Kinetic Mechanism of 1-N⁶-Etheno-2-aza-ATP Hydrolysis by Bovine Ventricular Myosin Subfragment 1 and Actomyosin Subfragment 1

THE NUCLEOTIDE BINDING STEPS*

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The large change in fluorescence emission of 1-N⁶-etheno-2-aza-ATP (ε-aza-ATP) has been used to investigate the kinetic mechanism of etheno-aza nucleotide binding to bovine cardiac myosin subfragment 1 (myosin-S1) and actomyosin subfragment 1 (actomyosin-S1). The time course of nucleotide fluorescence enhancement observed during ε-aza-ATP hydrolysis is qualitatively similar to the time course of tryptophan fluorescence enhancement observed during ATP hydrolysis. In single turnover experiments, the nucleotide fluorescence rapidly increases to a maximum level, then decreases with a rate constant of 0.045 s⁻¹ to a final level, which is about 30% of the maximal enhancement; a similar fluorescence enhancement is obtained by adding ε-aza-ADP to cardiac myosin-S1 or actomyosin-S1 under the same conditions (100 mM KCl, 10 mM 4-morpholinepropanesulfonic acid, 5 mM MgCl₂, 0.1 mM dithiothreitol, pH 7.0, 15 °C). The kinetic data are consistent with a mechanism in which there are two sequential (acto)myosin-S1 nucleotide complexes with enhanced nucleotide fluorescence following ε-aza-ATP binding. The apparent second order rate constants of ε-aza-ATP binding to cardiac myosin subfragment 1 and actomyosin subfragment 1 are 2–12 times slower than those for ATP. Actin increases the rate of ε-aza-ADP dissociation from bovine cardiac myosin-S1 from 1.8 to 110 s⁻¹ at 15 °C which can be compared to 0.3 and 65 s⁻¹ for ADP dissociation under similar conditions.

Although there are quantitative differences between the rate and equilibrium constants of ε-aza- and adenozine nucleotides to cardiac actomyosin-S1 and myosin-S1, the basic features of the nucleotide binding steps of the mechanism are unchanged.

Biochemical studies of the actomyosin ATPase cycle have provided a description of how the chemical reaction of ATP hydrolysis is coupled to the interaction of actin and myosin in solution (1–3). A minimal mechanism of hydrolysis of ATP by myosin and actomyosin is shown in Equation 1, where M represents a myosin head; A, actin; T, ATP; D, ADP; and P, phosphate. The essential features of the scheme are: (i) myosin and M-D bind tightly to and dissociate very slowly from actin (5). In contrast, the states M-T and M-D-P are weakly bound to actin and dissociate rapidly. As a result the kinetic pathway alternates between tight and weakly bound states (3). (ii) In the absence of actin, the rate-limiting step in the pathway occurs after product formation (1, 2). (iii) In the presence of actin, product release is accelerated at least 200-fold. The mechanism of hydrolysis of ATP by cardiac myosin-S1 and actomyosin-S1 is basically similar to that shown in Equation 1 for the skeletal proteins with differences in the rates of some of the steps (6–8). For example, the rate constant of ADP dissociation from rabbit skeletal actomyosin-S1 is too rapid to be measured by stopped-flow methods, whereas that for cardiac actomyosin-S1 is readily measured (8). Therefore, we have chosen to study the mechanism of cardiac rather than skeletal actomyosin-S1 ATP hydrolysis pathway, so that a more complete kinetic analysis can be made. In addition, such a study provides the background necessary to understand the molecular basis of the changes in cardiac contractility induced by adaptive or pathological conditions.

The intrinsic tryptophan fluorescence of the myosin head is enhanced during hydrolysis of ATP approximately 25% as denoted by the asterisks in Equation 1. This has allowed the kinetics of the hydrolytic pathway to be investigated. However, only kinetic steps that involve changes in the environment of the tryptophan chromophore can be observed. More detailed information about the mechanism of the reaction can be obtained by monitoring some spectroscopic property of the nucleotide during the reaction (for example, using 31P NMR (9) or the fluorescence of ATP analogues (10–12)). The reactions of the related fluorescent nucleotide 1-N⁶-etheno-ATP with myosin-S1 and actomyosin-S1 from skeletal and smooth muscle have been studied in detail (12). There are some interesting similarities and differences between the rates and apparent binding constants of this analogue and those for ATP. The steady state rate of hydrolysis of 1-N⁶-etheno-ATP

\[
\begin{align*}
K_{AT} & = K_{A} \\
K_{T} & = K_{T} \\
K_{H} & = K_{H} \\
K_{ADP} & = K_{ADP} \\
K_{DP} & = K_{DP} \\
K_{D} & = K_{D} \\
M & = M \\
\end{align*}
\]

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1 The following nomenclature is used to identify rate and equilibrium constants. Positive subscripts identify rate and equilibrium constants. Positive subscripts identify rate and equilibrium constants of association; negative subscripts identify rate equilibrium constants of dissociation. Single subscripts, A = actin, D = ADP, T = ATP, P = P, ε-ADP = ε-aza-ADP, ε-ATP = ε-aza-ATP, refer to the binding of the respective ligand to myosin S1, which is denoted as M. The subscript H refers to the hydrolysis of ATP to ADP and P. For multiple subscripts, the final letter of the string identifies the ligand associating (or dissociating) to myosin-S1 and all other letters refer
by skeletal myosin-S1 is about 6 times faster than that of ATP, while the apparent second order rate constant for 1-N'-etheno-ATP binding to skeletal myosin-S1 is similar to that of ATP. However, the maximum steady state rate of hydrolysis of 1-N'-etheno-ATP by skeletal actomyosin-S1 is about 10 times less than that for ATP (12). The chemical and functional properties of the related ATP analogue, c-aza-ATP, make it particularly useful to study the kinetics of nucleotide hydrolysis and to investigate the crossbridge cycle in muscle fibers (13), since it produces 75% of the isometric tension obtained with ATP. The fluorescence emission maximum is enhanced approximately 3-fold and shifted from 485 to 460 nm during steady state hydrolysis by skeletal heavy meromyosin (14). The shift of the fluorescence emission spectrum of c-aza-ATP on binding to myosin-S1 enables kinetic measurements to be made without the necessity of quenching the fluorescence of the unbound nucleotide with reagents such as acrylamide, as is required for etheno-ATP (12, 14). We show here that the large changes in fluorescence emission at 410-450 nm can be used to study the nucleotide binding steps of the hydrolytic pathway of bovine cardiac myosin-S1 and actomyosin-S1. Experiments were carried out at 15 °C, so that the kinetic data could be compared with that for ATP and ADP (6-8); measurements were also made at low temperature, 0 °C, to provide a set of kinetic parameters for the hydrolysis of the nucleotide under conditions where the reactions were likely to be slow enough to be measured in structured systems. The hydrolytic pathways of ATP and c-aza-ATP are qualitatively similar although there are significant differences (2-12-fold) in the values of some of the rate and equilibrium constants. Moreover, the maximum steady state rates of hydrolysis of ATP or c-aza-ATP by skeletal actomyosin-S1 (13) are the same. Thus, c-aza-ATP is suitable for use as a probe to compare the rate of certain steps in the hydrolytic pathway for bovine cardiac myosin-S1, actomyosin-S1, and myofibrils. The results of these experiments are described in the accompanying paper (16).

