Cross-linking Myosin Subfragment 1 Cys-697 and Cys-707 Modifies ATP and Actin Binding Site Interactions

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ABSTRACT Skeletal muscle myosin is an enzyme that interacts allosterically with MgATP and actin to transduce the chemical energy from ATP hydrolysis into work. By modifying myosin structure, one can change this allosteric interaction and gain insight into its mechanism. Chemical cross-linking with *N,N'-p*-phenylenedimaleimide (pPDM) of Cys-697 to Cys-707 of the myosin-ADP complex eliminates activity and produces a species that resembles myosin with ATP bound (Burke et al., 1976). Nucleotide-free pPDM-modified myosin subfragment 1 (S1) was prepared, and its structural and allosteric properties were investigated by comparing the nucleotide and actin interactions of S1 to those of pPDM-S1. The structural properties of the nucleotide-free pPDM-S1 are different from those of S1 in several respects. pPDM-S1 intrinsic tryptophan fluorescence intensity is reduced 28%, indicating a large increase of an internal quenching reaction (the fluorescence intensity of the related vanadate complex of S1, S1·MgADP·V_i, is reduced by a similar degree). Tryptophan fluorescence anisotropy increases from 0.168 for S1 to 0.192 for pPDM-S1, indicating that the unquenched tryptophan population in pPDM-S1 has reduced local freedom of motion. The actin affinity of pPDM-S1 is over 6,000-fold lower than that of S1, and the absolute value of the product of the net effective electric charges at the acto-S1 interface is reduced from 8.1 esu^2 for S1 to 1.6 esu^2 for pPDM-S1.

In spite of these changes, the structural response of pPDM-S1 to nucleotide and the allosteric communication between its ATP and actin sites remain intact. Compared to pPDM-S1, the fluorescence intensity of pPDM-S1·MgADP is increased 50% (compared to 8 and 31% increases, respectively, for MgADP and MgATP binding to S1). Compared to acto-pPDM-S1, the absolute value of the product of the net effective electric charge at the actin binding interface of acto-pPDM-S1·MgADP increases 7.3 esu^2 (compared to a 0.9 esu^2 decrease and an 11.0 esu^2 increase, respectively, for MgADP and MgATP binding to acto-S1). The interaction free energy for the ligands MgADP and actin, is −2.0 kcal/mol for pPDM-S1, compared to −1.2 kcal/mol for unmodified S1.

INTRODUCTION

The structural rearrangements of myosin during ATP hydrolysis are a critical feature of the chemomechanical energy transduction which drives the relative sliding of the thin and thick filaments in contracting muscle. The enzymatic moiety of myosin, which binds to actin and hydrolyzes ATP, can be isolated as a proteolytic fragment called subfragment 1 (S1). Much of the kinetic and structural information about force generation has been obtained from studies of the properties of purified S1, actin, and ATP in solution. Recently, it has been shown using in vitro assays that S1 is independently capable of using ATP to translocate actin filaments (Toyoshima et al., 1987). The high resolution crystal structure of actin (Kabsch et al., 1990) is known, and there is a preliminary report on that of S1 (Rayment, 1993). In order to interpret these static structures of actin and S1, it is important to obtain dynamic structural data on S1-nucleotide interactions and on the effects of nucleotides on S1-actin interactions.

While the molecular details of motility and force generation are unclear, most proposed models emphasize the control of the interactions at the actomyosin interface by the structure of the nucleotide bound at the myosin active site. It appears that the dissociation of P_i from the actin·S1·MgADP·P_i complex to give actin·S1·MgADP is correlated with force generation (Johnson and Taylor, 1978). For this step in the kinetic cycle, the affinity of S1 for actin increases about 1,000-fold (Highsmith and Murphy, 1992). The nucleotide site is 5 nm from the actin binding site (Tokunaga et al., 1987; Kasprzak et al., 1989); so there must be a pathway connecting the sites (Botts et al., 1984; Audemard et al., 1988). Nucleotides induce global as well as local structural changes in S1 (Aguirre et al., 1989; Highsmith and Eden, 1990, 1993; Wakabayashi et al., 1992), making it possible that the mechanism of communication between the two sites involves the segmental motions of domains, as well as more subtle localized structural changes.

Cys-697 and Cys-707, which are distant from both the nucleotide and the actin binding sites (Tokunaga et al., 1987; Sutoh et al., 1989), appear to be part of this pathway. An indication that Cys-697 and Cys-707 are involved in the communication between the ATP and actin sites is that their

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Abbreviations used: a, chemical activity; A1, myosin alkali light chain 1; A2, myosin alkali light chain 2; AEDANS, (*N*-acetyl-*N'*-1-sulfo-5-naphthyl)ethylenediamine; *c*, concentration; *I*, ionic strength; *K*, association constant; γ, activity coefficient; P_i, orthophosphate; pPDM, *N,N'-p*-phenylenedimaleimide; S1, myosin subfragment 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; V_i, orthovanadate; z_M, net effective electric charge at the actin binding site on myosin; z_A, net effective electric charge at the myosin binding site on actin.

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modification modulates the functions of both sites. When Cys-697 and Cys-707 are chemically cross-linked with *N,N'-p*-phenylenedimaleimide (pPDM), there is a complete loss of ATPase activity and a large reduction in actin affinity (Burke et al., 1976; Greene et al., 1986). Bound nucleotide accelerates the cross-linking reaction, and it has been shown that cross-linking traps the nucleotide in the active site (Wells and Yount, 1979). pPDM-myosin·MgADP has been suggested to be an analog of myosin·MgADP·P_i (Burke et al., 1976; Chalovich et al., 1983).

