Monitoring the Myosin ATPase Reaction Using a Sensitive Fluorescent Probe: Pyrene-Labeled ATP

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ABSTRACT A pyrene-labeled ATP (Pyr-ATP) in which a pyrene fluorophore is linked to the ribose moiety of ATP with a butyryl chain has been synthesized, together with the corresponding analog of ADP. The spectroscopic properties of two fluorescent analogs were found to be similar to those of 1-pyrenebutyric acid, making them photostable and highly sensitive probes for detecting changes in conformations around the nucleotide binding sites of proteins. Binding of Pyr-ADP to myosin subfragment-1 (S-1) resulted in a fluorescence quenching of about 70%. This binding was tight, with a dissociation constant (0.9 μM) similar to that of ADP itself. Formation of the stable ternary complex of Pyr-ADP with S-1 and orthovanadate could be monitored from the quench in pyrene fluorescence with a rate constant of 0.01 s^{-1}. The final fluorescence intensity was about 20% of that for Pyr-ADP alone. Pyr-ATP was hydrolyzed by S-1 1.3 times faster than was ATP. Hydrolysis of Pyr-ATP was accompanied by an initial quenching of pyrene fluorescence with a subsequent recovery of the fluorescence. The fluorescence changes could be used to monitor the hydrolysis reaction continuously and measure the turnover rates of the analog. The fluorescence assay was sensitive, particularly under single turnover conditions, allowing hydrolysis reactions to be monitored at concentrations of S-1 and the analog as low as 50 nM.

INTRODUCTION

The last few years have seen an exponential increase of research in the kinetics of the ATPase reactions of motor proteins, including myosins (Anson et al., 1995; Conibear et al., 1996; Franks-Skiba and Cooke, 1995; Funatsu et al., 1995; Ritchie et al., 1993; Rosenfeld et al., 1994; Sowerby et al., 1993; Woodward et al., 1991, 1995), kinesin and its families (Lockhart and Cross, 1996; Ma and Taylor, 1995; Sadhu and Taylor, 1992; Shimizu et al., 1995), and dynein (Inaba et al., 1989), employing fluorescent analogs of ATP. The introduction of a fluorescent ATP analog that more closely resembles ATP and has fluorescence signals that can report nucleotide binding has greatly contributed to the study of motor proteins. The popularity of this approach is due to its sensitivity as well as the relative simplicity of basic methodology and instrumentation.

Many ATP-requiring enzymes tolerate chemical modifications at the ribose ring of the ATP molecule (Yount, 1975). With this in mind, we have synthesized fluorescent ATP analogs, derivatized at the ribose moiety with various fluorophores, 2′(3′)-*O*-(2,4,6-trinitrophenyl)-ATP (Hiratsuka, 1976; Hiratsuka and Uchida, 1973), 2′(3′)-*O*-anthraniloyl-ATP, and 2′(3′)-*O*-(*N*-methylanthraniloyl)-ATP (mantATP) (Hiratsuka, 1983, 1984). MantATP and mantADP in particular are useful fluorescent nucleotide analogs. A relatively small molecular size of the fluorophore is a particular advantage, producing only minor perturbations in various biological systems. In most respects these analogs behave like ATP and ADP, respectively, not only for motor proteins, including myosin and its subfragments (Anson et al., 1995; Cremo et al., 1990; Franks-Skiba and Cooke, 1995; Hiratsuka, 1983, 1984; Ritchie et al., 1993; Rosenfeld et al., 1994; Woodward et al., 1991, 1995) and kinesin and its families (Lockhart and Cross, 1996; Ma and Taylor, 1995; Sadhu and Taylor, 1992; Shimizu et al., 1995), but also other enzymes (Hiratsuka, 1983).

Pyrene is a highly hydrophobic blue-emitting fluorophore with moderately high absorptivity and quantum yield (Haugland, 1992; Knopp and Weber, 1969; Weltman et al., 1973). However, the fluorophore has a high photostability and a high sensitivity to environmental factors (Haugland, 1992; Kierzek et al., 1993; Kouyama and Mihashi, 1981; Morrison et al., 1989). For example, modification of actin with pyrene led to a 25-fold fluorescence intensity increase upon polymerization of actin (Kouyama and Mihashi, 1981). The great advantage of the pyrene fluorophore is its extremely long fluorescence lifetime (Barrantes et al., 1975; Knopp and Weber, 1969; Weltman et al., 1973). Some of the physicochemical properties of large macromolecular entities, such as motor proteins, can be more conveniently studied with fluorophores having long excited-state life times (Barrantes et al., 1975; Haugland, 1992; Knopp and Weber, 1969; Weltman et al., 1973).

