Kinetic Mechanism of 1-N<sup>6</sup>-Etheno-2-aza-ATP and 1-N<sup>6</sup>-Etheno-2-aza-ADP Binding to Bovine Ventricular Actomyosin-S1 and Myofibrils

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The fluorescence emission of 1-N<sup>6</sup>-etheno-2-aza-ATP (ε-aza-ATP) at 410–460 nm is enhanced approximately 8-fold upon mixing substoichiometric concentrations of ε-aza-ATP with bovine cardiac actomyosin-S1 or myofibrils. The time course of nucleotide fluorescence measured in a front face stopped flow cell upon mixing ε-aza-ATP with bovine cardiac myofibrils ([Ca<sup>2+</sup>] < 10<sup>-7</sup> m) is essentially the same as that with bovine cardiac actomyosin subfragment-1. In single turnover experiments, the fluorescence rapidly rises to a maximum value, then decreases with a rate constant of 0.04 s<sup>-1</sup> at 0 °C to a final value that is approximately twice the level of the unbound nucleotide. At concentrations of ε-aza-ATP > 40 μM the kinetics of ε-aza-ATP binding is clearly biphasic for both actomyosin-S1 and myofibrils. At 0 °C, the rate of the more rapid phase is proportional to nucleotide concentration and has a second order rate constant of 1.7 × 10<sup>5</sup> M<sup>-2</sup>s<sup>-1</sup>; the rate of the slower phase extrapolates to a maximum of 4–5 s<sup>-1</sup> at high nucleotide concentration. The rate constants for dissociation of ε-aza-ADP from bovine cardiac actomyosin-S1 and myofibrils were measured from the decrease in ε-aza-ADP fluorescence enhancement observed upon displacement by ATP to be 20 and 18 s<sup>-1</sup>, respectively, at 0 °C. These results indicate that most of the cross-bridges in cardiac myofibrils are bound to actin and that the geometric constraints imposed upon the interaction of actin and myosin by the three-dimensional structure of the myofibril do not modify the kinetics of ε-aza-ATP binding or ε-aza-ADP dissociation.

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). The rationale for such biochemical studies is that the chemical intermediates of the actomyosin ATPase cycle in solution are related to the states of the cross-bridges in solution (1–3). The rationale for such biochemical studies is that the fundamental features of the hydrolytic pathway are unchanged, although the rate constants for the mechanism of ε-aza-ATP hydrolysis by bovine cardiac actomyosin-S1 differ by up to 10-fold from the values measured for ATP (4). Moreover, ε-aza-ATP can replace ATP and support the contraction of muscle fibers; ε-aza-ATP produces 75% of the isometric tension obtained with ATP and gives a similar maximum velocity of shortening as that of ATP in glycerinated rabbit psoas muscle fibers (5).

On the basis of these observations, ε-aza-ATP seemed an ideal substrate analogue with which to study the mechanism of the hydrolytic pathway of nucleotide triphosphate hydrolysis by myofibrils. The basic mechanism is a coupling of the hydrolysis of ε-aza-ATP to work production in muscle, which will tell us how ATP hydrolysis is coupled to work production in muscle. The following simplified version of the nucleotide

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The abbreviations used are: S1, subfragment 1 of myosin; MOPS, N-2-hydroxyethylpipecolonic acid; ε-aza-ATP, 1-N<sup>6</sup>-etheno-2-aza-adenosine-5′ triphosphate; ε-aza-ADP, 1-N<sup>6</sup>-etheno-2-aza-adenosine-5′ diphosphate; AdoA. P<sup>3</sup>P<sup>2</sup>-di(adenosine 5′)-pentaphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid.

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hydrolysis mechanism will be used as a working model for transient kinetic studies of myofibrils.\textsuperscript{2,3}

\[
\begin{align*}
\text{AM} & \Rightarrow \text{AM-T} \Rightarrow \text{([AM-T])} \Rightarrow \text{AM-D} \Rightarrow \text{AM} \\
\text{M-T} & \Rightarrow \text{([M-T])} \Rightarrow \text{M} \\
\end{align*}
\]

The states \([\text{AM-T} \text{ and M-T}]\) or \([\text{AM-D-P} \text{ and M-D-P}]\) are considered as single intermediates, \((\text{A})\text{-M-T} \text{ or } (\text{A})\text{-M-D-P}\), because these states are in rapid equilibrium at least in solution (9, 10) and the extent of the association between actin and myosin is not readily determined in myofibrils. The experiments described here compare the kinetics of e-aza-ATP binding to, and e-aza-ADP\textsuperscript{-}dissociation from, bovine ventricular actomyosin-S1 and myofibrils.

