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Antibody Directed against the 142-148 Sequence of the Myosin Heavy Chain Interferes with Myosin-Actin Interaction†

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ABSTRACT: It has been reported recently that the isolated and renatured 23-kDa N-terminal fragment of rabbit skeletal muscle myosin binds tightly to F-actin in an ATP-dependent manner [Muhlrad, A. (1989) *Biochemistry 28*, 4002-4010]. The binding to actin is of electrostatic nature and may involve a positively charged cluster of residues on the 23-kDa fragment stretching from Arg-143 to Arg-147. An octapeptide containing this positive cluster was synthesized and coupled to BSA through a cysteine residue added to the N-terminus of the peptide. Polyclonal antibody was raised against the BSA-coupled peptide in rabbits which recognized the N-terminal 23-kDa fragment of rabbit skeletal myosin subfragment 1, and a peptide comprised of residues 122-204 of the 23K fragment in Western blots. The purified antibody [IgG and F(ab)] inhibited the actin-activated ATPase activity of S1 without affecting its Mg^{2+}- and K^+(EDTA)-modulated ATPase activity. Both IgG and F(ab) decreased the binding of S1 to F-actin in a sedimentation assay, and actin inhibited the binding of both IgG and F(ab) to S1 in a competitive binding assay. The cysteine thiol of the synthetic octapeptide was labeled by the fluorescent thiol reagent monobromobimane, and the labeled peptide was found to bind to actin in a sedimentation assay. The results support the possibility that the positively charged Arg-143 to Arg-147 stretch of residues on the 23-kDa fragment participates in actin binding of myosin and may represent an essential constituent of the actin-S1 interface.

The interaction of myosin with actin and ATP is the basis of the molecular mechanism of muscle contraction and of many events involved in cell motility. The head segment of myosin, called subfragment 1 (S1),[1] contains two distinct sites responsible for actin and ATP binding. In order to understand the interaction between myosin, actin, and ATP, it is essential to characterize the two binding sites. The cleavage of S1 by trypsin into three major protease-resistant fragments—23, 50, and 20 kDa aligned in this order (Balint et al., 1978)—which are considered to be domains (Mornet et al., 1981), helps to

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SMPB, sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; MBB, monobromobimane; NTCB, 2-nitro-5-thiocyanobenzoic acid; LC, light chain; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; S1, myosin subfragment 1.

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locate the binding sites on the myosin head segment.

Various methods, including covalent cross-linking with photoaffinity analogues of ATP, electron microscopic imaging, and fluorescent energy-transfer measurements, have been used to locate the ATP binding site on S1. ATP analogues containing a photosensitive group on the adenine moiety were found to bind to the 23-kDa fragment (Szilagyi et al., 1979; Okamoto & Yount, 1985), while those containing a photosensitive group on the ribose moiety of ATP were found to attach to the 50-kDa fragment (Mahmood & Yount, 1984). By using an electron microscopic imaging technique, it was possible to locate the ATP binding site in the three-dimensional structure of myosin. The binding site (visualized with the help of a biotinylated ATP–avidin system) was 14 nm from the head–rod junction of myosin (Sutoh et al., 1986) and 4 nm from the actin binding site (Tokunaga et al., 1987). More recently, the location of the ATP binding site in the 3-D structure relative to other myosin functionalities has been accurately determined by fluorescent energy-transfer measurements (Botts et al., 1989). The comparison of homologue sequences of various ATP binding proteins also helps in assigning the ATP binding site of myosin. According to Walker et al. (1982) and Fry et al. (1986), all ATP binding proteins contain a highly homologous glycine-rich "consensus" ATP binding sequence. This glycine-rich sequence is located on the 23-kDa fragment (residues 178–185) (Tong & Elzinga, 1983). Both the results of comparative studies and of affinity labeling indicate that the 23-kDa fragment participates in ATP binding.

