Kinetic Characterization of Reductively Methylated Myosin Subfragment 1†

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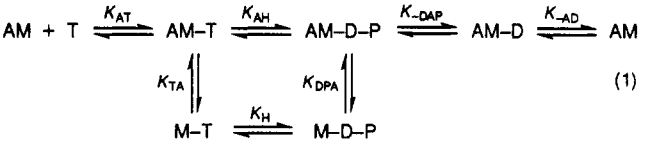
ABSTRACT: Reductive methylation of myosin-S1 converts 97% of lysine residues in native myosin-S1 to dimethyllysine without detectable modification of other amino acid side chains. RM-S1 is catalytically active, although the rate and equilibrium constants of many of the steps of the actomyosin ATP hydrolysis mechanism have been altered. The steady-state rate of MgATP hydrolysis by modified myosin-S1 (RM-S1) is increased 4–5-fold in the absence of actin. However, the maximum steady-state rate of RM-S1 at saturating actin, 0.59 s^{-1}, is less than one-tenth that observed for the unmodified protein, 7.4 s^{-1} (5 mM MOPS, 2 mM $MgCl_2$, pH 7, 20 °C). Under single-turnover conditions [S1] > [ATP], the observed rate of ATP hydrolysis by RM-S1 is fit by a single exponential that is no more than twice the steady-state rate, which indicates that the bond splitting state is at least partially rate limiting for RM-S1. Although a small decrease in intrinsic tryptophan fluorescence is observed upon ATP binding to RM-acto-S1, the large and rapid fluorescence enhancement produced by aza-ATP binding to RM-acto-S1 is nearly normal. ATP binds and dissociates modified RM-S1 from actin with a second-order rate constant that is more than twice that observed for control S1. The changes in the kinetic mechanism produced by reductive methylation of lysine are qualitatively and quantitatively similar to the changes that are induced by either SH1 modification or substrate analogues such as GTP.

In combination with structural studies, kinetic analysis offers one of the best routes to understanding the mechanism of action of a protein. It provides perhaps the most sensitive tool for assessing the changes in a protein brought about by site-directed mutagenesis or chemical modification. This is particularly important in those cases where chemical or genetic changes are necessary to provide a system that is more amenable for physical studies. A good example of this is found in the truncation of a polypeptide chain by proteolytic or genetic means as an approach to obtaining crystals suitable for a high-resolution X-ray structural analysis. This has been particularly useful for studying flexible, multidomain proteins such as antibodies and membrane-bound proteins. In such cases it was important to demonstrate that the modified system could be used as a model for the original state. Recently, an alternative approach to obtaining crystals of a protein was reported in the structure determination of myosin subfragment 1 (Rayment et al., 1993). In this case reductive methylation of essentially all of the lysine residues was the key step to obtaining crystals. So far this is the only approach that has yielded crystals of myosin-S1[1] that have been adequate to determine a high-resolution structure. For this structure to be of general use in understanding the molecular basis of motility, it is important to determine what if any changes the chemical modification has produced upon the structural and enzymatic properties of myosin.

To address this question, we report here a study of several key features of the mechanism of hydrolysis of myosin-S1 in which the lysine residues have been extensively modified by reductive methylation. Equation 1 contains a simplified scheme of the mechanism of actomyosin ATP hydrolysis in which all of the steps of ligand binding to myosin (A, T, D, P)[2] are shown here as single steps. A more complete scheme would include two-step ligand binding for each of these steps (Trybus & Taylor, 1982; Geeves, 1989).



(1)

ATP binding to AM is rapid and produces a rapid dissociation (>1000 s^{-1}) of myosin-S1 from actin in dilute solution (White & Taylor, 1976). The rate of M-ATP → M-D-P is approximately 100 s^{-1} at 20 °C (Sleep & Taylor, 1976). The steady-state rate of hydrolysis of myosin in the absence of actin is limited by slow release of products, ADP and phosphate (Bagshaw & Trentham, 1974). Actin increases the steady-state rate of ATP hydrolysis by increasing the rates of product release by more than 100-fold (Lymn & Taylor, 1971; Siemankowski & White, 1985). The transition between weakly bound states, AM–T and AM–D–P, and the strongly bound states, AM–D and AM, is thought to be associated with the movement and production of force (Eisenberg & Greene, 1980; Geeves, 1989). As described below, RM-S1

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[1] Abbreviations: aza-ATP, 1,N^6etheno-2-aza-ATP; S1, myosin subfragment 1; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; RM-S1, reductively methylated myosin subfragment 1.

[2] The following nomenclature is used to identify rate and equilibrium constants of the nucleoside hydrolysis mechanism. Positive subscripts identify rate and equilibrium constants of association; negative subscripts identify rate and equilibrium constants of dissociation. Single subscripts (A = actin, T = nucleoside triphosphate, D = nucleoside diphosphate, P = phosphate, H refers to the hydrolysis of T to D and P) refer to the binding of the respective ligand to myosin-S1, which is abbreviated M. For multiple subscripts the final letter of the string identifies the ligand associating (or dissociating) and all other letters refer to ligands already bound.

