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Nucleotide-Induced Changes in the Interaction of Myosin Subfragment 1 with Actin: Detection by Antibodies against the N-Terminal Segment of Actin†

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ABSTRACT: The binding of myosin subfragment 1 (S-1) to actin in the presence and absence of nucleotides was determined under conditions of partial saturation of actin, up to 80%, by $F_{ab}(1-7)$, the antibodies against the first seven N-terminal residues on actin. In the absence of nucleotides, the binding constant of S-1 to actin (2×10^7 M^{-1}) was decreased by 1 order of magnitude by $F_{ab}(1-7)$. The binding of S-1 to actin caused only limited displacement of F_{ab}, and between 30 and 50% of actin appeared to bind both proteins. In the presence of MgAMP·PNP, MgADP, and $MgPP_i$ and at low S-1 concentrations, the same antibodies caused a large decrease in the binding of S-1 to actin. However, the binding of S-1·nucleotide to actin in the presence of $F_{ab}(1-7)$ increased cooperatively with the increase in S-1 concentration. Also, in contrast to rigor conditions, there was no indication for the binding of $F_{ab}(1-7)$ and S-1·nucleotide to the same actin molecules. These results show a nucleotide-induced transition in the actomysin interface, most likely related to the different roles of the N-terminal segment of actin in the binding of S-1 and S-1·nucleotide. The possible implications of these findings to the regulation of actomyosin interactions are discussed.

The generation of force and the motile action of myosin and actin are believed to involve at least two different states in which these proteins bind to each other in different orientations or have different conformations (Cooke, 1990). Clearly, the transitions between the various structural states of actomyosin must be dominated by the nucleotides which are bound to it. This perception stimulated much interest in the ATP, ADP, and nucleotide analogue induced changes in actomyosin interactions in solution and in fibers. The eventual goal of such studies is to characterize actomyosin in a state-specific manner (i.e., in relationship to particular ATP hydrolysis steps).

Solution work on acto–subfragment 1 (acto–S-1)[1] and actomyosin complexes produced ample, albeit largely qualitative

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[1] Abbreviations: S-1, myosin subfragment 1; $F_{ab}(1-7)$, affinity-purified F_{ab} fragment of polyclonal peptide antibodies raised against the first seven N-terminal residues of α-skeletal actin; AMP·PNP, adenyl-5-yl imidodiphosphate; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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evidence for nucleotide-induced changes in these proteins (Cooke, 1986). This evidence includes among others the perturbation of S-1 tryptophan fluorescence (Bagshaw & Trentham, 1974), changes in the environment of extrinsic probes attached to the reactive cysteine residues on S-1, SH_1 (Seidel & Gergely, 1971; Aguirre et al., 1986; Miyanishi & Borejdo, 1989; Fajer et al., 1990; Ajtai at al., 1990), and SH_2 (Ajtai & Burghardt, 1989), changes in the chemical reactivities of these residues [e.g., see Doung and Reisler (1989)] and their distance from each other (Rajasekharan et al., 1989; Dalbey et al., 1983; Garland et al., 1988), alterations in the cross-linking of S-1 to actin (Yamomoto, 1989) and in the conformation of the cross-linked complex (Duong & Reisler, 1989), transitions in the state of the actin-bound nucleotide (Yanagida, 1981), etc. In most cases, these observations report on local changes in actin and S-1, and it is yet to be clarified which contact sites at the actomyosin interface are impacted by nucleotides. Interestingly, Highsmith (1990) has concluded recently that the binding of ADP to acto–S-1 increases the electric charge on the actin binding site of myosin.

