Reversible Inactivation of Myosin Subfragment 1 Activity by Mechanical Immobilization

Stefan Highsmith,* Kelly Duignan,* Kathy Franks-Skiba,# Katherine Polosukhina,* and Roger Cooke#
*Department of Biochemistry, School of Dentistry, University of the Pacific, San Francisco, California 94115, and #Department of Biophysics and Biochemistry, University of California, San Francisco, California 94143 USA

ABSTRACT The Mg-ATPase activity of skeletal muscle myosin subfragment 1 (S1) is reversibly eliminated when it is aggregated by the force of osmotic pressure dehydration using polyethylene glycol (PEG). Several experiments indicate nucleotides bind aggregated S1, but the effects of binding are attenuated. Compared with S1 in solution, ϵADP binds aggregated S1 with reduced affinity, and the bound ϵADP fluorescence intensity is more effectively quenched by acrylamide. When ATP binds aggregated S1, the tryptophan intensity increases to only 50% of the solution level. Chemical cross-linking of cys-707 to cys-697 by *p*-phenylenedimaleimide is less efficient for aggregated S1·MgADP. The data are consistent with aggregated S1 being able to bind nucleotide but not being able to complete the usual conformation change(s) in response to binding. If S1 is kept from aggregating by increasing the ionic strength at the same osmotic pressure, its Mg-ATPase activity and ATP-induced tryptophan fluorescence intensity increase are normal. The combined data are consistent with an ATP hydrolysis mechanism in which S1 segmental motion is coupled to its enzymatic activity. In this model, segmental motion is mechanically constrained by aggregation; the constrained S1 can bind ATP, but it cannot complete the hydrolysis mechanism.

INTRODUCTION

Hypothetical mechanisms of force generation by the actin-myosin-ATP system, which involve segmental motions of subdomains within the subfragment 1 (S1) domain of myosin, are widespread. In most cases, one segment of S1 is bound firmly to actin, and the reorientation of the other segment produces force. The details of the energetic roles of ATP binding and/or hydrolysis and of actin binding are not clear, but there are indications that nucleotide-induced segmental motions do occur for both actin-bound and for free S1.

Several reports indicate nucleotide-induced S1 segmental motions occur in solution. Differences between the shapes of S1·MgADP and S1·MgADP·X (where X is phosphate or a phosphate analog) have been detected by fluorescence anisotropy decay spectroscopy (Aguirre et al., 1989), transient electric birefringence (Highsmith and Eden, 1990, 1993), x-ray scattering (Wakabayashi et al., 1992), and neutron scattering (Mendelson et al., 1996). These results support the idea that S1 is energized by ATP, which by binding and/or being hydrolyzed produces a "cocked" S1 structure that has increased internal energy. Actin binding then facilitates the conversion of the internal energy into work, as the hydrolysis products dissociate and actin affinity increases.

Nucleotide-induced S1 segmental motion has also been observed for S1-actin complexes. Image reconstructions from electron micrographs of actin filaments saturated with smooth muscle S1 and S1·MgADP indicate that the segment of S1 distal to actin is displaced when ADP is removed (Whittaker et al., 1995). This actin-bound S1 segmental reorientation due to ADP removal has been confirmed by electron spin resonance spectroscopy for smooth muscle S1, but interestingly it is not observed for skeletal S1 (Gollub et al., 1996).

Computer graphic models of S1 bound to actin, based on their atomic structures, suggest that the portion of S1 that has the light chains bound to a long strand of heavy chain α-helix is the domain that is distal to actin, and is the segment that moves (Rayment et al., 1993a). This assignment of the moving portion of S1 is consistent with measurements on muscle fibers using orientation-reporting probes attached to the regulatory light chain, which suggest that it is part of the segment that moves during contraction (Allen et al., 1996; Ling et al., 1996).

If force production involves S1 segmental motion that is coupled to the ATP hydrolysis cycle, then it should be possible to inhibit S1 activity by constraining its segmental motion. Reported here are experiments in which the segmental motions of S1 in solution are reversibly immobilized by using osmotic pressure to force S1 to aggregate. The Mg-ATPase activity is completely but reversibly inhibited. In contrast to the activity, the changes in nucleotide affinity, tryptophan fluorescence intensity, ATP site accessibility, and cysteine chemical reactivity were changed only moderately by S1 immobilization.

MATERIALS AND METHODS

Proteins

Myosin was isolated from New Zealand rabbit dorsal muscle (Nauss et al., 1969). Myosin subfragment 1 was prepared from myosin using papain

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K. Polosukhina is on leave from the Laboratory of Biophysics, Kos'tsov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia.

Address reprint requests to Dr. Stefan Highsmith, Department of Biochemistry, University of the Pacific, 2155 Webster Street, San Francisco, CA 94115-2399. Tel.: 415-929-6670; Fax: 415-929-6654; E-mail: shighsmith@uop.edu.

