RNA–protein crosslinking to AMP residues at internal positions in RNA with a new photocrosslinking ATP analog

Celina Costas, Elizabeth Yuriev, Karen L. Meyer, Tina S. Guion¹ and Michelle M. Hanna*

Designer Genes, Inc., 8281 East Evans Road, Suite 104, Scottsdale, AZ 85260, USA and ¹Department of Chemistry and Biochemistry, University of Oklahoma, Norman, OK 73019, USA

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ABSTRACT

A new photocrosslinking purine analog was synthesized and evaluated as a transcription substrate for *Escherichia coli* RNA polymerase. This analog, 8-[(4-azidophenacyl)thio]adenosine 5'-triphosphate (8-APAS-ATP) contains an aryl azide photocrosslinking group that is attached to the ATP base via a sulfur-linked arm on the 8 position of the purine ring. This position is not involved in the normal Watson–Crick base pairing needed for specific hybridization. Although 8-APAS-ATP could not replace ATP as a substrate for transcription initiation, once stable elongation complexes were formed, 8-APAS-AMP could be site-specifically incorporated into the RNA, and this transcript could be further elongated, placing the photo-reactive analog at internal positions in the RNA. Irradiation of transcription elongation complexes in which the RNA contained the analog exclusively at the 3' end of an RNA 22mer, or a 23mer with the analog 1 nt from the 3' end, produced RNA crosslinks to the RNA polymerase subunits that form the RNA 3' end binding site (β, β'). Both 8-APAS-AMP and the related 8-azido-AMP were subjected to conformational modeling as nucleoside monophosphates and in DNA–RNA hybrids. Surprisingly, the lowest energy conformation for 8-APAS-AMP was found to be *syn*, while that of 8-azido-AMP was *anti*, suggesting that the conformational properties and transcription substrate properties of 8-azido-ATP should be re-evaluated. Although the azide and linker together are larger in 8-APAS-ATP than in 8-N₃-ATP, the flexibility of the linker itself allows this analog to adopt several different energetically favorable conformations, making it a good substrate for the RNA polymerase.

INTRODUCTION

Specific interactions between proteins and nucleic acids are involved in nearly all aspects of gene expression. Understanding

these interactions is important for determining the regulatory and catalytic mechanisms involved in gene expression. Unfortunately these interactions, which often occur within macromolecular complexes, can be weak and/or transient in nature and difficult to characterize. Photochemical crosslinking is a proven method for studying the RNA–protein and RNA–nucleic acid interactions that are involved in these cellular processes (1–3). In photochemical crosslinking, a chemically inert group is converted by photo-irradiation to a chemically reactive species that can then form a covalent bond with an adjacent molecule. This effectively traps the weak and/or transient molecular interactions present in the ribonucleoprotein complex. The crosslinked molecules can then be isolated and characterized to provide details of these molecular interactions.

While photochemical crosslinking of RNA to nucleic acids and/or proteins can be accomplished using direct irradiation with short wavelength ultraviolet light, this method can produce non-specific crosslinks, low crosslinking yields and photodamage to the proteins and nucleic acids in the complex. A more selective and efficient method involves the site-specific incorporation of a photocrosslinking moiety into the RNA molecule. Controlling the nature and position of the photocrosslinking analog facilitates the analysis of the crosslinked complexes. This can be accomplished by incorporation of a photocrosslinker-tagged nucleotide into the RNA *in vitro* by transcription with purified RNA polymerase (4–7), by chemical incorporation (8) or by post-transcriptional modification of the RNA (9–11).

There are several photocrosslinking analogs that serve as substrates for RNA polymerases and have been used to analyze ribonucleoprotein complexes. Several of these analogs, 4-thio-UTP (12), 5-azido-UTP (13), 5-bromo-UTP (1) and 8-azido-ATP (14–17), contain crosslinking groups directly on the nucleotide base and function essentially as 0 Å probes. Therefore a direct contact between the modified base and the protein is necessary for efficient crosslinking. Two pyrimidine analogs that have crosslinking groups tethered to a sulfur at the 5 position of the base are 5-APAS-UTP (4) and 5-APAS-CTP (5), both of which contain an aryl azide attached 10–13 Å from the base by a moderately flexible linker. These pyrimidine analogs have proven useful in photocrosslinking studies; however, they are

*To whom correspondence should be addressed. Tel: +1 480 596 9312; Fax: +1 480 991 4419; Email: mmh@designergenesinc.com

Present addresses:

Celina Costas, Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, University of Santiago, 15706-Santiago de Compostela, Spain
Elizabeth Yuriev, Protein Crystallography, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, OK 73104, USA

Figure 1. The structure of the photocrosslinking nucleotide analog 8-APAS-ATP is shown. The labels t1–t4 indicate the torsion angles that were varied during the conformational searching and molecular modeling.

not helpful for identification of interactions involving protein contacts with purine-rich regions of the RNA.

A purine analog, 8-azido-ATP, has been used to study the active site of *Escherichia coli* RNA polymerase, but it has been reported to not function as a transcription substrate (16). In our earlier work, we showed that it does function as an elongation substrate for *E. coli* RNA polymerase, but appeared to function as a chain terminator during *in vitro* transcription on a poly-dAT template (17). Its incorporation at internal positions in RNA has not been reported.

We report here the synthesis, characterization and evaluation of the transcription properties of a new purine analog, 8-[(4-azidophenyl)thio]adenosine 5'-triphosphate (8-APAS-ATP, Fig. 1). This analog contains the same photocrosslinking moiety present in 5-APAS-UTP and 5-APAS-CTP (4,5), but tethered to the 8 position of the purine base. Its substrate properties and its use for RNA–protein crosslinking are reported. In addition, molecular modeling of both 8-APAS-AMP and 8-azido-AMP, as nucleotides or incorporated into the RNA of a DNA–RNA hybrid was performed. The results suggest a need to re-examine either the conformational properties of 8-azido-AMP or the elongation substrate requirements for *E. coli* RNA polymerase.

