Transient Kinetics of the Interaction of 1,N^6-Ethenoadenosine 5′-Triphosphate with Myosin Subfragment 1 under Normal and Cryoenzymic Conditions: A Comparison with Adenosine 5′-Triphosphate†

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ABSTRACT: The kinetics of the interaction of the fluorescent analogue 1,N^6-ethenoadenosine 5′-triphosphate (ϵ-ATP) with myosin subfragment 1 (S1) were studied at 15 and −7.5 °C with 40% ethylene glycol as cryosolvent. Two techniques were used: fluorescence stopped flow and rapid flow–quench. When S1 is mixed with ϵ-ATP in a stopped-flow apparatus, biphasic fluorescence transients are obtained which are difficult to assign. Chemical sampling by the rapid-flow–quench method led to the chemical identity and the kinetics of interconversion of key intermediates, and by this method the optical signals were assigned and information about the cleavage and release of products was obtained. The data were interpreted by a shortened form of the Bagshaw–Trentham scheme for myosin adenosinetriphosphatase:

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M{\cdot}ATP \xrightarrow{k_2} M^*{\cdot}ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M^{**}{\cdot}ADP{\cdot}P_i \xrightarrow{k_4} M + ADP + P_i$$

The constants obtained were compared with those for ATP under identical conditions. In agreement with Rosenfeld and Taylor [Rosenfeld, S. S., & Taylor, E. W. (1984) *J. Biol. Chem. 259*, 11920–11929] we find that ϵ-ATP is bound tightly to S1 and that the chemical step is slower than with ATP. We show that the fast fluorescence transient is due to the tight binding of ϵ-ATP with K_1 = 32 μM and k_2 = 58 s^{-1} at 15 °C. With ATP these values are 8 μM and 16 s^{-1}, respectively. There is a large difference in the $\Delta H^{\ddagger}$ for k_2: 50 kJ·mol^{-1} for ϵ-ATP and 119 kJ·mol^{-1} for ATP. We suggest that the slow transient is due to a second type of nucleotide site which does not hydrolyze. At 15 °C with ϵ-ATP, $k_3 + k_{-3}$ = 1 s^{-1} and k_4 = 1.3 s^{-1}; with ATP the values are 22 s^{-1} and 0.12 s^{-1}, respectively. Thus, whereas with ATP k_4 is rate limiting, with ϵ-ATP no single step is clearly rate limiting. The kinetic constants were also obtained at −7.5 °C.

Muscle contraction consists of the cyclic attachment and detachment of myosin heads to actin filaments. The energy needed for these processes is supplied by the hydrolysis of ATP[1] by the myosin heads. Detailed studies carried out in several laboratories [for reviews, see Taylor (1979), Adelstein and Eisenberg (1980), and Hibberd and Trentham (1986)] have lead to a seven-step mechanism for the hydrolysis of ATP, the Bagshaw–Trentham pathway, as shown in Scheme I, where M represents myosin or one of its proteolytic fragments (subfragment 1 or heavy meromyosin). Different protein conformations are indicated by asterisks.

Scheme I

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M{\cdot}ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M^*{\cdot}ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M^{**}{\cdot}ADP{\cdot}P_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} M^*{\cdot}ADP{\cdot}P_i \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} M^*{\cdot}ADP \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} M{\cdot}ADP \underset{K_7}{\rightleftharpoons} M + ADP$$

A critical step of Scheme I is the ATP-induced conformational change of myosin, defined by K_1 and k_2. This irreversible step leads to the key intermediate M*·ATP (Geeves & Trentham, 1982). Therefore, it is important to determine fully the properties of this step, and to do this we must measure K_1 and k_2. They can be measured by the ATP chase method [e.g., Barman and Travers (1985)], but this method suffers from being expensive in materials and it is laborious. Attempts have been made to study the binding process by more economical and easier optical methods. Thus, there is an optical signal when ATP is mixed with myosin, but this is difficult to assign; it appears to be due mainly to M**·ADP·P_i, but there is also a contribution from M*·ATP [for a discussion of this problem, see Chock et al. (1979) and Biosca et al. (1984)].

A way of facilitating the study of nucleotide–protein interactions is to use fluorescent analogues. In the case of ATP there is ϵ-ATP, first described by Secrist et al. [1972; for a recent review see Leonard (1984)]. With its ultraviolet absorption band of lowest energy around 300 nm, its maximum emission well beyond protein fluorescence, and the high quantum yield, this useful analogue has been used as a substrate with a number of enzymes (Yount, 1975).

ϵ-ATP has been used as a substrate analogue with myosin and actomyosin. Mowery (1973) showed that it is hydrolyzed by myosin and that it supports the contraction of muscle fibers. McCubbin et al. (1973) found that whereas HMM hydrolyses ϵ-ATP more rapidly than ATP, the K_m values are similar and very low. This suggests that, as is ATP (Mannherz et al., 1974), ϵ-ATP is bound tightly to myosin. Willick et al. (1973) and Garland and Cheung (1976) showed that ϵ-ADP and ADP

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; ϵ-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; P_i, inorganic orthophosphate; S1, myosin subfragment 1; HMM, heavy meromyosin; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; ATPase, adenosinetriphosphatase.

