Myosin Regulatory Light Chain and Nucleotide Modulation of Actin Binding Site Electric Charge†

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ABSTRACT: The ionic strength dependence of skeletal muscle myosin subfragment 1 (S1) binding to actin in the presence of ADP and ATP was measured for S1 with either only an essential light chain [S1(elc)] or with both an essential and the regulatory light chains [S1(elc,rlc)] bound. The data were analyzed to determine the apparent association constant, K_A, for actin binding and the absolute value of the product of the net effective electric charges at the actin−myosin interface, $|z_M z_A|$. When MgADP is bound at the myosin active site, K_A values at 0 M ionic strength for S1(elc) and S1(elc,rlc) are 12 and 4.9×10^6 M^{-1}, respectively, and $|z_M z_A|$ values are 3.9 ± 0.3 and 3.6 ± 0.2 esu^2. In the presence of ATP, K_A values at 0 M ionic strength for S1(elc) and S1(elc,rlc) are 81 and 7.3×10^4 M^{-1}, respectively, and $|z_M z_A|$ values are 14.7 esu^2 for S1(elc) but only 6.4 esu^2 for S1(elc,rlc). The Michaelis constant, K_M, for the actin activation of S1 steady-state MgATPase activity was significantly smaller for S1(elc), consistent with its greater K_A and $|z_M z_A|$. These data indicate that the regulatory light chain can allosterically regulate the interactions of myosin and actin by modulating the electric charge at the actin binding site. K_A and $|z_M z_A|$ were also measured at 25 °C for S1(elc,rlc) binding to actin in the presence of the ATP analog ATPγS. At 0 M ionic strength, K_A is 8.0×10^4 M^{-1}, and $|z_M z_A|$ is 0, within experimental uncertainty, suggesting that for S1·MgATP the electric charge at the actin binding site is abolished. The results are interpreted in terms of possible roles of electrostatic interactions in mechanisms for S1·MgATP dissociating from one actin and S1·MgADP·P_i being guided electrostatically to bind to another.

The myosin ATP site has primary control over structural changes at the actin binding site. During the contractile cycle, the changing nucleotide structure induces changes in actin affinity and the overall structure of the actin−myosin−nucleotide complex. The structural changes generate force while myosin is bound to actin (Huxley, A. F., 1957; Huxley, H. E., 1969), and the affinity changes introduce a dissociation step into the contractile cycle (Lymn & Taylor, 1971). Although neither myosin light chain is required for actin-activated activity (Wagner & Giniger, 1981), the myosin regulatory light chain (rlc)[1] does exert control over the actin binding site structure. In smooth muscle (Trybus, 1991), molluscan muscle (Szent-Gyorgyi, 1996), and many nonmuscle myosins (Korn & Hammer, 1988), the rlc is the primary site of regulation of the contractile cycle. Control over myosin−actin interactions in these cases is due to calcium binding or phosphorylation of the regulatory light chain. Skeletal muscle is primarily regulated at the thin filament (Leavis & Gergely, 1984), but the rlc has important secondary regulatory roles involving modulation of the rate or duration of force development in response to phosphorylation (Sweeney et al., 1993) and changes in calcium concentration (Szczesna et al., 1996).

The interactions between the rlc, the ATP site, and the actin binding site on myosin provide an interesting allosteric system. The three interacting sites are distant from one another on the myosin motor domain (Botts et al., 1984; dosRemedios & Moens, 1995), and now that the atomic structure of myosin subfragment (S1) is known (Rayment et al., 1993b), the precise locations of the sites can be assigned (Figure 1). This spatial arrangement appears to be similar for many myosins, from muscle and nonmuscle sources, whether primary regulation is at the thin or thick filament.

It has long been known that nucleotide-driven structural changes at the actin−myosin interface involve both electrostatic and hydrophobic interactions (Tonomura et al., 1962). Electrostatic interactions appear to dominate in the earliest binding steps (Geeves & Conibear, 1995), and several electrically charged structures at the interface have been identified tentatively (Rayment et al., 1993a; Miller & Reisler, 1995). A portion of the S1 structure that is particularly likely to contribute to the electrical charge at the actin binding site is thought to be the 632−646 sequence of the myosin heavy chain. This presumably flexible loop has five positively charged amino acid side chains (Tong & Elzinga, 1990), is protected from trypsinolysis by actin binding (Mornet et al., 1979; Yamamoto & Sekine, 1979), and can be chemically cross-linked to actin (Mornet et al., 1981). Complementary structures that bind this segment inhibit actin binding exclusively to S1·MgADP·P_i (Chaussepied & Morales, 1988; Chausspied, 1989). On the basis of the actin activation and *in vitro* motility rates due to chimeric exchanges at this site on myosin, it has been postulated that it is the 632−646 loop in skeletal muscle

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[1] Abbreviations: S1, myosin subfragment 1; S1A1, S1 with only the larger alkali (or essential) light chain bound; S1A2, S1 with only the smaller alkali light chain bound; S1(elc), a mixture of S1A1 and S1A2; S1(elc,rlc), a mixture of S1 that has one alkali light chain and the regulatory light chain bound; K_A, apparent association constant; K_M, Michaelis constant; V_{max}, maximum steady-state MgATPase activity; $|z_M z_A|$, absolute value of the product of the net effective electric charges at the actin−myosin subfragment 1 binding interface.