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is catalytically active and maintains many of the essential characteristic properties of the myosin ATP hydrolysis mechanism; however, the rate and equilibrium constants of the steps of the actomyosin ATP hydrolysis mechanism are significantly altered.

MATERIALS AND METHODS

Proteins. Myosin was purified from adult white rock chicken pectoralis muscle according to a modification of the procedure of Margossian and Lowey (1985) (Smith and Rayment, manuscript in preparation). The major changes in the protocol include the use of a Cibacron Blue affinity column in place of DEAE cellulose and the incorporation of a dephosphorylation step in the early stages of the isolation protocol as first suggested by Perrie and Perry (1970). These changes in the isolation procedure do not affect the kinetic properties but lead to improvements in the crystals. Myosin isolated from chicken pectoralis muscle consists of a mixed population of two isozymes caused by the existence of two species of the essential light chain (Silberstein & Lowey, 1981). These light chains, referred to as A1 and A2, have molecular weights of 21 000 and 16 000, respectively. Amino acid sequence studies of the light chains have demonstrated that A1 and A2 are identical over their 142 C-terminal residues. The size difference is caused by an additional 41 amino acids present at the N-terminus of A1. These isozymes arise by alternative transcription and two modes of splicing from a single gene (Nabeshima et al. 1984). In this study the mixed isozyme preparation was used for the kinetic studies since this was the material used in the crystallographic analysis. Actin was prepared from rabbit muscle using a modification of the method of Spudich and Watt (1971).

Reagents. All solutions used in the preparation of myosin and in the reductive methylation were made from reagent grade water from a Milli-Q water purification system. The solutions used in the kinetic measurements were prepared from deionized, glass-distilled water. All experiments were performed at 4 °C except where noted. Dimethylamine–borane complex was obtained from Aldrich Chemical Co. A 1 M stock solution was prepared in water immediately prior to use. Formaldehyde was purchased as a 16% solution (methanol free) from Electron Microscopy Sciences and was diluted with water to form a 1 M stock solution immediately before use. All other reagents were reagent grade and purchased through VWR and Sigma.

Reductive Methylation. Prior to modification, 5 mg/mL myosin-S1 was dialyzed against 200 mM potassium phosphate, pH 7.5, and 1 mM $MgCl_2$. The reductive methylation was performed at 4 °C by the sequential addition of 20 μL of 1 M dimethylamine–borane complex dissolved in water and 40 μL of 1 M formaldehyde per milliliter of protein with rapid stirring. This was repeated after 2 h. A final aliquot of 10 μL/mL of dimethylamine–borane complex was added after a further 2 h whereupon the reaction mixture was allowed to sit overnight at 4 °C in the dark. The reaction was quenched by the addition of 3.8 M ammonium sulfate to a final concentration of 1 M and then dialyzed for 48 h against 2.5 M ammonium sulfate and 50 mM potassium phosphate at pH 6.7 to precipitate the protein. In an attempt to protect lysine residues in the nucleotide binding site, the chemical modification was also carried out on protein in which ADP had been previously trapped in the active site. Trapping was performed with either vanadate or beryllium fluoride (Goodno, 1982; Phan & Reisler, 1992). Stock solutions of 500 mM ADP, 100 mM $MgCl_2$, and 500 mM sodium vanadate were added to give final concentrations of 2 mM ADP, 1 mM $MgCl_2$, and 0.4 mM vanadate. Final concentrations of 2 mM $BeCl_2$ and 5 mM sodium fluoride replaced vanadate in beryllium fluoride trapping experiments. Proteins with trapped nucleotides were incubated on ice for 1 h before reductive methylation. Proteins that were reductively methylated in the absence of nucleotide are designated (A), in the presence of ADP/vanadate (B), and in the presence of ADP/BeF_3 (C). Proteins used for structural determinations were prepared in presence of ADP/vanadate. Additional details of the development of this procedure are presented in Rypniewski et al. (1993). The degree of modification was determined by amino acid analysis which was performed by Dr. Liane Mende-Mueller at the Protein and Nucleic Acid Facility, Medical College of Wisconsin, Milwaukee, WI 53226. Protein samples were exhaustively dialyzed prior to analysis against a 25 mM 4-ethylmorpholine–acetate buffer solution at pH 8.0.

Single-turnover measurements of ATP hydrolysis were made using a quenched-flow apparatus in pulse quench mode (Fersht, 1979). Approximately 0.2 mL each of ATP [containing 5000–10000 dpm of [^{32}P]ATP (Dupont)] and proteins were mixed and held in a delay line for the indicated time after which a second drive was used to expel the reaction mixture and mix it with an acid quench (2 N HCl, 0.35 M KH_2PO_4). Using this configuration, mixing times in excess of 150 ms could be obtained. The total number of counts was determined for each sample by directly counting 0.5 mL of the reaction mixture. One milliliter of the quenched reaction mixture was vortexed with 1 mL of a 10% charcoal slurry and then centrifuged using a table clinical centrifuge for 5 min. The supernatant contains radioactive phosphate from enzyme bound (M–D–P) and free phosphate. Unhydrolyzed ATP is bound to the charcoal. Control experiments indicate that more than 99% of the unhydrolyzed ATP is bound to the charcoal. The fraction of ATP hydrolyzed was obtained from a ratio of the number of counts in 1.0 mL of the supernatant after charcoal binding to the total number of counts in 0.5 mL of each reaction mixture. A more complete description of the quench-flow apparatus will be published elsewhere.

aza-ATP was synthesized and purified as previously described (Smith & White, 1985) and further purified by chromatography on Bio-Rad AG MP-1 anion resin equilibrated with 20 mM Tris, pH 7.5, and eluted with a 0–0.75 M KCl gradient. Samples were desalted by Sephadex G-10 chromatography and concentrated by lyophilization to just short of dryness. Concentrated stock solutions (5–50 mM) were stored frozen in solution at –20 °C.