MATERIALS AND METHODS

Chemicals and biochemicals

Unless stated otherwise, the starting materials for the chemical synthesis of the analogs were obtained from Aldrich (Milwaukee, WI), Sigma (St Louis, MO), Fisher (Pittsburgh, PA) or Fluka (Buchs, Switzerland). FPLC-purified ribonucleoside triphosphates from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden) were used in all transcription reactions. DNA templates 1 and 2 were DNA fragments prepared by the polymerase chain reaction from plasmid pAR1707, which contains the bacteriophage T7 A1 promoter (18). The primer end-points relative to the A1 promoter transcription initiation site are –149 and +153. Template 2 was synthesized with a 5'-biotinylated upstream primer.

Buffers

The buffers used were as follows. Buffer A, (5×): 315 mM Tris–HCl (pH 6.8), 250 mM DTT, 10% (w/v) SDS, 50% (v/v) glycerol, 0.1% (w/v) BPB; buffer B, 20 mM Tris–acetate (pH 7.8), 10 mM magnesium acetate, 50 mM potassium-glutamate, 4% (v/v) glycerol, 40 μM Na₂EDTA (pH 8.0),

10 mM β-mercaptoethanol, 0.2 mg/ml acetylated bovine serum albumin; buffer C, 20 mM Tris–acetate (pH 7.8), 10 mM magnesium acetate, 50 mM potassium-glutamate, 4% (v/v) glycerol, 40 μM Na₂EDTA (pH 8.0), 10 mM β-mercaptoethanol; buffer D, 90 mM Tris–borate, 2 mM Na₂EDTA (pH 8.0); buffer E, 7 M urea, 0.1% (w/v) bromophenol blue (BPB), 0.1% (w/v) xylene cyanol (XC); buffer F, 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS (pH 8.3).

Synthesis and characterization of 8-APAS-ATP

8-APAS-ATP was prepared in the dark from 8-SH-ATP. A solution of 8-SH-ATP (10 mg, 18 μmol) in H₂O (428 μl) was prepared as described (19,20) and mixed with APB (10 mg, 41 μmol) in 0.2 M K₂HPO₄ solution (428 μl). The reaction was stirred at room temperature for 3 h and then at 4°C overnight, after which it was extracted three times with water-saturated diethyl ether (300 μl). The aqueous layer was filtered through an Acrodisc 3 filter (0.45 μm). The filter was rinsed with 500 μl of H₂O and the rinse was combined with the filtrate. The product was purified and analyzed by high performance liquid chromatography (HPLC), as described below.

HPLC

HPLC analyses were performed with a Beckman LC system, which was equipped with a 126 solvent delivery module and a 168-diode array detector controlled with Beckman System Gold Software. A reverse-phase C18 column (Phenomenex Prodigy RP, 250 mm × 4.6 mm i.d.) and guard column (Upchurch, 1 cm × 4.3 mm i.d.) were used for analytical analyses. Semipreparative purifications were carried out on a Phenomenex Prodigy RP column (150 mm × 21.2 mm i.d.) and guard column (Upchurch, 1 cm × 4.3 mm i.d.). Sample loops of 1 ml and 100 μl and flow rates of 5 and 1 ml/min were used for semi-preparative and analytical analyses, respectively. 8-APAS-ATP was purified by preparative HPLC using 50 mM TEAB (pH 7.0) and a gradient of CH₃CN [0% (v/v) CH₃CN to 40% in 40 min, 40% CH₃CN to 100% in 6 min]. The purity of the 8-APAS-ATP was assessed by analytical RP HPLC using a gradient solvent system composed of triethylammonium buffers and acetonitrile: 100 mM TEAA (pH 7.0), CH₃CN [0% (v/v) CH₃CN to 50% in 20 min; 50% CH₃CN to 100% in 5 min]. UV absorption was monitored at 265 and 300 nm.

Spectrometry and photolysis

NOESY spectra were recorded in D₂O on a Varian 400 MHz spectrometer and the residual proton was used as the reference. The sample was prepared and loaded into the NMR in the dark. For irradiation for protein–RNA crosslinking, the samples were located 1–2 cm from a 302 nm medium-wavelength mercury vapor lamp (Spectroline Model XX-15B, 1800 μW/cm² at 15 cm).

Enzymatic characterization of 8-APAS-ATP

As a standard, 8-APAS-adenosine was chemically synthesized in two reactions, starting with bromoadenosine, and then characterized by NMR, UV and mass spectrometry. 8-APAS-ATP, 8-APAS-adenosine and ATP (5 nmol each) were individually treated at 37°C for 2 h with calf intestine phosphatase (CIP, 1 μl, 1 U/μl) in buffer A or snake venom phosphodiesterase (SVP, 1 μl, 0.1 U/μl) in 0.1 M Tris–HCl (pH 8.0) and 0.1 M NaCl (5 μl final reaction volumes). Control reactions without CIP or SVP were run in parallel. Digestion reactions were

analyzed by HPLC using a 30 min linear gradient from 0 to 100% (v/v) CH₃CN in 0.1 M TEAA buffer (pH 7.0) or by thin layer chromatography (TLC) on PEI-cellulose F plates, developed in 1.6 M LiCl.

Evaluation of 8-APAS-ATP as an initiation substrate for *E.coli* RNA polymerase

All reactions were performed in reduced light until irradiation for crosslinking or addition of DTT was completed. Part A: transcription was initiated by incubating RNA polymerase holoenzyme (16 nM) and DNA template 1 (8 nM) in buffer B for 2 min at 37°C (20 µl final volume). CTP, UTP and GTP (20 µM, [α -³²P]GTP = 4.4×10^6 c.p.m./pmol) were added, together with 8-APAS-ATP (200 µM) or 8-APAS-ATP that had been irradiated for 2 min before the assay. Heparin (0.1 mg/ml) was added immediately after the nucleotides, followed by incubation at 37°C for 1 min. Part B: transcription was initiated by incubating the holoenzyme (16 nM) with template 1 (8 nM) and ApU (200 µM) in buffer B at 37°C for 2 min. Nucleotides were added, as described for part A, and 8-APAS-ATP was included at various concentrations (20, 100, 200 µM). In both parts, a positive control, containing ATP (20 µM) and no analog, and a negative control, with no adenosine nucleotide present, were also performed.

