Volume 245, number 1,2, 9–13 FEB 06875 March 1989

Preparation of 2-azidoadenosine 3′,5′-[5′-^{32}P]bisphosphate for incorporation into transfer RNA

Photoaffinity labeling of *Escherichia coli* ribosomes

Lee A. Sylvers*°, Jacek Wower*, Stephen S. Hixson+ and Robert A. Zimmermann*°

*Departments of *Biochemistry and +Chemistry* and °*Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003, USA*

Received 6 January 1989

2-Azidoadenosine was synthesized from 2-chloroadenosine by sequential reaction with hydrazine and nitrous acid and then bisphosphorylated with pyrophosphoryl chloride to form 2-azidoadenosine 3′,5′-bisphosphate. The bisphosphate was labeled in the 5′-position using the exchange reaction catalyzed by T_4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Polynucleotide kinase from a T_4 mutant which lacks 3′-phosphatase activity (ATP:5′-dephosphopolynucleotide 5′-phosphotransferase, EC 2.7.1.78) was required to facilitate this reaction. 2-Azidoadenosine 3′,5′-[5′-^{32}P]bisphosphate can serve as an efficient donor in the T_4 RNA ligase reaction and can replace the 3′-terminal adenosine of yeast tRNA^{Phe} with little effect on the amino acid acceptor activity of the tRNA. In addition, we show that the modified tRNA^{Phe} derivative can be photochemically cross-linked to the *Escherichia coli* ribosome.

Photoreactive nucleotide; Polynucleotide kinase; RNA ligase; tRNA; Photoaffinity labeling; Ribosome

1. INTRODUCTION

The photoreactive compounds, 2-azidoadenosine ($2N_3A$) and 8-azidoadenosine ($8N_3A$), have been used extensively to probe nucleotide-binding sites in protein molecules [1,2]. Recently, we have used tRNAs containing $8N_3A$ to study tRNA-binding sites on the *Escherichia coli* ribosome [3]. In the latter work, $8N_3A$ was incorporated into tRNA as its 3′,5′-[5′-^{32}P]bisphosphate ([5′-^{32}P]-p$8N_3$Ap) using T_4 RNA ligase. Since the photoreactive portions of $2N_3A$ and $8N_3A$ lie on opposite sides of the purine ring, we anticipated that the use of $2N_3A$-modified tRNAs as probes of ribosome structure might provide information complementary to that obtained with the $8N_3A$ analog. Furthermore, we found that replacement of the 3′-terminal adenosine of tRNA^{Phe} with $8N_3A$ abolished the amino acid acceptor activity of this tRNA [3], and we were hopeful that the relocation of the azido group to the 2-position of adenosine would permit aminoacylation of the corresponding tRNA^{Phe} derivative. Accordingly, we have prepared 2-azidoadenosine 3′,5′-bisphosphate (p$2N_3$Ap). The identification of species cross-linked by azidonucleotide-modified RNAs is facilitated by the introduction of a radioactive label into the probe. For this purpose, we elected to use the T_4 polynucleotide kinase (PNK) exchange reaction to replace the 5′-phosphate of

Correspondence address: R.A. Zimmermann, Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, USA

Abbreviations: $2N_3A$, 2-azidoadenosine; $8N_3A$, 8-azidoadenosine; $2N_3$AMP, 2-azidoadenosine 5′-monophosphate; pAp, adenosine 2′,5′- and 3′,5′-bisphosphates (mixed isomers); p$2N_3$Ap, 2-azidoadenosine 2′,5′- and 3′,5′-bisphosphates (mixed isomers); p$8N_3$Ap, 8-azidoadenosine 2′,5′- and 3′,5′-bisphosphates (mixed isomers); AcPhe-, *N*-acetylphenylalanyl-; PNK, T_4 polynucleotide kinase; PEI, polyethyleneimine

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

p2N$_3$Ap with ^{32}P. While the PNK exchange reaction has been widely used for the 5′-end-labeling of oligonucleotides with an existing 5′-phosphate [4,5], the use of this reaction for the 5′-labeling of nucleoside 3′,5′-bisphosphates has, to our knowledge, never been reported.

