Osmotic Pressure Probe of Actin-Myosin Hydration Changes During ATP Hydrolysis

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ABSTRACT Osmotic stress in the 0.5–5 $\times$ 10^6 dyne/cm^2 range was used to perturb the hydration of actin·myosin·ATP intermediates during steady-state hydrolysis. Polyethylene glycol (PEG) (1000 to 4000 Da), in the 1 to 10 wt% range, which does not cause protein precipitation, did not significantly affect the apparent K_M or the V_{max} for MgATP hydrolysis by myosin subfragment 1 (S1) alone, nor did it affect the value for the phosphate burst. Consistent with the kinetic data, osmotic stress did not affect nucleotide-induced changes in the fluorescence intensities of S1 tryptophans or of fluorescein attached to Cys-707. The accessibility of the fluorescent ATP analog, ϵADP, to acrylamide quenching was also unchanged. These data suggest that none of the steps in the ATP hydrolysis cycle involve substantial hydration changes, which might occur for the opening or closing of the ATP site or of other crevices in the S1 structure. In contrast, K_M for the interaction of S1·MgADP·P_i with actin decreased tenfold in this range of osmotic pressure, suggesting that formation of actin·S1·MgADP·P_i involves net dehydration of the proteins. The dehydration volume increases as the size of the PEG is increased, as expected for a surface-excluded osmolyte. The measured dehydration volume for the formation of actin·S1·MgADP·P_i was used to estimate the surface area of the binding interface. This estimate was consistent with the area determined from the atomic structures of actin and myosin, indicating that osmotic stress is a reliable probe of actin·myosin·ATP interactions. The approach developed here should be useful for determining osmotic stress and excluded volume effects in situ, which are much larger than those of typical in vitro conditions.

INTRODUCTION

Water is involved in most biological reactions. In many cases water is a reactant or a product, and in a much larger group of reactions it is the solvent for the reactants, the products, and/or at least a portion of the catalysts. It is widely recognized that solvation, and changes in solvation, play important roles in determining the kinetic and thermodynamic parameters of reactions in solution. However, it is often difficult to obtain information about the role of hydration in biological reactions, because hydration equilibria are highly dynamic and for the most part involve nonspecific binding sites for the water molecules (Otting et al., 1991).

In a recent study, hydration changes that occur during substrate binding to hexokinase, and subsequent reactions, were investigated at various levels of osmotic stress, which were produced by osmolytes added to the solution (Rand et al., 1993). In this approach, osmotic work done to remove water from an osmolyte-excluded cavity where a substrate binds is estimated from the dependence of the apparent substrate affinity, or reaction rate, on osmotic pressure. The volume of water removed is then calculated from the osmotic work and the osmotic pressure. Osmolytes that are too large to penetrate into hydrated cavities and that do not bind to the proteins or substrates are used to generate the osmotic pressure on those cavities. The osmolyte-excluded cavity refers to any volume from which the osmolytes are excluded, which for some osmolyte structures includes a volume that extends from the surface of the protein (Bhat and Timasheff, 1992). The effect of the osmolytes is to vary the activity of bulk water, which accepts water of hydration from or donates it to the reactants, transition states, and products, which in turn changes the equilibrium or rate. Reported here are experiments that extend this approach to reactions of MgATP with myosin and actin in solution, to estimate the net changes of osmolyte-excluded volumes of hydration for several of the steps in the kinetic cycle that produces force in muscle.

In addition to any general interest in osmolyte-excluded hydration changes during enzymic reactions, there are specific features of actin-myosin structures and reactions that make knowledge about hydration changes of particular interest. First, the actin-myosin system uses ATP binding and hydrolysis to generate force, and osmotic stress techniques can be used to measure force generated between molecular surfaces (Parsegian et al., 1986). It is relevant that a force due to hydration changes may be involved in energy transduction by muscle (Oplatka, 1994), and a preliminary report indicates that osmotic stress increases actin binding to myosin (White et al., 1995). A second feature is that the association of actin and myosin to form the post-power-stroke rigor complex is known to be "entropy driven" (Highsmith, 1977); i.e., the decrease in standard free energy upon binding is due to $T\Delta S°$ being large enough to over-

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come a positive $\Delta H°$ of binding. This observation typically has been interpreted to indicate that surface-bound ions and/or waters of hydration are moved to the bulk solvent when actin and myosin form this lowest energy complex of the reaction cycle. Finally, the structures of actin and of the myosin motor domain, myosin subfragment 1 (S1), have been shown to include large crevices (Kabsch et al., 1990; Rayment et al., 1993b). The S1 ATP binding site has been hypothesized to close when ATP binds, and a larger S1 crevice may also close when S1 binds to actin (Rayment et al., 1993a). Such openings or closings of crevices in S1 or actin would very likely involve substantial changes in the volumes of hydration. These volumes would be perturbed by osmotic stress.