Pre-steady-state measurements of light scattering at 90° and fluorescence were made with an Applied Photophysics stopped-flow fluorimeter (Leatherhead, England) illuminated by a 100-W mercury arc lamp. The excitation wavelengths (295 or 365 nm) were selected with a 0.124 m monochrometer (Farrand Corp.). Emission wavelengths were either 320–380 nm (Schott UG-11) for tryptophan fluorescence or 430–470 nm (Corion LL-450 broad-band interference filter) for aza-ATP. A total of 1024 data points were collected with a Nicolet Explorer III digital oscilloscope and transferred to a Sharp 480-3 PC for permanent storage and analysis. Observed rate constants were obtained by fitting the data to a single-exponential equation by the method of moments (Dyson & Isenberg, 1971).

Steady-state nucleoside triphosphate hydrolysis was measured using a colorimetric procedure (White, 1982) from at least four time points per actin concentration by the method of initial rates. The data were then fit to the appropriate

steady-state kinetic equation using a Neader–Melder nonlinear least-squares simplex routine obtained from Dr. John Rupley at the University of Arizona. Standard deviations of the parameters are calculated by fitting a quadratic function to the surface about the minimum in parameter space and then using the properties of this function to calculate the variance–covariance matrix for the parameters.

RESULTS

Reductive alkylation of lysine residues involves the initial formation of a Schiff base between the ϵ-amino group of a lysine residue and either a ketone or aldehyde, which is then reduced to form a secondary or tertiary amine as indicated for formaldehyde in eq 2. With formaldehyde, the reaction

$$R\text{–}NH_2 + CH_2O \rightarrow R\text{–}N{=}CH_2 + H_2O \xrightarrow{\text{reduction}} R\text{–}NH\text{–}CH_3 \quad (2)$$

proceeds rapidly to form the dimethyllysine product since the pK_a of the monomethyllysine residue is lower than that of lysine itself. The initial attempts to alkylate the lysine residues in myosin subfragment 1 used sodium cyanoborohydride as the reducing reagent. Previous studies had demonstrated this reductant to be ideal for the labeling of proteins (Jentoft & Dearborn, 1979) since, in contrast to sodium borohydride (Means & Feeney, 1968), it was stable at pH 7.0. Consequently, many of the side reactions associated with the use of formaldehyde at higher pH could be avoided. Unfortunately, sodium cyanoborohydride ultimately proved unsuitable for the crystallization of myosin S-1 due to the inherent reaction of the cyanide ion with the initial Schiff base formation as indicated in eq 3 (Gidley & Sanders, 1982). This side reaction

$$R\text{–}N{=}CH_2 + CN^- + H^+ \rightarrow R\text{–}NH\text{–}CH_2CN \xrightarrow{\text{acid pH}} R\text{–}NH_3^+ + CH_2O + CN^- \quad (3)$$

reduced the incorporation of formaldehyde into the final modified protein by more than 20%, depending upon the concentration of sodium cyanoborohydride (Jentoft & Dearborn, 1979; Gidley & Sanders, 1982) and also presented three unacceptable problems in the preparation of material for crystallographic studies. First of all, it resulted in a heterogeneous population of molecules. Secondly, the slow hydrolysis of the *N*-cyanomethyl side product resulted in the release of formaldehyde, which then reacted during the crystallization experiments with other amino acid side chains such as cysteines and histidines. Finally, formation of the Schiff base between lysine and formaldehyde also facilitated the modification of adjacent tyrosine, tryptophan, histidine, asparagine, and cysteine residues (French & Edsall, 1945). In light of these problems, an alternative reduction strategy based on dimethylamine borane complex as a reducing agent was developed (Geoghegan et al., 1981; Cabacungan et al., 1982; Rypniewski et al., 1993). Table I shows the results of the amino acid analysis of the modified and control myosin subfragment 1. This shows that all except three to four of the lysine residues are modified. The extent of modification was the same within experimental error, 96.7 ± 1%, whether or not nucleotide was trapped in the active site. There is some discrepancy between the total number of lysine residues in the native and modified protein that may have arisen from a calibration error in the dimethyllysine standard. In addition, the number of lysine residues observed in the control is lower than that stated for the theoretical number. This latter discrepancy arises because the theoretical estimate was calculated from the sequences for the heavy chain, regulatory light chain, and a 50:50 mixture of the essential light chain isoforms. The A1 and A2 isoforms of the essential light chain contain 20 and 11 lysine residues, respectively. This estimate of the amino acid composition suffers from two sources of error. First, it assumes an equal distribution of light chain isoforms and, second, a full-length polypeptide chain for the A1 isoform. Based on polyacrylamide–SDS gel electrophoresis, the first assumption is approximately valid; however, papain cleaves a short peptide from the N-terminus of the A1 essential light chain that contains four lysine residues. This latter effect is the most likely source of the discrepancy and is also the reason for the difficulty is separating the isozymes of papain/Mg myosin S1 by ion exchange chromatography compared to the corresponding protein prepared by chymotryptic digestion that contains an intact A1 essential light chain. In this instance the relative number of residues observed in the control and modified protein is more important than the absolute number since this gives an estimate of how many residues, apart from the lysine, have been modified by the reductive methylation (samples A, B, and C were prepared from the same batch of protein). A comparison of the amino acid analysis of control and modified myosin-S1 in Table I indicates that there are no significant differences between the control and modified protein for any of the residues. This is particularly important for asparagine, methionine, tyrosine, and histidine that are known to have side reactions with formaldehyde and suggests that the reaction conditions described here avoid these problems.