RNA-protein photocrosslinking

After incubation, each reaction was divided into two parts. One aliquot from each reaction was kept in the dark at room temperature while the other was irradiated for 5 min at room temperature (in 6 mm $\times$ 50 mm borosilicate tubes placed 2 cm from a 302 nm UV light source, Spectroline model XX-15B, 1800 µW/cm² at 15 cm). DTT was then added to all reactions to a final concentration of 0.1 M. Samples were split for analysis of the RNA (7 M urea PAGE) or identification of crosslinked proteins (SDS-PAGE).

Gel electrophoresis

To analyze the RNA products, samples were adjusted to 1 $\times$ buffer E and heated for 3 min at 90°C. Short RNAs were analyzed on a 25% polyacrylamide/7 M urea gel (acrylamide/bisacrylamide = 12.5:1, 40 cm $\times$ 20 cm $\times$ 0.4 mm), or on a 20% polyacrylamide/7 M urea gel (acrylamide/bisacrylamide = 19:1, 30 cm $\times$ 20 cm $\times$ 0.5 mm). Long RNAs were analyzed on a 5% polyacrylamide/7 M urea gel (acrylamide/bisacrylamide = 19:1, 30 cm $\times$ 20 cm $\times$ 0.7 mm). High percentage gels were run in a discontinuous buffer system (0.5 $\times$ buffer D in the upper reservoir and 1.2 $\times$ buffer D in the lower one) at 700 W; low percentage gels were run in buffer D at 30 W until the XC had migrated ~25 cm from the top of the gel. After electrophoresis, an autoradiogram of the gel was obtained at -80°C with Kodak X-Omat AR-5 film and a Cronex Lightning Plus intensifying screen.

For identification of crosslinked RNA-protein complexes, the samples were treated with RQ1 RNase-Free DNase (1 µl, 1 U/µl) for 10 min at 37°C, adjusted to 1 $\times$ buffer A, heated at 90°C for 3 min, and resolved on a 5% SDS-polyacrylamide gel (30 cm $\times$ 20 cm $\times$ 0.5 mm) in buffer F. Crosslinked proteins were visualized by autoradiography and the Coomassie Blue staining of the gel was used to establish the position of the polymerase subunits.

Evaluation of 8-APAS-ATP as an elongation substrate for *E.coli* RNA polymerase

DNA template 2 (10 nM) was attached to Streptavidin-Magnosphere paramagnetic beads (100 µg, Promega) in buffer B at room temperature. ApU (150 µM) and RNA polymerase holoenzyme (20 nM) were added, and the reaction was incubated at 30°C for 2 min. ATP, CTP, [α -³²P]GTP (4.4×10^6 c.p.m./pmol) were then added to 20 µM, and the reaction (200 µl) was incubated at 30°C for 3 min to form immobilized A-20 transcription complexes (RNA sequence is 5'-AUCGAGAGGG₁₀AC-ACGGCGAA₂₀UAGCCAUCCC₃₀AAUCGA). The RNA polymerase is arrested at position 20 by omitting UTP (21,22). Unincorporated nucleotides were then removed from the beads, and the beads were washed three times with buffer C (200 µl) and re-suspended in 200 µl of buffer. The beads containing the transcription complexes with A-20 RNAs were divided. To one half, 20 µM UTP and 200 µM 8-APAS-ATP were added to elongate the RNA to 22 nt (A-22). To the other half, 20 µM UTP and GTP and 200 µM 8-APAS-ATP were added to elongate the RNA to 23 nt. These elongation reactions were incubated at 30°C for 10 min. Control reactions containing ATP (20 µM), or no adenosine nucleotide, were run in parallel.

Incorporation of 8-APAS-AMP into full-length RNA

Elongation complexes (A-20s) were prepared as described above. For elongation of the A-20 RNA to full length, 20 µM each UTP, CTP, [α -³²P]GTP (4.4×10^6 c.p.m./pmol), 100 µM 8-APAS-ATP and ATP (0, 1, 20 or 100 µM) were added, and the reactions (50 µl) were incubated for 10 min at 37°C. A control reaction with 20 µM ATP and no analog was carried out in parallel. After 10 min, heparin was added to 40 µg/ml and each sample was divided. One half was kept in the dark at room temperature, and the other half was irradiated as described above.

Molecular modeling

Structures of the nucleotide analogs 8-APAS-AMP and 8-azido-AMP, and of the analog-containing oligonucleotides, were optimized using the AMBER force field (23) implemented in HyperChem (v5.1, Hypercube Inc.), running on a COMPAQ PentiumII computer. Generation of the necessary force-field parameters and treatment of electrostatics were carried out as described for 5-SF-UTP (24). Details of the modeling protocol are provided in the Supplementary Material (available at NAR Online).

Monte-Carlo conformational searches of the nucleoside monophosphates were carried out by varying torsion angles in the APAS/azido groups, the phosphodiester backbone torsion angles β and γ , the glycosyl torsion angle χ , and applying torsional flexing to the sugar ring. For the APAS analog, torsion angles t1-t4 were varied (Fig. 1). For the azido analog, the rotation around the C8-N bond (torsion angle t1) was allowed during the search. During the conformational searches of analog-containing DNA-RNA hybrids, torsion angles t1-t4 (APAS) or t1 (azido) and the glycosyl torsion angle χ were varied and the positions of the APAS/azide groups and the adenine base were optimized.

RESULTS

Structural characterization of 8-APAS-ATP

The presence of the aryl azide was verified by analysis of the absorption spectrum of the compound before and after irradiation with a 302 nm light source. When 8-APAS-ATP was exposed to 302 nm light, the absorption at 300 nm, corresponding to the aryl azide, was significantly reduced, indicating the azide was present. The half-life of the azide group was <15 s under our irradiation conditions.

