2- and 8-Azido Photoaffinity Probes. 1. Enzymatic Synthesis, Characterization, and Biological Properties of 2- and 8-Azido Photoprobes of 2-5A and Photolabeling of 2-5A Binding Proteins†

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ABSTRACT: The 2- and 8-azido trimer 5′-triphosphate photoprobes of 2-5A have been enzymatically synthesized from [γ-^{32}P]2-azidoATP and [α-^{32}P]8-azidoATP by 2-5A synthetase from rabbit reticulocyte lysates. Identification and structural determination of the 2- and 8-azido adenylate trimer 5′-triphosphates were accomplished by enzymatic hydrolyses with T2 RNase, snake venom phosphodiesterase, and bacterial alkaline phosphatase. Hydrolysis products were identified by HPLC and PEI–cellulose TLC analyses. The 8-azido photoprobe of 2-5A displaces p_3A_4[^{32}P]pCp from RNase L with affinity equivalent to p_3A_3 (IC_{50} = 2 × 10^{-9} M in radiobinding assays). The 8-azido photoprobe also activates RNase L to hydrolyze poly(U)[^{32}P]pCp 50% at 7 × 10^{-9} M in core–cellulose assays. The 2- and 8-azido photoprobes and authentic p_3A_3 activate RNase L to cleave 28S and 18S rRNA to specific cleavage products at 10^{-9} M in rRNA cleavage assays. The nucleotide binding site(s) of RNase L and/or other 2-5A binding proteins in extracts of interferon-treated L929 cells were investigated by photoaffinity labeling. Dramatically different photolabeling patterns were observed with the 2- and 8-azido photoprobes. The [γ-^{32}P]2-azido adenylate trimer 5′-triphosphate photolabels only one polypeptide with a molecular weight of 185 000 as determined by SDS gel electrophoresis, whereas the [α-^{32}P]8-azido adenylate trimer 5′-triphosphate covalently photolabels six polypeptides with molecular weights of 46 000, 63 000, 80 000, 89 000, 109 000, and 158 000. Evidence that the photolabeling by 2- and 8-azido 2-5A photoprobes was highly specific for the p_3A_3 allosteric binding site was obtained as follows. Addition of 1 × 10^{-4} M or 1 × 10^{-5} M authentic p_3A_3 to incubation mixtures containing either the [^{32}P]2- or 8-azido photoprobe prevented the photolabeling. However, neither 2′,5′- or 3′,5′-trimer core molecules nor ATP prevented the photolabeling. This new class of 2-5A photoaffinity probes and their application for investigation of the nucleotide binding domain of RNase L and/or other 2-5A binding proteins in the antiviral/antiproliferative state of mammalian cells are discussed.

The 2-5A/RNase L system[1] is widely accepted to be part of the antiviral mechanism of interferon [for reviews, see Williams and Silverman (1985) and Lengyel (1982)] and may also play a role in the regulation of cell growth (Wells & Mallucci, 1985). 2-5A is synthesized from ATP by 2-5A synthetase and exerts its biologial effects by binding and activating its only known target enzyme, RNase L. The involvement of 2-5A has also been implicated in the regulation of mitogenesis or in cap methylation of viral mRNA through mechanisms different from RNase L activation (Kimchi et al., 1979; Sharma & Goswami, 1981; Leanderson et al., 1982; Schmidt et al., 1984). Numerous 2-5A analogues have been synthesized with the goal of characterization of the binding and activation processes of RNase L. Most recently, we have reported on the unique properties of 2-5A molecules in which R_P and S_P chirality have been introduced into the 2-5A backbone to form the phosphorothioate analogues of 2-5A (Karikó et al., 1987a,b; Suhadolnik et al., 1987). By chiral modification of the 2-5A backbone, it has been possible to examine the stereochemical requirements for binding to and activation of RNase L. Furthermore, we have reported that the metabolically stable S_PS_P trimer core and its 5′-monophosphate can bind to but not activate RNase L, thereby selectively shutting down the 2-5A/RNase L system in vitro and following microinjection into virus-infected mammalian cells in culture (Karikó et al., 1987b; Suhadolnik et al., 1988b). In order to understand the implication of these observations, it now becomes essential to ascertain the specific interactions

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[1] Abbreviations: 2-5A, 2′,5′-oligoadenylates [p_3A_n; in this paper the symbol A, rather than the more conventional Ap, is used to represent adenylic acid to permit more emphasis on the terminal triphosphate (refer to structures)]; p_3A_3 and p_3A_4, trimer and tetramer, respectively, of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-triphosphate; A_3, 5′-dephosphorylated p_3A_3; 2-N_3ATP, 2-azidoATP; 8-N_3ATP, 8-azidoATP; 2-azido-p_3A_3, 5′-*O*-triphosphoryl-2-azidoadenylyl(2′-5′)2-azidoadenylyl(2′-5′)2-azidoadenosine; 8-azido-p_3A_3, 5′-*O*-triphosphoryl-8-azidoadenylyl(2′-5′)8-azidoadenylyl(2′-5′)8-azidoadenosine; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; RNase L, 2-5A-dependent endoribonuclease; pCp, cytidine 3′,5′-diphosphate; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; PEI, poly(ethylene imine); TLC, thin-layer chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SVPD, snake venom phosphodiesterase; BAP, bacterial alkaline phosphatase; SCP, specific cleavage products.