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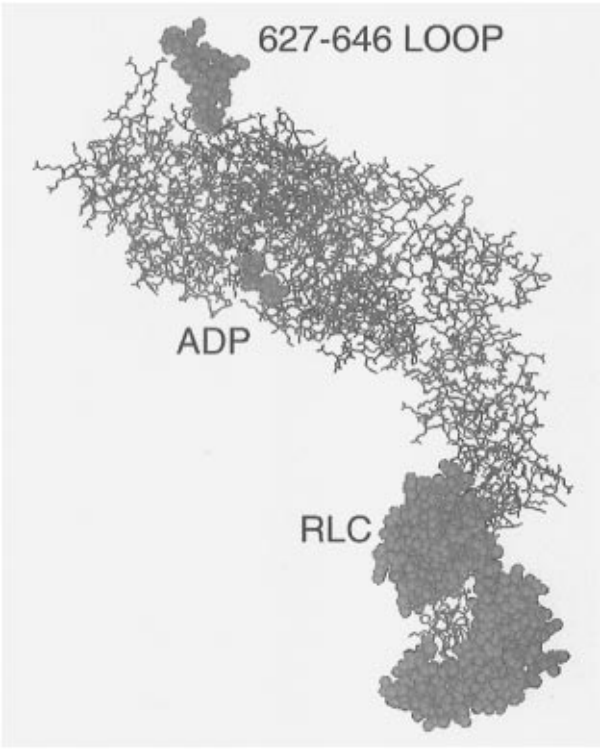

FIGURE 1: S1(elc,rlc) structure. The α-carbon coordinates from the Brookhaven Data Bank for chicken skeletal muscle myosin subfragment 1 [from Rayment et al. (1993b)] were modified by addition of heavy chain amino acids missing in the crystal structure but present in the primary sequence, of amino acid side chains, and of MgADP at the active site (Michael Lorenz, personal communication). The essential light chain is a truncated alkali light chain 2, consisting of 149 amino acids. The regulatory light chain has 166 amino acids. The structures of the regulatory light chain, ADP, and the heavy chain sequence 627−646 (a positively charged flexible loop that is part of the actin binding site) are the purple space-filled structures. The structures of the essential light chain and the rest of the heavy chain are the green lines.

myosin, and its equivalent in other myosins, that in fact determines the degree of actin-activated myosin ATPase activity for all myosins (Spudich, 1994).

The complementary cross-linked structure on actin is the 1−11 N-terminal sequence (Sutoh, 1982), which has a net electric charge of −5 (Collins & Elzinga, 1975). Antibodies to this structure block actin binding to S1·MgADP·P_i (DasGupta & Reisler, 1989). It has also been shown that the MgATPase activity of yeast S1 is increased more and less, respectively, by mutant actins with more and less negative electric charge at that location (Cook et al., 1992, 1993). Both sequences, 632−646 of myosin and 1−11 of actin, are too flexible for their coordinates to be determined by X-ray crystallography, but their positions can be localized in the atomic structures (Kabsch et al., 1990; Rayment et al., 1993b). Computer graphical docking of the S1 atomic structure onto an actin filament reconstructed from the actin−DNase 1 atomic structure suggests that myosin 632−646 and actin 1−11 are positioned to be among several potential electrostatic interactions (Rayment et al., 1993a).

The changes in the electrostatic interactions continue to be of interest because the formation of a primarily electrostatic and very flexible linkage between myosin 632−646 and actin 1−11 may be the first step in the sequence of reactions that generate force (Brenner et al., 1982; Raucher & Fajer, 1994; Geeves & Conibear, 1995; Thomas et al., 1996). For this reason, it is important to know the degree to which both the myosin ATP site and the its regulatory light chain affect the electric charge at the actin binding site. Quantitative measurements have been made of the electric charge at the interface of actin and S1 with only the larger essential light chain bound, S1A1 (Highsmith, 1990; Highsmith & Murphy, 1992). To gain more information about control of the electrostatic interactions at the interface, the ionic strength dependencies of actin binding to S1 with either only an essential light chain [S1(elc)] or with both an essential and the regulatory light chain [S1(elc,rlc)] were measured in the presence of ATP and ADP. The data are used to estimated changes in the actin affinity, K_A, and in the net effective electric charge, $|z_M z_A|$, at the actin−S1 interface, which are due to the presence of the rlc, for A·S1·MgADP·P_i and A·S1·MgADP. K_A and $|z_M z_A|$ were also measured for actin binding to S1(elc,rlc) in the presence of the ATP analog ATPγS, in order to obtain information relevant to the S1·MgATP complex.

MATERIALS AND METHODS

Proteins. Myosin was isolated from New Zealand rabbit dorsal muscle (Nauss et al., 1969). S1 with both elc and rlc bound was prepared from myosin in the presence of Mg^{2+} using papain (Margossian & Lowey, 1973) and purified by size exclusion chromatography (Sephacryl S-400) followed by anion-exchange chromatography (DE-52) (Weeds & Taylor, 1975). The final sample, S1(elc,rlc) is a mixture of S1 with one of either alkali light chain 1 or 2, and the regulatory light chain. Papain leaves the rlc intact but partially cleaves the heavy chain and removes entirely the N-terminus of the larger elc (Margossian et al., 1975). S1 with only an elc bound was prepared from myosin in the presence of EDTA using α-chymotrypsin and purified by the same method used for the papain preparation. The final sample, S1(elc), was a mixture of about equal amounts of S1 with either alkali chain 1 or 2, which was used to make comparisons with the S1(elc,rlc) mixture. Measurements were made using mixtures because methods for purifying adequate amounts of skeletal muscle S1 with rlc and one specific elc bound are not available. The S1 samples were dialyzed exhaustively against 10 mM MOPS and 2 mM Mg-$(OAc)_2$, pH 7 at 4 °C, and kept on ice until used in a measurement. Steady-state MgATPase activities were 0.040−0.050 s^{-1} at 25 °C, pH 7.0. F-Actin was isolated from acetone-extracted muscle tissue (Spudich & Watt, 1971) and purified by size exclusion chromatography (Sephadex G-150) (MacLean-Fletcher & Pollard, 1980). After polymerization, F-actin was pelleted by centrifugation at 80000*g* for 3 h, and the pellet was homogenized in the MOPS−$Mg(OAc)_2$ buffer before use. Protein concentrations were determined by absorbance measurements at 280 nm, correcting for light scattering. Chemicals were of the highest commercial grades available.

MgATPase Measurements. For determining K_M, steady-state MgATPase activities for S1 and actin−S1 were determined spectrophotometrically at 25 °C, pH 7.0, using a coupled assay system (Imamura et al., 1966). For verifying that adequate levels of ATP or ATPγS remained during a binding measurement, actin−S1 activities were determined using a phosphomolybdate method (Lin & Morales, 1977).