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(Margossian and Lowey, 1982) and purified by size exclusion chromatography (Sephacryl S-400) followed by anion exchange chromatography (DE-52) (Weeds and Taylor, 1975). Experiments were done using mixtures of S1 with its regulatory light chain plus either essential light chain 1 or essential light chain 2 bound. S1 Mg-ATPase activity at 25°C in 10–20 mM ionic strength solution was typically 0.04–0.06 s^{-1}. Creatine kinase and α-chymotrypsin were from Sigma Chemical Co., St. Louis, MO.

Chemicals

Chemicals used to prepare buffers were reagent grade. Polyethylene glycols (PEGs) were from Fluka Chemical, Buchs, Switzerland (PEG3, where the number is the average molecular weight/1000) and EM Sciences, Ft. Washington, PA (PEG4). Weight-percent (wt %) PEG stock solutions were prepared gravimetrically and diluted volumetrically to prepare solutions for experiments. Succinimidyl *N*-methylanthranilate was from Molecular Probes, Eugene, OR. The fluorescent ATP analog, ϵATP was synthesized as described (Franks-Skiba et al., 1994). The adsorption resin HA-ultragel (beads of hydroxyapatite cross-linked in an agarose matrix, from Sigma) was repeatedly washed with 10 M KH_2PO_4 buffer (pH 6.8) to remove the storage medium and then washed with 10.0 mM KH_2PO_4, 5.0 mM $MgCl_2$ (pH 6.0) before use in experiments.

Enzyme activities

Steady-state S1 Mg-ATPase activities for soluble S1 were determined at 25°C using a coupled assay system (Imamura et al., 1966). Control measurements, done in the presence of low [PEG] that did not induce S1 aggregation, indicated that the reactions of the coupled assay remain faster than those of S1.

For aggregated S1, the solutions can be turbid enough to interfere with the coupled assay absorbance measurements. For those cases, phosphate production in aliquots taken at five time points and diluted into a molybdate (Lin and Morales, 1977) or malachite green (Kodama et al., 1986) solution was used to measure the rate.

Measurement of the Mg-ATPase activity of S1 bound to hydroxyapatite beads is complicated by the effects of the hydroxyapatite on the coupled assay enzymes and by phosphate contamination from the hydroxyapatite on the molybdate assay. In this case, activity was determined by centrifuging the suspension gently to pellet the S1-hydroxyapatite complex, taking an aliquot and measuring the amount of ADP present using the coupled assay. The pellet was resuspended after each aliquot. The activity was determined from the dependence of [ADP] on time for five to seven time points.

α-Chymotrypsin esterase activity at 25°C was determined spectrophotometrically for *p*-nitrophenyl acetate hydrolysis by measuring the rate of the change of absorbance at 410 nm.

Nucleotide binding

The association constant for ϵADP binding to S1 in the presence of PEG was determined from the free ϵADP concentration, which was measured by the fluorescence intensity of the supernatant after removal of the aggregated S1 and bound ϵADP by centrifugation. The association constant in the absence of PEG was determined from the free ϵADP concentration in the filtrate after centrifuging the solution through a Centricon semipermeable membrane that retained S1 and bound ϵADP.

Fluorescence measurements

For S1 intrinsic tryptophan fluorescence, the excitation and emission wavelengths were 300 and 340 nm, respectively (Kirshenbaum et al., 1993). For the aggregated state, fluorescence intensity was measured using front-face detection (Franks-Skiba et al., 1994). Acrylamide quenching of fluorescence intensity of S1-bound ATP analogs was measured as described (Franks-Skiba et al., 1994).

Light-scattering measurements

The intensity of scattered light at 90° from the incident beam was measured at 25°C using a Perkin-Elmer MPF-44B fluorospectrophotometer. Both monochrometers were set at 400 nm. The spectral widths for the incident and scattered beams were 3 and 1 nm, respectively.

Chemical modification of S1

The cross-linking of the S1 heavy chain reactive thiols cys-707 and cys-697 with the bifunctional reagent *N,N′-p*-phenylenedimaleimide (pPDM) was done as described by previously (Wells and Yount, 1979; Kirshenbaum et al., 1993). The extent of cross-linking was monitored by measuring the residual Mg-ATPase activity of aliquots that were quenched with 1 mM β-mercaptoethanol and then diluted 10-fold into the coupled assay solution.

RESULTS

S1 aggregation by PEG

When the concentration of PEG is increased above a certain level, S1 aggregates. Larger PEG species are more effective at causing aggregation (Highsmith et al., 1996). In the experiments here, only PEG3 and PEG4 were used. The intensity of scattered light for 0.39 μM S1 in 10 mM ionic strength solutions containing PEG3 does not increase significantly until [PEG3] is near 10 wt % (Fig. 1 *A*). The midpoint is at 11 wt % PEG3, and the data suggest that above 15 wt % PEG3 the extent of S1 aggregation is constant. Solutions containing precipitated protein were visibly turbid. When solutions containing S1 in 20 wt % PEG3 were centrifuged at 90,000 $\times$ *g* for 60 min, the absorbance of the supernatant at 278 nm was less than 3% of the value measured before centrifugation, indicating that 0.39 μM S1 is at least 97% aggregated in 20 wt % PEG3.