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bind equally well to S1. Yanagida (1981) carried out experiments with glycerinated muscle fibers and found that as for ATP nearly two ϵ-ATP molecules were bound per myosin molecule.

Thus, from steady-state studies ϵ-ATP seems to behave very similarly to ATP with myosin and also with more complex muscle systems. Now, does this mean that we can rely on experiments with the convenient fluorescent ϵ-ATP to tell us about the mechanism of muscle contraction and, in particular, of the interaction of actin with the myosin ATPase intermediates?

Rosenfeld and Taylor (1984) carried out fluorescence stopped-flow experiments with S1 ϵ-ATPase. They obtained two transients; the data could not be fitted to successive intermediates, and a branched pathway for ϵ-ATP binding was proposed. The P_i burst was considerably smaller than that with ATP. This is evidence that the relative amounts of the various intermediates on the reaction pathways of S1 ATPase and ϵ-ATPase are different, a finding that confirms the danger in relying on steady-state parameters in determining the effect of a perturbant (Biosca et al., 1984). Rosenfeld and Taylor (1987) studied the dissociation of ϵ-ADP from regulated acto–S1, and again there were significant differences with respect to ADP.

In addition to its useful fluorescent properties, being an analogue, ϵ-ATP should perturb S1 and acto–S1 ATPases, and as with other perturbants, it is a way of obtaining mechanistic information on the system under study. Perturbation can be carried out by changing the solvent composition, the temperature (Douzou, 1977), or the pressure [e.g., Balny et al. (1985)]. One can also modify chemically the enzyme [for example, by site-directed mutagenesis, Knowles (1987)]. In particular, one can perturb by the use of substrate analogues. Dewar and Storch (1985) and, more specifically with myosin, Eccleston and Trentham (1979) have warned against the overinterpretation of experiments with substrate analogues; nevertheless, their use has led to important advances in the mechanism of enzyme action [for ATP handling enzymes, see Yount (1975) and Leonard (1984); for myosin, see Bagshaw et al. (1972)].

Here, we make an attempt at obtaining the details of the interaction of ϵ-ATP with S1. First, we assign the two fluorescence transients observed upon mixing ϵ-ATP with S1. The fast phase is due to an S1 that binds tightly and then hydrolyzes the nucleotide (by Scheme I). The slow phase could be due to a type of S1 that binds the nucleotide without hydrolysis (Tesi, Bachouchi, Barman, and Travers, unpublished results). Second, we conclude that whereas S1 catalyzes the hydrolysis of ϵ-ATP and ATP by the same reaction pathway (Scheme I), the individual kinetic constants are different. Thus, whereas with ATP step 4 is rate limiting, with ϵ-ATP no single step is clearly rate limiting.

Materials and Methods

Proteins and Reagents. The preparation and assay of myosin and S1 are given in Biosca et al. (1984). ϵ-ATP (Sigma) was purified by DEAE-cellulose chromatography and tested for purify by HPLC; by UV detection only ϵ-ATP (~98%) and ϵ-ADP (<2%) could be detected. [γ-^{32}P]-ϵ-ATP was prepared according to Glynn and Chappell (1964) and tested for radioactive purity by HPLC.

Experimental Conditions. Except where otherwise stated, experiments were carried out in 40% ethylene glycol, 50 mM Tris, 5 mM KCl, 2 mM magnesium acetate, and 1 mM DTT adjusted to pH 8 with acetic acid at 15 or −7.5 °C.

Quenched-Flow Experiments. Two apparatuses were used. With the first apparatus one takes samples in the 4–280-ms time range (Barman & Travers, 1985). For maximum precision with the apparatus one must sample rather large reaction mixture volumes (about 1 mL). The second apparatus permits the automatic sampling of reaction mixtures of ages 1 s up to at least $^1/_2$ h. Here the volumes sampled are 0.25 mL. With the apparatus a kinetic curve is obtained from a single reaction mixture (up to 30 time points); because it samples automatically, it is subject to less experimental error and is easier to use than the conventional flow–quench apparatus. A full description will appear elsewhere. Both apparatuses are thermostatically controlled.

In ATP chase experiments, S1 plus [γ-^{32}P]-ϵ-ATP reaction mixtures of ages t are quenched in a large molar excess (~1000-fold) of unlabeled ATP. The quenched reaction mixtures are incubated at 20–25 °C and after 3 min stopped by the addition of an equal volume of 22% (v/v) trichloroacetic acid containing 1 mM NaH_2PO_4, and the [^{32}P]P_i is determined by the method of Reimann and Umfleet (1978). By plotting [^{32}P]P_i against time t, we obtain a transient burst phase whose amplitude equals ATPase site concentration of the S1 used and kinetics $k = k_2[\epsilon\text{-ATP}]/(K_1 + [\epsilon\text{-ATP}])$. This is followed by the steady state. In experiments where $t \gg 1/k$ extrapolation of the steady state to zero time gives the ATPase site concentration (e.g., Figure 1). In P_i burst experiments reaction mixtures were quenched directly in the acid.