Not surprisingly, profound differences were found between the weakly bound acto–S-1 states in the presence of ATP or ATPγS and the rigor actomyosin complexes. Electron microscopy of cross-linked (Craig et al., 1985; Applegate, & Flicker, 1987) and un-crossed-linked (Frado & Craig, 1991) acto–S-1 and acto–HMM complexes suggested that the weakly bound myosin heads attach to actin over a wide range of angles. In agreement with this observation, the weakly bound S-1 was shown also to be mobile on a microsecond time scale (Svensson & Thomas, 1986; Bernett & Thomas, 1989) and accessible to chemical and proteolytic probes (Duong & Reisler, 1989). Clearly, the environment of the SH_1 cysteine on S-1 is changed upon binding of ATP or even ADP to actomyosin (Miyanishi & Borejdo, 1989; Fajer et al., 1990; Ajtai et al., 1990). Yet, it remains to be determined whether the SH_1 site is an actual component of the actomyosin interface (Suzuki et al., 1987; Keane et al., 1990; Chase et al., 1991) or is just located along the communication pathway between the nucleotide and actin binding sites on myosin (Botts et al., 1984).

More sharply defined, both in topographic and in functional terms, is the interaction between the N-terminal segment of actin and the lysine-rich residues 632–642 in the 50/20-kDa junction of the S-1 heavy chain. In spite of ready cross-linking of S-1 to actin by carbodiimide via these sites (Mornet et al., 1981; Sutoh, 1982a,b), the rigor actomyosin bond does not depend on the actual contact between them (Mejean et al., 1987; Miller et al., 1987). Yet, as shown by experiments with antibodies to the N-terminal segment of actin, $F_{ab}(1-7)$ (DasGupta & Reisler, 1989), and the work with antipeptide to residues 633–642 on S-1 (Chaussepied & Morales, 1988; Chaussepied, 1989) and with a labeled actin (Bertrand et al., 1989), the interaction between the N-terminal site on actin and residues 633–642 on S-1 appears important for actomyosin ATPase activity and actomyosin binding in the presence of ATP.

Further mapping of actomyosin interactions in the presence of ATP was achieved in the most recent proteolytic studies of Yamamoto (1991). In that work, Lys-640 and -641 in rabbit skeletal S-1 (Tong & Elzinga, 1990) were found to be particularly important for myosin·ATP interactions with actin. A recent chemical analysis of acto–S-1 cross-linking by carbodiimide revealed also a small shift in the cross-linking of the S-1 50/20-kDa junctional lysines to actin (Yamamoto, 1989).

In this work, we have extended the experiments on the interactions of myosin·ATP with the N-terminal segment of actin and shown a general nucleotide effect on the actomyosin interface as probed by $F_{ab}(1-7)$. While rigor binding of S-1 to actin is not changed much by $F_{ab}(1-7)$, the binding of S-1·nucleotide to actin is strongly inhibited by F_{ab} at low S-1 concentrations and is cooperatively "switched on" at higher S-1 levels. Furthermore, while S-1·nucleotide and $F_{ab}(1-7)$ bind to actin exclusively of each other, S-1 and F_{ab} can bind to the same actin in the absence of nucleotides. These results are discussed in terms of the acto–S-1 interface and the possible role of the N-terminal segment of actin in the regulation of actomyosin interactions.

Materials and Methods

Reagents. Distilled and Millipore-filtered water and analytical-grade reagents were used in all experiments. ADP, ATP, AMP·PNP, and TLCK-treated chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, MO). ELISA plates (Dynatech Immulon) were obtained from Fisher Scientific Co.

Preparation of Proteins. Rabbit skeletal muscle actin was prepared in G-actin buffer (0.5 mM mercaptoethanol, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 5 mM Tris, pH 7.6) by the procedure of Spudich and Watt (1971). G-Actin was polymerized by the addition of 2 mM $MgCl_2$ to the monomeric protein. Myosin was obtained as described by Godfrey and Harrington (1970). Subfragment 1 (S-1) was prepared by chymotryptic digestion of myosin according to the method of Weeds and Pope (1977). Antibodies directed against residues 1–7 from the N-terminus of α-skeletal actin and the F_{ab} fragments derived from them, $F_{ab}(1-7)$, were prepared and purified as described previously (Miller et al, 1987). Antibody titers were checked by ELISA using actin as the coating antigen (DasGupta et al., 1990).