MATERIALS AND METHODS

All solutions were prepared using glass distilled water. Ammonium sulfate was absolute grade (Research Fus Laboratories). The following chemicals were obtained from Sigma: ATP (Sigma grade vanadium-free, used for kinetic experiments and grade II used for preparative procedures), ADP, dicyclohexylammonium salt, A₆₅₀, chymotrypsin type 1-S, dithiothreitol, and lima bean trypsin inhibitor. MOPS was Ultrl grade from Calbiochem-Behring. Cardiac actin, myosin, and myosin-S1 were purified from the left ventricles of bovine hearts as described by Siemankowski and White (8). The concentration of bovine ventricular myosin and myosin-S1 was determined from the absorption at 280 nm using extinction coefficients (0.1%), w/v, 1 cm) of 0.55 and 0.64 (17). The concentration of bovine ventricular actin was determined using an extinction coefficient at 280 nm of 1.15 (0.1%, w/v, 1 cm).

Preparation of c-aza-ADP—c-aza-ADP was prepared by enzymatic hydrolysis of the triphosphate: 1 mM c-aza-ATP was incubated at 20 °C with 4 μM skeletal myosin-S1, 5 μM skeletal actin, 10 μM AP₆A (10 mM KCl, 10 mM MOPS, 5 mM MgCl₂, 0.1 mM dithiothreitol, pH 7.0); when the hydrolysis was complete as monitored by enhancement of fluorescence at 440 nm, the mixture was centrifuged at 100,000 × g for 2 hr to remove the actomyosin-S1. The supernatant containing the crude c-aza-ADP had 0.5-2.5% c-aza-ATP, which could be removed by chromatography on DEAE-52 as described above.

The concentration of the nucleotides was determined from the extinction coefficient at 354 nm of 1500 m²·cm⁻¹ (14).

Preparation of 1-N6-etheno-ATP—1-N6-etheno-ATP was prepared from 1-N6-etheno-2-aza-adenosine-5'-triphosphate, c-aza-ADP, 1-N6-etheno-2-aza-adenosine-5'-diphosphate, and c-aza-ATP (24). The synthesis and purification were monitored using thin layer and paper chromatography (19). The crude material was purified by ion exchange chromatography on DEAE-52-cellulose in 0.1 M NaCl, 10 mM Tris, pH 7.5, with a linear gradient to 0.4 M NaCl from each column separation. Fractions for which the ratio of ultraviolet absorption at 265 nm to that at 242 nm was less than 0.4 were analyzed using isotopechromatography. The leading electolyte was 18 mM β-alanine, 5 mM HCl, 0.5% hydroxypropyl methyl cellulose, and the terminating electrolyte was 5 mM α-capsaic acid. The nucleotide was detected using an ultraviolet monitor at 265 nm. The purity of the final product was 90-95% with a single major impurity as judged by comparison of the ratio of the areas under the peaks of the isotopechromatographs record (20). The nucleotide migrated as a single compound on paper chromatography and thin layer chromatography (19).

Etheno-aza Nucleotide Binding to Cardiac Myosin-S1 and Actomyosin-S1

Etheno-aza nucleotides were synthesized by the method of Foss (22). The synthesis and purification were monitored using thin layer and paper chromatography (19). The crude c-aza-ADP had 0.5-2.5% c-aza-ATP, which could be removed by chromatography on DEAE-52 as described above.

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The concentration of the nucleotides was determined from the extinction coefficient at 354 nm of 1500 m²·cm⁻¹ (14).
was adjusted until the difference between the data and simulation were a minimum. Computer fits of the data were made using a DEC Rainbow computer. Data were routinely fit to single or double exponentials by the Method of Moments using a modified version of a program written by Dr. Ken Johnson, Dept. of Biochemistry, Pennsylvania State University, which required approximately 3 min to fit 1024 points to two exponentials. Data were considered to be adequately fit by a single exponential (Equation 2a) if upon visual inspection the difference between the fit curve and the data did not contain long runs of positive or negative points or if the root mean square deviation of the data from the fit curve was not improved by at least a factor of two upon fitting to a double exponential equation. In some cases, data were also fitted by the method of Foss (22); data were averaged two at a time prior to fitting using a modified version of a program written by Duane Flamig in order to avoid memory overflow and reduce the amount of computer time to ~2 h for 512 points. The variability of the fits of the slow rate constant between the different fitting procedures was generally less than 5%. There was considerably more variability in fitting the fast rate constant, generally ~20%.