Stopped-Flow Experiments. These were carried out in an apparatus constructed in this laboratory (Markley et al., 1981) and adapted for fluorescence work at low temperature. The dead time of the apparatus is about 5 ms. The excitation and emission wavelengths depended on the type of experiment and are given in the figure legends.

Treatment of Data. Data fitting and simulations were carried out on an Apple IIe computer (Biosca et al., 1984). The data from stopped flow were stored in a Sord Mark III computer and transferred to the Apple for treatment. Further details for different types of experiments are given in the text.

Results

The interaction of ϵ-ATP with S1 was studied by chemical sampling (rapid flow–quench) and optical methods (fluorescence stopped flow). Chemical sampling led to the chemical identity and the kinetics of interconversion of certain key intermediates. The signals obtained from stopped-flow experiments were then assigned and the study was continued by this less laborious method.

Nucleotide Binding Experiments at 15 °C. Unlabeled ATP chase experiments with ϵ-ATP and ATP under steady-state conditions at 15 °C in 40% ethylene glycol are compared in Figure 1. With each there was a transient burst phase of tightly bound nucleotide followed by the steady state. Since the amplitudes of the phases are similar, it appears that ϵ-ATP is bound tightly to S1. S1 hydrolyzes ϵ-ATP more rapidly than ATP. These results agree with those of Rosenfeld and Taylor (1984), whose experiments where carried out in water.

The kinetics of the binding process (k) were now studied in the millisecond range, and a typical progress curve is given in Figure 2. As with ATP, the curve was cleanly first order but the process was considerably faster: 35 s^{-1} as compared with 16 s^{-1} (Barman et al., 1983).

The dependence of k on the ϵ-ATP concentration is hyperbolic (Figure 3); this confirms that as for ATP, S1 binds ϵ-ATP in a two-step process [see Barman et al. (1983) for a discussion].

The fluorescence signal obtained when ϵ-ATP was mixed with S1 at 15 °C in a stopped-flow apparatus is shown in

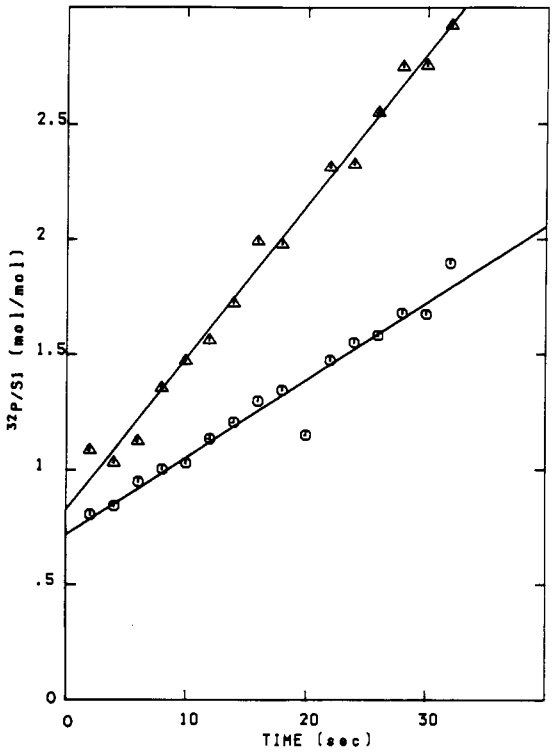

FIGURE 1: Steady-state time courses for S1 ATPase (O) and ϵ-ATPase (Δ) at 15 °C. The reaction mixtures (6.5 μM S1 + 23 μM [γ-^{32}P]ATP and 2.4 μM S1 + 14 μM [γ-^{32}P]-ϵ-ATP) were quenched in 60 mM unlabeled ATP at the times indicated, and the [^{32}P]P_i was determined as described in the text. The extrapolations of the steady states to zero time gave a ratio mol of active site/mol of S1 protein of 0.72 (±0.04) with ATP and 0.82 (±0.04) with ϵ-ATP. By dividing the steady states by the active site concentrations, k_{cat} for ATP = 0.047 s^{-1} and for ϵ-ATP = 0.09 s^{-1} [see Barman et al. (1983) for treatment].