Airfuge Binding Experiments. F-Actin (3.0 μM) was incubated for 20 min with various amounts of S-1 (between 0 and 16 μM in the absence of nucleotides and between 0 and 45 μM in the presence of nucleotides) and $F_{ab}(1-7)$ (between 0 and 9 μM) in 10 mM KCl and 10 mM imidazole, pH 7.0, at 23 °C. When present, the concentrations of MgADP, MgAMP·PNP, and $MgPP_i$ in these solutions were 4.0 mM. The protein mixtures were centrifuged at 140000*g* for 20 min in an air-driven ultracentrifuge (Beckman Instruments). The pelleted proteins were resolubilized in their buffer and then denatured and run on 15% SDS–polyacrylamide gels (Laemmli, 1970). Coomassie Blue R stained protein bands were scanned with a Biomed Instruments (Fullerton, CA) Model SLR 2D/1D soft laser scanning densitometer interfaced to a DTK computer. The densitomeric traces of the scanned proteins were analyzed to determine the molar ratios of S-1 and F_{ab} pelleted with actin, i.e., the binding of these proteins to F-actin. Molar ratios of bound proteins were calculated by using molar stain ratios obtained from the original uncentrifuged protein samples.

Concentration Determinations. Protein concentrations were determined spectrophotometrically by using the following extinction coefficients at 280 nm: actin, $E^{1\%} = 11.0\ cm^{-1}$; S-1, $E^{1\%} = 7.50\ cm^{-1}$; F_{ab}, $E^{1\%} = 16.0\ cm^{-1}$.

Results

Binding of S-1 to Actin in the Presence of F_{ab} (1–7). Earlier measurements of $F_{ab}(1-7)$ binding to actin revealed that approximately stoichiometric amounts of S-1 (with respect to actin) reduced by about 5- or 6-fold the affinity of these antibodies for actin (Miller et al., 1987). At the same time,

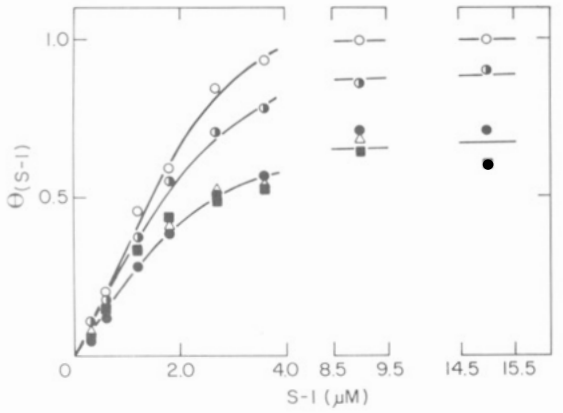

FIGURE 1: Binding of S-1 to actin in the absence of nucleotides and in the presence of $F_{ab}(1-7)$. F-Actin (3.0 μM) was incubated with S-1 (between 0 and 16.0 μM) in the absence (○) and the presence of $F_{ab}(1-7)$ set at 1.5 (◑), 3.0 (●), 6.0 (■), and 9.0 μM (△) concentrations in solutions containing 10 mM KCl and 10 mM imidazole, pH 7.0. The protein mixtures were incubated at 23 °C for 20 min and then pelleted in a Beckman airfuge and run on SDS–PAGE. The fraction of actin occupied by S-1, $\theta_{(S-1)}$, at different S-1 and F_{ab} concentrations was calculated from densitometric analysis of these gels. The solid curves correspond to theoretical binding of S-1 to actin calculated by assuming binding constants of 2×10^7, 5×10^6, and 2×10^6 M^{-1}, respectively, in a descending order of curves. From the apparent plateau levels of the binding data, 100, 90, and 70% of the high-affinity S-1 binding sites on actin were taken to be available for S-1 bining in the calculation of the respective curves.

