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Effect of Monoclonal Antibodies on the Properties of Smooth Muscle Myosin†

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ABSTRACT: Monoclonal antibodies were generated against turkey gizzard myosin, and their effects on some of the properties of myosin were assayed. Ca^{2+}- and Mg^{2+}-ATPase activities of myosin were enhanced by the anti-subfragment 2 antibodies at low ionic strength (i.e., with 10S myosin). Tryptic fragments of an anti-S2 IgM also activated these activities. Antibodies directed against subfragment 1 and light meromyosin had no effect. The Mg^{2+}-ATPase activity of heavy meromyosin also was activated by an anti-S2 antibody. Actin-activated ATPase activity of phosphorylated myosin was enhanced by the anti-S2 IgM fragments at low $MgCl_2$ concentrations. This increase was reflected by a 5-fold increase in V_{max} and a slight decrease in the apparent dissociation constant for actin. The actin-activated ATPase of dephosphorylated myosin was not affected by intact anti-S2 antibody or its fragments. The rates of phosphorylation and dephosphorylation of the 20 000-dalton light chains were increased by interaction of myosin with anti-S2 antibody. Limited proteolysis of myosin was used as a conformational probe. Interaction of anti-S2 antibody with 10S myosin increased the extent of cleavage at the S1–S2 junction. Proteolysis of 6S myosin was rapid and was not influenced by anti-S2 antibody. Our interpretation of these results is that interaction of the anti-S2 antibodies with myosin alters the conformation in the S2 region and this in turn modifies some of the properties of myosin. This is consistent with the hypothesis that the S2 region of smooth muscle myosin is a determinant of its biological properties.

Phosphorylation of myosin is an important regulatory component of smooth muscle. Although the relationship between the level of developed tension and phosphorylation is complex, it is accepted that the phosphorylation of myosin is required for contraction (Hartshorne, 1987). The sites of phosphorylation are the two 20 000-dalton light chains of myosin, and under most circumstances, the serine-19 residues are phosphorylated. The phosphorylation reaction is catalyzed by the Ca^{2+}- and calmodulin-dependent myosin light chain kinase (MLCK),[1] and dephosphorylation is achieved via a phosphatase, whose identity is not established.

The critical biochemical change elicited by phosphorylation is an increase of actin-activated ATPase activity, and this event is thought to reflect an increased rate of cross-bridge cycling in the intact tissue. Phosphorylation of the light chains thus initiates a conformational change that is transmitted to the myosin heads (S1) and results in modification of the active site(s). Details of the molecular events involved in this process are not established, but some interesting preliminary results have been obtained. These stem from the discovery that smooth muscle myosin can exist in two conformations, i.e., the extended (6S) and folded (10S) states (Suzuki et al., 1982), and that the conformation correlates with enzymatic activity (Ikebe et al., 1983). The simple hypothesis was developed (Ikebe et al., 1983) that the conformation of myosin determined enzymatic properties and that the critical conformational changes occurred as part of the 10S–6S transition. [Since myosin exists in thick filaments in both relaxed and contracting smooth muscle (Somlyo et al., 1981), it is unlikely that the entire folding transition is allowed in intact muscle.] According to this scenario, the role of phosphorylation is to alter conformation that in turn influences ATPase properties, and in support of this was the observation that under appropriate ionic conditions phosphorylation will convert 10S to 6S myosin (Ikebe et al., 1983; Craig et al., 1983; Onishi et al., 1983; Trybus & Lowey, 1984). The 6S–10S transition, however, could be a composite of several more subtle changes, and the problem is to identify those changes that affect biological properties. Using limited proteolysis as a conformational probe, it was shown that two sites are altered during the 10S–6S transition. These were designated sites A and B (Ikebe & Hartshorne, 1985a,1986) and are located at, or close to, the actin binding site and the S1–S2 junction, respectively. In the 6S state, site B is accessible to proteolysis (Ikebe & Hartshorne, 1984; Onishi & Watanabe, 1984), and the idea developed that changes in flexibility at the S1–S2 junction altered the mobility of the myosin heads. In the inactive state (10S equivalent), it was suggested that the heads are con-

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[1] Abbreviations: MLCK, myosin light chain kinase; HMM, heavy meromyosin; LMM, light meromyosin; S1, heavy meromyosin subfragment 1; S2, heavy meromyosin subfragment 2; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ELISA, enzyme-linked immunosorbent assay; IMDM, Iscove's modified Dulbecco's medium; HAT, hypoxanthine–aminopterin–thymidine; FBS, fetal bovine serum; NFDM, nonfat dry milk.

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strained and this hinders interaction with actin (Ikebe & Hartshorne, 1985a; Ikebe et al., 1988). Suzuki et al. (1985) also suggested that the orientation of the heads (in HMM) is important in determining ATPase activity.

