ATP Formation from Adenyl-5'-yl Imidodiphosphate, a Nonhydrolyzable ATP Analog*

(Received for publication, May 15, 1980, and in revised form, July 16, 1980)

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The purity of several preparations of adenyl-5'-yl imidodiphosphate (AMP-PNP) was analyzed using thin layer chromatography and the luciferin-luciferase assay. Three contaminants were identified: adenyl-5'-yl phosphoramidate, phosphorylated AMP-PNP, and ATP. The level of ATP contamination ranged from 0.02% to 0.3% in commercially obtained AMP-PNP preparations, and rose to 10% following incubation of AMP-PNP at 37°C for 3 weeks in aqueous solution. The chemistry of the phosphoramidate bond is reviewed briefly, and evidence for a simple mechanism for the spontaneous formation of ATP from AMP-PNP is presented.

Analogs of ATP are employed frequently to study mechanisms of ATP utilization by cellular enzymes (1). The analog AMP-PNP* bears a close structural resemblance to ATP, yet is resistant to hydrolysis by most ATPases (2). AMP-PNP has been used to investigate the roles of ATP binding in muscle contraction (3-5), actin polymerization (6, 7), and ciliary and flagellar motility (8-10). The utility of AMP-PNP is predicated on its freedom from ATP contamination. The absence of ATP would appear to be particularly critical for studies of dynein and myosin function, since the affinity of these two ATPases for ATP is on the order of 10^2-10^6-fold higher than their affinity for AMP-PNP (8, 11, 12).

In connection with our studies of the role of ATP binding to dynein in the flagellar motility of sea urchin spermatozoa, we have developed procedures for analyzing the purity of AMP-PNP. In this communication, we present evidence that AMP-PNP is consistently contaminated with ATP as well as two other nucleoside polyphosphates. This contamination is readily accounted for on the basis of the chemistry of the phosphoramidate bond. The possible implications of ATP contamination of AMP-PNP are considered with reference to the postulated roles of ATP binding in flagellar motility and muscle contraction.

* The abbreviations used are: AMP-PNP, adenylyl-5'-yl imidodiphosphate; AMP-PN, adenylyl-5'-yl phosphorylated phosphoramidate; P-AMP-PNP, phosphorylated AMP-PNP; AMP-PCP, adenylyl-5'-yl (β,γ-methyl) methylphosphonate; ATP-γ-S, adenosine-5'-O-(3-thiotriphosphate); ATP-γ-N, adenosine-5'-O-(3-amidotriphosphate); GMP-PNP, guanylyl-5'-yl imidodiphosphate.

**This work was supported by grants to S. M. P. from the Population Council (B79-2X), the National Science Foundation (PCM-7905611), and the New Jersey Osteopathic Education Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Materials and Methods

Biochemicals—AMP-PNP was purchased from Sigma Chemical Co., Boehringer Mannheim Corp., ICM Nutritional Biochemicals, and P-L Biochemicals. AMP-PCP and hexokinase were products of Boehringer Mannheim. ATP, ADP, AMP, alkaline phosphatase (type VII), firefly lantern extract, and luciferin were from Sigma. All other chemicals were reagent grade.

Thin Layer Chromatography—Ascending thin layer chromatograms were developed in one dimension on precoated polyethyleneimine-cellulose sheets (Bakerflex) at ambient temperature and distances of 12 to 15 cm from the origin. Nucleotides were localized under ultraviolet light; the proportions of individual components in heterogeneous mixtures were estimated. RF values are listed in Table 1.

Purification of Nucleotides—Nucleotides were eluted from thin layer chromatograms with 2.0 M Tris-HCl, 0.7 M MgCl2, pH 7.4, adjusted at 21°C (15). The eluate was diluted 3-fold with water, acidified with 7% perchloric acid, and mixed with 100 to 200 μg of acid-washed charcoal (Darco G-60). Following elution from charcoal with an aqueous solution of 10% ethanol, 1% ammonium hydroxide at 37°C for 30 min, the nucleotide was concentrated by liphosphatase or rotoevaporation. Yield was approximately 10%.

Preparation of AMP-PN—AMP-PN was prepared by digestion of 0.5 mM AMP-PNP with 0.5 mg/ml of alkaline phosphatase for 24 h at 37°C in 50 mM triethylenammonium bicarbonate, 1 mM MgSO4, 2 × 10^-3 M ZnCl2, pH 7.4, adjusted at 21°C (15). The eluate was diluted 3-fold with water, acidified with 7% perchloric acid, and mixed with 100 to 200 μg of acid-washed charcoal (Darco G-60). Following elution from charcoal with an aqueous solution of 10% ethanol, 1% ammonium hydroxide at 37°C for 30 min, the nucleotide was concentrated by liphosphatase or rotoevaporation. Yield was approximately 10%.