The first indication about the location of the actin binding site on S1 came from a limited proteolysis assay which showed that actin protects the "connector" region between the 50- and 20-kDa fragments of S1 from tryptic cleavage (Mornet et al., 1979). More recent studies using an "antipeptide" have supplied direct evidence about the participation of this "connector" region in actin binding (Chaussepied & Morales, 1988). Covalent cross-linking of S1 to actin showed that the N-terminal region of the 20-kDa fragment and a region on the 50-kDa fragment located 45 kDa from its N-Terminus are involved in forming the actin binding site of S1 (Mornet et al., 1981; Sutoh, 1983). Other studies using synthetic peptides corresponding to sequences around the SH_1 and SH_2 thiols (Suzuki et al., 1987; Keane et al., 1990) indicate that this region also participates in forming the actin binding site of S1.

Since the study of the isolated trypsin-resistant fragments of S1 can also contribute to the description of the actin binding site, we isolated and renatured the three tryptic heavy-chain fragments and found that all of them bind to actin [20 kDa (Muhlrad & Morales, 1984); 50 kDa (Muhlrad et al., 1986); 23 kDa (Muhlrad, 1989)]. It has been found that a shorter, recently isolated 21-kDa N-terminal fragment (containing residues 1–180) also binds to actin (Muhlrad, 1990). Our findings that the renatured N-terminal 23- and 21-kDa fragment binds tightly to actin were rather unexpected, since former cross-linking studies did not indicate the participation of the N-terminal region in forming the actin binding site. This unexpected result left us with two possibilities: (1) the binding site on the N-terminal domain is a part of the actin binding site of S1, and it is operational during the cross-bridge cycle; (2) the binding site on the N-terminal domain is buried in intact S1 by the other two domains, and, therefore, it reacts with actin only when the 23-kDa fragment is isolated from the rest of the S1 molecule. An approach to discriminating between these possibilities is to raise an antibody against the assumed binding site on the N-terminal domain and then study the effect of this antibody on the actin–S1 interaction. However, to do that, one has to guess at the binding site on the domain. The extreme sensitivity of actin binding of the isolated 23-kDa fragment to ionic strength and to various polyanions led us to conclude that the binding is based on electrostatic interactions between the N-terminal fragments and actin (Muhlrad, 1989, 1990). Since it is known that the negatively charged N-terminal region of actin interacts with S1 (Sutoh, 1982), we looked for a positively charged cluster on the N-terminal region is a putative binding site. The only positively charged stretch on the 23-kDa fragment is the 143–147 Arg-Gly-Lys-Lys-Arg sequence (Tong & Elzinga, 1983) which contains four positively charged residues. We have synthesized this peptide, demonstrated its binding to actin, and used it as antigen to raise polyclonal antibody. The obtained antibody reacts specifically with the region containing the 143–147 sequence in the 23-kDa fragment. Furthermore, this antibody inhibits the actin–S1 interaction, thus supporting a role for this positively charged sequence of the 23-kDa fragment in forming the actin bindig site of myosin. A preliminary report of this work has been published (Dan-Goor & Muhlrad, 1990).

Materials and Methods

Chemicals. ATP, PMSF, chymotrypsin, TPCK–trypsin, iodoacetic acid, NTCB, and protein A conjugated Sepharose CL-4B were from Sigma Chemical Co. Anti-rabbit IgG phosphatase conjugate was from KPL Laboratories. Nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, 4-chloro-1-naphthol alkaline phosphatase substrate, gelatin, and electrophoresis reagents were from Bio-Rad. Sephadex G-25 was from Pharmàcia. All other chemicals were of reagent grade.

Preparation of Proteins. Myosin and actin were prepared from back and leg muscle of rabbit (Tonomura et al., 1966; Spudich & Watt, 1971, respectively). S1 was prepared by digesting myosin with chymotrypsin, 1:100 (w/w) ratio to S1, at 25 °C for 10 min (Weeds & Taylor, 1975). The digestion was stopped by 1 mM PMSF. Protein concentrations were obtained by absorbance, using an $A(1\%)$ at 280 nm of 5.5, 7.5, 11.8, and 13.5 for myosin, S1, F(ab), and IgG and an $A(1\%)$ at 290 nm for actin of 6.3, respectively. Molecular masses were assumed to be 500, 115, 42, 60, and 180 kDa for myosin, S1, actin, F(ab), and IgG respectively.

