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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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(Lengyel, 1982). This mechanism is one of the mediators of the antiviral action of interferon. Induction of 2-5A synthetase in response to interferon treatment or alteration of the enzyme level during cell differentiation and cell growth has been described (Zilberstein et al., 1978; Jacobsen et al., 1983; Wells & Mallucci, 1985). Diagnostic value of the 2-5A synthetase has been suggested (Chousterman et al., 1983). Isoenzymes of 2-5A synthetase and their subcellular distribution have been described in mouse and human cells (St. Laurent et al., 1983; Chebath et al., 1987). 2-5A synthetase with a molecular weight of 110000 has been highly purified from rabbit reticulocyte lysates (Wu & Eslami, 1983; Wells et al., 1984; Rovnak & Ranu, 1987). Studies have appeared on the broad substrate specificity (Justesen et al., 1980b; Doetsch et al., 1981; Hughes et al., 1983) and stereoselectivity (Lee & Suhadolnik, 1985; Karikó et al., 1987a; Suhadolnik et al., 1987) of 2-5A synthetase, but to date no technique has been available to differentiate between the ATP binding and the 2′-adenylation processes of 2-5A synthetase. In the accompanying paper (Suhadolnik et al., 1988) we describe the enzymatic conversion of 2-N_3ATP and 8-N_3ATP photoreactive nucleotides to their corresponding 2- and 8-azido 2-5A trimer 5′-triphosphates by 2-5A synthetase and the photolabeling of 2-5A binding proteins in cytoplasmic extracts of L929 cells. These results initiated studies on the ATP binding process of 2-5A synthetase with 2-N_3ATP and 8-N_3ATP photoactive probes. Here we report the characterization of highly purified 2-5A synthetase from rabbit reticulocyte lysates by photoaffinity labeling. The present study focuses on (i) the kinetics of the binding process of 2-5A synthetase, (ii) the structural requirements of nucleotides for binding to the synthetase, and (iii) the influence of dsRNA on the binding of ATP to 2-5A synthetase. A preliminary report of this study has been presented (Li et al., 1987).

Materials and Methods

Materials. [γ-^{32}P]ATP (5000 Ci/mmol) and [α-^{32}P]ATP (410 Ci/mmol) were purchased from Amersham; nucleoside 5′-triphosphates and 5′-monophosphates were from Sigma; poly(rI)·poly(rC) was from Pharmacia. 2-N_3ATP, 8-N_3ATP, [γ-^{32}P]2-N_3ATP (7 μCi/nmol), and [α-^{32}P]8-N_3ATP (1.4 μCi/nmol) were synthesized as described (Czarnecki et al., 1982; Michelson, 1964; Suhadolnik et al., 1988). 2-5A synthetase was purified from rabbit reticulocyte lysates as described (Wu & Eslami, 1983).

Assays for 2-5A Synthetase Activity. Method A: The Synthesis of 2-5A. The reaction mixture (total volume 40 μL) contained 15 μL of 2-5A synthetase [specific activity 150 μmol (mg of protein)$^{-1}$ h^{-1}], 20 mM Tris-HCl, pH 8.0, 25 mM KCl, 20 mM $Mg(OAc)_2$, 7.5 mM creatine phosphate, 22.5 units/mL creatine phosphokinase, 50 μg/mL poly(rI)·poly(rC), 10 μCi [α-^{32}P]ATP, 5 mM ATP, and 8-N_3ATP at the concentrations indicated in Figure 1. Incubations were for 18 h at 30 °C. The activity of the 2-5A synthetase was determined by measuring the amount of ATP converted to 2-5A as described (Lee & Suhadolnik, 1985). In experiments with ATP, the yield of 2-5A was 18%.