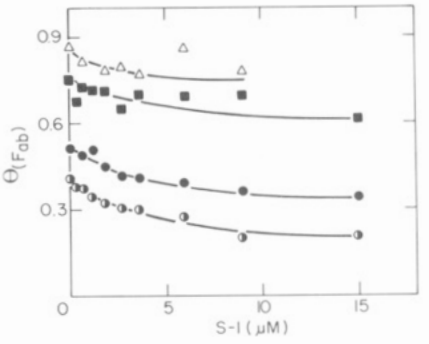

FIGURE 2: Binding of $F_{ab}(1-7)$ to actin in the presence of S-1 and the absence of nucleotides. The binding of F_{ab} to actin, $\theta_{(Fab)}$, was calculated from densitometric analysis of the same SDS–polyacrylamide gels which were used for the analysis of $\theta_{(S-1)}$ in the legend to Figure 1. All conditions and symbols are the same as in the legend to Figure 1.

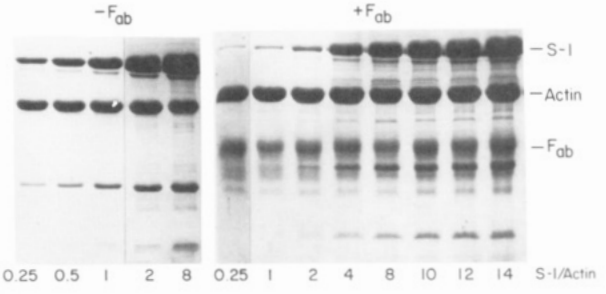

FIGURE 3: Binding of S-1·AMP·PNP to actin in the presence and absence of $F_{ab}(1-7)$. The representative electrophoretic patterns show airfuge-pelleted samples obtained from solutions of F-actin (3.0 μM), S-1 (between 0 and 45 μM), and, when present, $F_{ab}(1-7)$ (6.0 μM). Prior to centrifugation, these proteins were incubated in solutions containing 10 mM KCl, 4 mM MgAMP·PNP, and 10 mM imidazole, pH 7.0 at 23 °C, for 20 min. The molar ratios of S-1 to actin are indicated in the bottom line.

$F_{ab}(1-7)$ was found to decrease somewhat the binding of S-1 to actin under rigor conditions, but no attempt was made to quantify this effect. Because of the large difference between the effects of $F_{ab}(1-7)$ on the interactions of S-1 (Miller et al., 1987) and S-1·ATP (DasGupta & Reisler, 1989) with actin, the binding of S-1 to actin in the presence of these antibodies was examined in greater detail in the present work.

The rigor binding of S-1 to actin in the presence of different amounts of $F_{ab}(1-7)$ is shown in Figure 1. All binding data show a typical hyperbolic dependence on S-1 concentration irrespective of the presence or absence of $F_{ab}(1-7)$ in the solution. In the absence of F_{ab}, and in agreement with previous measurements (Greene & Eisenberg, 1980), the binding of S-1 to actin is represented best by a curve calculated for $K_a = 2 \times 10^7$ M^{-1}. In the presence of 1.5 μM F_{ab}, about 90% of actin sites could bind S-1 with a high affinity (Figure 1). The best fit for this binding was obtained with a theoretical curve corresponding to an apparent association constant $K_a = 5 \times 10^6$ M^{-1}. At higher F_{ab} concentrations, between 75% (at 3.0 μM F_{ab}) and 65% (at 9.0 μM F_{ab}) of actin sites had a high affinity for S-1 (Figure 1). Because of relatively small changes in the affinity of S-1 for actin over this range of F_{ab} concentrations (between 3.0 and 9.0 μM F_{ab}) and the limited resolution of the data, the binding of S-1 to actin was approximated by a single binding curve ($K_a = 2 \times 10^6$ M^{-1} for 70% of actin sites).

The results of parallel measurements of $F_{ab}(1-7)$ binding to actin in the presence of S-1 are shown in Figure 2. For all F_{ab} concentrations displayed here (1.5, 3.0, 6.0, and 9.0 μM in an ascending order of curves), the addition of S-1 caused partial dissociation of the antibodies from actin. In relative terms, the release of $F_{ab}(1-7)$ from actin appeared more pronounced at lower saturations of actin by antibodies and over the initial range of S-1 concentrations (up to 5 μM), where most of the myosin binding to actin was accomplished (Figure 1). The partial release of F_{ab} from actin in the presence of S-1 agrees with the previous finding of a severalfold decrease in the binding constant of F_{ab} for actin in the presence of S-1 (Miller et al., 1987). Notably, also, and as shown in that study, the combined binding of F_{ab} and S-1 to actin, $\theta_{(Fab + S-1)}$, exceeded significantly the molar ratio of 1.0 (F_{ab} + S-1) per actin monomer. At the higher concentrations of antibodies, the $\theta_{(Fab + S-1)}$ value was between 1.30 and 1.50, suggesting the binding of S-1 and F_{ab} to the same actin molecules.

