Photochemical Labeling of Bovine Pancreatic Ribonuclease A with 8-Azidoadenosine 3′,5′-Bisphosphate†

Jacek Wower,‡ Michael Aymie,‡ Stephen S. Hixson,§ and Robert A. Zimmermann*,‡

Departments of Biochemistry and Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

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ABSTRACT: A simple method has been developed for the preparation of 5′-^{32}P-labeled 8-azidoadenosine 3′,5′-bisphosphate ($p8N_3Ap$) for use in photoaffinity labeling studies. Irradiation of a complex between $p8N_3Ap$ and bovine pancreatic ribonuclease A (RNase A) with light of 300–350 nm led to the covalent attachment of the nucleotide to the enzyme. RNase A could also be labeled in the dark with prephotolyzed $p8N_3Ap$. In either case, the nucleotide reacted with the same tryptic peptide, encompassing amino acids 67–85 of the protein. The site of labeling was determined to be either Thr-78 or Thr-82, both of which are close to or at the pyrimidine binding site of the enzyme. This result is consistent with recent nuclear magnetic resonance and X-ray studies which indicate that 8-substituted adenine nucleotides interact with the pyrimidine binding site of RNase A.

The 8-azido analogues of adenosine nucleotides have proven to be useful probes of nucleotide binding sites in several proteins (Haley, 1983). To date, the most frequently utilized analogues have been 8-azidoadenosine 3′,5′-cyclic-monophosphate and 8-azidoadenosine 5′-triphosphate. We have developed a procedure for preparing ^{32}P-labeled 8-azidoadenosine 3′,5′-bisphosphate which involves alkaline hydrolysis of $8N_3cAMP$[1] to a mixture of 3′- and 5′-monophosphates followed by phosphorylation of the 3′-monophosphate with T4 polynucleotide kinase to produce 5′-^{32}P-labeled $p8N_3Ap$. This purine analogue may also be used for affinity labeling of proteins. In addition, it has proven to be a good substrate for T4 RNA ligase (Wower et al., 1988); it can therefore be incorporated into virtually any RNA molecule and used to study the topography of RNA–protein complexes.

In this paper, we describe a simple method for the preparation of $p8N_3Ap$ and demonstrate that this reagent can label RNase A in a site-specific manner. RNase A was used in the present study both because there is a sizable amount of structural data available for this enzyme and because it has the ability to bind both pyrimidine and purine nucleotides (Richards & Wyckoff, 1971; Blackburn & Moore, 1982). Earlier photochemical cross-linking studies showed that RNase A can be labeled with pUp when its noncovalent complexes with this nucleotide are irradiated with UV light (Sperling & Havron, 1976). Interestingly, the sites of cross-linking for pUp (Havron & Sperling, 1977) and $p8N_3Ap$ are located in the same region of RNase A. This fact provides additional evidence that 8-substituted adenosine nucleotides bind to the pyrimidine binding site of the enzyme (Arus et al., 1981, 1982; Borkakoti et al., 1983).

Experimental Procedures

Materials. RNase A and trypsin were purchased from Worthington. T4 polynucleotide kinase was the product of Pharmacia. 3′-AMP, 3′-CMP, $8N_3cAMP$, acrylamide, and *N*,*N*′-methylenebis(acrylamide) were obtained from Sigma. Crude [γ-^{32}P]ATP was purchased from ICN Biochemicals. CEL 300 and PEI–cellulose plates were from Macherey & Nagel. All other chemicals were of reagent grade. ^{3}H-Labeled ribosomal RNA from *Escherichia coli* was prepared as previously described (Zimmermann, 1979).

