Fluorescent Coumarin-Labeled Nucleotides to Measure ADP Release from Actomyosin

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ABSTRACT Several coumarin-labeled nucleotides have been synthesized, based on 2′(3′)-*O*-(2-aminoethyl)carbamoyl-ATP (edaATP). The fluorescent coumarins coupled with the free amino group are 7-diethylaminocoumarin-3-carboxylic acid (to give deac-edaATP), coumarin 343 (but-edaATP) and 7-ethylamino-8-bromocoumarin-3-carboxylic acid (mbc-edaATP). The carbamoyl linkage of these nucleotide analogs undergoes interconversion between 2′- and 3′-hydroxyl attachment very slowly, so that the 2′- and 3′-isomers were separated and stored with minimal equilibration. 3′-Deac-edaADP had fluorescence excitation and emission maxima at 430 nm and 477 nm, with a fluorescence quantum yield of 0.012. The equivalent data for 3′-but-edaADP are 445 nm, 494 nm, and 0.51, respectively, and for 3′-mbc-edaADP, 405 nm, 464 nm, and 0.62. The interaction with skeletal myosin subfragment 1 was measured in the absence and presence of actin. In each case the fluorescence was decreased when bound to subfragment 1, 3-fold for 3′-deac-edaADP, 7-fold for 3′-but-edaADP, and 11-fold for 3′-mbc-edaADP. Steady-state ATPase measurements and the kinetics of binding and release of nucleotides were similar to those reported for the natural nucleotide. Large fluorescence changes could be observed for the release of these analogs from actomyosin subfragment 1, enabling a direct measurement of the kinetics of this process. In the case of 3′-deac-edaADP a rate constant of 474 s^{-1} was measured (at pH 7.0, 20°C, and low ionic strength).

INTRODUCTION

The interconversion of purine nucleoside triphosphate (NTP) and diphosphate is a widespread reaction in biology and is mediated by a range of enzymes; examples include mitochondrial ATP synthase, kinases, and nucleoside triphosphatases such as actomyosin and signaling G proteins. In many triphosphatases, the energy of NTP hydrolysis is transduced via protein conformation changes to enable other functions of the system; e.g., actomyosin ATP hydrolysis couples with contraction. To understand such coupling or the enzymology of NTP-handling proteins, the kinetics of elementary processes (substrate binding, cleavage chemistry, conformation changes, and product release steps) need to be measured, often with time resolution in the millisecond range. Spectroscopic probes are useful for this purpose, and nucleotides specifically labeled with a fluorophore can give signals that relate directly to binding and release. In the case of actomyosin in muscle, product release steps are closely related to force generation and contraction (reviewed by Hibberd and Trentham, 1986), and hence these steps are particularly important targets for measurement. Here we describe the synthesis and characterization of novel coumarin-labeled nucleotides and their application to the actomyosin system in solution.

Many proteins that bind NTP and/or NDP are able to accommodate a variety of groups attached to the 2′- or 3′-position on the ribose with almost no change in affinity (for example, Neal et al., 1990). Such proteins include NTPases with the P-loop structure at the catalytic site but exclude many kinases that have high specificity with respect to ribose modification. The lack of specificity at these ribose positions has been rationalized from the crystal structures of several NTPases, in which the hydroxyl groups at these positions are exposed on the surface (Smith and Rayment, 1996). So groups attached to these hydroxyls are external to the binding site. In contrast, even small changes elsewhere in the nucleotide, such as to the base, can greatly alter the interaction with the protein binding site. This has been exploited using such ribose-modified analogs as 2′(3′)-*O*-(*N*-methylanthraniloyl)NTP (mantNTP) to study ATPases and GTPases. This mant fluorophore produces a large fluorescence change on binding (Hiratsuka, 1983; Eccleston et al., 1989). MantATP and mantADP have been used with actomyosin in solution and in muscle fibers. Some of the processes detected by light scattering in solution did not show a concomitant mant fluorescence change (Woodward et al., 1991). In muscle fibers the fluorescence change attributed to dissociation of mantADP from the ATPase sites was very small (Ferenczi et al., 1989).

A free amino group linked via the ribose ring provides a way to attach a variety of different groups to the nucleotide, and the approach chosen here is to use 2′(3′)-*O*-[*N*-(2-aminoethyl)carbamoyl]ATP (edaATP) (Hazlett et al., 1993), synthesized by methods based on the those of Cremo et al. (1990). Data are presented for three coumarin carboxylates attached by this route. The 7-diethylaminocoumarin-3-carboxylic acid gives deac-edaATP (Fig. 1) and is the same fluorophore that was used successfully to produce a sensor for inorganic phosphate (P_i) based on a phosphate-binding protein (Brune et al., 1994). This coumarin has a low fluorescence quantum yield in aqueous solution. The second label is one of the butterfly coumarins (Coumarin