\section*{MATERIALS AND METHODS}

Protein and Myofibril Preparation—Cardiac actin, myosin, and myosin-S1 were purified from the left ventricles of bovine hearts as described by Sienankowski and White (11). Crude cardiac myofibrils were obtained using the same procedure as that for the preparation of cardiac myosin. After 3 washes with 1% Triton X-100, the fluffy white superficial layer of the pellet was washed a further 3 times and stored at 100 mM KCl, 10 mM MOPS, 5 mM MgC\textsubscript{2}, 0.02% sodium azide, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0. Myofibrils were used within a few days or were stored at \(-20^\circ\text{C}\) in 50% glycerol. Examination of the preparation under a phase-contrast microscope showed single myofibrils that had fairly uniform dimensions of 1-2 \mu m in diameter and 10-40 \mu m in length. The concentration of bovine ventricular myosin and myosin-S1 were determined from the absorption at 280 nm using extinction coefficients \((0.1\% , w/v, 1 \text{ cm})\) of 0.55 and 0.64 (12). The concentration of myofibrillar protein is myosin and the molecular weight of myosin is 480,000.

\textsuperscript{2}The following nomenclature is used to 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\textsubscript{i}, a-ADP = e-aza-ADP, a-ATP = e-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\textsubscript{i}. For multiple subscripts, the final letter of the string identifies the ligand associating (or dissociating) to myosin-S1 and all other letters refer to ligands already bound. For example, \(k_{HT} \) refers to the binding of ATP to actomyosin-S1.

\textsuperscript{3}Experimentally measured rate constants of kinetic steps associated with myosin intermediates that are in rapid equilibrium with actin may represent combinations of several rate constants. For example, the \(k_{A-T}\) of the first fluorescence transition, which occurs after e-aza-ATP binds to actomyosin-S1:

\[
\begin{align*}
&\frac{k_{AT}}{k_{T}} \times \frac{[\text{AM-T}]}{[\text{AM}]} \\
&\frac{K_{TA}}{K_{A}} \times \frac{[\text{M-T}]}{[\text{M}]} \\
&\end{align*}
\]

is the weighted average of the rates of the processes that occur for M-T and AM-T.

\[
k_{obs} = k_{AT}(K_{TA}[A])/(1 + K_{TA}[A]) + k_{T}(1/(1 + K_{TA}[A])) + k_{AT}(1/(1 + K_{TA}[A])) + k_{T}(1/(1 + K_{TA}[A]))
\]

\textsuperscript{4}R. F. Siemankowski, unpublished data.
flow rate of approximately 12 ml/s. The drive was stopped in ~4 ms by holding the windings of the stepping motor in one position. The solutions were mixed with an 8-jet Berger ball mixer with a 3-mm orifice (Research Instrumentation). Temperature was regulated by running coolant through the syringe block and cell housing. The dead time of the stopped flow cell can be estimated from the cell volume (dimensions 7 × 15 × 1 mm) and the flow rate of 12 ml/s to be ~10 ms; this allows the measurement of rate constants as fast as 20 s⁻¹ with less than 10% loss of amplitude. The time course of the fluorescence enhancement observed for e-aza-ATP binding to bovine cardiac actomyosin-S1 was the same as that observed in a conventional stop flow with a dead time of 3 ms in which fluorescence emission is measured perpendicularly to excitation (7).

Analysis of the data were carried out as described in the accompanying paper (7).