The presence of the 5' phosphates on 8-APAS-ATP was verified by digestion with enzymes specific for the hydrolysis of nucleoside 5'-triphosphates to nucleosides (CIP) or cleavage of phosphodiester bonds to give 5'-monophosphates (SVP). When 8-APAS-ATP was subjected to enzymatic degradation with CIP and the reaction mixture was analyzed by RP HPLC, the resulting product eluted with the same retention time as the standard 8-APAS-adenosine.

TLC was used to analyze the digestion products of 8-APAS-ATP with CIP to form the nucleoside or with SVP to form the 5'-monophosphate. 8-APAS-ATP, which appeared as a single spot, exhibited an R_f value of 0.16. The spot turned brown when the plate was irradiated with a hand-held ultraviolet light. When the analog was applied to the TLC plate and the plate was irradiated before development, most of the product remained at the origin. This results from the covalent attachment of the analog to the plate, thereby preventing the migration of the compound. Both the color change and the crosslinking of the compound to the plate, which have been reported with other APAS analogs (4,5), are indicators of the presence of the azide group.

The digestion of 8-APAS-ATP with SVP and CIP resulted in the formation of two new products, 8-APAS-AMP and 8-APAS-A, the R_f values of which were 0.27 and 0.08, respectively. These results are slightly different from what has been found during the analyses of 5-APAS-CTP (5) and 5-APAS-UTP (4). This pyrimidine nucleoside 5'-triphosphates did not react with SVP. Control reactions with ATP show the conversion of ATP (R_f = 0.43) to AMP with SVP (R_f = 0.57) and to adenosine with CIP (R_f = 0.36). While the R_f values for the 8-APAS-labeled analogs are lower than the standard adenosine compounds, the order of mobility within the groups is the same: nucleoside, 5'-triphosphate and then 5'-monophosphate.

Evaluation of 8-APAS-ATP as an initiation substrate

The first nucleotide encoded by the *E. coli* RNA polymerase T7 A1 promoter is ATP. In this study, an attempt has been made to initiate transcription with 8-APAS-ATP, and it could not be incorporated at the 5' position of the nascent RNA by *E. coli* RNA polymerase (Fig. 2A, lane 7). Therefore, for *E. coli* RNA polymerase, 8-APAS-ATP does not serve as an initiation substrate.

Since the analog failed to initiate transcription, the dinucleotide ApU was used to initiate the synthesis of discrete transcripts of 4–8 nt. An RNA transcript 4 nt long was first synthesized with ApU, CTP, UTP and GTP (Fig. 2A, lanes 2 and 10). 8-APAS-ATP was then incorporated at position 5. This resulted in production of abortive RNA products (Fig. 2A, lanes 4 and 12, and B, lanes 2–4 and 7–9). DNA templates containing the bacteriophage

Figure 2. Evaluation of 8-APAS-ATP as a transcription substrate for *E. coli* RNA polymerase during initiation. (A) The autoradiogram of a 25% polyacrylamide/7 M urea gel is shown. Lanes 2–8 contain the non-irradiated control samples and lanes 9–15 contain the samples irradiated with 302 nm light. An aliquot of the nucleotide mix in buffer B was loaded in lane 1 to use as reference for single nucleotide mobility. In one set of reactions (lanes 2–4 and 10–12), transcription was initiated with the dinucleotide ApU (200 μ M) and 20 μ M CTP, UTP and [α - 32 P]GTP (lanes 2 and 10). In lanes 3 and 11, ATP (20 μ M) was also included, and lanes 4 and 12 contain 200 μ M 8-APAS-ATP and no ATP. In the second set of reactions (lanes 5–8 and 13–15) transcription was initiated with 20 μ M CTP, UTP and [α - 32 P]GTP (lanes 5 and 13), 20 μ M ATP, CTP, UTP and [α - 32 P]GTP (lanes 6 and 14), or with 200 μ M 8-APAS-ATP and no ATP (lanes 7 and 15). Lane 8 contains a reaction performed with 8-APAS-ATP pretreated with 302 nm light. RNA markers were loaded in lane 9. (B) The autoradiogram of a 20% polyacrylamide/7 M urea is shown. Lanes 1–4 contain the non-irradiated samples and lanes 5–9 contain the samples irradiated with 302 nm light. Lanes 1 and 6 show full-length RNA transcripts from the T7 A1 promoter (153 nucleotides). In lanes 2–4 and 7–9, the transcription reaction was initiated using 20 μ M CTP, UTP and [α - 32 P]GTP and increasing amounts of 8-APAS-ATP (20, 100 and 200 μ M). Lane 5 contains RNA markers, with sizes shown to the left (4mer, 5mer, 13mer and 22mer).

T7 A1 promoter form initial transcribing complexes (ITC), bearing chains of up to 8 nt, which are unstable and unable to continue elongation (25). Abortive initiation of the penta-nucleotide was increased when the RNA contained 8-APAS-AMP (compare lanes 3 and 4 in Fig. 2A). When ATP was present instead of 8-APAS-ATP, the majority of the transcripts produced were full length (Fig. 2A, lanes 3 and 11).