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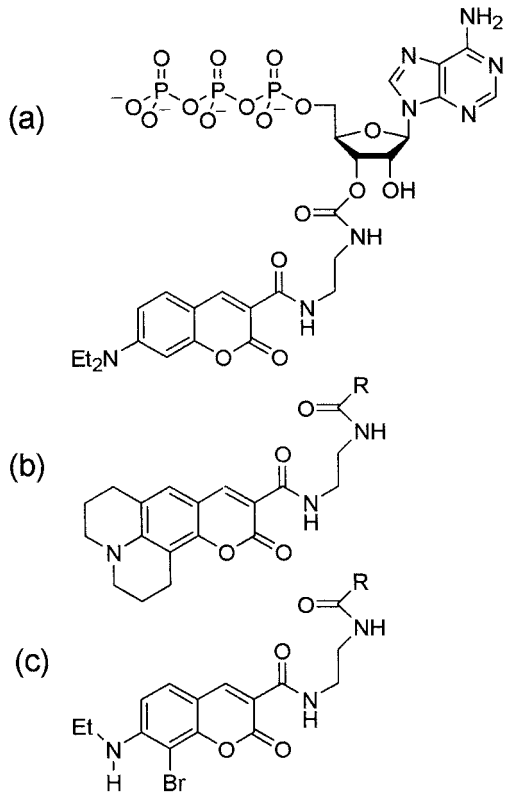

FIGURE 1 Structures of coumarin-labeled ATP analogs. (*a*) 3′-Deac-edaATP (3′-*O*-{*N*-[2-(7-diethylaminocoumarin-3-carboxamido)ethyl] carbamoyl}ATP); (*b*) 3′-But-edaATP (3′-coumarin 343-edaATP); (*c*) 3′-Mbc-edaATP (3′-*O*-{*N*-[2-(7-ethylamino-8-bromocoumarin-3-carboxamido)ethyl]carbamoyl}ATP), where R represents ATP with attachment to the ribose as in *a*.

343, 2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-[1]benzopyrano[6,7,8-*ij*]quinolizine-10-carboxylic acid) (to give but-edaATP), in which rotation of the amino group is constrained so that the nitrogen lone pair interacts maximally with the aromatic rings (Chaurasia and Kauffman, 1990). This interaction results in a much higher quantum yield in aqueous solution than the diethylaminocoumarin, but the substituent is more bulky and hydrophobic. The third, 7-ethylamino-8-bromocoumarin-3-carboxylic acid was used to prepare mbc-edaATP and has a single ethyl substituent on the amine and a bromine on the coumarin (Corrie et al., 2000). This also has a high fluorescence quantum yield in aqueous solution.

The linkage of ribose-modified nucleotides such as edaNTP and mantNTP undergoes base-catalyzed exchange between 2′- and 3′-hydroxyl positions on the ribose. The two isomers are chemically distinct and may interact differently with proteins with respect to affinities, fluorescence properties, etc. In the case of mantATP this exchange is too fast to be able to isolate and use each isomer: for mantGDP the half-time is 7 min at pH 7.4 and 20°C (Eccleston et al., 1991) and 8 min at pH 7.0 and 37°C (Rensland et al., 1991). The 2′-deoxy-3′-mant derivatives have been used to circumvent this problem, as no such interconversion is possible. As will be shown for these coumarin nucleotides and has been reported previously for another derivatized edaATP (Oiwa et al., 2000), the isomers have very low interconversion rates at neutral pH, and so the individual isomers can be isolated and used without significant re-equilibration. Except where indicated, measurements were done with the 3′-isomers at 20°C.

Data on the interaction with skeletal actomyosin subfragment 1 (acto-S1) are given using each nucleotide. However, deac-edaATP and -edaADP have better physiological properties with skeletal fibers than the but-edaATP and -edaADP (S. Millar, M. A. Ferenczi, and M. R. Webb, unpublished result), and so more emphasis here is given to deac-edaATP. Mbc-edaATP has not yet been fully characterized in fibers.

MATERIALS AND METHODS

Protein preparation

Skeletal myosin subfragment 1 (S1) was prepared by chymotryptic digestion of rabbit skeletal myosin (Weeds and Taylor, 1975) and stored in liquid nitrogen at ~1 mM. Concentrations were calculated on the basis of a molecular weight of 115,000 and $E^{1\%}(280\ \text{nm}) = 7.9\ \text{cm}^{-1}$. F-actin from rabbit skeletal muscle was prepared essentially as described (Lehrer and Kerwar, 1972). Concentrations were measured from the absorbance spectra assuming $E^{1\%}(290\ \text{nm}) - E^{1\%}(310\ \text{nm}) = 6.2\ \text{cm}^{-1}$. Smooth myosin subfragment 1 was prepared from chicken gizzards using *Staphylococcus aureus* protease (Ikebe and Hartshorne, 1985). Concentrations were calculated on the basis of a molecular weight of 130,000 and $E^{1\%}(280\ \text{nm}) = 7.5\ \text{cm}^{-1}$.

Synthesis of coumarin-labeled nucleotides

The nucleotides were synthesized from edaATP (Hazlett et al., 1993), which was prepared using methods based on those of Cremo et al. (1990). ATP (Sigma Chemical Co., St. Louis MO; 500 μmol) was converted to the tributylammonium salt by passing a solution through a Dowex-50 column in the free acid form. The eluate was mixed with tributylamine (0.48 ml, 2 mmol). After rotary evaporation, several batches of dimethylformamide (dried over Molecular Sieves 4A, Merck, Poole, UK) were evaporated from the residue, which was then dissolved in dry dimethylformamide. 1,1′-Carbonyldiimidazole (Aldrich, Milwaukee, WI; 420 mg, 2.5 mmol) was added to the anhydrous solution, which was stirred in a sealed vessel overnight at 5°C. Excess carbonyldiimidazole was removed by adding methanol (144 μl, 3.6 mmol) and leaving for 30 min, also at 5°C. A threefold excess of 1,2-ethylenediamine (81 μl, 1.5 mmol) was added while the solution was stirred rapidly. A white precipitate formed immediately, and the solution was stirred for 3 h at 5°C. Water (80 ml) was added.