The objective of the present study was to continue studies on the structure–function relationship of smooth muscle myosin and to use monoclonal antibodies as probes directed against specific regions of the myosin molecule. Several monoclonal antibodies were generated, and selected ones were assayed for their effects on the properties of myosin and HMM. In particular, our interests were focused on the effects obtained with antibodies directed against the S2 region.

Materials and Methods

Protein Preparations. Myosin (Ikebe & Hartshorne, 1985b) and MLCK (Walsh et al., 1983) were isolated from frozen turkey gizzard. HMM was prepared from turkey gizzard myosin following proteolysis with α-chymotrypsin (Onishi & Watanabe, 1979). Myosin rod was prepared from gizzard myosin as follows: gizzard myosin (9.3 mg/mL) in 0.2 M KCl, 30 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1.0 mM dithiothreitol, and 0.2 mM EGTA was digested with papain (1:6000 weight ratio papain to myosin) at 25 °C for 95 min. The reaction was stopped by addition of iodoacetic acid to 5 mM, and the digest was dialyzed versus 15 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), and 0.2 mM dithiothreitol. The precipitate, myosin rod, was collected by centrifugation at 10000*g* for 15 min. There was no myosin detectable in this preparation. S2 was isolated from myosin rod (1 mg/mL) in 0.3 M KCl and 30 mM Tris-HCl (pH 7.5) by digestion with α-chymotrypsin (1:100 protease:rod weight ratio) at 25 °C for 80 min, followed by dialysis against 15 mM $MgCl_2$ and 30 mM Tris-HCl (pH 7.0). The insoluble material (LMM) was removed by centrifugation at 10000*g* for 20 min, and the supernatant was used as S2. This contained two components, a major band of M_r 40 000 and a minor band of M_r 34 000. S1 was prepared by digestion of gizzard myosin with *Staphylococcus aureus* protease (Ikebe & Hartshorne, 1985a). Actin was prepared from chicken skeletal muscle (Driska & Hartshorne, 1975). Calmodulin was isolated from bull testes (Walsh et al., 1983). Phosphatase was isolated from fresh chicken gizzards according to Onishi et al. (1982).

Procedures. ATPase activities were assayed as described previously (Ikebe & Hartshorne, 1985a). Conditions are given in the figure legends. Phosphorylation of myosin was determined by the procedure of Walsh et al. (1983) under the conditions given in the figure legends. Electrophoresis was carried out on 7.5–20% polyacrylamide gradient slab gels using the discontinuous buffer system of Laemmli (1970). Conditions for papain digestion of myosin in the presence and absence of antibodies were as described previously (Ikebe & Hartshorne, 1984). The time courses of papain digestion were monitored by $NaDodSO_4$–polyacrylamide electrophoresis, and the concentrations of myosin heavy chain and 120-kDa myosin rod were estimated from scans of these gels using a GS 300 scanning densitometer (Hoefer Scientific Instruments) attached to a LCI-100 laboratory computing integrator (Perkin-Elmer). The electrophoretic transfer of proteins from the polyacrylamide gels to nitrocellulose sheets (Bio-Rad, 0.45 μm) was carried out according to Tsang et al. (1983) at 0.1 A for several hours. Cross-reactivity of the monoclonal antibodies was detected by using horseradish peroxidase conjugated to goat anti-mouse immunoglobulin (American Qualex) usually at a 1:500 fold dilution in 1% gelatin, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.05% Tween 20 and the subsequent application of 0.5 mg/mL 4-chloro-1-naphthol and 0.015% H_2O_2 in 0.5 M NaCl and 20 mM Tris-HCl (pH 7.5). Protein bands on the nitrocellulose paper were detected by staining with 0.1% naphthol blue black in 5% methanol and 10% acetic acid. Protein concentrations were measured by the BCA protein assay reagent (Pierce).