Preparation of [5′-^{32}P]-8-Azidoadenosine 3′,5′-Bisphosphate. $8N_3cAMP$ (9.3 mg) was dissolved in 50 μL of 0.3 M NaOH and 1 mM EDTA, hydrolyzed at 37 °C for 5 h, and then neutralized with HCl. Under these conditions about 40% of the $8N_3cAMP$ was converted to its 3′-monophosphate. The resulting solution was kept at −20 °C in the dark as a stock. Storage for up to 1 year under these conditions caused no decrease in the ability of the 3′-monophosphate to act as a substrate for T4 polynucleotide kinase. [5′-^{32}P]-8-Azidoadenosine 3′,5′-bisphosphate was prepared by a modification of the procedure of England et al. (1980). A 20-μL reaction mixture, containing 25 mM K-CHES, pH 9.5, 5 mM $MgCl_2$, 1 mM $8N_3cAMP$ hydrolysate, 5 μM [γ-^{32}P]ATP, and 250 units/mL T4 polynucleotide kinase, was incubated on ice overnight and then for 30 min at 37 °C. When [5′-^{32}P]-$p8N_3Ap$ was to be used for the labeling of RNase A, the concentration of ATP was increased to 400 μM in order to drive the phosphorylation reaction to completion and thereby increase the yield of the nucleoside bisphosphate. The products were analyzed by thin-layer chromatography on PEI–cellulose plates using 0.75 M KH_2PO_4 adjusted with HCl to pH 3.5 as the mobile phase (Reeve & Huang, 1983).

[5′-^{32}P]pAp and [5′-^{32}P]pCp were prepared by phosphorylation of 3′-AMP and 3′-CMP as described by England et al. (1980).

Irradiation. Solutions containing 30 μM RNase A and 30 μM [5′-^{32}P]$p8N_3Ap$ were prepared in appropriate buffers as indicated in the text. The 20–100-μL samples of these solutions were deposited as single droplets on Petri dishes partially immersed in ice to maintain a temperature of 0 °C. The depth of the droplets was approximately 1 mm. The Petri dishes were placed in a Rayonet photochemical reactor (Model RPR-100) and irradiated with four 2537-Å, 3000-Å, or

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‡ Department of Biochemistry.

§ Department of Chemistry.

[1] Abbreviations: $8N_3cAMP$, 8-azidoadenosine 3′,5′-cyclic-monophosphate; $p8N_3Ap$, 8-azidoadenosine 3′,5′-bisphosphate; Asx, asparagine or aspartic acid; Glx, glutamine or glutamic acid; PEI, poly(ethylenimine); RNase A, bovine pancreatic ribonuclease A.

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3500-Å lamps, depending on the experiment. The distance between the lamps and the samples was approximately 5 cm. Aliquots of 10 μL of the irradiated enzyme–nucleotide mixtures were analyzed on 15% polyacrylamide slab gels according to Laemmli and Favre (1973). Labeled bands were located by autoradiography and excised from the gel, and the radioactivity in each was determined by Čerenkov counting. Results were expressed as mole percent of nucleotide incorporated into RNase (moles of nucleotide/mole of RNase × 100).

Ribonuclease Activity. RNase activity was assayed by a modification of the method of Kalnitsky et al. (1959). A 20-μL sample of the irradiated RNase–nucleotide mixture was mixed with 20 μL of 200 mM sodium acetate, pH 5.0, and 40 μL of a 1% (w/v) solution of ^{3}H-labeled ribosomal RNA in 100 mM sodium acetate, pH 5.0. After incubation for 10 min at 37 °C, the reaction was terminated by the addition of 40 μL of 25% perchloric acid containing 0.75% uranyl acetate. This mixture was allowed to stand in an ice bath for 10 min and then clarified by centrifugation. Aliquots of 60 μL of the supernatant were added to 10 mL of Aquasol (New England Nuclear) and analyzed for radioactivity by scintillation counting.

Oxidation of RNase. When needed, RNase A was denatured by oxidation with performic acid as described by Hirs (1967).

Protease Digestion and Peptide Analysis. Covalent complexes between RNase A and [5′-^{32}P]p8N_3Ap were extracted from polyacrylamide gel slices, precipitated with 5 volumes of cold acetone in the presence of 20 μg of unlabeled RNase A at −20 °C for 10–12 h, and then subjected to performic acid oxidation. The oxidized RNase A was dissolved in 0.5% morpholine–formate buffer, pH 8.0, and digested by incubation for 5 h at 37 °C with trypsin (final concentration, 2 μg/mL). The digest was lyophilized and then fingerprinted on CEL 300 thin-layer plates with the two-dimensional electrophoresis/chromatography system of Hitz et al. (1977). The plates were then subjected to autoradiography, and the labeled peptides were extracted with 50% (v/v) acetic acid and lyophilized. The pattern of unlabeled peptides was checked by staining with ninhydrin. Each radioactive peptide was analyzed for its amino acid composition and subjected to N-terminal sequence analysis according to the procedure of Chang et al. (1978). Amino acid analyses were performed with a Durrum D-500 amino acid analyzer as described by Hitz et al. (1977). Peptides were identified by reference to the complete amino acid sequence of RNase A (Smyth et al., 1963).