Following the addition of 8-APAS-ATP to form the 5mer, the reaction mixture was irradiated with a 302 nm light. After electrophoresis and autoradiography, the band for the 5mer that contains 8-APAS-AMP at the 3' end was visible only in the control reaction (no irradiation, Fig. 2A, lane 12, and B, lanes 7–9). Disappearance of the RNA transcript is consistent with the incorporation of the photocrosslinking group. Once the photocrosslinking group is activated, it can form covalent bonds with adjacent molecules (i.e., proteins, DNA or small

Figure 3. Specific incorporation of 8-APAS-AMP during elongation by *E. coli* RNA polymerase. (A) Sequence of the first 30 nt in the RNA from the T7 A1 promoter. (B) The autoradiogram of a 25% polyacrylamide/7 M urea is shown. Lanes 1–6 contain the non-irradiated samples and lanes 7–12 contain the samples irradiated with 302 nm light. Paused transcription complexes, containing RNA 20 nt long (lanes 1, 4, 7 and 10), were prepared from the T7 A1 promoter with ApU, [α - 32 P]GTP, CTP and ATP. These A-20 complexes were separated from unincorporated nucleotides and then incubated with ATP, CTP, GTP and UTP (20 μ M) and 8-APAS-ATP (200 μ M) as follows: lanes 2 and 8, UTP + ATP; lanes 3 and 9, UTP + ATP + GTP; lanes 5 and 11, UTP + 8-APAS-ATP; lanes 6 and 12, UTP + 8-APAS-ATP + GTP.

solvent molecules). These new crosslinked products have different mobilities than the 5mer, producing a diffuse band or complete disappearance of the band.

When 8-APAS-ATP was irradiated with 302 nm light before its use as a transcription substrate, full-length RNA was synthesized (Fig. 2A, lane 8). This is consistent with photoconversion of the analog to a product that destabilizes ITC less (causing less abortive initiation) and can be incorporated into RNA at adjacent positions (producing more full-length RNA). These same results are obtained when the analog is treated with thiol prior to transcription (data not shown). When reactions containing analog that had not been incorporated were irradiated, full-length RNA was synthesized as well (Fig. 2A, lanes 12 and 15).

Incorporation of 8-APAS-AMP internally and at the 3' end of RNA

To determine if ATP could be specifically replaced by 8-APAS-ATP during transcription with *E. coli* RNA polymerase, transcription complexes containing RNA 20 nt long were isolated. A biotinylated DNA template was used for immobilization of the ternary complexes on streptavidin-coated beads to facilitate removal of unincorporated nucleotides from the reaction. The nucleotides encoded after position 20 in this RNA are UA₂₂GC. The addition of UTP and ATP (Fig. 3, lanes 2 and 8) or UTP and 8-APAS-ATP (Fig. 3, lanes 5 and 11) to complexes containing the 20mer RNA resulted in elongation of the 20mer RNA to a 22mer. Addition of GTP resulted in

Figure 4. Crosslinking of analog-substituted RNA to RNA polymerase. The autoradiogram of a 5% SDS/polyacrylamide is shown. Lanes 1–5 contain the non-irradiated samples and lanes 6–10 contain the samples irradiated with 302 nm light. The first five lanes include the A-20 complexes (lanes 1 and 6) and the elongation products after addition of UTP and ATP (A-22, lanes 2 and 7) or UTP, ATP and GTP (G-23, lanes 3 and 8) or 8-APAS-ATP instead of ATP (lanes 4, 5, 9 and 10). The location of the polymerase subunits was determined by utilizing broad range protein molecular weight markers (Bio-Rad, Richmond, CA) which were visualized by Coomassie Blue staining of the gel before autoradiography (not shown).

elongation of the RNA to a 23mer (Fig. 3, lanes 3 and 9, containing ATP, lanes 6 and 12, containing 8-APAS-ATP).

The RNA products of 22 and 23 nt long (containing 8-APAS-AMP at position 22) failed to migrate into the gel after irradiation with 302 nm light (Fig. 3, lanes 11 and 12), which is indicative of photocrosslinking. When the RNA is crosslinked to proteins, it is retained in the wells of the high percentage gels. RNA, containing no analog, was unaffected by irradiation (Fig. 3, lanes 7–10). The double band observed in lanes 2 and 8 is probably due to deamination of A to I (which replaces G), which, in this case, allows the elongation of the RNA to 23 nt. This double band did not appear when ATP was replaced by 8-APAS-ATP (Fig. 3, lanes 5 and 11), suggesting either a higher K_m for incorporation just beyond the analog or that RNA polymerase cannot incorporate two 'analog' sequentially.

Verification of crosslinking of analog-modified RNA to protein

To determine if any of the RNA polymerase subunits were crosslinked to the RNA polymerase by the irradiation, the transcription reactions were analyzed on 5% SDS-PAGE gels. The 22mer, with 8-APAS-AMP at position 22, was crosslinked to the β, β' subunits, but not to σ (Fig. 4). This result is consistent with previous observations for RNAs of similar length containing 5-APAS-CMP (16mer) or 5-APAS-UMP (21mer) at the 3' end (21,26).

Incorporation of 8-APAS-AMP into the full-length RNA

Complete replacement of ATP by 8-APAS-ATP in the reaction did not result in elongation of the A-20 RNA to full-length transcript (Fig. 5, lane 1), therefore a small amount of ATP had to be added to the nucleotide mixture, as has been observed with the related pyrimidine analogs. This was due to the inability of the RNA polymerase to incorporate two 8-APAS-AMP analogs sequentially, as is the case for the related pyrimidine analogs. To allow elongation through regions of the DNA that encode two or more sequential AMP residues,

Figure 5. Internal incorporation of 8-APAS-AMP into the full-length transcript. An autoradiogram of a 5% polyacrylamide/7 M urea gel is shown. Samples loaded in lanes 1–5 were kept in reduced light conditions while lanes 6–10 contain the samples irradiated with 302 nm light. A-20 ternary transcription complexes were synthesized and the RNA was elongated with CTP, GTP ($[\alpha\text{-}^{32}\text{P}]\text{GTP}$), UTP and ATP (20 μM , lanes 5 and 10) or 8-APAS-ATP instead of ATP (100 μM , lanes 1 and 6). In lanes 2–4 and 7–9 a combination of 8-APAS-ATP (100 μM) and ATP (1 μM in lanes 2 and 7; 20 μM in 3 and 8; and 100 μM in 4 and 9) was included in the elongation reactions.