Monoclonal Antibody Production. The procedures used are modified from the Oi and Herzenberger (1980) method. BALB/c mice were injected (intraperitoneal) with 12 μg of gizzard myosin plus the Ribi adjuvant system (Ribi Immunochem Research). After 30 days, the serum titer was tested by ELISA in 96-well A/2 EIA plates, and the mice with the highest titer were boosted by intravenous injection of 50 μg of myosin in isotonic saline. After 3 days, the spleens were aseptically removed, and fusion was achieved with P3x63-Ag8.653 myeloma cells, pretreated with demecolcine (to synchronize cells). Fusions were carried out in PEG 1500, and cells were plated in 96-well plates in Iscove's modified Dulbecco's medium (IMDM) containing hypoxanthine–aminopterin–thymidine (HAT), 15% fetal bovine serum (FBS), 2 mM glutamine, 1% antibiotic, and 10 μg/mL *Salmonella typhimurium* mitogen (Ribi Immunochem Research) to which is added 15% (by volume) of IMDM and 15% FBS medium conditioned by J774A.1 macrophages. When visible colonies were present, the wells were screened for antibody production by ELISA techniques (Butler et al., 1980) using the peroxidase-linked second antibody. Cells in positive wells were transferred to 24-well plates and then to 25 cm^2 flasks containing 5 mL of IMDM–HAT and 15% FBS plus 15% macrophage-conditioned medium. Supernatants were screened by ELISA and Western blotting. Cloning was accomplished by limiting dilution techniques in 96-well plates. After visible colonies were detected, supernatants were screened by ELISA techniques and the positive colonies expanded and cloned a second time. Cells derived from the second clones were then injected into BALB/c mice for production of monoclonal antibodies by ascites tumors. The ascites fluid was collected, and antibodies were purified by the method of Boonekamp and Pomp (1986). The immunoglobulin subclasses of the monoclonal antibodies were classified by using a mouse Typer Sub-Isotyping kit (Bio-Rad). For the characterization of the antibodies, both a-ELISA (amplified ELISA; Butler et al., 1980) and Western blots were used. With the former, the wells were coated overnight at 4 °C with either 200 ng of gizzard myosin, 110 ng of HMM, 79 ng of S1, or 93 ng of myosin rod, each dissolved in 0.05 M sodium carbonate (pH 9.6). The wells were washed 3 times with 1% nonfat dry milk (NFDM is a relatively inert material that was more effective in blocking nonspecific protein binding sites in ELISA and Western blots than either gelatin or serum albumin), in phosphate-buffered saline [0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4 (pH 7.3) plus 0.05% Tween 20], incubated with antibody, washed with saline, and subjected to the anti-mouse immunoglobulin conjugated with horseradish peroxidase (American Qualex) at a 1:1000 dilution in 1% NFDM in phosphate-buffered saline–Tween 20 solvent for 90 min at room temperature. The wells were washed 3 times with 1% NFDM in phosphate-buffered saline plus Tween 20, and color development was achieved after 7 min with 1 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and 0.015% H_2O_2. ELISA plates were read at 405 nm using a Titertek Multiskan.

Fragments of IgM were prepared by the procedure of Matthew and Reichardt (1982). The tryptic digest of IgM was dialyzed against 0.1 M KCl and 20 mM Tris-HCl (pH 7.5) and applied to a Sephacryl S-300 column (2.5 × 90 cm)

Table I: Characterization of the Anti-Gizzard Myosin Monoclonal Antibodies

monoclone designation	immuno-globulin type	myosin fragment specificity[a]
B8C	IgG_1	S1 (50 kDa)[b]
C5C	IgM	S2
D10F	IgM	S2
E10D	IgM	S2
B6G	IgG_{2a}	LMM
D7E	IgG_1	LMM
E8F	IgG_1	LMM
E5F	IgG_{2a}	LMM

[a] Specificity determined by ELISA and Western blots. [b] The anti-S1 antibody was specific for the central domain (i.e., 50 kDa) of S1.

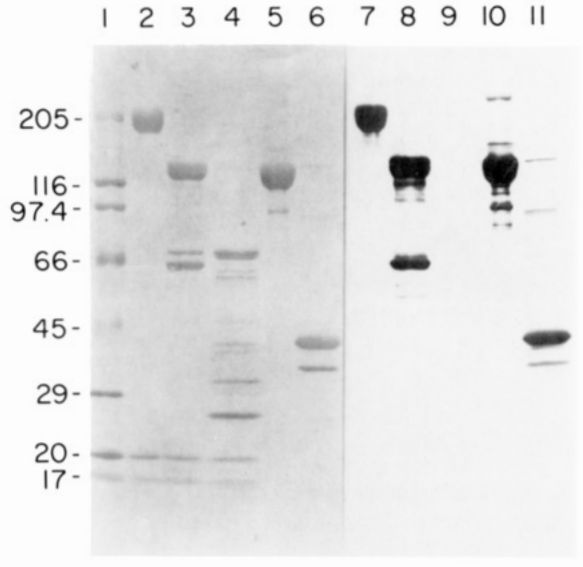

FIGURE 1: Reactivity of the anti-S2 antibody, D10F, as determined by electrophoresis and immunoblots. Following electrophoresis, proteins were transferred to nitrocellulose and either stained (for protein) with naphthol blue black (lanes 1–6) or subjected to D10F antibody and peroxidase staining for second antibody (lanes 7–11; see Materials and Methods). Approximately 10 μg of protein was applied in each lane. Lane 1, molecular weight standards; lanes 2 and 7, myosin; lanes 3 and 8, HMM; lanes 4 and 9, S1; lanes 5 and 10, myosin rod; lanes 6 and 11, S2.

equilibrated with the same solvent. The fractions containing predominantly 110-kDa components (determined by electrophoresis in the absence of reducing agents) were collected, freeze-dried, dissolved in 50 mM KCl and 30 mM Tris-HCl (pH 7.5), and used for assays. The yield of the 110-kDa component was approximately 40% of the starting IgM, and the 110-kDa component comprised greater than 80% of the fraction used in assays.