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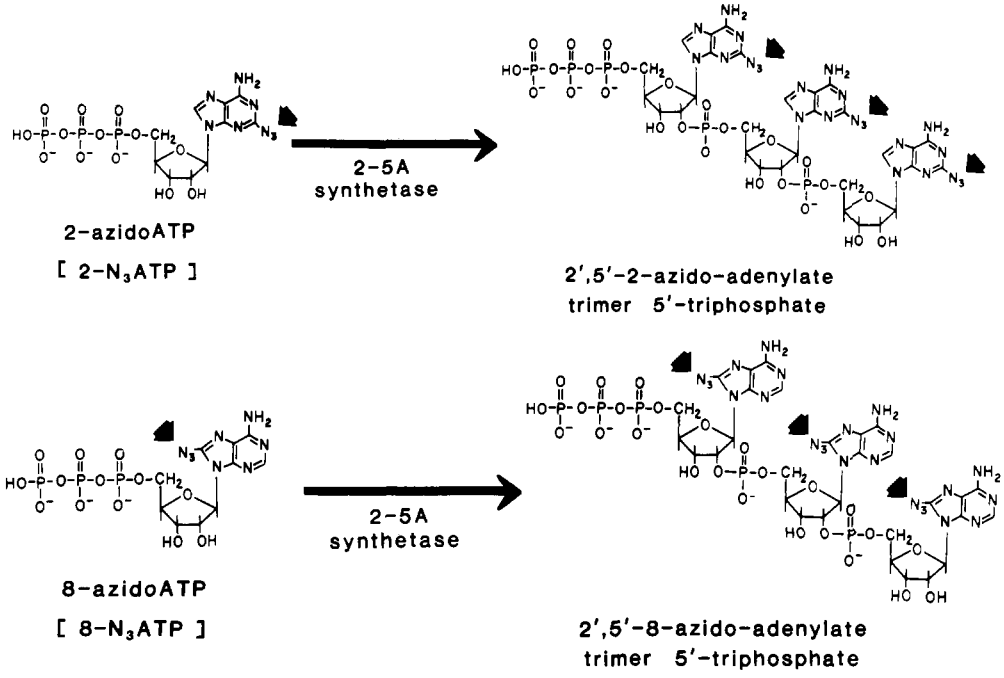

FIGURE 1: Structures of the photoaffinity probes of 2-5A enzymatically synthesized from 2-N_3ATP and 8-N_3ATP. The arrows indicate the location of the photoreactive azido groups.

occurring in the nucleotide binding domain of RNase L and/or other 2-5A binding proteins. We have approached this goal by employing the technique of photoaffinity labeling using enzymatically synthesized 2- and 8-azido photoprobes of 2-5A.

Photoaffinity azido probes form covalent bonds to proteins through reactive nitrene intermediates generated by low-intensity ultraviolet (UV) light (Knowles, 1972; Hoyer et al., 1980). Photolabeled proteins are detected with great sensitivity and high specificity. 2- and 8-azido purine nucleotides have captivated the interest of the biochemist and organic chemist and have been widely used as site-directed photoprobes to identify nucleotide binding proteins in crude cell extracts (Evans et al., 1985; Malkinson et al., 1986). In addition, these azido nucleotides have also been used to map nucleotide binding domains and to study enzyme kinetics of purified proteins (Abraham et al., 1983; Boulay et al., 1985; Hegyi et al., 1986; Garin et al., 1986; Xue et al., 1987).

In an effort to examine the salient features of 2-5A binding to RNase L and other 2-5A binding proteins, we have synthesized and characterized the 2- and 8-azido trimer 5'-triphosphate photoprobes of 2-5A (Figure 1). In this paper we describe the enzymatic synthesis, structural characterization, and biological properties of these photoprobes of 2-5A and their application in photolabeling of RNase L and/or other 2-5A binding proteins. These two photoprobes represent our initial efforts to develop a new class of 2-5A analogues for use in (i) the identification of the amino acids in the binding domain of cytoplasmic 2-5A binding proteins and (ii) the elucidation of differences in RNase L and/or other 2-5A binding proteins in normal, interferon-treated, neoplastic, and virus-infected cells. A preliminary report of this work has been presented (Li et al., 1987). In the accompanying paper we describe the photolabeling of highly purified 2-5A synthetase with 2- and 8-azidoATP (Suhadolnik et al., 1988a).

Materials and Methods

Materials. 2',5'-Adenylate trimer and tetramer 5'-triphosphates, 2',5'- and 3',5'-adenylate trimer cores, and poly(U) were obtained from Pharmacia; [α-^{32}P]ATP (410 Ci/mmol), p_3A_4[^{32}P]pCp (3000 Ci/mmol), and [^{32}P]pCp (3000 Ci/mmol) were from Amersham; media and sera for cell culture were from GIBCO; T4 RNA ligase was from Bethesda Research Laboratories; rabbit reticulocyte lysates were from Green Hectares. 2-5A core–cellulose was a generous gift from Dr. R. Silverman. Mouse interferon (1.5×10^7 units/mg of protein) was generously supplied by Dr. D. Murasko.

Cell Culture. L929 cells were maintained in monolayer culture in Dulbecco's modified Eagle medium supplemented with 5% bovine serum. Monolayers were treated where indicated with 200 units/mL mouse interferon for 20 h.

Cell Extracts. L929 cells were collected by trypsinization, washed once with PBS, and lysed in glycerol buffer as described (Karikō & Ludwig, 1985).

Chemical Synthesis of 2-AzidoATP and 8-AzidoATP. 2-N_3ATP was synthesized from 2-chloroadenosine (Sigma) (Czarnecki et al., 1982). 8-N_3ATP was synthesized from 8-N_3AMP by the method of Michelson (1964). 8-N_3AMP was synthesized from AMP as previously described (Czarnecki et al., 1979). [γ-^{32}P]2-N_3ATP and [α-^{32}P]8-N_3ATP were prepared and purified as reported (Haley, 1977; Potter & Haley, 1982). Purity of the 2- and 8-N_3ATP was determined by HPLC and TLC analysis (Czarnecki et al., 1982). Following purification by DEAE-cellulose column chromatography and HPLC, the 2- and 8-N_3ATP were shown to be free of any contaminating ATP, 8-bromoATP, 8-aminoATP, or nucleotide 3'(2')-phosphates.