RESULTS

Emission Spectra of the Steady State Complex during $\epsilon$-aza-ATP Hydrolysis—The fluorescence emission maximum of $\epsilon$-aza-ATP is shifted from 485 to 450 nm and is enhanced (approximately 5-fold in the 415–445 nm region for ratios of $\epsilon$-aza-ATP/myosin-S1 < 1) during the steady state hydrolysis by cardiac myosin-S1. The fluorescence emission spectrum of the myosin-S1-$\epsilon$-aza-ATP complex can be obtained from the maximum fluorescence at each wavelength observed on addition of stoichioimetric amounts of $\epsilon$-aza-ATP to myosin-S1. Such a spectrum for cardiac myosin-S1 is shown in Fig. 1. The spectrum of the nucleotide bound to cardiac myosin-S1 is similar to that observed for the complex of the nucleotide with skeletal heavy meromyosin (14). The time course of enhancement of fluorescence at 440 nm observed upon adding $\epsilon$-aza-ATP to cardiac myosin-S1 is shown in Fig. 2. Addition of amounts of nucleotide substoichiometric to the number of myosin heads produces a rapid increase in fluorescence that is followed by a slow decay. $k_{\text{cat}}$ of $\epsilon$-aza-ATP hydrolysis obtained from the half-time of the exponential decrease in fluorescence enhancement is 0.02 s$^{-1}$ at 0 °C. The fluorescence remains enhanced to about 30% of maximum even after hydrolysis has occurred; a similar fluorescence enhancement can be obtained by adding $\epsilon$-aza-ADP to myosin-S1 under the same conditions, suggesting that this complex, which has 30% of the maximal fluorescence enhancement obtained with $\epsilon$-aza-ATP, corresponds to M-$\alpha$-ADP*. On addition of ATP, the fluorescence intensity decreases to approximately that of the myosin-S1 plus that of the unbound nucleotide which was added (shown on the right). The rate at which $\epsilon$-aza-ADP is displaced from the nucleotide-myosin-S1 complex (M-$\alpha$-ADP*) by ATP is 0.05 s$^{-1}$ at 0 °C. The time course of fluorescence enhancement observed upon addition of $\epsilon$-aza-ATP to cardiac myosin-S1 at 15 °C is similar to that at 0 °C (data not shown); the steady state rate of $\epsilon$-aza-ATP hydrolysis which can be determined from the rate of decay of the fluorescence enhancement in single turnover experiments is 0.045 s$^{-1}$.

Kinetics of Binding $\epsilon$-aza-ATP to Cardiac Myosin-S1—The rate of the rapid transient enhancement of fluorescence of $\epsilon$-aza-ATP when mixed with cardiac myosin-S1 can be measured in a stopped flow fluorometer. Fig. 3 shows examples of such data at 15 °C. The fluorescence transient can be fit to a single exponential, which is shown by the solid line through the trace (Fig. 3A). However, from 0 to 100 μM $\epsilon$-aza-ATP, the observed transients are better fit by two exponentials, as judged by the root mean square deviations of the data from the fit which are approximately 50% lower for the fit to two exponentials (Equation 2b) than for the single exponential fit (Equation 2a). In this concentration range, the fitted rate constants ($k_{\text{obs}}$ and $k_{\text{cat}}$) differ by less than a factor of three making unique determination of the rate and amplitude data difficult; this can be seen by comparison of two possible combinations of values for $k_{\text{obs}}$ and amplitude coefficients for the data shown in Fig. 3B. Therefore, where $\epsilon$-aza-ATP < 100 μM, $k_{\text{obs}}$ was obtained from single exponential fits of this data. At $\epsilon$-aza-ATP concentrations greater than 100 μM (Fig. 3C), the values for the rate constants obtained by double exponential fits of the data are unambiguous. The dependence of $k_{\text{obs}}$ on

![Fig. 1. Fluorescence emission spectra of $\epsilon$-aza-ATP in the presence and absence of cardiac myosin-S1. Both in the presence (dashed line) and absence (solid line) of cardiac myosin-S1 (16 μM), the concentration of $\epsilon$-aza-ATP was 9 μM. The time course of fluorescence enhancement at each wavelength was monitored on addition of stoichiometric amounts of $\epsilon$-aza-ATP cardiac myosin-S1. An example of such data is shown in Fig. 2. The maximum fluorescence at each wavelength was plotted to give the spectrum. Excitation at 364 nm; band width; 10 nm. Conditions: 100 mM KCl, 10 mM MOPS, 5 mM MgCl$_2$, 0.1 mM dithiothreitol, pH 7.0, 0 °C.](image1)

![Fig. 2. Time course of enhanced fluorescence of $\epsilon$-aza-ATP in the presence of cardiac myosin-S1. At the arrow marked "ATP" is added and $\epsilon$-aza-ATP (final concentration 8 μM) as injected into a stopped flow cuvette containing 1.5 ml of 100 mM KCl, 16 mM MOPS, 5 mM MgCl$_2$, 0.1 mM dithiothreitol, pH 7.0, 0 °C. The arrow marked "ATP," 20 μl of 10 mM ATP was added to give a final concentration of 80 μM. The emission expected from the noninteracting mixture of myosin-S1 and $\epsilon$-aza-ATP is equal to the sum of the signals measured with myosin-S1 and $\epsilon$-aza-ATP, which is indicated by the dashed lines at the bottom of the figure. Excitation at 365 nm, emission at 440 nm, 10-nm band width.](image2)
The apparent second order rate constant for the enhancement upon the concentration of ε-aza-ATP is shown in Fig. 4. A rapid phase of the reaction corresponding to 40–50% of the fluorescence enhancement increases linearly with ε-aza-ATP concentration and is described by an apparent second order rate constant of ~2 × 10^6 M^−1 s^−1 at 15 °C. The slower component of the fluorescence enhancement corresponds to 50–60% of the total amplitude. The relationship between $k_{ob}$ for this slow component and the concentration of ε-aza-ATP can be described by a hyperbola with a maximum rate of 13.3 s^−1 as estimated by linear least squares analysis of the data.

The fluorescence transients observed on mixing ε-aza-ATP with cardiac myosin-S1 were also monitored at 0 °C and 15 °C. The data in B is fit by either of two double exponential equations with rate constants (i) 19.97 s^−1 (0.17) and 4.63 s^−1 (0.83) with a root mean square deviation of 0.0312. The data in A is fit by a single exponential with a rate constant of 13.3 s^−1 shown by the solid line segments. The dotted line segments represent regions of the theoretical curve that cannot be assigned exclusively to either physical process. Final myosin-S1 concentration was 100 PM could be unambiguously analyzed (data not shown).