Results

Characterization of Monoclonal Antibodies. Eight monoclonal antibodies were chosen and characterized by ELISA and Western blots with respect to their reactivity and immunoglobulin type. These are listed in Table I. Many of the studies described in this presentation were carried out with the anti-S2 antibody D10F. As an example of the characterization procedures that were used, the relative reactivity of myosin and fragments with D10F is shown in Figure 1 using Western blots. The protein staining patterns are shown in lanes 2–6, and the antibody reactivity is shown in lanes 7–11. Only those fragments containing S2 gave a positive reaction, i.e., myosin, HMM, myosin rod, and S2. The isolated S2 contained two components, M_r 40 000 and 34 000 and both reacted with the D10F antibody.

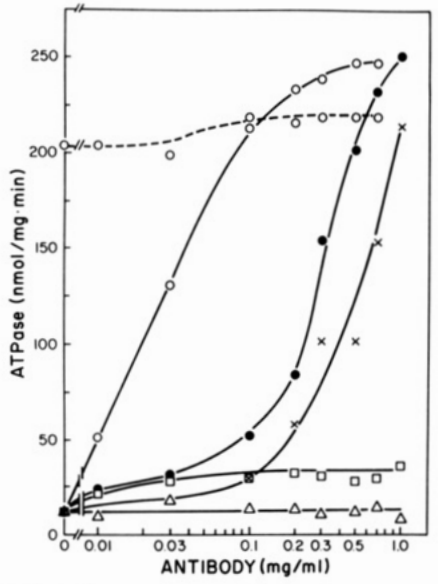

FIGURE 2: Effect of monoclonal antibodies on Ca^{2+}-ATPase of dephosphorylated myosin. Assay conditions (solid lines): 7 mM $CaCl_2$, 1 mM ATP, 0.1 M KCl, 30 mM Tris-HCl (pH 7.5), and 0.1 mg/mL myosin. Assay conditions (dashed line): same except 0.4 M KCl. Antibodies: anti-S2 D10F (○); anti-S2 D10F fragments (×); anti-S2 C5C (●); anti-LMM E8F (□); anti-S1 B8C (△).

Effect of Antibodies on Myosin ATPase Activity. The transition in smooth muscle myosin from 6 S to 10 S is accompanied by a decrease in Ca^{2+}- and Mg^{2+}-ATPase activities. These activities can therefore be used as indicative of myosin conformation. The effects of various antibodies on the Ca^{2+}-ATPase activity of gizzard (dephosphorylated) myosin are shown in Figure 2. In 0.1 M KCl (and the other conditions of the ATPase assay), dephosphorylated myosin exists as 10 S and has a relatively low ATPase activity. Addition of the D10F antibody (anti-S2) caused a marked activation. Two other anti-S2 antibodies also increased Ca^{2+}-ATPase activity. The effect of C5C is shown in Figure 2, but the data on E10D are not presented. One concern with the use of IgM was that the changes in ATPase activity could reflect the formation of aggregates, rather than a conformational change in myosin. It was not possible to prepare Fab fragments from these IgM molecules, but smaller active fragments were prepared by digestion with trypsin (Matthew & Reichardt, 1982). The addition of such fragments, derived from D10F, to 10S myosin also increased Ca^{2+}-ATPase activity (Figure 2) although the fragments were less effective than the parent IgM molecule. This probably reflected a reduced titer for the fragments. In 0.4 M KCl, the gizzard myosin exists as 6S myosin and exhibits a higher level of ATPase activity. The addition of D10F to 6S myosin did not alter Ca^{2+}-ATPase activity (Figure 2). Other antibodies were also assayed, and these were the anti-S1 antibody (B8C) and the anti-LMM antibodies E8F, B6G, and D7E. None of these antibodies had any effect on the Ca^{2+}-ATPase of 10S gizzard myosin (Figure 2).

The effects of various antibodies on the Mg^{2+}-ATPase activity of myosin are shown in Figure 3. For dephosphorylated myosin (Figure 3A), activation was obtained with the two anti-S2 antibodies (D10F and C5C) and for the fragments of D10F. Activation was also observed with E10D (data not shown). (These assays were carried out at 0.1 M KCl where dephosphorylated myosin is in the 10S conformation.) The anti-S1 antibody (B8C) and the anti-LMM antibody (E8F) had no influence on Mg^{2+}-ATPase activity. With phosphorylated myosin (approximately 2 mol of P/mol of myosin),