Enzymatic Synthesis and Isolation of 2- and 8-Azido Photoprobes of 2-5A. The synthesis of the 2- and 8-azido photoprobes of 2-5A was performed in the absence of UV irradiation with 2-5A synthetase from rabbit reticulocyte lysates, basically as described for 2-5A from this laboratory (Suhadolnik et al., 1983; Lee & Suhadolnik, 1985). The reaction mixture (150 μL) contained 8.5 mM HEPES, pH 7.5, 17 mM $Mg(OAc)_2$, 42.6 mM KCl, 5% glycerol, and 2.5 mM [γ-^{32}P]2-N_3ATP (7.0 μCi/nmol) or 2.5 mM [α-^{32}P]8-N_3ATP (1.4 μCi/nmol) (DTT was omitted to avoid reduction of the azido residues). Incubations were for 18 h at 30 °C. The 2',5'-oligonucleotides synthesized were separated by DEAE-cellulose column chromatography as described (Doetsch et al., 1981). The isolated oligonucleotides were analyzed by HPLC,

and 250-μL fractions were collected for determination of radioactivity. The fractions containing the trimer photoprobes were dialyzed, lyophilized, and stored in absolute methanol at −70 °C. Storage of the 2-azido analogues of 2-5A in methanol at −70 °C prevents formation of the tetrazolo tautomers (Czarnecki, 1984).

High-Performance Liquid Chromatography. HPLC analysis was performed with two Waters Associates Model 6000 pumps controlled by a Model 660 solvent programmer. Reverse-phase chromatography was performed with a Waters radial compression system (Z-module) and a C_{18} μBondapak Radial Pak cartridge (8 mm × 10 cm). For separation, 50 mM ammonium phosphate, pH 7.0 (buffer A) and methanol:water (1:1) (buffer B) were used in a linear gradient (t = 1 min, 10% B; t = 31 min, 20% B) at a flow rate of 1 mL/min.

Structural Elucidation of the 2- and 8-Azido Photoprobes of 2-5A. The structural elucidation of the 2- and 8-azido analogues of 2-5A was accomplished by enzymatic hydrolyses (T2 RNase, SVPD, BAP) followed by HPLC and PEI–cellulose TLC analyses as described (Karikó et al., 1987a).

Radiobinding Assays. Radiobinding assays were performed with L929 cell extracts as the source of RNase L (50 μg of protein/assay) (Knight et al., 1981). In control experiments, 55% (5000 dpm) of the added p_3A_4[^{32}P]pCp was bound to RNase L.

Core–Cellulose Assays. Core–cellulose assays for RNase L activation were performed as previously described (Karikó et al., 1987a). Poly(U)[^{32}P]pCp was synthesized from poly(U) by using T4 RNA ligase (Karikó et al., 1987a).

Ribosomal RNA Cleavage Assays. Ribosomal RNA cleavage assays were performed according to the procedure of Wreschner et al. (1982) with L929 cell extracts [150 μg of protein/assay, 15-μL final volume as prepared and described by Karikó and Ludwig (1985)].

Photoaffinity Labeling. Ten microliters of L929 cell extract (100 μg of protein/assay) were combined with 10 μL of buffer (140 mM NaCl and 35 mM Tris-HCl, pH 7.5) and were incubated in the presence of 1×10^{-6} M [γ-^{32}P]2-azido-p_3A_3 (0.9 μCi/nmol; 60 000 dpm), 1×10^{-6} M [α-^{32}P]8-azido-p_3A_3 (0.3 μCi/nmol; 20 000 dpm), or 1×10^{-8} M p_3A_4[^{32}P]pCp (400 μCi/nmol) in a 30-μL final volume. In competition photolabeling experiments with the 2- and 8-azido photoprobes of 2-5A, p_3A_3 (1×10^{-4} or 1×10^{-5} M), 2′,5′-A_3 (5×10^{-4} M), 3′,5′-A_3 (5×10^{-4} M), or ATP (1×10^{-4} M) were also added. After incubation in microcentrifuge tubes at 0 °C for 90 min, the samples were transferred to ice-cold porcelain spot plates and photolyzed for 60 s with a 254-nm UVG-11 Mineralight lamp (UltraViolet Products, Inc.) at a distance of 2 cm (1.0 J/m^2). After photolabeling, 60 μL of a reductive, protein solubilizing mixture (15.4 mg/mL DTT, 25% w/v sucrose, 2.5% SDS, 25 mM Tris-HCl, pH 8.0, and 0.0025% pyronin Y) was added to the samples. The photolabeled proteins were analyzed by 8% SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The Coomassie blue stained dried gels were subject to autoradiography at −70 °C with X-Omat film (Kodak).

Densitometric Tracing. Autoradiograms were traced on a Hoefer scanning densitometer Model GS350.

Radioactive Measurements. A Beckman LS-100C liquid scintillation spectrometer was used for all radioactive measurements (counting efficiency, 99% for ^{32}P).