Immunoblot. Sample buffer (100 mM Tris-HCl, pH 6.5, 5% 2-mercaptoethanol, 5% SDS, 50% glycerol, and 0.02% bromophenol blue) was mixed with the protein sample in a 1:3 ratio. The samples were loaded on a 7–18% SDS–polyacrylamide gradient slab gel and were electrophoresed. The peptide bands were electrophoretically transferred from the gel to a nitrocellulose membrane in Tris–glycine buffer and 20% methanol, pH 8.4. The nitrocellulose was cut into three parts, following the transfer: one part was stained by amido black for the peptide bands, and the other two parts were immunostained by the anti-peptide antibody and by a monoclonal antibody reacting with the N-terminus of the S1 heavy chain (Dan-Goor et al., 1990). The second antibodies were anti-rabbit and anti-mouse IgG–alkaline phosphatase conjugates for the anti-peptide and anti-N-terminus antibodies, respectively. The reacting bands were visualized by nitroblue tetrazolium and 5-bromo-4 chloro-3-indolyl phosphate.

Preparation of the Octapeptide. The peptide was synthesized by Dr. O. Goldberg in the Weizmann Institute of Science, Rehovot, Israel. The sequence of the synthetic octapeptide was NH_2-Cys-Tyr-Arg-Gly-Lys-Lys-Arg-Gln, which corresponds to the 142–148 sequence of the 23-kDa tryptic fragment of S1 (Tong & Elzinga, 1983).

Raising the Antibody against the Octapeptide. The octapeptide was coupled to BSA with SMBP according to Sutoh (1987). Ten milligrams of BSA in 0.1 M phosphate buffer was modified by 3 mg of SMPB in dimethylformamide for 30 min at room temperature. The solution was filtered through a Sephadex G-25 column to remove the free sulfo-SMPB. The protein was eluted by 0.15 M NaCl/50 mM phosphate, pH 7.0. Fractions were read at 280 nm, and those containing the modified BSA were mixed with 10 mg of the synthetic octapeptide for 3 h. The coupled peptides were loaded on SDS–PAGE to verify the coupling. Rabbits were immunized subcutaneously at multiple sites with 2 mg of peptide–BSA conjugate emulsified with an equal volume of complete Freund's adjuvant (final volume 2 mL). A booster injection was performed 6 weeks later with 0.4 mg of conjugate in incomplete Freund's adjuvant (final volume 1 mL). After 10 days, blood was collected, and the serum was stored at −20 °C.

IgG Preparation. The IgG fraction was collected from the antiserum by ammonium sulfate precipitation. The anti-peptide antibody was further purified by affinity chromatography on an immobilized S1 column, which was prepared by coupling S1 to CNBR–Sepharose 4B. IgG (6 mL) was loaded on a Sepharose 4B-S1 column and then washed with 10 mL of PBS. The elution of the antibody was performed with 50 mM boric acid/150 mM NaCl, pH 1.9, 2-mL fractions were collected into vials which contained 50 μL of 2 M Tris, pH 9.0.

Preparation of the F(ab) Fragment. F(ab) fragment was prepared by digesting IgG with papain according to Miller et al. (1987). The undigested IgG and Fc fragment were removed from the preparation by adsorption onto protein A–Sepharose CL-4B. For the ATPase measurements, the phosphate was removed by dialyzing the sample against 10 mM imidazole, pH 7.0, for 2 days at 4 °C.

Immunological Assays. Dynathech microtiter plates were coated with 100 μL of 20 μg/mL S1 in PBS, overnight at 4 °C, blocked with 1% gelatin at 37 °C for 2 h, and incubated with 100 μL of anti-peptide antibody overnight at 4 °C. Antibody binding was detected by the binding of anti-rabbit IgG–alkaline phosphatase conjugate and visualized with *p*-nitrophenyl phosphate substrate in diethanolamine buffer, pH 8.4. In competitive ELISA assays, 15 μg of F-actin was also present during the incubation with the anti-peptide antibody.

