Real-Time Measurement of Myosin–Nucleotide Noncovalent Complexes by Electrospray Ionization Mass Spectrometry

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ABSTRACT Nanoelectrospray ionization mass spectrometry has been used to measure the binding of ATP and ADP to the active site of rabbit skeletal myosin-S1. Increases in the molecular mass of myosin-S1 of 425 ± 10 Da were obtained with the binding of ADP to the active site and by 530 ± 10 Da with either ATP or hydrolysis products ADP and phosphate. Active site titrations of myosin-S1 with ADP gave a stoichiometry of ~1 ADP/S1 with an affinity in the micromolar range. The binding of ATP to myosin-S1 could be observed in the presence of up to 60 μM of excess MgATP without nonspecific binding of MgATP to the myosin. Conversion of the nucleotide complex containing an equilibrium mixture of ATP and ADP-Pi bound to myosin-S1 to one containing only bound ADP occurs at a rate consistent with that of the known steady-state rate of ATP hydrolysis. We expect this method to be of considerable use in the analysis of ligand binding and hydrolysis by the active sites of expressed myosin and myosin subfragments, which are not available in sufficient quantities for conventional methods of measurement of ligand binding.

INTRODUCTION

Most cellular processes are performed by protein complexes that involve noncovalent molecular interactions that are often weak and transient. Myosins are a family of motor proteins that interact with actin to produce force and motility in many eukaryotic cells, most notably in muscle. Skeletal muscle myosin is composed of a coiled-coil polypeptide tail attached to two globular heads. Each molecule contains two heavy chains, two regulatory light chains, and two essential light chains. Each globular head of myosin has two unique binding sites: one for binding adenosine triphosphate (ATP) and the other for actin. The molecular origin of muscle force involves a gross change in conformation of the head of the myosin molecule that is associated with the dissociation of the hydrolysis products ADP and phosphate while it is attached to actin. A major challenge in biophysics and cell biology is to understand how the free energy of ATP hydrolysis is coupled to the production of mechanical work. This is important in many cellular processes in addition to muscle contraction, including membrane trafficking, cell motility, and signal transduction (1,2).

To investigate noncovalent complexes between myosin and its substrate and products, an analytical technique must be capable of observing these interactions under conditions in which the protein is catalytically active. Electrospray ionization-mass spectrometry (ESI-MS) (3) was first reported as a method for the accurate mass analysis of proteins following denaturation at low pH in aqueous/organic solvent mixtures. Shortly afterward, the ability of this technique to monitor biological molecules in their functionally active form was demonstrated by the observation of noncovalently bound complexes when the analysis was performed under near-physiological conditions (4–6). Recent advances in the characterization of biomolecules using this technique and the low-flow-rate version nano-ESI (7) have involved analyses of noncovalent macromolecular complexes in excess of 1 MDa (8–11). ESI-MS can also be used to measure ligand binding to biologically functional proteins in the native state under carefully controlled conditions in which low concentrations of volatile buffers such as ammonium bicarbonate or ammonium acetate are used to maintain pH in the physiological range (12,13). The accurate mass measurement of noncovalently bound complexes often allows the stoichiometry of such species to be determined. An additional attractive feature is that because of the submicroliter per minute flow rates of nano-ESI, very little sample is consumed. Although the protein is desolvated in the vacuum system of the mass spectrometer, ligands, especially those bound by electrostatic forces, remain bound if mild ionization conditions and instrumental parameters are chosen and the desolvation effectively quenches the reaction in a dehydrated form. Methods based on ESI-MS for determining noncovalent protein-protein and protein-ligand complexes have been reviewed comprehensively (8,12,14,15).

ESI-MS has been used successfully to analyze noncovalent myosin complexes arising from calcium binding to the full motor domain of myosinVI (16,17). It has also been used to deduce the stoichiometry of noncovalently bound protein kinase–cyclic guanosine monophosphate complexes consisting of the protein homodimer bound to four nucleotide molecules (15). Here we have applied nano-ESI-MS

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to measure the binding of ADP and ATP to the active site of the proteolytic subfragment of rabbit skeletal myosin, myosin-A1-S1.