RESULTS

Time Course of e-Aza-ATP Fluorescence during Hydrolysis by Cardiac Actomyosin-S1 and Myofibrils—The time courses of enhancement of nucleotide fluorescence emission observed upon mixing substoichiometric amounts of e-aza-ATP with bovine cardiac actomyosin-S1 and myofibrils are shown in Fig. 2, A and B, respectively. The time course of enhanced nucleotide fluorescence observed upon addition of e-aza-ATP to myofibrils (Ca²⁺ < 10⁻⁷ M), (Fig. 2B) is virtually identical to that observed for actomyosin-S1 as is shown in Fig. 2A. The emission intensity rapidly increases to a maximum and then slowly decays to a final steady value. The nucleotide fluorescence remains enhanced by approximately 30% of maximum even after hydrolysis is complete. The fluorescence intensity is the same as that obtained by mixing e-aza-ADP with actomyosin-S1 or myofibrils (shown by the dotted lines in Fig. 2, A and B). If the actomyosin-S1 or myofibrils are mixed with 1 mM ATP prior to mixing with e-aza-ATP, the nucleotide fluorescence is the same as that measured for the same concentration of e-aza-nucleotide in the absence of actomyosin-S1 or myofibrils (shown by the dashed lines in Fig. 2, A and B). Greater than stoichiometric amounts of e-aza-ATP produce a steady state enhancement of fluorescence with a duration proportional to the amount of nucleotide added (data not shown). The kcat for steady state hydrolysis of e-aza-ATP, determined from the duration of the fluorescence enhancement or from the rate of the fluorescence decay in a single turnover (Fig. 2A) is 0.04 s⁻¹ at 0 °C. These results are consistent with the maximum enhancement of the nucleotide fluorescence on addition to actomyosin-S1 and myofibrils being due to a mixture of the steady state actomyosin-e-aza-nucleotide intermediates, (A)-M-a-ATP and/or (A)-M-a-ADP-P, with the intermediate level of enhancement corresponding to (A)-M-a-ADP.

Spectrum of the Steady State Intermediate of Myofbrillar e-Aza-ATP Hydrolysis—The fluorescence emission spectrum of the steady state complex formed during the hydrolysis of e-aza-ATP by myofibrils can be obtained from the maximum fluorescence at each wavelength observed upon addition of substoichiometric amounts of e-aza-ATP as was shown for 440-460 nm emission in Fig. 2B. Such a point by point spectrum for bovine cardiac myofibrils is shown in Fig. 3. During steady state hydrolysis by stirred suspensions of cardiac myofibrils (Ca²⁺ < 10⁻⁷ M), the fluorescence emission of e-aza-ATP at the maximum is increased ~2-3-fold and the maximum is shifted from 485 to 460 nm. The fluorescence emission spectrum of the myofibril-e-aza-ATP complex is very

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**Fig. 2.** Time course of the fluorescence emission during single turnover hydrolysis of e-aza-ATP by cardiac actomyosin-S1 and myofibrils. Actomyosin-S1 (A) or myofibrils (B) containing ~20 μM myosin heads were mixed with 10 μM e-aza-ATP in the front face stopped flow cell described in the legend to Fig. 1 and under “Materials and Methods.” The solid lines drawn through the experimental data are the best fits to the equation I(t) = I0e⁻kcatt + Ipte⁻kt + C. The fit rate constants are kcat = 1.0 s⁻¹, kobs = 0.04 s⁻¹, and corresponding amplitude coefficients are I1 = 1.0, I2 = -0.7. The dotted lines are the fluorescence intensity measured upon adding e-aza-ADP to actomyosin-S1 or myofibrils. The dashed lines are the fluorescence intensity expected from noninteracting solutions of (A) actomyosin-S1 and 10 μM e-aza-ATP or (B) myofibrils and 10 μM e-aza-ATP. The same fluorescence intensities were obtained with actomyosin-S1 (A) or myofibrils (B) to which 1 mM ATP was added before mixing with e-aza-ATP. Experimental conditions: 100 mM KCl, 10 mM MOPS, 5 mM MgCl₂, 10 μM A₂₃₈₀, 0.1 mM EGTA, 0.1 mM dithiothreitol, pH 7.0 °C. Excitation was at 365 nm and emission at 440-460 nm.

**Fig. 3.** Fluorescence emission spectra of e-aza-ATP during hydrolysis by cardiac myofibrils. The time course of fluorescence enhancement at each wavelength was monitored on addition of substoichiometric amounts of e-aza-ATP to cardiac myofibrils at 0 °C. Experimental conditions were similar to those used in Fig. 2 except that 20 μl of 0.9 mM e-aza-ATP was added to a stirred suspension of 2.0 ml of 10 mg/ml cardiac myofibrils (16 μM myosin heads) in a Spex fluorolog spectrofluorometer in which the emission wavelength could be varied as indicated. The maximum emission at each wavelength minus the value obtained with a blank prior to the addition of e-aza-ATP is plotted to give the point by point spectrum (dashed line). The emission spectrum of 8 μM e-aza-ATP in the absence of myofibrils is shown for comparison (solid line). The fluorescence enhancement at 440 nm was measured in triplicate and the mean ± S.E. is shown.
similar to that obtained with myosin-S1 (7). The increase in fluorescence is greatest in the 410–460-nm region of the emission spectrum. In this region the maximum increase in fluorescence is 5–fold for ε-aza-ATP binding and approximately 2–3-fold for ε-aza-ADP binding. The large fluorescence enhancement provides a high selectivity for bound compared to free nucleotide; a 25% fluorescence enhancement at 450 nm is observable with a 20-fold molar excess of ε-aza-ATP to myosin binding sites.