Structural data on pPDM cross-linked S1 are limited. It appears that the binding of nucleotide to S1 decreases the distance between the two cysteines and increases the chemical reactivity of Cys-697 (Burke and Knight, 1980; Dalbey et al., 1983; Cheung et al., 1985; Garland et al., 1988; Huston et al., 1988). Cross-linking with pPDM causes a small increase in the S1·MgADP radius of gyration (Wakabayashi et al., 1992) and a small decrease in the temperature at which S1 thermally denatures (Levitsky et al., 1992). A preliminary report indicates cross-linking decreases S1 fluorescence intensity (Perkins et al., 1981). Otherwise the structural effects of cross-linking are not well characterized.

pPDM-S1 binding to actin has been shown to be nucleotide-dependent (Greene et al., 1986). Based on the idea that pPDM-S1 is better considered a structurally compromised energy transducer than an analog of a particular steady state kinetic intermediate, we have investigated the effect that cross-linking S1 with pPDM has on the nucleotide binding site, on the actin binding site, and also on the communication between the two sites (Papp et al., 1993). Our strategy is to obtain structural data on the binding sites that can be interpreted quantitatively to elucidate their interaction. The tryptophan fluorescence intensity and anisotropy changes caused by pPDM modification itself, and changes in the response to nucleotide binding, were compared to those of native S1 (Papp et al., 1992). pPDM cross-linking rigidifies tryptophan-containing structural elements of S1 that are involved in the thermally sensitive association and dissociation of nucleotides. The direct effects of pPDM, and its modulation of nucleotide effects, on the acto-S1 interface were also determined, using electrolyte solution theory to interpret the ionic strength dependence of binding (Highsmith, 1990). Cross-linking greatly reduces the contributions to actin binding of both the nonelectrostatic and electrostatic actin recognition elements on S1. In spite of these major changes at the active site and at the actin binding site of pPDM-S1, allosteric interaction between the sites, although modified, are maintained.

MATERIALS AND METHODS

Proteins and chemicals

Myosin was isolated from New Zealand albino rabbit dorsal muscle, and subfragment 1 was prepared by α-chymotryptic digestion of myosin and purified using a DE-52 anion-exchange resin (Bio-Rad) eluted with a linear gradient of 0–0.12 M NaCl (Weeds and Taylor, 1975). The isoenzyme fractions obtained containing either alkali light chain 1 or 2 (S1A1 or S1A2, respectively) were each precipitated with 60% ammonium sulfate and then dialyzed exhaustively to obtain stock solutions that were used in the experiments. Chromatographic separation of the isoenzymes was verified by SDS-PAGE (data not shown). S1A1 and S1A2 MgATPase activities (data not shown) were typically 0.040 ± 0.010 s^{-1} when measured at 20°C, pH 7.0, for steady state conditions, using a coupled assay method (Imamura et al., 1966). F-Actin was prepared from acetone-extracted muscle tissue (Spudich and Watt, 1971). Protein concentrations were determined by absorbance measurements at 280 nm, and by using a commercial protein assay (Bio-Rad protein assay (Bradford, 1976)), in order to compare extinction coefficients. pPDM was from Aldrich Chemical Co. All other chemicals were reagent grade and used without further purification.

Protein modification

Cross-linking of the S1 heavy chain reactive thiols Cys-707 and Cys-697 with pPDM in the presence of MgADP was performed essentially as described (Wells and Yount, 1979). S1 (40 μM) was allowed to react with excess pPDM for 1 h at 0°C in the presence of 0.1 mM ADP to yield pPDM-S1 with trapped MgADP. The course of the reaction was followed by the decrease in MgATPase activity of successive aliquots. The reaction was complete in less than 30 min. For fluorescence measurements, the pPDM-S1·MgADP was diluted to ~1 μM and used within a few hours. For these conditions, the trapping of MgADP is stoichiometic and dissociation has a half time of 5 days (Wells and Yount, 1979); so the samples are greater than 95% pPDM-S1·MgADP. For actin binding measurements, the free nucleotide and pPDM were removed by dialysis for 24–36 h. This procedure reduces the level of bound MgADP to about 85%. However, in the presence of actin, nucleotides are rapidly exchanged (Perkins et al., 1981; Greene et al., 1986), and the actin binding measurements were made with nucleotide in excess to ensure that the active site of pPDM-S1 was saturated.

Trapped nucleotide was removed from pPDM-S1·MgADP by incubating in the presence of actin and making repeated additions of the anion exchange resin DE-52 (Bio-Rad), followed by decanting and centrifugation to remove the ion exchange resin and actin, respectively, in a modification of an earlier procedure (Greene et al., 1986). The ratio of bound nucleotide to protein was determined using [^{3}H]ADP radioactivity and optical density measurements, respectively. The bound [^{3}H]ADP was reduced to undetectably low levels in the pPDM-S1 samples by this procedure. This is an improvement over reductions to only ~20% bound, obtained using Dowex 1X-8 (Greene et al., 1986). pPDM-S1 was also prepared by allowing S1 to react with pPDM overnight at 0°C in the same buffer used above but in the absence of nucleotide. Under these conditions, a significant portion of S1 was not cross-linked. pPDM-S1 was purified by centrifuging in the presence of actin (5:1, S1:actin) to remove the non-cross-linked S1 (Chalovich et al., 1983). Control measurements on parallel preparations that had only actin (no S1) indicated that residual actin after centrifugation and dilution made a negligible fluorescence intensity contribution to the pPDM-S1 measurements. pPDM-S1 prepared this way has no detectable MgATPase activity and has the same extinction coefficient at 280 nm ($\epsilon^{0.1\%}$ = 0.75 cm^2/mg) as unmodified S1.