Binding of S-1 to Actin in the Presence of Nucleotides and $F_{ab}(1-7)$. Figure 3 shows representative SDS gels of S-1·AMP·PNP pelleted with actin in the presence and absence of $F_{ab}(1-7)$. In the absence of antibodies, the binding of S-1·AMP·PNP to actin followed the expected pattern and increased progressively with S-1 concentrations. Surprisingly, $F_{ab}(1-7)$ not only inhibited the binding of S-1·AMP·PNP to actin but also changed its dependence on the concentration of S-1. These effects could be assessed better by examining the results of binding measurements of S-1·AMP·PNP to actin shown in Figure 4. In the absence of antibodies, the binding of S-1·AMP·PNP to actin was adequately described by a theoretical curve corresponding to a binding constant of 1.2×10^6 M^{-1} (Greene & Eisenberg, 1980). The binding of S-1–AMP·PNP to actin was greatly changed in the presence of F_{ab} (6 μM). This binding was inhibited strongly at low S-1 levels (about 50–100-fold) and was cooperatively "activated" or "switched on" with the increase in S-1 concentrations. Virtually the same sigmoidal pattern of S-1 binding to actin in the presence of F_{ab} was observed also for solutions containing 4.0 mM MgADP or $MgPP_i$ (Figure 4). In these latter two cases, the actual binding data and the cooperative effects were only marginally different from those corresponding to S-1·AMP·PNP interaction with actin in the presence of $F_{ab}(1-7)$.

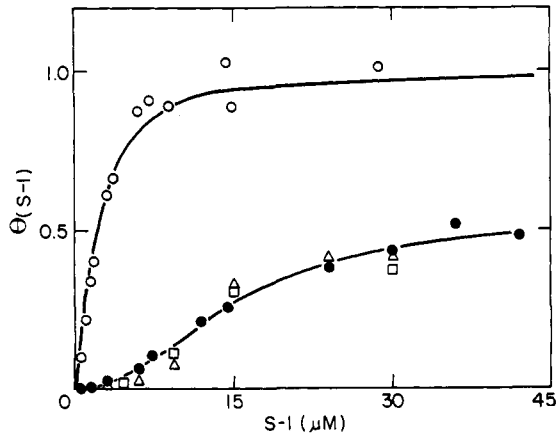

FIGURE 4: Binding of S-1·AMP·PNP, S-1·ADP, and S-1·PP_i to actin in the presence of $F_{ab}(1-7)$. F-Actin (3.0 μM) was incubated with S-1 (between 0 and 45 μM) in the absence (○) or presence of 6.0 μM F_{ab} (●, △, □) in solutions containing 10 mM KCl, 10 mM imidazole, pH 7.0, and 4.0 mM MgAMP·PNP (○, ●), MgADP (□), and $MgPP_i$ (△), respectively. These solutions were incubated at 23 °C for 20 min and then pelleted in a Beckman arifuge. The binding of S-1 to actin in the presence of nucleotides, $\theta_{(S-1)}$, was calculated from densitometric analysis of the pelleted samples run on SDS–PAGE. The upper curve (in the absence of F_{ab}) corresponds to a theoretical binding of S-1 to actin calculated by assuming a binding constant of 1.2×10^6 M^{-1}. The lower curve is a best fit to experimental $\theta_{(S-1)}$ values for the binding of S-1·AMP·PNP to actin in the presence of F_{ab} (6.0 μM).