Here, we describe the preparation of p2N$_3$Ap, the successful 5′-^{32}P labeling of this bisphosphate with 3′-phosphatase-free PNK, and the use of the radiolabeled product for constructing photoreactive tRNAs that can be both aminoacylated and cross-linked to the P site of *E. coli* ribosomes.

2. MATERIALS AND METHODS

2.1. *Materials*

Adenosine, 2-chloroadenosine, adenosine 5′-monophosphate, adenosine 3′-monophosphate, adenosine 2′,5′-bisphosphate, adenosine 2′,5′- and 3′,5′-bisphosphate (mixed isomers), anhydrous hydrazine and pyrophosphoryl chloride were purchased from Sigma. Yeast tRNA^{Phe} (specific amino acid acceptance, 1100 pmol/A_{260} unit) and T_4 polynucleotide kinase (wild-type and 3′-phosphatase-free) were products of Boehringer Mannheim. T_4 RNA ligase was obtained from Pharmacia. Crude [γ-^{32}P]ATP (7000 Ci/mmol) was purchased from ICN Biochemicals. PEI-cellulose plates were from Macherey & Nagel. All other reagents were of reagent grade and were obtained from Fisher.

2.2. *Synthesis of p2N$_3$Ap*

2-Azidoadenosine 3′,5′-bisphosphate was synthesized as depicted in fig.1. 2-Chloroadenosine was reacted sequentially with hydrazine and nitrous acid as described by Schaeffer and Thomas [6], and the identity of the product was confirmed by its characteristic ultraviolet spectrum and by the response of the spectrum to changes in pH [7]. 2-Azidoadenosine was then bisphosphorylated with pyrophosphoryl chloride [8]. After chromatography of the reaction products on a DEAE-cellulose column, a mixture of 2′,5′- and 3′,5′-bisphosphates was obtained.

2.3. *^{32}P labeling of 2-azidoadenosine 3′,5′-bisphosphate*

Reaction mixtures containing 0.25 mM 2-azidoadenosine 2′,5′- and 3′,5′-bisphosphates, 0.25 mM ADP and 83 μCi [γ-^{32}P]ATP were incubated with 10 units of 3′-phosphatase-free PNK for 1 h at 37°C in 50 mM imidazole-HCl (pH 6.6), 10 mM $MgCl_2$, and 0.1 mM EDTA. The enzyme was then inactivated by heating at 100°C for 1 min. Products were analyzed by thin-layer chromatography on PEI-cellulose plates using 0.75 M KH_2PO_4, adjusted to pH 3.5 with HCl, as the mobile phase [8].

2.4. *Incorporation of [5′-^{32}P]p2N$_3$Ap into tRNA^{Phe}*

2-Azidoadenosine 3′,5′-[5′-^{32}P]bisphosphate was incorporated into the 3′-position of yeast tRNA^{Phe} lacking its 3′-terminal adenosine [tRNA^{Phe}(– A76)] using T_4 RNA ligase as in [3]. Since nucleoside 2′,5′-bisphosphates are neither substrates for, nor inhibitors of, RNA ligase [10], separation of the nucleotide isomers was not necessary. To determine the extent of incorporation, aliquots were precipitated with trichloroacetic acid and analyzed in a scintillation counter [11].