In all the experiments reported below, pPDM-S1 had the same properties whether MgADP was removed from pPDM-S1·MgADP that had been prepared using methods described by others (Riesler et al., 1974; Wells and Yount, 1979; Greene et al., 1986), or the cross-linking was done in the absence of the nucleotide. All the data are consistent with the complexes being identical, and it seems very likely that it is Cys-707 and Cys-697 of S1 that are being cross-linked when nucleotide is absent, as well as when it is present (Burke and Knight, 1980). Cross-linking in the absence of ADP and removing unmodified S1 and non-cross-linked pPDM-modified S1 species by centrifuging in the presence of actin is more convenient than removing MgADP from pPDM-S1·MgADP.

Fluorescence measurements

Steady-state fluorescence intensity and anisotropy measurements were made using a Perkin-Elmer MPF-44B fluorospectophotometer as described

earlier (Papp et al., 1992). Temperature was maintained to ± 0.1°C and monitored during the measurements directly in the cell compartment using a small thermocouple inserted to a cuvette. The excitation and emission wavelengths were 300 and 340 nm, respectively, in order to maintain the selective excitation of the tryptophans at the "red edge" (Papp et al., 1992).

Association constants

The fractions of bound and free S1 in the presence of F-actin were determined by a centrifugation method except that intrinsic tryptophan fluorescence was used to determine [S1] instead of the radioactivity of an extrinsic label (Marston and Weber, 1975). pPDM-S1 and unmodified S1 were dialyzed exhaustively against 4-morpholinepropanesulfonc acid (MOPS) buffer (10 mM MOPS, 3 mM $MgCl_2$, 0.05 mM EGTA, 5 mM KOAc, pH 7.2). F-Actin was pelleted by centrifugation at 80,000 × g for 3 h, and the pellet was homogenized in the same buffer immediately before use. S1 or pPDM-S1 at a final concentration of 0.5–5 μM, and F-actin in the range 20–40 μM, were prepared from stock solutions to obtain a final volume of 175 μl. Higher ionic strengths were obtained by including the appropriate volume of 1 M KOAc (in MOPS buffer, pH 7.2). For measurements in the presence of MgADP or MgATP, the nucleotide (10 mM in MOPS buffer, pH 7.2), was included to obtain a final concentration of 1.4 mM. MgATPase activities were measured to ensure that [MgATP] remained high enough to saturate the S1 active site throughout the course of the measurement. Samples were incubated at 25°C for 10 min and then centrifuged for 30 min at 25°C (Beckman Airfuge, 30 p.s.i.). Free [S1] was determined by fluorescence intensity of 75-μl aliquots taken from the acto-S1 samples (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 unsedimented actin, respectively. K_A was calculated from {[total S1] − [free S1]}/{[free S1]x[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

$$K(0) = a_{MA}/a_M a_A = (c_{MA}/c_M c_A)(\gamma_{MA}/\gamma_M \gamma_A) \tag{2}$$

where 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

$$\ln K(0) = \ln K_A - \ln \gamma_\pm, \tag{3}$$

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

The original theory of Debye and Huckel (1923) for the effect of ionic strength on the chemical activity of ions was modified (Pitzer, 1979) to be applicable at all ionic strengths. 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 ln is the natural logarithm (2.3 log), $|z_M z_A|$ 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 are determined experimentally for specific electrolytes (Pitzer, 1979). 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 $K(0)$ and $|z_M z_A|$ for acto-S1 interactions (Highsmith, 1990; Highsmith and Murphy, 1992). The method of Bevington (1969) (and see Highsmith and Murphy (1992)) was used for error analysis. Myosin and actin are very large compared to the ionic species for which the theory has been tested (Pitzer, 1979); so, z_M and z_A are net effective electric charges at the myosin and actin surfaces at the interface.

RESULTS

pPDM-S1 binding to actin

The apparent association constants (K_A) for the binding of pPDM-S1A2 to F-actin in solutions containing increasing concentrations of KOAc were measured at 25°C. The results are shown as semilogarithmic plots of K_A as a function of $I^{1/2}$ (Fig. 1 *A*). The solid line is the best fit of the data to Eqs. 3 and 4, and the values obtained for $K(0)$, presented as log $K(0)$, and $|z_M z_A|$ are in Table 1. Identical results were obtained, within experimental error, using pPDM-S1A2 that was prepared with no nucleotide present, or by removing MgADP from pPDM-S1A2·MgADP, as described above in Materials