This coupling produces a modification to the terminal phosphate, which can be removed by an acid treatment (Maeda et al., 1977). There is also a modification, presumably on the purine, that is removed by base treatment: the rate of this base-catalyzed hydrolysis is slower for guanine nucleotides than adenine (M. R. Webb, unpublished result). The clear solution was first taken to pH 2.5 using concentrated HCl and stirred for ~20 h at 5°C with occasional adjustment of pH. The progress of reaction was checked by high-performance liquid chromatography (HPLC) using a Partisil SAX column (Whatman, 0.4 cm × 25 cm) with mobile phase 0.4 M $(NH_4)_2HPO_4$, adjusted to pH 4.0 with concentrated HCl, running at 2 ml min^{-1}. The acid treatment took the main peak from 2.5 to 7 min, similar

to that for ATP itself. The solution was then raised to pH 9.0 using 5 M NaOH and stirred at 20°C for 90 min. The progress of reaction was followed by HPLC as above, with the formation of two overlapping peaks centered at 5.5 min, corresponding to 2′- and 3′-edaATP. The edaATP was purified at 5°C on a DEAE-cellulose column (200 ml) pre-equilibrated in 10 mM triethylammonium bicarbonate, pH 7.6. The product was eluted by a linear gradient of this buffer from 10–450 mM (1.5 L). Eluted material was analyzed by HPLC, and fractions were pooled that contained pure edaATP (mixed isomers, eluted at 240 mM bicarbonate) in ~50% yield from ATP. The isomers were not well separated by this procedure. The buffer was removed by repeated rotary evaporation of methanol and edaATP was stored at −20°C as a concentrated solution at pH 7.0.

To prepare deac-edaATP, 7-diethylaminocoumarin-3-carboxylic acid (Corrie, 1994) was first activated using isobutyl chloroformate (Corrie, 1994). The coumarin (46 mg, 200 μmol) was dissolved in dry dimethylformamide (1 ml) with tributylamine (70 μl, 290 μmol) and isobutyl chloroformate (Aldrich; 28 μl, 215 μmol). This was left on ice for 50 min, and then edaATP in aqueous solution (1 ml, 100 μmol) was added. More dimethylformamide (0.8 ml) was added to obtain a clear solution, and this was left for 2 h at 20°C. Deac-edaATP was purified on DEAE-cellulose, as above, but using a gradient up to 500 mM. Fractions were analyzed by HPLC on a Partisphere SAX column (Whatman, 12.5 cm × 0.45 cm). The mobile phase at 1.5 ml min^{-1} was 0.35 M $(NH_4)_2HPO_4$, adjusted to pH 4.0 with HCl (75% v/v), with methanol (25%). The 2′-isomer eluted at 8.3 min, the 3′-isomer at 7.2 min. The order of elution of isomers is reversed relative to DEAE-cellulose, from which the 2′-isomer elutes at 340 mM bicarbonate and the 3′-isomer at 380 mM. Fractions containing pure 2′- and 3′-isomers were concentrated as above and stored at −80°C. The total yield of deac-edaATP from edaATP was 52%, with the pure 3′-isomer yield 23%, the remainder being 2′- and mixed isomers.

The butterfly coumarin carboxylic acid, Coumarin 343 (Acros, Loughborough, UK) was used to prepare 3′-but-edaATP (Fig. 1) by the same method, except for the following. Because of much lower solubility, the coumarin (25 mg, 73 μmol) was dissolved in dry dimethylformamide (2 ml) with tributylamine (35 μl, 145 μmol) and isobutyl chloroformate (14 μl, 108 μmol). Elution from the DEAE-cellulose required a gradient of up to 750 mM triethylammonium bicarbonate. The 2′-isomer of but-edaATP, etc. was not isolated in a pure form, due to a co-eluting impurity. For HPLC on the Partisphere SAX column the phosphate concentration was increased to 0.5 M; otherwise the conditions were the same. The 3′-isomer elutes at 4.5 min, and the 2′-isomer elutes as an asymmetric peak, with the maximum varying between 5.5 and 6.5 min depending on loading. The 3′-isomer was obtained pure in 12% yield from edaATP.

The 2′- and 3′- isomers of mbc-edaATP were also prepared by the route as for deac-edaATP from the equivalent carboxylic acid (Corrie et al., 2000), but with the following differences. The coumarin (20 mg, 62 μmol) was dissolved in dry dimethylformamide (1.4 ml) with tributylamine (30 μl, 124 μmol) and isobutyl chloroformate (14 μl, 108 μmol), and 70 μmol of eda-ATP was used. After purification on DEAE-cellulose using a gradient up to 700 mM triethylammonium bicarbonate, the mbc-edaATP was obtained in 54% yield. The 2′- and 3′-isomers were obtained in separate pure fractions with the 3′-isomer in 29% yield. HPLC as for deac-edaATP was used to analyze the mbc-edaATP. The 3′-isomer elutes at 12.4 min, and the 2′-isomer elutes as an asymmetric peak, with the maximum varying between 13.5 and 16.5 min depending on loading.

