18–28 on actin suggest the involvement of this region in acto–S-1 binding. However, given the size of IgG, the inhibition of binding may arise from steric exclusion of S-1 from a different site on actin. Also, in contrast to the solution approach taken in this work, some shortcomings are inherent in solid-phase ELISA procedures. For example, it is difficult to ascertain the three-dimensional orientation of epitopes or protein binding sites with respect to the solid surface. In our system, for example, certain orientations may hinder or block the interaction of solution S-1 or antibody with the immobilized actin. Possible changes in protein conformation brought about by binding of actin to the ELISA plate may also affect S-1 and antibody interaction. In addition, ELISA incubations carried out at 37 °C will alter S-1 ATPase properties (Setton & Muhlrad, 1984) and possibly interaction with actin.

In contrast to the above, the interpretation of solution studies presented in this work is less ambiguous. Formation of ternary complexes between actin, S-1, and Fab directed against the N-terminal portion of actin demonstrates that the N-terminal acidic residues on actin do not contribute significantly to acto–S-1 binding interaction. More generally, the approach taken in this work may prove useful in studying the interaction of the N-terminal segment of actin with other actin binding proteins.

ACKNOWLEDGMENTS

We thank Janet White for skillful assistance in experiments with the hydroxylamine-cleaved actin and James Lessard for the gift of C4 antibody.

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A Synthetic 33-Residue Analogue of Bovine Brain Calmodulin Calcium Binding Site III: Synthesis, Purification, and Calcium Binding†

Ronald E. Reid
Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2
Received January 20, 1987; Revised Manuscript Received May 13, 1987

ABSTRACT: The sequential solid-phase synthesis of a peptide analogue of bovine brain calmodulin calcium binding site III covering residues 81–113 of the natural sequence is described. Methionine-109 is replaced by a leucine residue to avoid complications in the synthesis and purification. In an attempt to relate the structure of the calcium binding sites in the naturally occurring calcium binding protein to the calcium affinity of these sites, the synthetic analogue is examined for calcium binding by circular dichroism spectroscopy. The calcium binding characteristics are compared to those of a synthetic analogue of the homologous calcium binding site III in rabbit skeletal troponin C. The K_d of the calmodulin site III fragment for Ca^{2+} is determined as 878 μM whereas the K_d of the troponin C fragment is 30 times smaller at 28 μM. Structural changes induced in the peptides by Ca^{2+} and trifluoroethanol are similar. This study supports our contention that the single synthetic calcium binding site is a reasonable model for the study of the structure–activity relationships of the calcium binding sites in calcium-regulated proteins such as calmodulin and troponin C.

The function of calmodulin (CaM)[1] at the molecular level has proven to be an active area of inquiry [for a recent review, see Cox et al. (1984)]. A description of the mechanism by which calmodulin can regulate several different enzyme systems, some of which are mutually antagonistic, in response to changes in intracellular calcium levels, is a major barrier to be overcome. Current thought on the subject suggests that calcium binds to the four sites on CaM with differing affinities resulting in a stepwise change in structure dependent on calcium concentration. Different enzymes recognize different conformations of CaM and are thereby differentially regulated depending on the calcium concentration of the medium (Klee et al., 1986). Since difficulties can arise in the interpretation of experimental results from studies on the natural protein, which binds 4 mol of calcium/mol of protein, several authors have attempted to simplify the experimental conditions by reducing the number of calcium binding sites through the use of fragments of the natural protein obtained by controlled enzymic cleavage (Newton et al., 1984; Ni & Klee, 1985; Thulin et al., 1984; Malencik & Anderson, 1984; Dalgarno et al., 1984; Krebs et al., 1984; Guerini et al., 1984; Ikura et al., 1984; Minowa & Yagi, 1984; Aulabaugh et al., 1984; Wall et al., 1981; Kuznicki et al., 1981; Head et al., 1982; Brzeska et al., 1983; Vogel et al., 1983). Although several sound hypothetical arguments can be put forward against extrapolating from fragment studies on a protein to the biological function of the intact protein, many areas of research such as synthetic antigens (Atassi, 1984) and hormone analogues (Gysin & Schwyzer, 1984) have shown that such studies contribute substantially to an understanding of the biological

† Funded by a grant from the Medical Research Council of Canada.

[1] Abbreviations: CaM, calmodulin; TnC, troponin C; DIEA, diisopropylethylamine; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenztriazole; Tris, tris(hydroxymethyl)-aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; IE-HPLC, ion-exchange high-pressure liquid chromatography; RP-HPLC, reversed-phase high-pressure liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/87/0426-6070$01.50/0