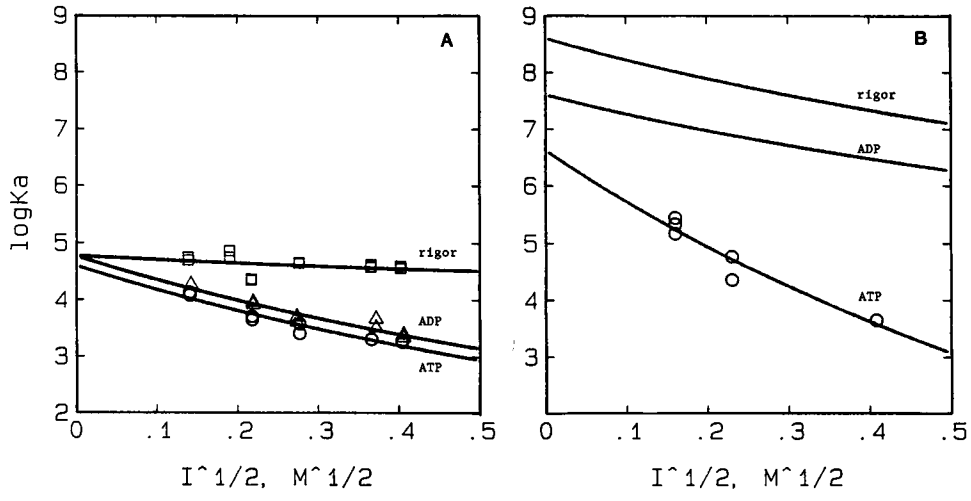

FIGURE 1 Ionic strength dependence of F-actin binding to pPDM-S1 (*A*) and S1 (*B*) measured at 25°C in solutions containing increasing amounts of KOAc. The apparent association constant, K_A, was calculated from the fractions of free and actin bound S1 in the presence of MgATP (○), MgADP (△), and in rigor (□), as described under Materials and Methods, and is plotted as logK_A. Solid lines through symbols are best fits to Eqs. 3 and 4 in the text. Solid lines without symbols, for S1 binding in the presence of MgADP and in rigor, are from (Highsmith, 1990; Highsmith and Murphy, 1992).

TABLE 1 Auto-S1 and acto-pPDM-S1 interactions

Ligand	$\lvert Z_M Z_A \rvert$		Log $K(0)$	
	No pPDM	pPDM	No pPDM	pPDM
None	8.1 ± 1.0	1.6 ± 1.3	8.6 ± 0.2	4.8 ± 0.2
MgADP	7.2 ± 2.0	8.9 ± 1.1	7.7 ± 0.1	4.8 ± 0.1
MgATP	19.1 ± 2.0	8.9 ± 0.9	6.6 ± 0.2	4.6 ± 0.1

The absolute values of the product of the net effective electric charge on myosin and actin at their binding interface, $Z_M Z_A$, are given in units of esu^2. $K(0)$ is the association constant obtained by extrapolating to zero ionic strength (Fig. 1), and is given as log $K(0)$. [ATP] was in excess for S1 and pPDM-S1. For the S1 case, S1·MgADP·P_i binds to actin, but for the pPDM-S1 case, it is probably pPDM-S1·MgADP (see text). Data for unmodified S1 with MgADP and no nucleotide are from earlier work (Highsmith, 1990; Highsmith and Murphy, 1992). Conditions are given under Materials and Methods.

and Methods. Measurements made on pPDM-S1A1 gave quantitatively similar values for $K(0)$ and $\lvert z_M z_A \rvert$ (data not shown). Results obtained from measurements of S1·MgADP·P_i binding to actin, along with data obtained earlier for S1 and S1·MgADP (Highsmith, 1990) are shown in Fig. 1 *B* and Table 1, for comparison. At zero ionic strength, the affinity for actin of pPDM-S1 is reduced by a factor of $10^{-3.8}$ compared to S1, which is the equivalent of 5.2 kcal/mol less energy of stabilization for pPDM-S1 binding. The size of the decreased binding energy suggests that both the electrostatic and nonelectrostatic interactions have been reduced by cross-linking. Increasing the ionic strength has only a small effect on the affinity of pPDM-S1 for actin (Fig. 1 *A*). The absolute value of the product of the net effective electric charges on the actin and myosin binding sites is 1.6 esu^2 for pPDM-S1, compared to 8.1 esu^2 for unmodified S1 (Table 1).

pPDM-S1-nucleotide complexes binding to actin

The binding of pPDM-S1A2·MgADP to actin was measured in the presence of 1.4 ADP and 3 mM $MgCl_2$. The presence of MgADP in the active site of pPDM-S1A2 increased the ionic strength dependence of its binding to actin. The affinity at zero ionic strength is not changed, but $\lvert z_M z_A \rvert$ for pPDM-S1A2·MgADP binding to actin is increased to 8.9 esu^2, compared to 7.1 esu^2 for unmodified S1·MgADP (Fig. 1 and Table 1). Quantitatively similar results were obtained using pPDM-S1A1·MgADP (data not shown). Given the small change in the electrostatic contribution to binding, the nonelectrostatic actin recognition pattern must be altered greatly by cross-linking S1·MgADP, in order to account for the $10^{-2.9}$-fold reduced affinity compared to unmodified S1·MgADP.

In the presence of actin, the ADP bound to pPDM-S1 is exchangeable (Greene et al., 1986). In an attempt to investigate the actin interactions of pPDM-S1·MgATP or pPDM-S1·MgADP,P_i, the actin affinity of pPDM-S1·MgADP was measured in the presence of excess free MgATP in the solution, instead of excess MgADP. The association constants measured for actin and pPDM-S1 in the presence of MgATP were insignificantly smaller, at all ionic strengths, than those measured in the presence of excess MgADP (Fig. 1 *A*). Likewise, the binding constants at zero ionic strength for pPDM-S1·MgADP and the putative pPDM-S1·MgATP are not significantly different (log $K(0)$ = 4.8 + 0.1 and 4.6 + 0.1, respectively). $\lvert z_M z_A \rvert$ values for the two cases were identical, within experimental uncertainty (Table 1). When pPDM cross-linking is done in the presence of MgATP, the phosphate is lost during the multi-hour trapping and isolation procedure, and one obtains pPDM-S1·MgADP (Wells and Yount, 1979). The observations that the actin interactions are identical quantitatively, within experimental uncertaintly, for pPDM-S1 in the presence of excess ADP and ATP (Fig. 1 and Table 1) suggest that pPDM-S1·MgADP is present in both cases at shorter times as well.