PEG4 is somewhat more effective at precipitating S1 than is PEG3, as expected for a larger excluded volume contribution from a larger osmolyte (Bhat and Timasheff, 1992). The midpoint for 50 μM S1 aggregation at 10 mM ionic strength is near 7 wt % PEG4 (Fig. 1 *B*). When ADP or ATP is included in the solution, the midpoint of the transition to aggregated S1 is moved progressively to higher [PEG4] (Fig. 1 *B*). The transition also becomes progressively less steeply dependent on [PEG4]. These results suggest that S1·MgADP and S1·MgADP·P_i are more soluble in PEG-containing solutions, presumably because they have structures requiring greater osmotic pressures for aggregation.

Increasing the ionic strength reduces the extent of aggregation. For 0.18 μM S1 in 20 wt % PEG3, increasing the ionic strength with KCl from 10 to 510 mM reduces the aggregated S1 from 95% to 5% of the total S1 (Fig. 2 *A*). The ionic strength inhibition of aggregration is overcome if [S1] is increased. In 510 mM ionic strength solutions, increasing [S1] from 0.18 to 80 μM causes a steady increase in S1 aggregation (Fig. 2 *B*). It appears that there are

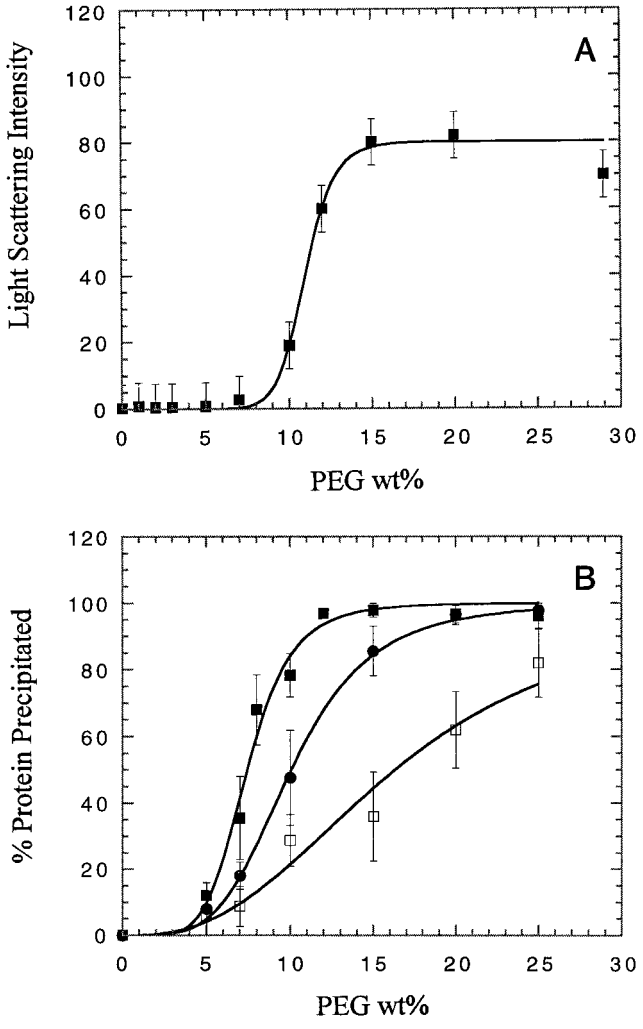

FIGURE 1 PEG-induced aggregation of S1, S1 · MgADP and S1 · MgADP.P_i (*A*) S1 aggregation monitored by light scattering. Aliquots of concentrated S1 were diluted to 0.39 μM in 10 mM MOPS, 1 mM $MgCl_2$, pH 7.0 solutions at 25°C that contained between 0 and 30 wt % PEG3. The increase in the intensity of scattered light at 90° from the incident beam was measured, using a Perkin-Elmer MPF-44B fluorospectrophotometer. The intensity is given in arbitrary units. The midpoint of the transition is 11 wt % PEG3. The midpoint of the transition is 8 wt % when PEG4 is used (data not shown). The experimental uncertainty is ±15%. (*B*) Effects of nucleotides on S1 aggregation. Solutions containing 50 μM S1 with increasing amounts of PEG4 and no nucleotide (■), 5 mM MgADP (●), or 5 mM Mg-ATP (□) were centrifuged at 90,000 × *g* for 10 min, and the [S1] remaining in solution was determined by absorption at 280 nm.