The equivalent diphosphate of each analog was prepared from the parent diphosphate or by S1-catalyzed hydrolysis of the triphosphate. During chemical synthesis of edaADP, the unwanted modification to the phosphate is much more resistant to the acid treatment than is the case with edaATP. Therefore, 16 h at pH 2.0 and 20°C was used.

Steady-state ATPase activity

The extent of hydrolysis was measured by HPLC analysis, as described above, of aliquots removed at specific times from a solution containing 10 mM PIPES, pH 7.0, 1 mM $MgCl_2$, 1 mM ATP or analog, with sufficient S1 or acto-S1 for complete hydrolysis in ~40 min; see text for details.

Other measurements

Absorbance spectra were obtained on a Beckman DU460 spectrophotometer. Concentrations of nucleotide were determined from the coumarin absorbance as well as the absorbance of adenosine at 260 nm after correction for absorbance due to the coumarin. The two measurements generally were within 10% of each other. The coumarin extinction coefficients were those obtained for the parent coumarins carboxylic acids as simple esters or amides. For deac these values are 46,800 M^{-1} cm^{-1} at 430 nm and 12,800 M^{-1} cm^{-1} at 260 nm (Corrie, 1994); for mbc, 34,500 M^{-1} cm^{-1} at 410 nm and 10,300 M^{-1} cm^{-1} at 260 nm (Corrie et al., 2000); for but, 45,000 M^{-1} cm^{-1} at 436 nm and 7660 M^{-1} cm^{-1} at 260 nm (Fletcher and Bliss, 1978). The coumarin maximum wavelength shifts depending on chemical environment, but it is assumed that the extinction coefficient is unaltered.

Fluorescence measurements were obtained on a Perkin-Elmer LS50B fluorimeter with xenon lamp. Stopped-flow experiments were carried out in a HiTech SF61MX apparatus, with a mercury lamp and HiTech IS-2 software. There was a monochromator and 5-nm slits on the excitation light (436 nm) and a 455-nm cutoff filter on the emission. All measurements were in 10 mM PIPES, pH 7.0, 1 mM $MgCl_2$ at 20°C, unless otherwise stated. Data were fitted using the HiTech software or Grafit (Leatherbarrow, 1992).

NMR data were collected at 500 MHz as described (Oiwa et al., 2000), using sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal reference. The nucleotide as the triethylammonium salt at pH 7.0 was lyophilized and redissolved in D_2O. Selective total correlation spectroscopy measurements were used to confirm assignments in the isomer mixture by establishing connectivity between ribose protons (Xu and Evans, 1996).

RESULTS

The syntheses of the three coumarin-labeled nucleotides via edaATP resulted in a mixture of 2′- and 3′-isomers in similar ratios (~40:60) in each case. These could be isolated as single isomers as they separate from each other on ion-exchange chromatography. The presence of a single isomer is an important factor when interaction with proteins is considered; it is shown below that the 2′- and 3′-isomers interact differently with myosin subfragment 1. But-edaGDP mbc-edaGDP, and deac-edaGDP bind tightly to the small G protein rac but only as the 3′-isomer; the 2′-isomers do not bind when rac is presented with the equilibrium mixtures of isomers (A. Shutes, A. R. Newcombe, and M. R. Webb, unpublished result).

The identity of the 2′- and 3′-isomers of deac-edaATP was determined by ^{1}H-NMR by comparison with *N*-acetyl-edaATP (Cremo et al., 1990; Oiwa et al., 2000). The 2′-isomer has ribose proton chemical shifts centering on 6.04 (H1′), 5.60 (H2′), 4.65 (H3′), and 4.28 (H4′). The shifts for the 3′-isomer are 5.77 (H1′), 4.82 (H2′), 5.26 (H3′), and 4.45 (H4′). The ribose linkage of other coumarin nucleotide isomers was inferred by analogy. All the nucleotides show similar chromatographic behavior with the reversal of elution order between two ion-exchange chromatography media, DEAE-cellulose (2′-isomer first), and Partisphere SAX (3′ first).

The interconversion rate between isomers was determined for deac-edaATP by following the progress toward equilibration of each isomer by HPLC. At pH 8.0 (100 mM Tris/HCl) and 22°C, only ~10% conversion was observed over 24 h for 2′ to 3′ or vice versa, equivalent to a rate constant (for forward plus reverse reactions) of 0.004 h^{-1}. At pH 7.1 (100 mM PIPES) there was 1.5% conversion. These rates are considerably slower than were reported for *N*-acetyl-edaATP, 0.24 h^{-1} at pH 8.4 and 22°C (Oiwa et al., 2000). Currently there is no explanation for this difference, but the group attached to the eda moiety possibly has a role in facilitating or inhibiting the interconversion. Thus, it was possible to purify the separate isomers of the coumarin nucleotides by chromatography at pH 7.5 and 5°C and then remove the volatile buffer triethylammonium bicarbonate under vacuum, with minimal equilibration of isomers. In practice, 2′-but-edaATP and other 2′-nucleotides with this coumarin were contaminated with a co-eluting coumarin species. Because the 3′-isomers seem to be of greater use, the further purification of these 2′-isomers of butterfly coumarin nucleotides was not pursued. Except where indicated, all measurements are with the 3′-isomers.