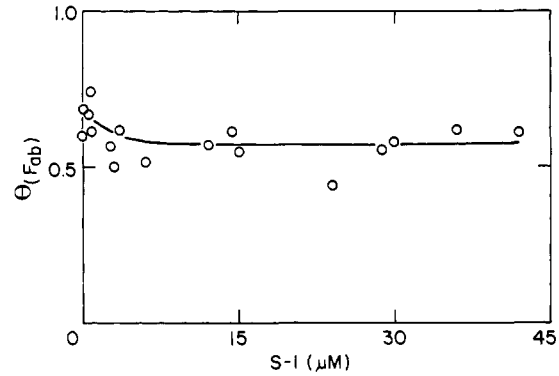

FIGURE 5: Binding of $F_{ab}(1-7)$ to actin in the presence of S-1 and MgAMP·PNP. The binding of F_{ab} to actin, $\theta_{(Fab)}$, was calculated from densitometric analysis of the same SDS–polyacrylamide gels which were used for the analysis of S-1·AMP·PNP binding to actin in the legend to Figure 4. All conditions are the same as in the legend to Figure 4.

In all cases, the binding of S-1·nucleotide to actin in the presence of F_{ab} (6.0 μM) appeared to saturate at between 0.4 and 0.5 S-1 bound per actin. However, this value depended on the amount of F_{ab} bound to actin (not shown).

Measurements of F_{ab} binding to actin in the presence of S-1·AMP·PNP revealed a small displacement of F_{ab} from actin by S-1 (Figure 5), not much different from that observed at similar F_{ab} concentrations in the absence of nucleotides (Figure 3). At high S-1·AMP·PNP levels, the binding of F_{ab} (6.0 μM) to actin decreased to about 0.6 F_{ab}/actin. Similar F_{ab} binding curves were obtained also in the presence of S-1·ADP and S-1·PP_i, with F_{ab} binding to actin leveling off at between 0.5 and 0.6 F_{ab}/actin at high S-1·nucleotide concentrations.

In contrast to the binding equilibria between F_{ab}, S-1, and actin under rigor conditions (no nucleotides), the combined binding of F_{ab} and S-1·nucleotide to actin saturated at close to molar ratios of 1.0 (F_{ab} + S-1) per actin monomer (Figures 4 and 5). This relationship between F_{ab} and S-1·nucleotide binding to actin was maintained at various levles of actin saturation by F_{ab} (not shown).

Discussion

Earlier chemical cross-linking (Sutoh, 1982a,b) and NMR studies (Moir et al., 1987) mapped one of the rigor actomyosin interaction sites to the N-terminal acidic residues on actin. Subsequent immunochemical work (Mejean et al., 1987; Miller et al., 1987) and experiments with S-1 coupled to residues 1–28 on actin (Labbe et al., 1990) have revealed that while this particular contact between myosin and actin may contribute to their rigor binding to each other, it is not essential for such binding. This semiquantitative conclusion has been verified in the present work by direct measurements of S-1 binding to actin in the presence of $F_{ab}(1-7)$. The antibodies decreased the binding of S-1 to actin, but not by a large factor. At 75–80% saturation of actin by F_{ab}, the binding constant of S-1 to actin is decreased by about 10–20-fold, a change which is frequently observed upon single-residue modification of these proteins. The fact that a similar decrease in S-1 binding to actin is noted already at 40% saturation of actin by F_{ab} (at 3.0 μM F_{ab}) suggests an overall, F_{ab}-induced change in the affinity of actin for S-1. However, the differences between the affinities of S-1 for actin bound to F_{ab} and its F_{ab}-free neighbors may be beyond the resolution of the present data. On the basis of the overall binding of F_{ab} and S-1 to actin [$\theta_{(Fab + S-1)} > 1.0$], our results confirm also the earlier suggestion that F_{ab} and S-1 can bind to the same actin monomers in the filament (Miller et al., 1987).