Results

Preparation of [5′-^{32}P]-8-Azidoadenosine 3′,5′-Bisphosphate. 8N_3cAMP was hydrolyzed with 0.3 N NaOH in the presence of 1 mM EDTA to yield a mixture of 8-azidoadenosine 3′- and 5′-monophosphates. The nucleoside 3′-monophosphate present in the hydrolysate was then phosphorylated with T4 polynucleotide kinase using [γ-^{32}P]ATP to yield [5′-^{32}P]p8N_3Ap. The products of the synthesis were analyzed by thin-layer chromatography on PEI–cellulose plates. Figure 1a shows that all of the ^{32}P in [γ-^{32}P]ATP is converted to [5′-^{32}P]p8N_3Ap under the conditions of the experiment shown. When the reaction mixture was exposed to UV light after deposition on the plate but prior to chromatography, [5′-^{32}P]p8N_3Ap remained fixed at the origin (Figure 1, lane b). This demonstrated that the azidonucleotide is photoreactive in the presence of UV light. Figure 1 (lane d) shows that [γ-^{32}P]ATP is not cross-linked to the plate under our conditions. We have demonstrated that the crude [5′-^{32}P]p8N_3Ap preparation can be used without further purification for the incorporation of the modified nucleotide into tRNA with T4 RNA ligase (Wower et al., 1988). Experiments described below show that the crude preparation of [5′-^{32}P]p8N_3Ap can also be used for affinity labeling of proteins.

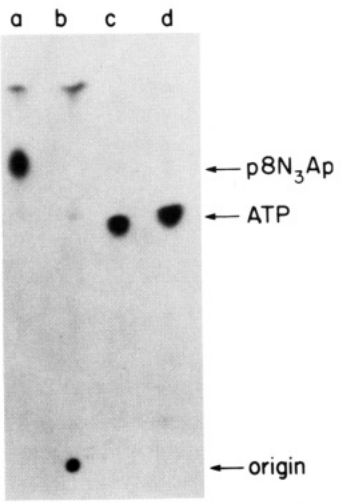

FIGURE 1: Thin-layer chromatography of [5′-^{32}P]p8N_3Ap on PEI–cellulose. Aliquots of the reaction mixtures containing (a) [5′-^{32}P]p8N_3Ap and (c) [γ-^{32}P]ATP were applied to a PEI–cellulose sheet and developed according to Reeve and Huang (1983). To test the photoreactivity of [5′-^{32}P]p8N_3Ap, equivalent aliquots of (b) [5′-^{32}P]p8N_3Ap and (d) [γ-^{32}P]ATP were applied to a PEI–cellulose sheet and irradiated in situ with 300-nm light prior to chromatography. Note that photolysis fixed p8N_3Ap to the origin.

Irradiation of [5′-^{32}P]p8N_3Ap–RNase Complexes with UV Light. The exposure of [5′-^{32}P]p8N_3Ap to UV light in the presence of RNase A resulted in the covalent attachment of the nucleotide to the protein. Since the irradiation of RNase A with UV light at 254 nm leads to a rapid inactivation of the enzyme (Schulz et al., 1975), the incorporation of p8N_3Ap into RNase A was studied at longer wavelengths. When 300-nm light was used for irradiation, labeling was essentially complete within 20 min. At that time the incorporation of ^{32}P-labeled nucleotide into RNase A was approximately 40% (Figure 2). Irradiation at 350 nm led to somewhat lower levels of nucleotide incorporation which reached about 25% after a 20-min exposure. The extent of labeling varied with pH and was maximal at pH 7.2, for an equimolar p8N_3Ap–RNase mixture. The specificity of [5′-^{32}P]p8N_3Ap labeling was tested by employing RNase denatured by performic acid oxidation as a substrate. As indicated in Figure 2, [5′-^{32}P]p8N_3Ap incorporation into denatured RNase A was no more than 6% and 4% after 20 min of irradiation with 300- and 350-nm light, respectively. This result indicates that the native enzyme structure is required for efficient photoincorporation of p8N_3Ap into RNase A.