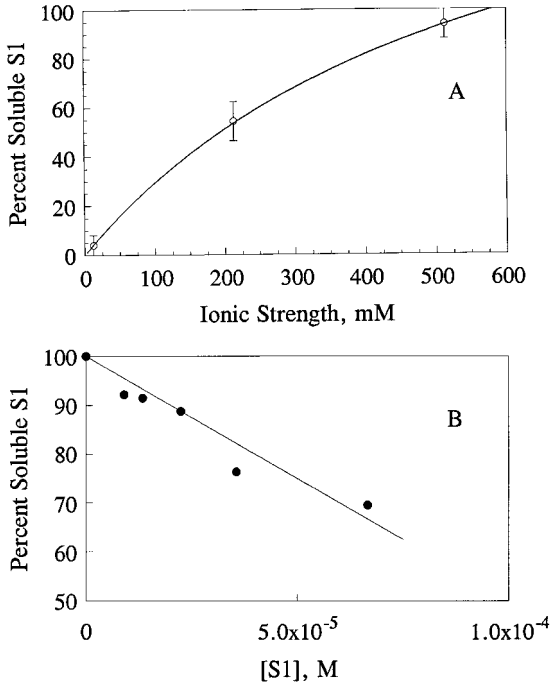

FIGURE 2 Ionic strength and [S1] effects on aggregation in PEG3. (*A*) Increasing ionic strength. Aliquots of concentrated S1 were diluted to 0.18 μM in 20 wt % PEG3, 10 mM MOPS, 1 mM $MgCl_2$, pH 7.0 solutions at 25°C that contained between 0 and 500 mM KCl. The samples were centrifuged for 30 min at 90,000 × *g* to remove the aggregated S1, and the remaining [S1] was determined from the tryptophan fluorescence intensity. (*B*) Increasing [S1]. Aliquots of 200 μM S1 were diluted to the concentrations shown in 10 mM MOPS, 1 mM $MgCl_2$, 500 mM KCl, pH 7.0 solutions at 25°C that contained 20 wt % PEG3. The fraction of S1 remaining in solution after centrifugation for 60 min at 90,000 × *g* was determined by measuring tryptophan fluorescence intensity.

attractive electrostatic intermolecular interactions between S1 molecules. Increasing the ionic strength reduces them, but even without these electrostatic interactions S1 can be forced to aggregate.

Aggregation reversibly inactivates S1

Aggregation inactivates S1 (Fig. 3). The Mg-ATPase activity of the aggregated material at 25°C is 0 ± 0.005 s^{-1}. The apparent increase in activity observed in the presence of 1–7 wt % PEG3 in an earlier study (Highsmith et al., 1996) was eliminated when the PEG-containing coupled assay solution was incubated at 25°C for 10 min before the S1 was added. There may be some direct oxidation of NADH by impurities in the PEG that are depleted by the incubation. The aggregation and inhibition are reversed when the aggregated sample is diluted to reduce the [PEG] to the 3–5 wt % level.

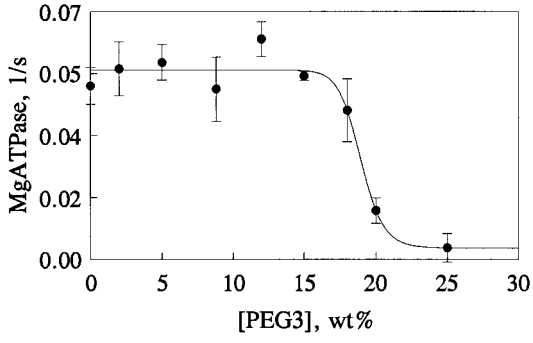

FIGURE 3 Effects of PEG3 on S1 Mg-ATPase activity. (*A*) Steady-state Mg-ATPase activity of 1 μM S1 was measured at 25°C in 1 mM MgATP, 10 mM MOPS, 1 mM $MgCl_2$, pH 7.0, with increasing amounts of PEG3 present. The activity decreases steeply above 15 wt % PEG3 and remains near 0 s^{-1} above 20 wt %.

When S1 is kept in an aggregated state (20 wt % PEG3) for 5 to 120 min and then diluted to 2 wt % PEG3, the average activity was 120 ± 24% of control after dilution, suggesting that no irreversible reactions are occurring while S1 is aggregated.

If S1 is kept from aggregating in the presence of 20 wt % PEG3, by including KCl, the activity is not inhibited. The S1 Mg-ATPase activities at 25°C in solutions containing 500 mM KCl are 0.040 ± 0.006 s^{-1} and 0.041 ± 0.004 s^{-1} in the absence and presence of 20 wt % PEG3, respectively (Table 1). KCl has little effect on PEG osmotic pressure (Rau and Parsegian, 1992), and the lack of inhibition in the presence of KCl indicates that S1 is not being inactivated by osmotic pressure-driven S1 intramolecular conformational changes.