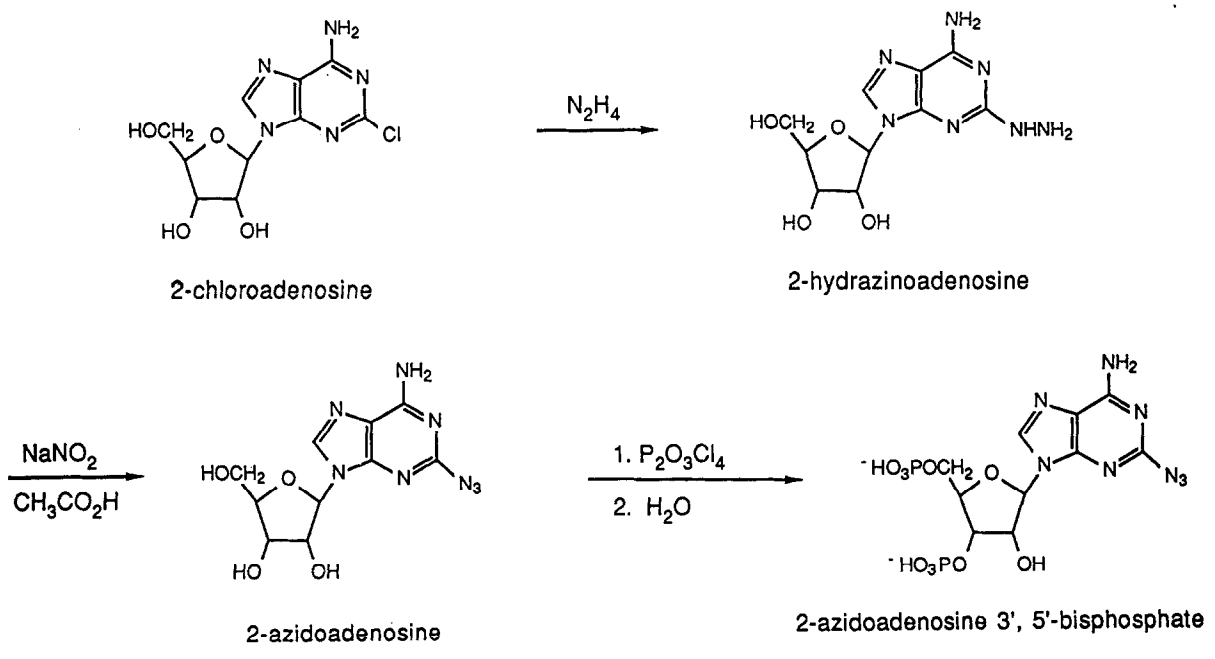

Fig.1. Synthesis of 2-azidoadenosine 3′,5′-bisphosphate from 2-chloroadenosine.

2.5. *Aminoacylation of [2N₃A76]tRNA^{Phe}*

[2N₃A76]tRNA^{Phe} was aminoacylated and then acetylated according to Wower et al. [3].

2.6. *Formation and irradiation of tRNA-ribosome complexes*

The noncovalent binding of [2N₃A76]tRNA^{Phe} and Ac[^{3}H]-Phe-[2N₃A76]tRNA^{Phe} to *E. coli* 70 S ribosomes and subsequent irradiation of the complexes with 300-nm light were carried out as described [3].

3. RESULTS AND DISCUSSION

We investigated the 5′-^{32}P labeling of p2N₃Ap via the PNK-catalyzed exchange reaction using both the wild-type and 3′-phosphatase-free enzymes. When the wild-type enzyme was utilized, a small amount of [5′-^{32}P]2N₃AMP was formed with most of the [γ-^{32}P]ATP remaining unreacted (fig.2a, lane 2). Thus, although the wild-type PNK evidently carried out the 5′-exchange reaction, both the substrate (p2N₃Ap) and the product ([5′-^{32}P]p2N₃Ap) were dephosphorylated to 2N₃AMP by the 3′-phosphatase activity of the enzyme. As a consequence, the exchange reaction was terminated and yielded no [5′-^{32}P]p2N₃Ap. Our results are consistent with the observation of Cameron and Uhlenbeck [12] that the 3′-phosphatase activity of PNK is maximal at the optimum pH of the exchange reaction. By contrast, when the exchange reaction was performed with 3′-phosphatase-free PNK, efficient 5′-^{32}P labeling of p2N₃Ap was accomplished (fig.2a, lane 3).

The fate of the 2′,5′-bisphosphate isomer of 2-azidoadenosine present in the p2N₃Ap preparation was assessed by testing the ability of adenosine 2′,5′-bisphosphate to serve as a substrate for the exchange reaction. We found that this compound is not a substrate when either the wild-type or mutant enzyme is used for catalysis, and we assume that the azido analog is similarly unreactive. This finding is not surprising in view of an earlier demonstration by Richardson [13] that the presence of a 3′-phosphate is an absolute requirement for 5′-phosphorylation by PNK. We also observed that, unlike adenosine 3′,5′-bisphosphate, the 2′,5′ isomer was not dephosphorylated by either form of PNK. The high regiospecificity of the 3′-phosphatase activity can therefore be of use for the selective removal of the 3′-phosphate from nucleotides.