The actin activation of the steady-state rate of ATP hydrolysis by RM-S1 and unmodified S1 is shown in Figure

Table I: Amino Acid Analysis of Modified and Native Myosin Subfragment 1

amino acid	theoretical	native[a]	modified[a]
aspartate + asparagine	114.5	118.9	119.2
threonine	60	60.4	59.5
serine	59	53.3	52.6
glutamate + glutamine	153	161.6	161.4
proline	48	51.2	50.5
glycine	80	78.1	78.0
alanine	91.5	86.0	85.4
valine	66.5	67.1	66.9
methionine	39	38.9	38.6
isoleucine	69	60.7	60.9
leucine	90.5	93.6	93.2
tyrosine	37	40.4	40.2
phenylalanine	72	74.1	73.9
histidine	24	23.7	23.2
methionine	39	39.4	38.9
arginine	46	47.5	47.2
lysine	103	96.2	4.2
Me_1-Lys	0	0	0
Me_2-Lys	0	0.6	96.7
Me_3-Lys	3	3.6	3.4
total lysine	106	100.4	104.3

[a] This is based on a total of 1157 amino acid residues. This number was calculated from the amino acid sequences of the heavy chain, regulatory light chain, and the average values for the two essential light chain isoforms. It includes all of the residues except cysteine and tryptophan that were lost during hydrolysis. These analyses were performed on protein derived from the same preparation and represent the data for modification in the absence of nucleotide (sample A). The amino acid composition of protein modified in the presence of trapped nucleotide (B and C) was statistically identical to the result shown above. An amino acid analysis of modified protein from a different preparation was performed after performic acid oxidation to determine the status of the cysteine residues. This revealed that the cysteine residues are not chemically modified (data not shown) by the reductive methylation procedure.

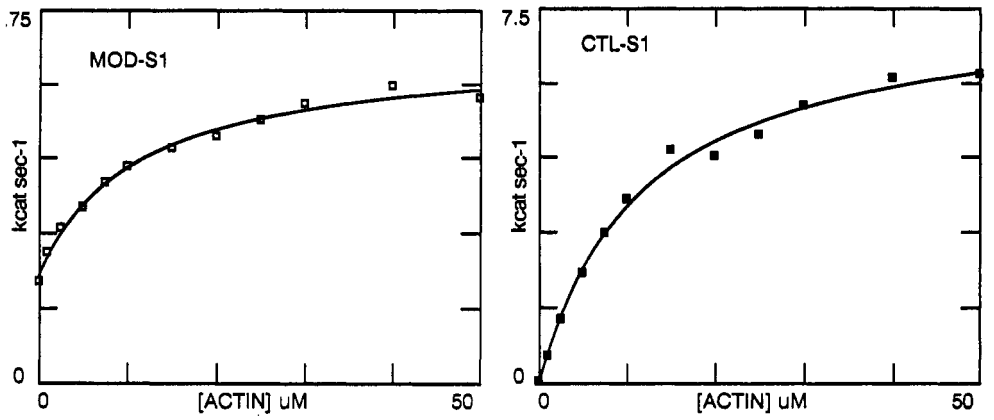

FIGURE 1: Steady-state hydrolysis by control and modified actomyosin-S1. Data were fit to a hyperbolic equation: $V = k_{cat}/(1 + K_{app}/[NTP]) + k_0$. For control S1 (solid symbols), $k_0 = 0.04$ s^{-1}, $K_{app} = 11.8$ μM, and $k_{cat} = 7.7$ s^{-1}. For modified S1 (open symbols), $k_0 = 0.2$ s^{-1}, $K_{app} = 10.2$ μM, and $k_{cat} = 0.68$ s^{-1}. Experimental conditions: 0.2 mg/mL control or modified S1, 5 mM MOPS, 2 mM $MgCl_2$, 0.2 mM DTT, 1 mM ATP and the indicated concentration of actin, pH 7, 20 °C.