EXPERIMENTAL PROCEDURES

Myosin preparation and characterization

Rabbit skeletal myosin was prepared as previously described (18). Rabbit myosin-S1 is composed of two polypeptide chains: a heavy chain of ~92 kDa and a mixture of two light chains of ~16 and ~21 kDa (18). The two isomers were separated, and the A1 (~21 kDa) light chain fraction was used in this study (19). Nonvolatile salts and buffers were removed by extensive dialysis against ammonium bicarbonate (10 mM; pH 7.9; four changes of 1000× volume, 12 h each). We have also explored the use of Nanosep 3K Omega membranes (resuspending and spinning a 50-μl protein sample of 2 mg/ml myosin-A1-S1 six times with 0.5 ml of the same buffer) (16,17). This procedure also produced myosin-S1 suitable for MS and is a more rapid alternative method to exhaustively desalting labile proteins by dialysis. Steady-state ATP hydrolysis was measured by colorimetric determination of phosphate (19).

Noncovalent binding myosin experiments

Apomyosin-A1-S1 (4 μM) in 10 mM ammonium bicarbonate was brought to a final concentration of 60 μM $MgAc_2$ and analyzed by nano-ESI-MS. ADP titration experiments were performed by the addition of up to 9 μM ADP. The steady-state hydrolysis of mysosin-S1 with ATP (60 μM) was also monitored by nano-ESI-MS to detect the addition of ATP and its subsequent hydrolysis to ADP and phosphate. Preliminary experiments determined that 1–4 μM protein produced the largest signal. Higher concentrations of the protein, up to 20 μM, reduced the amplitude of the observed signal.

Electrospray ionization–mass spectrometry

Nano-ESI-MS was carried out on a Q-T of quadrupole–orthogonal acceleration time-of-flight mass spectrometer (Waters, Manchester, UK) fitted with a standard Z-spray source in positive ion mode. Three microliters of protein in solution (1–20 pmol μl^{-1}; 1–20 μM) was sprayed from a gold-plated borosilicate nanospray vial using a capillary voltage of 1 kV and a sampling cone voltage of 50 V. Data over the *m*/*z* range of 500–7000 were acquired over 1 min at a scan time of 3 s and combined to produce a single spectrum. An external calibration was applied using horse heart myoglobin. The *m*/*z* spectra were processed using the MassLynx software provided with the instrument. For Fig. 1, the *m*/*z* spectra were smoothed using the Savitzky Golay algorithm. For Figs. 2–4, the *m*/*z* spectra were transposed onto a molecular mass (i.e., zero charge) scale using Maximum Entropy Techniques (20,21). The ATP hydrolysis reaction (Fig. 3) was monitored continuously in real time. The spectra shown are the results of data acquired over a 1-min interval, each interval centered on the time indicated on the spectra ±30 s.

Data in Figs. 3 and 4 were fit using the nonlinear least-squares algorithms in the Scientist software package (ver 2.01, Micromath, St. Louis, MO). In both cases the first mass spectrum (1.5 min in Fig. 3 or 0 ADP in Fig. 4) was taken as a template for the pattern of mass distribution. It was assumed that, during the course of either ATP hydrolysis (Fig. 3) or ADP binding (Fig. 4), the mass spectra would be a combination of two components, the original spectra and the spectra generated by either the dissociation of phosphate (Fig. 3) or from ADP binding (Fig. 4). Initially, the data were fit by varying the mass difference and the fraction of the second component produced by ATP hydrolysis or ADP binding. The fraction of the bound components was then fit to determine the rate of ATP hydrolysis or the stoichiometry and equilibrium constant of ADP binding.

