γ-Amido-ATP stabilizes a high-fluorescence state of myosin subfragment 1

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Abstract On binding to myosin subfragment 1 (S1), the γ-amido derivative of ATP ($ATP\gamma NH_2$), an isomer of adenosine 5′-[β,γ-imido]-triphosphate (AMPPNP), induces a larger increase in the intrinsic (tryptophan) fluorescence than is seen with ATP. A binding constant of 1.7×10^7 M^{-1} was measured for $ATP\gamma NH_2$, compared to $2.1–2.4\times10^7$ M^{-1} for AMPPNP. $ATP\gamma NH_2$ was hydrolyzed only very slowly by S1. $ATP\gamma NH_2$ appears to stabilize the 'closed' conformation of S1, and does so without cleavage of the β–γ phosphate bond. The dissociation of actin-S1 by $ATP\gamma NH_2$ and that of S1.$ATP\gamma NH_2$ by actin are both strikingly slow.

Key words: γ-Amido-adenosine 5′-triphosphate; Myosin; Actomyosin; Tryptophan fluorescence

1. Introduction

ATP analogs have played a major role in the investigation of the molecular mechanism of muscle contraction. Different states of myosin subfragment 1 (S1) are signalled by changes in its intrinsic fluorescence, reflecting changes in the environment of tryptophan residues within the protein [1–5]. Much interest is currently focussed on two crystallographically identified states ('open' and 'closed'; see [4]) and on how the transition between them is related to the cleavage step in ATP hydrolysis. The closed form has higher fluorescence, achieved particularly when the products of ATP hydrolysis ($ADP.P_i$) are bound. γ-Amido adenosine 5′-triphosphate ($ATP\gamma NH_2$) is an isomer of adenosine 5′-[β,γ-imido]-triphosphate (AMPPNP) that was first described over 20 years ago [6]. We show here that the complex of myosin with $ATP\gamma NH_2$ has a fluorescence even higher than that seen with ATP ($ADP.P_i$) bound, providing especially direct evidence that hydrolysis is not required for the high-fluorescence state.

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Abbreviations: HPLC, high pressure liquid chromatography; $ATP\gamma NH_2$, γ-amido adenosine 5′-triphosphate; AMPPNP, adenosine 5′-[β,γ-imido]-triphosphate; mantATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)-adenosine 5′-triphosphate; $GTP\gamma NH_2$, γ-amido-guanosine 5′-triphosphate; PP_i, inorganic pyrophosphate; A, actin; S1, myosin subfragment 1; M, myosin; N, nucleotide

2. Materials and methods

$ATP\gamma NH_2$ was prepared using the reaction of adenosine-5′-trimetaphosphate with ammonia/water by analogy with [7]. The nucleotide was purified by chromatography on a Super Q (TosoHaas) and/or a DEAE Sephadex column (Pharmacia) using triethylammonium bicarbonate buffer; the latter was removed from collected fractions by evaporation under vacuum, followed by addition of methanol and repeated evaporation, if necessary several times. 2′(3′)-*O*-(*N*-Methylanthraniloyl)-adenosine 5′-triphosphate (mantATP) was prepared according to [8]. Nucleotides were analyzed by reversed phase high pressure liquid chromatography (HPLC). S1 from rabbit and chicken skeletal muscle and rabbit actin were prepared as described [9,10].

For slow fluorescence measurements an SLM 8000C fluorimeter was used, with an excitation wavelength for tryptophan fluorescence of 302–305 nm and an emission wavelength of 342 nm. Stopped flow experiments were performed on a Hi-Tech SF-61DX2 instrument, using a 75 W Xe/Hg lamp and a monochromator; intrinsic fluorescence was excited at 295 nm and mant-nucleotide fluorescence (via energy transfer from tryptophan) at 288 nm and emission was detected using WG320 and KV389 filters respectively. Light scattering was measured at 364 nm. Curves were fitted using the software provided with the stopped flow instrument or the program 'Origin'. Temperature-jump fluorescence measurements were made as described [11].