The ionic strength dependence of the binding of unmodified S1A2·MgADP·P_i to actin (Fig. 1 *B*) gives values for $K(0)$ and $\lvert z_M z_A \rvert$ that are in good agreement with those obtained previously using unresolved (S1A1/S1A2)·MgADP·P_i (Highsmith and Murphy, 1992), which were obtained using an SDS-PAGE method (Furukawa and Arata, 1984). As for pPDM-S1, the affinity for S1·MgADP·P_i of actin is greatly reduced. However, the results in Fig. 1 *A* and Table 1 indicate that the electrostatic component of the binding is much smaller for pPDM-S1 that it is for S1·MgADP·P_i. This conclusion in not consist with that of an earlier investigation of the ionic strength effects on pPDM-S1·MgADP binding to actin, perhaps because the previous measurements were made over a much smaller range of ionic strength (Greene et al., 1986).

pPDM-induced changes in S1 tryptophan fluorescence intensity and anisotropy

The steady-state intrinsic fluorescence intensity of S1A1 decreases by 28% as result of pPDM cross-linking. Quantitatively similar large decreases were observed when S1A2 was cross-linked. The fluorescence anisotropy of S1A1 increases from 0.168 to 0.192 when its Cys-697 and Cys-707 are cross-linked by pPDM (Table 2). No differences in intensity of anisotropy could be detected between pPDM-S1 prepared in the absence of nucleotide and pPDM-S1 prepared by removing MgADP after cross-linking. Adding MgADP or MgATP

TABLE 2 Tryptophan fluorescence intensity and anisotropy of S1 and pPDM-S1

Ligand	I_f		r_f	
	No pPDM	pPDM	No pPDM	pPDM
None	100 ± 3.5%	72 ± 3.2%	0.168 ± 0.003	0.192 ± 0.002
MgADP	108 ± 1	108 ± 1	0.180 ± 0.001	0.190 ± 0.001
MgADP,P_i	131 ± 5	108 ± 2	0.191 ± 0.002	0.190 ± 0.002

The Tryptophan fluorescence intensity and anisotropy were measured at 340 nm, using 300 nm irradiation. Intensities are given in arbitrary relative values. Nucleotides were present at saturating concentrations. For pPDM-S1, "MgADP,P_i" refers to S1 that was cross-linked in the presence of MgATP and had 100 μM ATP present during the measurements. No significant differences between S1A1 and S1A2 were detected. See Materials and Methods for experimental details.

to pPDM-S1 after cross-linking did not change the values of the intensity or anisotropy significantly, consistent with the very slow rates of association and dissociation for nucleotide binding to pPDM-S1 in the absence of actin (Burke et al., 1976; Wells and Yount, 1979).

Cross-linking S1·MgADP with pPDM does not change its tryptophan fluorescence intensity (Table 2). However, when compared to the value for pPDM-S1, the increase in tryptophan intensity due to the inclusion of MgADP in the active site is remarkably large (+50%). It seems that the substantial quenching of tryptophan fluorescence intensity caused by pPDM in the absence of nucleotide is reversed when nucleotide is present, and that a fluorescence increase similar to the effect of MgADP on S1 (Werber et al., 1972; Torgerson, 1984) also occurs. The fluorescence anisotropy of pPDM-S1, on the other hand, is not significantly changed by the presence of MgADP in the active site (Table 2). Experiments also were done using S1 that had been cross-linked in the presence of MgATP. As was the case with the actin binding experiments described above, the fluorescence intensity and anisotropy values were the same for S1 cross-linked by pPDM in the presence of ATP or ADP (Table 2), consistent with the conclusion that phosphate is lost (Wells and Yount, 1979), or that MgATP/MgADP, P_i cannot be trapped. The equality of the intensities for S1 cross-linked in the presence of ADP and ATP suggest that the ADP:S1 stoichiometries are identical, rather than lower for cross-linking in the presence of ATP, as reported (Wells and Yount, 1979). There is no obvious explanation for this difference. The addition of 100 μM ATP to solutions of pPDM-S1 or pPDM-S1·MgADP had no effect on the fluorescence intensity or anisotropy, as expected. The addition of MgADP or MgATP to the unmodified S1 results in about 8 or 31% increases in the fluorescence intensity, respectively (Table 2).

The effects of changing temperature on S1 and pPDM-S1 anisotropy

The interpretation of tryptophan fluorescence anisotropy changes in terms of specific protein structural changes is better founded than the interpretation of fluorescence intensity changes. It has been shown that fluorescence anisotropy increases with increased restriction of local tryptophan rotational mobility (Rholam et al., 1984; Weber, 1989). To exploit this advantage, the temperature dependencies of the tryptophan fluorescence anisotropy of unmodified S1A1 and of pPDM-S1A1 with and without MgADP were determined. Measurements made with S1A2 gave the same results, within experimental error (data not shown). For S1, the gradual decrease in the anisotropy values, with a characteristic transition around 15°C, is observed (Fig. 2), as reported for S1 in the presence of MgADP, MgATP, or no nucleotide (Papp et al., 1992). Cross-linking eliminates this transition (or moves it out of the 0–25°C range) with or without MgADP bound (Fig. 3). Similar anisotropy values and lack of temperature sensitivity were observed for S1·MgADP·V_i (Papp et al., 1992), which also has the nucleotide trapped in the active site (Goodno and Taylor, 1982).