Steady-state fluorescence properties

Fluorescence properties of the three pure coumarin-labeled ADPs were measured in the presence and absence of skeletal S1. Emission spectra are shown in Fig. 2. The deac-edaADP has a much lower fluorescence intensity than but-edaADP or mbc-edaADP, and this is reflected in the fluorescence quantum yields (Table 1). The quantum yield is greater in each case when the nucleotide is free in solution rather than bound to S1: <2-fold for 2′-deac-edaADP, 3-fold for 3′-deac-edaADP, 7-fold for 3′-but-edaADP, and 11-fold for 3′-mbc-edaADP.

Kinetics of interaction with skeletal S1

Steady-state ATPase measurements showed that the nucleotide analogs had similar k_{cat} values to ATP (Table 2). The deac 2′-isomer also had a similar value. The binding and release kinetics for the analogs with S1 were measured using stopped-flow fluorimetry. When 3′-deac-edaADP is displaced from its complex with S1 by a large excess of ATP in a stopped-flow apparatus, there is an increase in fluorescence (Fig. 3) with a rate constant (for nucleotide dissociation) of 1.3 s^{-1} at 20°C. A similar value was found for the 2′-isomer (Table 2). The same measurement with but-edaADP and mbc-edaADP gives 0.40 s^{-1} and 1.1 s^{-1}, respectively, but with a much larger signal change, as expected from the relative quantum yield changes. These rate constants are similar to those obtained for ADP or fluorescent nucleotides such as mantADP (Trentham et al., 1976; Woodward et al., 1991).

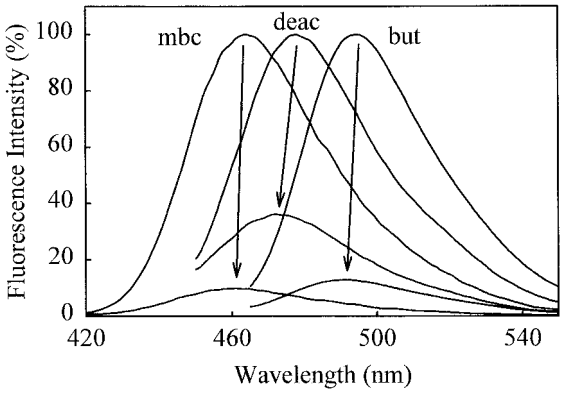

FIGURE 2 Fluorescence spectra of coumarin-labeled ADPs in absence and presence of skeletal myosin subfragment 1. The spectra are normalized so that for each nucleotide in the absence of S1 the intensity is set to 100%. Sufficient S1 was added to bind all nucleotides. The 1.8 μM deac-edaADP (deac) was excited at 430 nm and 14 μM S1 added; 1 μM but-edaADP (but) was excited at 450 nm and 4 μM S1 added; 1 μM mbc-edaADP (mbc) was excited at 408 nm and 10 μM S1 added.

Association kinetics were measured under pseudo-first-order conditions by mixing various concentrations of excess S1 with a low concentration of nucleotide or by varying excess nucleotide with low protein. As previously described for mantADP (Woodward et al., 1991), traces were biphasic with a small slow-phase component. The 2′-deac-edaADP and -ATP gave signals with a larger proportion of a slow phase than the 3′-isomers, and overall they gave smaller signals, so they are not described in detail here. The 3′-deac-edaADP and -edaATP gave similar rate constants to each other, and to the other 3′-isomers in the triphosphate state.

Measurements with 3′-isomers in excess over protein gave better fits to single exponentials. The observed rate constants increased over the range of concentrations examined, with indication that the rates become saturated at high

TABLE 1 Fluorescence properties of coumarin-labeled ADPs in absence and presence of skeletal myosin subfragment 1

	Quantum yield		Fluorescence	Excitation maximum (nm)	Emission maxima (nm)	
	−S1	+S1	(−S1/+S1)*	−S1	−S1	+S1
3′-Deac-edaADP	0.038	0.012	2.7	430	477	473
2′-Deac-edaADP	0.046	0.028	1.5	430	479	470
3′-But-edaADP	0.51	0.067	6.8	445	494	491
3′-Mbc-edaADP	0.62	0.056	10.5	405	464	461

Values were calculated relative to the fluorescence quantum yield of Coumarin 314 in ethanol, 0.83 (Fletcher and Bliss, 1978). For quantum yield determinations, emission spectra were obtained using solutions with absorbance <0.05 cm^{-1} and then corrected for the photomultiplier profile and baseline.

*Ratio of fluorescence intensities at the wavelength of maximum emission in the absence of S1.