Photolysis of p8N_3Ap in the presence of RNase A resulted in the rapid and irreversible loss of enzymatic activity (Figure 3). From 40% to 55% of the activity was lost in 20 min when equimolar mixtures of p8N_3Ap and RNase A were irradiated at 300 and 350 nm. To find out how much of this decrease was due to UV light inactivation of RNase A, the enzyme was irradiated in the presence of a nonphotolabile nucleotide, pAp, under the same conditions. Only a small amount of photoinactivation was observed when irradiation was carried out at 300 nm. Essentially no loss of enzyme activity occurred when the pAp–RNase mixture was irradiated at 350 nm even for a period as long as 60 min. As expected, no inactivation of the enzyme was observed when the complex was incubated in the absence of UV light. The fact that photoincorporation of

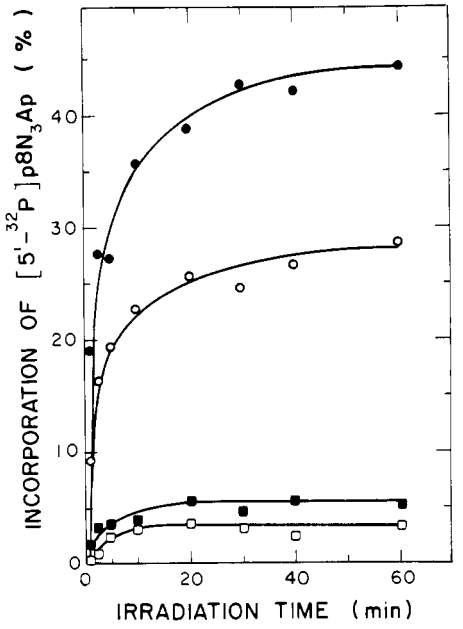

FIGURE 2: Covalent incorporation of [5′-^{32}P]p8N_3Ap into RNase A as a function of time. Mixtures of 30 μM [5′-^{32}P]p8N_3Ap and 30 μM RNase A were irradiated at 300 (●) and 350 nm (○) for the times indicated. To determine the extent of nonspecific labeling, mixtures of [5′-^{32}P]p8N_3Ap with performic acid oxidized RNase A were also irradiated at 300 (■) and 350 nm (□). Photoincorporation was expressed as mole percent of nucleotide incorporated into RNase A. For details, see Experimental Procedures.

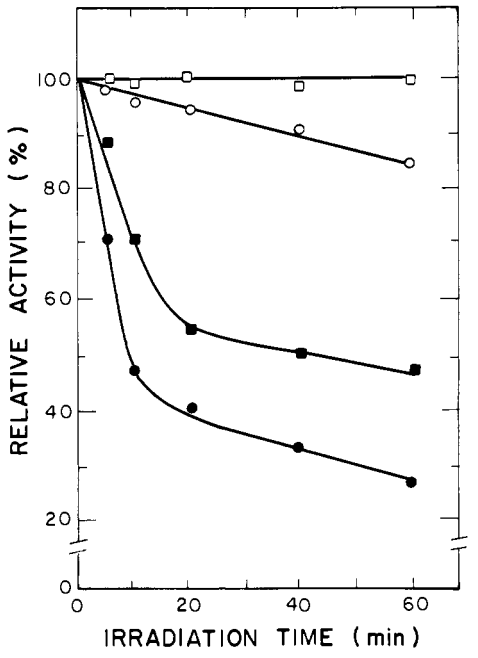

FIGURE 3: Kinetics of UV-induced inactivation of RNase A in the presence of p8N_3Ap and pAp. Solutions containing 30 μM p8N_3Ap and 30 μM RNase A were irradiated at 300 (●) and 350 nm (■) for the times indicated. As a control, solutions containing 30 μM pAp and 30 μM RNase A were irradiated at 300 (○) and 350 nm (□) for the same times. Enzymatic activity was assayed as described under Experimental Procedures. RNase incubated with p8N_3Ap or pAp in the dark did not undergo any change in activity (not shown).

p8N_3Ap into RNase A blocks its enzymatic activity substantiates the site-specific character of the labeling reaction.