Association Constants. The fractions of bound and free S1 in the presence of F-actin were determined by a cosedimentation method, except that intrinsic tryptophan

fluorescence was used to determine [S1] instead of the radioactivity of an extrinsic label (Marston & Weber, 1975). Solutions with final concentrations of 0.5−5 μM S1 and 2−50 μM F-actin were prepared from stock solutions to obtain a final volume of 400 μL. Higher ionic strengths were obtained by including the appropriate volume of 1 M KOAc [in Mops−$Mg(OAc)_2$ buffer]. For measurements in the presence of MgATP or MgATPγS, the magnesium-nucleotide complex [100 mM in MOPS−$Mg(OAc)_2$ buffer] was included to obtain an adequate final concentration. MgATPase or MgATPγS activities were measured for identical preparations to ensure that [MgATP] or [MgATPγS] remained high enough to saturate the S1 active site throughout the course of the binding measurement. Samples were prepared at 25 °C and centrifuged at 50 000 rpm for 15−60 min at 25 °C in a TLA-100 rotor using a temperature-controlled desktop ultracentrifuge (Beckman) to remove actin and bound S1. The free [S1] in the supernate was determined by fluorescence intensity of 200 μL aliquots taken from the acto−S1 samples and diluted to 1 mL (excitation at 279 nm, emission at 335 nm). Standard samples containing only S1 and only actin were included in every centrifugation run and used to determine the fluorescence intensity for total [S1] and the correction for any unsedimented actin, respectively. The apparent association constant, K_A, was calculated from {[total S1] − [free S1]}/{[free S1][free actin]}.

Data Analysis. The reversible equilibrium binding of the solutes M and A

$$M + A = M{\cdot}A \tag{1}$$

is described by the expression

$$K = a_{MA}/a_M a_A = (c_{MA}/c_M c_A)(\gamma_{MA}/\gamma_M\gamma_A) \tag{2}$$

where K is the association constant, a is chemical activity, c is concentration, and γ is the activity coefficient. The ratio of the concentrations is K_A, the apparent association constant, which usually is measured. For ions M and A binding in solution, $\gamma_{MA} = 1$ and $\gamma_M\gamma_A$ is represented as $\gamma_\pm$, which has values that decrease from 1 as the ionic strength increases. Thus, eq 2 can be written

$$\ln K = \ln K_A - \ln \gamma_\pm \tag{3}$$

which describes the lowered K_A in terms of K, the value of K_A at 0 M strength = 0, and the activity coefficient of the ions. The greater the charges on M and A, the stronger the dependence of K_A on the ionic strength of the solution, I.

The original theory of Debye and Huckel for the effect of ionic strength on the chemical activity of ions in low ionic strength solution was modified to be applicable at all ionic strengths (Pitzer, 1979). It has the following expression relating $\gamma_\pm$ and I:

$$\ln \gamma_\pm = -0.392|z_M z_A|[I^{1/2}/(1 + bI^{1/2}) + (2/b)\ln(1 + bI^{1/2})] + m\{2\beta(0) + [2\beta(1)/\alpha^2 I][1 - (1 + \alpha I^{1/2} - \alpha^2 I/2)\exp(-\alpha I^{1/2})]\} + m^2(3C/2) \tag{4}$$

where $|z_M z_A|$ is the absolute value of the product of the net effective electric charges on myosin (z_M) and actin (z_A) at their binding sites, m is the molar concentration of the solution, $b = 1.2$ and $\alpha = 2.0$ for all electrolytes, and $\beta(0)$, $\beta(1)$, and C are parameters that were determined experimentally for KOAc (Pitzer & Mayorga, 1973). Equation 4 can be used with eq 3, above, to fit data from measurements of K_A that are made over any range of I and has been used to obtain estimates of the values of K at 0 M ionic strength and $|z_M z_A|$ for acto−S1 interactions (Highsmith, 1990; Highsmith & Murphy, 1992; Kirshenbaum et al., 1993). Myosin and actin are very large molecules compared to the ionic species for which the theory has been tested rigorously (Pitzer, 1979), which is why z_M and z_A are net effective electric charges at the myosin and actin surfaces of the interface.

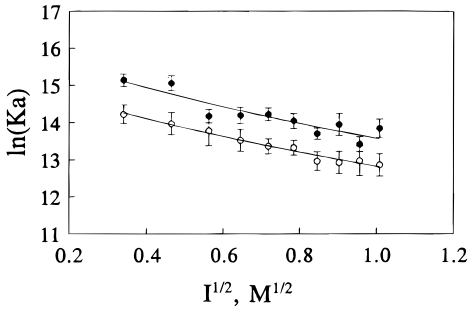

FIGURE 2: Ionic strength dependence of S1(elc) and S1(elc,rlc) binding to F-actin in the presence of MgADP. The fractions of free and bound S1−nucleotide complexes in the presence of actin were determined by a cosedimentation technique and used to calculate the apparent association constant, K_A (see Materials and Methods). The natural logarithm of K_A for the binding of S1(elc)·MgADP (filled circles) and S1(elc,rlc)·MgADP (open circles) is plotted against the square root of the solution ionic strength. KOAc was added to ~1 μM S1 and 3−5 μM actin in 10 mM MOPS, 1 mM ADP, and 2 mM $MgCl_2$, pH 7 at 25 °C, to adjust the ionic strength. The solid lines are best fits of the equation of Pitzer (Pitzer, 1979; Highsmith, 1990) to obtain the product of the net effective electric charges on actin and S1 at the interface, and the apparent association constant at zero ionic strength, where the electrostatic interactions are least shielded by counterions. The best fit to the data gave $|z_M z_A| = 3.9$ and 3.6 esu^2, and $K_A(0\ M) = 1.2 \times 10^7\ M^{-1}$ and $4.9 \times 10^6\ M^{-1}$, for S1(elc)·MgADP and S1(elc,rlc)·MgADP, respectively.

RESULTS

Light Chain Effects on S1·MgADP·P and S1·MgADP Binding to Actin. The association constant for F-actin binding to S1 when MgADP was bound in the ATP site was determined in solutions that had the ionic strength adjusted between 0.1 and 1.0 M by added KOAc. The data were analyzed as described above in Materials and Methods. K_A at 0 M ionic strength is $1.2 \times 10^7\ M^{-1}$ for S1(elc) and $4.9 \times 10^6\ M^{-1}$ for S1(elc,rlc). A comparable reduction in K_A due to the presence of the rlc was observed over the entire ionic strength range (Figure 2). This decrease in K_A corresponds to a small, 7−8%, decrement in the standard free energy of actin binding to S1·MgADP when the rlc is present.