THEORY

If osmotic work is involved in an equilibrium, and if one assumes that the free energies for binding, $\Delta G_{\text{binding}}$, and for osmotic work, $\Delta G_{\text{osmotic work}}$, are independent, then for the reaction

$$E + S \rightleftarrows E \cdot S, \tag{1}$$

the total free energy change is

$$\Delta G_{\text{total}} = \Delta G_{\text{binding}} + \Delta G_{\text{osmotic work}}, \tag{2}$$

with

$$\Delta G_{\text{binding}} = \Delta G°_{\text{binding}} + RT \ln(K), \tag{3}$$

which is the standard binding expression. Furthermore,

$$\Delta G_{\text{osmotic work}} = \Pi \Delta V, \tag{4}$$

where Π is the osmotic pressure due to added osmolyte, and ΔV is the change in osmolyte-excluded volume of products relative to reactants. At equilibrium, $\Delta G_{\text{total}} = 0$, and K is the apparent equilibrium constant, K_A, at a particular osmotic strength. Therefore, at equilibrium,

$$\Delta G°_{\text{binding}} = -RT \ln(K_A) - \Pi \Delta V = \text{constant}, \tag{5}$$

independent of T, Π, and K_A.

If Π is changed, K_A will change, and for any two values of Π, one can set $RT \ln(K_{A1}) + \Pi_1 \Delta V = RT \ln(K_{A2}) + \Pi_2 \Delta V$, to obtain the expression

$$RT \ln(K_{A1}/K_{A2}) = -(\Pi_1 - \Pi_2)\Delta V. \tag{6}$$

This is equivalent to the equation of Rand et al. (1993), which is used to estimate ΔV, the volume of water moved by osmotic work, from the measured changes in the apparent affinity at different osmotic strengths. Notice the assumptions: the first is that the effect of [osmolyte] can be assigned to osmotic work done to remove water from, or add water to, the binding site or other osmolyte-excluded volume that is sensitive to binding; the second is that the free energy of binding is independent of the free energy of osmotic work, i.e., of hydration change. The osmolyte exerts its effect only on the bulk solvent.

A second approach to relating ligand binding and hydration is to include the hydration change explicitly in the reaction equation, and to treat the effects of the osmolytes in terms of their effects on the activity of water. In this case one starts with

$$E(H_2O)_n + S \rightleftarrows E \cdot S + nH_2O, \tag{7}$$

and the binding equation is

$$\Delta G = \Delta G°_{\text{binding}} + RT \ln(a_{E\cdot S} a^n_{H_2O}/a_E a_S), \tag{8}$$

where $a = c \times \gamma$ (i.e., activity = concentration × activity coefficient). At equilibrium $\Delta G = 0$, and

$$\Delta G°_{\text{binding}} = -RT \ln(a_{E\cdot S}/a_E a_S) - nRT \ln(a_{H_2O}) \tag{9}$$

$$= -RT \ln(c_{E\cdot S}/c_E c_S) - RT \ln(\gamma_{E\cdot S}/\gamma_E \gamma_S) - nRT \ln(a_{H_2O}). \tag{10}$$

Assuming that all of the protein-protein and nonosmolyte solute-protein interactions are in $\gamma_{E\cdot S}$, γ_E, and γ_S,

$$\Delta G°_{\text{binding}} = -RT \ln(K_A) - nRT \ln(a_{H_2O}), \tag{11}$$

if the reactants and products, other than water, are sufficiently dilute that their activities can be assumed to be unity. Now, making the assumption that only the activity of the bulk water is sensitive to osmotic strength, the change of osmolyte-excluded hydration volume can be expressed as osmotic work:

$$\Pi \Delta V = nRT \ln(a_{H_2O}). \tag{12}$$

When Π is varied, one obtains from Eq. 11

$$RT \ln(K_{A1}/K_{A2}) = -(\Pi_1 - \Pi_2)\Delta V, \tag{13}$$

which is the same as Eq. 6. This derivation is more model dependent than the previous one, but in it the role of the water of hydration is clearer. The number of waters, n, is introduced stoichiometrically in this case, although it is unlikely that this corresponds to n specific binding sites for water. It is more likely that the reactants contain, on the average, n more water molecules in osmolyte-excluded volumes than the products do.

MATERIALS AND METHODS

Proteins

Myosin was isolated from New Zealand rabbit dorsal muscle and purified (Nauss et al., 1969). Myosin subfragment 1 was prepared from myosin using papain (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. Actin was prepared and purified from rabbit dorsal muscle (Spudich and Watt, 1971). F-actin pellets were suspended in osmolyte-free buffer and dispersed using a glass homogenizer

to make actin stock solutions. S1 MgATPase activity was typically 0.050 s^{-1} and increased 20- to 100-fold in the presence of actin. S1 was modified at Cys-707 with the fluorescent probe 5-(iodoacetamido)-fluorescein by standard methods (Lin and Cheung, 1991).