Table I: Kinetic Constants for S1 ATP and ϵ-ATPases[a]

	−7.5 °C		15 °C	
constant[b]	ATP	ϵ-ATP	ATP	ϵ-ATP
K_1 (μM)	1	65	8	32
k_2 (s^{-1})	0.17	10	16	58
k_2/K_1[c]	0.17	0.15	2.0	1.8
k_3 (s^{-1})	0.015	0.004	11	0.21
k_{-3} (s^{-1})	0.15	0.01	11	0.8
K_3	0.1	0.4	1	0.26
k_4 (s^{-1})	0.03	0.1	0.12	1.3
k_6 (s^{-1})	0.0016	0.0018	0.15	0.7
k_{cat} (s^{-1})	0.0013	0.0014	0.043	0.09

[a] The data for ATP are from Biosca et al. (1984). For ϵ-ATP, K_1 and k_2 were obtained from ATP chase and stopped-flow experiments. k_3, k_{-3}, k_4, and k_6 were obtained by computer simulation using the data from P_i burst experiments. For full details, see text. [b] For the sake of clarity errors are not given. For K_1 ±SD is 10–20% (see text); for the other constants ±SD is better than 10%. [c] M^{-1} s^{-1} × 10^{-6}.

Figure 2. As found by Rosenfeld and Taylor (1984), there were two exponential processes, but under the experimental conditions used here the slow phase had a very low amplitude and it was difficult to exploit. The fast transient gave k_{obsd} = 37 s^{-1}. This compares with 35 s^{-1} found by ATP chase under identical conditions (Figure 2). As shown in Figure 3, the dependence of the kinetics of the fast fluorescence transient and that of the ATP chase kinetics on the ϵ-ATP concentration at 15 °C are within experimental errors identical. The constants for the binding of ϵ-ATP and ATP to S1 are compared in Table I.

Nucleotide Binding Experiments at −7.5 °C. It was important to assign the slow phase of the fluorescence change, but conditions had first to be found under which the two phases are distinct and more easily to exploit than at 15 °C.

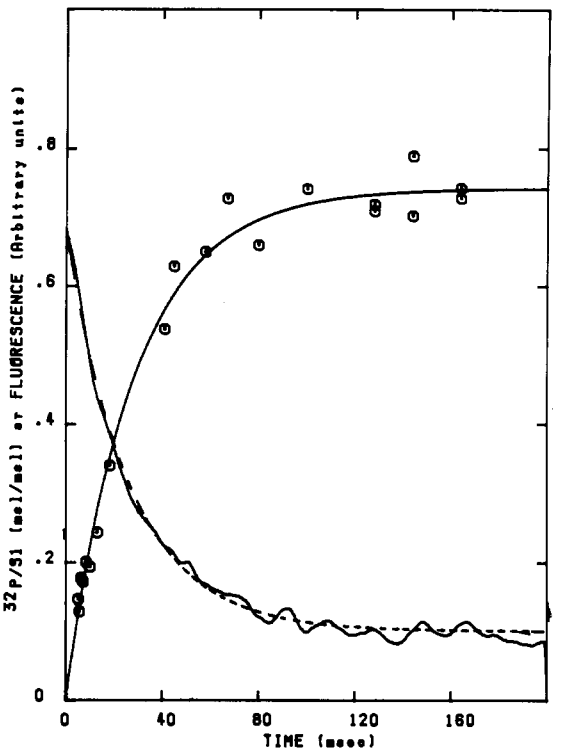

FIGURE 2: Binding of ϵ-ATP to S1 at 15 °C measured by ATP chase and fluorescence stopped flow. For ATP chase (O) the reaction mixture was 13.5 μM S1 + 47.5 μM [γ-^{32}P]-ϵ-ATP. The solid line was computer fitted to k = 35 (±2) s^{-1} and a plateau of 0.74 (±0.02) [^{32}P]P_i/S1 (mol/mol). The continuous line represents the fluorescence transient given by 7.5 μM S1 + 50 μM ϵ-ATP with excitation at 290 nm and emission at 340 nm. The dashed curve was fitted to a single exponential of k = 37 (±1) s^{-1}. This fast phase was followed by a slow phase (not seen on time scale of figure).

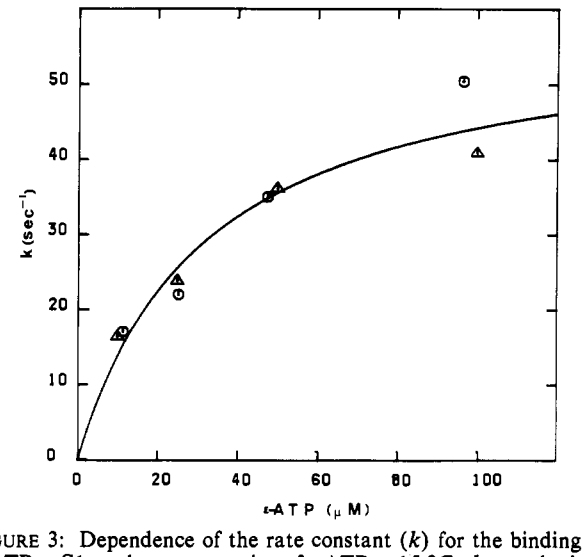

FIGURE 3: Dependence of the rate constant (k) for the binding of ϵ-ATP to S1 on the concentration of ϵ-ATP at 15 °C. k was obtained from ATP chase experiments (O) and fluorescence stopped-flow experiments (Δ). The curve was obtained by computer fitting to a hyperbola with K_1 = 32 (±8) μM and k_2 = 58 (±7) s^{-1}.