S1 tryptophan fluorescence intensity

Steady-state tryptophan fluorescence intensity is a sensitive monitor of the S1 structural response to ATP binding and hydrolysis (Werber et al., 1972). The effect of PEG on this physical response to ATP was determined for S1 in low and high ionic strength solutions. When S1 is aggregated, and inactive, the fluorescence intensity increase in the presence of ATP is half of normal (Table 1). This diminished ATP-induced increase in fluorescence intensity for aggregated S1 is independent of [ATP] from 0.25 to 1.0 mM, indicating that the available ATP sites are saturated. These intrinsic fluorescence data suggest that aggregated S1 binds ATP but does not form the same more fluorescent S1 · MgADP · P_i intermediate formed by soluble S1.

ϵADP binding to aggregated S1

The fluorescent analog of ADP, ϵADP, was used to compare the association constant for nucleotide binding to S1 in solution and to 20 wt % PEG4-induced aggregated S1. The amounts of free and bound ϵADP were determined by co-sedimentation of the bound nucleotide with the aggregated S1. In the absence of PEG, the association constant is (1.0 ± 0.2) × 10^5 M^{-1}, somewhat smaller than reported previously (Rosenfeld and Taylor, 1984). In the presence of 20 wt % PEG4, the association constant for the aggregated S1 is reduced to (2 ± 1) × 10^4 M^{-1} and the stoichiometry is 1.2 ± 0.1 ϵADP per S1. The weaker ϵADP binding to aggregated S1 is consistent with the reduced tryptophan fluorescence intensity increase when ATP binds (see above). The stoichiometry indicates that the active site is accessible in the aggregated state.

TABLE 1 S1 tryptophan intensity response to ATP

[PEG4] (wt %)	[KCl] (mM)	ATP-induced % increase in tryptophan fluorescence intensity	Mg-ATPase activity (s^{-1})
0	0	24 ± 3	0.048 ± 0.005
20	0	12 ± 5	0 ± 0.005
0	500	19 ± 2	0.040 ± 0.004
20	500	18 ± 2	0.041 ± 0.005

S1 samples were irradiated at 295 nm and the fluorescence intensity monitored at 340 nm. Detection was at 90° for the soluble S1 samples, and front-face detection was used for the aggregated samples. The fluorescence intensity observed by front-face detection for PEG4-aggregated S1 with no nucleotide was increased compared with the same [S1] in the absence of PEG. This appears to be due to scattering of the irradiating beam by the aggregates, which increases the volume of irradiated sample. The increases reported above are percent increases over the observed S1 intensity in the absence of nucleotide.

Acrylamide quenching of S1 · MgϵADP fluorescence intensity

The solvent accessibility of ϵADP bound in the active site was monitored by measuring the acrylamide quenching of ϵADP fluorescence intensity. In the aggregated state, ϵADP fluorescence intensity is quenched more effectively than it is in solution. The Stern-Volmer quenching constants from linear fits to the points for soluble S1 and aggregated S1 were 1.5 ± 0.07 M^{-1} and 3.7 ± 0.2 M^{-1}, respectively (Fig. 4). The curvature in the low [acrylamide] range for S1 in 10 wt % PEG4 is due to the increase in free [ϵADP], in agreement with the ϵADP binding results, described above. For comparison, the data for S1 in 7 wt % [PEG4], where

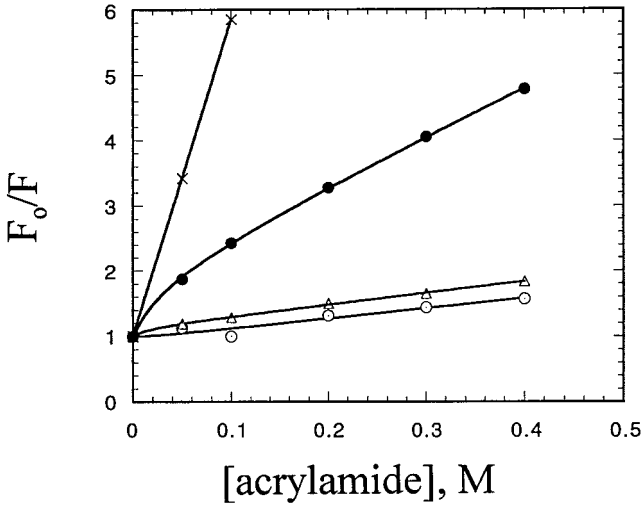

FIGURE 4 Acrylamide quenching of MgϵADP bound to S1. The ratio of the fluorescence intensity of ϵADP in 20 mM ionic strength buffer at 10°C in the absence, F_o, and presence, F, of acrylamide is plotted as a function of the acrylamide concentration (a Stern-Volmer plot). Data are for free ϵADP (×) and for 25 μM ϵADP in the presence of 65 μM S1, in solution in the absence of PEG (○), not aggregated in the presence of 7 wt % PEG4 (△), and aggregated in the presence of 10 wt % PEG4 (^). To obtain quenching constants (K_{SV}), the data were fitted to a two-component Stern-Volmer equation in which one component was free nucleotide as determined from parallel sedimentation measurements, and the second component was bound nucleotide. For free ϵADP, K_{SV} = 54 ± 4 M^{-1} (×). For bound ϵADP, K_{SV} = 1.5 ± 0.07 in the absence of PEG (○), 1.6 ± 0.1 in the presence of 7 wt % PEG4 (△), and increased to 3.7 ± 0.2 M^{-1} in the presence of 10 wt % PEG4 (●).