Chemicals

Chemicals used to prepare buffers were reagent grade. 5-(Iodoacetamido)-fluorescein was purchased from Molecular Probes. Polyethylene glycols (PEGn has an average molecular weight n × 1000) were from Fluka Chemical (PEG1 and 3), EM Sciences (PEG4), and Sigma Chemical (PEG1.45), and were used without further purification. PEG stock solutions were prepared gravimetrically and diluted volumetrically to prepare solutions used for experiments. The fluorescent ATP analog ϵATP was synthesized as described (Franks-Skiba et al., 1994).

MgATPase measurements

Steady-state S1 MgATPase activities were determined at 25°C by measuring the decreased absorbance at 340 nm resulting from decreasing concentrations of the reduced form of nicotinamide adenine dinucleotide during the 5 min after the addition of ATP, using a coupled assay system (Imamura et al., 1966). Control measurements were done in the presence of PEG to ensure that the reactions of the coupled assay remained faster than those of the ATPase. Values for K_M and V_{max} were determined from best statistical fits to plots of activity versus [ATP], assuming Michaelis-Menten kinetics. The apparent K_M values for MgATP association with S1 were in the high range, $\sim 1 \times 10^{-6}$ M. These are not true K_M values, as expected for kinetic measurements made with enzyme concentrations high in comparison to the K_M (Hackney and Clark, 1985).

For actin-activated S1 MgATPase activities, a molybdate assay of phosphate production at five time points was used to measure the rate (Lin and Morales, 1977). As for S1, values for K_M and V_{max} were determined from best statistical fits to plots of activity versus [actin], assuming Michaelis-Menten kinetics. Typical ATPase data are shown in Figs. 1 *A* and 5. The [PEG] in the molybdate assay solution is diluted (compared with the reaction mixture) to concentrations that do not interfere with the assay. For dilute [PEG], where both coupled and molybdate assays could be done, the measured rates were the same, within experimental error.

Phosphate burst

The production of phosphate by S1 in the presence and absence of PEG4 was determined as a function of time and fitted to a straight line. The burst of P_i production, which occurs before the first data point, was taken as the intercept of the fitted line with the ordinate. Data were measured at 1 s using a home-built, quenched-flow apparatus (Myburgh et al., 1995). Data at 3 s and 10 s were obtained by mixing ATP and S1 with rapid vortexing, followed at the appropriate time by an injection of perchloroacetic acid to quench the reaction. [S1] was 30 μM and [ATP] was 100 μM in 40 mM MOPS, 5 mM $MgCl_2$, 1 mM EGTA, pH 7 at 25°C.

Fluorescence measurements

Fluorescence intensities were measured at 25°C using a Perkin-Elmer MPF-44B fluorospectrophotometer. For S1 intrinsic tryptophan fluorescence, the excitation and emission wavelengths were 300 and 340 nm, respectively. For fluorescein-modified S1 fluorescence, the excitation and emission wavelengths were 490 and 520 nm, respectively.

Acrylamide quenching of fluorescence intensity of S1-bound ATP analogs was measured using front-face detection of fluorescence intensity as previously described (Franks-Skiba et al., 1994). The fluorescence of ϵADP in the presence of S1 displays a downward concavity characteristic of the quenching of multiple species with different values for the quenching constant K_Q. In this case at least two species of nucleotide exist, the free nucleotide and the bound nucleotide. The data were fitted using

$$F_0/F = \{F_1/(1 + K_1[Q]) + F_2/(1 + K_2[Q]) \ldots\}^{-1}, \quad (14)$$

which is the appropriate extension of the Stern-Volmer equation for samples having multiple components (Lakowicz, 1983). F_1, F_2, etc. represent the fluorescent intensities of components 1, 2, etc. in the absence of quenchers, and K_1, K_2, etc. represent their respective quenching constants.