Kinetics of the Fluorescence Enhancement Observed upon Mixing ε-Aza-ATP Binding with Cardiac Actomyosin-S1 and Myofibrils—The rapid increase in ε-aza-nucleotide fluorescence observed upon mixing ε-aza-ATP with cardiac actomyosin-S1 or myofibrils at [Ca\(^{2+}\)] < 10\(^{-7}\) M in a front face stopped flow is shown in Fig. 4. At concentrations of ε-aza-ATP < 20 \(\mu\)M the data for both actomyosin-S1 and myofibrils can be approximately fitted to single exponential indicated by the solid line through the data (Fig. 4, A and B). At concentrations of ε-aza-ATP > 20 \(\mu\)M the fluorescence enhancement is biphasic for both actomyosin-S1 and myofibrils (Fig. 4, C and D). A double exponential equation is required to fit the data well, indicating that two processes with different time courses are occurring. In the example shown in Fig. 4C, where the final concentrations of actomyosin-S1 and ε-aza-ATP were 10 and 85 \(\mu\)M, respectively, the fast reaction contributed \(\sim 47\%\) of the total fluorescence amplitude. When myofibrils were used, the observed rate constants were very similar to those obtained with actomyosin-S1 for the same concentration of nucleotide and myosin heads; in this case (Fig. 4D) the fast reaction contributed \(\sim 39\%\) of the total fluorescence amplitude. The dependence of the fit rate constants upon ε-aza-ATP concentration is shown in Fig. 5. For both actomyosin-S1 and myofibrils, the rate of the more rapid phase of fluorescence enhancement is proportional to nucleotide concentration with a second order rate constant of 1.7 \(\times\) \(10^5\) M\(^{-1}\) s\(^{-1}\). The first order rate constant of the slower component of the reaction shows a much smaller dependence upon nucleotide concentration with a rate of 2.7 s\(^{-1}\) at the maximum concentration of nucleotide used, 85 \(\mu\)M. The line drawn through the slow phase of the data is for an apparent binding constant of 25 \(\mu\)M and a maximum rate of 4.0 s\(^{-1}\). Data obtained with actomyosin-S1 under identical conditions from fluorescence measured at 90° in a conventional stopped flow were also biphasic and could be fit by a similar set of rate constants. The rate constants of ε-aza-ATP binding to cardiac myosin-S1 (7), shown by the (———) line in Fig. 5, are 2–5 times slower than the rate constants of ε-aza-ATP binding to cardiac actomyosin-S1 and myofibrils at concentrations of ε-aza-ATP < 100 \(\mu\)M. However, the maximum rate for myosin-S1 was also found to plateau at 4–5 s\(^{-1}\) at 0 °C at higher nucleotide concentrations (>300 \(\mu\)M) and is probably due to the same step of the mechanism as for actomyosin-S1.

No changes in light intensity were detected in experiments in which ATP was mixed with actomyosin-S1 or myofibrils using the same excitation and emission filters that were used to measure ε-aza-ATP fluorescence enhancement. Such experiments indicate that rejection of scattered light is sufficiently good that light scattering changes could not contribute to the biphasic fluorescence transients that we observed. Thus, the biphasic fluorescence enhancement is due to the kinetic mechanism rather than optical artifact.

