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SYNTHESIS AND PROPERTIES OF A NEW FLUORESCENT ANALOG OF ATP: ADENOSINE-5'-TRIPHOSPHORO-γ-1-(5-SULFONIC ACID) NAPTHYLAMIDATE

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SUMMARY

An analog of ATP has been synthesized which contains the fluorophore, 1-aminonapthalene-5-sulfonate attached via a γ-phosphoamidate bond. This analog is strongly fluorescent (quantum yield = 0.63) with an emission maximum at 460 nm; the excited state lifetime is 20 nsec. It is a substrate for DNA-dependent RNA polymerase of E. coli and wheat germ RNA polymerase II. It is also a substrate for E. coli valyl t-RNA synthetase, venom phosphodiesterase, and potato apyrase. Cleavage of the α-β phosphoryl bond as a result of RNA synthesis or by venom phosphodiesterase produces a 15 nm red shift in the fluorescence emission spectrum. This property should make this nucleotide useful for studies of the mechanisms of enzymatic reactions involving cleavage of the α-β phosphoryl bond.

INTRODUCTION

Nucleotides play important roles in many biological processes. These processes include DNA and RNA synthesis, protein synthesis, and energy transduction. A number of nucleotide analogs have been synthesized and used to study the role of nucleotides in various systems (1). These analogs have included those with altered chemical reactivity (AMPPnP and ATP-γ-S), altered chromophoric ring structures (6-thioguanosine triphosphate), and fluorescent derivatives such as ε-ATP and formycin triphosphate (2-7).[1]

The use of fluorescent nucleotides, ε-ATP and formycin triphosphate, has often been limited by the properties of the enzyme or by the spectroscopic properties of the nucleotide. For example, ε-ATP contains a bridge group which prevents normal hydrogen bonding of the purine ring. This analog is

[1]Abbreviations used are: (γ-AmNS)-ATP, adenosine-5'-triphosphoro-γ-1-(5-sulfonic acid) napthylamidate; AMPPnP, adenylyl-imidodiphosphate; ATP-γ-S, adenosine-5'-O-(3-thiotriphosphate); and ε-ATP, 1,N^6-ethenoadenosine triphosphate.

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neither a substrate nor inhibitor for DNA-dependent RNA polymerase of E. coli an enzyme in which hydrogen bonding to the template evidently plays a key role (L. Yarbrough, unpublished observations). Formycin triphosphate is a substrate for DNA-dependent RNA polymerase, however, its excited state lifetime is only about 1 nsec and its quantum yield is very low (Q = 0.054) thus its application has been somewhat limited.

Grachev and Zaychikov (7) have reported the synthesis of an ATP analog containing aniline bound to the γ-phosphate via a phosphoamidate linkage. This analog is a good substrate for DNA-dependent RNA polymerase of E. coli. This suggested that it should be possible to prepare the analogous derivative containing 1-aminonapthalene-5-sulfonate. Here I report the synthesis of this fluorescent derivative of ATP, adenosine 5'-triphosphoro-γ-1-(5-sulfonic acid) napthylamidate, (γ-AmNS)-ATP, and some of its spectroscopic and enzymatic properties.

MATERIALS AND METHODS

Chemicals - The following chemicals were purchased from the sources listed in parenthesis: ATP, grade 1 (Sigma), [^{3}H] ATP (New England Nuclear), poly d (A-T) (P and L Labs), 1-aminonapthalene-5-sulfonate (Tridom), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce).

Enzymes - E. coli RNA polymerase was purified as described previously (8). Wheat germ RNA polymerase II was purified as described by Jendrisak and Burgess (9). Purified valyl t-RNA synthetase was a gift of Drs. Ann Collins and George Marchin, Kansas State University. E. coli alkaline phosphatase, adenylate kinase, and apyrase were from Sigma; Acetate kinase was from Boehringer; cAMP dependent protein kinase from bovine heart was a gift of Dr. Ora Rosen, Albert Einstein College of Medicine, N.Y. Venom phosphodiesterase was purified from crude venom of Crotalus adamanteus by incubation for 3 hr at 37° and pH 3.6.