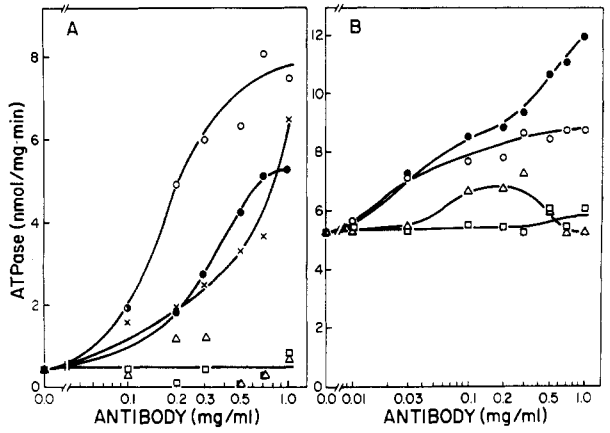

FIGURE 3: Effect of monoclonal antibodies on Mg^{2+}-ATPase of dephosphorylated (A) and phosphorylated (B) myosin. Assay conditions (dephosphorylated myosin): 5 mM $MgCl_2$, 1 mM ATP, 50 mM KCl, 30 mM Tris-HCl (pH 7.5), and 0.3 mg/mL myosin. Assay conditions (phosphorylated myosin): 10 mM $MgCl_2$, 1 mM ATP, 100 mM KCl, 30 mM Tris-HCl (pH 7.5), and 0.3 mg/mL myosin. Myosin was prephosphorylated to 1.7 mol of P/mol of myosin in 1 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM $CaCl_2$, 85 mM KCl, 30 mM Tris-HCl (pH 7.5), 2 mg/mL myosin, 10 μg/mL MLCK, and 10 μg/mL calmodulin for 15 min at 25 °C. Reaction stopped by addition of EGTA to 2 mM. Antibodies: anti-S2 D10F (○); anti-S2 D10F fragments (×); anti-S2 C5C (●); anti-LMM E8F (□); anti-S1 B8C (△).

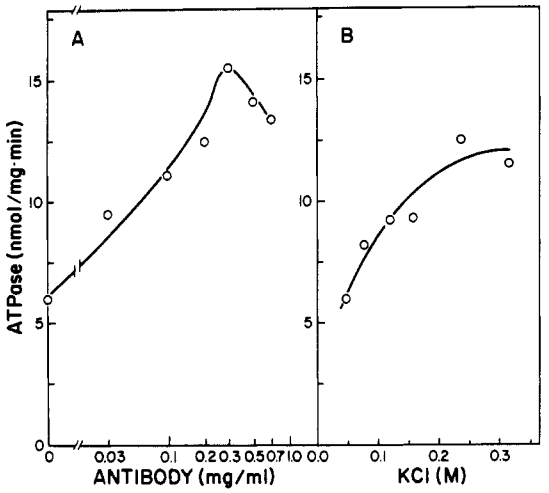

FIGURE 4: Effect of anti-S2 antibody D10F on Mg^{2+}-ATPase of HMM. Antibody effect (A); KCl effect in the absence of antibody (B). Assay conditions: 4 mM $MgCl_2$, 1 mM ATP, either 50 mM KCl (A) or varied KCl (B), 30 mM Tris-HCl (pH 7.5), and 0.1 mg/mL HMM.

both anti-S2 antibodies activated Mg^{2+}-ATPase, and there was little effect of the anti-S1 and anti-LMM antibodies (Figure 3B). The extent of activation with phosphorylated myosin is less compared to the dephosphorylated myosin since the conformation of phosphorylated myosin at this ionic strength is intermediate between the 6S and 10S states; i.e., it is partially activated. It is interesting that the C5C antibody showed an apparently higher titer with the phosphorylated myosin than with dephosphorylated myosin.

The effect of D10F on the Mg^{2+}-ATPase of gizzard HMM was determined as shown in Figure 4. Increasing concentrations of D10F activated Mg^{2+}-ATPase (Figure 4A), and the range of activation was similar to that induced by KCl via the conformational transition (Figure 4B) from 8.6 S to 7 S (Suzuki et al., 1985; Ikebe et al., 1988).

Next, the influence of D10F and its fragments was assayed for the actin-activated ATPase activities of phosphorylated and dephosphorylated myosins (Figure 5). The level of antibody was constant [at a relatively high concentration where maximum effects on Mg^{2+}-ATPase activity were observed (Figure 3)], and the $MgCl_2$ concentration was varied. The actin-activated ATPase of phosphorylated gizzard myosin has a marked dependence on the $MgCl_2$ concentration, as shown by the control curve in Figure 5A. In the presence of D10F (the anti-S2 antibody), a slight activation was observed at low $MgCl_2$ concentrations and an inhibition (relative to the control curve) at higher $MgCl_2$ levels. A considerably larger activation of actin-activated ATPase was induced at low $MgCl_2$ concentrations by the D10F fragments. The difference between the effects obtained with the IgM molecule and its fragments could be due to steric interference at the actin–myosin binding site by the mass of the intact molecule.