varying amounts of ATP were added to the transcription reactions that contained a fixed amount of analog. Control reactions were done at these different ATP concentrations in the absence of analog. This is to ensure that it is not the ATP alone that is responsible for the production of the full-length RNA, even when analog is present. At 99% analog (ATP at 1 μM , 8-APAS-ATP at 100 μM) full-length RNA was not synthesized (lane 2). At 80 or 50% 8-APAS-ATP (with ATP at 20 or 100 μM) full-length RNA was synthesized (lanes 3, 4, 8 and 9). To determine the minimal amount of ATP that needed to be present for production of full-length RNA, the ATP was varied from 1 to 100 μM (data not shown). For this transcript, the optimal ratio of 8-APAS-ATP to ATP was 20:1 (95% analog, 5 μM ATP), similar to our observation in previous work with the UTP analog 5-APAS-UTP (21). The presence of analog in the full-length transcript can be verified by the change in appearance on the autoradiogram of the RNA band after irradiation. Because of reaction of the nitrene upon irradiation (to solvent, RNA, DNA or protein) RNA bands that contain analog often disappear or appear diffuse after irradiation (lanes 8 and 9). Interestingly, if the 8-APAS-ATP is photo-activated before addition to the transcription reaction, full-length RNA can be synthesized even in the absence of added ATP (lanes 6 and 7).

Modeling of nucleoside monophosphates

Both 8-azido-AMP and 8-APAS-AMP were subjected to conformational analysis, which permitted an estimation of the effect of base substitution on the flexibility of the nucleotides. While the lowest energy conformation of 8-azido-AMP was found to adopt the *anti* orientation about the glycosyl bond ($\chi = -143^\circ$), characteristic of nucleotides (27), 8-APAS-AMP assumed the *syn* orientation ($\chi = 45^\circ$). This orientation avoids the steric clash between the sulfur atom of the APAS group and the sugar and phosphate group that would otherwise occur in the *anti* conformation. However, the energy of the *anti* conformation, in which 8-APAS-AMP adopts a more conventional glycosyl torsion angle ($\chi = -137^\circ$), was found to be only 0.8 kcal/mol higher.

Figure 6. Lowest energy *anti* conformations of analog nucleotides. The lowest energy *anti* structures for nucleotides 8-azido-AMP (left) and 8-APAS-AMP (right) are shown. First row, front view; second row, top view. The nucleotides are oriented in such a way that the base is on the right of the sugar and the phosphate is on the left of the sugar allowing defining of the APAS group position as being 'in front of' or 'behind' the base. Color coding: base, light blue; sugar, light gray; azide group, dark blue; C, dark gray; O, red; S, yellow; P, orange; the hydrogens have been omitted for clarity.

The lowest energy *anti* conformations of nucleotides 8-azido-AMP and 8-APAS-AMP are presented in Figure 6. The orientations of the APAS/azido groups in the nucleotides adopting the conventional *anti* conformation are shown. For 8-azido-AMP (left), this structure features the direction of the azido group away from the sugar in the plane of the base. For 8-APAS-AMP (right), the APAS group is positioned behind the base and its phenyl ring is π -stacked with the adenine base. This is probably due to the steric clash between phosphorous, sulfur and 3'-carbon atoms that a conformation with the front position would produce.

Modeling of DNA–RNA analog-modified hybrids

Oligonucleotides, containing 8-azido-AMP and 8-APAS-AMP at the 3' end or 1 nt away from the 3' end of the RNA strand, were modeled to investigate the conformational flexibility of the analogs while in the DNA–RNA hybrid, which is formed during transcription (24). Figure 7 shows the models of the 22mer oligonucleotides with the analogs in the terminal positions. In the case of 8-azido-AMP (left), the analog adopts the lowest energy conformation (yellow), in which glycosyl is *anti* and the azide group points up and away from the sugar. This conformation represents a significantly deep energy minimum, stabilized by the hydrogen bonding of the base with the complementary strand, which compensates for the steric demands of the azido group. Only two other conformations were found and they featured the *syn* glycosyl and two possible orientations of the azido group with respect to the sugar: up and away ($\Delta E = 5.76$, red) and down and towards ($\Delta E = 10.32$ kcal/mol, green). The above findings show the preference of 8-azido-A for the *anti* conformation, at least once incorporated into the RNA transcript. With respect to the photocrosslinking abilities of this analog, it could be seen that, if the analog assumes the *syn* conformation, the azido group is buried deep inside the

Figure 7. Conformations of DNA-RNA hybrids with analogs at terminal positions. The low energy conformations of 22mer DNA-RNA hybrids are shown. The RNA strand is shown in blue and the DNA strand in white, with the arrow tip corresponding to the 3' end of the DNA or RNA chain. The bottom row shows solvent accessible surfaces of the DNA and RNA strands. Left, rA22 = 8-azido-AMP; right, rA22 = 8-APAS-AMP. Analog conformation color coding: lowest energy, yellow; second in energy, red; third in energy, green. The hydrogens have been omitted for clarity.

duplex, while in the *anti* conformation it projects outside of the RNA surface.

Similar to 8-azido-AMP, 8-APAS-AMP (right) adopts *anti* glycosidic orientation in the lowest energy conformation (yellow). However, unlike 8-azido-AMP, 8-APAS-AMP demonstrates significant flexibility and produces 14 conformations within 2 kcal/mol above the lowest energy structure. These 14 conformations fall into three groups with respect to *syn/anti* orientation and the position of the APAS moiety. Namely, it projects into the solution in the 5' direction of the RNA and makes close contacts with residues rU21 and rA20 (lowest energy, yellow) or π -stacks with the adenine base of rA22 outside the helix ($\Delta E = 0.79$ kcal/mol, red; $\Delta E = 0.86$ kcal/mol, green). The representatives of these three groups are schematically depicted in Figure 8A. One very interesting attribute of all three groups is that the APAS group occupies the position in front of the base, unlike that found for the nucleoside monophosphate (Fig. 6). Once the analog is incorporated into the RNA transcript, the space behind the base gets occupied by another nucleobase and, therefore, becomes inaccessible. It is even more interesting to note that, notwithstanding the steric repulsion caused by the orientation in front of the base (see above), the lowest energy conformation of the analog is still *anti*, probably due to the stabilization by the hydrogen bonding with the complementary strand.