The observed rate of decrease in fluorescence is equal to the rate constant of ε-aza-ADP dissociation ($k_{ob}$), if $k_{d}[ATP] > k_{d} + k_{o}[ε-aza-ADP]$, i.e. if the binding of ATP to myosin-S1 is more rapid than the dissociation of ε-aza-ADP from the complex. Fig. 3D shows an example of the data from such an
experiment. ε-Aza-ADP is displaced from the complex at a rate of 1.9 s⁻¹, at 15 °C. This is ~6 times faster than the rate constant measured for ADP dissociation from bovine cardiac myosin-S1 (7).

**Kinetics of the Fluorescence Enhancement on Binding ε-Aza-ATP to Cardiac Actomyosin-S1**—ε-Aza-ATP binding to actomyosin-S1 may be measured from the enhancement of nucleotide fluorescence. Fig. 5 shows the increase in fluorescence that occurs upon mixing cardiac actomyosin-S1 with ε-aza-ATP. The fluorescence data at 15 °C could be fit reasonably by a single exponential for concentrations of ε-aza-ATP <10 μM (Fig. 5A) and 40–80 μM (Fig. 5C). In the range of 10–40 μM ε-aza-ATP (Fig. 5B), the data were best fit by two exponential terms with amplitude coefficients of opposite sign. At concentrations of ε-aza-ATP >100 μM, the fluorescence enhancement was biphasic (Fig. 5D) and was best fit by two exponential terms with amplitude coefficients of the same sign. The excellent signal to noise ratio of the data, from approximately 0.5% root mean square at low ε-aza-ATP concentrations to 4% at the highest concentrations, enabled us to make reliable double exponential fits of the fluorescence data.

Fig. 6 shows the dependence of $k_{obs}$ for the fluorescence upon the concentration of ε-aza-ATP. The slower component of the fluorescence (open squares) reaches a maximum rate of ~15 s⁻¹ at ε-aza-ATP concentrations >100 μM. The rate of the more rapid component (open circles) increases with ε-aza-ATP concentration over the experimentally accessible range of concentration; the highest measured rate is in the range of 60–90 s⁻¹ at 400 μM. At concentrations of ε-aza-ATP >400 μM, the signal to noise ratio was not good enough to obtain reliable double exponential fits of the data. A maximum rate can be estimated to be ~100 s⁻¹ from the curvature of the dependence of the rapid component upon ε-aza-ATP concentration. The apparent second order rate constant of ε-aza-ATP binding to actomyosin-S1 can be estimated from the slope of the dependence of $k_{obs}$ on ε-aza-ATP concentration to be $5 \times 10^{9} M^{-1} s^{-1}$.

The kinetic data for the fluorescence enhancement observed on ε-aza-ATP binding to bovine cardiac actomyosin-S1 were modeled by the three-step mechanism shown in Equation 4a. The observed rate constants, $k_1$, $k_2$, and $k_3$, were calculated for a given set of rate constants, $k_1$, $k_{-1}$, $k_2$, $k_{-2}$, $k_3$, $k_{-3}$, nucleotide concentrations and relative fluorescence enhancements using a computer program. If the initial binding step is in rapid equilibrium ($k_{-1} \gg k_1 + k_{-2}$) and no fluorescence change occurs upon the formation of the collision intermediate then its amplitude coefficient is very small and the observed rate constant of the rapid component of the solution is too fast to measure with a stopped flow. In this case the solution reduces to the sum of two exponentials as has been analytically described by Trybus and Taylor (24). The dependences of the observed rate constants upon ε-aza-ATP concentrations were used to make initial estimates of the rate constants for the steps in the mechanism. The values for $k_5 + k_3$ and $k_2 + k_3$ were estimated from the hyperbolic dependence of the rates

$$I(t) = L_0 e^{-k_1 t} + L_1 e^{-k_2 t} + L_2 e^{-k_3 t} + C$$

Fig. 5. Stopped flow measurements of the fluorescence enhancement observed upon ε-aza-ATP binding to cardiac actomyosin-S1. Final concentrations of protein and nucleotide in the stopped flow cell were: A, 1.5 μM actin, 1.0 μM myosin-S1, and 5.0 μM ε-aza-ATP; or (B-D) 2.5 μM actin and 2.0 μM myosin-S1; and either B, 20 μM; C, 60 μM; or D, 400 μM ε-aza-ATP. Computer fit curves to the data by the Method of Moments are shown as smooth lines through the data. Single exponential fits were made in A, 0.78 s⁻¹; B, 5.39 s⁻¹; and C, 12.2 s⁻¹. The dashed curve in B is an example of a single exponential that did not satisfactorily describe the data. Double exponential fits were made for B, 11.4 s⁻¹ (−0.51) and 6.29 s⁻¹ (1.51), not shown and D, 80.6 s⁻¹ (0.40) and 15.5 s⁻¹ (0.69). The numbers in parenthesis are fractional amplitude coefficients described in the legend to Fig. 3. Excitation was at 395 nm and emission from 415 to 445 nm. Experimental conditions are the same as those described in Fig. 3.
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of the slow and fast processes upon nucleotide concentration. The value of \( k_1k_2/k_{-1} \) was estimated from the second order rate constant obtained from the experimental data at low nucleotide concentrations. A systematic investigation of the dependence of the observed rate constants and amplitude coefficients upon the microscopic rate constants defined by Equation 4a resulted in the following conclusions for the general model. 1) The initial assumption that the maximum rate of the fast component at high nucleotide concentration is \( k_2 + k_{-2} \) is correct. If \( k_2 > k_{-2} \), the model predicts that observed rate constants and amplitude coefficients are essentially independent of \( k_{-2} \). Solutions in which \( k_{-2} > k_2 \) are not ruled out on kinetic grounds, but the intermediate (A)M-a-ATP** occurs indicating that the equilibrium constant for its formation (\( K_5 \)) cannot be unfavorable. 2) The initial assumption that the maximum rate of the fast component at high nucleotide concentration is \( k_2 + k_{-2} \) is correct. During the modeling, the rate constants of the fast processes were found to be insensitive to the values of \( k_{-2} \). In contrast, the concentration of etheno-aza-ATP at which the slow component of the fluorescence enhancement reached one-half of its maximum rate was found to depend on the value of \( k_{-2} \). The dependence of the relative amplitude coefficients of the slow and fast components upon etheno-aza-ATP concentration is also very insensitive to the values chosen for \( k_{-2} \). No attempt was made to obtain the best fit of the rate constants by covariance methods. The solid lines drawn through the data in Fig. 6 were calculated for the values \( k_1 = 10^4 \text{ M}^{-1} \text{s}^{-1}, k_{-1} = 2 \times 10^4 \text{ s}^{-1}, k_2 = 100 \text{ s}^{-1}, k_{-2} = 0.2 \text{ s}^{-1}, k_3 + k_{-3} = 15 \text{ s}^{-1} \) and the fluorescence enhancements relative to etheno-aza-ATP (zero) were AM-a-ATP = 0, AM-a-ATP = 0.5, and AM-a-ATP** = 1.0. The dependence of the calculated rates and amplitude coefficients is essentially independent of the values of \( k_1 \) and \( k_{-1} \). 