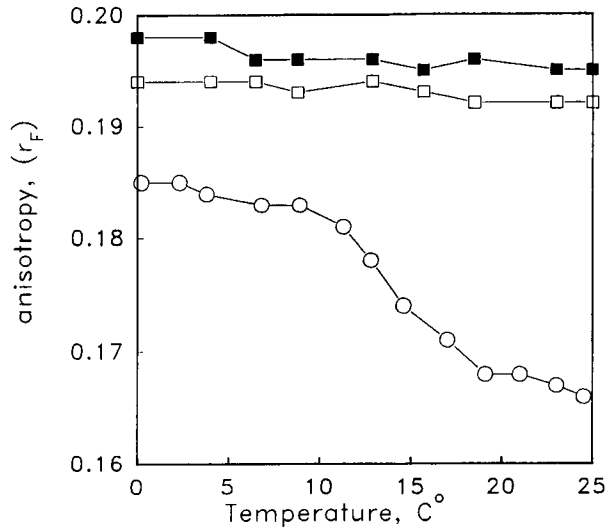

FIGURE 2 Temperature dependence of the average steady-state tryptophan fluorescence anisotropy of S1 (○), pPDM-S1 (□), and pPDM-S1·MgADP (■) in 100 mM KOAc, 10 mM MOPS (pH 7.0), 1 mM $MgCl_2$. The presence of added free nucleotide had no detectable effect on pPDM-S1 or pPDM-S1·MgADP anisotropy.

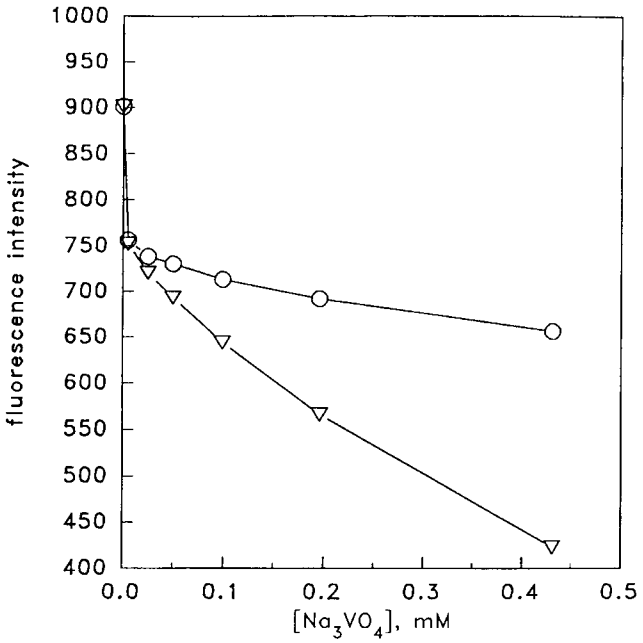

FIGURE 3 Effect of added free vanadate on the steady-state fluorescence intensity of tryptophans of S1·MgADP·V_i before (▽) and after (○) correction for the vanadate inner filter effect. Conditions are as in Fig. 2, with 1 mM ADP and V_i added as indicated. Fluorescence intensity is in arbitrary units.

The S1-ADP-vanadate complex has other similarities to pPDM-S1. The tryptophan fluorescence intensity of S1·MgADP·V_i is quenched, presumably by an energy transfer mechanism (Werber et al., 1992); although vanadate also can reduce tryptophan fluorescence intensity by

an inner filter effect, or by collisional quenching. Because pPDM modification and orthovanadate binding both trap MgADP (Wells and Yount, 1979; Goodno and Taylor, 1982), increase tryptophan anisotropy (Papp et al., 1992), and also reduce tryptophan fluorescence intensity (Werber et al., 1992), experiments were done to clarify the contributions of free vanadate inner filter effect and collisional quenching to the total vanadate quenching of S1·MgADP·V_i fluorescence. S1·MgADP·V_i was prepared and diluted to reduce the free [V_i] to less than 10 μM. The tryptophan fluorescence intensities of S1·MgADP in the absence of vanadate and of S1A1·MgADP·V_i in the presence of free orthovanadate between 0.007 and 0.430 μM are shown in Fig. 3. After correcting for the vanadate inner filter effect, there is limited quenching due to free vanadate. Extrapolation of the corrected fluorescence intensity for S1A1·MgADP·V_i back to zero free [V_i] indicates that the intensity of complex itself is reduced to 83% of the value of S1 without nucleotide (Fig. 3). This is comparable to the reduction to 72% caused by pPDM cross-linking of S1 (Table 2), a case for which energy transfer seems unlikely.