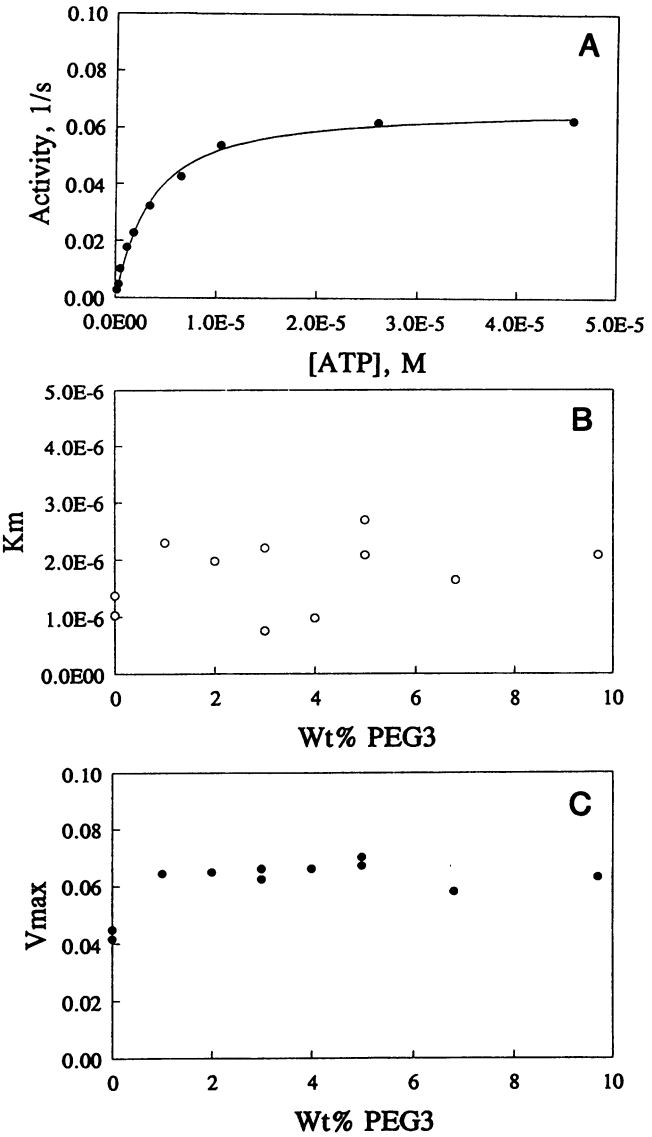

FIGURE 1 The effect of osmotic stress on S1 hydrolysis of MgATP. S1 MgATP activity was measured at 25°C in the presence of varying [ATP] in 10 mM KCl, 10 mM MOPS (pH 7), 1 mM $MgCl_2$, and varying amounts of PEG3, using the coupled assay described in the text. (*A*) Activity versus [ATP]. Typical data are shown for 1 μM S1 with 5 wt% PEG3 present. The solid line is the best fit to the data using a Michaelis-Menten kinetic scheme, with V_{max} and K_M as the variable parameters. (*B*) Apparent Michaelis constants. K_M values (M) for ATP hydrolysis by S1 in 0 to 10 wt% PEG3. There is no significant [PEG3] dependence. (*C*) Maximum velocities. V_{max} values (s^{-1}) for ATP hydrolysis by S1 in solutions containing 0 to 10 wt% PEG3 also show no significant dependence on [PEG3].

Osmotic pressures

Osmotic pressures for PEG solutions were measured using a vapor pressure osmometer or from the force between phospholipid membranes, and were kindly provided to us by Nola Fuller, Chuck Reid, and Peter Rand at Brock University (at http://aqueous.labs.brocku.ca/osfile.html).

RESULTS

Steady-state S1 MgATPase activity

Polyethylene glycol of molecular weights ranging from 1000 to 4000 was included at concentrations between 1 and 10 wt% in S1 MgATPase assay buffers, and steady-state activities were measured with increasing concentrations of ATP present. PEG was chosen as an osmolyte because it does not interact with proteins or protein hydration spheres directly (Bhat and Timasheff, 1992). For higher molecular weight PEG and/or at concentrations greater than 10 wt%, S1 solubility was often a problem. Light scattering intensity at 90° was used to monitor for aggregation (data not shown), and only data for solutions are reported. In all cases, the activity versus [ATP] data were well approximated by Michaelis-Menten kinetics (Fig. 1 *A*). The values observed for K_M (Fig. 1 *B*) and V_{max} (Fig. 1 *C*) measured in solutions containing up to 10 wt% PEG3 were independent of [PEG3] within experimental error. These data suggest that for osmotic pressures up to 5×10^6 dyne/cm^2, S1 does not undergo a significant net hydration change for the reactions involving the binding and hydrolysis of ATP, or for the reaction step that produces the transition state for phosphate release.

Phosphate burst data

Myosin S1 binds ATP and undergoes hydrolysis in a series of steps that are relatively rapid when compared to the release of products. This produces a burst of acid-labile phosphate production in times less than 1 s, followed by a slower rate of steady-state hydrolysis. Fig. 2 shows the number of phosphates hydrolyzed per S1 as a function of time. The data were fit by straight lines, giving intercepts at time 0 of 0.60 ± 0.1 in the absence of PEG, and 0.63 ± 0.1 in the presence of 5 wt% PEG4. The steady-state rate of hydrolysis is 0.04 s^{-1} in the absence of PEG, which is appropriate for S1 at 25°C. These data show that the binding of ATP and the steps preceding the hydrolysis of ATP at the active site of myosin are not altered in the presence of 5 wt% PEG4.