As shown in Figure 4A the binding of ϵ-ATP to S1 was clearly biphasic at −7.5 °C. The kinetics of the fast phase gave a k = 0.63 s^{-1}; by the ATP chase under identical conditions k = 1 s^{-1} (curve not shown). The ϵ-ATP concentration dependencies of the two phases are given in Figure 5; in both cases they were hyperbolic. The maximum rate constants for the two transients (k_2, k_2') differ by a factor of 9, but the apparent dissociation constants (K_1, K_1') are almost identical (Table II).

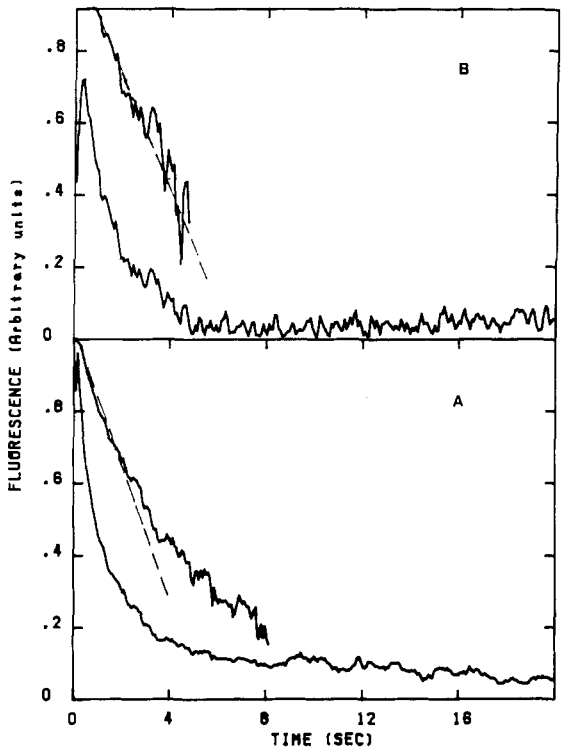

FIGURE 4: Fluorescence transients for the binding of ϵ-ATP to S1 at −7.5 °C. (A) Multiturnover experiment with 2 μM S1 + 12.5 μM ϵ-ATP (excitation at 290 nm and emission at 340 nm). The transient was fitted to two exponentials: a fast phase (k = 1.16 s^{-1}) and a slow phase (0.07 s^{-1}). The amplitude of the fast phase was about 80% of the total. (B) Single-turnover experiment with 7.5 μM S1 nucleotide sites + 2 μM ϵ-ATP (excitation at 315 nm and emission at 412 nm). The transient was fitted to a single exponential (k = 0.63 s^{-1}). The dashed lines are log plot fits (multiturnover experiment: only fast phase shown in log plot).

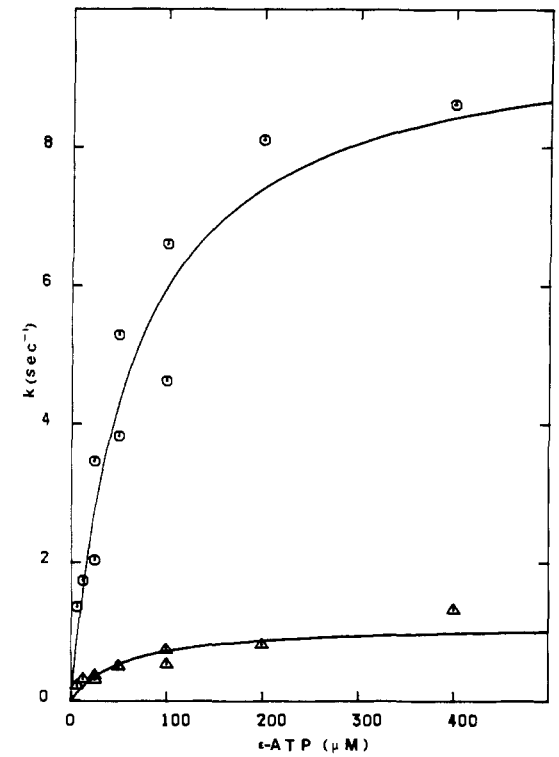

FIGURE 5: Concentration dependencies of the kinetics of the two fluorescence phases observed upon the binding of ϵ-ATP to S1 at −7.5 °C. The data were fitted to hyperbolas. The fast phase (O) gave K_1 = 65 (±19) μM and k_2 = 10 (±1) s^{-1}, and the slow phase (Δ) gave K_1' = 55 (±22) μM and k_2' = 1.1 (±0.2) s^{-1}.