Taken together, the binding study results show that the N-terminal segment of actin does not make a major contribution to rigor actomyosin binding. Yet, the F_{ab}-induced change in S-1 binding to actin observed in this work and the reduction of F_{ab} binding to actin by S-1 (Miller et al., 1987) are not inconsistent with some direct or indirect involvement of the actin's N-terminus in rigor actomyosin interactions. It remains to be clarified whether the blocking of actomyosin contact at that site by F_{ab} does not result in any secondary changes at other postulated myosin binding sites on actin (Labbe et al., 1990).

An obvious concern when using F_{ab} as a probe of macromolecular interactions is the antibody's size and its possible steric interference with the interacting proteins. Steric exclusion between F_{ab} and S-1 and the "crowding" of these proteins on actin may indeed be responsible for the apparent loss of up to 25 or 35% of the high-affinity binding sites for S-1 on actin in the presence of F_{ab} (Figure 1). Obviously, this loss could be due to conformational changes induced in actin by F_{ab}. That geometric factors do not rule out the combined binding of S-1 and F_{ab} to actin should be perhaps attributed in part to the surface location (DasGupta et al., 1990; Kabsch et al., 1990; Holmes et al., 1990) and the flexibility (Barden & DosRemedios, 1983) of the N-terminal segment on the actin filament. The fact that F_{ab} can assume different orientations with respect to the actin filament (DasGupta et al., 1990) may help also in accommodating S-1 and F_{ab} on actin.

More importantly, the antibodies reveal a nucleotide-induced change in the acto–S-1 interface. As observed earlier for the binding of S-1·ATP to actin (DasGupta & Reisler, 1989), F_{ab} inhibits strongly the binding of all S-1·nucleotide complexes to actin. Strikingly, this inhibition is pronounced at low S-1 concentrations and is partially and cooperatively released with the increase in S-1 concentrations without much displacement of F_{ab}. Because of this, the inhibition of S-1·nucleotide binding to actin by $F_{ab}(1-7)$ cannot be explained by steric factors. The simplest analogy to the present system is that of actin "switched off" or "down-regulated" by regulatory proteins

(Bremel et al., 1972; Greene, 1982; Williams & Green, 1983; Lehrer & Morris, 1982). The inhibition of S-1·nucleotide binding to regulated actin (at low S-1 concentrations) is reversed cooperatively upon fractional saturation of S-1 binding sites on actin by S-1·nucleotide (Greene, 1982). The implication of this analogy to the $F_{ab}(1-7)$, S-1·nucleotide, and actin system is that the antibodies mimic in a way the function of regulatory proteins and "switch off" the affinity of actin for S-1·nucleotide (and much less so for S-1). As with regulated actin, the "inhibited" actin can be "switched-on" by the binding of S-1. In contrast to the regulated actin, the inhibitory effect of F_{ab} is not propagated along the filament by another protein (tropomyosin) but must result from changes induced directly on actin.

Another important difference between the binding of S-1 and S-1·nucleotide to actin in the presence of $F_{ab}(1-7)$ is in the overall binding saturation of actin, $\theta_{(Fab + S-1)}$, by these proteins. In the presence of nucleotides, $\theta_{(Fab + S-1)}$ saturates at values close to 1.0. This suggests (but does not prove) that S-1·nucleotide complexes, unlike S-1, do not bind readily to actin molecules bound to $F_{ab}(1-7)$. The sigmoidal changes in the binding of S-1·nucleotide to actin would appear then to occur mostly (if not exclusively) on the F_{ab}-free actin molecules in the filament (between 40 and 50% of total actin in Figures 4 and 5). Irrespective of the validity of this interpretation, the binding experiments in the presence of F_{ab} show a nucleotide-induced transition in the acto–S-1 interface. Either a direct contribution of actin's N-terminal segment or that of another part of actin perturbed by $F_{ab}(1-7)$ is considerably more important for the binding of S-1 to actin in the presence than in the absence of nucleotides. A similar conclusion was reached earlier with respect to S-1·ATP binding to actin (DasGupta & Reisler, 1989).