Results and Discussion

Analysis of AMP-PNP Preparations—AMP-PNP routinely showed two or three components on thin layer chromatograms (Fig. 1, A and B). These three components were...
purified and their structures were determined on the basis of their ultraviolet spectra, susceptibility to digestion by alkaline phosphatase, and behavior on thin layer chromatography. All gave ultraviolet spectra identical to that of an ATP standard, indicating they were adenosine nucleotides. Treatment of the major spot \((R_F = 0.39, \text{Solvent } A)\) with alkaline phosphatase resulted in its slow conversion to material which co-chromatographed with the major spot \((R_F = 0.24)\).

The relative rates of alkaline phosphatase digestion of the major and trailing components were estimated by treating unpurified AMP-PNP \((11 \text{ mM})\) with alkaline phosphatase \((0.3 \text{ mg/ml})\) at \(37^\circ\text{C}\) and analyzing the reaction mixture at various times by thin layer chromatography in Solvent B. The trailing spot disappeared within 5 min, the earliest time examined.

After 24 h, approximately 50% of the material in the major spot had been converted to the front-running spot. The major and trailing components comprised an estimated 85% and 5%, respectively, of the total material in the reaction mixture, as judged from thin layer chromatograms. Therefore, the difference in digestion times indicated that the trailing component was hydrolyzed a minimum of 30-fold faster than the major component. Since the terminal phosphoramide bond in AMP-PNP is known to be less susceptible to hydrolysis by alkaline phosphatase than phosphate ester bonds (2), it was concluded that the trailing component differed from the major component in having a fourth phosphate joined in ester linkage to the terminal phosphosphate of AMP-PNP. These results indicated that the major, front-running, and trailing components (Fig. 1B) corresponded, respectively, to AMP-PNP, AMP-PN, and AMP-PNP-P. This confirmed an earlier analysis of the front-running impurity (8). The trailing impurity had not been identified previously.

**Identification of ATP**—Attempts were made to visualize ATP on thin layer chromatograms in Solvent A, which resolves ATP from AMP-PNP (Table I). Despite the application of large quantities of AMP-PNP (up to 100 \(\mu\)g), no ATP was detected on thin layer chromatograms.

In order to detect possible ATP contamination, the more sensitive luciferin-luciferase assay was used. It was found that 1 to 5 \(\text{mM}\) AMP-PNP produced a signal in the luciferin-luciferase assay which was similar in intensity and decay kinetics to that given by ATP at micromolar concentrations. The signal was abolished when AMP-PNP was pretreated with hexokinase, indicating it was not due to AMP-PNP, which is insensitive to hexokinase (2). In order to confirm that the signal in the firefly assay was due to ATP, AMP-PNP was fractionated by thin layer chromatography in Solvent A, and a small quantity of material was recovered from the region of the chromatogram corresponding to the \(R_F\) value for ATP (Table I). This material produced a signal in the luciferin-luciferase assay which was kinetically indistinguishable from that of an ATP standard.

**Quantitation of ATP Contamination in Commercial AMP-PNP Preparations**—Using the firefly assay, we analyzed six commercial preparations of AMP-PNP. All of them were found to contain ATP (Table II). ATP levels varied more than 10-fold among different preparations, from about 0.02% to 0.3%. In two samples whose ages were estimated from the manufacturer’s specifications sheet, the level of ATP contamination was higher in the older sample.

In general, it was found that the levels of AMP-PN and AMP-PNP-P impurities which were estimated from thin layer chromatograms correlated positively with the level of ATP contamination determined by the firefly assay. For example, the AMP-PNP sample in Fig. 1A, which was comprised of

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**Table I**

| Nucleotide      | \(R_F\) value | Solvent A | Solvent B |
|-----------------|---------------|-----------|-----------|
| AMP-PNP         | 0.39          | 0.24      |           |
| AMP-PN          | 0.71          | 0.60      |           |
| AMP-PNP-P       | 0.14          | 0.06      |           |
| ATP             | 0.18          | 0.21      |           |
| ADP             | 0.38          | 0.39      |           |
| AMP             | 0.63          | 0.60      |           |
| AMP-PCP         | 0.59          | 0.33      |           |

*Performed as described under "Materials and Methods."

1.2 \(\text{mLCl}\) (13).

0.75 \(\text{mKH}_2\text{PO}_4, \text{pH} 3.4, \text{with phosphoric acid at } 21^\circ\text{C}\) (14).
approximately 98% AMP-PNP and 2% AMP-PN, containing 0.02% ATP, while the sample in Fig. 1B, which contained approximately 8% AMP-PNP, 1% AMP-PN, and 6% AMP-PNP-P, contained 0.2% ATP.

Generation of ATP from AMP-PNP—Because the level of ATP appeared elevated in older preparations of AMP-PNP (Table II), we tested the possibility that ATP was generated as a decomposition product of AMP-PNP. AMP-PNP was incubated in aqueous solution (pH 6) at 37°C, and the ATP concentration was determined at weekly intervals by the firefly assay. Fig. 2 shows that the concentration of firefly assay substrate increased dramatically over a 3-week period. Analysis by thin layer chromatography confirmed the presence of ATP, as well as at least seven other components (Fig. 1C).