Results

Enzymatic Synthesis and Structural Characterization of 2- and 8-Azido Photoprobes of 2-5A. In the absence of UV irradiation, the 2-5A synthetase from rabbit reticulocyte lysates converted [γ-^{32}P]2-N_3ATP to a putative trimer 5′-triphosphate, i.e., [γ-^{32}P]2-azido-p_3A_3, in a yield of 3.0%. [α-^{32}P]8-N_3ATP was also converted to a putative trimer 5′-triphosphate, i.e., [α-^{32}P]8-azido-p_3A_3, in a yield of 0.7%. These yields compare to 13.0% for formation of authentic [α-^{32}P]p_3A_3 from [α-^{32}P]ATP. The 2- and 8-azido photoprobes were isolated from the incubation mixtures and purified by DEAE-cellulose column chromatography and analyzed by HPLC (Karikó et al., 1987a). The elution time of the putative [α-^{32}P]8-azido-p_3A_3 was 9.0 min, which was identical with authentic p_3A_3. The elution time of the putative [γ-^{32}P]2-azido-p_3A_3 was also 9.0 min. The elution time for ATP and 8-N_3ATP was 4 min. Some incubations with [γ-^{32}P]2-N_3ATP resulted in the formation of a second radioactive product (elution time 13.0 min, which coeluted with p_3A_4). The molar ratio of putative trimer:tetramer 5′-triphosphates was 70:30.

The chain length of the putative [α-^{32}P]8-azido-p_3A_3 was confirmed by charge separation. All of the radioactivity from [α-^{32}P]8-azido-p_3A_3 had a charge of 6− (R_f = 0.2, PEI–cellulose TLC), which is equivalent to that of authentic 2′,5′-adenylate trimer 5′-triphosphate. There was no hydrolysis of the putative [α-^{32}P]8-azido-p_3A_3 by T2 RNase, thus establishing the presence of 2′,5′-phosphodiester internucleotide linkages. SVPD hydrolysis of the 8-azido trimer 5′-triphosphate photoprobe of 2-5A yielded 8-N_3AMP (elution time 20 min) and no 8-aminoAMP or AMP (elution times 30 and 12 min, respectively). When the SVPD hydrolysis product (i.e., 8-N_3AMP) was reduced by DTT and further analyzed by HPLC, all of the radioactivity resided in the region equivalent to authentic 8-aminoAMP (elution time 30.0 min). BAP hydrolysis of the putative [α-^{32}P]8-azido-p_3A_3 (525 000 dpm) followed by PEI–cellulose TLC resulted in the formation of [^{32}P]2′,5′-8-azido adenylate trimer core with an R_f of 0.7 and charge of 2−, which is identical with that of 2-5A trimer core. Furthermore, the ratio of inorganic ^{32}P to [^{32}P]8-azido-A_3 was 1:2. The proof of structure of the [γ-^{32}P]2-azido-p_3A_3 was accomplished by analogous enzymatic hydrolyses and chromatographic analyses. The results from these determinations support the structural assignment of the trimer 5′-triphosphate photoprobes from 2-azidoATP and 8-azidoATP as 5′-*O*-triphosphoryl-2-azidoadenylyl(2′-5′)2-azidoadenylyl(2′-5′)2-azidoadenosine and 5′-*O*-triphosphoryl-8-azidoadenylyl(2′-5′)8-azidoadenylyl(2′-5′)8-azidoadenosine, respectively (Figure 1).

Binding Affinity of the 8-Azido Photoprobe of 2-5A to RNase L. The ability of 8-azido-p_3A_3 to compete with p_3A_4[^{32}P]pCp for binding to the RNase L in L929 cell extracts was compared to that of p_3A_3 by using radiobinding assays. A dose-dependent displacement of the radioactive probe from RNase L was observed (Figure 2A). 8-Azido-p_3A_3 (●) binds to RNase L with an IC_{50} of 2×10^{-9} M, which is similar to the IC_{50} of authentic p_3A_3 (▲).

Activation of RNase L by 2- and 8-Azido Analogues of 2-5A. The recently developed core–cellulose assay, which involves the immobilization and partial purification of RNase L on 2-5A-core–cellulose, was used to measure the ability of 8-azido-p_3A_3 to activate RNase L. The 8-azido-p_3A_3 (●) was equal to authentic p_3A_3 (▲) in its ability to activate RNase L to hydrolyze poly(U)[^{32}P]pCp to acid-soluble fragments (IC_{50}, $7–9 \times 10^{-9}$ M) (Figure 2B). The activation of RNase L by the 2- and 8-azido photoprobes in the rRNA cleavage assay was consistent with that measured in the core–cellulose assay. In the most specific functional assay (rRNA cleavage assay), 2- and 8-azido-p_3A_3 could activate RNase L in a

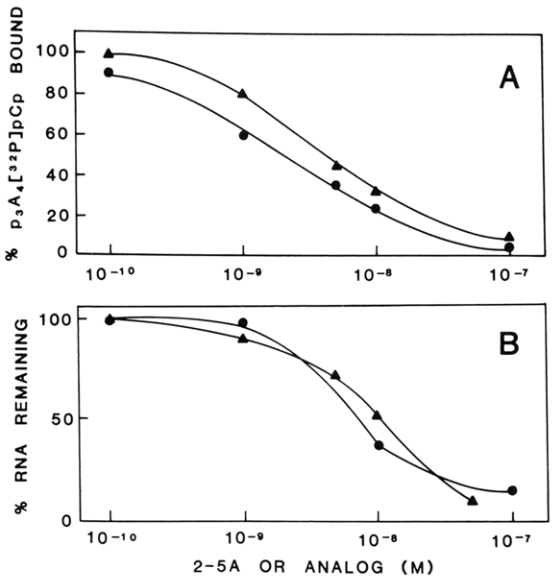

FIGURE 2: Binding (A) and activation (B) of RNase L by the 8-azido photoprobe of 2-5A. (A) The ability of 8-azido-p_3A_3 (●) to compete with p_3A_4[^{32}P]pCp for binding to the RNase L in L929 cell extracts was compared to that of p_3A_3 (▲) by using radiobinding assays. (B) The ability of 8-azido-p_3A_3 (●) to activate the partially purified RNase L from L929 cell extracts was compared to that of p_3A_3 (▲) in core–cellulose assays. A quantitative measure of RNase L activation was determined by the conversion of poly(U)[^{32}P]pCp to acid-soluble fragments. Data represent an average of duplicate determinations.