Figure 9 shows the low energy structures of the 23mer oligonucleotides with the analogs positioned 1 nt away from the

Figure 8. Schematic representation of the 8-APAS-AMP conformations in DNA-RNA hybrids. Horizontal arrows symbolize nucleobases with the RNA base on the left and DNA base on the right. Head-to-head arrows represent the standard Watson-Crick arrangement, in which both nucleotides are in the *anti* conformation (dotted line indicates hydrogen bonding); head-to-tail arrows represent an arrangement in which ribonucleotide rA22 adopts the *syn* conformation and disables Watson-Crick base pairing. (A) The analog is in the terminal position. Three representative conformations of 8-APAS-AMP are as follows. First (represented by the lowest energy structure) is characterized by the *anti* conformation and the APAS group contacts with rU21 and rA20. Second and third feature the APAS orientation, where it is π -stacked with the adenine base of rA22 outside the helix, and assume the *syn* and *anti* orientations, respectively. (B) The analog is 1 nt away from the 3' end. Two representative conformations of 8-APAS-AMP are as follows. In the first, represented by the lowest energy conformation, the APAS group is projected into solution towards the 5' end of the RNA and makes close contacts with rA20 and rU21. In the second, the APAS group is projected into solution towards the 3' end of the RNA and contacts residue rG23.

3' end of the RNA. For both analogs, the lowest energy conformations featured the *anti* orientation. In the case of 8-azido-AMP, two *syn* conformations were also found with ΔE being 6 and 11 kcal/mol. In the case of 8-APAS-AMP, the *syn* conformations were located as well, but represented only a small portion of all the conformations found and had $\Delta E = 6.6$ kcal/mol and above. For the hybrid, modified with 8-azido-AMP (left), the lowest energy conformation (yellow) features the azido group pointing up and away from the sugar in the plane of the base (similar to the analog at the terminal position). For the hybrid, modified with 8-APAS-AMP (right), nine conformations within 2 kcal/mol of the lowest energy structure have resulted, which fall into two groups differing in the orientation of the APAS group. Namely, it projects into the solution in either the 5' or 3' direction of the RNA. In the first case (lowest energy, yellow), it makes close contacts with the residues rU21 and rA20 and in the second case ($\Delta E = 1.42$ kcal/mol, red), it contacts residue rG23. The representatives of these two groups are schematically depicted in Figure 8B. Thus, the *syn/anti* conformational behavior of this analog has been shown to be similar both at the internal and terminal positions. However, the orientations of the photocrosslinker at the internal position of this analog differ from that at the terminal position and also from the orientations of the fluorescent group of the internally

Figure 9. Conformations of DNA–RNA hybrids with analogs at positions 1 nt away from the 3' end. The low energy conformations of 23mer DNA–RNA hybrids (structures within 2 kcal/mol of the lowest energy structure) are shown. Left, rA22 = 8-azido-AMP; right, rA22 = 8-APAS-AMP. Color coding is similar to Figure 7.

incorporated 5-thioacetamidofluorescein-uridine 5'-monophosphate (5-SF-UMP), which has a longer and more flexible linker (24). Namely, the length of the linker in the APAS group does not allow its phenyl group to π -stack with the terminal base when 8-APAS-AMP is 1 nt away from the 3' end of the RNA.

NMR characterization of 8-azido-ATP conformation

There was no NOE observed between the aromatic proton of the adenine base (H2) and sugar protons. The lack of an NOE between the sugar protons and the aromatic H2 proton indicates the absence of the close contact between these atoms (28). This may be interpreted as the *anti* conformation being favored or both *anti* and *syn* conformations being in rapid equilibrium in solution due to free rotation around the N9–C1' bond.

DISCUSSION

The synthesis and characterization of a novel photocrosslinking adenosine triphosphate is described. We have isolated this nucleotide analog by RP HPLC and identified it as 8-APAS-ATP. The structure was verified by enzymatic digestion with CIP to form the corresponding nucleoside, 8-APAS-adenosine. A comparison of both the ultraviolet and HPLC analyses of the enzymatically obtained and the chemically prepared nucleosides verified that the compounds were identical. Enzymatic degradation with SVP and the ability of the analog to serve as a substrate for transcription established the presence of the triphosphate moiety. Photolytic and crosslinking studies of both the nucleotide and the labeled RNA transcripts confirmed

the presence of the azide moiety. In addition, we have shown that this nucleotide can be incorporated at 3' terminal or internal positions within an RNA molecule by *E.coli* RNA polymerase but not at the 5' terminal position.

This compound represents the first reported photocrosslinking purine analog modified on a non-base pairing position which, following enzymatic incorporation into the nascent RNA by *E.coli* RNA polymerase, allows elongation of the transcript. The photoaffinity label of this analog is located at the 8 position of the adenosine base. This position was chosen because it is not involved in Watson–Crick base pairing. Therefore, normal secondary structure should form, providing there is no interference from the crosslinking arm. In our previous reports on the pyrimidine analogs, this indeed was the case (29,30). When the APAS moiety is attached to the 5 position of the pyrimidine base, a non-Watson–Crick base-pairing site, formation of the normal secondary structure of the nascent RNA was observed. Recently, the synthesis of another photocrosslinking analog of adenosine containing an aryl azide at the 8 position was reported (31). However, the ability of this compound, 8-[*N*-[(2-nitro-5-azidobenzoyl)-4-aminobutyl]amino]-adenosine 5'-triphosphate, to serve as a substrate for RNA polymerases has not been demonstrated.