Table II: Comparison of Kinetic Parameters for ATP Hydrolysis by Normal and Reductively Methylated Actomyosin-S1

myosin-S1	temp (°C)	k_0[a] (s^{-1})	k_t[b] (s^{-1})	k_{app} (μM)	k_{cat}[a] (s^{-1})
control	20	0.05	0.063	11.8	7.7
RM-S1-A	20	0.20	0.49	12.0	0.61
RM-S1-B	20	0.18	0.30	10.2	0.68
RM-S1-C	20	0.15	0.25	16.5	0.71
control	10	0.03	0.025	ND	ND
RM-S1-A	10	0.115	0.095	ND	ND
RM-S1-B	10	0.102	0.209	ND	ND
RM-S1-C	10	0.072	0.138	ND	ND

[a] Determined by steady-state rate measurements. [b] Determined by single-turnover measurements.

1. In the absence of actin, the rate of MgATP hydrolysis is increased from 0.05 s^{-1} for control S1 to 0.15–0.2 s^{-1} by RM-S1. Actin increases the rate of hydrolysis of control S1 by approximately 150-fold to a maximum of 7.4 s^{-1} but increases the maximum rate of hydrolysis by RM-S1 to a maximum rate of 0.6–0.7 s^{-1}. The value of K_{app}, which represents a complex set of rate and equilibrium constants, is unchanged within experimental error. Table II contains a list of kinetic parameters for RM-S1 prepared under slightly different procedures described under Materials and Methods. Similarly enhanced rates of nucleoside hydrolysis in the absence of actin and reduced rates in the presence of actin have been observed for SH1 (Cys 707 in chicken skeletal myosin) modified myosin-S1 (Sleep et al., 1981; Ostap et al., 1993) and for unmodified myosin-S1 for substrates such as GTP in which the 6-amino group of the purine has been changed to a hydroxyl group (Eccleston & Trentham, 1979; White et al., 1993). In the latter two cases the changes in the observed kinetics were associated with a decrease in the rate of the hydrolysis of M–T to M–D–P.

One of the characteristics of the hydrolysis of ATP by myosin is the rapid pre-steady-state burst of hydrolysis of ATP, which is illustrated in the right half of Figure 2. The rapid initial rate of hydrolysis of ATP corresponds to the rapid binding and hydrolysis of ATP to form M–D–P in eq 1. At the relatively low concentrations of ATP and protein used in Figure 2, the rate of the rapid burst is limited by the rate of ATP binding and thus does not measure the rate of the hydrolysis step. The slower hydrolysis of the remainder of the ATP is due to an equilibrium constant for M–ATP ↔ M–D–P that only partially favors products followed by rate-limiting product dissociation. The slow phase of phosphate production that occurs at a rate similar to the steady-state rate and thus represents the breakdown of the steady-state intermediates M–T and M–D–P. However, the ratio of the amplitudes of the two phases can be used to measure the equilibrium constant of the hydrolytic step to be 2.3 for unmodified S1. The single-turnover hydrolysis of ATP by RM-S1 shown in the left-hand

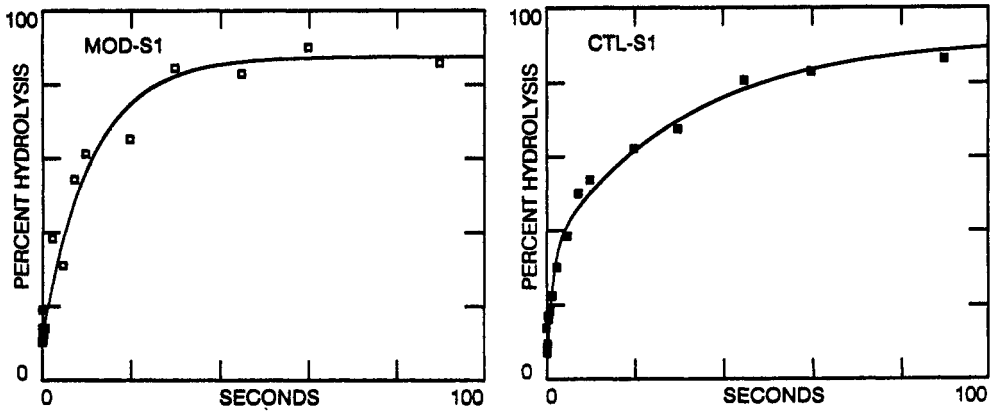

FIGURE 2: Quench-flow measurements of single turnovers of ATP hydrolysis by control and modified myosin-S1. 2 μM either of control (solid symbols) or of modified (open symbols) S1 was mixed with 1 μM ATP. The fraction of ATP hydrolyzed was determined as described under Materials and Methods. Experimental conditions: 5 mM MOPS, 2 mM $MgCl_2$, and 0.2 mM DTT, pH 7, 20 °C. Data obtained with modified S1 were fit to a single-exponential equation: $[P(t) = P_0e^{-k_{obs}t} + C]$, where $k_{obs} = 0.25$ s^{-1}. The data obtained with control S1 were fit to a two-exponential equation: $P(t) = P_ae^{-k_at} + P_be^{-k_bt} + C$, where $P_a = 0.28$, $k_a = 0.061$ s^{-1}, $P_b = 0.63$, and $k_d = 4.3$ s^{-1}, pH 7, 20 °C.