SDS–PAGE. The SDS gel electrophoresis was carried out according to Mornet (1981). It was performed on a 7–18% polyacrylamide gradient slab gel. Molecular masses of the bands were estimated by comparing the electrophoretic mobility of the bands with that of authentic markers.

Binding of S1 to Actin in the Sedimentation Assay. Different concentrations of the anti-peptide antibody, in the 0–10 μM range, were added to 3 μM S1 and 10 μM F-actin and sedimented at 180000*g* for 30 min at 20 °C. S1 control without F-actin was also run. The final volume of the sample was 300 μL in 10 mM sodium phosphate/60 mM NaCl, pH 7.0. A sample was taken from the supernatant, and it was mixed with protein sample buffer in a 1:3 ratio and loaded on a 7–18% gradient slab gel. After being stained with Coomassie blue, the band intensities were quantified by extracting the band slices with 1 mL of 20% (v/v) pyridine at room temperature fo 48 h, and the S1 concentration was taken to be proportional to the absorbance of the eluted dye to 605 nm.

ATPase Assay. Actin-, Mg^{2+}-, and K^+(EDTA)-activated ATPase activities (micromoles of per milligram of S1 per minute) were calculated from the inorganic phosphate (P_i) produced. The assay was performed as described previously (Muhlrad & Morales, 1984). S1 was preincubated with the anti-peptide antibody at 0 °C for 3 h before the assay. The reaction was performed at 25 °C on 1-mL aliquots taken at various time intervals. Incubation time was chosen so that not more than 15% of the ATP was hydrolyzed. The assay contained 0.1 μM S1, 0.5 μM F-actin, 2 mM $MgCl_2$, 1 mM ATP, and 20 mM imidazole, pH 7.0, for the actin-activated ATPase, and no actin for the Mg^{2+}-modulated ATPase. For the K^+(EDTA)-activated ATPase, 0.2 μM S1, 5 mM EDTA, 600 mM KCl, and 50 mM Tris-HCl, pH 8.0, were used.

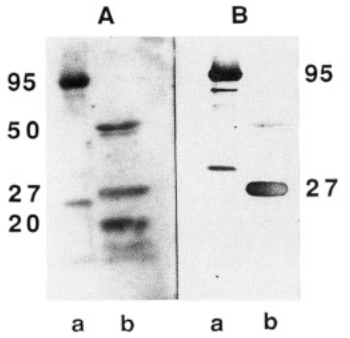

FIGURE 1: Reaction of the anti-peptide antibody with S1 and trypsin-digested S1 in Western blot. S1 and trypsin-digested S1 were electrophoresed on an SDS–PAGE 7–18% gradient gel and then transferred into a nitrocellulose membrane. The blot was stained with amido black to visualize all peptides (A). Blot reacted with the anti-peptide antibody (B). (Lanes a) S1; (lanes b) trypsin-digested S1. Vertical numbers are the apparent molecular mass, based on electrophoretic mobility, in kilodaltons. 23 kDa has an anomalous electroporetic mobility; it runs as 27 kDa. Therefore, the 27-kDa band in all Western blots corresponds to the 23-kDa fragment.

Preparation and Chemical Cleavage of the 23-kDa Fragment. Preparation of the 23-kDa fragment was carried out according to Muhlrad (1989). Chemical cleavage was performed by NTCB, which cleaves peptide bonds specifically at cysteine residues. The 23-kDa fragment, which contains a single cysteine residue, was cleaved by NTCB at Cys-112 by the method of Peyser et al. (1989).

Labeling of the Octapeptide with Monobromobimane. The N-terminal cysteine residue of the peptide was modified with the fluorescent thiol reagent monobromobimane by the following procedure: To 10 mM octapeptide in 50 mM $NaHCO_3$ was added monobromobimane (dissolved in acetonitrile) in 13.6 mM final concentration. The reaction mixture was incubated in the dark at room temperature for 1 h. Finally, the reaction was quenched by a 100-fold excess of 2-mercaptoethanol over monobromobimane, and the product was lyophilized. The labeled peptide was dissolved in 0.1% trifluoroacetic acid and further purified by HPLC on a Hi-pore RP318 reverse-phase column (Bio-Rad) with a gradient from 0 to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. The concentration of MBB–octapeptide was obtained by using a 1:1 stoichiometry and an $E_{390} = 5.3 \times 10^3\ M^{-1}\ cm^{-1}$ extinction coefficient.