ATP binding site-related structural changes

The possibility of significant but compensating changes in S1 hydration cannot be excluded by the above data. For example, if the ATP site were dehydrated while another site were hydrated, net hydration would not change. In such a case there would be associated structural changes that could be perturbed by osmotic pressure

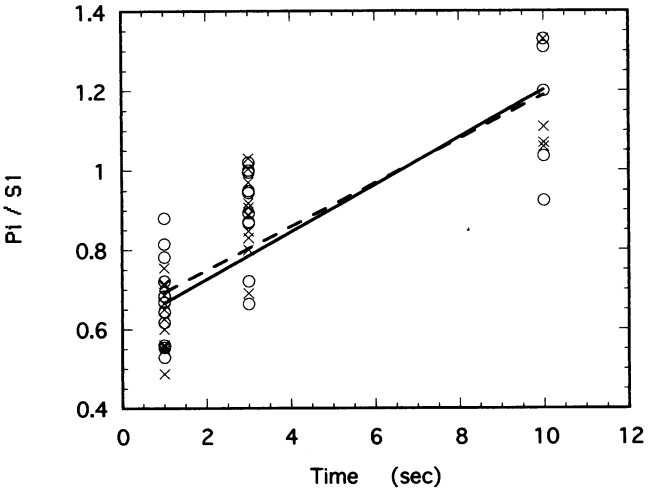

FIGURE 2 The production of phosphate by S1 as a function of time. S1 was mixed rapidly with ATP using a simple stopped flow apparatus (data at 1 s), or by vortexing in a test tube (data at 3 and 10 s), and the reactions were quenched by the addition of acid. The production of phosphate was measured by its reaction with malachite green. The intercepts at $t = 0$ are 0.60 ± 0.1 and 0.63 ± 0.1 mol phosphate/mol S1, in the absence (○) and presence (×), respectively, of 5 wt% PEG4.

when the ATP site is empty or when it is occupied by ADP rather than ADP·P_i. However, several experiments, discussed below, suggested that no substantial osmotic pressure-sensitive structural changes occurred for the reactions that produce S1·MgADP·P_i, S1·MgADP, or S1, during ATP hydrolysis and product release.

S1 tryptophan fluorescence intensity is an intrinsic probe that reports structural changes that occur when MgATP binds and is converted to MgADP·P_i (Werber et al., 1972). When the osmotic pressure is increased from 0 to 5×10^6 dyne/cm^2, using 1–10 wt% PEG1.45, tryptophan fluorescence intensity in the absence of ATP remains unchanged, within experimental uncertainty (Fig. 3 *A*). The increase in tryptophan fluorescence intensity for the S1 to (S1·MgATP $\rightleftharpoons$ S1·MgADP·P_i) transition is also constant within experimental error (Fig. 3 *A*). The increase in fluorescence intensity of S1·MgADP is also unchanged by 10 wt% PEG1.45 (data not shown).

If S1 is chemically modified at Cys-707 with the fluorescence probe 5-(iodoacetamido)-fluorescein, its fluorescence intensity decreases by 30% when MgADP binds to form S1·MgADP (Aguirre et al., 1986). This extrinsic probe was used because it might be more sensitive than the intrinsic tryptophan signal to putative PEG-induced structural changes due to ADP binding; however, the decrease in fluorescence intensity of S1·Mg·ADP, compared to S1, was not significantly changed by 1 to 10 wt% PEG3, which increases the osmotic pressure by 2×10^6 dyne/cm^2 (Fig. 3 *B*).

The accessibility of the fluorescent ADP analog ϵADP, when bound in the ATP site, was examined by measuring the rate of collisional quenching by acrylamide (Ando and Duke, 1982; Franks-Skiba et al., 1994). The accessibility of