The values of $|z_M z_A|$ for the actin interface when S1(elc) and S1(elc,rlc) are bound are 3.9 and 3.6 esu^2, respectively (Table 1). Although $|z_M z_A|$ is somewhat larger for S1(elc), as is K_A, the $|z_M z_A|$ values are nearly equal. Taken together, the K_A and $|z_M z_A|$ values indicate that the presence of the rlc has a small or negligible effect on the actin binding site when MgADP is in the active site.

K_A at 0 M ionic strength for S1(elc) binding to actin is equal, within experimental error, to the value measured under similar conditions for S1A1 (S1 with only the larger essential light chain) (Highsmith, 1990; Highsmith & Murphy, 1992). The value of $|z_M z_A|$ for S1(elc), on the other hand, is substantially smaller than 7 esu^2, which was measured for

Table 1: Regulatory Light Chain Effects on Actin Binding Site[a]

	S1(elc)	S1(elc,rlc)
N = ADP		
K_A(0 M) (in M^{-1})	$(1.2 \pm 0.2) \times 10^7$	$(4.9 \pm 0.6) \times 10^6$
$\|z_M z_A\|$ (in esu^2)	3.9 ± 0.3	3.6 ± 0.2
N = ATP		
K_A(0 M) (in M^{-1})	$(8.1 \pm 0.6) \times 10^5$	$(7.3 \pm 0.8) \times 10^4$
$\|z_M z_A\|$ (in esu^2)	14.7 ± 0.9	6.4 ± 0.5

[a] The ionic strength dependencies of S1(elc) and S1(elc,rlc) binding to actin at 25 °C in the presence of MgADP and MgATP were measured (Figures 2 and 3) and analyzed to determine the apparent association constant, K_A, at 0 M ionic strength and the absolute value of the product of the net electric charge at the actin−S1 binding site, $|z_M z_A|$ (see text).

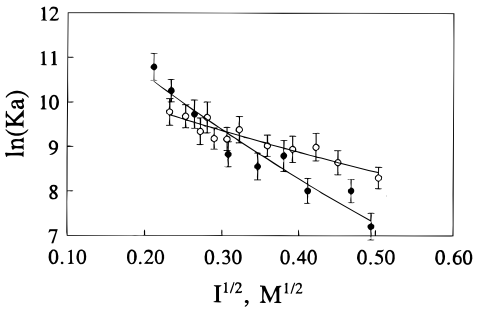

FIGURE 3: Ionic strength dependence of S1(elc) and S1(elc,rlc) binding to F-actin in the presence of MgATP. The natural logarithm of the apparent association constant for the binding of S1(elc)·MgADP·P_i (filled circles) and S1(elc,rlc)·MgADP·P_i (open circles) is plotted against the square root of the solution ionic strength. KOAc was added to ~5 μM S1 and ~50 μM actin in 10 mM MOPS, 2 mM $MgCl_2$, and 6−10 mM MgATP, pH 7 at 25 °C, in order to adjust the ionic strength. The analysis of the data to obtain $|z_M z_A|$ and K_A was as in Figure 2 (see text). The best fit to the data gave $|z_M z_A| = 14.7$ and 6.4 esu^2, and $K_A(0\ M) = 8.1 \times 10^5\ M^{-1}$ and $7.3 \times 10^4\ M^{-1}$, for S1(elc)·MgADP and S1(elc,rlc)·MgADP, respectively.

S1A1 (Highsmith, 1990; Highsmith & Murphy, 1992). This reduction of ~3 esu^2 in $|z_M z_A|$ is likely due to the inclusion of S1A2 in the S1(elc) preparation, which appears to have a much lower net effective electric charge. Actin binding to S1A2 is less sensitive to ionic strength than S1A1 (Chalovich et al., 1984), which is consistent with the observed lower $|z_M z_A|$ value for the mixture.

When ATP was present at concentrations high enough to maintain the steady state intermediate S1·MgADP·P_i during the actin binding measurement, the presence of the regulatory light chain had a significant effect on both K_A and $|z_M z_A|$. At 0 M, K_A is $8.1 \times 10^5\ M^{-1}$ for S1(elc) and $7.3 \times 10^4\ M^{-1}$ for S1(elc,rlc), but at high ionic strengths, the relative affinities are reversed (Figure 3). At 0 M ionic strength, the difference in the association constants for S1(elc)·MgADP·P_i and S1(elc,rlc)·MgADP·P_i corresponds to a standard free energy change of interaction, $\Delta\Delta G°$, of 1.3 kcal/mol for the interaction between the rlc binding site and the actin binding site, if one assumes that rlc is a ligand for S1(elc)·MgADP·P_i.

The values of $|z_M z_A|$ are 14.7 esu^2 for S1(elc)·MgADP·P_i and 6.4 esu^2 for S1(elc,rlc)·MgADP·P_i (Table 1). S1·MgADP·P_i has more electric charge than S1·MgADP at the actin binding site for both S1(elc) and S1(elc,rlc), but the addition of the rlc to S1(elc) when ATP is bound to the active site reduces the electric charge on the actin site by a factor of 2.3. Unlike the ADP case, the rlc has a significant allosteric effect on K_A and $|z_M z_A|$ when ADP·P_i is bound to S1.

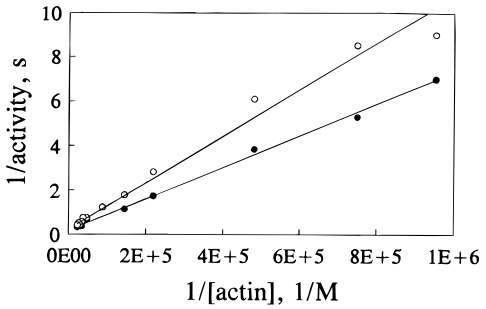

FIGURE 4: Michaelis−Menten plots for the actin activation of S1(elc) and S1(elc,rlc). The MgATPase activities of 1 μM S1(elc) or S1(elc,rlc) in 10 mM MOPS, 10 mM KOAc, 2 mM $MgCl_2$, 0.25 mM EDTA, and 90 μM ATP (maintained by a backup system), pH 7 at 25 °C, were measured in the presence of increasing [actin]. The solid lines are best fits of the Lineweaver−Burk equation to the data to obtain K_M, the free [actin] that activates the MgATPase activity to 50% of maximum (V_{max}). For S1(elc) (filled circles), K_M and V_{max} were $(1.2 \pm 0.3) \times 10^{-6}$ M and $5.9 \pm 0.8\ s^{-1}$, respectively, and for S1(elc,rlc) (open circles), they were $(2.5 \pm 0.5) \times 10^{-6}$ M and $4.1 \pm 0.7\ s^{-1}$.