RESULTS

Localization of the Epitope of the Anti-Peptide Antibody in the Primary Structure. The reaction of S1 and its tryptic fragments (23, 50, and 20 kDa) with the antibody was analyzed by immunoblot, following SDS–PAGE (Figure 1). The anti-peptide antibody reacted with the S1 heavy chain (95 kDa) and with the 23-kDa fragment, which contains the sequence of the synthetic octapeptide. The other two fragments (50 and 20 kDa) of the S1 heavy chain and the LC_1 and LC_3

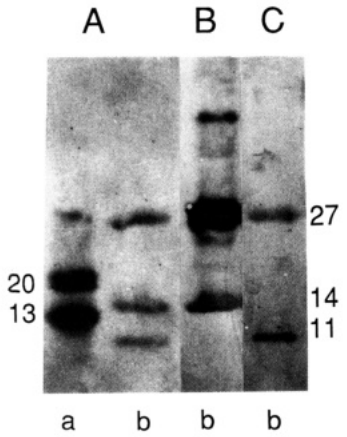

FIGURE 2: Western blot of NTCB-treated 23-kDa fragment. Nitrocellulose membrane stained by amido black (A); immunostained by "anti N-terminus" (B) and by "anti-peptide" antibody (C). (Lane a) Molecular mass markers (cytochrome *c*, 13 kDa; soybean trypsin inhibitor, 20 kDa); (b) NTCB-treated 23-kDa fragment. Vertical numbers are the apparent molecular mass in kilodaltons.

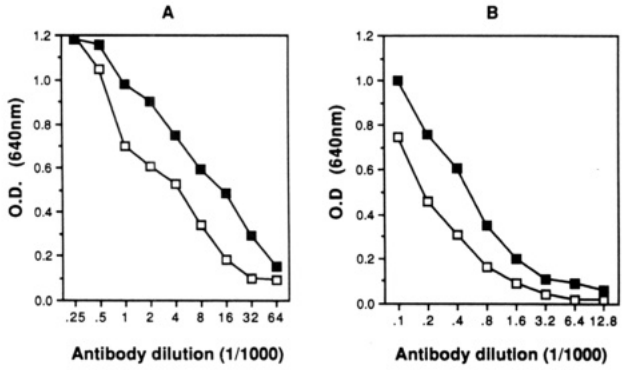

FIGURE 3: Effect of actin on the binding of the anti-peptide antibody to S1. Antibody binding was measured by a competitive ELISA assay (see details under Materials and Methods). Binding of the purified IgG (A) and its F(ab) fragment (B). Assay in the presence (□) and in the absence of (■) F-actin.

light chains did not react with the antibody. Isolated 23-kDa fragment was treated by NTCB which cleaves peptide bonds at cysteinyl residues; 23 kDa contains only one cysteine, Cys-122, and the NTCB treatment giving rise to two peptides: one contains residues 1–121 (14-kDa molecular mass) including the N-terminus, and the other stretches from 122 to 204 (11-kDa molecular mass) containing the C-terminus (Figure 2). The anti-octapeptide antibody recognizes only the 11-kDa peptide (Figure 2C), which contains the Arg-143–Arg-147 stretch, while the anti-N-terminus antibody reacts only with the 14-kDa peptide, which contains the N-terminus.

Accessibility of S1 Epitopes to the Antibody in the Acto–S1 Complex. The reaction of the anti-peptide antibody and its F(ab) fragment with S1 and the effect of complex formation between actin and S1 on the reaction were studied by a competitive ELISA assay. Microtiter plates were coated with S1, and the binding of purified IgG and the F(ab) fragment was measured both in the presence and in the absence of F-actin (Figure 3). We found decreased accessibility of the epitope on S1 both to the whole antibody and to its F(ab) fragment in the presence of actin, which was expressed as a leftward shift of the antibody binding curve.