The biphasic fluorescence enhancement observed upon ε-aza-ATP binding to bovine cardiac actomyosin-S1 and myofibrils is most easily explained by two sequential transitions.
between states that have different levels of fluorescence emission as indicated by the single and double asterisks in Equation 2. The lines in Fig. 5 are theoretical curves for the dependence of the observed rate constants of the slow and fast components of the fluorescence enhancement upon nucleotide concentration calculated from the mechanism shown in Equation 2. The upper panel of Fig. 5 shows the expected dependence of the amplitude of the fast component upon nucleotide concentration.

\[ 1.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \]
\[ \text{AM} \rightleftharpoons \text{AM}\text{-a-ATP} \rightleftharpoons \frac{[\text{AM}\text{-a-ATP}]}{[\text{M}\text{-a-ATP}]} = 4 \text{ s}^{-1} \]
\[ (\text{AM}\text{-a-ATP}) \rightleftharpoons \frac{[\text{AM}\text{-a-ATP}]}{[\text{M}\text{-a-ATP}]} = 4 \text{ s}^{-1} \]

The slower fluorescence change may correspond either to the transition to a second (AM)M-a-ATP intermediate or to the hydrolytic step producing (AM)M-a-ADP-P** (17, 18).

**Fig. 5. The dependence upon \( e\text{-aza-ATP} \) of the observed rate constant \( k_{\text{obs}} \) of \( e\text{-aza-ATP} \) binding to cardiac myosin-S1, actomyosin-S1, and myofibrils. Experimental conditions were identical to Fig. 2 except that \( e\text{-aza-ATP} \) concentration was varied as indicated. Open symbols are for data obtained with actomyosin-S1; solid symbols are for myofibrils. Single exponential fits of data are indicated by triangles and the fast and slow components of double exponential fits are indicated, respectively, by circles and squares. The solid line drawn through the data of the fast phase of the reaction is for a second order rate constant of \( 1.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \). The dashed line drawn through the slow phase of the reaction extrapolates to a maximum rate of 4 \text{s}^{-1}. The fractional amplitude coefficient of the fast component to the total amplitude. The upper limit of 10% shown at 21 \text{pM} \text{e-aza-ATP} \) is the smallest amount of a fast component that could be detected. Single exponential fits of data for \( e\text{-aza-ATP} \) binding to bovine cardiac myosin-S1 over this nucleotide concentration range, obtained as described in the accompanying paper (7), are indicated by a dotted line, -----. The solid line segments correspond to the nucleotide binding step and first fluorescence transition \( (\text{AM} + \text{a-ATP} \rightleftharpoons \text{AM}\text{-a-ATP} \rightleftharpoons \text{AM}\text{-a-ATP}^*) \) and the dashed line segments to the transition \( (\text{AM}\text{-a-ATP} \rightleftharpoons \text{AM}\text{-a-ATP}^*) \). The observed rate constants cannot be assigned to individual transitions at concentrations shown by dotted lines, -----.**

**Fig. 6. Kinetics of the displacement of \( e\text{-aza-ADP} \) by ATP from cardiac actomyosin-S1 and myofibrils. Equal volumes of either (A) cardiac actomyosin-S1 or (B) myofibrils containing \( ~20 \mu\text{M} \) myosin heads and \( 50 \mu\text{M} e\text{-aza-ADP} \) were mixed with \( 2 \text{mM ATP} \). The solid lines through the experimental data are fitted curves corresponding to rate constants of (A) 20 and (B) 18 \text{s}^{-1}. Fluorescence emission of \( e\text{-aza-ADP} \) bound to actomyosin-S1 was measured at 90° to excitation in a 2-cm path length cell; emission of \( e\text{-aza-ADP} \) bound to myofibrils was measured using the front face cell described in the legend to Fig. 1 and under “Materials and Methods.” Experimental conditions were otherwise identical to those described in the legend to Fig. 2.**

Displacement of \( e\text{-aza-ADP} \) Bound to Cardiac Actomyosin-S1 and Myofibrils by ATP—We have previously shown that the kinetic mechanism of \( e\text{-aza-ADP} \) dissociation from bovine cardiac actomyosin-S1 is quantitatively accounted for by Equation 3 in which \( e\text{-aza-ATP} \) is a competitive inhibitor of the ATP binding sites of actomyosin-S1 (7).

\[ \text{AM}\text{-a-ADP} \rightleftharpoons \frac{K_{\text{AD}}}{k_{\text{AD}}} \text{AM} \rightleftharpoons \frac{K_{\text{AT}}}{k_{\text{AT}}} \text{AM}\text{-a-ATP} \rightleftharpoons \frac{k_{\text{TA}}}{k_{\text{TA}}} \text{A} + \text{M-ATP} \]