Table II: Comparison of Kinetic Constants for the Binding of ATP and ϵ-ATP to S1 at −7.5 °C[a]

constant	ATP	ϵ-ATP	constant	ATP	ϵ-ATP
K_1 (μM)	1	65	k_{-2}' (s^{-1})	0.003	nd[c]
k_2 (s^{-1})	0.15	10	k_2/K_1[b]	0.15	0.15
K_1' (μM)	0.15	55	k_2'/K_1'[b]	0.067	0.02
k_2' (s^{-1})	0.01	1.1			

[a] The binding data were interpreted by the equations

$$M + NTP \overset{K_1}{\rightleftharpoons} M{\cdot}NTP \xrightarrow{k_2} M^*{\cdot}ATP \rightarrow \text{products}$$

$$M' + NTP \overset{K_1}{\rightleftharpoons} M'{\cdot}NTP \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} M^\circ{\cdot}ATP$$

where M and M′ are different types of myosin head and NTP is nucleoside triphosphate. The signs * and ° indicate different protein conformations. The data for ATP are from Tesi et al. (unpublished experiments), and those for ϵ-ATP were obtained from stopped-flow experiments (for errors see text). [b] M^{-1} s^{-1} × 10^{-6}. [c] nd, not determined.

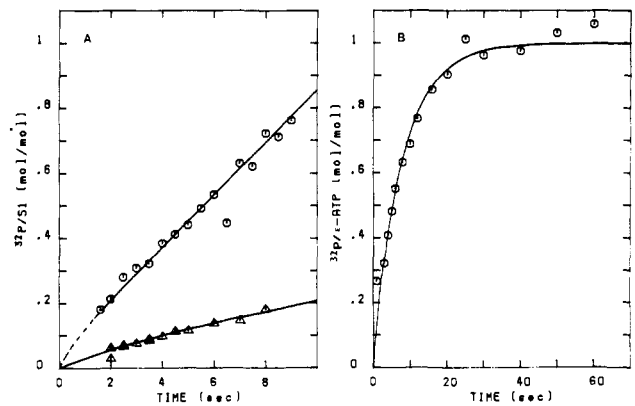

FIGURE 6: Time courses of the hydrolysis of ϵ-ATP by S1 under multiturnover and single-turnover conditions at 15 °C. (A) Multiturnover experiment. Reaction mixtures (10 μM S1 + 18 μM [γ-^{32}P]-ϵ-ATP were quenched in 20% trichloroacetic acid, and the [^{32}P]P_i was determined. (B) Single-turnover experiment. Reaction mixtures (4.6 μM S1 + 1 μM [γ-^{32}P]-ϵ-ATP) were quenched in 20% trichloroacetic acid, and the [^{32}P]P_i was determined. In both experiments the curves were obtained by computer simulation using Scheme I and the constants of Table I. For points (O) the time scale is as indicated, and for (Δ) it is divided by 5.

Experiments carried out under single-turnover conditions aided in assigning the slow phase. A typical experiment is illustrated in Figure 4B: with a ratio of S1 to ϵ-ATP of 6 the fluorescent transient was virtually monophasic. Thus by inversion of the reagent concentrations, monophasic fluorescent transients are obtained.

Temperature Dependence of the Binding Process. From the binding experiments carried out at 15 and −7.5 °C (Table I) the ΔH^* of k_2 for ϵ-ATP is 50 kJ·mol^{-1}. This compares with 119 kJ·mol^{-1} for ATP in the same temperature range (Biosca et al., 1983). For both nucleotides, K_1 is relatively insensitive to temperature.

Studies on the Postbinding Steps of ϵ-ATPase. A typical P_i burst experiment at 15 °C with ϵ-ATP is given in Figure 6A. Unlike with ATP under the same conditions (Biosca et al., 1984), the amplitude was very low: it was discerned only by taking samples over a large time range. Rosenfeld and Taylor (1984) also found a low P_i burst with S1. However, with myosin Onishi et al. (1973) observed a large P_i burst of 1.3 mol of P_i/mol of myosin—presumably because 1 M KCl was used as solvent (Biosca et al., 1984).

A P_i burst experiment carried out with ϵ-ATP under single-turnover conditions is given in Figure 6B. The curve appeared to be monophasic with $K_{obsd} \sim k_{cat}$ (about 0.1 s^{-1}). This