I report here that a pyrene-labeled ATP (Pyr-ATP) (Fig. 1), in which pyrene (2′(3′)-*O*-(1-pyrenebutyryl) in derivatives of ATP and ADP) is coupled with a butyryl linker to the ribose moiety of ATP, has favorable characteristics for studies of nucleotide binding and kinetics of the myosin ATPase. In particular, the fluorescence changes that accompany hydrolysis of the ATP analog can be used to monitor the hydrolysis reaction continuously and measure its rates of single turnovers. The fluorescence assay is sensitive allow-

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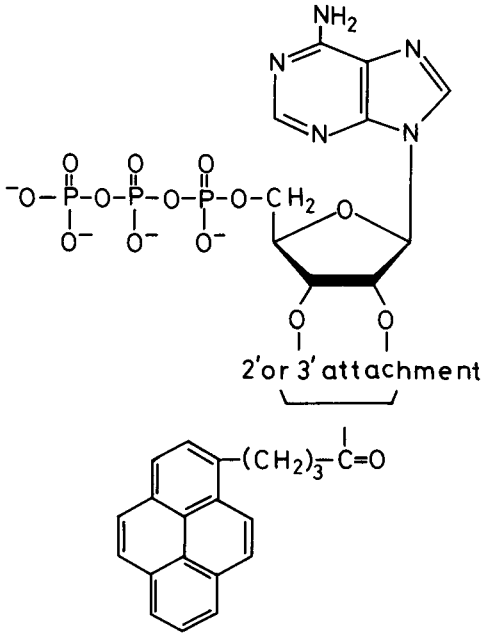

FIGURE 1 The proposed structure of Pyr-ATP.

ing the reactions to be monitored at concentrations of myosin subfragment 1 (S-1) and Pyr-ATP as low as 50 nM.

MATERIALS AND METHODS

Materials

ATP and ADP were purchased from Yamasa Shoyu Co. 1-Pyrenebutyric acid (PBA) was from Tokyo Kasei Kogyo Co. TLC plates (Silica gel 60 F-254) were from Merck Co. Other reagents were of reagent or biochemical research grade.

TLC

Thin-layer chromatography (TLC) was performed with 1-butanol:acetic acid:water (5:2:3, v/v/v) on silica gel. Compounds were detected on chromatograms under a Camag Deluxe UV lamp. The blue fluorescence of the pyrene derivatives on chromatograms under UV light allowed them to be distinguished from the dark-colored unreacted nucleotides.

Protein preparation

Rabbit skeletal myosin was prepared by the method of Perry (1955) with slight modifications. S-1 was prepared by chymotryptic digestion of myosin (Weeds and Taylor, 1975). S-1 was assumed to have a molecular weight of 120,000 (Weeds and Taylor, 1975). Concentrations of S-1 were determined from the extinction coefficient ($A^{1\%}_{1cm}$) at 280 nm of 7.5 (Wagner and Weeds, 1977).

Buffer systems

All experiments with S-1 samples were carried out at 25°C in buffer A (25 mM HEPES, pH 8.0, 30 mM KCl, and 2 mM $MgCl_2$). Absorption and fluorescence spectra of the fluorescent analogs in the absence of S-1 were measured in buffer B (50 mM Tris-HCl, pH 8.0) and buffer C (2 mM Tris-HCl, pH 8.0), respectively.

Spectral measurements

Absorption spectra were measured at room temperature with a Shimadzu spectrophotometer MPS-2000. Fluorescence emission spectra (uncorrected) were recorded at 25°C in a thermostated Hitachi fluorescence spectrophotometer (model F-4010). The slit widths on excitation and emission monochromators were 1.5 and 5 nm, respectively. Excitation wavelengths were 340 or 350 nm (pyrene fluorescence) and 290 nm (tryptophan fluorescence).