Comparing actin binding for the mixture S1(elc) to that of purified S1A1 in the presence of ATP, the value for K_A at 0 M ionic strength is somewhat smaller for S1(elc), but $|z_M z_A|$ is reduced from 17−19 esu^2 for S1A1 (Highsmith & Murphy, 1992; Kirshenbaum et al., 1993) to 14.5 esu^2 for S1(elc) (Table 1). This decreased electrostatic charge for S1(elc) compared to S1A1 in the presence of ATP is similar in magnitude to that observed with ADP (see above) and is also likely due to S1A2 having less positive charge at the actin binding site. The magnitudes of the $|z_M z_A|$ differences between S1A1 and S1(elc) are similar for the MgADP and MgADP·P_i cases.

Light Chain Effects on K_M for Actin Activation of S1 MgATPase Activity. The Michaelis constant, K_M, for the actin activation of S1 MgATPase activity is not a binding constant, but it can be used to estimate the strength of the interaction of S1·MgADP·P_i and actin. K_M measured at 25 °C in 10 mM MOPS, 2 mM $Mg(OAc)_2$, and 10 mM KOAc, pH 7.0, was 1.2×10^{-6} M for S1(elc) and 2.5×10^{-6} M for S1(elc,rlc) (Figure 4). The increased K_M for S1(elc,rlc) is consistent with its reduced K_A for actin binding in the presence of ATP at low ionic strength as determined by cosedimentation (Figure 3). The change in K_M demonstrates that the rlc modulation of the electrostatic charge at the actin binding site has functional kinetic consequences that may be related to the contractile cycle. Removing light chains from myosin has also been shown to change K_M and V_{max} for actin activation (Lowey et al., 1993), but the myosin results cannot be compared directly to those reported here.

Actin Site Electric Charge for S1(elc,rlc)·ATPγS. Myosin·MgATP is the first species that is formed when ATP binds to free myosin, and it, or a structure similar to it, is also the intermediate that dissociates from actin when ATP binds the actin−myosin rigor complex at the end of a contractile cycle. S1·MgATP is not stable, but the slowly hydrolyzed ATP analog, ATPγS, can be used to approximate S1·MgATP. S1(elc,rlc) is the S1 preparation that is structurally the most similar to the S1 portion of myosin *in vivo*, and the above data indicate that the absence of the rlc makes a significant difference in the nucleotide and actin interactions; so S1(elc,rlc) was used to investigate the electrostatic interactions of actin and S1·MgATPγS for comparison to the ADP and ADP·P_i results. Data were collected for S1(elc,rlc) in the presence of 1 mM ATPγS at 25 °C in solutions in the

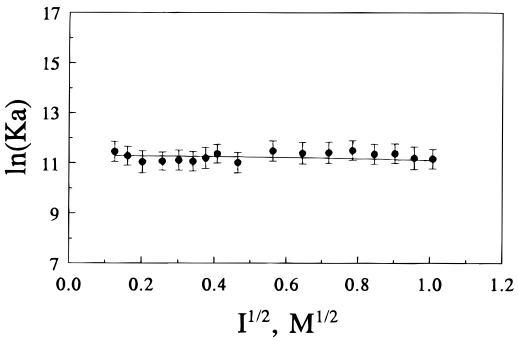

FIGURE 5: Ionic strength dependence of S1(elc,rlc) binding to F-actin in the presence of MgATPγS. The natural logarithm of K_A for the binding of S1(elc,rlc)·MgATPγS to actin is plotted against the square root of the solution ionic strength. KOAc was added to ~3 μM S1 and ~50 μM actin in 10 mM MOPS, 2 mM $MgCl_2$, and 1 mM MgATPγS, pH 7 at 25 °C, in order to adjust the ionic strength. The analysis of the data was as in Figure 2 (see text). The best fit to the data gave $|z_M z_A| = 0$ and $K_A(0\ M) = 8.0 \times 10^4\ M^{-1}$.

Table 2: Nucleotide Effects on Actin Binding Site of S1(elc,rlc)[a]

	nucleotide		
	ATPγS	ADP·P_i	ADP
K_A(0.2 M) (in M^{-1})	$(8.0 \pm 1) \times 10^4$	$(8.1 \pm 2) \times 10^3$	$(1.2 \pm 0.7) \times 10^6$
$\lvert z_M z_A \rvert$ (in esu^2)	0.0 ± 0.2	6.4 ± 0.5	3.6 ± 0.2

[a] The ionic strength dependencies of S1(elc,rlc) binding to actin at 25 °C in the presence of MgATPγS, MgATP, MgADP were measured (Figures 2, 3, and 5) and analyzed to determine the apparent association constant, K_A, at 0.20 M ionic strength and the absolute value of the product of the net electric charge at the actin−S1 binding site, $|z_M z_A|$ (see text).

0.10−1 M ionic strength range. Increasing [ATPγS] to 2 mM had only an ionic strength effect, indicating that the ATP site was saturated when S1 was bound to actin. The data were analyzed as described above for ATP and ADP. At 0 M ionic strength K_A for S1(elc,rlc)·MgATPγS is 8.0×10^{-4} M (Figure 5), which is near the value obtained for S1(elc,rlc)·MgADP·P_i. At higher ionic strengths, however, the actin affinity of S1(elc,rlc)·MgADP·P_i decreases while that of S1(elc,rlc)·MgATPγS does not (Figures 3 and 5). The value for $|z_M z_A|$ for S1(elc,rlc)·MgATPγS is 0, within experimental error (Table 2). This striking result suggests that when ATP binds to myosin, and presumably to actin−myosin, the electric charge on the myosin side of the actin−myosin interface is abolished.