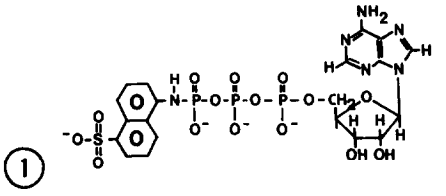
Spectroscopic measurements - Absorption spectra were obtained with a Cary model 118-C recording spectrophotometer. Fluorescence measurements were made at 25° with a Perkin-Elmer MPF-44 recording fluorescence spectrophotometer equipped with a microprocessor corrected spectra attachment. Samples had an absorbance of $\leq$ 0.1 absorbance unit to obviate significant inner filter effects. Excited state lifetime measurements were obtained with the Ortec model 9200 single photon counting system. Data was deconvoluted and fit to a single or double exponential by the method of moments.

Enzymatic Digestion of (γ-AmNS)-ATP - Reactions in 0.05 mL contained: 0.05 M Tris HCl, pH 8, 10^{-2}M $MgCl_2$, 10^{-4}M dithiothreitol and 2 mM ATP or (γ-AmNS)-ATP. Samples were incubated at 37° for 3 hr with 25 μg of Crotalus adamanteus phosphodiesterase, 25 μg of E. coli alkaline phosphatase, or 25 μg of each.

RESULTS

Synthesis and Spectroscopic Properties of (γ-AmNS)-ATP. (γ-AmNS)-ATP was synthesized from 1-aminonapthalene-5-sulfonate, [^{3}H]ATP (500 cpm/nmole), and the water soluble carbodiimide, 1-ethyl-3-(3-dimethylamino propyl)carbodiimide, using a modification of the procedure of Babkina et al. (10). Details of the synthesis procedure will be presented in a subsequent communication. The reaction product, (γ-AmNS)-ATP (Fig. 1), was purified by chromatography on DEAE cellulose. The purified nucleotide showed a single intense blue fluorescent spot with a mobility about one-half that of ATP when chromatographed on polyethyleneimine cellulose according to the procedure of Gonzales and Geel (11). Incubation of (γ-AmNS)-ATP in 0.5 N HCl for 30 minutes at 37° resulted in the complete disappearance of the original material and the appearance of two new chromatographic species. One strongly absorbed ultraviolet light and migrated with authentic ATP; the other exhibited a yellow-green fluorescence and migrated with 1-aminonapthlene-5-sulfonate.

The absorption spectrum of (γ-AmNS)-ATP is shown in Fig. 2. It exhibits a maximum at about 243 nm, a shoulder at 260 nm, and a broad band centered at about 315 nm. Based on radioactivity measurement of [^{3}H]ATP, a molar extinction coefficient at 315 nm of 5580 M^{-1} cm^{-1} can be calculated. 1-aminonapthalene-5-sulfonate shows a similar band at about 330 nm with ε = 6000. These results are consistent with a conjugate containing 1 mole of ATP and 1 mole of 1-aminonapthalene-5-sulfonate. The corrected fluorescence excitation spectrum is also shown in Fig. 2. Excitation maxima are observed at 243 and 315 nm. Following acid hydrolysis, the long wave length excitation maximum is shifted to 330 nm, the same as found for 1-aminonapthalene-5-sulfonate. The corrected fluorescence emission spectrum of (γ-AmNS)-ATP is shown in Fig. 3. It exhibits a broad maximum at 460 nm. The quantum yield was calculated to be 0.63 using quinine sulfate in 0.1 N H_2SO_4 as standard (Q = 0.55). Measurements of the excited state lifetime show a single component with a lifetime of 20 nsec. 1-aminonapthalene-5-sulfonate shows a lifetime of about 5 nsec under the same conditions.