While substitution at the 8 position does not interfere with base pairing it does have varying effects on compound substrate ability with RNA polymerase. For example, the spin-labeled analog, 8-amino(2,2,6,6-tetramethylpiperidine-*N*-oxyl)-adenosine 5'-triphosphate, is reported to be a good substrate for *E.coli* RNA polymerase. It is incorporated ~60% as effectively as ATP (32). 8-Azidoadenosine 5'-triphosphate, however, is an example of an analog that is incorporated by *E.coli* RNA polymerase at the 3' position but then appears to inhibit further elongation of the nascent RNA (17). Previously, these differences in activity were attributed to a steric hindrance or a transcription-‘incompatible’ *syn* nucleotide conformation resulting from the modification (17).

Analysis of the literature shows that no direct conformation determination for 8-azidoadenosine and its phosphates, neither in the solid state nor in solution, has been carried out. However, *syn* conformation seems to be a common notion (see for example 33), mainly based on the assumption that the steric demands of the 8-azido group are like those of the 8-bromo substituent (see for example 28,34) and that the steric effect of the substitution prevents them from adopting the *anti* conformation. This assumption is surprising since the van der Waals radii of N and Br atoms are significantly different: 1.5 and 1.95 Å, respectively (35). With regard to 8-Br purine nucleos(t)ides, their preference for *syn* conformation had been well documented by experimental evidence: X-ray (36), CD (37–39), NMR (40,41), enzymatic (42) and electronic energy calculations (43,44). However, 8-Br-ATP had been also found to adopt the *anti* conformation while complexed with enzymes (45,46 and references therein). Additionally, polymerization of 8-Br-ADP by polynucleotide phosphorylase had been achieved in the presence of ADP and GDP (47). Therefore, even if 8-azido nucleotides did prefer the *syn* conformation, by analogy to 8-Br nucleotides they should be able to undergo the conformational change in enzymatic reactions. Another group of experimental evidence in favor of 8-azido analogs' ability to adopt *anti* conformation is the findings of 8-azido-ATP binding to enzymes possessing ATP binding sites (33,48). Since ATP

preference for *anti* conformation is well known, it is reasonable to assume that enzyme recognition of 8-azido-ATP may be, at least in part, based on its *anti* conformation. Thus, this analysis calls for re-evaluation of the conformational properties of 8-azidoadenosine nucleotide, as well as the ability of 8-azido-ATP to serve as a transcription substrate.

In this work we have shown that 8-APAS-ATP can be used as an internal analog. It has been demonstrated in CD (38) and NMR (41) studies and also enzymatically (49) that 8-thio-purine substituents do cause the *syn* conformation of the adenosine analogs due to steric and electronic effects. Thus, we have demonstrated that the nucleotide analog with the established tendency for *syn* conformation can serve as a substrate for an internal incorporation.

In order to further investigate the conformational properties of 8-azido and 8-APAS adenosine nucleotides, we have carried out molecular modeling studies of these analogs both free and incorporated into DNA-RNA hybrids. This analysis resulted in surprising (compared to the accepted view on the conformational properties of 8-azidoadenosine nucleotides), but not entirely unexpected results. Specifically, it indicated the preference of 8-azido-AMP for the *anti* conformation and the preference of 8-APAS-AMP for the *syn* conformation. These modeling findings were strongly supported by the lack of an NOE between the H2 and sugar protons in the NOESY spectrum of 8-azido-ATP, which indicates that the azido group is not bulky enough to cause steric hindrance (28). Based on these results and on the above analysis of the previous work, we propose the *anti* conformation for 8-azidoadenosine nucleotide. We are speculating that either this nucleotide can be used as an internal analog, provided appropriate transcription conditions exist, or that the transcription substrate properties of nucleotide analogs can be determined by not only their conformational preference.

With respect to the 8-APAS-AMP, we found that it has a preference for the *syn* conformation, as expected. However, once incorporated into an RNA transcript, it adopts the *anti* conformation, both in terminal and internal positions in DNA-RNA hybrids. Also, although 8-APAS-AMP adopts *syn* in its lowest energy conformation, the energy increase to the *anti* conformation is only 0.8 kcal/mol. The preference of free 8-APAS-AMP to adopt the *syn* conformation may explain its inability to serve as an initiation substrate, because, in general, the substrate specificity for initiation is more stringent than for elongation.

The photoaffinity group chosen for the 8-APAS-ATP analog is *p*-azidophenacyl. This moiety, which is stable in the dark at room temperature, is easily activated with long wavelength UV light, thereby avoiding the damage to nucleic acids and proteins caused by irradiation with shorter wavelengths. The highly reactive nitrene species that is formed upon irradiation of the azide reacts rapidly and non-specifically. Most amino acids are believed to interact with this species (50). This non-specificity is particularly important when probing unknown interactions between nucleic acids and proteins. Since a specific amino acid does not need to be involved for covalent bond formation, the possibility that an interaction will go undetected is reduced. In addition, this non-specificity allows for a wider range of applications for this analog.

Previously, we utilized two pyrimidine analogs, 4-APAS-UTP and 4-APAS-CTP, to study various protein-RNA interactions occurring during transcription. The same photocrosslinking

moiety, used in 8-APAS-ATP, is present in these analogs. The arm for APAS is ~11 Å from the base. This allows one to probe an area within 2 nt 5' to and 1 nt 3' to the modified nucleotide when present in the nascent RNA. The length of the arm on these compounds is important to consider especially when used in mechanistic studies. If the arm is too long, extraneous crosslinks may occur which will complicate the analysis. When zero length groups are used, covalent bonds can only form when the two compounds are in direct or very close contact. In this case important interactions may go undetected. 6-Thioguanosine 5'-triphosphate is an example of this latter group, which has been recently reported as a substrate for T7 RNA polymerase (51). We developed the 8-APAS-ATP as a supplement to our photocrosslinking analogs. The availability of this purine analog will ensure that, even when studying purine-rich sequences, the detection of the nucleic acid-protein interactions is possible.

SUPPLEMENTARY MATERIAL

The Supplementary Material available at NAR Online contains modeling protocol (force field modification, charge derivation and optimization details) and atomic coordinates for the molecular models described in the paper.

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