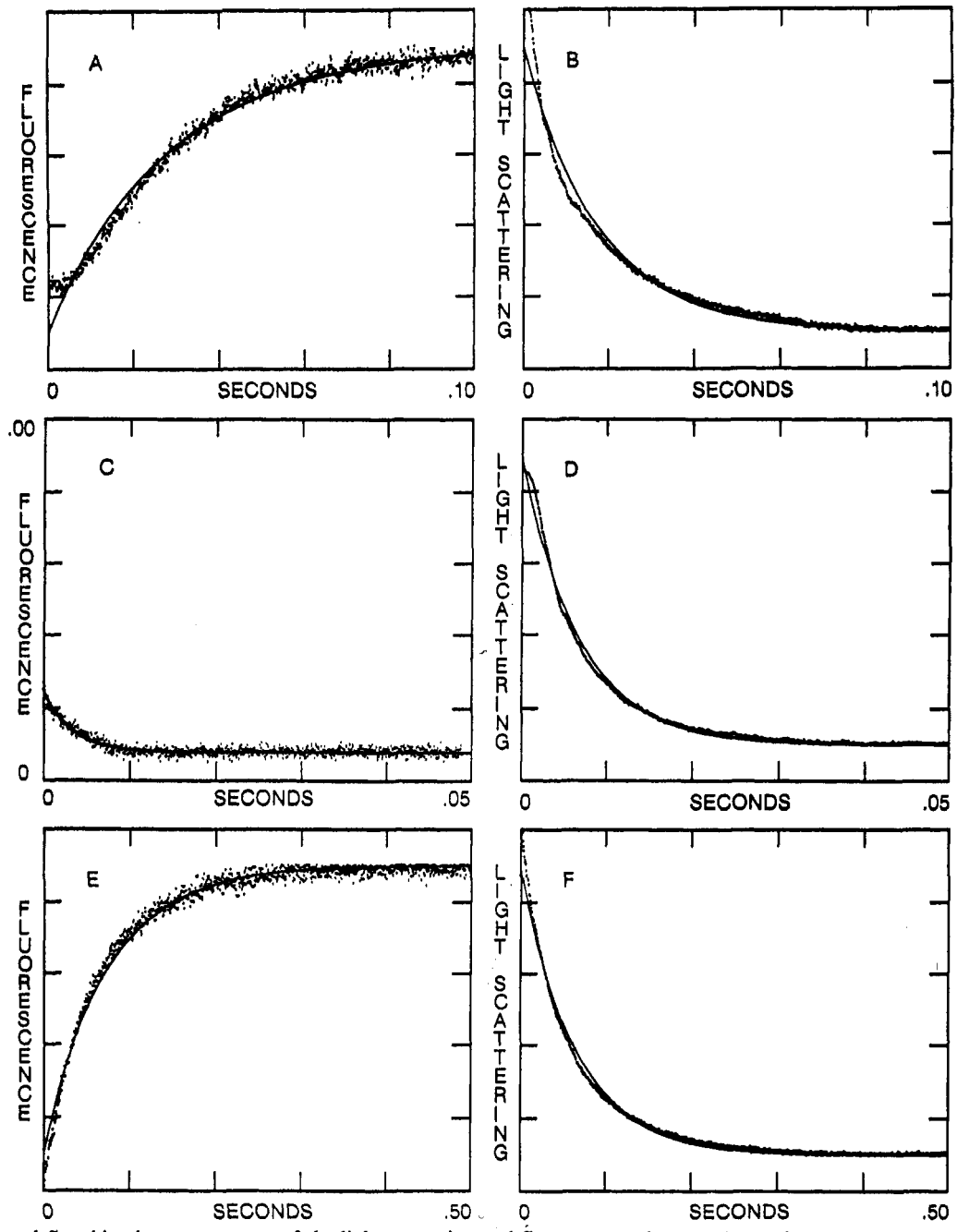

FIGURE 3: Stopped-flow kinetic measurement of the light scattering and fluorescence changes observed upon mixing control and modified actomyosin-S1 with ATP and aza-ATP. Shown are light scattering and fluorescence changes observed upon mixing equal volumes of either 50 μM ATP (A–D) or aza-ATP (E–F) with 2.5 μM F-actin and 2 μM control (A and B) or modified (C–F) myosin-S1 in a stopped fluorimeter. The excitation wavelength was either 295 (A–D) or 365 nm (E–F). Emission was the same as excitation for light scattering (B, D, and F), 320–380 nm (A and B), or 420–450 nm (F). The solid lines through the data were for the k_{obs} obtained by fitting the data to the single-exponential equation $I(t) = I_0e^{-k_{obs}t} + C$, where k_{obs} = 45.2 s^{-1} (A), 57.4 s^{-1} (B), 173 s^{-1} (C), 340 s^{-1} (D), 13.6 s^{-1} (E), and 15.7 s^{-1} (F). Experimental conditions: 2.5 μM actin, 2 μM control or modified S1, 5 mM MOPS, 2 mM $MgCl_2$, and 0.2 mM DTT, pH 7, 20 °C.