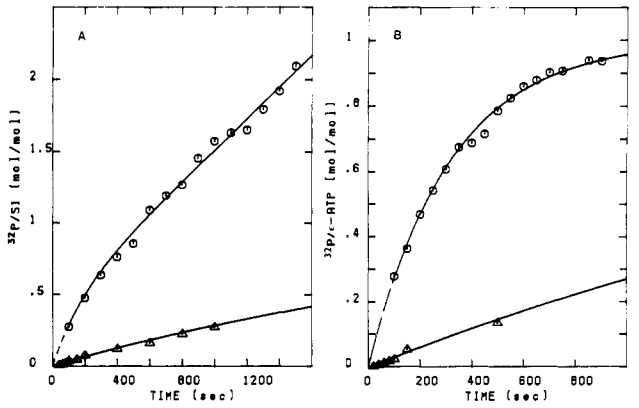

FIGURE 7: Time courses of the hydrolysis of ϵ-ATP by S1 under multiturnover and single-turnover conditions at −7.5 °C. (A) Multiturnover experiment. The reaction mixture was 4 μM S1 + 11 μM [γ-^{32}P]-ϵ-ATP. (B) Single-turnover experiment with 4 μM S1 + 1.7 μM [γ-^{32}P]-ϵ-ATP. In both experiments the curves were obtained by computer simulation using Scheme I and the constants in Table I. For points (○) the time scale is as indicated, and for (△) it is divided by 10.

situation is very different from that obtained with ATP under identical conditions: a large rapid rise in P_i followed by $k_0 = k_4K_3/(1 + K_3)$ (Bagshaw & Trentham, 1973; Biosca et al., 1984). It suggests that with ϵ-ATP no single step is clearly rate limiting, and this makes it difficult to obtain directly the constants for the chemical step and release of products.

We obtained estimates of the postbinding constants for ϵ-ATP by computer simulation. Thus, curves were plotted by using Scheme I and adjusting the individual constants until the best fit to the experimental points and k_{cat} were obtained. This is illustrated in Figure 6. We believe that the constants obtained are unique. They are summarized in Table I.

In Figure 7 are illustrated P_i burst experiments under multiturnover and single-turnover conditions carried out at −7.5 °C. In the multiturnover experiment the P_i burst amplitude was larger than at 15 °C, but it remained low. Further, no rapid rise in P_i could be detected in the single-turnover experiments. We conclude that as at 15 °C no single step is clearly rate limiting. Estimates for the individual constants were obtained as for 15 °C, and they are summarized in Table I.

Discussion

The main purpose of our work was to ascertain the quality of ϵ-ATP in replacing ATP as a substrate for myosin. But before discussing this, we must assign the two fluorescence transients observed upon mixing the analogue with myosin in a stopped-flow apparatus.

Assignment of the Two Fluorescence Transients Observed by Stopped Flow. Under identical conditions, the kinetics of the fast fluorescence transient and of ATP chase experiments were very similar (Figure 2). Further, the dependencies of the two kinetics upon the ϵ-ATP concentration were within experimental errors identical (Figure 3). These experiments were carried out at 15 °C, but at −7.5 °C too the kinetics obtained by the two methods were similar. Taken together, these results show that the fast phase obtained in stopped-flow experiments is a manifestation of the tight binding of ϵ-ATP to S1 (i.e., steps 1 and 2 of Scheme I). Thus, the binding process can be studied by stopped flow rather than the rapid-flow–quench method. However, because of the slow fluorescence transient, stopped-flow experiments are less accurate than the cleaner chemical experiments.

To what process can we assign the slow phase? Garland and Cheung (1979) carried out stopped-flow experiments with ϵ-ATP and S1 and obtained two phases. They interpreted their results by a scheme involving two successive intermediates, each of which gives a fluorescent signal—a slow and a fast phase. Now, in such a situation only the fast phase (representing the first intermediate) should be sensitive to the ϵ-ATP concentration: the kinetics of the slow phase (i.e., of the succeeding intermediate) should be relatively insensitive. Since Rosenfeld and Taylor (1984) found that the slow transient was sensitive to the ϵ-ATP concentration, they exclude a scheme involving two successive intermediates.

Here biphasic fluorescence transients were obtained at −7.5 °C (Figure 4A). The kinetics of each phase varied with the concentration of ϵ-ATP—indeed, the dissociation constants derived from the dependence curves were almost identical (Figure 5). This confirms that a scheme involving successive intermediates must be excluded.

Another explanation for the slow phase is that it is due to a fluorescent impurity in the ϵ-ATP which binds to S1. It is unlikely that self-stacking of ϵ-ATP is a problem as the concentrations needed for this (>0.5mM) are much higher than those used here (Sigel et al., 1986). Yet another explanation is due to Rosenfeld and Taylor (1984), who proposed a branched pathway to explain the fluorescence transients.

We put these two explanations to the test. The two transient phases were obtained under multiturnover conditions (i.e., ϵ-ATP > S1): with a fluorescent impurity or branched pathway the two phases should remain upon inverting the reagent concentrations (i.e., ϵ-ATP < S1). But single-turnover experiments were monophasic—a typical experiment is given in Figure 4B. We conclude that the slow phase is not due to an impurity or to a branched pathway.