Method B: The Determination of Pyrophosphate Release from ATP. This assay was as described (Wells et al., 1984). The reaction mixture (10 μL final volume) was the same as in method A with the exceptions that it contained 3.5 μL of 2-5A synthetase and [γ-^{32}P]ATP (455 μCi/mmol, 0.1–4 mM) replaced [α-^{32}P]ATP. Samples were incubated for 90 min at 30 °C, and the reaction was terminated by the addition of 10 μL of 50 mM EDTA. Samples were then mixed with 1 mL of 25 mM phosphate buffer, pH 7.0, 25 mM sodium pyrophosphate, 10 mM EDTA, and 40 mg of acid-washed Norit A charcoal (Pfanstiehl Laboratories) and centrifuged (5 min, 15000 rpm, Beckman Microfuge E). The activity of 2-5A synthetase was determined by measuring the [^{32}P]pyrophosphate in the supernatant. Results include a correction for [^{32}P]pyrophosphate in the [γ-^{32}P]ATP as determined in a control assay.

Photolabeling. Fifteen microliters of 2-5A synthetase was incubated with [α-^{32}P]8-N_3ATP or [γ-^{32}P]2-N_3ATP (at the concentrations indicated in Figures 2 and 3), 20 mM Tris-HCl, pH 8.0, 25 mM KCl, 20 mM $Mg(OAc)_2$, and 50 μg/mL poly(rI)·poly(rC) (or at concentrations indicated in Figure 6) in a final volume of 30 μL for 20 min on ice. In competition experiments, nucleoside 5′-monophosphates, 5′-triphosphates, or NAD^+ was added to the incubation mixtures at the concentrations indicated in Figures 4 and 5. The samples were UV-irradiated for 60 s (or as indicated) at a distance of 2 cm (1.0 J/m^2) by using a 254-nm UVG-11 Mineralight lamp (UltraViolet Products, Inc.). After photolysis, 30 μL of a protein-solubilizing mixture (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) was added to the samples. The photolabeled 2-5A synthetase was analyzed by 8% SDS–PAGE as described (Laemmli, 1970). The Coomassie blue stained dried gels were subject to autoradiography at −70 °C with X-Omat film (Kodak).

Quantitative Analysis of Photolabeling of 2-5A Synthetase by 2- and 8-AzidoATP. Autoradiograms were quantitated on a Hoefer scanning densitometer Model GS350. Peak areas were determined by integration with a computer program of the Hoefer GS350 scanning system. Alternatively, the radioactive protein bands were cut from dried gels and processed as described (Karpel et al., 1987). The radioactivity was then compared to the value obtained in the densitometric tracing.

Radioactive Measurements. A Beckman LS-100C liquid scintillation spectrometer was used. Counting efficiency was 99% for ^{32}P.

Data Analysis. Saturation labeling data were analyzed according to the method of Cantor and Schimmel (1980) using a ligand binding analysis program developed by EMF Software.

Results

Competitive Inhibition of the Enzymatic Conversion of ATP to Authentic 2-5A. The enzymatic synthesis of authentic 2-5A was determined by measuring the release of [^{32}P]pyrophosphate from the [γ-^{32}P]ATP incorporated into 2-5A. The formation of 2-5A was competitively inhibited by 2-N_3ATP and 8-N_3ATP in a concentration-dependent manner. The K_m for ATP was 5.0 mM as determined from Lineweaver–Burk plots. The Lineweaver–Burk plots of 2-5A formation in the presence of either 2-N_3ATP or 8-N_3ATP indicate that 2-N_3ATP and 8-N_3ATP act as competitive inhibitors and inhibit the formation of 2-5A. The K_i for 2-N_3ATP is 1.9 mM and for 8-N_3ATP is 0.8 mM.

Photoincorporation of 8-AzidoATP into 2-5A Synthetase and Subsequent Inactivation of the Enzyme. 2-5A synthetase converted 18% of the ATP to 2-5A (referred to as 100% enzyme activity). One-minute UV irradiation of the 2-5A synthetase in the *absence* of either 2- or 8-azidoATP did not inhibit 2-5A synthetase activity. However, in the absence of UV light but in the presence of 0.15 mM 8-N_3ATP, there was a 10% decrease in the enzymatic conversion of ATP to 2-5A (Figure 1, ○). On the basis of competitive inhibition data, this 10% decrease in enzyme activity was likely due to the competitive inhibition of ATP for the 2-5A synthetase by 0.15 mM 8-N_3ATP and *not* to inactivation by the photoprobe