The slow fluorescence measurements described refer to rabbit muscle chymotryptic S1A1. S1A2, chicken muscle S1 and papain S1 behaved essentially similarly. Stopped flow experiments were done with rabbit muscle S1A1 or S1A2. The S1 concentration (after mixing) was 1 μM in all fluorescence experiments. Light scattering was measured with 5 or 2.5 μM actin/S1. All measurements were done in TP buffer (30 mM K^+, 70 mM propionic acid, Tris (about 55 mM), 2 mM $MgCl_2$, pH 7.8, 0.02% NaN_3) at 20°C except where otherwise stated. This buffer was also used with 12 mM $MgCl_2$. The modifying group at the γ phosphate in $ATP\gamma NH_2$ may influence the binding of Mg^{2+} ions. The analogous γ-amido-guanosine 5′-triphosphate ($GTP\gamma NH_2$) binds Mg^{2+} with a K_{ass} of 0.82 mM^{-1} obtained by isothermal titration calorimetry [12]; unphysiologically high Mg^{2+} levels (>10 mM) are necessary in order to approach a saturation of $ATP\gamma NH_2$ with Mg^{2+}. For stopped flow measurements, traces of ATP and ADP were removed by treatment of a 100 mM solution of $ATP\gamma NH_2$ in TP buffer with 2 U/ml of alkaline phosphatase (calf intestine, Roche) for 1 day at room temperature. In some of the experiments the stock solution of $ATP\gamma NH_2$ was supplemented with a stoichiometric amount of $MgCl_2$.

3. Results

3.1. Fluorescence measurements

The binding of $ATP\gamma NH_2$ to S1A1 led to an approximately 30–32% increase in the intrinsic fluorescence of the protein when measured at an excitation wavelength of 295 nm. The corresponding value for ATP was 26%. For both nucleotides, the fluorescence (F_{MN}) relative to that of S1 alone (F_M) varied significantly with excitation wavelength, peaking at 302–305 nm, and the percentage difference between the two nucleotides

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was also maximal here (Fig. 1A). The absolute signal at this wavelength, however, was less than 30% of its level at 295 nm. The fluorescence intensity with ATPγNH$_2$ bound was studied as a function of temperature from 3 to 30°C, using MOPS buffer as in [11] because of the lower temperature sensitivity of its pH. The plot (Fig. 1B) coincided with that for the M.ADP.AlF$_4^-$ complex at higher temperatures and deviated slightly at lower temperatures; also shown are the plot for ATP, which lay lower and curved downwards markedly in the cold, and the strikingly flat plot seen with AMPPNP.

3.2. Hydrolysis

The enzymatic hydrolysis of ATPγNH$_2$ by S1 (10 μM) was measured using HPLC over a concentration range of 20 μM–2 mM in TP buffer and under slightly different conditions (pH 6.8, 8 mM Mg^{2+}; up to 100 mM KCl). A constant rate of $2–3\times10^{-4}$ s^{-1} was found. The total ADP (bound+free) at the first measured time-point (15 s) was, within the accuracy of the measurements, as expected for a linear rise from zero; any 'burst' must be less than 3% of the bound nucleotide, so that $>97\%$ of sites were occupied with an unsplit phosphate chain. The hydrolysis was slower in the presence of actin, particularly at low actin concentrations: e.g. 0.2×10^{-4} s^{-1} with 10 μM actoS1 and 20 μM ATP. ATPγNH$_2$ was cleaved only very slowly by alkaline phosphatase (0.1–0.2 μmol U^{-1} h^{-1}), providing a way of removing traces of ATP from ATPγNH$_2$ solutions.

ATPγNH$_2$ has a non-negligible rate of spontaneous hydrolysis, which however was still much lower than the enzymatic rate. The primary hydrolysis product is ATP. At a pH above 7 (e.g. TP buffer) 1–2% of the nucleotide was split within 10 days at room temperature. The hydrolysis was faster at lower pH: in 0.1 M MES buffer (pH 6.35) and in 0.1 M KH_2PO_4 (pH 4.4) the corresponding values were 7.3% and 85% respectively over 10 days.

3.3. Stopped flow experiments

In stopped flow experiments, mixing ATPγNH$_2$ and S1 (1 μM) led to an increase in fluorescence which could be fitted to a single exponential. The observed rate constant varied linearly with ATPγNH$_2$ concentration up to about 150 μM. The amplitude did not vary significantly between the lowest ATPγNH$_2$ concentration tested (10 μM) and about 400 μM. Higher concentrations led to a small decrease in amplitude. Sometimes the fluorescence signal displayed a second, much slower phase of low amplitude. This effect may be related to the slow release of inorganic pyrophosphate (PP$_i$) present as a contaminant in some S1 preparations, and could be induced in other S1 samples by addition of traces of PP$_i$.