The dependence of the fractional amplitude coefficient \( I_{0a} \) on etheno-aza-ATP concentration agreed well with that predicted by the three-step sequential mechanism calculated using these parameters. In particular the model predicts that the fractional amplitude coefficient, \( I_{0a} \), on etheno-aza-ATP concentration is less than zero for concentrations of etheno-aza-ATP in the range 10–40 \( \mu \text{M} \). It should be noted that an exponential term with a negative coefficient does not necessarily indicate that there is a decrease in fluorescence intensity associated with one step of the mechanism; rather, a negative amplitude coefficient can also occur for a two-step reaction in which the fluorescence enhancement of one step of the reaction is intermediate between the initial and final fluorescence intensities. At nucleotide concentrations above 100 \( \mu \text{M} \) the mechanism predicts biphasic fluorescence data with the fast component accounting for 40–50% of the fluorescence signal at etheno-aza-ATP (zero) were AM-a-ATP = 0, AM-a-ATP = 0.5, and AM-a-ATP** = 1.0. The dependence of the calculated rates and amplitude coefficients is essentially independent of the values of \( k_1 \) and \( k_{-1} \). One of the observed rate constants, which corresponds to nucleotide binding and the first fluorescence transition (AM + T ⇔ A-M-T ⇔ A-M-T**), is shown by the solid line segments in Fig. 6. The other observed rate constant corresponding to the nucleotide binding steps (AM-T** ⇔ AM-T**') is shown by the dashed line segments at \( k_{obs} \approx 15 \text{ s}^{-1} \) in Fig. 6. At nucleotide concentrations \( \approx 40 \mu \text{M} \), shown by the dotted line segments in Fig. 6, the two observed rate constants are nearly the same and assignment of either to a single physical process cannot be made.

The dependence of the fractional amplitude coefficient \( I_{0a} \) on etheno-aza-ATP concentration agreed well with that predicted by the three-step sequential mechanism calculated using these parameters. In particular the model predicts that the fractional amplitude coefficient, \( I_{0a} \), on etheno-aza-ATP concentration is less than zero for concentrations of etheno-aza-ATP in the range 10–40 \( \mu \text{M} \). It should be noted that an exponential term with a negative coefficient does not necessarily indicate that there is a decrease in fluorescence intensity associated with one step of the mechanism; rather, a negative amplitude coefficient can also occur for a two-step reaction in which the fluorescence enhancement of one step of the reaction is intermediate between the initial and final fluorescence intensities. At nucleotide concentrations above 100 \( \mu \text{M} \) the mechanism predicts biphasic fluorescence data with the fast component accounting for 40–50% of the fluorescence signal at etheno-aza-ATP > 200 \( \mu \text{M} \). At concentrations <10 and 40–80 \( \mu \text{M} \) the data are predicted to be essentially single exponential curves either because the amplitude coefficient of the fast component is small (etheno-aza-ATP <10 \( \mu \text{M} \)) or because the rate constants of the two components differ by less than 50% (40–80 \( \mu \text{M} \)) such that the two components cannot be resolved.

The fluorescence data at 0 °C can be fitted by the same model. The rate constants at 0 °C used were \( k_1 = 10^6 \text{ M}^{-1} \text{s}^{-1}, k_{-1} = 3 \times 10^4 \text{ s}^{-1}, k_2 = 50 \text{ s}^{-1}, k_{-2} = 0.1 \text{ s}^{-1} \), and \( (k_3 + k_{-3}) = 5 \text{ s}^{-1} \). Again, the amplitude coefficients of the two components and the rate constants measured at high nucleotide concentration predict the negative amplitude coefficient of the fast exponential term at intermediate nucleotide concentration.

The solution for the branched pathway mechanism, shown in Equation 5, is the sum of three exponentials if the nucleotide binding step is in rapid equilibrium, \( k_{-1} \gg k_2 + k_{-2} + k_{-3} + k_{-6} \).

\[
\text{AM} \xrightleftharpoons[k_1]{k_{-1}} \text{AM} \xrightleftharpoons[k_3]{k_{-3}} \text{AM-a-ATP}^* \xrightleftharpoons[k_5]{k_{-5}} \text{AM-a-ATP}^**
\]

We have evaluated the observed rate constants and amplitude coefficients for a model in which the fluorescence transitions...
have the same rates but occur in opposite order in the upper and lower pathways. The dependence of two of the observed rate constants and amplitude coefficients obtained for the branched pathway mechanism were nearly the same as those previously obtained for the linear mechanism. The additional term in the branched pathway mechanism has an observed rate constant of $k_1 + k_2$, 100.2 s$^{-1}$, that is independent of nucleotide concentration. The amplitude coefficient increases linearly from <0.01 at low nucleotide concentration to 0.1 at 400 μM. Such a small additional component would be too small to measure. Thus the linear and branched mechanisms are essentially indistinguishable. The data, which we have presented here for e-aza-ATP binding to bovine cardiac actomyosin-S1 can be explained by the simpler linear mechanism, although a more complex branched pathway mechanism of the type suggested by Rosenfeld and Taylor (12) for the mechanism of 1-Ν²-etheno-ATP binding to rabbit skeletal and chicken smooth muscle myosin-S1 cannot be excluded. Another possible class of mechanisms is illustrated by Equation 4.

$$\text{AM}_1 = \text{AM}_1 \cdot \text{a-ATP} = \text{AM}_1 \cdot \text{a-ATP}^*$$

$$\text{AM}_2 = \text{AM}_2 \cdot \text{a-ATP} = \text{AM}_2 \cdot \text{a-ATP}^*$$

Such mechanisms would account for the dependence of the rates of the slow and fast processes upon nucleotide concentration but predict that either single exponential or biphasic kinetics would occur at all nucleotide concentrations. The type of kinetic behavior observed in Fig. 5B, in which there is a rapid component with a negative amplitude coefficient would not occur for this class of mechanism. Therefore the simplest mechanism that will explain the data of Fig. 6 for e-aza-ATP binding to cardiac actomyosin-S1 is a linear mechanism of the class described in Equation 4a.