Effect of Antibody on the Binding of S1 to F-Actin in the Sedimentation Assay. Purified IgG or F(ab) was added to a solution containing 5 μM F-actin and 3 μM S1, and the reaction mixture was centrifuged at 180000*g* for 30 min.

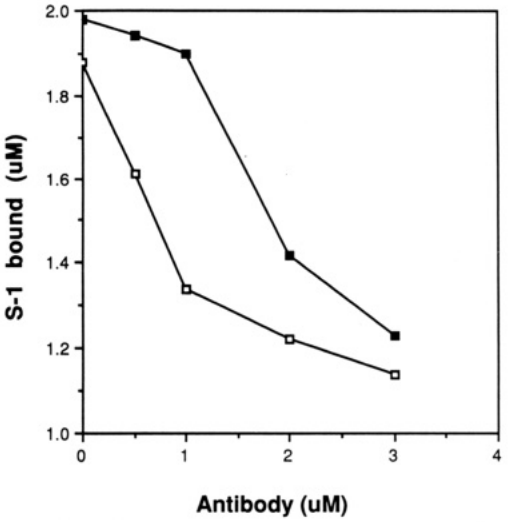

FIGURE 4: Effect of the anti-peptide antibody on the binding of S1 to F-actin in sedimentation assay. Purified IgG (■) or its F(ab) (□) fragment was added to a mixture of F-actin and S1, and then it was centrifuged at 180000*g* for 30 min. S1 was measured in the supernatant by extracting the Coomassie blue stained bands of SDS–PAGE (see the details of the procedure under Materials and Methods).

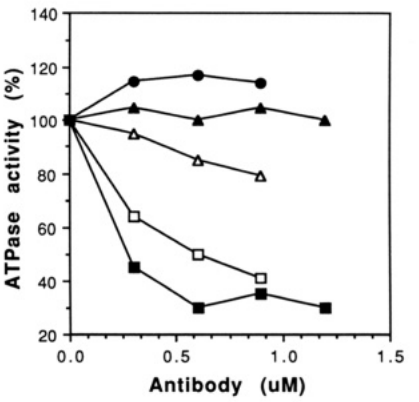

FIGURE 5: Effect of the anti-peptide antibody on the ATPase activity of S1. S1 was incubated with the antibody for 3 h in the cold, and then the ATPase activity was measured at 25 °C (for details, see Materials and Methods). K^+(EDTA)-activated ATPase, triangles; Mg^{2+}-modulated ATPase, circles; actin-activated ATPase, squares. Activity in the presence of IgG and F(ab) is closed and open symbols, respectively.

F-Actin and the acto–S1 complex were pelleted by centrifugation. Free S1 which remained in the supernatant was estimated by extracting the stained band slices of SDS–PAGE. The actin-bound S1, which was estimated by subtracting free S1 from the total S1 added, was presented versus antibody or F(ab) concentrations (Figure 4). The results show that the actin-bound fraction of S1 significantly decreases with increasing concentration of antibody or F(ab) fraction, indicating that the antibody inhibits the formation of the acto–S1 complex.

Effect of the Antibody on the ATPase Activity of S1. The effect of the anti-peptide antibody and its F(ab) fragment on the Mg^{2+}-, actin-, and K^+(EDTA)-activated ATPase activity of S1 was also studied (Figure 5). Our antibody strongly inhibits the actin-activated ATPase activity (maximal inhibition 70%) with little or no effect on the Mg^{2+}- and the K^+(EDTA)-ATPase activity of S1. Similarly, the F(ab) fragment was found to strongly inhibit the actin-activated ATPase activity of S1 (up to 65%), and in this case, a slight (maximally 23%) inhibition of the K^+(EDTA)-activated S1 ATPase was also observed.