Ligand binding measurements

The dissociation constant and number of ADP analog-binding sites of S-1 were determined at 25°C as follows. The fluorescence of Pyr-ADP (1 μM) was measured at 400 nm in the presence and absence of S-1 at various concentrations (0.6–14 μM). The amount of bound analog was calculated from the decrease in fluorescence due to the binding of analog, and the dissociation constant and number of the binding sites were calculated by Scatchard analysis as described previously (Hiratsuka, 1990).

Kinetic measurements

For fluorescence measurements of turnover rates of Pyr-ATP under single and multiple turnover conditions, a solution containing the analog was preincubated in buffer A at 25°C, and the reaction was started by rapid mixing with an aliquot of S-1. The pyrene fluorescence was monitored at 400 nm upon excitation at 340 nm. For the measurements with ATP and dATP, S-1 was preincubated, and the reaction was started by mixing with the substrate. The tryptophan fluorescence was monitored at 340 nm upon excitation at 290 nm. Under single turnover conditions, single-exponential fit gave a reasonably good fit of the data in all cases. Under multiple turnover conditions, rate constants of turnover of Pyr-ATP were calculated from the fluorescence recovery curve according to the method of Chance (1957).

ATPase measurements

The Mg^{2+}-ATPase activities were measured in buffer A at 25°C in 0.1 mM ATP or its analog. Liberated P_i was determined by the method of Fiske and Subbarow (1925).

Synthesis of Pyr-ATP and Pyr-ADP

PBA (0.1 mmol) and *N,N'*-carbonyldiimidazole (0.3 mmol) were dissolved in 0.7 ml of *N,N*-dimethylformamide. The solution was incubated at 25°C for 30 min with continuous stirring. To this solution, ATP or ADP (0.05 mmol in 0.75 ml of water) was added, and the reaction mixture was stirred at 25°C for 48 h. The progress of reactions was followed by TLC. The reaction mixture was then left overnight in ice and centrifuged at 26,000 × *g* for 10 min. The supernatant was concentrated to about 0.3 ml at 25–30°C. The solution was chromatographed on a column of Sephadex LH-20 (1.7 × 34 cm) eluted with water (Hiratsuka, 1983; Hiratsuka and Uchida, 1973). Fractions of 0.6 ml were collected at a flow rate of 36 ml/h. A separation profile was obtained after assays by TLC; portions of each fraction (1–2 μl) were spotted on a silica gel plate. The R_F values were Pyr-ATP, 0.45; Pyr-ADP, 0.51; PBA, 0.86; ATP, 0.08; ADP, 0.10. Pyr-ATP and Pyr-ADP eluted after the unreacted nucleotides as well as the mant nucleotide analogs (Hiratsuka, 1983). Unreacted PBA eluted after the fluorescent nucleotide analogs. Peak fractions of the fluorescent analog were pooled and evaporated to dryness at 20°C. The residue was dissolved in 2–3 ml of water and finally clarified by filtration through a Millipore filter (0.65 μm). Analogs were stored frozen in aqueous solution (1–1.5 mM) at −20°C and showed no significant degradation over several months, as judged by TLC analysis. Concentrations of Pyr-ATP and Pyr-ADP were based on A_{342} = 42,000 M^{-1} cm^{-1} for the 1-pyrene derivatives (Knopp and Weber, 1969; Morrison et al., 1989; Somerharju et al., 1985; Weltman et al., 1973).

RESULTS AND DISCUSSION

Characterization

Purities of Pyr-ATP and Pyr-ADP were checked by TLC on silica gel. The analogs were chromatographically pure, as indicated by a single fluorescent spot, and free from starting materials.

The analogs are stable at neutral pH in solution and can be stored for at least several months at −20°C without significant degradation.

The proposed structure of Pyr-ATP is shown in Fig. 1. It should be noted that the product may be present as a mixture of 2′ and 3′ isomers similar to mantATP (Cremo et al., 1990). It has been established in the actomyosin systems that the 3′ isomer of the fluorescent analog of ATP in which the mant fluorophore (Woodward et al., 1991) or fluorescein (Conibear et al., 1996) is linked to the ribose moiety has kinetics similar to those of the 2′ isomer. For this reason, I have used preparations of pyrene-labeled nucleotides without further separation. The pyrene fluorophore is considerably bulkier than the mant fluorophore (Hiratsuka, 1983, 1984). Steric hindrance factors would be expected to be more prominent. To minimize perturbations of such a bulky fluorophore in the interactions with various ATP-requiring enzymes, including S-1, the strategy involved the placement of the fluorophore at an appropriate distance from the binding site of the parent ATP moiety, thereby making it possible to overcome constraints on the size of the attached fluorophore. Thus, Pyr-ATP in which the pyrene fluorophore was kept remote from the ATP moiety by an alkyl chain (—$CH_2CH_2CH_2$—) was designed and synthesized. Introduction of such a chain between the ribose moiety and the fluorophore resulted in increased affinity for S-1 (see below).