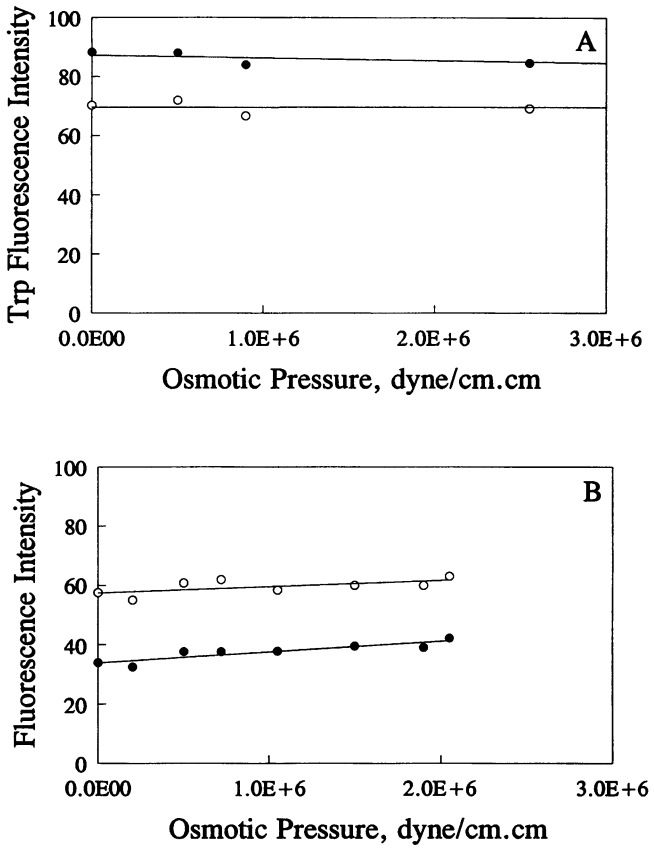

FIGURE 3 S1 and S1-fluorescein fluorescence intensities in the presence of osmolytes. (*A*) Tryptophan intensity. The tryptophan intensity, red edge excitation corrected for background and dilution, of S1 (○) and S1·MgADP·P_i (●) were measured in solutions containing 100 mM KCl, 10 mM MOPS, 1 mM $MgCl_2$, pH 7.0. When ATP is present the fluorescence intensity increases by about 23% for solutions containing 0 to 10 wt% PEG1.45. (*B*) Fluorescence intensity of S1-(acetamido)-fluorescein. The fluorescence intensity at 25°C of 5-(iodoacetamido)-fluorescein-modified S1 was measured in the absence (○) and presence (●) of 1 mM MgADP. The decrease in intensity was about 30% in the presence of 0 to 10 wt% PEG3. The conditions are as in Fig. 1.

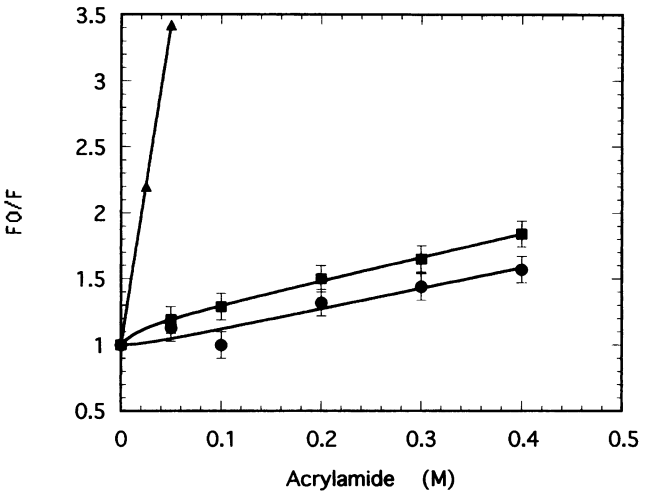

FIGURE 4 Acrylamide quenching of S1·MgϵADP. Stern-Volmer quenching of S1·MgϵADP by acrylamide was unchanged within experimental error, in the absence and presence of 7 wt% PEG4. The data are plotted and fitted as described in the text, with 25 μM ϵADP in the presence of 60 μM S1 in 100 mM KCl, 10 mM MOPS, 1 mM $MgCl_2$, pH 7, at 25°C. K_{SV} was 1.4 ± 0.2 M^{-1} for S1·MgϵADP in the absence of PEG (●) and was 1.6 ± 0.2 M^{-1} in the presence of PEG (■), compared to 54 M^{-1} for the quenching of free ϵADP (▲).

ϵADP was unchanged by the presence of 7 wt% PEG4 (Fig. 4). This result is consistent with the data above from intrinsic and extrinsic probes, which indicate no hydration-sensitive, nucleotide-induced protein conformational changes for the reaction S1 + MgADP ⇄ S1·MgADP (Fig. 3). Taken together, these data suggest that the structure of S1, by itself or for the reactions that produce S1·MgADP or S1·MgADP·P_i, does not undergo hydration changes that can be effected by osmotic strengths in the 1–5 × 10^6 dyne/cm^2 range.

Steady-state actin-S1 MgATPase activity

When actin-S1 MgATPase activities were measured at constant [ATP] with increasing amounts of actin at different osmotic pressures, V_{max} varied between 3 and 9 s^{-1}, but the variation did not depend on [PEG] in the 1–10 wt% range (data not shown). K_M for actin binding, on the other hand, depended strongly on [PEG], as originally reported by White et al. (1995); it decreased by an order of magnitude in the 5 × 10^{-4} to 5 × 10^{-6} M range (Fig. 5). The V_{max} and K_M values in the absence of PEG are in reasonable agreement with those expected for S1 under the conditions used (Chalovich et al., 1984). When $\ln(K_M/K_{M,\pi})$ is plotted against osmotic pressure, the positive slope indicates that PEG-induced dehydration promotes the binding of actin to the S1·MgADP·P_i intermediate. This slope increased with increasing molecular weight of PEG (Fig. 6), indicating that the change in osmolyte-excluded volume, ΔV, increases

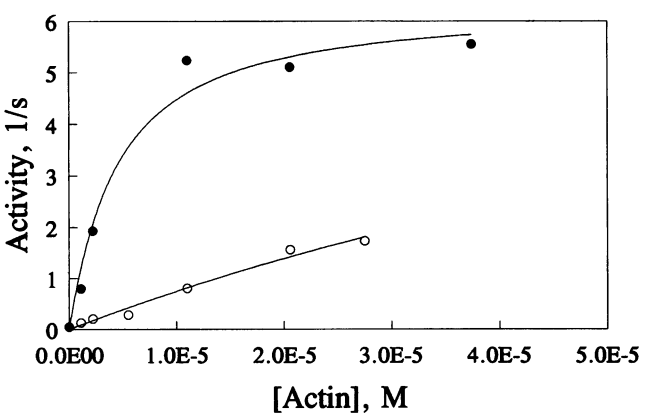

FIGURE 5 Osmotic stress effects on actin-S1 hydrolysis of MgATP. S1 MgATPase activities at 25°C were measured as a function of increasing [actin] in solution containing added PEG3. Conditions are as in Fig. 1. Data for 1 μM S1 in the presence of 1.5 wt% (○) and 7.5 wt% (●) PEG3 are shown, where the solid lines are the best fits using Michaelis-Menten kinetics, with V_{max} and K_M as the variable parameters.