TABLE 2 Rate constants determined for nucleotides with skeletal S1 and acto-S1 at 20°C

	S1			Acto-S1		
Coumarin	k_{cat}* (s^{-1})	k_{ass}† (M^{-1} s^{-1})	k_{diss} (s^{-1})	k_{cat}‡ (s^{-1})	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})
3′-Deac	0.069	4.1×10^6	1.3	13.3	4.5×10^6	474
2′-Deac	0.050		1.0	8.1		
3′-But	0.063	4.3×10^6	0.4	8.8	5.1×10^6	158
3′-Mbc	0.053	5.9×10^6	1.1	8.1		220

The association rate constant for the triphosphate (k_{ass}) and dissociation rate constants for the diphosphates (k_{diss}) were obtained as described for Figs. 3 and 4.

*The measurements were done concurrently with a similar measurement using ATP for which the k_{cat} was 0.071 s^{-1}. The 2′-deac- and 3′-mbc-analogs showed a marked decrease in rate above ~20% hydrolysis, possibly due to product inhibition.

†The rate constants were derived from the linear fit to data in Fig. 4 *b* at <8 μM nucleotide. Data were also obtained for 3′-deac-edaADP and gave a rate constant of 5.7×10^6 M^{-1} s^{-1}.

‡These measurements were done at 41 μM actin concurrently with a similar measurement using ATP for which the k_{cat} was 7.9 s^{-1}.

concentrations (Fig. 4). However, up to 8 μM the dependence is fairly linear, and this analysis gives an estimate of the second-order association rate constants (Table 2). Measurements were also done with 3′-deac-edaADP, and these were very similar to those with the triphosphates.

Kinetics of interaction with skeletal acto-S1

Steady-state ATPase activity was measured at a single concentration of analog and actin (Table 2). The actin-activated rates of the 3′-analogs are similar to that of ATP.

The equivalent association and dissociation measurements to those described above, but using acto-S1, are hampered by the much weaker binding of ADP to S1 when actin is present. Furthermore, the actin tends to cause a deterioration of the fluorescence signal, for example, by light scattering. However, fluorescence signals were observed for association and dissociation from acto-S1 for both nucleotide analogs.

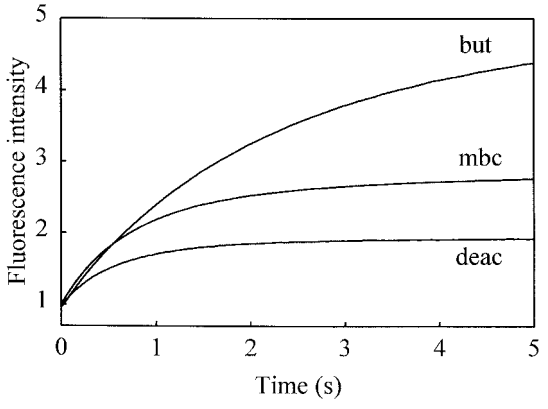

FIGURE 3 Displacement of coumarin-labeled ADPs from skeletal myosin subfragment 1 by ATP. 0.25 μM deac-edaADP (deac), 0.1 μM but-edaADP (but) or 0.5 μM mbc-edaADP (mbc) was pre-mixed with 2 μM S1 and then mixed with 50 μM ATP in a stopped-flow apparatus. (All concentrations are those in the mixing chamber.) The data are normalized for starting fluorescence.

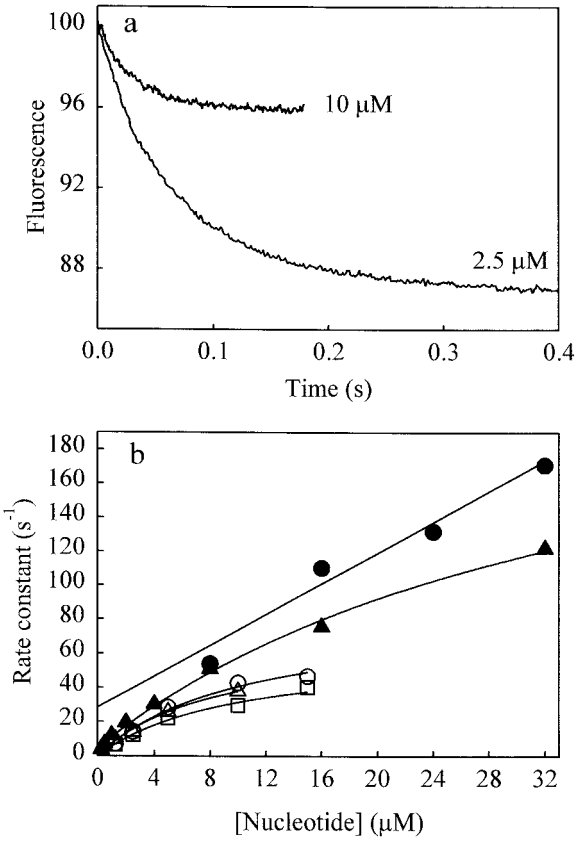

FIGURE 4 Binding kinetics for coumarin-labeled nucleotides to skeletal S1 and acto-S1. (*a*) 3′-Deac-edaADP (at concentrations shown) binding to 0.5 μM S1; (*b*) Dependence of observed rate constant for S1 binding on concentration of 3′-deac-edaADP (□), 3′-deac-edaATP (○), 3′-but-edaATP (△), 3′-mbc-edaATP (▲), and for the binding of 3′-deac-edaATP to actoS1 (●). The fluorescence binding curves were fitted to single exponentials, and each point is the average of at least three curves. For clarity the S1 data are shown fit to a two-step binding model but are analyzed (Table 2) in terms of a single-step binding (linear fit). For the S1 measurements, the nucleotide at various concentrations was mixed with S1 at a concentration that was varied so that it was maintained at 25% of nucleotide concentration. The acto-S1 measurements were performed similarly. The linear fit gives a slope of 4.5×10^6 ($\pm$ 0.5×10^6) M^{-1} s^{-1} and intercept of 28.4 ($\pm$ 11.6) s^{-1}.