Typical values for the rate constants of the ATP hydrolysis mechanism of fast rabbit skeletal myosin at 20°C are shown in Eq. 1 (20). ATP binding and hydrolysis and ADP dissociation are fast relative to the steady-state rate, which is limited by slow dissociation of phosphate, k_{ss}. As a result, the steady-state rate of ATP hydrolysis is significantly dependent only on the value of phosphate dissociation and is not significantly dependent on the exact values of the other rate constants.

$$\mathrm{M} + \mathrm{ATP} \underset{2\,\mu\mathrm{M}^{-1}\,\mathrm{s}^{-1}}{\rightarrow} \mathrm{M\text{-}ATP} \underset{70\,\mathrm{s}^{-1}}{\rightleftarrows} \mathrm{M\text{-}ADP\text{-}Pi} \underset{k_{ss}}{\rightarrow} \mathrm{M\text{-}ADP} \underset{1\,\mathrm{s}^{-1}}{\rightleftarrows} \mathrm{M} \quad (1)$$

The time dependence of the concentration of the intermediates during steady-state ATP hydrolysis was determined by numerically evaluating the differential equations describing the catalytic mechanism in Eq. 1 using routines in Scientist. The dissociation constant of ADP from myosin is ~1 μM and at the myosin-S1 concentration used in these experiments, 4 μM, the concentration of apomyosin-S1 (M) is low, and M-ATP, M-ADP-Pi, and M-ADP are the predominant steady-state intermediates. The fraction

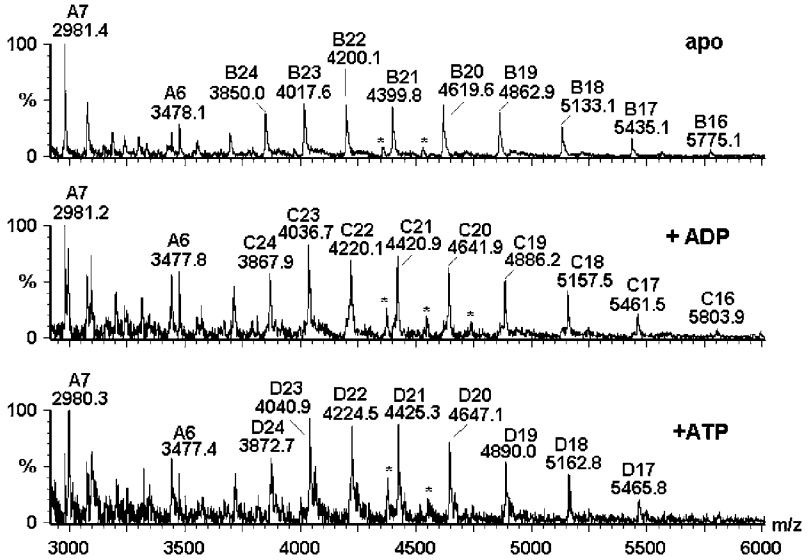

FIGURE 1 ESI-MS *m*/*z* spectra of myosin-A1-S1 in the absence of nucleotide (*upper*) and in the presence of ADP (*middle*) or ATP (*lower*). Experimental conditions during the measurement: 4 μM A1-S1 and either no additions (*upper*) or 20 μM ADP (*middle*) or 60 μM ATP (*lower*), 10 mM NH_4HCO_3, 60 μM $MgAc_2$ pH 7.9, 20°C. Masses of the predominant species are: A (A1 light chain) = 20,863 Da; B (heavy chain) = 92,382 Da, C (heavy chain + ADP) = 92,807 Da; D (heavy chain + ATP) = 92,912 Da; and * (B, C, or D + light chain). For each spectrum, data were acquired over 1 min and then combined to produce a single spectrum.

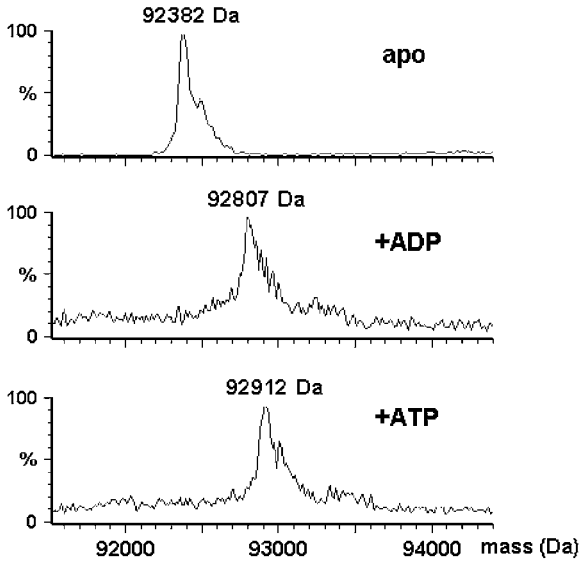

FIGURE 2 Mass profiles generated by Maximum Entropy (25,29) processing of the ESI-MS *m/z* spectra of the spectra shown in Fig. 1. Experimental conditions are as described in Fig. 1.