At high concentrations of ATP such that \( K_{\text{AT}} k_{\text{TA}}[\text{ATP}] \gg k_{\text{AD}} [\text{e-aza-ADP}] + k_{\text{AD}}, \) the observed rate constant is equal to \( k_{\text{AD}}, \) the rate constant of \( e\text{-aza-ADP} \) dissociation from actomyosin-S1. Measurements of the dissociation of \( e\text{-aza-ADP} \) from bovine cardiac actomyosin-S1 and myofibrils were made by stopped flow measurements of the decrease in enhancement of \( e\text{-aza-ADP} \) fluorescence observed upon mixing with ATP (Fig. 6). The solid lines through the data are single exponential fits of 20 \text{s}^{-1} for actomyosin-S1 (Fig. 6A) and 18 \text{s}^{-1} for myofibrils (Fig. 6B). The observed rate constant of \( e\text{-aza-ADP} \) dissociation was unchanged upon increasing the ATP concentration from 0.5 to 1.0 mM and is therefore a good measure of \( k_{\text{AD}}. \)**
Although the kinetic mechanism of hydrolysis of ATP by myosin-S1 and actomyosin-S1 in solution has been studied in considerable detail, relatively little is known about the hydrolytic pathway in the lattice of actin and myosin filaments which make up the contractile apparatus of muscle. Actomyosin-S1 in solution differs in several significant ways from the actin and myosin filaments in muscle. 1) Myosin-S1 is a proteolytic fragment of myosin. Therefore, study of the mechanism of myosin-S1 or actomyosin-S1 ATP hydrolysis would not reveal any modification of the catalytic properties of the myosin head by the S2 or rod portion of myosin, by the regulatory light chains (which are frequently partially proteolyzed or missing in myosin-S1) or by interaction between the myosin heads. For example, the calcium-sensitive regulation of both scallop and smooth muscle actomyosin does not occur with actomyosin-S1(19, 20). 2) Several hundred myosin molecules are organized into each thick filament in muscle. Intrafilamentary interactions between tails of the myosin molecules in the thick filament or between the heads on the surface of the filament could alter the kinetic behavior of the catalytic site or its interaction with actin. 3) The unequal helical repeat of the actin and myosin filaments in vertebrate striated muscle requires that some cross-bridges are constrained to geometries that may not be optimal for myosin to bind to actin (4). Recent optical reconstruction of electron micrographs of insect flight muscle revealed two classes of rigor cross-bridge structures, which differ in their apparent angle of attachment to actin by nearly 30° (21). The precise details of the distribution of cross-bridge binding angles are likely to be different in vertebrate striated muscle and insect flight muscle because of the different axial repeats of the actin and myosin lattice positions; however, it would be surprising if the cross-bridges were to have a uniform geometry of attachment in vertebrate striated muscle.

Detailed analysis of the kinetics of ε-aza-ATP binding to myofibrils (7) could not be made because the experiments had to be carried out at high protein concentration and a restricted range of nucleotide concentration to obtain adequate signal to noise ratio. However, at a somewhat less detailed level, the kinetics of ε-aza-ATP binding to the myosin cross-bridges in bovine cardiac myofibrils are essentially the same as that to actomyosin-S1. The data obtained in these experiments is summarised in Equation 4.

The differences in the rates of nucleotide binding to and dissociation from cardiac myosin-S1 and actomyosin-S1 (7) allow us to distinguish kinetically between free and actin bound cross-bridges in myofibrils. Since the rate constant of ε-aza-ADP dissociation from cardiac actomyosin-S1, 20 s⁻¹, is 400 times greater than that from cardiac myosin-S1, 0.05 s⁻¹ (7), the similarity between the rates of dissociation of ε-aza-ADP from cardiac actomyosin-S1 and myofibrils observed here suggests that most of the nucleotide was bound to attached cross-bridges. In addition, we have found that the rate constants of the fast and slow components of the fluorescence enhancement of ε-aza-ATP binding to cardiac actomyosin-S1 and myofibrils are the same, but are as much as 10 times faster at low nucleotide concentrations (<100 μM) than the observed rate of fluorescence enhancement at corresponding nucleotide concentrations with cardiac myosin-S1 (7). This kinetic evidence that most of the cross-bridges in bovine cardiac myofibrils behave as if they were bound to actin is in agreement with several additional pieces of physical and biochemical evidence. The affinity of ADP for rabbit skeletal and bovine ventricular myofibrils is similar to the affinity of ADP for the corresponding actomyosin-S1; less than 10% of the sites have affinities for ADP that would be expected for myosin-S1 (22). This is consistent with the observation that more than 95% of the proteolytic digestion products from myosin in skeletal myofibrils have the peptide composition that is characteristic of that obtained with actomyosin-S1 rather than that obtained with myosin-S1 (23). Moreover, in glycerinated muscle fibers more than 90% of the myosin heads are bound to actin as indicated by the end spectra of spin labeled myosin (24).