Association rates were measured as described for S1 alone, but the scatter on data was greater. Results for 3′-deac-edaATP are in Fig. 4 and are fitted to a one-step binding model (linear fit). The slope gives the second-order binding constant whereas the intercept is a measure of the dissociation kinetics. Rate constants obtained from this treatment are in Table 2 for this nucleotide and 3′-but-edaATP.

The dissociation kinetics were measured by displacing 3′-deac-edaADP from its acto-S1 complex using a large excess of ATP at different concentrations of actin. At the highest concentration, a rate constant of 474 s^{-1} was observed, but most of the signal was lost during the dead time of the stopped-flow instrument (Fig. 5). An alternative approach is to displace the diphosphate using actin itself; nucleotide is released because of the much weaker binding of ADP to acto-S1 than to S1 alone. However, the process is more complex than the previous protocol, and other slow processes may limit the exchange, such as a conformation change following actin binding to S1·ADP. When actin is mixed with 1 μM 3′-deac-edaADP plus 2 μM S1, the observed fluorescence changes are good fits to single exponentials (Fig. 5). The rate constant remains at ~180 s^{-1} as actin is reduced from 60 to 15 μM and only reduces to 144 s^{-1} at 7.5 μM actin and 63 s^{-1} at 3.8 μM actin. Thus, it is likely that a process with rate constant 180 s^{-1} is limiting the nucleotide dissociation following actin binding. The rate constant for displacement of but-edaADP or mbc-edaADP by ATP was also measured (Fig. 5 *b* and Table 2); the fluorescence change was much larger, but significantly slower.

Measurements with smooth muscle S1 showed that the observed fluorescence change were dependent on the type of myosin. There was an ~2-fold fluorescence increase when 3′-deac-edaADP binds to S1 from smooth muscle, and this change was reduced by addition of skeletal actin. In the case of but-edaADP the increase with smooth S1 is smaller, and subsequent addition of actin caused a fluorescence change back almost to the level of free nucleotide. Displacement of 3′-deac-edaADP from smooth S1 with 50 μM actin by excess ATP gave a rate constant for release of ~20 s^{-1}. These measurements were done at high enough [S1] (5 μM) so that the nucleotide should remain predominantly bound, assuming the analog has similar affinity as ADP. However, we cannot discount a much weakened binding of deac-edaADP for this muscle type, although the similar ADP release kinetics to that found with ADP itself (Siemankowski et al., 1985; Cremo and Geeves, 1998) makes this possibility unlikely.

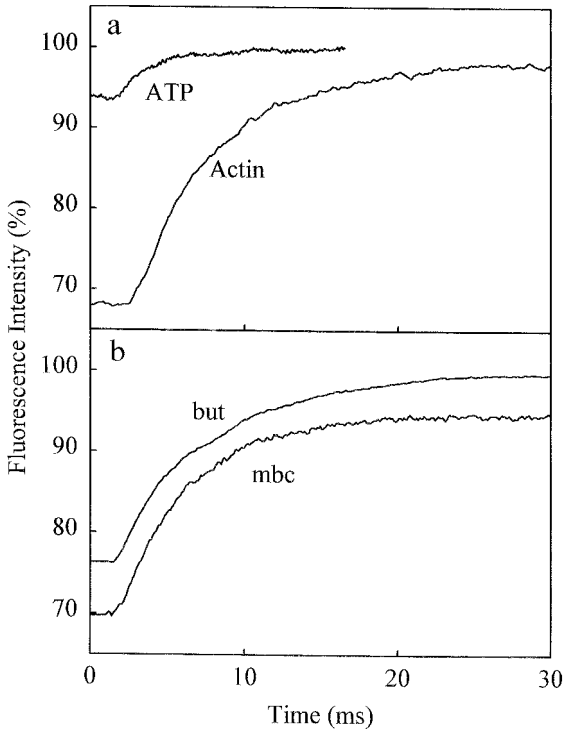

FIGURE 5 Dissociation of coumarin-labeled nucleotides from acto-S1. (*a*) ATP: 2 mM ATP was used to displace 4 μM deac-edaADP from its complex with acto-S1 (40 μM actin, 20 μM S1); actin: 60 μM actin was used to displace 1 μM deac-edaADP from its complex with 2 μM S1. (*b*) 2 mM ATP was used to displace 1 μM but-edaADP (but) or mbc-edaADP (mbc) from its complex with acto-S1 (40 μM actin, 20 μM S1). The mbc trace is offset by 5% for clarity. By varying the concentration of ATP it was shown that ATP binding did not contribute to rate limitation (data not shown). Fluorescence is shown as percent of maximum. The approximately horizontal initial parts of each trace are due to the dead time of the stopped-flow instrument, during which the reaction is not observed.