precipitation is minimal, are also shown in Fig. 4. The increase in the rate of quenching, which is consistent with the active site being in a more open conformation, is observed only when aggregation occurs. A similar increase in accessibility to quencher was observed in the presence of high [PEG] using the fluorescent nucleotide mant-ADP trapped on the S1 by AlF_4 (Franks-Skiba and Cooke, 1995). The data for the bound mant-ADP could be fit by a single component with a quenching constant $0.24 \pm 0.04\ M^{-1}$ in the absence of PEG and $0.73 \pm 0.02\ M^{-1}$ in the presence of 10 wt % PEG4 (data not shown).

Control measurements to determine the effectiveness of acrylamide to penetrate the PEG-induced aggregate were made using 10 wt % PEG4-aggregated creatine kinase, which had been labeled with the extrinsic fluorophore succinimidyl *N*-methylanthranilate. The Stern-Volmer plot in the absence of PEG displayed two components, a minor one (24% of the total) having a quenching constant $0.07 \pm 0.02\ M^{-1}$ and a major one having a quenching constant $8.6 \pm 1.6\ M^{-1}$. When the labeled creatine kinase was precipitated by 10 wt % PEG4, the minor and major component quenching constants were $0.09 \pm 0.02\ M^{-1}$ and $7.0 \pm 1.5\ M^{-1}$, respectively. The differences are not statistically significant. The important point of this control experiment is that precipitation by PEG does not change the accessibility of a probe bound to the surface of creatine kinase.

Cross-linking aggregated S1 · MgADP with pPDM

The binding of MgADP to S1 induces a conformation change in solution that greatly increases the chemical reactivity of cys-697 and cys-707 to the bifunctional cross-linking reagent pPDM (Burke et al., 1976; Hiratsuka, 1992). The cross-linked S1 is inactive. These cysteines are near the ATP binding site, but not in it (Rayment et al., 1993b). Their response to nucleotide may be part of the allosteric interaction between the active site and the actin binding site. Intramolecular cross-linking of S1 · MgADP traps the nucleotide in the active site (Wells and Yount, 1979) and modifies the S1 tryptophan fluorescence (Kirshenbaum et al., 1993). The effects of nucleotide binding on the distance between the two cysteines and their chemical reactivity are well documented (see Barnett and Schoenberg, 1993, and references therein).

S1 was aggregated by 20 wt % PEG3, and the degree of pPDM cross-linking in the presence of MgADP was monitored by assaying the loss of ATPase activity after quenching the unreacted pPDM with β-mercaptoethanol and diluting to dissociate the aggregated S1. Aggregation by PEG3 reduced the degree of pPDM cross-linking of S1 · MgADP after 20 min to $46 \pm 5\%$ of the level obtained in solution in the absence of PEG. These data suggest that the cross-linkable S1 · MgADP structure is not as prevalent for aggregated S1 as it is for S1 in solution.

Ca^{2+}- and K^+-ATPase activities of aggregated S1

S1 hydrolyzes ATP in the absence of Mg^{2+}, but the hydrolysis does not support contraction. The Ca^{2+}- and K^+-ATPase activities were measured in the absence and presence of 20 wt % PEG4, which aggregated over 95% of the S1. Rather than being inhibited, these non-Mg-ATPase activities were somewhat activated when aggregated (Table 2). These results suggest that substrates that do not support contraction do not have an ATP hydrolysis mechanism that is sensitive to mechanical constraint.

Activity of aggregated α-chymotrypsin

α-Chymotrypsin can also be aggregated by PEG, but higher [PEG3] is required for α-chymotrypsin than for S1; 77% and 85% of 0.32 mg/ml α-chymotrypsin were removed by centrifugation in 18 and 40 wt % PEG3, respectively. By measuring the rate of *p*-nitrophenyl acetate hydrolysis by α-chymotrypsin in the presence of varying [PEG3] and determining the amount of aggregate present, the esterase activity of aggregated α-chymotrypsin was determined to be $51 \pm 9\%$ of control. The activity is reduced when α-chymotrypsin is aggregated, but remains substantial.