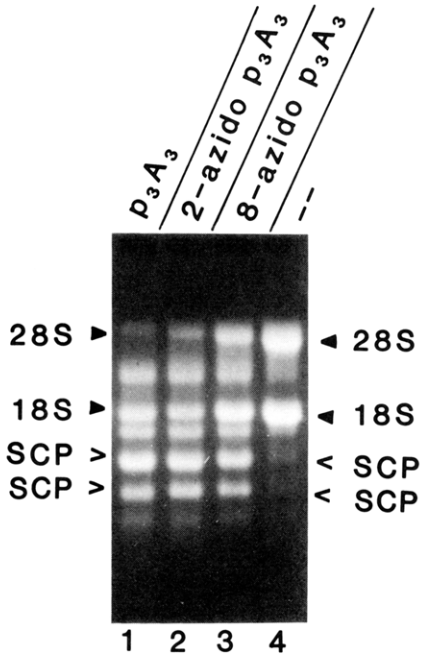

FIGURE 3: Ribosomal RNA cleavage assays with the 2- and 8-azido photoprobes of 2-5A. L929 cell extracts were incubated in the absence (lane 4) or presence of p_3A_3 (lane 1), 2-azido-p_3A_3 (lane 2), or 8-azido-p_3A_3 (lane 3) at 1×10^{-8} M final concentration in the absence of UV light. The positions of 28S and 18S rRNA and specific cleavage products (SCP) are indicated.

concentration-dependent manner to degrade rRNA to highly characteristic, specific cleavage products (SCP). The 2-azido-p_3A_3 was as active as authentic p_3A_3, while 8-azido-p_3A_3 appeared less active, with significant cleavage of 28S and 18S rRNA to SCP observed at 10^{-8} M (Figure 3). Slight cleavage of 28S and 18S rRNA to SCP was also observed at 1×10^{-9} M with 2- and 8-azido-p_3A_3 (data not shown).

Photoaffinity Labeling of RNase L and/or 2-5A Binding Proteins by 2- and 8-Azido Photoprobes of 2-5A. Interferon-treated L929 cell extracts were incubated with either [γ-^{32}P]2-azido-p_3A_3 or [α-^{32}P]8-azido-p_3A_3 prior to UV irradiation. After UV irradiation, the photoinsertion of the ^{32}P-labeled photoprobes was determined by SDS–PAGE and subsequent autoradiography. Photoincorporation was UV-irradiation-dependent and was found to be maximal after a 60-s UV exposure (Figures 4 and 5). Photolabeling of L cell extracts by [γ-^{32}P]2-azido-p_3A_3 resulted in the specific labeling of one protein band with an apparent molecular weight of 185 000 (Figure 4, lane 1, protein *a*). Authentic p_3A_3 at 1×10^{-5} and 1×10^{-4} M competes for the photoinsertion of [γ-^{32}P]2-azido-p_3A_3 (Figure 4, lanes 2 and 3). Other photolabeled protein bands were also observed with [γ-^{32}P]2-azido-p_3A_3, but such photolabeling was not competed out by authentic p_3A_3 in a dose-dependent manner (Figure 4 compare lane 1 with lanes 2 and 3). A possible explanation may be the presence of two proteins migrating at this position in the gel, one being specifically labeled and the other nonspecifically labeled. When photolabeling of L929 cell extracts was done with [α-^{32}P]8-azido-p_3A_3, dramatically different results were obtained (Figure 5). With [α-^{32}P]8-azido-p_3A_3, six proteins in interferon-treated L929 cell extracts were photolabeled (Figure 5, lane 2). To determine whether the photolabeling was 2-5A dependent, competition experiments were performed by the simultaneous addition of 1×10^{-4} M p_3A_3 and 1×10^{-6} M [α-^{32}P]-8-azido-p_3A_3 following a 60-s UV irradiation. Under these conditions, photolabeling of proteins *a–f* by [α-^{32}P]8-azido-p_3A_3 was competed out by authentic p_3A_3 (Figure 5A, lane 3). This photoincorporation of [α-^{32}P]8-azido-p_3A_3 into proteins *a–f* was specific for p_3A_3 because neither 5×10^{-4}