A situation involving different types of sites for nucleotide would explain the biphasic stopped-flow kinetics. Rosenfeld and Taylor (1984) did not exclude this possibility to explain their data. There is evidence for variable amounts of different types of ATP binding sites in S1 preparations: One type hydrolyzes ATP by the Bagshaw–Trentham mechanism (tight binding of nucleotide followed by hydrolysis) and is determined in unlabeled ATP chase experiments. The second type appears to bind ATP relatively weakly without hydrolysis; this site is not determined in chase experiments. Such a situation would explain the variability of the ATPase site concentrations of different S1 preparations (Barman et al., 1983; Tesi et al., unpublished results). Now, a situation involving variable amounts of different sites should result in two phases of variable amplitudes in stopped-flow experiments, but we were unable to show this because of the difficulty in determining accurately the amplitudes.

We summarize in Table II the two-site scheme (see equations in footnote *a*) together with values for the kinetic constants. It is noteworthy that the ratios K_1/K_1' and k_2/k_2' are relatively independent of the experimental conditions: at −7.5 °C in 40% ethylene glycol they are 1.2 and 9, respectively (here), and at 20 °C in water, 2 and 6 (Rosenfeld & Taylor, 1984). Thus, the constants defining k_{on} for ϵ-ATP are not only of the same order of magnitude, but they also have very similar thermodynamic properties. The difference between the sites resides mainly in the very large difference in k_{off} (k_{-2}).

All the experiments reported here were carried out in 40% ethylene glycol. Whereas the use of this solvent causes changes to certain of the individual kinetic constants of Scheme I and the mechanism shown in Table II, we have no reason to suspect that the overall mechanisms change (Biosca et al., 1984; Barman et al., 1986).

Comparison of the Reaction Pathways of S1 ϵ-ATP and ATPases. A key feature of the S1 ATP pathway is that it has a clearly rate limiting step (k_4) which aided Bagshaw and Trentham (1973) in elaborating a reaction scheme (Scheme I). Essentially, ATP is bound and cleaved rapidly whereas the products are released slowly. Thus, there is a large P_i burst (the kinetics give $k_3 + k_{-3}$), and in single-turnover experiments there is a rapid rise (giving K_3) followed by a slow $k_0 = k_4K_3/(1 + K_3)$.

With ϵ-ATP the situation is different: the P_i burst is low (Figure 6A), and in single-turnover experiments a rapid rise could not be discerned (Figure 6B). Thus, here P_i burst experiments yielded relatively little information, and unlike with ATP k_3, k_{-3}, and k_4 could not be obtained directly—computer simulation had to be used. However, because of the specificity of the ATP chase method (and also the subsequent fluorescence stopped-flow experiment) we were able to show that S1 binds ϵ-ATP in two steps, and K_1 and k_2 were measured in terms of Scheme I.

Certain of the constants of the S1 ATP and ϵ-ATPase pathways at −7.5 and 15 °C are compared in Table I. There are several noteworthy features.

K_1 is higher for ϵ-ATP than for ATP. This is compensated for by a higher k_2, and at low concentrations of nucleotide both are bound at the same rate with k_2/K_1 about 2×10^6 M^{-1} s^{-1} at 15 °C and 1.5×10^5 M^{-1} s^{-1} at −7.5 °C.

The difference in $\Delta H^{\ddagger}$ of k_2 for the nucleotides is large: 50 and 119 kJ·mol^{-1} for ϵ-ATP and ATP, respectively. Bagshaw and Trentham (1974) have pointed out that large activation energies can be associated with cooperative structural transitions of macromolecules although an explanation involving solvent cannot be excluded. Therefore, the difference in $\Delta H^{\ddagger}$ could indicate a difference in the conformational change induced by ϵ-ATP and ATP. Since ϵ-ATP is hydrolyzed much less rapidly than ATP, one might argue that the "quality" of the induced fit with ϵ-ATP is lower than that with ATP. A poor substrate-induced fit could lead to an improper alignment of the amino acid residues involved in cleavage and therefore slower hydrolysis. Alternatively, the analogue is hydrolyzed slowly because it does not completely fill the active site. Here the cleavage step would be less effective because it occurs in solution rather than in the gas phase as with the natural substrate ATP (Dewar & Storch, 1985).

It is indeed at the chemical step that ϵ-ATP and ATP differ the most: at 15 °C this step becomes near to being rate limiting with ϵ-ATP. The situation is very different with ATP where $k_3 + k_{-3}$ is about 500 times as large as k_{cat}.

In conclusion, we return to the question asked in the introduction: can the experimentally more convenient ϵ-ATP replace ATP in studies on myosin and actomyosin? The answer depends on what one wants to study. Thus, the data obtained from transient experiments with ϵ-ATP can be fitted to the reaction pathway of S1 ATPase. In particular, as is ATP, ϵ-ATP is bound tightly in two steps. However, there are important differences in the individual rate constants and equilibria. For example, with ATP the rate-limiting step at 15 °C is k_4 and at −7.5 °C k_6, but with ϵ-ATP no single step is rate limiting at either temperature. This could explain the relative ineffectiveness of actin as an activator of S1 ϵ-ATPase (Rosenfeld & Taylor, 1984): with ATP actin accelerates k_4 and k_6 but probably not $k_3 + k_{-3}$.

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