DISCUSSION

Nucleotide-free pPDM cross-linked S1 is a new species that has several striking structural features, compared to S1, which are manifested at both the ATP and the actin binding sites. The tryptophan fluorescence properties, usually associated with nucleotide binding, are changed dramatically in ways that suggest two populations of tryptophan(s) respond to bound nucleotide. The actin recognition pattern also is greatly modified. Nonetheless, the communication between the ATP and actin sites is maintained, making pPDM-S1 itself useful for the investigation of myosin allosteric interactions. Several investigations of pPDM-S1·MgADP have been made, since it was first synthesized (Riesler et al., 1974). Most of them were aimed at evaluating pPDM-S1·MgADP as a stable analog of S1·MgADP·P_i (Burke et al., 1976; Chalovich et al., 1983; Greene et al., 1986), or at measuring changes in the distance between Cys-697 and Cys-707 that occur when MgADP binds (Riesler et al., 1974; Burke and Reisler, 1977; Wells and Yount, 1979; Cheung et al., 1985; Garland et al., 1988; Huston et al., 1988; Chantler et al., 1991). pPDM-S1 itself has not be investigated previously. Its luminescence properties are discussed first.

S1 luminescence

There are remarkable changes in the pPDM-S1 tryptophan fluorescence intensity and anisotropy, which are usually associated with nucleotide binding in the ATP site (Werber et al., 1972; Papp et al., 1992). There is a large (28%) decrease in pPDM-S1 tryptophan fluorescence intensity compared to S1 (Table 2). The reduced intensity for pPDM-S1 is consistent with increased solvent exposure for some tryptophans, or with some effective internal quenching mechanism, perhaps a Cys that is moved close to a tryptophan by cross-linking. The latter arrangement has been suggested based on fluorescence energy transfer from a tryptophan (which was speculated to be Trp-510) to fluorophores attached to Cys-697 on S1 (Hiratsuka, 1992). The presence of MgADP in the active site of pPDM-S1 reverses the quenching of tryptophan fluorescence intensity observed for pPDM-S1 (Table 2). The increase in intensity is 50% for the pPDM-S1 to pPDM-S1·MgADP transition (which we detect by isolating pPDM-S1 and pPDM-S1·MgADP), the largest nucleotide-induced fluorescence increase for any ATPase of which we are aware. It seems possible that MgADP binding increases the fluorescence intensity by actual physical protection from the putative nearby internal quencher, which would be expected to be in a solvent protected location (Werber et al., 1972; Morita and Ishigami, 1977; Torgerson, 1984). Recent ^{3}H NMR measurements on [2-^{3}H]ADP bound to S1 are consistent with the adenine of ADP being physically close to a tryptophan when it is bound (Highsmith et al., 1993). Relief from this internal quenching, as it occurs in unmodified S1, also may be occurring when ATP binds and increases its fluorescence intensity. The identification of the tryptophans in the S1 crystal structure (Rayment, 1993) that are near sidechains expected to be effective quenchers may be an approach to identify which tryptophan(s) increase in fluorescence intensity when ATP binds.

pPDM cross-linking also increases S1 tryptophan fluorescence anisotropy. The increases reported here (Table 2 and Fig. 3) are quantitatively similar to the increases observed when MgADP·P_i or MgADP·V_i is bound to S1 (Papp et al., 1992). Increased fluorescence anisotropy is associated with reduced motional freedom of the tryptophan indole moiety in proteins in general (Lakowicz and Weber, 1980; Rholam et al., 1984; Weber, 1989). Increased S1 tryptophan fluorescence anisotropy is correlated with S1-nucleotide complexes or conditions for which the rate of nucleotide dissociation is slow (Papp et al., 1992). Actin binding accelerates the rate of ADP dissociation from pPDM-S1·MgADP (Wells and Yount, 1979; Chalovich et al., 1983) and of P_i from S1·MgADP·P_i (Taylor, 1979), and also decreases the S1·MgADP·P_i tryptophan anisotropy (Andreev et al., 1989). The pPDM-induced increase in anisotropy, the loss of the thermal transition at 15°C to lower anisotropy values (Table 2 and Fig. 2) and the inability of nucleotides to bind to pPDM-S1 are consistent with the idea that there is a local rigidified tryptophan-containing S1 structural domain that must become less rigid for nucleotide entry or exit from the ATP site (Papp et al., 1992). This proposed change in the internal dynamic rigidity of a local structural domain during ligand binding is analogous the domain loosening that must occur for CO binding to myoglobin (Ansari et al., 1992). In S1, Trp-130 has been shown to interact with ATP analogs (Okamoto and Yount, 1985) and is near to what appears to be the entrance to the ATP binding site (Rayment, 1993). Trp-130 may be in the local domain of S1 that is hypothesized to loosen for nucleotide association and dissociation. The increase in tryptophan fluorescence anisotropy, which is associated with hindered access to the ATP site, oc-

curs whether the fluorescence intensity decreases (as for pPDM-S1 and S1·MgADP·V_i) or increases (as for pPDM-S1·MgADP and S1·MgADP·P_i), consist with the notion that the ATP-induced anisotropy and intensity changes of S1 are from different tryptophan(s).

Actin binding

The electrostatic and the nonelectrostatic actin recognition elements of S1 are both severly compromised by cross-linking, as indicated by the low values for $|z_M z_A|$ and $K(0)$, respectively (Table 1). The reduced electrostatic contribution to actin binding ($|z_M z_A| = 1.6$ esu^2 for pPDM-S1 compared to 8.1 esu^2 for S1) may be due to some distortion of the interaction of the "clusters of negative and positive charge" (Yamamoto, 1989) that are at the acto-S1 interface (Sutoh, 1982, 1983; Chaussepied and Morales, 1988), or by an actual removal of positive charge on the actin binding site of S1, perhaps by folding it into an interior S1 location. The fact that pPDM cross-linking causes decreases in affinity with either a decrease (as for S1) or an increase (as for S1·MgADP) in electrostatic interaction suggests that the nonelectrostatic actin recognition pattern is also changed by cross-linking (Table 1). There may be a pPDM-induced structural distortion (or rearrangement) of the acto-S1 binding interface, or it may be that pPDM interferes sterically because it is bound to S1 at the actin binding site.