Absorption and fluorescent properties

Pyr-ATP exhibits absorption maxima at 264, 275, 300, 313, 326, and 342 nm (Fig. 2 *a*). The characteristic absorption bands of the pyrene moiety in the intermediate PBA (Knopp and Weber, 1969) are conserved in the final derivative Pyr-ATP.

Because the fluorescence of the pyrene fluorophore attached to actin was greatly changed upon polymerization of actin (Kouyama and Mihashi, 1981), it was of interest to study the fluorescent properties of Pyr-ATP in known environments. Upon excitation at 340 nm, Pyr-ATP fluoresces with emission maxima at 377 and 397 nm in aqueous solutions (Fig. 2 *b*). In the presence of 20% and 40% ethanol, the fluorescence intensity increases 1.4- and 1.6-fold, respectively, with a slight blue-shift in the emission maxima (1 nm). At a higher concentration of ethanol (80%), the fluorescence was quenched by 40% with a slight blue-shift of the emission maxima (2 nm). These results suggest that Pyr-ATP shows effects other than solvent polarity on the fluorescence intensities. Thus careful interpretation of changes in fluorescence intensities is required when Pyr-ATP is used as a fluorescent probe for the ATP-binding sites of various proteins.

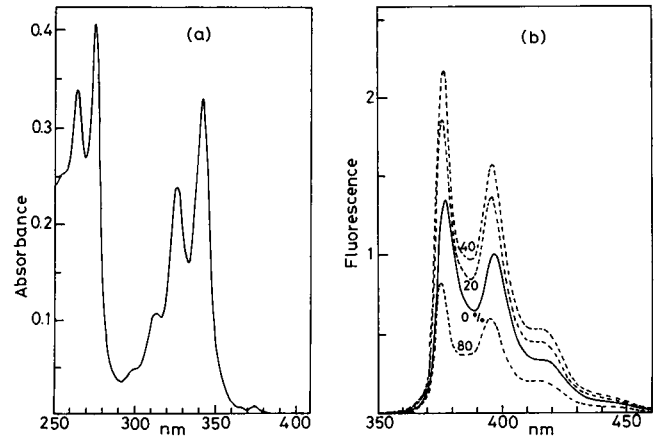

FIGURE 2 Absorption (*a*) and fluorescence emission (*b*) spectra of Pyr-ATP. The absorption spectrum of the analog (7.9 μM) was measured in buffer B. Fluorescence emission spectra were measured in buffer C in the presence (- - -) and absence (———) of ethanol. Samples (1 μM) were excited at 340 nm.

For Pyr-ADP, the absorption and fluorescent properties were essentially the same as those of Pyr-ATP (data not shown).

Interaction of Pyr-ADP with S-1

Although the shapes of the fluorescence spectra of Pyr-ADP were insensitive to the environment, the intensities of fluorescence were sensitive. When Pyr-ADP was bound to S-1, the fluorescence of Pyr-ADP was quenched to 28% of the original value (Fig. 3 *a*). At the same time, the emission maxima were blue-shifted by only 1 nm. This fluorescence quenching was fully reversed by the addition of a large excess of ATP, indicating the displacement of the ADP analog from the nucleotide-binding site of S-1. Furthermore, this result suggests the absence of a nonspecific binding of Pyr-ADP to S-1.

The fluorescence quenching of Pyr-ADP upon binding to S-1 enables us to determine the dissociation constant and the number of binding sites for the complex of the ADP analog with S-1. Fig. 3 *b* is a Scatchard plot depicting the binding data. The intercept and slope of this line gave a value of 1.0 binding site per S-1 with a dissociation constant of 0.9 μM. This value of the dissociation constant is similar to that (0.6 μM) obtained with ADP (Konrad and Goody, 1982).