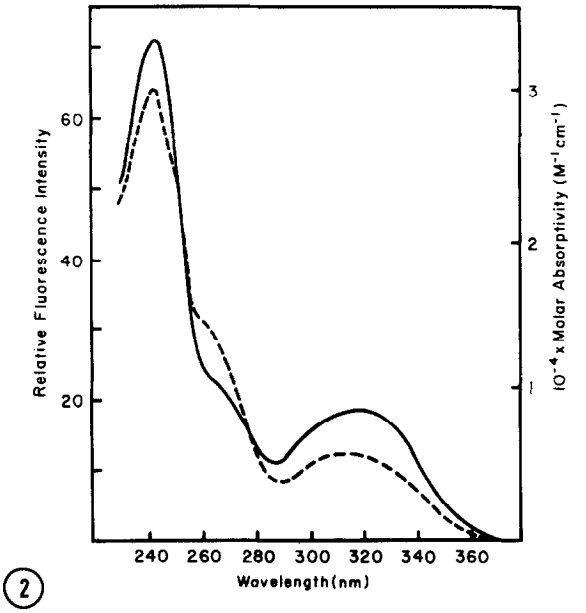
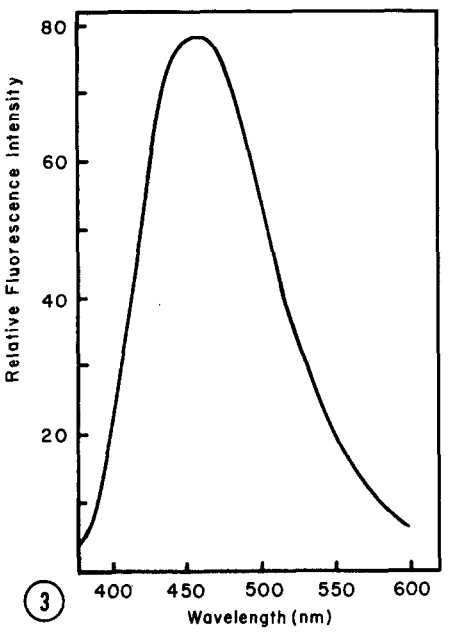


Fig. 1. Structure of (γ-AmNS)-ATP

Fig. 2. Absorption (---) and corrected fluorescence excitation (——) spectra of (γ-AmNS)-ATP. Measurements were performed at 25° in 0.05 M Tris.HCl, pH 8, 0.05 M NaCl, 10^{-2}M $MgCl_2$, 10^{-4} EDTA. For the absorption spectrum the nucleotide concentration was 4.3 x 10^{-5}M. For the fluorescence excitation spectrum, the nucleotide concentration was 2 x 10^{-6}M. Emission was measured at 460 nm through a 350 nm cut-off filter.

Enzymatic Properties of (γ-AmNS)-ATP. The ability of (γ-AmNS)-ATP to substitute for ATP in a number of enzymatic reactions was examined. Table 1 shows that this nucleotide is a good substrate for DNA-dependent RNA polymerase isolated from E. coli and wheat germ (polymerase II). It is also a substrate for valyl t-RNA synthetase from E. coli. Thus this nucleotide appears to be an effective substrate for reactions involving cleavage of the α-β phosphoryl bond.

(γ-AmNS)-ATP is not a substrate under the conditions tested for any of the following kinases: acetate kinase, adenylate kinase, or cAMP-dependent protein kinase. It is degraded by potato apyrase and venom phosphodiesterase of Crotalus adamanteus but not by bacterial alkaline phosphatase.



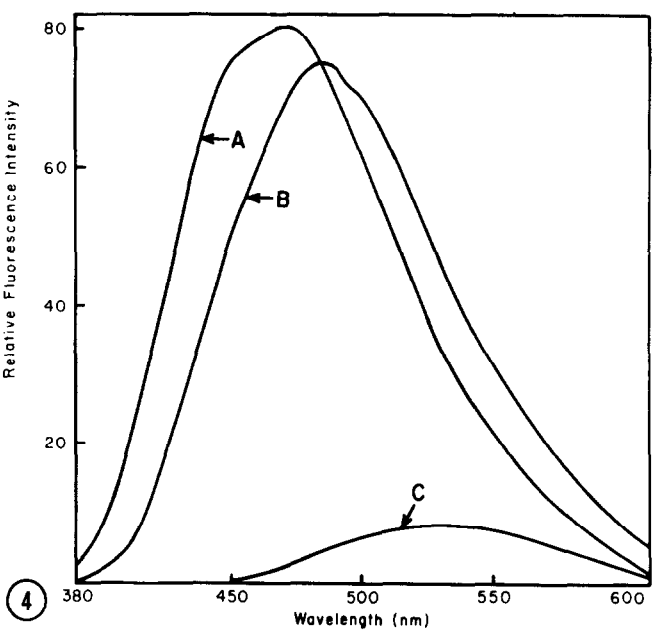


Fig. 3. Corrected fluorescence emission spectrum of (γ-AmNS)-ATP. Conditions were the same as in Fig. 2 except that no cut-off filter was used. Excitation was at 320 nm.

Fig. 4. Alterations in the fluorescence emission spectrum of (γ-AmNS)-ATP produced by enzymatic utilization. Measurements were performed as described in Fig. 3.