Kinetcs of the Dissociation of Cardiac Actomyosin-S1 by e-Aza-ATP—The decrease in light scattering intensity observed upon mixing cardiac actomyosin-S1 with e-aza-ATP at 15 °C is shown in Fig. 7. The dissociation of the actomyosin-S1 complex by e-aza-ATP was complete as judged by comparison of the extent of the light scattering change accompanying the reaction with that observed for ATP. At low concentrations of e-aza-ATP, the light scattering changes are fit reasonably well by a single exponential equation as shown in Fig. 7, A–C. The second order rate constant measured from the light scattering at e-aza-ATP <100 μM, $5 \times 10^5$ M$^{-1}$ s$^{-1}$, is the same as that determined for the fluorescence enhancement. At nucleotide concentrations <60 μM, the decrease in light scattering intensity can be fit to a single exponential giving values for $k_{obs}$ similar to those of the fast fluorescence transient. However, at higher nucleotide concentrations better fits of the data could be obtained by fitting to two exponentials. For example, at 400 μM e-aza-ATP, the more rapid component of the light scattering signal is 172 s$^{-1}$ and accounts for 55% of the amplitude of the light scattering change (Fig. 7D). The slower component of the light scattering has a value of 38 s$^{-1}$ and contributes 45% of the signal.

The light scattering data at 0 °C are qualitatively similar. The apparent second order rate constant estimated from the dependence of $k_{obs}$ on e-aza-ATP concentration (at values <40 μM) is $2 \times 10^5$ M$^{-1}$ s$^{-1}$. The data are also biphasic at higher nucleotide concentrations. For example, at 400 μM e-aza-ATP the light scattering transients are fit by two exponentials; the faster component at 92 s$^{-1}$ accounting for 45% of the signal and the slower component at 18 s$^{-1}$ accounting for 55% of the signal.
tering or fluorescence transients were biphasic giving fast and intermediate concentrations of ATP, the observed light scatter by observing changes in either nucleotide fluorescence or light shown by closed circles in Fig. 8. The values for in enhancement of fluorescence of t-aza-ADP on mixing the ADP at 0 °C. The dissociation of 6-aza-ADP from cardiac acto- myosin-S1 could also be followed by monitoring the decrease in light scattering as the actomyosin-S1-t-aza-ADP complex dissociates upon mixing with ATP in a stop flow. At 0 °C, the light scattering data can be fairly well fitted to a single exponential at concentrations of ATP < 50 μM. The dependence of the rate of dissociation of cardiac actomyosin-S1 upon the concentration of ATP, in the absence or presence of c-aza-ADP (50 μM final concentration) is shown in Fig. 8. In the absence of c-aza-ADP, the apparent second order rate constant for the dissociation of cardiac actomyosin-S1 by ATP is 8 × 10^5 M⁻¹ s⁻¹ at 0 °C. In the presence of c-aza-ADP (data shown by crosses), the apparent second order rate constant for dissociation is reduced to 2.5 × 10^4 M⁻¹ s⁻¹. At higher concentrations of ATP (> 200 μM), the rate constant for dissociation of the actomyosin-S1-c-aza-ADP complex reaches a plateau at 20 s⁻¹. This behavior is similar to that of the cardiac actomyosin-S1-c-aza-ADP complex and can be quantitatively described by a model shown in Equation 8, in which c-aza-ADP acts as a competitive inhibitor of ATP binding at the active site of actomyosin-S1.

\[
\frac{k_{AD}}{k_{AD}[\text{c-aza-ADP}]} = \frac{AM\cdot\text{ATP}}{AM\cdot\text{ADP}*} \quad (8)
\]

The second slower phase of the light scattering is from the dissociation of AM-a-ATP* and AM-a-ATP**: In such a mechanism the affinity for actin would decrease in the order M-a-ATP, M-a-ATP*, M-a-ATP**.

**Kinetics of the Dissociation of Cardiac Actomyosin-S1-c-Aza-ADP by MgATP**—The rate constant of c-aza-ADP dissociation from cardiac actomyosin-S1 can be measured from the rate at which c-aza-ADP is displaced from the complex by ATP. The reaction can be observed by monitoring the decrease in light scattering as the actomyosin-S1-c-aza-ADP complex dissociates upon mixing with ATP in a stop flow. At 0 °C, the light scattering data can be fairly well fitted to a single exponential at concentrations of ATP < 50 μM. The dependence of the rate of dissociation of cardiac actomyosin-S1 upon the concentration of ATP, in the absence or presence of c-aza-ADP (50 μM final concentration) is shown in Fig. 8. In the absence of c-aza-ADP, the apparent second order rate constant for the dissociation of cardiac actomyosin-S1 by ATP is 8 × 10^5 M⁻¹ s⁻¹ at 0 °C. In the presence of c-aza-ADP (data shown by crosses), the apparent second order rate constant for dissociation is reduced to 2.5 × 10^4 M⁻¹ s⁻¹. At higher concentrations of ATP (> 200 μM), the rate constant for dissociation of the actomyosin-S1-c-aza-ADP complex reaches a plateau at 20 s⁻¹. This behavior is similar to that of the cardiac actomyosin-S1-c-aza-ADP complex and can be quantitatively described by a model shown in Equation 8, in which c-aza-ADP acts as a competitive inhibitor of ATP binding at the active site of actomyosin-S1.

\[
\frac{k_{AD}}{k_{AD}[\text{c-aza-ADP}]} = \frac{AM\cdot\text{ATP}}{AM\cdot\text{ADP}*} \quad (8)
\]

The second slower phase of the light scattering is from the dissociation of AM-a-ATP* and AM-a-ATP**: In such a mechanism the affinity for actin would decrease in the order M-a-ATP, M-a-ATP*, M-a-ATP**.