To evaluate the photoreactivity of [5′-^{32}P]-p2N₃Ap, the compound was irradiated at 300 nm for 10 min after deposition on a PEI-cellulose TLC plate. After chromatography two new spots were evident (fig.2b, lane 2). [5′-^{32}P]p2N₃Ap that had become cross-linked to the PEI-cellulose remained at the origin. The spot that migrated slightly slower than [5′-^{32}P]p2N₃Ap represents an additional photoproduct, the identity of which is currently under investigation. It has been demonstrated that between pH 3 and 11, solutions of 2N₃A nucleotides exist in an approximately equimolar mixture of photoreactive azide and nonphotoreactive tetrazole tautomeric forms [7]. Our observation that all of the p2N₃Ap reacted when irradiated may indicate that the tetrazoles are converted to the azide during the course of the photolysis.

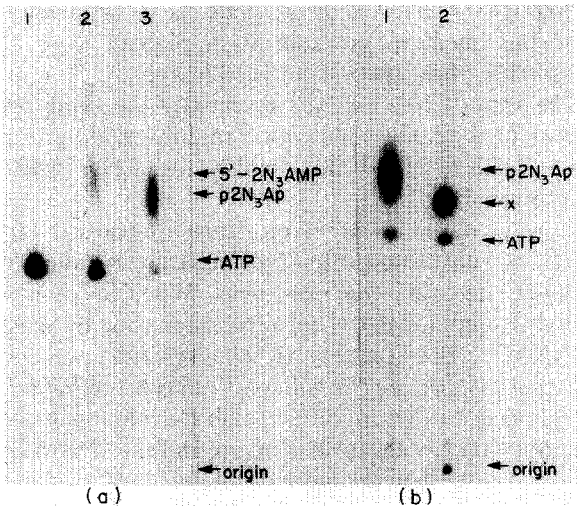

Fig.2. Synthesis and photoreactivity of [5′-^{32}P]p2N₃Ap. (a) Analysis of the exchange reaction by TLC on PEI-cellulose. A sample of [γ-^{32}P]ATP (1) and equivalent aliquots from exchange reaction mixtures containing either the wild-type PNK (2) or the 3′-phosphatase-free PNK (3) were applied to a PEI-cellulose sheet and developed according to Reeve and Huang [9]. (b) Analysis of the photoreactivity of [5′-^{32}P]p2N₃Ap by TLC. Two aliquots of [5′-^{32}P]p2N₃Ap were applied to the origin and either kept in the dark (1) or irradiated at 300 nm for 10 min (2) prior to chromatography. Product x represents an additional photoproduct yet to be characterized.

The efficacy of [5′-^{32}P]p2N₃Ap as a donor in the RNA ligase reaction was tested using tRNA^{Phe} lacking its 3′-terminal adenosine as the acceptor. The kinetics of addition of [5′-^{32}P]p2N₃Ap to the truncated tRNA^{Phe} molecule was compared with

that of [5′-^{32}P]pAp. In each case the incorporation of the bisphosphate rose to a plateau level of 88–95% of the theoretical yield in approx. 50 min. The rate of ligation was essentially the same for both of the donors indicating that the azido group at position 2 did not inhibit the RNA ligase reaction.

In a recent study from this laboratory [3], tRNAs substituted with 8N_3A were cross-linked to the P site of *E. coli* ribosomes. When 8N_3A was used to replace the 3′-terminal adenosine of yeast tRNA^{Phe}, the resulting tRNA derivative was no longer a substrate for aminoacyl tRNA synthetase. This may be due to the fact that 8-azidoadenosine adopts the *syn* conformation around the glycosidic bond rather than the more usual *anti* conformation [14]. Therefore, it was of interest to see if tRNA^{Phe} with 2-azidoadenosine in place of the 3′-terminal adenosine could be aminoacylated. As demonstrated in fig.3, the maximum charging capacity of [2N_3A76]tRNA^{Phe} was 83% of that found for tRNA^{Phe} prepared by ligation of pAp to tRNA^{Phe}(– A76). Although the yield of Phe-tRNA^{Phe} could most likely be improved by the addition of 5 mM dithiothreitol or 2-mercaptoethanol, these thiols must be avoided in the aminoacylation of [2N_3A76]tRNA^{Phe} for they lead to reduction of the azido group [15] and thus destroy its photoreactivity. The fact that [2N_3A76]tRNA^{Phe} but not [8N_3A76]tRNA^{Phe} could be aminoacylated, and that 2N_3A exists in the *anti* conformation [16], may indicate that the conformation adopted by the 3′-terminal nucleotide of tRNA is important for aminoacyl tRNA synthetase recognition.