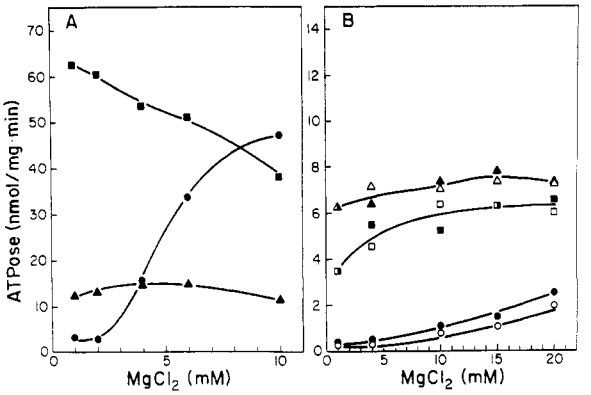

FIGURE 5: Effect of anti-S2 antibody D10F and its fragments on actin-activated ATPase of phosphorylated (A) and dephosphorylated (B) myosin. Assay conditions: $MgCl_2$ (as indicated), 1.3 mM ATP, 0.06 mM $CaCl_2$, 85 mM KCl, 30 mM Tris-HCl (pH 7.5), 0.3 mg/mL myosin, 0.68 mg/mL actin, and 1.2 mg/mL D10F or D10F fragments. Myosin phosphorylated to 1.9 mol of P/mol of myosin as in Figure 4. Control actin-activated ATPase (●); actin-activated ATPase plus D10F (▲); actin-activated ATPase plus D10F fragments (■); myosin ATPases (B): control (○); plus D10F (△); plus D10F fragments (□).

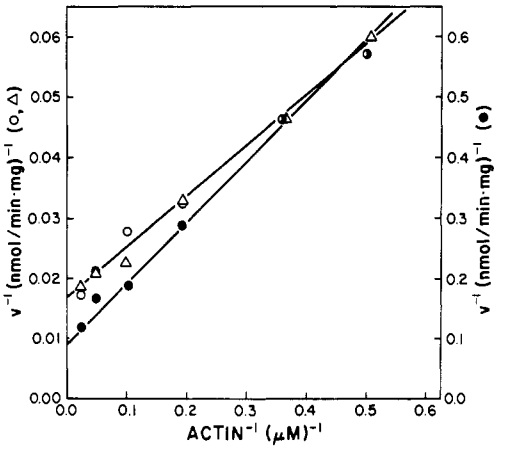

FIGURE 6: Actin concentration dependence of Mg^{2+}-ATPase of phosphorylated myosin in the presence of anti-S2 D10F fragments. Assay conditions as in Figure 5, except that [$MgCl_2$] was either 2 mM (○, ●) or 10 mM (△). Control ATPase (●, △); ATPase in presence of antibody fragments (○).

With dephosphorylated myosin (Figure 5B), neither D10F nor its fragments activated actin-activated ATPase. The increase in ATPase activity, compared to the control actin-activated level, was due to an increase in myosin ATPase (i.e., not actin-moderated) induced by the anti-S2 antibody and fragments.

Activation of the actin-activated ATPase of phosphorylated myosin by the D10F fragments was examined as a function of actin concentration with the results shown in Figure 6. At 2 mM $MgCl_2$ (total) in the absence of antibody, the apparent

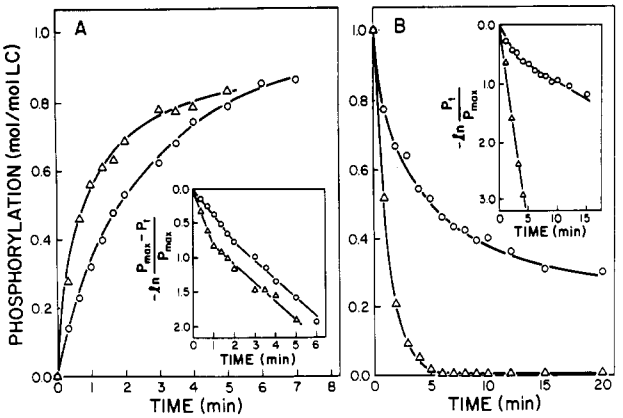

FIGURE 7: Effect of anti-S2 antibody D10F on rates of phosphorylation (A) and dephosphorylation (B) of the 20000-dalton light chain. Assay conditions (A): 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM $CaCl_2$, 85 mM KCl, 30 mM Tris-HCl (pH 7.5), 1 mg/mL myosin, 2 μg/mL (control) or 0.4 μg/mL (plus antibody) MLCK, 5 μg/mL calmodulin, and 1 mg/mL D10F, 25 °C. Assay conditions (B): 2.5 mM $MgCl_2$, 91 mM KCl, 1 mM EGTA, 30 mM Tris-HCl (pH 7.5), 1 mg/mL phosphorylated myosin (2 mol of P/mol of myosin), 12.5% (v/v) crude phosphatase, and 1 mg/mL D10F. Conditions for prephosphorylation as in Figure 4, using 3 μg/mL MLCK and 5 μg/mL calmodulin. From each assay, individual time points are also expressed in semilogarithmic plots as shown in insets and initial rates calculated. Myosin plus antibody (▲); myosin control (○).

binding constant for actin, K_A, was 11 μM, and V_{max} was 12 nmol of P_i released min^{-1} (mg of myosin)$^{-1}$. In the presence of the D10F fragments, V_{max} increased to 61 nmol of P_i min^{-1} mg^{-1} and K_A was 5 μM. For comparison, at 10 mM $MgCl_2$, where there was little effect of the D10F fragments, the values of K_A and V_{max} also were 5 μM and 61 nmol of P_i min^{-1} mg^{-1}, respectively (i.e., in the absence of antibody). Thus, the binding of the antibody fragments to the S2 region alters both the binding affinity for actin and the enzymatic activity. The more dominant of these two effects is the increase in V_{max}.