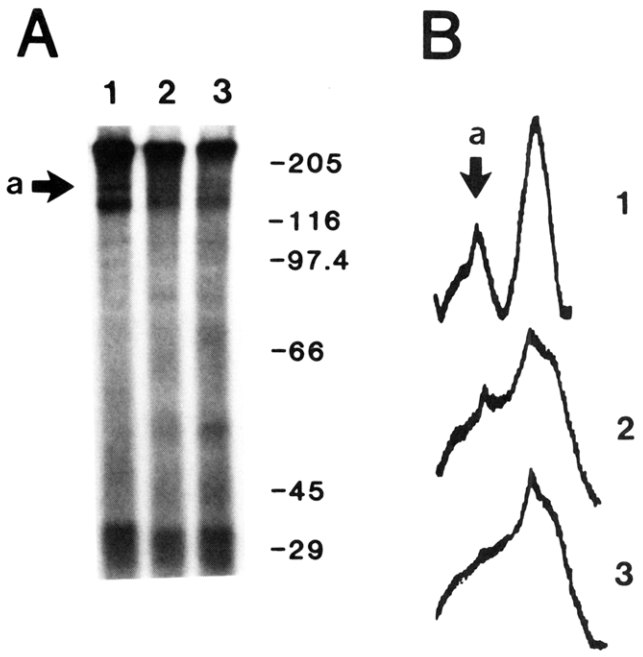

FIGURE 4: Photoaffinity labeling of cytoplasmic L929 cell extracts by 2-azido-p_3A_3. (A) Interferon-treated L929 cell extracts were incubated with [γ-^{32}P]2-azido-p_3A_3 at 1×10^{-6} M in the absence (lane 1) or presence of p_3A_3 at 1×10^{-5} and 1×10^{-4} M (lanes 2 and 3, respectively). Samples were UV-irradiated for 60 s at 0 °C and analyzed by 8% SDS–PAGE as described under Materials and Methods. Labeled proteins were detected by autoradiography. Molecular weight standards ($\times 10^3$) are indicated. The size markers used were myosin (205 000), β-galactosidase (116 000), phosphorylase B (97 400), bovine serum albumin (66 000), ovalbumin (45 000), and carbonic anhydrase (29 000). In the absence of UV irradiation, there was no photolabeling (not shown). (B) Densitometric traces of a portion of the autoradiogram. Authentic p_3A_3 (lanes 2 and 3) competes for the photoinsertion of [γ-^{32}P]2-azido-p_3A_3 into a protein of molecular weight 185 000 (protein *a*) indicated by the arrow.

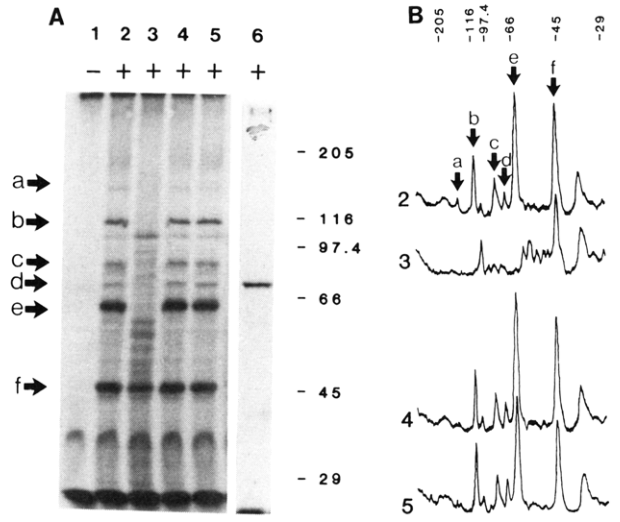

FIGURE 5: Photoaffinity labeling of cytoplasmic L929 cell extracts by 8-azido-p_3A_3. (A) Interferon-treated L929 cell extracts were incubated with [α-^{32}P]8-azido-p_3A_3 at 1×10^{-6} M in the absence (lanes 1 and 2) or presence of p_3A_3 at 1×10^{-4} M (lane 3), 2′,5′-A_3 at 5×10^{-4} M (lane 4), or 3′,5′-A_3 at 5×10^{-4} M (lane 5). Photolabeling of the cell extract was also done with p_3A_4[^{32}P]pCp (lane 6). In the absence of UV irradiation, there was no photolabeling (lane 1). Samples (lanes 2–6) were UV-irradiated for 60 s and analyzed by SDS–PAGE as described under Materials and Methods. The ^{32}P-labeled proteins were detected by autoradiography. (B) Densitometric traces of lanes 2–5 of the autoradiogram in (A). The arrows indicate the positions of those proteins whose photolabeling was prevented by p_3A_3 (proteins *a–f*). Molecular weight standards ($\times 10^3$) are shown on the right.

M 2-5A trimer core (lane 4), 5×10^{-4} M 3-5A trimer core (lane 5), nor 1×10^{-4} M ATP (data not shown) could protect against photolabeling by [α-^{32}P]8-azido-p_3A_3. Under the same photolabeling conditions as in lanes 2–5, p_3A_4[^{32}P]pCp was photoinserted into one protein with a molecular weight of 80 000 (Figure 5A, lane 6).

Discussion

In continued investigations of the binding and activation processes of RNase L, we have synthesized and characterized photoreactive azido probes of 2-5A. Our aim in this study and in the following paper in this issue has been the rational development of effective photoaffinity probes of RNase L and/or 2-5A binding proteins and 2-5A synthetase. The 2-5A photoprobes described herein are valuable biochemical tools that can be used to explore the nucleotide binding site(s) of RNase L and/or other 2-5A binding proteins and to permit the selective identification and isolation of 2-5A binding proteins from cell extracts. Furthermore, the strategic placement of azido groups on carbon-2 and carbon-8 of the adenine ring of the 2-5A molecule has provided new biochemical probes to examine nucleotide binding sites of cytoplasmic 2-5A binding proteins.

The enzymatic synthesis, structural characterization, and biological properties of the [^{32}P]-labeled 2- and 8-azido 2-5A photoprobes from [γ-^{32}P]2-N_3ATP and [α-^{32}P]8-N_3ATP are described here. 2-N_3ATP and 8-N_3ATP are substrates for 2-5A synthetase, extending the reported broad substrate specificity of the enzyme (Doetsch et al., 1981; Hughes et al., 1983; Lee & Suhadolnik, 1985; Karikō et al., 1987a). The observation that 2-N_3ATP is a better substrate for the 2-5A synthetase than is 8-N_3ATP may be explained by either the replacement of hydrogen with the azido group on C-2 or C-8 of ATP or a change in the conformation of the nucleotide about the N-glycosidic linkage from the preferential anti conformation to the reported syn conformation (Czarnecki, 1984; Sarma et al., 1974). The syn conformation of 8-N_3ATP could render 8-N_3ATP a less suitable substrate for 2-5A synthetase than 2-N_3ATP. This suggestion may be supported by studies on 2-5A synthetase with 2-chloroATP (anti conformation) and 8-bromoATP (syn conformation). 2-ChloroATP was converted to 2′,5′-oligonucleotides by 2-5A synthetase as efficiently as was ATP; however, 8-bromoATP was 10 times less efficient a substrate (Hughes et al., 1983).