To determine whether the pyrimidine or purine binding site of RNase A is involved in the UV light dependent reaction, labeling of the enzyme with [5′-^{32}P]p8N_3Ap was measured in the presence of increasing amounts of either pAp or pCp. A 5-fold molar excess of pCp over p8N_3Ap abolished the

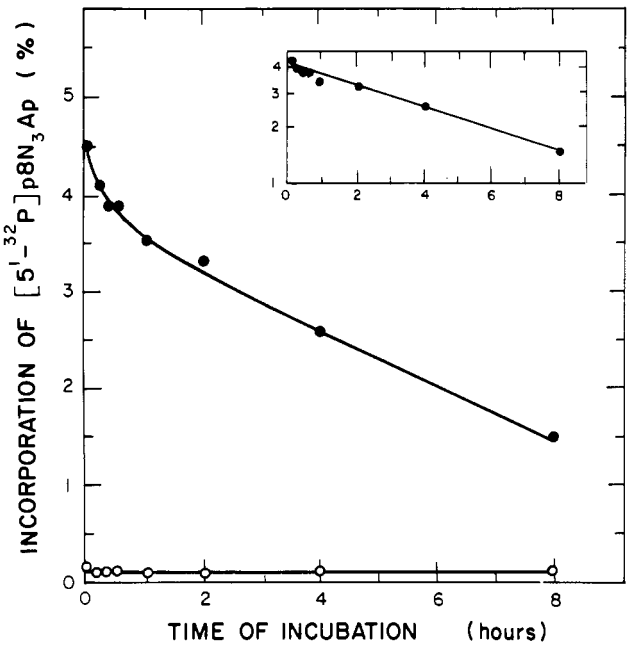

FIGURE 4: Covalent attachment of prephotolyzed [5′-^{32}P]p8N_3Ap to RNase A. Samples containing 30 μM [5′-^{32}P]p8N_3Ap in 20 mM Tris-HCl, pH 7.2, were irradiated at 300 nm for 10 min and then incubated in the dark with (○) or without (●) 10 mM dithiothreitol. At various times, 20-μL aliquots of prephotolyzed [5′-^{32}P]p8N_3Ap were added to 2-μL aliquots of 300 μM RNase, and after an additional 10-min incubation, covalent incorporation of [5′-^{32}P]p8N_3Ap into RNase was analyzed as described under Experimental Procedures. Inset is semilog plot of the data for covalent [5′-^{32}P]p8N_3Ap–RNase A complexes.

labeling of RNase A. In contrast, addition of a 5-fold molar excess of pAp only decreased the incorporation of p8N_3Ap from 40% (without pAp) to 24%. The decreased photoincorporation of p8N_3Ap in the latter case was most probably the result of an internal filter effect since a similar decrease in incorporation was observed when RNase was irradiated in the presence of a 6-fold molar excess of p8N_3Ap relative to the enzyme. These experiments demonstrate that p8N_3Ap and pCp, but not pAp, compete for the same site on RNase A and suggest that p8N_3Ap becomes attached to the pyrimidine binding site.

Covalent Attachment of Prephotolyzed [5′-^{32}P]p8N_3Ap to RNase A. The covalent incorporation of [5′-^{32}P]p8N_3Ap into RNase A also occurred in the dark following prephotolysis of the nucleotide at 300 nm, indicating that a long-lived reactive intermediate had been formed. [5′-^{32}P]p8N_3Ap prephotolyzed with 350-nm light labeled RNase A less efficiently. Denaturated RNase failed to react with [5′-^{32}P]p8N_3Ap prephotolyzed with either 300- or 350-nm light. We investigated the possibility of intercepting the long-lived intermediate with dithiothreitol and 2-mercaptoethanol. Dithiothreitol was found to totally abolish the covalent labeling of RNase when used at a final concentration of 10 mM.