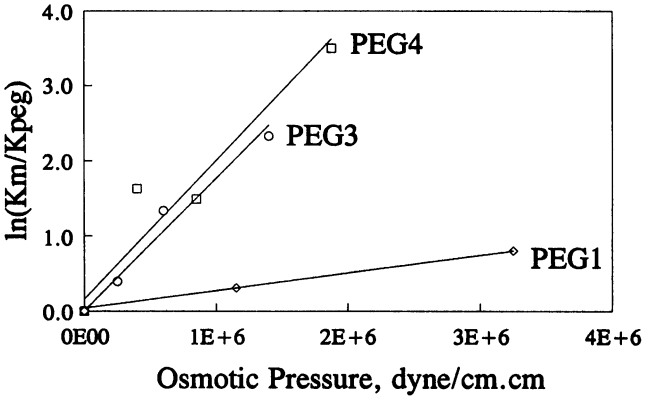

FIGURE 6 PEG size effects on apparent hydration volumes. The natural logarithm of the ratio of the Michaelis constant in the absence and presence of PEG1, PEG3, and PEG4 is plotted against the osmotic pressure increase due to added PEG (see Eq. 6). The positive slopes indicate that actin and S1·MgADP·P_i are dehydrated when they bind. The slope increases with the size of PEG because PEGs are excluded from the volumes near the surfaces of proteins, and the larger the PEG, the larger the excluded volume (Bhat and Timasheff, 1992).

when larger PEG molecules are used. This behavior has been reported for PEG-induced protein aggregation (Bhat and Timasheff, 1992). The slopes and volumes obtained using PEG1, PEG3, and PEG4 are shown in Table 1.

The experimentally determined osmolyte-excluded volume of hydration was used to estimate the area of the binding interface. The PEG average length was assigned to be the thickness of excluded volumes extending from the surfaces of actin and S1·MgADP·P_i, which are dehydrated upon binding. The PEG average end-to-end length, $\langle r \rangle$, was calculated from $\langle r \rangle^2 = nl^2$ (Cantor and Schimmel, 1980), where n is the number of flexibly attached carbon and oxygen atoms in the PEG polymer, and l was taken as 1.54 Å for the C-C and C-O bond lengths. The dehydrated area on each surface is $\Delta V/2\langle r \rangle$. The estimated area is smaller for the smallest PEG, suggesting that it may not be probing surfaces identical to those probed by the larger PEG3 and PEG4.

DISCUSSION

All of the data reported here for S1, in the absence of actin, are consistent with nucleotide-induced structural changes that do not involve significant changes in S1 hydration. The relatively small increase in osmotic stress used here, to avoid precipitation, does not appear to perturb S1·MgADP or S1·MgADP·P_i formation, or the structure of S1 itself, as monitored by steady-state kinetics, phosphate burst values, intrinsic tryptophan fluorescence intensity, and the fluorescence intensity of an extrinsic probe that is sensitive to nucleotide binding. Measurements of the effects of PEG on the accessibility to acrylamide of an ATP site-bound ATP analog indicate that the ATP site is not specifically being opened or closed significantly by osmotic stress.

It was anticipated that MgATP binding at the S1 active site would displace bound water, which would be detected. The atomic structure of S1 (Rayment et al., 1993b) has several crevices, the ATP site among them, that could open or close. The original application of osmotic stress to measure ligand-induced dehydration of hexokinase included data for higher pressures, although the effects of osmotic stress on the hexokinase system were evident at the lower pressures used in the present study (Rand et al., 1993). Significant S1 structural changes due to ATP binding and/or hydrolysis have been observed (Aguirre et al., 1989; Wakabayashi et al., 1992; Highsmith and Eden, 1993), but the data here suggest that those conformational changes do not involve significant net changes in active site hydration.

The larger crevice that bisects the actin-binding site has a volume of 8200 Å^3, estimated from molecular graphics depiction of the atomic structure of S1 (Rayment et al., 1993b). Using Eq. 6, one can estimate that a net hydration change of this volume for 1 to 10 wt% [PEG] would change K_M or the other measured kinetic and structural parameters by 50%. The S1-ATP K_M data are not precise enough to measure such a change. However, the V_{max}, P_i burst, fluorescence intensity, and acrylamide quenching data are precise enough, and none are consistent with crevice closure.