Error Analysis. Stock solution protein concentrations were determined spectrophotometrically and are accurate within 1−2%. Dilution errors in making up the working solutions contribute another 2−3%. The individual activity measurements have ~5% error (SD). The error for K_M determinations is ~20%. For the association constant measurements, the random error was reduced to 10−15% by repeating measurements four to seven times. The error for the extrapolated K_A at 0 M ionic strength is larger, in the 15−60% range. There are several potential sources of systematic errors for the K_A measurements. One is structural modification of S1 by the different proteolytic enzymes used for its preparation. This error cannot be readily estimated quantitatively, and its possible effects are discussed below. Another potential source of systematic error is the exhaustion of the ligand in the ATP and ATPγS cases. Error from this source is small, as adequate ATP and ATPγS concentrations were verified after the actin-bound S1−nucleotide complex had been removed by centrifugation. For ATPγS, the fact that $|z_M z_A|$ is zero indicates that contributions from ATP or ADP contamination are negligible. For ATP, any error due to too low [ATP] would make the observe K_A too large, but it is in the range reported in earlier studies (Chalovich et al., 1981; Highsmith & Murphy, 1992). An additional potential problem for the ATP case is the possible contribution of actin-bound species other than S1·MgADP·P_i, which exist in the kinetic cycle. If other bound species do contribute, then the measured value of K_A is larger and the measured value of $|z_M z_A|$ is smaller than the true values, but contributions from other bound intermediates are thought to be small (Highsmith & Murphy, 1992). The statistically determined error for $|z_M z_A|$ is 7−10%.

DISCUSSION

Regulatory Light Chain Allosteric Interactions. The data indicate that the addition of the rlc to the S1(elc) complex modifies the nucleotide control of the actin binding site structure. The actin affinity for S1(elc,rlc)·MgADP is reduced by 60% compared to S1(elc)·MgADP, and the electric charge at the actin binding site is almost unchanged (Table 1). The effect the rlc on the S1(elc)·MgADP·P_i complex is greater. The actin affinity for S1(elc,rlc)·MgADP·P_i is reduced by 90% compared to S1(elc)·MgADP·P_i, and $|z_M z_A|$ is reduced by 57%. If the reasonable assumption is made that both S1(elc)·MgADP·P_i and S1(elc,rlc)·MgADP·P_i bind at the same site(s) on actin, then the reduction in $|z_M z_A|$ that occurs when the rlc is added indicates that the electric charge at the actin binding site of S1(elc,rlc)·MgADP·P_i is less than half of that of S1(elc)·MgADP·P_i. The decreased value of K_M measured for S1(elc) in low ionic strength solution, compared to S1(elc,rlc) (Figure 4), is consistent with S1(elc,rlc) having less electric charge at the actin site and lower actin affinity.

The changes in K_M, K_A, and actin binding site electric charge, caused by the presence or absence of the rlc, cannot be compared directly to an *in vivo* regulatory mechanism for myosin. *In vivo*, the rlc is always present. Its effects on the ATP and actin binding sites are due to rlc conformational changes caused by calcium binding or phosphorylation, which affect the conformation of the rest of the heavy chain−essential light chain complex. Nonetheless, the results shown here indicate that the rlc communicates with the ATP and actin binding sites. The rlc modulates their allosteric interactions, as well as the structure of the actin binding site, as others have reported [see Sweeney (1995) and Szczesna et al. (1996) and references therein]. The fact that the rlc effect is greater for S1·MgADP·P_i, the intermediate that first binds to actin, is consistent with rlc involvement in regulation of the initiation of the contractile cycle.

It is possible that proteolytic artifacts contribute to the differences shown here for the mixtures of S1(elc) and S1(elc,rlc). Such effects would be difficult to quantitate; however, unlike trypsin, neither papain nor α-chymotrypsin appears to damage the actin binding site (Yamamoto & Sekine, 1980); so decreases in K_M and K_A due to cleaving the 632−646 loop (Bobkov et al., 1996) are not likely to be a problem here. The observation that S1(elc,rlc) prepared with or without actin bound, to protect the actin binding site, has the same actin affinity (Highsmith, 1976) is consistent with papain not modifying the actin binding site. Nonetheless, the possibility that proteolysis contibutes the differences

observed for S1(elc) and S1(elc,rlc) cannot be ruled out entirely. Papain does remove part of the N-terminal portion of the larger elc, A1 (Margossian et al., 1975). This segment can be cross-linked to actin (Sutoh, 1982), suggesting that it may spend some time in the actin binding site. Related to this, a drag effect caused by A1 positive charge binding to actin has been proposed (Sweeney, 1996) to explain why S1A1 is less activated by actin (Wagner & Weeds, 1977) and has lower *in vitro* motility (Lowey et al., 1993) than S1A2. Solution studies suggest that the A1−actin interaction may only occur for rigor binding at low ratios of S1 to actin (Andreev & Borejdo, 1995), but some positive charge contribution from S1A1 in the S1(elc) preparation, which is missing in the S1(elc,rlc) preparation, seems possible. However, papain proteolytic effects on the heavy chain or A1 positive charge contributions cannot simply produce a static structural difference; for the ADP case, the difference in $|z_M z_A|$ is 0 esu^2, while for the ATP case, the difference is 8 esu^2 (Table 1). Even if proteolysis is contributing charge to the actin binding site, the amount of charge is being controlled allosterically by nucleotide and the rlc.

It should also be emphasized that S1(elc) and S1(elc,rlc) are both mixtures of isozymes having either alkali light chain 1 or 2 bound. The data are therefore averages for two isozymes in each case. This is not ideal. At least for S1(elc), it is known that the essential light chain does affect actin binding (Wadzinski et al., 1979). The actin affinity of S1A1 is greater than that of S1A2 by a fixed ratio when ADP, ATP, or AMPPNP is present, and the enhanced binding appears to be largely ionic (Chalovich et al., 1984). It is not known if there are comparable essential light chain effects for S1(elc,rlc) as it is not yet feasible to purify these isozymes. If the essential light chain of S1(elc,rlc) is contributing to the differences observed in Figures 2 and 3, its quantitative effects cannot readily be assessed. However, the use of mixtures does not change the major conclusion drawn from the data: that the presence of the regulatory light chain modulates the effect that nucleotides have on the electrostatic charge at the actin binding site. It is not possible to rationize the observed nucleotide-dependent differences between S1(elc) and S1(elc,rlc) electrostatic interactions with actin (Figures 2, 3, and 5) using the nucleotide-independent differences of the S1(elc) isozyme−actin interactions (Chalovich et al., 1984).