The lifetime of the dark-reactive species was examined in the following way. [5′-^{32}P]p8N_3Ap was irradiated at 300 nm for 10 min and then kept at 0 °C in the dark for various periods of time. Next, RNase A was added and covalent attachment of the prephotolyzed nucleotide was measured 10 min later. The labeling reaction was terminated by the addition of 10 mM DTT. The amount of p8N_3Ap attachment decreased steadily with time after irradiation although a small fraction of the prephotolyzed nucleotide was still able to label the protein after 16 h (Figure 4). From the time-dependent decrease in covalent attachment, the half-life of the photo-

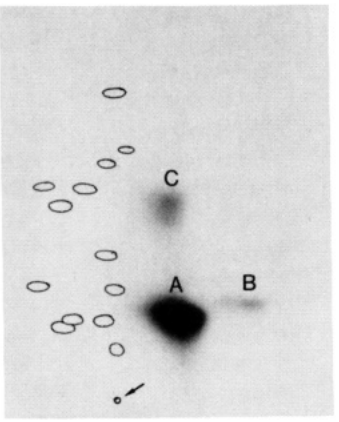

FIGURE 5: Two-dimensional peptide map obtained after tryptic digestion of [5′-^{32}P]p8N_3Ap–RNase A complexes. Fractionation in the first dimension was by electrophoresis (cathode left and anode right) and in the second by chromatography (bottom to top). The point of sample application is indicated by the arrow. The figure depicts an autoradiogram of the CEL 300 thin-layer plate over which the positions of the ninhydrin-stained spots have been traced. A, B, and C indicate radioactive spots.

product responsible for the labeling of RNase A in the dark was estimated to be approximately 5 h in 20 mM Tris-HCl, pH 7.2.

Determination of the Site of Labeling in RNase A. Covalent nucleotide–RNase complexes, obtained either by irradiation of RNase A in the presence of [5′-^{32}P]p8N_3Ap or by incubation of RNase A with preirradiated nucleotide, were digested with trypsin, and the products were fractionated by chromatography on Polygram CEL 300 thin-layer plates. Autoradiography of the chromatograms revealed the presence of three or four radioactive spots (Figure 5). In both digests, over 90% of the label was attached to a peptide in spot A. This peptide was extracted from the plates and subjected to amino acid analysis and to N-terminal sequence analysis by the double-coupling method of Chang et al. (1978). Amino acid analysis of peptide A indicated the following composition: Asx(3.1), Gly(1.4), Glx(1.8), Thr(1.8), Cys(1.6), Tyr(1.8), Ser(2.8), Met(0.8), Ile(0.8), and Arg(1.0), where the numbers in parentheses give the relative molar ratios. N-Terminal sequence analysis of peptide A gave Asn-Gly-Gln-Thr-. Both the amino acid composition and the N-terminal sequence are consistent with the structure of the tryptic peptide encompassing residues 67–85 of RNase A. The absence of one Thr residue from the amino acid composition of peptide A indicates that this amino acid was attached to p8N_3Ap. Since no radioactivity was released with Thr-70 during the N-terminal analysis of peptide A, p8N_3Ap must have labeled either Thr-78 or Thr-82.

Discussion

Because the published procedures for the preparation of nucleoside 3′,5′-bisphosphates could not be easily adapted for the preparation of ^{32}P-labeled p8N_3Ap (Richards & Laskowski, 1969; Barrio et al., 1978), we devised a new method based on hydrolysis of 8N_3cAMP and addition of ^{32}P to the 5′-position of the resulting 3′-nucleosides using T4 polynucleotide kinase. The final product, [5′-^{32}P]p8N_3Ap, may be used without purification in photochemical labeling studies of nucleases, as demonstrated here, or for incorporation into RNA molecules with T4 RNA ligase as reported elsewhere (Wower et al., 1988).

In the present work, we describe the highly specific reaction of p8N_3Ap with RNase A. In order to maximize the yield of covalent nucleotide–protein complexes, labeling was studied at a number of pH values. The highest level of labeling was observed at pH 7.2 although at this pH the association constant for the nucleotide–RNase A interaction would be expected to be 5–10 times lower than that at pH 5.5 (Anderson et al., 1968). The greater yield of nucleotide attachment at pH 7.2 than at pH 5.5 must therefore be ascribed to the nature of the labeling reaction.