**Kinetics of the Dissociation of Cardiac Actomyosin-S1-c-Aza-ADP by MgATP**—The rate constant of c-aza-ADP dissociation from cardiac actomyosin-S1 can be measured from the rate at which c-aza-ADP is displaced from the complex by ATP. The reaction can be observed by monitoring the decrease in light scattering as the actomyosin-S1-c-aza-ADP complex dissociates upon mixing with ATP in a stop flow. At 0 °C, the light scattering data can be fairly well fitted to a single exponential at concentrations of ATP < 50 μM. The dependence of the rate of dissociation of cardiac actomyosin-S1 upon the concentration of ATP, in the absence or presence of c-aza-ADP (50 μM final concentration) is shown in Fig. 8. In the absence of c-aza-ADP, the apparent second order rate constant for the dissociation of cardiac actomyosin-S1 by ATP is 8 × 10^5 M⁻¹ s⁻¹ at 0 °C. In the presence of c-aza-ADP (data shown by crosses), the apparent second order rate constant for dissociation is reduced to 2.5 × 10^4 M⁻¹ s⁻¹. At higher concentrations of ATP (> 200 μM), the rate constant for dissociation of the actomyosin-S1-c-aza-ADP complex reaches a plateau at 20 s⁻¹. This behavior is similar to that of the cardiac actomyosin-S1-c-aza-ADP complex and can be quantitatively described by a model shown in Equation 8, in which c-aza-ADP acts as a competitive inhibitor of ATP binding at the active site of actomyosin-S1.

\[
\frac{k_{AD}}{k_{AD}[\text{c-aza-ADP}]} = \frac{AM\cdot\text{ATP}}{AM\cdot\text{ADP}*} \quad (8)
\]

In the limiting case where \( k_{AT}/k_{TA}[\text{ATP}]/k_{AT} \gg k_{AD}[\text{c-aza-ADP}] + k_{AD} \), then at least 90% of the total amplitude of the signal is associated with an observed rate constant \( k_{obs} \), which depends upon nucleotide concentration as described by Equation 10.

\[
k_{obs} = k_{TA}/(1 + K_{AT}[\text{ATP}])/(1 + [\text{c-aza-ADP}]/K_{AD}) \quad (10)
\]

The association constant of c-aza-ADP for cardiac actomyosin-S1, \( K_{AD} \), calculated from Equation 10 is 4.4 × 10^4 M⁻¹ at 0 °C. The dissociation of c-aza-ADP from cardiac actomyosin-S1 could also be followed by monitoring the decrease in enhancement of fluorescence of c-aza-ADP on mixing with the complex with ATP in the stop flow as indicated by the data shown by closed circles in Fig. 8. The values for \( k_{obs} \) obtained by observing changes in either nucleotide fluorescence or light scattering are similar at a given concentration of ATP. At intermediate concentrations of ATP, the observed light scattering or fluorescence transients were biphasic giving fast and slow rates of approximately the same amplitude as shown by the boxed data points. More complete solutions to Equation 10, which are multi-exponential (8) predict that the reaction is biphasic over this range of ATP concentrations.

Similar experiments were carried out at 15 °C, monitoring the decrease in light scattering on mixing cardiac actomyosin-S1-c-aza-ADP with ATP. At this temperature, the binding of c-aza-ADP to actomyosin-S1 is about 3 times weaker than that at 0 °C, so that the fluorescence signal observed on dissociation of the actomyosin-S1-c-aza-ADP complex by ATP is too small to measure accurately; however, values of \( k_{obs} \) obtained from the light scattering transients also gave good fits to the fluorescence data at a given concentration of ATP. At high concentrations of ATP, the rate constant for dissociation reached a plateau at 110 s⁻¹. The association constant of c-aza-ADP for cardiac actomyosin-S1, \( K_{AD} \), calculated from Equation 10 is 1.4 × 10^4 M⁻¹. The second order rate constant for c-aza-ADP binding to cardiac actomyosin-S1 \( (r_{AD}) \) estimated from this data is 1.5 × 10³ M⁻¹ s⁻¹. Under similar conditions, ADP binds to cardiac actomyosin-S1 with an apparent second order rate constant 1 × 10^5 M⁻¹ s⁻¹ (8).

**DISCUSSION**

Table I summarises the data obtained in these experiments for the binding of c-aza-ATP, and the dissociation of c-aza-ADP from, cardiac myosin-S1 and actomyosin-S1 and compares the values of equilibrium and rate constants with those for ATP and ADP. The rate constants of c-aza-ATP binding to cardiac myosin-S1 and actomyosin-S1 are 5–10-fold less slower than those for ATP and ADP.
Etheno-aza Nucleotide Binding to Cardiac Myosin-S1 and Actomyosin-S1

**Table I**

Generalized scheme of the hydrolytic pathways for ATP and e-aza-ATP

\[
\begin{align*}
& \text{AM} \\
\text{AM-T1} \xrightarrow{h_{\text{ATP}}} \text{AM-T2} \xrightarrow{h_{\text{ADP}}} \text{AM-D-P} \xrightarrow{h_{\text{DP}}} \text{AM-D-P**} \xrightarrow{h_{\text{AD}}} \text{AM} \\
\text{M} \\
\text{M-T1} \xrightarrow{h_{\text{ATP}}} \text{M-T2} \xrightarrow{h_{\text{ADP}}} \text{M-D-P**} \xrightarrow{h_{\text{DP}}} \text{M-D-P} \xrightarrow{h_{\text{AD}}} \text{M}
\end{align*}
\]