The Actin Binding Site of S1(elc,rlc)·MgATPγS. The ATPγS data indicate that, for S1(elc,rlc)·MgATPγS, the actin binding site has no detectable electric charge (Figure 5, Table 2). This result suggests that the electrostatic interaction is abolished when ATP binds to A·M and that electric charge is not redisplayed at the actin binding site until ATP is hydrolyzed and S1·MgADP·P$_i$ is formed. The conclusion that S1·MgATP is electrically neutral is supported by the ionic strength independence of the kinetically determined association constant for S1·MgATP binding actin (White & Taylor, 1976). The apparent absence of electric charge at the actin binding site of S1·MgATP, followed by a substantial increase when S1·MgADP·P$_i$ forms, is consistent with contractile cycle mechanisms which have an electrostatic attraction as the first step. As is well known, and clear from the data in Tables 1 and 2, electrostatic interactions are not particularly important in determining the strength of the actin−myosin bond. It is more likely that the highest level of electric charge is on S1·MgADP·P$_i$ in order to provide longer range forces to guide it to complementary electric charge on actin. It is also possible that the equilibrium between Mg·ATP and Mg·ADP·P$_i$ in the active site (Trentham et al., 1976) provides a blinking on and off of positive electric charge at the actin binding site. Related to this possible blinking electric charge at the actin binding site, a formalism for a novel model of force producing motion by a periodically charged enzymic particle in a static electric field was recently developed (Zhou & Chen, 1996).

The absence of electric charge at the actin binding site of S1·MgATP may also have a role in its dissociation from actin and its ability to move to a new actin further along the filament. If an increase in actin binding site electric charge is, in fact, the trigger for actin binding to begin the contractile cycle, as discussed above, then the existence of an uncharged S1·MgATP would reduce the probability of rebinding to the same actin and give the dissociating cross-bridge time to move to a new site. The rate of ATP hydrolysis is 150 s^{-1} (White & Taylor, 1976), which makes the average lifetime of the electrically neutral S1·MgATP about 7 ms. At a shortening velocity of 2 μm/s (Cooke et al., 1988), the electrically neutral motor domain would move about 14 nm away from the actin from which it dissociated before the positive charge appeared. Although K_A values are equal for S1·MgATPγS and S1·MgADP·P$_i$ at 0 M ionic strength, at so-called physiological ionic strengths S1·MgATPγS binds 10-fold less strongly than S1·MgADP·P$_i$ (Table 2). This surprising result may reflect the importance of eliminating the electric charge on S1·ATP in order for it to dissociate from actin.

There is always some risk in using an analog, because it must be different from the true species. In the present case, there are data to support using ATPγS and ATP as substrates to compare the intermediates S1·MgATP and S1·MgADP·P$_i$. S1·MgATPγS is the major primary species present; the hydrolysis of S1-bound ATPγS is very slow and is not significantly activated by actin (Goody & Mannherz, 1975; Resetar & Chalovich, 1995). For ATP, on the other hand, at 25 °C the ratio of S1·MgADP·P$_i$ to S1·MgATP is greater than 10 (Trentham et al., 1976; White & Taylor, 1976). Relevant to this point, the applicability of S1·MgATPγS as an analog of S1·MgATP was recently reviewed; it was concluded that complications can occur with ATPγS, when regulated actin is used, or when the slowly formed [ADP] is high enough to compete with MgATPγS (Resetar & Chalovich, 1995). Neither is a likely problem in the present case.

Structural Mechanism of Actin Binding Site Electric Charge Changes. There are several nucleotide or rlc-induced S1 conformational changes that could be responsible for the observed changes in electrostatic charge at the actin site (Tables 1 and 2). One possibility is that protons on lysine and/or arginine side chains at the actin binding site of S1·MgADP·P$_i$ dissociate to reduce the charge when S1·MgATP or S1·MgADP form. The lack of any pH dependence for S1·MgADP·P$_i$ binding to actin makes this an unlikely explanation (Duignan & Highsmith, unpublished results). Another possibility is that S1·MgADP and S1·MgATP bind more anions at the actin binding site than S1·MgADP·P$_i$ does. The overall net electric charge of myosin is nucleotide sensitive, apparently due to chloride binding (Bartels et al., 1993), suggesting that anion binding may occur at the actin binding site. The [Cl$^-$] is low in the present measurements, but acetate may also bind. Finally, positive charges at the actin binding site of S1·MgADP·P$_i$ could move to form

neutral ion pairs by binding nearby glutamates and/or aspartates for the S1·MgATP and S1·MgADP cases.

The last mechanism would be facilitated by a flexible structure such as the 632−646 stretch of the myosin heavy chain (Figure 1), which is located near a crevice in the myosin structure, which may be able to open and close (Rayment et al., 1993b). For the many reasons given in the introduction, myosin 632−646 and actin 1−10 probably are the structures involved in forming the earliest contact. It is true that the largest value measured for $|z_M z_A|$ when both light chains are bound is 6.4 esu^2, which is less than the 25 esu^2 expected if the five positive charges in the myosin 632−646 loop (Tong & Elzinga, 1990) and the five negative changes in the actin 1−11 strand (Collins & Elzinga, 1975) were all involved. The data here suggest that a net charge of 2−3 is contributed by each protein, and whatever the mechanism for changing charge on the actin binding site is, only 2−3 positive charges are involved. This does not argue again the myosin loop and actin N-terminus; in fact, these values are consistent with the number of cross-links formed between myosin 632−646 and actin 1−10 (Yamamoto, 1990).