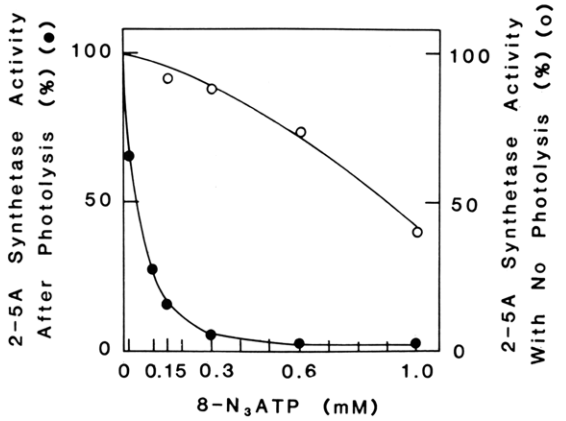

FIGURE 1: Photoincorporation of 8-N_3ATP into 2-5A synthetase. The 2-5A synthetase photolabeling mixtures were UV irradiated for 60 s in the presence of 8-N_3ATP at 0, 0.02, 0.1, 0.15, 0.30, 0.60, and 1.0 mM. [α-^{32}P]ATP (5 mM) was then added to the assay to determine enzyme activity (●) as determined by the formation of 2′,5′-oligoadenylates as described under Materials and Methods (method A). Decreased synthesis of 2-5A with increasing concentrations of 8-N_3ATP in the *absence* of UV irradiation (○) was due to competition for ATP by 8-N_3ATP. In control experiments where incubation samples were not photolyzed, 18% of the ATP was converted to 2-5A by 2-5A synthetase in the absence of 8-N_3ATP. This value is referred to as 100% enzyme activity.

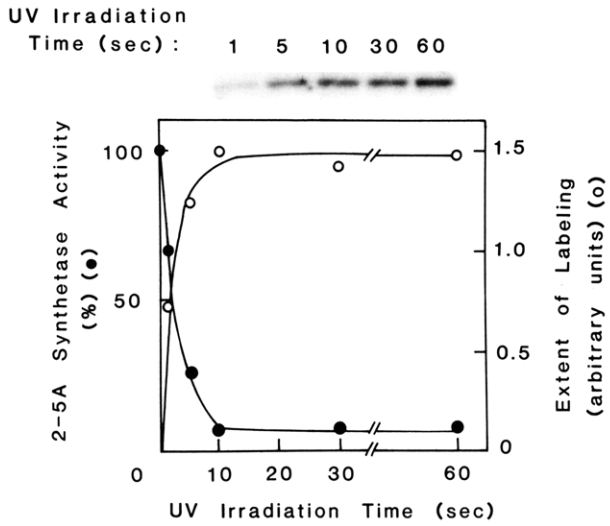

FIGURE 2: Photoincorporation of 8-N_3ATP into 2-5A synthetase and subsequent inactivation of the enzyme. The 2-5A synthetase photolabeling mixtures were UV-irradiated in the presence of 0.15 mM 8-N_3ATP (at 0 °C) and assayed for enzyme activity (●), or the irradiation was done in the presence of 0.15 mM [α-^{32}P]8-N_3ATP (114 μCi/μmol) and analyzed for photolabeling (○). 2-5A synthetase activity was determined by detection of 2-5A formation from [α-^{32}P]ATP as described under Materials and Methods (method A). The activity of the enzyme without UV irradiation was referred to as 100%. Photolabeling of [α-^{32}P]8-N_3ATP into 2-5A synthetase was analyzed by 8% SDS–PAGE as described under Materials and Methods. The relative extent of photolabeling was quantitated by densitometric traces of the autoradiogram.

(Figure 1, ○). However, 1-min UV irradiation in the presence of 0.02 mM 8-N_3ATP resulted in a 35% decrease in the activity of 2-5A synthetase; the presence of 0.15 mM 8-N_3ATP resulted in an 85% decrease in enzyme activity (Figure 1, ●). These data indicate that the inhibition of the enzyme following UV irradiation must be due to the photoinsertion of 8-N_3ATP into the 2-5A synthetase (see Figure 3D). This observation is confirmed in Figure 2. The extent of photolabeling of 2-5A synthetase by [α-^{32}P]8-N_3ATP increased from 1 to 10 s of photolysis (Figure 2, ○ and autoradiogram insert). Further irradiation of the reaction mixture (up to 60 s) showed no