Both nonaminoacylated [2N_3A76]tRNA^{Phe} and Ac[^{3}H]Phe-[2N_3A76]tRNA^{Phe}, with a ^{32}P label adjacent to the 2N_3A moiety, were bound to poly(U)-programmed ribosomes at a molar tRNA ribosome input ratio of 1:4. The noncovalent tRNA-ribosome complexes were irradiated with 300 nm light for 12 min at which time cross-linking reached its maximum. It was found that 11% of the nonaminoacylated tRNA and 13% of the aminoacylated tRNA had become covalently bound to ribosomes. Cross-linking was poly(U)-dependent; when poly(U) was omitted, only 0.3–0.7% of either tRNA was cross-linked to the ribosome. Treatment of the covalent tRNA^{Phe}-ribosome complexes with puromycin resulted in the release of

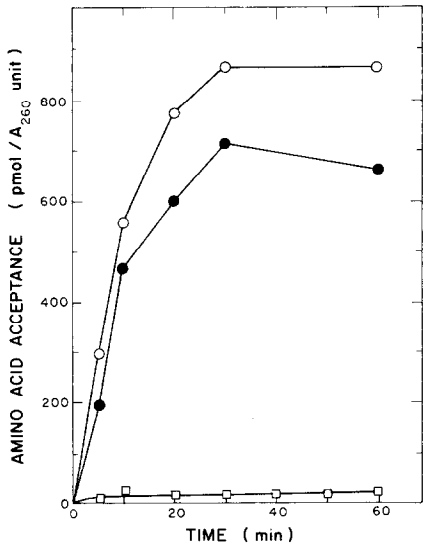

Fig.3. Aminoacylation of reconstructed native tRNA^{Phe} and [2N_3A76]tRNA^{Phe}. (○) tRNA^{Phe}; (●) [2N_3A76]tRNA^{Phe}; (□) tRNA^{Phe}(– A76) as a control.

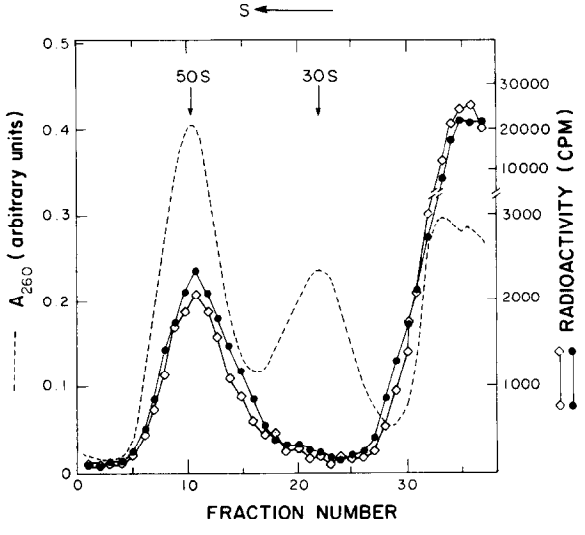

Fig.4. Cross-linking of AcPhe-[2N_3A76]tRNA^{Phe} and [2N_3A76]tRNA^{Phe} to the 50 S subunit. Noncovalent complexes of AcPhe-[^{32}P][2N_3A76]tRNA^{Phe} and [^{32}P][2N_3A76]tRNA^{Phe} with poly(U) and 70 S ribosomes were formed and irradiated [3]. The irradiated complexes were centrifuged through 10–30% sucrose gradients in 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 0.25 mM $MgCl_2$, and 0.05% 2-mercaptoethanol at 40000 rpm for 100 min at 4°C in a Beckman VTi50 rotor. (–) A_{260}; (◇) AcPhe-[2N_3A76]tRNA^{Phe}; (●) [2N_3A76]tRNA^{Phe}.