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REFERENCES

Andreev, O. A., & Borejdo, J. (1995) *Biochemistry 34*, 14829−14833.
Bartels, E. M., Cooke, P. H., Elliott, G. F., & Hughes, R. A. (1993) *Biochim. Biophys. Acta 1157*, 63−73.
Bobkov, A. A., Bobkova, E. A., Lin, S. H., & Reisler, E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2285−2289.
Botts, J., Takashi, R., Torgerson, P., Hozumi, T., Muhlrad, A., Mornet, D., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2060−2064.
Brenner, B., Schoenberg, M., Chalovich, J. M., Greene, L. E., & Eisenberg, E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7288−7291.
Chalovich, J. M., Stein, L. A., Greene, L. E., & Eisenberg, E. (1984) *Biochemistry 23*, 4885−4889.
Chaussepied, P. (1989) *Biochemistry 28*, 9123−9128.
Chaussepeid, P., & Morales, M. F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7471−7175.
Collins, J. H., & Elzinga, M. (1975) *J. Biol. Chem. 250*, 5915−5920.
Cook, R. K., Blanke, W. T., & Rubenstein, P. A. (1992) *J. Biol. Chem. 267*, 9430−9436.
Cook, R. K., Root, D., Miller, C., Reisler, E., & Rubenstein, P. A. (1993) *J. Biol. Chem. 268*, 2410−2415.
Cooke, R., Franks, K., Lucianni, G., & Pate, E. (1988) *J. Physiol. 395*, 77−97.
DasGupta, G., & Reisler, E. (1989) *J. Mol. Biol. 207*, 833−836.
dosRemedios, C. G., & Moens, P. D. J. (1995) *Biochim. Biophys. Acta 1228*, 99−124.
Geeves, M. A., & Conibear, P. B. (1995) *Biophys. J. 68*, 194S−201S.
Goody, R. S., & Mannherz, H. G. (1975) in *Protein−Ligand Interactions* (Sund, H., & Blauer, G., Eds.) pp 109−127, de Gruyter, Berlin.
Highsmith, S. (1976) *Arch. Biochem. Biophys. 180*, 404−408.
Highsmith, S. (1990) *Biochemistry 29*, 10690−10694.
Highsmith, S., & Murphy, A. J. (1992) *Biochemistry 31*, 385−389.
Huxley, A. F. (1957) *Prog. Biophys. Biophys. Chem. 7*, 255−318.
Huxley, H. E. (1969) *Science 164*, 1356−1366.
Imamura, K., Tada, M., & Tonomura, Y. (1966) *J. Biochem. (Tokyo) 59*, 280−289.
Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37−44.
Kirshenbaum, K., Papp, S., & Highsmith, S. (1993) *Biophys. J. 65*, 1121−1129.
Korn, E. D., & Hammer, J. A., III (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 23−45.
Leavis, P. C., & Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235−305.
Lin, T. I., & Morales, M. F. (1977) *Anal. Biochem. 77*, 10−17.
Lowey, S., Waller, S., & Trybus, K. M. (1993) *Nature 365*, 454−456.
Lymn, R. W., & Taylor, E. W. (1971) *Biochemistry 10*, 4617−4624.
MacLean-Fletcher, S., & Pollard, T. D. (1980) *Biochem. Biophys. Res. Commun. 96*, 18−27.
Margossian, S. S., & Lowey, S. (1973) *J. Mol. Biol. 74*, 313−330.
Margossian, S. S., Lowey, S., & Barshop, B. (1975) *Nature 258*, 163−168.
Marston, S., & Weber, A. (1975) *Biochemistry 14*, 3868−3873.
Miller, C. J., & Reisler, E. (1995) *Biochemistry 34*, 2694−2700.
Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 925−932.
Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Biochemistry 20*, 2110−2120.
Nauss, K. M., Kitagawa, S., & Gergely, J. (1969) *J. Biol. Chem. 244*, 755−765.
Pitzer, K. S. (1979) in *Activity Coefficients in Electrolyte Solutions* (Pytkowitc, Ed.) pp 157−208, CRC Press, Boca Raton, FL.
Pitzer, K. S., & Mayorga, G. (1973) *J. Phys. Chem. 77*, 2300−2308.
Raucher, D., & Fajer, P. G. (1994) *Biochemistry 33*, 11993−11999.
Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., & Milligan, R. A. (1993a) *Science 261*, 58−65.
Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., & Holden, H. M. (1993b) *Science 261*, 50−58.
Resetar, A. M., & Chalovich, J. M. (1995) *Biochemistry 34*, 16039−16045.
Spudich, J. A. (1994) *Nature 372*, 515−518.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866−4871.
Sutoh, K. (1982) *Biochemistry 21*, 3654−3661.
Sweeney, H. L. (1995) *Biophys. J. 68*, 112s−119s.
Sweeney, H. L., Bowman, B. F., & Stull, J. T. (1993) *Am. J. Physiol. 264*, C1085−C1095.
Szczesna, D., Zhao, J., & Potter, J. D. (1996) *J. Biol. Chem. 271*, 5246−5250.
Szent-Gyorgyi, A. G. (1996) *Biophys. Chem. 59*, 357−363.
Thomas, D. D., Ramachandran, S., Roopnarine, O., Hayden, D. W., & Ostap, E. M. (1995) *Biophys. J. 68*, 135s−141s.
Tong, S. W., & Elzinga, M. (1990) *J. Biol. Chem. 265*, 4893−4901.
Tonomura, Y., Tokura, S., & Sekiya, K. (1962) *J. Biol. Chem. 237*, 1074−1081.
Trentham, D. R., Eccleston, J. F., & Bagshaw, C. R. (1976) *Q. Rev. Biophys. 9*, 217−281.
Trybus, K. M. (1991) *Cell Motil. Cytoskel. 18*, 81−85.
Wadzinski, L., Botts, J., Wang, A., Woodward, J., & Highsmith, S. (1979) *Arch. Biochem. Biophys. 198*, 397−402.
Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455−470.
Wagner, P. D., & Giniger, E. (1981) *Nature 292*, 560−562.
Weeds, A. G., & Taylor, R. S. (1975) *Nature 257*, 54−56.
White, H. D., & Taylor, E. W. (1976) *Biochemistry 15*, 5818−5826.
Yamamoto, K. (1990) *Biochemistry 29*, 844−848.
Yamamoto, K., & Sekine, T. (1979) *J. Biochem. (Tokyo) 86*, 1863−1868.
Yamamoto, K., & Sekine, T. (1980) *J. Biochem. (Tokyo) 87*, 219−226.
Zhou, H. X., & Chen, Y. (1996) *Phys. Rev. Lett. 77*, 194−197.

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