of the myosin, f_{ss}, with ADP bound relative to ATP (and hydrolysis products ADP and Pi) was calculated using Eq. 2.

$$f_{ss} = [\text{M-ADP}]/([\text{M-ATP}] + [\text{M-ADP-Pi}]) \quad (2)$$

Equation 3 can be used to determine f_D, the fraction of myosin-S1 heavy chain with bound ADP, where M_0 is the concentration of myosin-S1 nucleotide binding sites, K_{ADP} is the equilibrium constant for binding, and ADP_0 is the concentration of added ADP.

$$f_D = 1/(1 + K_{ADP}/(ADP_0 - f_D M_0)) \quad (3)$$

solving for f_D:

$$f_D = (x - (x^2 - 4(M_0)(ADP_0)^{1/2})/(2)(M_0) \quad (4)$$

where $x = K_{ADP} + M_0 + ADP_0$.

Global fitting routines using the nonlinear least-squares algorithms in Scientist were used to determine values for either k_{ss} (steady-state ATP hydrolysis) or K_{ADP} and M_0 (ADP binding) by nonlinear least-squares fitting of the experimental mass distributions in Figs. 3 and 4 to theoretical mass distributions calculated from the values for f_{ss} and f_D.

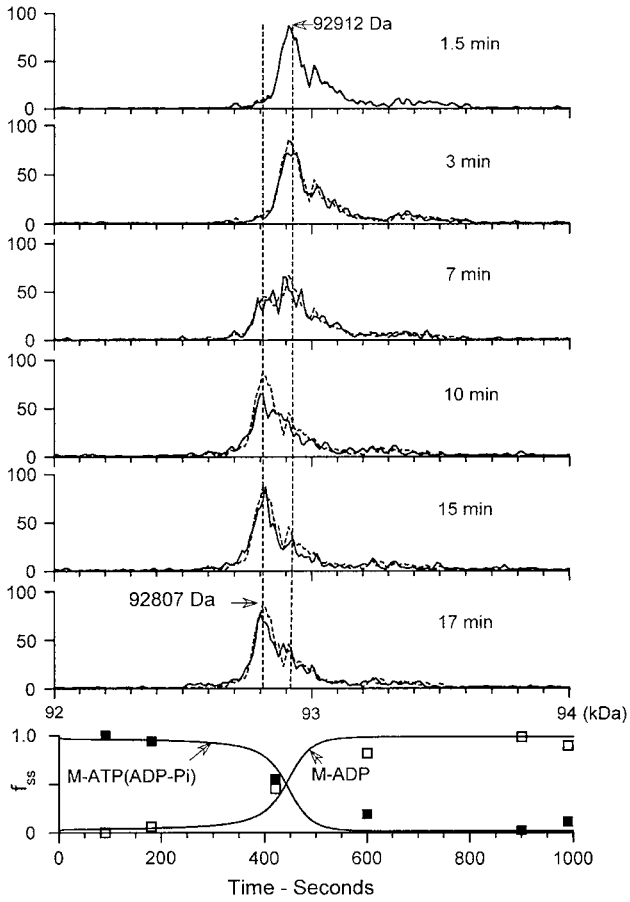

FIGURE 3 Mass profiles generated by maximum entropy (25,29) processing of the ESI-MS *m/z* spectra acquired during the real-time hydrolysis of ATP by myosin-S1 (*upper six graphs*). Experimental conditions were similar to those in Fig. 1 except that [myosin-A1-S1] = 4 μM and [ATP] = 60 μM at time zero. The hydrolysis reaction was monitored continuously in real time; the spectra shown are the result of combining data over a 1-min interval centered on the time indicated on the spectra $\pm$ 30 s. The solid lines show the experimentally measured mass spectral data; the dotted lines represent a global fit of the entire data set to the mechanism in Eqs. 1 and 2 to determine the steady-state rate of hydrolysis, $k_{ss} = 0.043\ s^{-1}$. The lower figure shows the time dependence of the fraction of the mass spectra corresponding to either M-ATP/M-ADP-Pi (*solid squares*) or M-ADP (*open squares*). The solid lines through the data are the theoretical time dependence calculated using the steady-state rate constant of $0.043\ s^{-1}$ measured by global fitting of the data.