The fluorescence properties of (γ-AmNS)-ATP are altered when P-O or P-N bonds are broken. The intact nucleotide has a fluorescence emission maximum at 460 nm (curve A, Fig. 4). Following extensive RNA synthesis during which most of the nucleotide was utilized, the emission maximum shifted to 475 nm (curve B, Fig. 4). The excited state lifetime decreased from 20 nsec to 16 nsec. A similar change in fluorescence properties was produced by digestion with venom phosphodiesterase.

Although (γ-AmNS)-ATP is not digested by bacterial alkaline phosphatase alone, when the nucleotide is digested with a combination of venom phosphodiesterase and alkaline phosphatase, the fluorescence emission maximum shifts to 520 nm, the same as found for free 1-aminonapthalene-5-sulfonate (curve C, Fig. 4). Analysis of the reaction products by thin layer chromatography

Table 1. Utilization of (γ-AmNS)-ATP by DNA-dependent RNA polymerase and valyl t-RNA synthetase.

Enzyme	Activity (%)	
	ATP	(γ-AmNS)-ATP
RNA polymerase (E. coli)	100	60
RNA polymerase II (wheat germ)	100	27
valyl t-RNA synthetase (E. coli)	100	20

Assays for RNA polymerase contained in 0.1 mL: 0.05 M Tris HCl, pH 8, 10^{-2}M $MgCl_2$, 10^{-3}M dithiothreitol, 10^{-4} M UTP, 10^{-4} M [^{3}H]ATP (4980 cpm/nmole) or (γ-AmNS)[^{3}H]ATP (500 cpm/nmole), 20 nmoles of poly d (A-T), and 8 pmoles of E. coli holo enzyme. Assays for wheat germ RNA polymerase contained 16 nmoles of denatured calf thymus DNA in lieu of poly d (A-T), 3 mM $MnCl_2$ instead of $MgCl_2$, [^{3}H]GTP (11,300 cpm/nmole), 0.05 M $(NH_4)_2SO_4$, 5 µg of purified wheat germ RNA polymerase, and other components as described above. Samples were incubated for 10 min at 37°, precipitated with 5% trichloracetic acid, and the precipitate collected on glass fiber filters. The filters were dried and counted in a toluene based liquid scintillation fluid. Assays for valyl t-RNA synthetase contained in 0.1 mL: 0.1 M Tris.HCl, pH 7.3, 10^{-2}M $MgCl_2$, 10^{-2} M KCl, 10^{-4} M dithiothreitol, 10^{-4} M [^{3}H]valine (35 cpm/pmole), 50 µg t-RNA, 2 mM ATP or (γ-AmNS)-ATP and 0.1 µg of purified E. coli valyl t-RNA synthetase. Following a 15 min incubation at 37°, samples were precipitated with 5% trichloroacetic acid, the precipitates collected, and counted by liquid scintillation as described above.

revealed the presence of two species. One absorbed u.v. light and migrated with adenosine; the other was fluorescent and migrated with 1-aminonapthalane-5-sulfonate. No (γ-AmNS)-ATP was detected following digestion.

DISCUSSION

(γ-AmNS)-ATP should be an excellent probe for many ATP requiring enzymes, especially those which cleave the α-β phosphoryl bond such as nucleic acid polymerases and t-RNA synthetases. This nucleotide has an absorption band in the region 300-350 nm which permits its selective excitation. Since this is the region in which tryptophan fluorescence occurs, it is also a potential acceptor for resonance energy transfer from intrinsic fluorophores of proteins. In addition, the relatively long excited state lifetime makes it potentially useful for studies of fluorescence polarization. The quantum yield is high and

is altered when P-O or P-N bonds are broken. This property may allow one to study the dynamics of the phosphoryl bond breaking step.

The reaction used to synthesize (γ-AmNS)-ATP can be used to synthesize other nucleotide analogs. For example, we have already synthesized the comparable GTP analog. In addition, it should be possible to synthesize other ribo- as well as deoxyribonucleoside mono, di, or triphosphate derivatives.

It has not yet been determined whether (γ-AmNS)-ATP is capable of being incorporated into the 5' terminus of RNA chains, i.e., acting as an initiator. It appears that it can however, since Grachev and Zaychikov (7) have shown that the corresponding derivative, ATP-γ-anilidate, can initiate RNA chains. If studies show that (γ-AmNS) can initiate, we plan to use it to study the dynamics of RNA chain initiation.

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