side of Figure 2 is qualitatively and quantitatively different. The hydrolysis is fit reasonably well by a single-exponential curve. A comparison of the first and second columns of Table I shows that the rate constant of the bond splitting step, measured in a series of RM-S1 preparations, is no more than two times the steady-state rate of ATP hydrolysis. Increasing the concentrations of S1 and ATP 5-fold only produced a 30% increase in the rate of a single turnover of ATP hydrolysis catalyzed by RM-S1 (data not shown). This indicates that the observed rate is not limited by slow binding of ATP to the active site and that the bond splitting step is at least partially rate limiting. The slow rate of the bond splitting step measured here for RM-S1 can be compared with the rate of the bond splitting step that has been previously measured to be 50–100 s^{-1} for unmodified actomyosin and myosin-S1 at 20 °C (Sleep & Taylor, 1976). The close but not identical values obtained by single-turnover and steady-state measurements for RM-S1 could be explained if the hydrolysis step was rate limiting but only half of the preparation was catalytically active or if another step was also partially rate limiting. An alternative explanation of the single-turnover and steady-state data is a rapid hydrolysis step with an equilibrium constant favoring M–T, followed by slow product dissociation. In either case, the rate constants of the bond splitting step are altered so that M–T rather than M–D–P is the predominant steady-state intermediate.

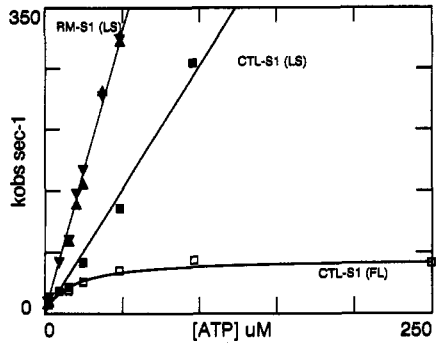

FIGURE 4: Dependence of k_{obs} for light scattering and tryptophan fluorescence enhancement of control and RM actomyosin-S1 upon the concentration of ATP. The values for k_{obs} were obtained from experiments similar to those shown in Figure 3A–D except that the concentrations of ATP were varied as indicated. The solid lines fit to the data for RM-S1 (triangles) and control S1 (solid squares) correspond to apparent second-order rate constants of 6.4×10^6 M^{-1} s^{-1} and 2.4×10^6 M^{-1} s^{-1}, respectively, for the binding and dissociation of acto-S1 by ATP. Upward triangles were obtained with protein reductively methylated in the presence of ADP and BeF_3; downward triangles were obtained with protein reductively methylated in the absence of nucleotides. The observed rate of intrinsic fluorescence enhancement (open squares) was fit to the hyperbolic equation $k_{obs} = k_{max}/(1 + K_0/[ATP])$, where $k_{max} = 62.3$ s^{-1} and $K_0 = 16.3$ μM.

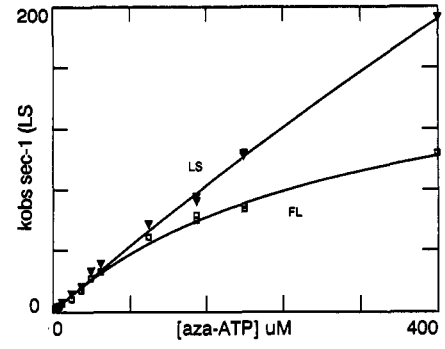

FIGURE 5: Dependence of k_{obs} for the fluorescence enhancement and dissociation of actomyosin-S1 upon the concentration of aza-ATP. The values for k_{obs} were obtained from experiments similar to those shown in Figure 3E–F except that the concentrations of aza-ATP were varied as indicated. The light scattering data (▼) were fit by a second-order rate constant of 5.1×10^5 M^{-1} s^{-1}. The fluorescence data (□) were fit by a hyperbolic equation, $k_{obs} = k_{max}/(1 + K_0/[NTP])$ in which $k_{max} = 182 \pm 12$ s^{-1} and $K_0 = 313 \pm 35$ μM.

The enhancement in intrinsic tryptophan fluorescence has been used to measure the binding and hydrolysis of ATP by actomyosin and myosin. An increase in tryptophan fluorescence of approximately 25% is observed with ATP binding to unmodified acto-S1 as shown in Figure 3A. ATP binding to RM acto-S1, however, resulted in either no change or a small (approximately 5%) decrease in fluorescence as shown in Figure 3C. Approximately one-third of the increase in tryptophan fluorescence in unmodified myosin-S1 by ATP is associated with the M–ATP intermediate and the remainder with the M–D–P (Johnson & Taylor, 1978). Depending upon experimental conditions, M–D–P is usually the predominant steady-state intermediate during ATP hydrolysis. The reduced fluorescence enhancement by SH1 modified myosin, or if GTP is the substrate for unmodified myosin, has been at least in part explained by M–T being the predominant steady-state intermediate. The data in Figure 3 are consistent with a mechanism in which M–T rather than M–D–P is the predominant steady-state intermediate of ATP hydrolysis by RM-S1. An alternative explanation for the decrease in the tryptophan fluorescence is that the structural change normally associated with nucleoside triphosphate binding and hydrolysis has been altered in the RM-S1.