The biological activities of the 2- and 8-azido photoprobes of 2-5A were shown to be similar to that of authentic 2-5A in rRNA cleavage assays (Figure 3). These results suggest that azido substitution on either carbon-2 or carbon-8 of the adenine rings of 2-5A does not alter the binding or the activation process of RNase L (Figure 3). The nearly identical biological activities of authentic p_3A_3 compared to that of the 2- and 8-azido trimer 5′-triphosphate photoprobes also indicate that the three-dimensional arrangement of the photoprobes in the allosteric site of RNase L is similar to that of authentic p_3A_3. Binding to the allosteric site is essential for the formation of a productive complex between the allosteric modifier (2-5A), the enzyme (RNase L), and its substrate (RNA) followed by efficient catalysis. This observation suggests that the 2- and 8-azido photoprobes of 2-5A are ideally suited for use in the identification of the amino acids in the three-dimensional binding domain of RNase L and/or 2-5A binding proteins.

There are several interesting aspects of the findings reported here with the 2- and 8-azido trimer 5′-triphosphate photoprobes. The 2-azido photoprobe can specifically photolabel one protein with an apparent molecular weight of 185 000, whereas the 8-azido photoprobe specifically labels six proteins of molecular weights 46 000, 63 000, 80 000, 89 000, 109 000, and 158 000 (Figures 4 and 5). However, under the same photolabeling conditions p_3A_4[^{32}P]pCp photolabeled only one protein with a molecular weight of 80 000 (Figure 5, lane 6). It has been reported that cytoplasmic cell extracts contain a protein of molecular weight 77 000–85 000 that covalently linked with the cytosine residue of 2-5A[^{32}P]pC(p) either by photoirradiation (Floyd-Smith et al., 1982) or by dialdehyde–Schiff base formation (Wreschner et al., 1982). This photolabeled protein appears to be one form of RNase L. In view of the knowledge that the 2- or 8-adenine azides are nearly 7000 times more sensitive to UV light than the pyrimidine bases (Evans et al., 1986), photolysis of 2- and 8-azido-substituted 2-5A molecules should cause fewer undesirable perturbations than photolysis of pyrimidine-substituted 2-5A molecules such as p_3A_4[^{32}P]pCp. This property of the 2- and 8-azido 2-5A photoprobes makes it possible to study the recognition contacts of 2-5A binding proteins more effectively. The contrasting photolabeling results achieved with p_3A_4[^{32}P]pCp vs the 2- and 8-azido photoprobes of 2-5A can be further explained in view of the strategic location of the photosensitive groups on the 2-5A molecule. In p_3A_4[^{32}P]pCp, the photoreactive cytosine is covalently linked at the 3′-terminus of 2-5A and is therefore removed from the adenine binding domain of the 2-5A molecule. Precedents for difficulty in interpretation of photolabeling data have been reported when the azido group is separated from the binding domain "by a long arm" (i.e., arylazido groups coupled to the 3′-ribose of nucleotides) (Boulay et al., 1985, and references cited therein). However, in the studies presented here, the photosensitive azido groups are located on C-2 and C-8 of the adenine rings of the 2-5A molecule. Furthermore, in the p_3A_4[^{32}P]pCp molecule, the 3′-phosphocytidine residue is linked by a 3′,5′-phosphodiester bond, whereas in the 2- and 8-azido 2-5A photoprobes

all internucleotide linkages are 2′,5′. The difference in the linkage of the phosphodiester bonds may result in altered photolabeling due to modified base stacking in p_3A_4[^{32}P]pCp compared with the 2- and 8-azido photoprobes of 2-5A. Such different relative orientations of the aglycon have been shown in X-ray studies of dimer A2′p5′C and A3′p5′C by Ts'o and co-workers (Kondo et al., 1970). However, although the UV-mediated cross-linking of pyrimidine nucleotides (i.e., dTTP and p_3A_4pCp) to substrate and/or allosteric binding sites requires larger doses of UV irradiation plus a longer time exposure and is less efficient, covalent adduct formation does occur (Floyd-Smith et al., 1982; Pandey & Modak, 1988).

Because the photosensitive groups on our 2- and 8-azido 2-5A photoprobes are attached directly to the purine rings (Figure 1) and because the biological properties of the 2- and 8-azido 2-5A trimer 5′-triphosphates are very similar to those of authentic p_3A_3 (Figures 2 and 3), it might be anticipated that the 2- and 8-azido 2-5A probes would photolabel the same proteins. However, experimentally, dramatically different photolabeling results were obtained with the 2- and 8-azido trimer 5′-triphosphates. The [γ-^{32}P]2-azido-p_3A_3 specifically photolabeled only *one* protein with an apparent molecular weight of 185 000 (Figure 4, lane 1, protein *a*), whereas [α-^{32}P]8-azido-p_3A_3 photolabeled *six* 2-5A-dependent protein species with molecular weights from 46 000 to 158 000 (Figure 5, proteins *a*–*f*). These results are similar to the report of Czarnecki et al. (1982), where 2-azidoADP was an effective inhibitor of ADP tight binding to chloroplast thylakoid membranes, whereas the 8-azidoADP was unable to displace tightly bound nucleotides. The protein of molecular weight 185 000 that is photolabeled by 2-azido-p_3A_3 in cytoplasmic L929 cell extracts may be similar to the 2-5A-dependent RNase identified by gel filtration by Lengyel and co-workers in cytoplasmic EAT cell extracts (Slattery et al., 1979). Several explanations can be proposed for the contrasting photolabeling patterns observed with the 2- and 8-azido-p_3A_3. If 2- and 8-azido-p_3A_3 are in the same conformation in the 2-5A binding domain, the orientation of the azido groups on these photoprobes would likely be different. In substrate binding studies of RNA polymerase with 5-N_3UTP and 8-N_3ATP, differences in photolabeling were attributed to a difference in the orientation of the azido groups on these two photoprobes (Woody et al., 1988). In addition, the distance between C-2 and C-8 in the purine ring is about 6 Å (Garin et al., 1986). Therefore, the 2- and 8-azido groups would be in juxtaposition to different amino acid residues in the three-dimensional 2-5A binding domain and might form covalent bonds with different amino acids following UV irradiation. An added advantage for mapping studies is that the 2- and 8-azido adenylate trimer 5′-triphosphate photoprobes contain *three* azido groups capable of reacting with more than one amino acid in the 2-5A binding domain.