RESULTS AND DISCUSSION

Analysis of myosin-A1-S1 by nano-ESI-MS

Myosin-A1-S1 in the presence of magnesium acetate was analyzed by nano-ESI-MS at neutral pH to produce a *m/z* spectrum showing three major myosin components of molecular masses 20,863, 92,382, and 113,216 Da, consistent with the A1 light chain, the S1 heavy chain, and an A1-S1 complex of the two components, respectively (*upper spectrum*, Fig. 1). Approximately 80% of the myosin-A1-S1 signal intensity corresponded to the heavy-chain constituents of the myosin from which the light chain had dissociated during the MS analysis. This is perhaps not surprising because the light chains are bound to the IQ region of the myosin heavy chain by a mixture of ionic and hydrophobic forces, and the latter have difficulty surviving the ESI-MS analysis, which involves solvent removal (21,22). The mass of 20,863 Da measured here is consistent with that expected from the sequence of the A1 light chain with two Mg^{2+} bound to the high-affinity divalent cation binding sites (20,866 Da) (23). Unfortunately, there is no DNA sequence available for fast rabbit skeletal myosin-S1 heavy chain with which to compare the mass determined here for the molecule in the absence of ligands bound to the active site. Moreover, the available sequence information determined from protein sequencing

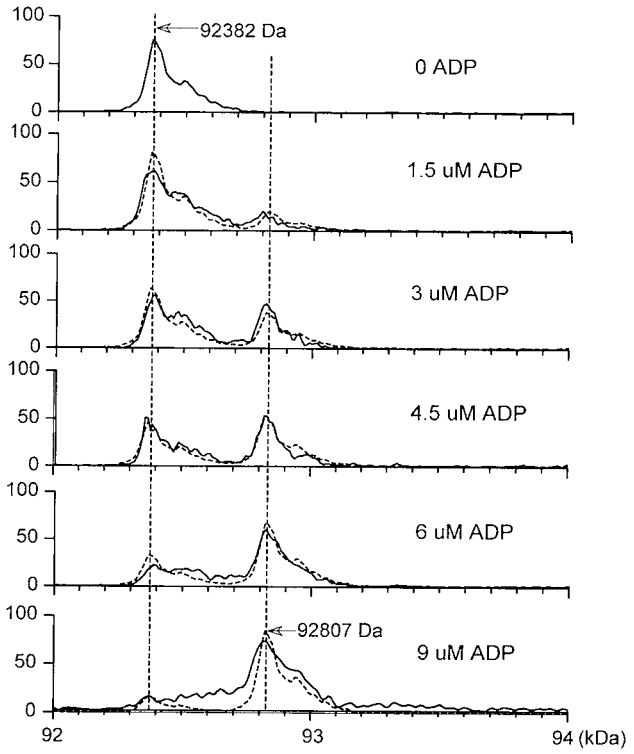

FIGURE 4 Mass profiles generated by maximum entropy (25,29) processing of the ESI-MS *m/z* spectra of the titration of 4 μM myosin-S1 with the indicated concentrations of ADP. Final experimental concentrations were otherwise similar to those in Fig. 1. The solid lines show the experimentally measured mass spectral data; the dotted lines represent a global fit of entire data set to determine the concentration of active site $M_0 = 3.2$ μM and association constant $K_{ADP} = 0.93$ μM.