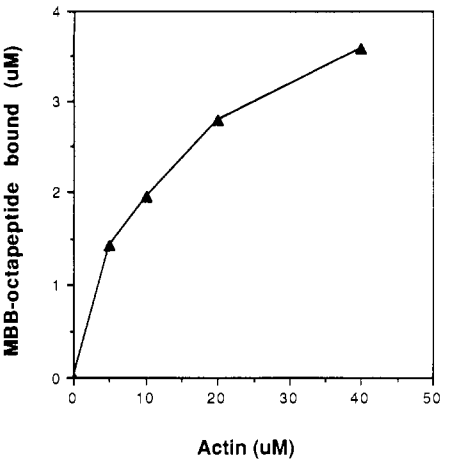

FIGURE 6: Binding of the synthetic octapeptide to F-actin in sedimentation assay. To 5 μM monobromobimane-labeled octapeptide were added increasing concentrations of F-actin and centrifuged at 180000*g* for 30 min. Octapeptide content in the supernatant was measured by fluorometry, and the actin-bound fraction was estimated as described in the text.

Binding of Octapeptide to F-Actin. Monobromobimane-labeled octapeptide was added in constant concentration (5 μM) to increasing concentrations of F-actin in 1 mM $MgCl_2$ and 10 mM imidazole, pH 7.5. The mixture was centrifuged at 180000*g* for 45 min at 20 °C, and the MBB–octapeptide concentration in the supernatant was measured by fluorometry. Control MBB–octapeptide in the absence of actin was also run. The amount of MBB–octapeptide bound to F-actin was calculated from the decrease in fluorescence following centrifugation, and presented in Figure 6. The obtained binding curve was used to estimate the affinity between the MBB–octapeptide and F-actin. A Scatchard plot was derived from the binding curve, and the association constant was calculated as 1.1×10^5 M^{-1} with 1.1 MMB–octapeptide binding per actin monomer. The MBB–octapeptide was successfully cross-linked to both F- and G-actins with EDC (results not shown) which also indicates the considerble affinity of the peptide to actin.

Discussion

Recently, we have found that the isolated and renatured 23-kDa (Muhlrad, 1989) and 21-kDa (Muhlrad, 1990) N-terminal fragments of S1 bind tightly to F-actin. Since the 23-kDa–actin and 21-kDa–actin complexes can be easily dissociated by increasing the ionic strength, by polyphosphate or polyglutamate anions, we have thought that there is a Coulombic interaction between the isolated N-terminal fragments of S1 and actin, in which the positively charged fragments react with the negatively charged actin (Muhlrad, 1989, 1990). The only positively charged cluster of residues on the N-terminal domain is the 143–147 sequence, which contains two lysine and two arginine residues; it was assumed that this sequence participates in the actin binding of myosin. This assumption was supported by the findings of nitrocellulose overlay experiments, which showed that the actin binding site of the 23-kDa fragment resides on the 130–204 sequence (Muhlrad, 1989). On the basis of these results, it seemed to us of interest to obtain a synthetic peptide containing the 143–147 sequence, in order to study the reaction of the peptide with actin and to raise an antibody against it which can be used to describe the role of the 143–147 sequence in the actin–S1 interaction.

The 143–147 sequence of S1 is highly conserved; it can be found essentially without any change in 10 different myosin heavy chains from sources of great evolutionary distances including *Acanthamoeba*, *Saccharomyces*, and rabbit skeletal muscle [for a review, see Mornet et al., (1989)]. Even the few alterations that appear are conserved and do not change the positive charge of the sequence. Sequences, which are highly conserved during the evolution, are generally functionally significant. Therefore, it is plausible to assume that the 143–147 stretch has an important role in myosin function.

A peptide was synthesized with the sequence NH_2-Cys-Tyr-Arg-Gly-Lys-Lys-Arg-Gln which is identical with the 142–148 sequence of the rabbit skeletal muscle myosin S1 heavy chain (Tong & Elzinga, 1983). The addition of cysteine to the N-terminus was necessary for coupling the peptide to the fluorescent thiol reagent MBB or to BSA. The MBB-labeled peptide was found to bind to F-actin with a significant affinity (association constant 1.1×10^5 M^{-1}) in sedimentation assays and was cross-linked to F-actin by EDC. The finding that the synthetic octapeptide containing the 143–147 sequence of S1 binds to actin supports the possibility that this region can be a part of the actin binding site of myosin.