Superscripts are used to identify rate and equilibrium constants for multiple intermediates with the same ligands. For example, $K_{\text{ATP}}$ is the equilibrium constant for the formation of the complex between actomyosin-S1 and nucleotide triphosphates; $h_{\text{ATP}}$ and $h_{\text{ADP}}$ are rate constants for the transition between AM-T1 to AM-T2. Intermediates with enhanced fluorescence for e-aza-nucleotides are indicated by asterisks; two asterisks indicate approximately double the fluorescence enhancement of one. We have not yet determined if the fluorescence intermediate observed during e-aza-ATP hydrolysis is an additional triphosphate intermediate AM-T2** as has been shown by Rosenfeld (12) to occur during the hydrolysis of 1-N'-etheno-ATP by skeletal myosin-S1 or if hydrolysis of the enzyme bound nucleotide triphosphate is completed and the intermediate is AM-D-P** (or an equilibrium mixture of the two). Experimental conditions are 100 mM KCl, 10 mM MOPS, 5 mM MgCl₂, 0.1 mM dithiothreitol, pH 7.0, unless otherwise indicated.

| Parameter | Units | Temperature 0°C | Temperature 15°C |
|-----------|-------|-----------------|-----------------|
| $K_{\text{ATP}}$ | M⁻¹ s⁻¹ | $5.0 \times 10^6$ | $1.1 \times 10^6$ | (a) | $2.0 \times 10^6$ | $1.7 \times 10^6$ | (23) |
| $k_{\text{ATP}}$ | s⁻¹ | $4.6 (5.1)$ | $0.006$ | (a) | $13.3 (15.2)$ | $63$ | (23) |
| $k_{\text{DP}}$ | s⁻¹ | $0.02$ | $0.045$ | $0.015$ | (23) |
| $k_{\text{ADP}}$ | s⁻¹ | $0.05$ | $1.9$ | $0.34$ | (6) |
| $K_{\text{ATP}}$ | M⁻¹ s⁻¹ | $1.7 \times 10^6$ | $8.0 \times 10^5$ | (a) | $5.0 \times 10^6$ | $2.0 \times 10^6$ | (8) |
| $k_{\text{ATP}}$ | s⁻¹ | $50$ | | 100 | |
| $k_{\text{ADP}}$ | s⁻¹ | $0.1$ | | $0.2$ | |
| $K_{\text{ADP}}$ | M⁻¹ | $4.0 \times 10^4$ | $3.5 \times 10^4$ | (8) | $1.4 \times 10^4$ | $1.5 \times 10^4$ | (8) |

* S. Smith, unpublished data.
* The number in parentheses is the rate constant of the slow phase of the fluorescence observed upon mixing e-aza-ATP with actomyosin-S1.
* Limiting rate of tryptophan fluorescence at high concentrations of ATP.
* Experimental conditions are 100 mM KCl, 10 mM Tris, 5 mM MgCl₂, 0.1 mM dithiothreitol, 20 °C, pH 8.0.
* Obtained from light scattering data.

than the corresponding values for ATP binding. Actin increases the rate of e-aza-ADP dissociation from cardiac myosin-S1 ~60-fold at 15 °C and 400-fold at 0 °C. A similar increase in the rate of dissociation of ADP from myosin-S1 has been observed in the presence of actin (5, 8).

E-aza-ATP binding to bovine cardiac myosin-S1 and actomyosin-S1 is a useful system for studying the mechanism of nucleotide triphosphate binding. The large fluorescence enhancement provides data with extremely good signal to noise ratio that are required to make reliable double exponential fits. In addition, the maximum rates of the reactions are sufficiently slow <100 s⁻¹ that amplitude data can be obtained without making large corrections for signal loss due to the dead time of the stopped flow. In contrast, when the intrinsic tryptophan fluorescence of the myosin head is used to monitor ATP binding, the relatively small signal size and large rate of the rapid phase of the reaction make such detailed analysis of the data difficult. The mechanism of e-aza-ATP binding and hydrolysis by cardiac actomyosin-S1 is kinetically complex. Work presented here indicates the existence of 2 sets of transitions between nucleotide bound actomyosin-S1, and between the corresponding myosin-S1 states. This is the first indication of this phenomenon with cardiac proteins, although similar observations have been made for the hydrolysis of 1-N⁶-etheno-ATP by skeletal and smooth muscle proteins (12).

Several common features are apparent for the binding and hydrolysis of these two analogues, although a detailed comparison of the kinetic mechanism of the hydrolytic pathways of 1-N⁶-etheno-ATP by skeletal and gizzard actomyosin-S1 with the hydrolytic pathway of e-aza-ATP hydrolysis by cardiac actomyosin-S1 is not justified. For both nucleotides, complex kinetics are observed, which require mechanisms more complicated than a simple two-step binding mechanism in which a collision intermediate is followed by a conversion to a more tightly bound intermediate. These mechanisms may result in fluorescence data that are obviously the sum or difference of two exponentials (e-aza-ATP binding to cardiac myosin-S1 and actomyosin-S1; 1-N⁶-etheno-ATP binding to skeletal and smooth muscle myosin-S1 and smooth muscle actomyosin-S1) or data which are approximately fit by a single exponential equation (1-N⁶-etheno-ATP binding to skeletal actomyosin-S1). The extent of deviation of the observed data from single exponential kinetics will be determined by the rate constants of each step of the mechanism and the fluorescence amplitudes of the transitions. Complex kinetics are observed for various different nucleotides under a variety of different experimental conditions (11, 24, 26). This suggests that such mechanisms, which are not of the simple two-step type, are a general feature of nucleotide binding to myosin-S1 and actomyosin-S1, although they may not be obvious under a particular set of experimental conditions. For example, a rapid initial phase of tryptophan fluorescence enhancement has been observed during ATP binding to skeletal (26) and cardiac myosin-S1, but only at temperatures below 10 °C.

The biochemical and physiological properties of e-aza-ATP make it very useful as a substrate with which to investigate the cross-bridge cycle in muscle. A complete description of the kinetics of hydrolysis of e-aza-ATP in vitro should facilitate interpretation of the data from such studies on whole muscles or muscle fibers (13). Another approach to investi-
gating the actomyosin ATP hydrolysis mechanism in vivo is to adapt biochemical techniques to study simple structured systems (27). The similarity of the overall hydrolysis mechanisms for ε-aza-ATP and ATP makes this analogue useful to compare the rates of some of the steps of the nucleotide hydrolysis mechanism for myosin-S1, actomyosin-S1, and myofibrils. The results of such studies on cardiac contractile proteins are described in the accompanying paper (16) and provide a direct test of the rationale that the biochemical intermediates of nucleotide triphosphate hydrolysis which occur in solution are related to those in a structured system.

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