more than 96% of the Ac[^{3}H]Phe, indicating that the cross-linked Ac[^{3}H]Phe-[2N$_3$A76]tRNA^{Phe} was located in the P site. We assume that [2N$_3$A76]tRNA^{Phe} was bound to the P site as well, since it is well documented that nonaminoacylated tRNAs bind with a strong preference for the P site [17,18], and because binding of nonaminoacylated [2N$_3$A76]tRNA^{Phe} was carried out under the same conditions as used for the aminoacylated tRNA. Separation of the covalent tRNA-ribosome complexes into subunits by sucrose gradient centrifugation at 0.25 mM Mg^{2+} revealed that all of the cross-linked tRNA was attached exclusively to 50 S ribosomal particles, as indicated by the distribution of the ^{32}P label (fig.4). Distribution of the label between the components of the 50 S subunit is currently under investigation.

The incorporation of photoreactive, ^{32}P-labeled nucleotides into RNA molecules provides a valuable tool for studying RNA-RNA and RNA-protein interactions in ribonucleoprotein complexes. The simple procedure described here for the preparation and labeling of p2N$_3$Ap should therefore be generally applicable to many experimental problems. In addition, the PNK exchange reaction should prove amenable to the labeling of a wide variety of nucleoside 3′,5′-bisphosphates.

Acknowledgement: This work was supported by Research Grant GM22807 from the National Institutes of Health.

REFERENCES

[1] Haley, B.E. (1983) Fed. Proc. 42, 2831–2836.
[2] Cusack, N.J. and Born, G.V.R. (1976) Proc. R. Soc. Lond. B 197, 307–311.
[3] Wower, J., Hixson, S.S. and Zimmermann, R.A. (1988) Biochemistry 27, 8114–8121.
[4] Van de Sande, J.H., Kleppe, K. and Khorana, H.G. (1973) Biochemistry 12, 5050–5055.
[5] Chaconas, G., Van de Sande, J.H. and Church, R.B. (1975) Biochem. Biophys. Res. Commun. 66, 962–969.
[6] Schaeffer, J.H. and Thomas, J.H. (1958) J. Am. Chem. Soc. 80, 3738–3742.
[7] MacFarlane, D.E., Mills, D.C.B. and Srivastava, P.C. (1982) Biochemistry 21, 544–549.
[8] Barrio, J.R., Barrio, M.C.G., Leonard, N.J., England, T.E. and Uhlenbeck, O.C. (1978) Biochemistry 17, 2077–2081.
[9] Reeve, A.E. and Huang, R.C. (1983) Anal. Biochem. 130, 14–18.
[10] England, T.E. and Uhlenbeck, O.C. (1978) Biochemistry 17, 2069–2076.
[11] England, T.E., Bruce, A.G. and Uhlenbeck, O.C. (1980) Methods Enzymol. 65, 65–74.
[12] Cameron, V. and Uhlenbeck, O.C. (1977) Biochemistry 16, 5120–5126.
[13] Richardson, C.C. (1971) in: Proc. Nucleic Acid Res. (Cantoni, G.L. and Davies, D.R. eds) vol.2, pp.815–828.
[14] Sarma, R.H., Lee, C.H., Evans, F.E., Yathindra, N. and Sundaralingam, M. (1974) J. Am. Chem. Soc. 96, 7337–7348.
[15] Cartwright, I.L., Hutchinson, D.W. and Armstrong, V.W. (1976) Nucleic Acids Res. 3, 2331–2339.
[16] Czarnecki, J.J. (1984) Biochim. Biophys. Acta 800, 41–51.
[17] De Groot, N., Panet, A. and Lapidot, Y. (1971) Eur. J. Biochem. 23, 523–527.
[18] Wurmbach, P. and Nierhaus, K.H. (1979) Proc. Natl. Acad. Sci. USA 76, 2143–2147.