Influence of Antibodies on Phosphorylation and Dephosphorylation. The phosphorylation time course of myosin was followed in the presence and absence of D10F (Figure 7A). In the presence of anti-S2 antibody, the phosphorylation rate was considerably faster (the MLCK concentrations in the presence and absence of antibody were different). The difference in phosphorylation rates was more marked for the initial phase of phosphorylation, as shown in the inset of Figure 7A. For the fast (initial) phase of phosphorylation, k_{cat}/K_m values were 291 and 25.5 min^{-1} μM^{-1} in the presence and absence of antibody, respectively (assuming a molecular weight of 130000 for MLCK).

Time courses of dephosphorylation of gizzard myosin are shown in Figure 7B, in the presence and absence of D10F antibody. Clearly, there was a marked influence of the anti-S2 antibody on the kinetics of dephosphorylation. The presence of D10F increased the rate of dephosphorylation by approximately 4-fold (on the basis of the initial phase of dephosphroylation in the absence of antibody) as estimated from the semilogarithmic plot (inset of Figure 7B).

Effect of Antibodies on the Conformation of Myosin. Many of the techniques that are used conventionally to monitor myosin conformation are not suitable in the presence of antibody, particularly IgM. These include viscosity and sedimentation velocity measurements. However, limited proteolysis has been used to investigate the 6S–10S transition (Ikebe & Hartshorne, 1984; Onishi & Watanabe, 1984), and since the products of myosin degradation can be identified and quantitated (Ikebe & Hartshorne, 1984), this procedure is applicable in the presence of other proteins, i.e., antibodies.

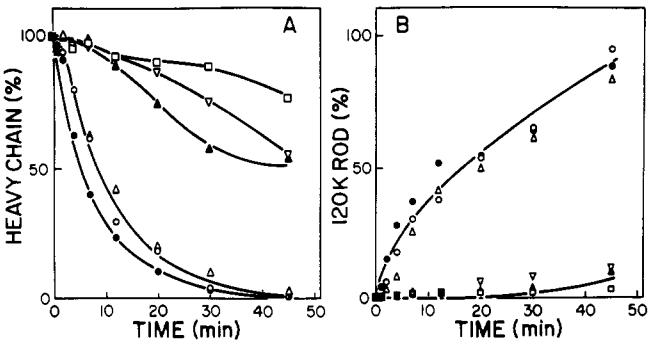

FIGURE 8: Effect of antibodies on the time course of papain digestion of myosin. The disappearance of the myosin heavy chain (A) and the generation of myosin rod (B) were calculated from scans of $NaDodSO_4$ gels. Conditions of proteolysis for dephosphorylated myosin: 5 mM $MgCl_2$, 1 mM ATP, 50 mM KCl, 30 mM Tris-HCl (pH 7.5), 0.25 mg/mL myosin, 1 mg/mL antibody, and 1:10 papain to myosin (w/w). For phosphorylated myosin (1.8 mol of P/mol of myosin), conditions the same except 10 mM $MgCl_2$ and 100 mM KCl. Myosin prephosphorylated as in Figure 4. Dephosphorylated myosin (10 S) plus anti-LMM E8F (□); dephosphorylated myosin (10 S) plus anti-S1 B8C (▽); dephosphorylated 10S myosin (▲); dephosphorylated myosin (10 S) plus anti-S2 D10F fragments (△); phosphorylated myosin (6 S) plus anti-S2 D10F fragments (○); phosphorylated 6S myosin (●).

The effect of various antibodies on the pattern of papain digestion of myosin, therefore, was determined as shown in Figure 8. The disappearance of the myosin heavy chain (Figure 8A) and the appearance of the 120-kDa myosin rod (Figure 8B) were monitored [cleavage of gizzard myosin heavy chain by papain is predominantly at the S1–S2 junction to liberate S1 plus myosin rod (S2 plus LMM)]. Proteolysis of myosin in the absence of antibodies is faster for the 6S conformation compared to the 10S conformation (Figure 8A,B). Addition of the anti-S1 antibody B8C to 10S myosin slightly decreased the rate of papain digestion, and a similar situation was observed with the anti-LMM antibody E8F (Figure 8A,B). The reduction in rate of proteolysis, compared to control, was probably due to an increase in the total protein concentration. In contrast to these results, addition of the fragments of the anti-S2 antibody, D10F, to 10S myosin caused an increase in the rate of proteolysis. The rates of proteolysis of 10S and 6S myosins both in the presence of D10F fragments were indistinguishable (Figure 8A) and were similar to the rate of proteolysis of 6S myosin in the absence of antibody. As shown in Figure 8B, this increased rate of proteolysis was due essentially to more rapid cleavage at the S1–S2 junction. Similar results were obtained for the intact D10F antibody (results not shown).