In summary, the 2- and 8-azido photoprobes of 2-5A will be powerful investigative probes for mapping the amino acids in the three-dimensional binding domain of RNase L and/or other 2-5A binding proteins. Our results raise the possibility that the proteins photolabeled by [α-^{32}P]8-azido-p_3A_3 are either proteolytic degradation products or multimeric subunit forms of RNase L. In view of the unique results obtained with the 2- and 8-azido 2-5A photoprobes, we have included in our studies the examination of the nucleotide binding site(s) of 2-5A synthetase using 2- and 8-N_3ATP (see accompanying paper). Considering what is known about the ubiquitous occurrence of the 2-5A molecule in mammalian cells, the 2- and 8-azido photoprobes of 2-5A have great potential to identify, isolate, and characterize 2-5A binding proteins in normal, interferon-treated, neoplastic, and virus-infected cells and to elucidate the role of 2-5A in the regulation of cell metabolism.

Because the S_PS_P 2,5-phosphorothioate trimer core and its 5′-monophosphate can bind to but *not* activate RNase L, whereas the R_PR_P trimer core and 5′-monophosphate can bind to and activate RNase L (Karikó et al., 1987a,b), we are currently synthesizing the corresponding 8-azido 2,5-phosphorothioate trimer diastereomeric cores and 5′-monophosphates. Photolabeling technology will permit us to compare the amino acids covalently linked to chiral phosphorothioate 8-azido 2-5A photoprobes with the amino acids covalently linked to 2- and 8-azido-p_3A_3. Furthermore, the metabolically stable S_PS_P 2,5-phosphorothioate 8-azido trimer 5′-monophosphate, when introduced into the cell and photolyzed, could selectively shut down RNase L in the cell. Under such conditions, the effects of biological response modifiers such as interferon, tumor necrosis factor, and interleukins on cellular reactions could be examined independent of the 2-5A/RNase L antiviral system.

Registry No. 2-N_3ATP, 72884-75-4; 8-N_3ATP, 53696-59-6; [γ-^{32}P]-2-N_3ATP, 117145-98-9; [α-^{32}P]-8-N_3ATP, 117145-99-0; 2-azido-p_3A_3, 117146-00-6; 8-azido-p_3A_3, 117146-01-7; [γ-^{32}P]-2-azido-p_3A_3, 117146-02-8; [α-^{32}P]-8-azido-p_3A_3, 117146-03-9; RNase L, 76774-39-5; 2-5A synthetase, 69106-44-1.

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2- and 8-Azido Photoaffinity Probes. 2. Studies on the Binding Process of 2-5A Synthetase by Photosensitive ATP Analogues†

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ABSTRACT: The photoaffinity probes [γ-^{32}P]2-azidoATP (2-N_3ATP) and [α-^{32}P]8-azido-ATP (8-N_3ATP) were used to investigate the binding of ATP to highly purified 2-5A synthetase. 2-N_3ATP and 8-N_3ATP are substrates for 2-5A synthetase [Suhadolnik, R. J., Karikō, K., Sobol, R. W., Jr., Li, S. W., Reichenbach, N. L., & Haley, B. E., preceding paper]. In this study we show that 2- and 8-N_3ATP are competitive inhibitors of the enzymatic conversion of ATP to 2-5A. Ultraviolet irradiation results in the photoinsertion of 2-N_3ATP and 8-N_3ATP into the enzyme. The covalent photoinsertion of [α-^{32}P]8-N_3ATP into the 2-5A synthetase is proportional to the inactivation of the enzyme as UV irradiation is increased. Photolabeling of 2-5A synthetase is saturated at 1.5 mM 2-N_3ATP and 2.0 mM 8-N_3ATP. Computer analysis of the curvilinear Scatchard plots of the 2-5A synthetase suggests the presence of high-affinity and low-affinity binding sites that may correspond to the acceptor and the 2′-adenylation sites of the enzyme. The competition of nucleotides for the covalent photoinsertion of 8-N_3ATP into the binding site(s) of the synthetase was as follows: ATP > 2′dATP = 3′dATP > CTP > ITP > AMP > NAD^+ > UTP > UMP > CMP. Photoinsertion of 8-N_3ATP into 2-5A synthetase increases with the addition of poly(rI)·poly(rC). Without the addition of poly(rI)·poly(rC) to the synthetase, the [α-^{32}P]8-N_3ATP is photoinserted into the enzyme; however, in the absence of dsRNA and in the absence of UV irradiation, the synthetase cannot convert ATP to 2-5A. The findings suggest that the formation of the enzyme/substrate complex can occur in the absence of dsRNA but dsRNA is essential to activate the 2-5A synthetase to form the productive complex needed for synthesis of 2-5A from ATP.

2-5A[1] synthetase is a dsRNA-dependent enzyme that catalyzes the synthesis of 2′,5′-oligoadenylates from ATP in what appears to be a nonprocessive (dissipative) manner (Justesen et al., 1980a). The best-characterized function of 2-5A is the activation of the 2-5A-dependent endoribonuclease (RNase L) and subsequent degradation of viral and cellular RNA

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[1] The abbreviations used are as listed in the accompanying paper (Suhadolnik et al., 1988).

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