Kouyama and Mihashi (1981) have used the pyrene fluorophore-labeled Cys-373 of actin as a fluorescent probe to detect conformational changes of actin. The fluorescence intensity of the pyrene-labeled G-actin was greatly enhanced to 25-fold upon polymerization of the actin. On the other hand, the fluorescence of pyrene-labeled F-actin was quenched to 25% of the original value upon binding to S-1.

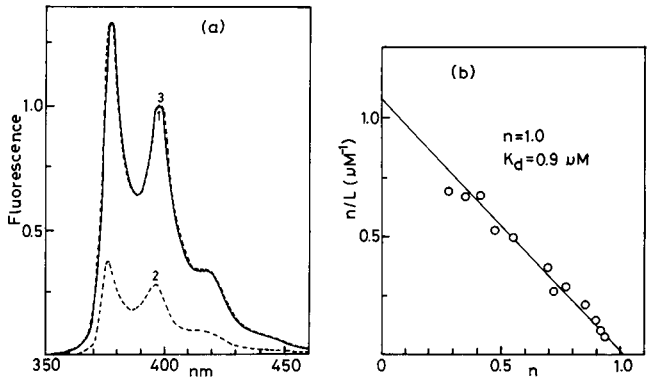

FIGURE 3 Changes in the fluorescence emission spectrum of Pyr-ADP induced by the addition of S-1 (*a*), and a Scatchard plot depicting the binding of Pyr-ADP to S-1 (*b*). (*a*) The fluorescence spectra were recorded in buffer A at 25°C. (*Curve 1*) 1 μM Pyr-ADP. (*Curve 2*) 1 μM Pyr-ADP and 14 μM S-1. The final intensity of fluorescence represents 28% of that of Pyr-ADP alone. (*Curve 3*) After completion of the binding of Pyr-ADP to S-1, ATP (0.8 mM) was added, and the spectrum was recorded again after 15 min. (*b*) Binding of Pyr-ADP to S-1 was measured from the fluorescence decrease and analyzed by a Scatchard plot, as described in Materials and Methods.

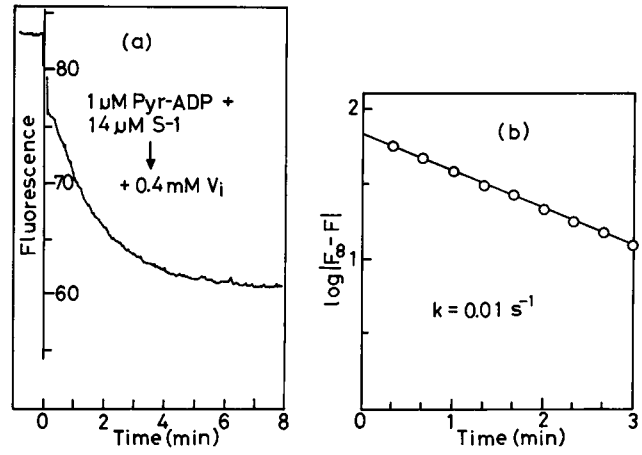

FIGURE 4 Formation of the ternary complex between Pyr-ADP · S-1 and V_i monitored by pyrene fluorescence (*a*) and its first-order plot (*b*). (*a*) Pyr-ADP (1 μM) was initially mixed with S-1 (14 μM) in buffer A at 25°C. After completion of the binding reaction, ternary complex formation was initiated by the addition of 0.4 mM V_i. The final intensity of fluorescence represents 20% of that of Pyr-ADP alone. (*b*) The rate constant was determined from a semilog plot of fluorescence changes against time. The data were taken from *a*.

This quenching extent is similar to that (28%) of Pyr-ADP bound to S-1 (Fig. 3 *a*). Quenching of pyrene fluorescence has also been observed when pyrene is covalently attached to the 5′ end of an oligoribonucleotide. It has been shown that pyrene fluorescence is quenched to 14% of the original value upon conjugation to the 5′ amino RNA oligomers (Kierzek et al., 1993). There may be a number of different factors influencing the fluorescence of pyrene (Kierzek et al., 1993; Kouyama and Mihashi, 1981; Morrison et al., 1989). Whatever these factors, however, present data together with the previous results (Kierzek et al., 1993; Kouyama and Mihashi, 1981; Morrison et al., 1989) clearly show that the pyrene fluorophore has a high sensitivity to environmental factors, making it a sensitive probe for detecting changes in conformations not only of RNA, but of proteins as well.