Data corroborating the osmotic stress results can be obtained from measurements of partial specific adiabatic compressibility, which should be greater for protein structures that are more hydrated, especially at locations in crevices that can close. Recent measurements of the partial specific adiabatic compressibilities for S1·MgADP·P_i and S1 are $(5.7 \pm 1.3) \times 10^{12}$ and $(4.2 \pm 0.6) \times 10^{12}$ cm^2/dyne, respectively (Tamura et al., 1993). These data suggest that the compressibilities of S1·MgADP·P_i and S1 are not sig-

TABLE 1 Actin·S1·MgADP·P dehydration probed with osmotic stress

PEG M.W.	Slope (cm^2/dyne)	ΔV (Å^3)	n H_2O	$\langle r \rangle$ (Å)	Calculated area (Å^2)
1000	2.4×10^{-7}	10,000	330	12.9	390
3000	1.8×10^{-6}	74,000	2,500	22.1	1,700
4000	1.8×10^{-6}	74,000	2,500	25.5	1,500

Volumes of osmolyte-excluded water of hydration that are transferred to the bulk solvent by the reaction actin + S1·MgADP·P_i ⇄ actin·S1·MgADP·P_i were estimated from the data in Fig. 6 using Eq. 6, as described in the text. The experimental uncertainty is ±10%. The numbers of waters were calculated assuming 30 Å^3/water molecule. The volume, ΔV, increases with the size of the PEG, as reported by Bhat and Timasheff (1992). The average end-to-end lengths, $\langle r \rangle$, of the PEGs were calculated assuming that PEG is a flexible polymer (see text), and the area of the binding interface was estimated from $\Delta V/2\langle r \rangle$.

nificantly different, which is consistent with small differences in the amount of internally bound water.

In contrast to S1 binding ATP, the interaction of actin and S1·MgADP·P_i is sensitive to the levels osmotic stress induced by 1–10 wt% PEG in the 1000–4000 Da range. Dehydration favors the formation of actin·S1·MgADP·P_i, as first reported by White et al. (1995). This confirms that the approach of Rand et al. (1993), using osmotic stress to estimate hydration volume changes associated with ligand binding (Garner and Rau, 1995), also can be used for protein-protein binding. The increase in the measured hydration volume with increasing PEG size is also consistent with the excluded volume mechanism of PEG-induced protein-protein binding, reported by Bhat and Timasheff (1992) for nonspecific reversible aggregation of identical proteins, and shows that this method is applicable to specific binding of heterogeneous subunits.

The numbers of water molecules that dissociate as actin·S1·MgADP·P_i forms, as estimated from the osmotic work, are larger than the partial specific volume changes that have been determined from the effects of hydrostatic pressure on reaction rates and equilibria. Increased hydrostatic pressure decreases the rate, but not the extent, of actin·myosin superprecipitation, indicating a 59–84 $Å^3$/myosin molecule increase in the solution volume for ATP binding (Rainford et al., 1965). Similarly, the effects of decreased hydrostatic pressure on actin·myosin in solution indicated a 166-$Å^3$/myosin molecule increase upon binding (Coates et al., 1985). However, the hydrostatic pressure-induced volume change of the solution is not directly comparable to the osmotic pressure-induced volume change of hydration, although the two pressures can be related (Robinson and Sligar, 1994). For PEG, the excluded volumes are defined by any impenetrable volume plus a region at the surfaces of the proteins extending out a distance equal to the average length of the PEG polymer (Bhat and Timasheff, 1992). This explains the large volumes, and their PEG size dependence, shown in Table 1.

An estimate of the area of the binding interface was made from the measured PEG3-excluded volume for actin·S1·MgADP·P_i formation and the length of the PEG, $\langle r \rangle$ (= 22.1 Å; see Table 1). Assuming that the PEG3 is excluded from a cylinder two PEG lengths long between the surfaces, the area of each surface is ~1700 $Å^2$. This simple calculation overestimates the area because of edge effects. Nonetheless, the calculated area is consistent with estimates of the actual interface area at 1100 $Å^2$ (estimated by Dr. Robert Mendelson (unpublished data) using the Rayment et al. (1993a) model of S1, with amino acid side chains modeled by Dr. Michael Lorentz).

Our results confirm the usefulness of the approach of Rand and his colleagues (Rand et al., 1993) of using osmotic pressure to probe hydration changes during enzymic reactions. They also are consistent with the theory of Bhat and Timasheff (1992) of the mechanism of action of surface-excluded osmolytes. The effects of osmolytes on the actin·myosin·ATP interactions must be studied in greater detail, because osmotic strength in situ is much greater than in solutions typically used for biochemical and biophysical measurements (Minton, 1981). Experiments on muscle fibers are under way, and preliminary data (Cooke, unpublished data) indicate that tension is increased and velocity is decreased for fiber contraction in the presence of osmotic pressure comparable to those used here.

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