To avoid UV light induced inactivation of RNase A (Aktipis & Iammartino, 1972; Schulz et al., 1975), the photoincorporation of p8N_3Ap into RNase A was instigated by irradiation with light of wavelength longer than 254 nm. Irradiation at 300 nm proved to induce effective labeling of RNase A without itself provoking significant inactivation of the enzyme. This finding makes p8N_3Ap an attractive probe for investigating the structure of macromolecular complexes such as ribosomes that are particularly sensitive to UV-induced inactivation.

In the course of our studies, we observed that preirradiated [5′-^{32}P]p8N_3Ap became covalently attached to RNase A even after a delay of up to 16 h prior to mixing the reactants. This indicates that a long-lived intermediate was formed upon irradiation. Although we have not yet been able to identify the reactive intermediate, we were able to intercept it with 10 mM dithiothreitol. From the time-dependent decrease in covalent bond formation, the half-life of the photoproduct responsible for the labeling was determined to be 5 h in 20 mM Tris-HCl, pH 7.2. Our observations are in contrast to the report of Walter and Greengard (1983) that preirradiated 8N_3cAMP was unable to covalently label proteins. The long-lived intermediate is certainly not a nitrene. Furthermore, such nitrene-derived metastable intermediates as azirines or heterocumulenes also seem unlikely candidates for a species with a 5-h half-life in neutral aqueous solution (Platz, 1984). Possibly a product derived from the reaction of water with one of these three transient species is responsible for the dark-labeling reaction.

Several lines of evidence indicate the labeling reaction is active site specific. [5′-^{32}P]p8N_3Ap reacted only slightly with RNase A denatured by performic acid oxidation, showing that the native structure of the enzyme is a prerequisite for efficient labeling. In all cases, the attachment of the nucleotide to RNase A led to the loss of its enzymatic activity. Moreover, the presence of excess [5′-^{32}P]p8N_3Ap did not lead to nonspecific labeling of native RNase although a decrease in incorporation was observed that most likely resulted from the competitive absorption of UV light by the unbound nucleotide. Finally, the inhibition of labeling by pCp suggests that p8N_3Ap interacts with the pyrimidine binding site of the enzyme (Richards & Wyckoff, 1971).

The site of photoaffinity labeling by p8N_3Ap is located within a tryptic peptide that encompasses amino acids 67–85 of the RNase A chain. Analysis of this peptide indicated that p8N_3Ap was attached to either Thr-78 or Thr-82. These two residues are part of the sequence spanning amino acids 78–82 which forms the "bottom" of the pyrimidine binding site (Richards & Wyckoff, 1971, 1973). At this point, it is impossible to resolve which of the two residues is actually labeled with p8N_3Ap. Close examination of the X-ray structure of bovine RNase A complexes with 8-oxoguanosine 2′-monophosphate (Borkakoti et al., 1983), cytidine-N(3)-oxide 2′-monophosphate (Palmer et al., 1984), and cytidine 2′-monophosphate (Howlin et al., 1987) suggests that the more likely site of attachment is Thr-82. This amino acid, together with Ser-80 and Ile-81, was earlier identified as a residue modified by photolyzed pUp (Havron & Sperling, 1977). Our photo-

chemical labeling results therefore support the nuclear magnetic resonance (Haar et al., 1974; Arus et al., 1981, 1982) and X-ray diffraction studies (Pavlovskii et al., 1978; Borkakoti et al., 1983) which demonstrate that 8-substituted adenine nucleotides interact with the pyrimidine binding site of bovine pancreatic RNase A.

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Registry No. 8N_3cAMP, 31966-52-6; [5′-^{32}P]p8N_3Ap, 116234-52-7; p8N_3Ap, 116234-51-6; RNase A, 9001-99-4; [γ-^{32}P]ATP, 2964-07-0; ATP, 56-65-5; L-Thr, 72-19-5; 8-azidoadenosine 3′-monophosphate, 117985-22-5.

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