Discussion

The above results demonstrate that interaction of antibodies with the S2 region of myosin induces an alteration of biological properties. Since the active site, or ATP binding site, is within the S1 moiety, it is reasonable to propose that the binding of antibodies causes a conformational change that influences the active site. The use of limited proteolysis offers evidence consistent with a conformational change at the S1–S2 junction. The alteration in the kinetics of phosphorylation and dephosphorylation suggests that the conformation around the N-terminal sequence of the 20000-dalton light chain also is affected by the binding of antibody to S2. These findings, therefore, support the general shape–activity hypothesis, in which the conformation of myosin is an important factor in determining enzymatic activity (Ikebe et al., 1983). Similar results to ours were described in a preliminary report by Higashihara and Ikebe (1988) using a monoclonal antibody

to the 17 000-dalton light chain. It is therefore possible that binding of antibody to either S2 or the 17 000-dalton light chain induces similar changes at the S1–S2 junction.

The simplest interpretation of the shape–activity hypothesis is that enzymatic activity is determined entirely by conformation and that the only role of phosphorylation is to modify conformation. This appears to be valid for Ca^{2+}-, Mg^{2+}-, and K^+-EDTA-activated ATPases of myosin alone but is not as obvious for the actin-activated ATPase activity. The major uncertainty is whether phosphorylated 10 S is identical with the dephosphorylated species. The evidence pro and con is not overwhelming, but there is some indication, based on the effects of Ca^{2+}, that the two species are distinct. It was shown (Ikebe & Hartshorne, 1985b) that myosin in the dephosphorylated 10S state was not influenced by Ca^{2+} and the effects of Ca^{2+} on ATPase and conformation were observed only with phosphorylated 10S myosin. The effect of the anti-S2 antibodies on actin-activated ATPase also favors the view that phosphorylated and dephosphorylated 10S myosins are different, the key point being that the actin-activated ATPase activity was activated by the antibody fragments only with phosphorylated myosin at low $MgCl_2$ concentrations, i.e., conditions favoring the 10S conformation. Our interpretation of these data is that only phosphorylated 10S myosin can be activated (with respect to actin-activated activity) via the conformational change in the S1–S2 region.

In the above discussion, the assumption is that the interaction of antibody with the S2 region of myosin induces a conformational change that is reflected at the active site. Another possibility that was considered is that the interaction of antibody with myosin leads to aggregate formation and that the increase in enzymatic activity could be due to this altered physical state. The importance of filament formation to the ATPase activity of gizzard myosin has been stressed by Wagner and Vu (1987). However, we do not think that the increased activity of the antibody–myosin complex reflects aggregate formation. The reasons for this are as follows: (1) The antibodies to S1 and LMM did not increase ATPase activity but did form aggregates at the higher levels of antibody. Similarly, the proteolysis rate of 10S myosin was not increased by interaction either with anti-S1 or with anti-LMM antibodies. (2) The fragments of the anti-S2 IgM antibody also increased ATPase activity and were considerably less effective in forming aggregates than the parent molecule. (3) The Mg^{2+}-ATPase activity of the soluble myosin fragment, HMM, was increased by interaction with antibody, and it is expected that HMM has a reduced tendency for aggregation. (4) The most important factor in the increased actin-activated ATPase activity (by the anti-S2 fragments) was a shift in V_{max} rather than in K_A. The latter would be expected to be dominant if the activation was due entirely to aggregation.

The most convincing evidence in favor of an antibody-induced conformational change comes from the limited proteolysis experiments. On the basis of earlier work (Ikebe & Hartshorne, 1984; Onishi & Watanabe, 1984), it was established that the S1–S2 junction is modified during the 10S–6S transition. In the 10S state, this site is blocked and becomes accessible (to proteolysis) when the 6S conformation is formed. This is thought to reflect the conversion from a state in which the heads are restricted (10S) to one in which they are more mobile (6S), and this is due to an increased flexibility of the S2 region (Ikebe et al., 1988). It is therefore reasonable to propose that the interaction of antibody with S2 of 10S myosin alters its flexibility, or the orientation of the two myosin heads, and this accounts for the change in enzymatic activity. It is important to note that when myosin is in the 6S state (at high salt concentration) there is no influence of any of the antibodies on enzymatic activity.

These studies support the concept that alterations in the S2 region of myosin can influence enzymatic activity. The evidence for this is based on the application of monoclonal antibodies to S2. The epitope(s) of these anti-S2 antibodies is (are) not identified, and future studies are required to establish whether different parts of S2 are more critical than others with respect to the flexibility of the S2 units.

Registry No. ATPase, 9000-83-3.

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