Dissociation of acto-S1 by ATP was measured from the decrease in light scattering in the same experiments as the fluorescence enhancement. Both control and RM acto-S1 are dissociated by ATP in dilute solution as shown by the decrease in light scattering in Figure 3B,D. The solid lines represent the best computer fit to a single-exponential equation. The RM acto-S1 (Figure 3B) is dissociated more rapidly and is better fit by a single-exponential equation than the unmodified protein (Figure 3D). Figure 4 shows the dependence of the observed rate of dissociation of RM (triangles) and control (solid squares) acto-S1 upon ATP concentration. Over the range of ATP used in these experiments, the rate of dissociation increases linearly with ATP as is expected for a pseudo-first-order reaction. The second-order rate of dissociation of RM-acto-S1, 6.4×10^6 M^{-1} s^{-1}, is more than twice that of control acto-S1, 2.4×10^6 M^{-1} s^{-1}. The observed rate of the tryptophan fluorescence enhancement measured for control S1 only (open squares) has a maximum rate of 62.3 s^{-1} at saturating ATP. The maximum rate of fluorescence enhancement has been shown to be equal to the sum of the forward and reverse rate constants, $k_{+H} + k_{-H}$, of the bond splitting steps (Sleep & Taylor, 1976; Stein et al., 1979).

An alternative way in which to observe the interaction between the nucleoside triphosphate and protein is to measure changes in nucleotide fluorescence using a fluorescent nucleotide. There is a large enhancement in fluorescence of aza-ATP during steady-state hydrolysis (Smith & White, 1985), which indicates that the environment of the nucleotide base during steady-state hydrolysis is in a considerably more hydrophobic environment than in solution. Moreover, the kinetics of aza-ATP binding to myosin-S1 and actomyosin-S1 is proceeded by a complex kinetic pathway that was interpreted as being evidence for at least two first-order processes. Nucleotide fluorescence and light scattering signals observed upon mixing aza-ATP with RM acto-S1 are shown in Figure 3E,F. There is a large increase in nucleotide fluorescence, similar to that previously observed with unmodified protein, and the data are reasonably well fit by a single-exponential (Figure 3E) process with little evidence for a second slower step as has been observed with control acto-S1 (Smith & White, 1985). The decrease in light scattering observed with RM acto-S1 is also fit reasonably well by a single-exponential equation (Figure 3F). The dependence of the observed rate constants of the fluorescence and light scattering measurements upon aza-ATP concentration is shown in Figure 5. Both observed rate constants increase linearly with aza-ATP concentration at low concentrations, but, at aza-ATP concentrations above 100 μM, k_{obs} for fluorescence is slower than the light scattering and is fit to a maximum rate of 182 ± 12 s^{-1}. The rate measured here is similar to that measured for the rapid phase of the fluorescence enhancement with unmodified acto-S1, but the previously measured slow phase of 15–20 s^{-1} was not observed.

DISCUSSION

Chemical modification has proven to be one of the most powerful tools available for studying the biochemical properties of myosin. Much of what is known about the chemical, enzymatic, and physical properties of myosin has arisen from such studies. Although the approach is likely to perturb the native structure, it has always been justified in that frequently it has been the only way to introduce a required functional group into the molecule or demonstrate the role or importance

of a functional group. At the time the crystals of myosin subfragment 1 were grown, there was no precedent for the use of chemical modification as a crystallization technique. Thus it was essential to establish what type of structural and enzymatic changes are produced in myosin-S1 by reductive methylation of lysine residues. In the preceding paper in this issue (Rypniewski et al., 1993), it is demonstrated that reductive methylation produces only very small changes in the structure of lysozyme.

Here we have demonstrated that myosin-S1 retains most of the fundamental features of the actomyosin ATP hydrolysis mechanism after reductive methylation: the modified enzyme is catalytically active; ATP binds to and dissociates RM-S1 from actin in dilute solution; actin increases the steady-state rate of ATP hydrolysis by RM-S1. The rapid dissociation of RM-S1 by ATP from actin indicates that ATP must bind tightly to the active site of RM-S1 and generate structural transformations similar to those that occur in unmodified protein. In addition, the kinetics of dissociation and fluorescence enhancement observed upon the binding of aza-ATP to RM acto-S1 suggest that the nucleotide undergoes a similar structural transition when it binds to modified and unmodified acto-S1. However, there are significant quantitative changes in the rate and equilibrium constants of all of the steps of the mechanism that we have measured. Although the thiol groups have not been modified by reductive methylation (Table I), the reduction of the rate and equilibrium constants of the cleavage step observed upon methylation of the lysine residues are qualitatively and quantitatively similar to changes that are induced by either SH1 modification or substrate analogues such as GTP, which reduce the rate and equilibrium constants for the bond splitting step. In addition, modification of a single lysine residue (Lys 83) by 2,4,6-trinitrobenzenesulfonate in the N-terminal 25K domain of myosin S1 has also been shown to produce similar alterations in the kinetic mechanism (Muhlrad, 1983).

RM-S1 is catalytically active although the rate and equilibrium constants of many of the steps of the actomyosin ATP hydrolysis mechanism have been altered. Although the use of chemical modification is an unprecedented method to induce protein crystallization and might appear drastic at first sight, it is no different from any other chemical approach to understanding the behavior of proteins.

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