Because of the low affinity for Mg^{2+}, attention was paid to the Mg^{2+} concentration in these experiments. From a plot of the observed rate constant versus ATPγNH$_2$ concentration, a second order rate constant of $0.6\pm0.2\times10^5$ M^{-1} s^{-1} was calculated ($n=10$). In a 12 mM Mg^{2+} TP buffer this value was slightly higher ($0.74\pm0.02\times10^5$ M^{-1} s^{-1}; $n=4$). For comparison, measurements with AMPPNP under identical conditions (except for 2 mM Mg^{2+}) yielded an apparent binding rate of $1.7–1.9\times10^5$ M^{-1} s^{-1}.

The dissociation rate of ATPγNH$_2$ from myosin was measured by displacement of ATPγNH$_2$ from S1 with mantATP (2 μM ATPγNH$_2$/100 μM mantATP) or PP$_i$ (at least a 50–100-fold excess over ATPγNH$_2$) using either the stopped flow or the SLM instrument. Dissociation rates of 3.8×10^{-3} s^{-1} and 4.6×10^{-3} s^{-1} were found with PP$_i$ and mantATP respectively. The corresponding values for AMPPNP were 7.9×10^{-3} s^{-1} and 8.1×10^{-3} s^{-1} respectively. Using the value at high Mg^{2+} concentration, a binding constant of 1.7×10^7 M^{-1} was calculated for ATPγNH$_2$. The value for AMPPNP (at 2 mM Mg^{2+}) was $2.1–2.4\times10^7$ M^{-1}.

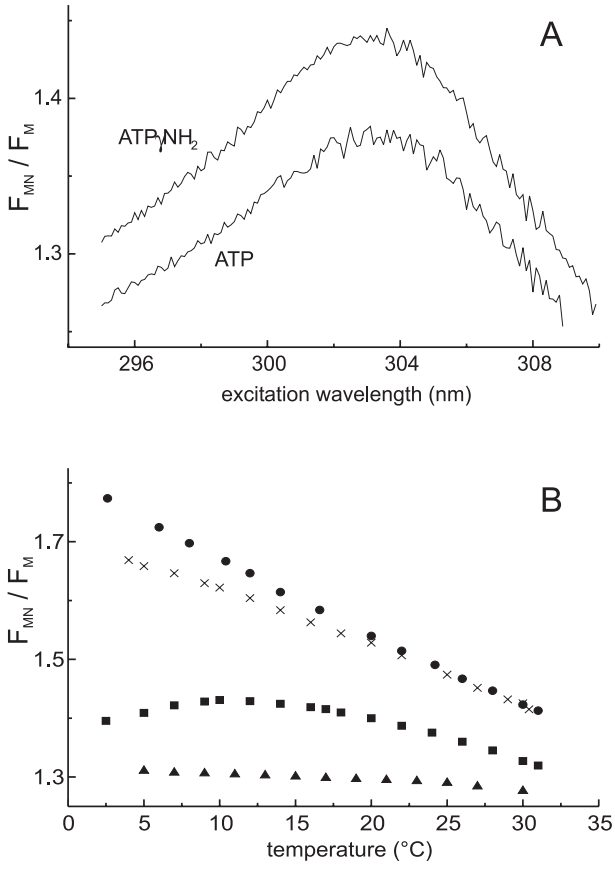

Fig. 1. Fluorescence properties of rabbit S1A1–nucleotide complexes. A: Quotient of the excitation spectra of S1–nucleotide complexes and nucleotide-free S1, for the complex with ATPγNH$_2$ (100 μM) and for the complexes present during steady-state hydrolysis of ATP at 20°C (each curve is the average of five measurements). Emission wavelength 342 nm. B: Temperature-dependence of the fluorescence intensity for S1 in the presence of 100 μM ATPγNH$_2$ (crosses), 500 μM ADP, 10 mM KF and 1 mM $AlCl_3$ (circles), 1 mM ATP (squares) and 200 μM AMPPNP (triangles) (all relative to that for nucleotide-free S1); buffer composition 70 mM potassium propionate, 12 mM MOPS, 2 mM magnesium acetate, 1 mM NaN_3, 5 mM dithioerythritol, pH 7.0. Excitation wavelength 302 nm, emission 342 nm.

These binding constants were compared with results obtained by measuring the fluorescence of S1 in the presence of ATPγNH$_2$ with various amounts of AMPPNP added. The ratio [S1.ATPγNH$_2$]/[S1.AMPPNP] (obtained from the fluorescence values) and the ratio [ATPγNH$_2$]/[AMPPNP] in the solution gave a value for $K_{ATP\gamma NH_2}/K_{AMPPNP}$ of about 0.1 at 2 mM Mg^{2+} and 0.5 ± 0.2 at 12 mM Mg^{2+}, showing that the nucleotide was not saturated with Mg^{2+} under all conditions. A similar experiment led to a value of 0.15 for $K_{ATP\gamma NH_2}/K_{PP_i}$, thus justifying the use of PP$_i$ in the displacement experiments.