S-1 · Pyr-ADP · V_i complexes

Orthovanadate (V_i) acts as a phosphate analog that forms the stable trapped complex analogous to the M · ADP · P_i** state (where M denotes myosin or S-1) (Goodno and Taylor, 1982). Formation of the M · Pyr-ADP · V_i complex was monitored from the quench in pyrene fluorescence. In a typical experiment, 1 μM Pyr-ADP was added to 14 μM S-1 at pH 8.0. V_i (0.4 mM) was then added, and the fluorescence was monitored (Fig. 4 *a*). The time course fitted an exponential profile yielding a rate constant of 0.01 s^{-1} (Fig. 4 *b*). The final fluorescence intensity was about 20% of that for Pyr-ADP alone.

This rate constant was similar to that for trapping ADP (0.008 s^{-1}), which was obtained with spin-labeled myosin at pH 8.0 (Wells & Bagshaw, 1984), but fivefold slower than the corresponding value (0.05 s^{-1}) that was measured at pH 7.0 (Goodno and Taylor, 1982). Taking into account the fact that the rate of formation of the ternary complex of myosin with ADP and V_i is faster at lower pH (Goodno and Taylor, 1982), the present result suggests that Pyr-ADP forms the ternary complex with S-1 and V_i at a rate similar to that of ADP at pH 8.0.

Hydrolysis of Pyr-ATP by S-1

Before kinetic studies, hydrolysis of Pyr-ATP by S-1 was checked by TLC analysis. When the ATP analog (0.1 mM) was incubated with S-1 (0.1 μM) at room temperature overnight, the analog was fully hydrolyzed to produce Pyr-ADP, as indicated by the conversion of the fluorescent spot of Pyr-ATP (R_F = 0.45) to the spot corresponding to Pyr-ADP (R_F = 0.51) (data not shown).

The hydrolysis of Pyr-ATP by S-1 was also confirmed by the assay of P_i released (data not shown). The assays were carried out with S-1 (0.6 μM) in the presence of a large excess of the ATP analog (0.1 mM). Liberated P_i was determined by the method of Fiske and Subbarow (1925). For purposes of comparison, the assays were also carried out with ATP and dATP as substrates under the same conditions. The values of k_{cat} were Pyr-ATP, 0.073; ATP, 0.056; dATP, 0.087 s^{-1}. The hydrolysis rate of Pyr-ATP was 1.3 times higher than that of ATP and intermediate between those of ATP and dATP.

Fluorescence measurements of Pyr-ATP under multiple turnover conditions

Under multiple turnover conditions (i.e., [Pyr-ATP] > [S-1]), the addition of S-1 to Pyr-ATP results in an initial

quenching of pyrene fluorescence with a subsequent recovery of the fluorescence (Fig. 5). However, the extent of fluorescence changes was small. Typically, the minimum fluorescence observed corresponds to a quench of 2–3% of the fluorescence of Pyr-ATP alone. The pyrene fluorescence remained at a quenched level during the steady state but recovered when Pyr-ATP was exhausted. The time at which the recovery occurs was delayed with an increasing amount of Pyr-ATP added. The value of τ, the time required for the fluorescence to recover to one-half of its maximum amplitude, was 300 and 648 s upon the addition of 2 (Fig. 5 *a*) and 4 μM (Fig. 5 *b*) Pyr-ATP, respectively.

Because the ATPase reaction catalyzed by myosin obeys the simple Michaelis-Menten relationship and a sufficiently higher concentration of Pyr-ATP is used in these measurements, a rate constant of turnover of Pyr-ATP can be calculated from the fluorescence recovery curve according to the method of Chance (1957). The calculated values of the rate constant were 0.07 and 0.06 s^{-1}, respectively, when 2 and 4 μM Pyr-ATP were used. These values were similar to the value of k_{cat} of Pyr-ATP by S-1 (0.073 s^{-1}), which was determined from the P_i assays.