The most interesting question raised by the results of this work is how far can the analogy between the inhibition of actomyosin ATPase activity by regulatory proteins and $F_{ab}(1-7)$ be extended. There is evidence from chemical cross-linkings, NMR studies, and immunochemical work that troponin I (Grabarek & Gergely, 1987; Levine et al,. 1988), tropomyosin (Grabarek & Gergely, 1990), and caldesmon (Patchell et al., 1989; Bartegi et al., 1990; Adams et al., 1990; Levine et al., 1990) ineract with the N-terminal segment of actin. These findings prompted the speculation that the N-terminal segment of actin is involved in the regulation of actomyosin interactions. The fact that antibodies which bind to the same region on actin induce some of the changes and responses elicited by troponin and tropomyosin and compete effectively with caldesmon (Adams et al., 1990) adds credence to such speculations.

Registry No. MgAMPPNP, 69977-25-9; MgADP, 7384-99-8; MgPPi, 20768-12-1.

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Functional Characterization of Lanthanide Binding Sites in the Sarcoplasmic Reticulum Ca^{2+}-ATPase: Do Lanthanide Ions Bind to the Calcium Transport Site?†

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ABSTRACT: Gd^{3+} binding sites on the purified Ca^{2+}-ATPase of sarcoplasmic reticulum were characterized at 2 and 6 °C and pH 7.0 under conditions in which $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ specifically labeled the calcium transport site and the catalytic site of the enzyme, respectively. We detected several classes of Gd^{3+} binding sites that affected enzyme function: (a) Gd^{3+} exchanged with $^{54}Mn^{2+}$ of the ^{54}MnATP complex bound at the catalytic site. This permitted slow phosphorylation of the enzyme when two Ca^{2+} ions were bound at the transport site. The Gd^{3+} ion bound at the catalytic site inhibited decomposition of the ADP-sensitive phosphoenzyme. (b) High-affinity binding of Gd^{3+} to site(s) distinct from both the transport site and the catalytic site inhibited the decomposition of the ADP-sensitive phosphoenzyme. (c) Gd^{3+} enhanced 4-nitro-2,1,3-benzoxadiazole (NBD) fluorescence in NBD-modified enzyme by probably binding to the Mg^{2+} site that is distinct from both the transport site and the catalytic site. (d) Gd^{3+} inhibited high-affinity binding of $^{45}Ca^{2+}$ to the transport site not by directly competing with Ca^{2+} for the transport site but by occupying site(s) other than the transport site. This conclusion was based mainly on the result of kinetic analysis of displacement of the enzyme-bound $^{45}Ca^{2+}$ ions by Gd^{3+} and vice versa, and the inability of Gd^{3+} to phosphorylate the enzyme under conditions in which GdATP served as a substrate. These results strongly suggest that Ln^{3+} ions cannot be used as probes to structurally and functionally characterize the calcium transport site on the Ca^{2+}-ATPase.

The Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR)[1] utilizes the magnesium-ATP complex as a physiological substrate to drive active transport of Ca^{2+} across the SR membrane (Vianna, 1975; Martonosi & Beeler, 1983). For the rapid turnover of the ATPase, high-affinity binding of 3 mol of divalent cations is minimally required, of which 2 mol is Ca^{2+} ions bound at the calcium-specific transport site while the remainder is 1 mol bound at the catalytic site as a component of the divalent cation-ATP complex (Shigekawa et al., 1983b; Ogurusu et al., 1991). The divalent cation at the catalytic ATP site remains bound until the phosphoenzyme intermediate is hydrolyzed. This divalent cation presumably determines the catalytic rate of each reaction step of ATP hydrolysis (Shigekawa et al., 1983b; Ogurusu et al., 1991). In addition to these high-affinity sites, several classes of low-affinity sites for divalent cations have been reported for the Ca^{2+}-ATPase (Ikemoto, 1974; Kalbitzer et al., 1978; Guillain et al., 1982; Loomis et al., 1982; Champeil et al., 1983; Highsmith & Head, 1983; Wakabayashi et al., 1986, 1987, 1990b), although the functional roles of some of these sites remain unclear.

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[1] Abbreviations: SR, sarcoplasmic reticulum; Ln^{3+}, lanthanide ion(s); Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole.

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