This latter possibility has been suggested, based on the interference of acto-S1 interactions by oligopeptides that mimic S1 structure near Cys-697 and Cys-707 (Katoh et al., 1985; Suzuki et al., 1987); although, there is some controversy as to whether any charged oligopeptide will interfere at least with binding (Chase et al., 1991; Katoh and Morita, 1993). It has been shown that modifying the two Cys residues without cross-linking has only a small effect on actin affinity (Burke et al., 1976). More useful for comparison to our data, when Cys-707 and Cys-697 are modified with AEDANS and -S-CH_3, the modified S1 binds to actin with about 85% of the affinity of native S1 at 0.28 M ionic strength (Botts et al., 1979). AEDANS plus -S-CH_3 is nearly isovolumic with pPDM, which reduces the association constant for actin binding to less than 0.02% that of S1 (Table 1). This comparison suggests that the pPDM effect on actin binding is not steric. It seems more likely that the actin binding site (or sites, the approach used here cannot distinguish between one and two acto-S1 interfaces) is being transformed allosterically by pPDM attached to S1 at sites distant from either the ATP and the actin sites. The locations of the three sites, as determined by electron microscopy, are consistent with this interpretation (Tokunaga et al., 1987; Sutoh et al., 1989). The quantitative relationship of the changes in net effective electric charge, determined here (Table 1) using electrolyte solution techniques, to the sums of the electrostatic charges at the binding sites, as determined from crystal structures of S1 and actin, should be of interest when the crystal structure data are available.

Allosteric interactions

Although the rates of nucleotide binding and dissociation are extremely slow for pPDM-S1, it is clear that cross-linking does not eliminate communication between the active and actin sites. In general, ligands in the active site of S1 or pPDM-S1 change either the electrostatic and/or the nonelectrostatic actin recognition elements at the acto-S1 interface. There is no indication that the control over the two classes of elements is coupled.

MgADP binding to S1 decreases the electrostatic contribution to actin binding with no apparent change in the nonelectrostatic contribution (Highsmith, 1990). MgADP in the ATP site of pPDM-S1 increases the contribution of the electrostatic actin recognition elements at the acto-pPDM-S1 binding interface, as indicated by the increase in $|z_M z_A|$ from 1.6 to 8.9 esu^2 (Table 1). The affinity for actin of pPDM-S1 with and without MgADP is the same at zero ionic strength, where the electrostatic interactions are maximum. This suggests that the contribution to actin binding of the nonelectrostatic elements must be diminished by MgADP to a degree equal to the increased contribution of the electrostatic elements. These effects of MgADP on pPDM-S1, to decrease nonelectrostatic and increase electrostatic interactions with an overall decrease in affinity, are qualitatively the same as the effects of MgATP on unmodified S1 (Table 1 and Highsmith and Murphy (1992)).

The communication between allosteric sites can be assessed quantitatively by the interaction free energy, $\Delta\Delta G'^{o} = -RT\ln(K/K')$, where K is the association constant for binding in the absence of ligand and K' is the constant in the presence of ligand (Highsmith, 1976). When MgADP is the ligand, the interaction free energies for S1 and pPDM-S1 binding to actin are −1.2 and −2.0 kcal/mole, respectively, at 25°C and 0.20 M ionic strength (Fig. 1). The ATP and actin binding sites on S1 still communicate when Cys-697 and Cys-707 are cross-linked, but the message is somewhat distorted. MgADP is actually more effective at reducing the binding of pPDM-S1 than the binding of S1, to actin. For comparison, the value of the interaction free energy when MgATP is the ligand, for S1 binding to actin, is −5.4 kcal/mol, for the same conditions.

Weak binding state analog

Because of its weak binding to actin, pPDM-S1·MgADP has been considered to be an analog of the kinetic intermediate S1·MgADP·P_i (Burke et al., 1976; Chalovich et al., 1983). Because the modification is covalent, pPDM-S1·MgADP has the added virtue of being stable. From the perspective of actin binding, cross-linking S1·MgADP with pPDM does decrease the actin affinity to a level similar to that of S1·MgADP·P_i, but by different means than P_i does when it present with MgADP in the ATP site. The loss of nonelectrostatic binding interactions is greater and the increase of electrostatic interactions is smaller for pPDM-S1·MgADP than it is for S1·MgADP·P_i. The actin affinities are equal for the two S1 complexes only in the 0.25 M ionic strength range

(Fig. 1). From the perspective of MgADP dissociation rates, cross-linking has an effect that is closer to that of V_i rather than P_i being bound in the active site (Wells and Yount, 1979; Goodno and Taylor, 1982). As discussed above, pPDM cross-linking causes the same tryptophan environment rigidification and the same loss of the thermal transition at 15°C that is observed for V_i binding to S1·MgADP (Papp et al., 1992). Finally, the shape of pPDM-S1·MgADP has been shown to be different from that of S1·MgADP·P_i (Wakabayashi et al., 1992). In conclusion, although pPDM-S1·MgADP is similar in some respects to S1·MgADP·P_i, caution should be exercised when considering it as an analog.

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