Monitoring slow processes such as the formation of the stable ternary complex of S-1 · Pyr-ADP · V_i (Fig. 4) and the hydrolysis reaction of Pyr-ATP by S-1 under multiple turnover conditions (Fig. 5) requires stability against photodegradation to allow data to be collected for long times. The single exponential characters of the curve (Fig. 4) indicate that the pyrene fluorophore is stable for more than 3 min. Indeed, control experiments in the absence of protein indicate that virtually no photobleaching of the pyrene occurred over the 40-min period upon excitation at 340 nm. Thus the properties with negligible photodegradation also make the pyrene-labeled nucleotides ideal for monitoring slow processes concerning the binding and hydrolysis of nucleotides.

Fluorescence measurements of Pyr-ATP under single turnover conditions

Under multiple turnover conditions (Fig. 5), only a limited molar excess of Pyr-ATP (≦40-fold) can be used to detect changes in fluorescence. Otherwise, the observed quench in fluorescence becomes negligible relative to the background signal. Thus the changes in fluorescence accompanying the formation of the intermediate are better studied under single turnover conditions (i.e., [S-1] ≧ [Pyr-ATP]) than multiple turnover conditions, so as to minimize the contribution from free Pyr-ATP. Single turnover experiments were then carried out to measure the turnover rates more accurately.

The addition of S-1 (50 nM) to an equal amount of Pyr-ATP results in an initial quench of pyrene fluorescence with a subsequent recovery of fluorescence (Fig. 6 *a*), similar to results under multiple turnover conditions (Fig. 5). However, the relative amplitude of the quenching phase is greater, and hence the initial state is quenched by as much as 25–30%. The recovery phase in the fluorescence change of Pyr-ATP was fitted to a single exponential curve with a rate constant of 0.082 s^{-1} (Fig. 6, *inset*). This fluorescence profile was similar to that observed at higher concentrations (0.1 μM) of S-1 and Pyr-ATP (Fig. 6 *b*), reflecting a single turnover of the ATP analog. In this case, a rate constant of

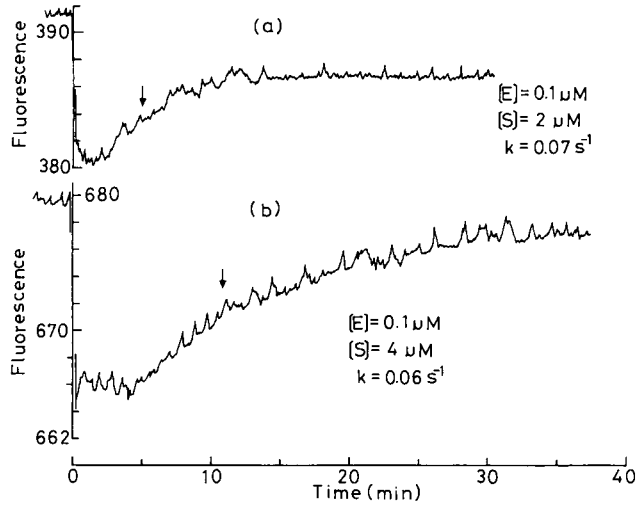

FIGURE 5 Time course of fluorescence changes, showing the hydrolysis of Pyr-ATP by S-1 under multiple turnover conditions. The measurements were performed in buffer A at 25°C. (*a*) 0.1 μM S-1 and 2 μM Pyr-ATP. (*b*) 0.1 μM S-1 and 4 μM Pyr-ATP. The values of τ (*arrows*), the time required for the fluorescence to recover to one-half of its maximum amplitude, is 300 s (*a*) and 648 s (*b*). The rate constants of turnover of Pyr-ATP were calculated from the fluorescence recovery curves according to the method of Chance (1957).