Mg^{2+}-ATPase activity of S1 immobilized on hydroxyapatite beads

The effect on activity of removing S1 from solution by a method different from aggregation was investigated by adsorbing S1 onto beads of hydroxyapatite cross-linked in an agarose matrix. The fraction of S1 bound was determined from the ATPase activity and/or tryptophan fluorescence intensity of the supernatant after centrifuging the sample to pellet the beads. When 600 mg of hydroxyapatite was added to 1.0 ml of 2 μM S1 in 10.0 mM KH_2PO_4, 5.0 mM $MgCl_2$ (pH 6.0) and the suspension was shaken gently for 15 min, no free S1 could be detected. The adsorbed S1 could be removed by adding high [ATP] and had normal activity. If 200 μM ATP were added, 90–93% of the S1 remained adsorbed, and the free [ATP] was $\sim$50 μM. The rate of ADP production was measured as described in Materials and Methods, and the activity of the adsorbed S1 was calculated for the observed activity, V_{OBS}, using the equation $V_{OBS} = V_{FREE} \times f_{FREE} + V_{BOUND} \times f_{Bound}$, where f_{Bound} and f_{FREE} are the fractions of bound and free S1, respectively. V_{BOUND} was 39% of control S1 activity (Table 3). When 10 wt % PEG3 was added to enhance S1 adsorp-

TABLE 2 Ca^{2+}- and K^+-ATPase activities

Activity	0 PEG	20 wt % PEG4
Ca^{2+}-ATPase	$0.60 \pm 0.12\ s^{-1}$	$1.15 \pm 0.22\ s^{-1}$
K^+-ATPase	$0.24 \pm 0.07\ s^{-1}$	$0.67 \pm 0.13\ s^{-1}$

Activities were assessed in the absence and presence of 20 wt % PEG4 for 2 μM S1 at 23°C in 0.10 M KCl, 50 mM MOPS (pH 7.0), 4.0 mM ATP, and either 4 mM $CaCl_2$ or 5 mM EDTA.

TABLE 3 Mg-ATPase activity of S1 adsorbed on hydroxyapatite

Addition to solution	State of S1	Mg-ATPase activity (% of control)
None	Soluble	100
10 wt % PEG3	Soluble	105 ± 9
Hydroxyapatite	Adsorbed	39 ± 17
Hydroxyapatite plus 10 wt % PEG3	Adsorbed	13 ± 5

Mg-ATPase activities of S1 in solution and in the presence of PEG3 and/or hydroxyapatite were measured as described in the text. S1 is soluble in 10 wt % PEG, and its activity is changed very little (Fig. 3 and Highsmith et al., 1996). Less than 3% of the S1 was free in the presence of the hydroxyapatite beads.

tion to the hydroxyapatite beads, V_{BOUND} was reduced to 13% of control.

DISCUSSION

Characterization of reversible S1 aggregation by PEG

PEG aggregates polymers by osmotic pressure, which reduces the pool of water available for hydration. This forces the polymers together. Very large forces can be generated. For example, PEG induces aggregation of DNA molecules under conditions for which they repel one another strongly (Podgornik et al., 1995). Twenty weight percent of PEG3 exerts $\sim 7 \times 10^6$ dyne/cm^2 of osmotic pressure (http://aqueous.labs.brocku.ca/osfile.html). Low [PEG] forces S1 to bind to actin more strongly (Highsmith et al., 1996) at its normal binding site (White et al., 1995). Higher [PEG] forces S1 to aggregate (Fig. 1). The inhibition of S1 aggregation by KCl (Fig. 2 *A*) indicates that electrostatic interactions between S1 molecules contribute to S1-S1 interactions. That the KCl inhibition can be overcome by increasing [PEG] or [S1] (Fig. 2 *B*) is consistent with all the observed aggregation phenomena being reversible mass action equilibria.

The structure of the aggregate is not well defined. However, they do contain sufficient water to allow free access of substrates, quenchers, and other small molecules to the active and other sites on the surface of S1. This conclusion is based on the observation that the rate of acrylamide quenching of succinimidyl *N*-methylanthranilate attached to the surface of a control protein, creatine kinase, is unchanged by PEG-induced aggregation.

The inhibition of S1 aggregation by nucleotide is not as easily explained. There may be a contribution from negatively charged nucleotide screening attractive positive charge on the S1 surface, or from changing the electric charge more globally on the surface (Bartels et al., 1993). It is also possible that changes in S1 shape and/or dynamics induced by nucleotide binding make it more soluble.

Nucleotide interactions with aggregated S1

The data strongly support the loss of Mg-ATPase activity occurring because S1 aggregates (Fig. 3). There is no partial inactivation before aggregation occurs, in agreement with previous observations (Highsmith et al, 1996). Inactivation is not due to osmotic pressure causing an intramolecular conformational change to a less hydrated structure. When S1 in the presence of 20 wt % PEG3 is kept in solution by increasing [KCl], it retains full activity and full tryptophan fluorescence intensity increase upon ATP binding and hydrolysis (Table 1). The osmotic pressure is not reduced by KCl; contributions from PEG and monovalent salts are additive (Rau and Parsegian, 1992). Aggregation, the close packing, and perhaps specific and/or nonspecific contact of S1 sites seems to be the cause of inactivation.