3.4. Temperature-jump experiments

From temperature-jump experiments it is known that S1

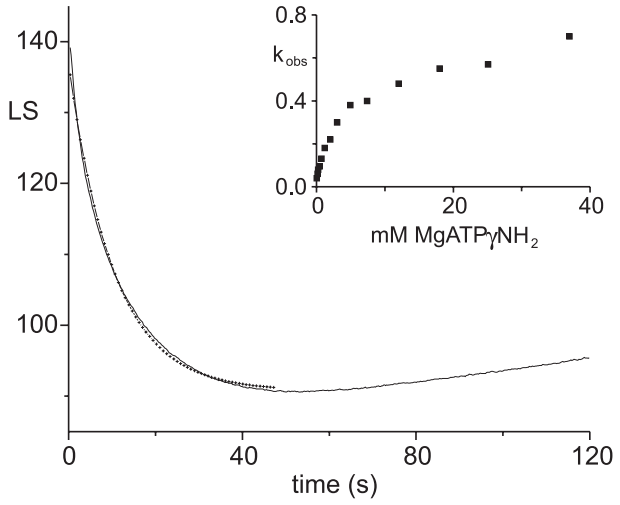

Fig. 2. Light scattering signal on mixing 2.5 μM actomyosin with 0.74 mM ATPγNH$_2$; a single exponential is fitted to the initial part of the signal, with a rate constant of 0.096 s^{-1}. Inset: observed rate constants for the series of ATPγNH$_2$ concentrations.

with AMPPNP bound exhibits a rapid equilibrium ($\tau = 0.1$ ms [11]) between a high- and a low-fluorescence state. Corresponding experiments with ATPγNH$_2$ under the same conditions did not display any relaxation process between 3 and 30°C (not shown).

3.5. Dissociation of actoS1 by ATPγNH$_2$

The dissociation of acto S1 by ATPγNH$_2$ was investigated in several series of stopped flow experiments by light scattering. It was expected that high Mg^{2+} would be needed, but that it would cause problems due to aggregation (e.g. formation of actin paracrystals). The buffer for these experiments contained 12 mM Mg, plus an amount of Mg equal to the ATPN concentration. ATPγNH$_2$ induced approximately the expected exponential decrease in light scattering. This phase was followed, within 5–10 min, by a slow increase in the signal at nucleotide concentrations up to about 1 mM (Fig. 2). At higher nucleotide concentrations this increase was not visible within the observation time (up to 15 min); instead, a slow linear decline was seen. Dissociation rates (k_{obs}) obtained by fitting a single exponential to the initial fall of the scattering curve are shown in Fig. 2 (insert) and are for orientation only. The rate constant approached a range of 0.6–0.8 s^{-1} at the highest concentration tested (37 mM ATPγNH$_2$). The dissociation of actoS1 was complete at about 3 mM ATPγNH$_2$ with 5 μM or 2.5 μM actoS1 (based on the effect of ATP under identical conditions). Corresponding experiments using AMPPNP (2 mM Mg^{2+}) led to a maximal value for the dissociation rate constant in the range 12–20 s^{-1}, essentially in agreement with published data [13].

The converse measurement of light scattering on mixing 2.5 or 5 μM actin with S1.ATPγNH$_2$ (2 mM Mg^{2+}, 18 or 300 μM ATPγNH$_2$) gave a k_{obs} of 0.005–0.008 s^{-1} at a 1:1 ratio of actin to S1 ($n = 3$); this value was unchanged at 12 mM Mg^{2+} (0.005 and 0.006 s^{-1}). With a 10-fold molar excess of actin, values of 0.02 and 0.03 s^{-1} were found.

The association constant (K_{ass}) for the binding of ATPγNH$_2$ to actoS1 was estimated using stopped flow by mixing about 20 μM ATP with actoS1 and various concentrations of ATPγNH$_2$ (at 2 mM Mg^{2+}) using the equation $k_{obs} = k_o/(1+[N]K_{ass})$ [14]. A value of $0.7 \pm 0.3 \times 10^3$ M^{-1} ($n = 5$) was found.