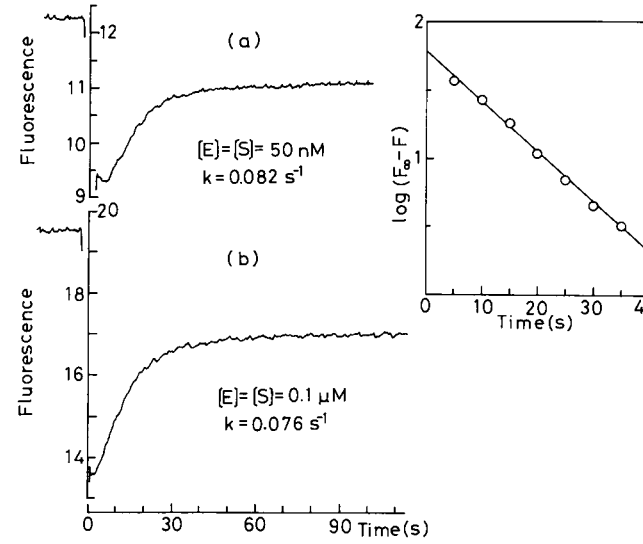

FIGURE 6 Fluorescence transients observed upon the addition of S-1 to Pyr-ATP under single turnover conditions. A solution containing Pyr-ATP was preincubated at 25°C in buffer A, and the reaction was started by rapid mixing with an aliquot of S-1. (*a*) 50 nM Pyr-ATP and 50 nM S-1. (*b*) 0.1 μM Pyr-ATP and 0.1 μM S-1. The inset shows a semilog plot of fluorescence changes in a recovery phase against time, where the data are taken from *a*.

single turnover was determined to be 0.076 s^{-1}. These rate constants obtained from fluorescence measurements were similar to the value of k_{cat} for Pyr-ATP (0.073 s^{-1}), suggesting that the K_m for Pyr-ATP is not more than 50 nM.

The fluorescence value in a final state was quenched by about 10–15% in both cases, indicating that a significant amount of the resultant Pyr-ADP remained bound to S-1. These fluorescence values were fully reversed upon the addition of a large excess of ATP (40 μM), suggesting a displacement of Pyr-ADP from S-1 by ATP. On the other hand, the addition of Pyr-ADP caused only fluorescence changes that were observed in final states with Pyr-ATP, without a recovery phase (data not shown).

These experiments indicate that Pyr-ATP is also useful for probing rapid processes of the hydrolysis reaction. Probing of rapid processes of the reaction by a fluorescent ATP analog requires sufficient fluorescence intensity to provide adequate signal-to-noise over a wide range of concentrations. The results in Fig. 6 demonstrate that hydrolysis reactions under single turnover conditions can be monitored with Pyr-ATP and S-1 at concentrations as low as 50 nM. These results, together with those in Figs. 4 and 5, indicate that the pyrene-labeled nucleotides are useful for probing both rapid and slow processes of the interactions between nucleotide and protein.

Comparison of turnover rates of Pyr-ATP determined from P_i assays and fluorescence measurements

The above results indicate that the turnover rates of Pyr-ATP can be measured directly from changes in pyrene fluorescence. To confirm this point further, turnover rate constants obtained from fluorescence measurements were compared with those obtained from P_i assays (Table 1).

TABLE 1 Turnover rate constants for the hydrolysis of ATP and its analogs by S-1 ATPase*

	ATP (s^{-1})	dATP (s^{-1})	Pyr-ATP (s^{-1})
P_i assay*	0.056	0.087	0.073
Fluorescence assay§			
A	ND	ND	0.082 ± 0.002
B	0.058 ± 0.003	0.083 ± 0.003	0.076 ± 0.005
C	ND	ND	0.072 ± 0.003

*Measurements were performed in buffer A at 25°C. Other conditions are described in Materials and Methods.

#P_i assays were carried out in the presence of 0.1 mM substrate and 0.6 μM S-1. Liberated P_i was determined by the method of Fiske and Subbarow (1925).

§Fluorescence assays were performed under single turnover conditions using tryptophan fluorescence (ATP and dATP) and the pyrene fluorescence (Pyr-ATP) as described in Materials and Methods. A, [S-1] = [substrate] = 50 nM; B, [S-1] = [substrate] = 0.1 μM; C, [S-1] = 0.2 μM, [substrate] = 0.1 μM. Results are the mean ± SD of three to five traces. ND, value not determined.

As shown in Table 1, not only the rate constants of ATP and dATP, which are determined from changes in tryptophan fluorescence, but also those of Pyr-ATP, which are determined from changes in pyrene fluorescence, are essentially identical to those obtained from P_i assays.

In conclusion, the properties of Pyr-ATP suggest its applicability to structural and kinetic studies of ATP-requiring proteins that exploit the sensitivity and versatility of fluorescence techniques. The results presented here clearly show that the use of Pyr-ATP as a substitute for ATP enables us to follow the myosin ATPase reactions directly from changes in pyrene fluorescence. This new fluorescent analog of ATP should greatly facilitate further studies of the ATPase mechanisms of various motor proteins, including myosin.

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