indicates that rabbit fast skeletal muscle myosin-S1 preparations, such as the one used in this work, are microheterogeneous at 19 positions in the protein sequence (24). In the absence of nucleotide, the major heavy-chain species having a mass of 92,382 Da is accompanied by a minor species, seen as a partially resolved shoulder, with a mass of 92,494 Da. The two components are consistent with myosin-S1 being the product of at least two genes or splice variations. The mass observed in which the A1 light chain is bound to the heavy chain was measured to be 113,239 Da and is in good agreement with that predicted from adding the individually determined masses of the A1 light chain to that of the heavy chain (113,244 Da). On addition of ADP, the A1 light chain was observed again at 20,863 Da, and the myosin-S1 heavy chain at 92,807 Da, consistent with the binding of a single molecule of ADP, some 425 Da higher (Fig. 1, *middle spectrum*). On addition of ATP, the A1 light chain was again observed at the same mass (20,863 Da), but the myosin S1 heavy chain was observed at a mass of 92,912 Da, some 530 Da higher, which is consistent with the mass increase expected from ATP binding to the S1 heavy chain (Fig. 1, *lower spectrum*). Fig. 2 shows the molecular mass profiles (i.e., zero charge state profiles) generated by maximum entropy (25) processing of the *m/z* spectra obtained from the analysis of the heavy chain of myosin-S1 with no added nucleotide, in the presence of ADP or ATP (*upper, middle, and lower traces, respectively*). The increase in mass relative to the apoprotein by ATP of 530 Da is that expected from having either MgATP (529 Da) or MgADP and phosphate (546 Da) bound to the active site during steady-state hydrolysis of ATP. Under the experimental conditions in Fig. 1–3, at times less than or equal to 3 min after the addition of ATP, it would be expected that the bound nucleotide would be a rapidly interconverting mixture of approximately 30% M-ATP and 70% M-ADP-Pi (20). The mass of myosin-S1 in the presence of ATP (92,912 Da) is ~100 Da greater than that observed in the presence of ADP (92,807 Da), and thus it is possible to detect the presence of phosphate bound to the active site in addition to ADP by nano-ESI-MS. The minor species of 92,494 Da has similar mass increases to the major peak in the presence of ADP and ATP. In all cases only a single ATP or ADP molecule bound to the protein is detected (i.e., a one-to-one stoichiometry of myosin-S1 to nucleotide).

Time course of the hydrolysis of ATP by myosin-S1

Steady-state kinetics of myosin-S1 hydrolysis of ATP was monitored over time after addition of ATP to A1-S1 and sampling the reaction at the indicated times (Fig. 3). The molecular mass of the myosin-S1–ATP complex was 92,912 Da, indicating the additional mass of ATP or ADP + Pi, at the earliest time point measured (90 s). The mass of the major peak decreased to 92,807 Da at times greater than 15 min, which is consistent with the mass of myosin-S1 with ADP noncovalently bound, indicating that all of the ATP had been hydrolyzed. At 7 min, the myosin-S1–ATP and myosin-S1–ADP peaks are of approximately equal size. The solid lines represent the measured mass distribution of the data scaled to make the total amplitudes equal at the various time points. Dotted lines through the data represent a global fit of the entire data to determine the steady-state rate of hydrolysis, $k_{ss} = 0.043 \pm 0.002$ s^{-1} set as described in the Methods section. This is in good agreement with the rate measured by colorimetric phosphate determination under the same experimental conditions (0.045 ± 0.003 s^{-1}) and literature values for MgATP hydrolysis (0.05 ± 0.01 s^{-1}) at 20°C (25). The bottom trace in Fig. 3 shows that the expected time dependence of the myosin-S1-ATP (*solid squares*) and myosin-S1-ADP (*open squares*) determined by fitting the mass spectra at individual time points is in good agreement with that determined by global fitting (*solid lines*).