Reduced S1 activity in the aggregated state is not due to unavailability of the active site. The unchanged Stern-Volmer quenching constant for a fluorophore attached to the surface of creatine kinase, when aggregated, suggests that small molecules can diffuse freely into the aggregates. Stronger evidence is that the S1 active site can bind ATP and ϵADP and that the stoichiometry of ϵADP binding is not reduced. The structural response to nucleotide binding appears to be partial. Tryptophan fluorescence increases only 50% of expected when ATP binds (Table 1). ϵADP binding is only 20% (Fig. 4). The quenching of bound ϵADP by acrylamide is enhanced (Fig. 4), which suggests that the active site of aggregated S1 is not providing as much protection of the adenine portion of the nucleotide as it does when S1 is in solution. All of the nucleotide data are consistent with the S1 ATP site being able to bind nucleotide but not being able to complete the normal binding mechanism.

A reasonable interpretation of the data is that S1-S1 interactions in the aggregate inhibit S1 segmental motions that are required for the hydrolysis and/or product release steps of the ATP hydrolysis cycle. The asymmetric shape of S1 (Rayment et al., 1993b) and its well documented nucleotide-induced shape changes make such a coupling of segmental motion and hydrolysis plausible. The fact that S1 is a motor protein with moving parts is consistent with the idea that constraining the motions would inhibit activity. Enzymatic inhibition by aggregation-induced immobilization is not unprecedented. The Ca-ATPase activity of the membrane-bound calcium pump in sarcoplasmic reticulum is inhibited by the binding of melittin (Mahaney and Thomas, 1991) or phospholamban (Voss et al., 1994), which reduce rotational mobility by inducing aggregation.

The intrinsic tryptophan fluorescence intensity of S1 reports conformational changes that occur when nucleotides bind (Werber et al., 1972). For S1 with only the essential light chain, there is a 16% increase when ATP binds, followed by an additional 20% increase upon hydrolysis (Johnson and Taylor, 1978). For S1 with both the essential and regulatory light chains bound the increases are somewhat smaller (Johnson and Taylor, 1978; Papp et al., 1992).

When S1 is aggregated, the tryptophan fluorescence intensity response to ATP is reduced by 50%, which suggests that the aggregated S1-nucleotide conformation is not converted fully into the solution S1 · MgADP · P_i conformation. The diminished increase in fluorescence intensity (Table 1) is quantitatively consistent with aggregated S1 binding ATP but then being arrested in a conformation that precedes the formation of S1 · MgADP · P_i (Johnson and Taylor, 1978).

S1 adsorption on hydroxyapatite

The activity of S1 immobilized by hydroxyapatite is also inhibited. In the presence of PEG3, at concentrations that do not aggregate S1, the inhibition is 87% (Table 3). This increased inhibition is likely due to PEG enhancing the nonspecific binding interactions of S1 by osmotic stress, which decreases the range of S1 segmental motion. The inactivation is reversible. This reduction of S1 activity by adsorbing it on a surface supports the interpretation of the loss of aggregated S1 activity being due to the restraint of segmental flexibility.

Enzymic activities that do not require segmental motion

If the observed inhibition of S1 Mg-ATPase activity is due to restraint of segmental motion, then enzymic activity that does not require segmental motion for activity should be less inhibited by aggregation. This is what is observed for S1 Ca^{2+}- and K^+-ATPase activities. For these substrates, hydrolysis is not coupled to force generation. Assuming that product release is the rate-determining step for the hydrolysis of Ca-ATP and K-ATP, the observed increased activities (Table 2) suggest that product release is enhanced when S1 is aggregated. This interpretation of the non-Mg-ATPase kinetic data is consistent with observed weaker binding of ϵADP and its greater exposure to the bulk solvent (Fig. 4). It is also important that the normal rates of Ca-ATP and K-ATP hydrolysis are much higher than that of Mg-ATP, and the observed increases indicate that there is no significant effect on activity due to a reduction in the rate of the diffusion of substrate to the active site when S1 is aggregated.

The structure of α-chymotrypsin is more symmetrical than that of S1, and segmental motions are not thought to be an important component of its mechanism of action. Therefore, α-chymotrypsin should be less inhibited by aggregation. This is what was observed; it retains 51% of its esterase activity when it is aggregated. Some inhibition might be expected, as some of the active sites may not be available in this case. But the complete loss of S1 Mg-ATPase activity is consistent with a qualitative difference between the effects of aggregation on S1 and α-chymotrypsin.

Implications for mechanism of action of myosin

The interpretation of the PEG and hydroxyapatite effects on S1 in terms of mechanical restraint is reasonable given that S1 is a part of a motor, the moving part. The data suggest that the mechanical and enzymatic properties are coupled, which is not surprising. This interpretation is consistent with ATP binding and/or hydrolysis "cocking" S1 into an energized structure (Aguirre et al., 1989; Highsmith and Eden, 1990; Wakabayashi et al., 1992; Rayment et al., 1993a; Mendelson et al., 1996) before it binds to actin, the formation of which requires segmental rearrangements that cannot take place when S1 is immobilized by aggregation or adsorption.

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