The biochemical and physiological properties of ε-aza-nucleotides make them valuable tools to investigate the kinetics of the cross-bridge cycle in muscle. The data that we have obtained here about the kinetics of ε-aza-nucleotide binding and hydrolysis by actomyosin-S1 in solution and in the myofibril are relevant to an interpretation of such studies. The spectrum of the steady state intermediate that we observe in cardiac myofibrils is similar to that obtained with myosin-S1 and actomyosin-S1. This suggests that the same steady state intermediates, presumably (A)M-a-ATP and (A)M-a-ADP-P, are present in the simple structured system and in solution. Nagano and Yanagida (8) have used ε-aza-ATP as a probe to study the states of the cross-bridge nucleotide complex during contraction of glycerinated rabbit psoas muscle fibers; their observations indicate that these same intermediates are present in skeletal muscle fibers during isometric contraction or shortening. In addition, we have found that the second order rate constants of ε-aza-ATP binding to skeletal or cardiac actomyosin-S1 and cardiac myofibrils are approximately 10-fold slower than the corresponding rate constant for ATP binding (7). This can be related to the observation that the concentration of ε-aza-ATP required for a muscle fiber to attain maximal isometric tension under relaxing conditions is 10 times that required for ATP (8). Such results can be explained if the rates of ε-aza-ATP binding to the muscle fibers are 10-fold slower than the rates of binding of ATP, so that a 10-fold higher concentration of nucleotide is necessary to obtain the same steady state concentration of force producing cross-bridge intermediate when ε-aza-ATP is substrate for the cycle.

On the basis of measurements of the rate of ADP dissociation from actomyosin-S1, isolated from a variety of vertebrate muscles, one of us has previously proposed that ADP dissociation from cross-bridges in a muscle may be sufficiently slow to limit the maximum rate of shortening in that muscle (11, 25). For such a hypothesis to be correct, the rates of ADP dissociation from actomyosin-S1 in solution and from the nucleotide binding site of cross-bridges must be the same. The rate constant of ε-aza-ADP dissociation from bovine cardiac actomyosin-S1, 20 s⁻¹ at 0 °C, is similar to that measured for ADP dissociation, 15 s⁻¹ at 0 °C, under identical conditions (7, 11). These studies show that the rate of ε-aza-ADP dissociation from bovine cardiac actomyosin-S1 and myofibrils is the same, suggesting that the rate of this process and probably also that of ADP dissociation is not affected by...
geometric constraints imposed upon the cross-bridges in myofibrils.

The rate constants of most of the steps in the hydrolytic pathway for the analogue \(\varepsilon\)-aza-ATP are different from those for the physiological substrate, ATP; the exception is that the rate constants of both \(\varepsilon\)-aza-ADP and ADP dissociation from cardiac actomyosin-S1 are similar. Moreover, these processes have the same temperature dependence (7). These observations allow a further indirect test of whether the velocity of unloaded shortening of muscle is limited by the rate at which ADP dissociates from the cross-bridge. This model predicts that if \(\varepsilon\)-aza-ATP were used as a substrate for a muscle, the velocity of unloaded shortening should be the same as that for ATP. This has been found to be the case for rabbit psoas muscle fibers (8). A proper test would, however, require that shortening velocity measurements and kinetic measurements of the rate of \(\varepsilon\)-aza-ATP dissociation be made on the same type of muscle.

We have shown here that the time courses of the fluorescence enhancement of \(\varepsilon\)-aza-nucleotides associated with \(\varepsilon\)-aza-ATP binding, \(\varepsilon\)-aza-ADP dissociation, and single turnovers of \(\varepsilon\)-aza-ATP hydrolysis are essentially the same for bovine cardiac actomyosin-S1 and bovine cardiac myofibrils. These results suggest that the rates of these reactions are not dependent upon the angle of cross-bridge attachment and/or cross-bridge strain since these would be expected to show a distribution of values in myofibrils.

REFERENCES