Titration of myosin S1 with MgADP

Myosin-S1 (4 μM) was analyzed by nano-ESI-MS in the presence of 0 to 9 μM ADP. Two major peaks, corresponding

to myosin-S1 (92,382 Da) and myosin-S1 with noncovalently bound ADP (92,807 Da) were detected (Fig. 4). The fraction of myosin-S1 bound to ADP increases with ADP concentration until it approaches saturation at 9 μM ADP. Dotted lines through the data are global fits of the data to determine the dissociation constant and active site concentration as described in the Methods section. Fig. 5 shows the fraction of myosin-S1 with ADP noncovalently bound, f_D, plotted against the concentration of added ADP, ADP_0. The theoretical curve through the data corresponds to a stoichiometry of ~1 ADP/myosin-S1 and an affinity of 0.93 ± 0.5 μM, which is in agreement with values previously obtained by kinetic and spectroscopic methods that are in the 1–2 μM range (26–28).

Nano-ESI-MS has been used to measure the binding of substrate ATP and ADP to the active site of rabbit skeletal myosin-S1, the proteolytic subfragment of myosin, in volatile buffers. Mass measurements of species >90 kDa are achievable, as is the resolution of a mass difference HPO_4^{2-} (96 Da) between the two species of ~93 kDa, myosin-ADP and myosin-ATP. This is significant, especially when we are taking into account the noncovalent nature of these complexes for which analysis in aqueous solutions at neutral pH is a prerequisite. Together these features permit real-time monitoring of ATP hydrolysis by myosin-S1 using nano-ESI-MS. Up to 60 μM magnesium and nucleotide may be added without measurable nonspecific binding. The complete disappearance of the mass species corresponding to the apoprotein indicates that all of the protein is capable of binding and hydrolyzing the substrate and that nano-ESI-MS can preserve these particular noncovalent interactions. The molecular mass of the steady-state complex (M-MgATP ⇄ M-MgADP-Pi) is, as would be expected, ~100 Da greater than the ADP complex. The stoichiometry and affinity of ADP binding measured by MS are comparable to the values measured by solution and spectroscopic methods. Single determinations of substrate and product binding require only a few picomoles of protein and may be suitable for determining the functionality of expressed myosins that are only available in small quantities. Although the data are consistent with the expected substrate MgATP being bound and hydrolyzed to MgADP-Pi, the mass accuracy measured under these conditions is not sufficient to determine whether Mg (24 Da) is bound to the substrate and product or to measure the increase in mass (17 Da) that occurs on the hydrolysis of the MgATP to MgADP, although the dissociation of phosphate from the steady-state complex is readily determined. In some regards, fast rabbit skeletal myosin-S1, although stable and widely available, was not the ideal myosin for these experiments because of the heterogeneity of the heavy chain, which undoubtedly leads to additional uncertainty in the mass measurements, which were in the range of ± 5 to ± 10 Da of the total mass of the myosin heavy chain ~93 kDa. It is possible that the use of more homogeneous preparations of expressed myosins may provide higher accuracy and detail of the hydrolysis mechanism.

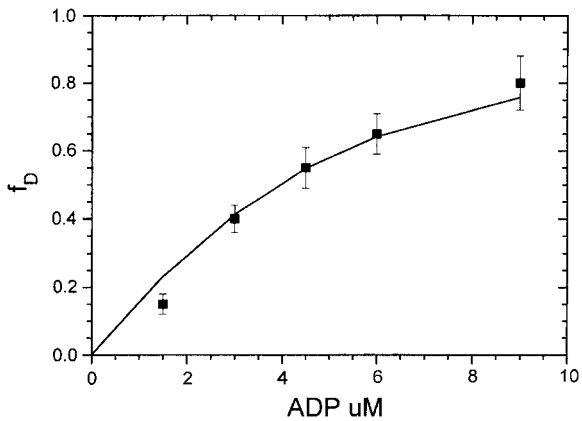

FIGURE 5 Dependence of the fraction of myosin-S1 containing bound MgADP. Experimental conditions are shown in Fig. 4. The solid line fit through the data is the best fit to Eq. 4 in the text where $M_0 = 3.2 \pm 1$ μM and $K_{ADP} = 0.93 \pm 0.5$ μM.

Nano-ESI-MS has, however, been demonstrated to be a valuable and reliable biophysical tool for real-time monitoring of binding and hydrolysis of ATP/ADP by rabbit skeletal myosin-S1 and thus presents a viable method for determining ligand binding at the active sites of myosin subfragments, which are unavailable in sufficient quantities for conventional methodologies to be employed.

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