4. Discussion

High fluorescence of myosin S1 was originally interpreted as due to a conformation specific to the M.ADP.P$_i$ complex with a cleaved β–γ bond [2]. More recent evidence [5,11] supports the suggestion [4] that the fluorescence rise is not due to cleavage itself but to a conformational change which precedes cleavage and is required for it, and that the change is from the 'open' to the 'closed' form. For a number of ligands, the two conformations appear to be in equilibrium. With AMPPNP the equilibrium constant was close to 1, rising significantly with temperature; for the complexes present during steady-state hydrolysis of ATP, the average occupancy of the closed state may be less than 100% even at high temperature, and falls greatly in the cold. The high fluorescence form was most strongly stabilized in the M.ADP.AlF$_4^-$ complex [11]. The present results demonstrate for the first time the ability of an adenine nucleotide with an uncleaved triphosphate chain to stabilize a high-fluorescence state of S1. The fluorescence with ATPγNH$_2$ bound was comparable to that of M.ADP.AlF$_4^-$ over the temperature range studied and no temperature-jump transient was seen. The simplest interpretation of our results is that the closed form predominates unusually strongly in M.ATPγNH$_2$ also; however, it is conceivable that an alternative high-fluorescence conformation exists, and studies using other spectroscopic signals (e.g. [15]) are needed. If crystals of S1 with ATPγNH$_2$ bound can be obtained, comparison of the atomic structure with that for AMPPNP [16] may suggest why these analogs stabilize different conformations.

In stopped flow experiments the magnitude of the fluorescence rise did not vary significantly within the range 10–400 μM ATPγNH$_2$, indicating that even at the lowest concentration the protein was saturated with the nucleotide. The fluorescence rise resembled that for AMPPNP in showing only a single phase, which we interpret as reflecting the second step in the binding reaction: M+N ↔ MN → MN** [3]. From the stopped flow experiments, the binding constant of ATPγNH$_2$ to S1 (about 1.7×10^7 M^{-1}) was only slightly smaller than that found for AMPPNP (2.3×10^7 M^{-1}). The latter agrees well with values in the literature measured under slightly different conditions ($3–4 \times 10^7$ M^{-1} [13]). The value of $K_{ATP\gamma NH_2}/K_{AMPPNP}$ obtained by kinetic measurements (0.7) agrees, within the likely limits of error, with that from equilibrium measurements at 12 mM Mg^{2+} (0.5). These results gave evidence of the low affinity of ATPγNH$_2$ for Mg^{2+}.

It is not yet known whether the primary enzymatic cleavage step is the hydrolysis of the amide group or the cleavage of the β–γ phosphate bond to yield inorganic amidophosphate. In nuclear magnetic resonance studies of the enzymatic hydrolysis of the analogous GTPγNH$_2$ by p21, which in some respects resembles myosin, no amidophosphate could be detected [12]. The inhibition of the enzymatic cleavage of ATPγNH$_2$ by actin would result from the binding of myosin to actin if only unbound myosin is enzymatically competent.

The need to use high Mg^{2+} concentrations introduced an uncertainty in light scattering experiments involving actin: high Mg^{2+} induces the bundling of actin filaments, and possibly of actomyosin filaments also, and resulted in variable

ionic strengths. Further measurements are required to take account of these effects.

The interaction of ATPγNH_2 with actomyosin suggests comparison with AMPPNP and ADP [13,17,18]. The ability of a nucleotide to dissociate actomyosin does not depend on its absolute affinity for actomyosin or myosin but on its relative affinities for myosin and for actomyosin. AMPPNP dissociates AM significantly better than ADP does, but far less well than ATP (ADP.P_i). The extent of dissociation by saturating concentrations of ATPγNH_2 is not given reliably by our present data, but is probably close to that of AMPPNP. Both the dissociation reaction and the reassociation of M.ATPγNH_2 with actin are however strikingly slow: they are probably too fast to proceed entirely by dissociation of AM to A+M or of MN to M+N, and the ternary complexes AMN and A-MN (see [4]) are therefore involved. An interesting possibility is that the ternary complex A-M.ATPγNH_2 is unfavorable, for reasons related to the high fluorescence of M.ATPγNH_2: for example, if the closed conformation is in some way incompatible with binding of myosin to actin. Moreover, the stabilization of the closed conformation by a nucleotide which binds much less tightly than ATP is notable, and requires explanation in terms of the atomic structure. The properties of ATPγNH_2 thus raise important questions for understanding actomyosin and perhaps other systems.

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