This hypothesis was tested by raising a site-directed antibody to the region containing the 143–147 sequence and determining its effect on binding of S1 to actin. Affinity-purified IgG and its F(ab) fragment obtained from the antiserum were reacted with S1 in a solid-phase ELISA assay. The reaction was significantly inhibited in the presence of F-actin, which indicates that the accessibility of the S1 epitope to the antibody was decreased by actin. In solution, both the affinity-purified antibody and its F(ab) fragment inhibited the interaction between S1 and actin under rigor conditions (strongly attached state) in sedimentation assays. In the ATPase activity measurements, both IgG and F(ab) inhibited the actin-activated ATPase activity of S1, but they had virtually no, or a very slight, effect on the intrinsic Mg^{2+}- or K^+(EDTA)-activated ATPase activities. This implies that the antibody does not directly affect the ATPase activity of S1 but inhibits the actin-activated ATPase activity by preventing the actin–S1 interaction. The findings show that the antibody inhibits the actin–S1 interaction both in strongly and in weakly attached states, in rigor and in the presence of ATP, respectively. All these results can be easily interpreted by assuming that actin and the antibody compete for the same binding site of S1, e.g., for the 143–147 positive cluster. The inhibition caused by the antibody is not complete, because the actin–S1 interface is rather extended and the 143–147 stretch constitutes only one part of the actin binding site of S1. However, it is also possible that the interference in the actin–S1 interaction is caused by steric hindrance introduced by the attachment of the bulky antibody to its epitope, which is proximal to the actin binding site in the three-dimensional structure of S1. The finding that the F(ab) fragment, whose molecular mass is only one-third of the complete IgG, similarly affects the actin–S1 interaction as the whole antibody does not exclude the possibility of steric hindrance but makes it less plausible. In light of the foregoing, it is highly probable that the observed actin binding by the renatured N-terminal 23-kDa (Muhlrad, 1989) fragment is physiological and the 143–147 stretch is directly involved in forming the actin binding site of S1. Further studies by cross-linking are in progress in order to unambiguously resolve this problem.

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Registry No. ATPase, 9000-83-3; NH_2-Cys-Tyr-Arg-Gly-Lys-Lys-Arg-Gln, 130469-77-1.

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A Photochemically Induced Dynamic Nuclear Polarization Study of Denatured States of Lysozyme†

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ABSTRACT: Photochemically induced dynamic nuclear polarization (photo-CIDNP) techniques have been used to examine denatured states of lysozyme produced under a variety of conditions. ^{1}H CIDNP difference spectra of lysozyme denatured thermally, by the addition of 10 M urea, or by the complete reduction of its four disulfide bonds were found to differ substantially not only from the spectrum of the native protein but also from that expected for a completely unstructured polypeptide chain. Specifically, denatured lysozyme showed a much reduced enhancement of tryptophan relative to tyrosine than did a mixture of blocked amino acids with the same composition as the intact protein. By contrast, the CIDNP spectrum of lysozyme denatured in dimethyl sulfoxide solution was found to be similar to that expected for a random coil. It is proposed that nonrandom hydrophobic interactions are present within the denatured states of lysozyme in aqueous solution and that these reduce the reactivity of tryptophan residues relative to tyrosine residues. Characterization of such interactions is likely to be of considerable significance for an understanding of the process of protein folding.

Recent advances in the fields of protein engineering, X-ray crystallography, and NMR spectroscopy have increased dramatically our knowledge of the structures of proteins in their native states and of the factors which stabilize them (Eisenberg & Hill, 1989; Shortle, 1989; Wright, 1989). By contrast, relatively little is known about the denatured states of proteins, primarily because of the problems involved in studying them. It is particularly difficult to characterize denatured proteins because the unfolded polypeptide chain interconverts rapidly between many different conformational states of similar energies (Tanford, 1968). As a consequence, crystallization of

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