DISCUSSION

Fluorescence change on binding to S1

The three types of coumarin-modified nucleotides described here represent a range of properties. All three adenosine nucleotides show a similar fluorescence quantum yield to the equivalent coumarin carboxylic acids as simple esters or amides free in aqueous solution (Corrie et al., 2000; Brune et al., 1998; Fletcher and Bliss, 1978). They all show a fluorescence decrease on binding in the skeletal myosin active site. It has not been possible to determine whether there is a change in fluorescence on cleavage or P_i release, although any such change is likely to be very small compared with that on binding. The fluorescence change on binding to S1 is affected by the type of myosin; binding to smooth S1 causes an increase in fluorescence.

The physico-chemical basis of the fluorescence decrease on binding to skeletal S1 is not clear. In the case of the phosphate sensor, MDCC-PBP, which is also based on a coumarin fluorophore, there is a fluorescence increase associated with the protein conformation change on binding P_i. This conformation change caused the localization of the coumarin in a hydrophobic cleft and the restriction of rotation of the diethylamino group such that the nitrogen lone pair then interacts with the coumarin aromatic system

(Brune et al., 1998; Hirshberg et al., 1998). It does not seem possible for a similar mechanism to apply to the fluorescence change when these coumarin nucleotides bind to S1. They all show a decrease on binding, and a large change occurs with but-edaATP where the nitrogen is prevented from rotation by the constraints of the ring system, independent of external constraints on the molecule.

It is more likely that the decrease in fluorescence on binding to S1 arises from a feature common to the three analogs. This could be a constraint imposed on the coumarin due to its proximity to the protein surface or a more specific interaction of the protein with the coumarin. For example, hydrogen bonding between an amino acid and the 2-carbonyl group of the coumarin may occur, and this might replace a hydrogen bond between this carbonyl and the 3-carboxamido group. Alternatively, if the 3-carboxamido group is slightly distorted by the protein, and therefore no longer coplanar with the coumarin ring, the fluorescence would be reduced. However a large distortion might be expected to affect the absorbance spectrum of the coumarin; the presence of S1 has only minimal affect on this spectrum.

Interaction with S1

Whatever the mechanism of the fluorescence change on binding to S1, these coumarin nucleotides have several features that make them useful to investigate actomyosin ATPase kinetics and potentially other systems. There is a several-fold change in fluorescence intensity on binding to S1. The excitation (405–430-nm maxima, depending on coumarin) and emission (461–491 nm) ranges are well away from protein and natural nucleotide absorbance wavelengths. Single isomers are available and are stable under most likely experimental conditions and measurement times. Perhaps most important is the high degree to which such analogs mimic natural nucleotides in their protein interactions; this factor can put severe limitations on the extent of interpretation of fluorescence data, in terms of relating it to the natural situation.

In all the biochemical processes measured for S1 and acto-S1 (tri- and diphosphate binding, diphosphate release, steady-state ATPase activity) there are minor variations between coumarin analogs, but all have rates within a factor of three of those for the natural nucleotides. An estimate of K_d comes from the ratio of the dissociation and association rate constants (Table 2). Association kinetics were measured for all three triphosphate analogs, and a similar rate constant was obtained for the one diphosphate measured (3′-deac-edaADP). We have therefore assumed that the association rate constant for all the coumarin diphosphates is $5.1 \times 10^6\ M^{-1}\ s^{-1}$, this value being the average of those reported in Table 2 for the triphosphates. So for deac-edaADP this treatment gives K_d as 0.3 μM; for but-edaADP, 0.1 μM; and for mbc-edaADP, 0.2 μM. For ADP itself the equivalent rate constants have been determined at pH 8.0 as $1.3\ s^{-1}$ and $2 \times 10^6\ M^{-1}\ s^{-1}$, giving K_d as 0.7 μM (Trentham et al., 1976; Geeves, 1989).

Diphosphate dissociation from acto-S1

With acto-S1 a direct measurement of ADP release can be obtained as these analogs give a fluorescence change on binding to and release from acto-S1. Previous measurements have relied on light scattering and shown that the rate constant of ADP release is highly dependent on myosin type, being 20–30 s^{-1} for smooth and $> 500\ s^{-1}$ for skeletal (Siemankowski et al., 1985). Comparable rate constants were obtained using the coumarin nucleotides. These nucleotide analogs overcome the problem with the widely used mant nucleotides that the fluorescence change on binding to S1 is reduced to almost zero when actin is present. This may be similar to the situation of one coumarin nucleotide (but-edaADP) with smooth muscle S1, where an increase in fluorescence on binding to S1 is then reversed on addition of actin, so overall there is little or no change in fluorescence on binding to acto-S1 with smooth S1.

These results suggest that different nucleotide analogs may be preferred for different situations such as measurement of particular kinetic events within the ATPase pathway or with specific myosins. The availability of such a series, with similar overall properties but differing in detail and in fluorescence response, may enable a wider applicability. The nucleotides are currently being applied to measure the strain-dependent nucleoside diphosphate release kinetics in glycerinated muscle fibers during active contraction.

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