The possibility that the multicomponent mixture in Fig. 1C might have been generated by contaminating microorganisms was ruled out by the inclusion of 0.1% NaN₃ in the incubation mixture. Also, control samples of AMP-PNP from which NaN₃ was omitted generated ATP, eliminating the possibility that ATP formation resulted from catalysis by NaN₃ (data not shown). Further controls incubated in parallel with AMP-PNP showed that no ATP arose from AMP-PCP, while pure ATP, not unexpectedly, broke down to ADP and AMP (Fig. 2). No change in ATP contamination was detected in AMP-PNP incubated at -20°C for 4 weeks, within the error of the assay (Fig. 2).

Four of the nucleotides generated by incubation of AMP-PNP at 37°C were identified by co-migration with known standards as AMP-PNP-P, ATP, AMP, and AMP-PN (Fig. 1C). A fifth spot could have been due to a mixture of ADP and AMP-PNP, which were not resolved in Solvent System A (Table I). Three compounds representing over 50% of the material in the mixture were not identified (Fig. 1C).

A discrepancy was noted between the ATP concentration determined by the firefly assay (21%, Fig. 2) and by visual inspection of a chromatogram of the same mixture (approximately 3%, Fig. 1C). The flash height given in the firefly assay was reduced only by 65% while the rate of flash decay was not changed when the decomposition mixture was pretreated with hexokinase, suggesting that about one-third of the signal may have been due to a compound(s) other than ATP. It is conceivable that other nucleotide(s) in this mixture was capable of acting as substrates in the luciferin-luciferase assay in addition to ATP. In this regard, we mention our observation that the ATP analog ATP-γ-S gives a positive signal in the firefly assay with 75% lower intensity and 5-fold slower decay kinetics than ATP. A structurally analogous phosphoramide containing a nitrogen instead of a sulfur atom bonded to the terminal phosphate of ATP (ATP-γ-N) could conceivably have been generated by decomposition of AMP-PNP and then acted as a substrate in the firefly assay (Fig. 2). Whether one of the three unidentified components in Fig. 1C corresponded to amidated ATP was not determined.

Mechanism of ATP Formation—Our results are explained readily by the chemistry of the phosphoramidate (P-N) bond. The phosphoramidate bond has a high phosphate transfer potential, as was first recognized for creatine phosphate (18). The reactivity of phosphoramidates was exploited by Khorana and his co-workers to synthesize specific nucleoside diphosphates from nucleoside 5'-phosphoramidates and phosphoric acid (19-21). Their synthesis was based on phosphorolysis (20), in which phosphate attacks the electrophilic phosphorus in the phosphoramidate bond, and the nitrogen acts as a leaving group. The preparation of specific nucleoside polyphosphates is limited only by the availability of appropriate nucleoside phosphoramidates (20-22).

We propose that ATP is generated from AMP-PNP in a simple two step reaction sequence involving the spontaneous hydrolysis of AMP-PNP to AMP-PN, followed by phosphorolysis of AMP-PN:

**Hydrolysis:**

\[ \text{AMP-PNP} + \text{H}_2\text{O} \rightarrow \text{AMP-PN} + \text{P}_i \]

**Phosphorolysis:**

\[ \text{AMP-PN} + \text{P}_i \rightarrow \text{AMP} + \text{NH}_3 \]

Evidence for the first step, the spontaneous hydrolysis of the phosphoramidate bond, is found in the literature (2, 20) and in Fig. 1. Evidence for the second step of our proposed mechanism, the spontaneous phosphorolysis of AMP-PN in aqueous solution, is presented in Fig. 3. Incubation of purified AMP-PN with orthophosphate resulted in the synthesis of ATP, and the reaction was enhanced at low pH, as expected for phosphorolysis (20). The identity of ATP as the reaction product was confirmed by the sensitivity of the signal obtained in the firefly assay in Fig. 3 to pretreatment of either reaction product with 8 M urea.
ATP in Adenyl-5'-yl Imidodiphosphate

We have not analyzed guanylyl-5'-yl imidodiphosphate. However, we would predict that GTP is present in GMP-PNP preparations at levels comparable to the ATP levels we have found in AMP-PNP, because the mechanism of ATP generation by coupled hydrolysis-phosphorolysis proposed here does not depend on the structure of the pyrimidine base.

In view of the routine contamination of AMP-PNP by ATP, we have re-examined the effects of AMP-PNP on the relaxation of rigor wave sea urchin sperm flagella. While a detailed report of our findings is in preparation, we note here that, contrary to two earlier reports (8, 10) but in agreement with a third (9), we now find that axonomal rigor waves are not relaxed by purified AMP-PNP. AMP-PNP has also been used extensively to study muscle contraction (3–5), and perhaps it would be of interest to re-examine the effect of purified AMP-PNP on rigor muscle. In any event, the chemical reactivity of nucleoside phosphoramidates reported here suggests the need for continual monitoring for the spontaneous generation of nucleoside triphosphates.

Acknowledgments—We thank Dr. L. Dickson for helpful discussions regarding chemical mechanisms of polyphosphate synthesis and Dr. G. Witman for his stimulating interest in this work.

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*S. M. Penningroth, A. Cheung, and K. Olehnik, manuscript in preparation.