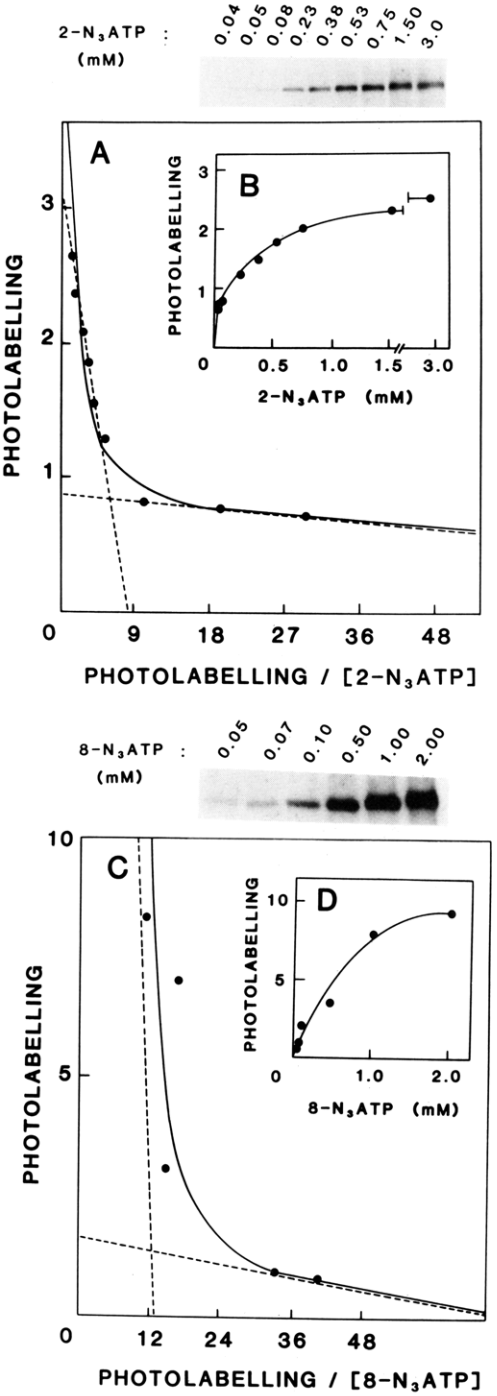

FIGURE 3: Saturation photolabeling of 2-5A synthetase. The 2-5A synthetase photolabeling mixtures, with increasing concentrations of [γ-^{32}P]2-N_3ATP (36 μCi/μmol) or [α-^{32}P]8-N_3ATP (45 μCi/μmol), were incubated, UV-irradiated, and analyzed on 8% SDS–PAGE as described under Materials and Methods. The autoradiograms were quantitated by densitometry. Saturation curves of 2-5A synthetase by [γ-^{32}P]2-N_3ATP [(B) and autoradiogram insert] and [α-^{32}P]8-N_3ATP [(D) and autoradiogram insert] and Scatchard plots from saturation labeling experiments with [γ-^{32}P]2-N_3ATP (A) and [α-^{32}P]8-N_3ATP (C) are shown. Photolabeling is expressed in arbitrary units.

additional photolabeling (○) or decrease in 2-5A synthetase activity (●).

Saturation Labeling of 2-5A Synthetase. When 2-5A synthetase was treated with increasing concentrations of either [γ-^{32}P]2-N_3ATP or [α-^{32}P]8-N_3ATP, the enzyme was saturated at 1.5 and 2.0 mM, respectively (Figure 3B,D and autoradiogram inserts). Fifty percent of the maximal extent of photolabeling occurs at 0.25 mM with 2-N_3ATP and at 0.5

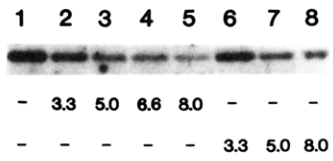

FIGURE 4: Inhibition of photolabeling of 2-5A synthetase by ATP and GTP. The 2-5A synthetase photolabeling mixtures were incubated in the presence of 0.15 mM [α-^{32}P]8-N_3ATP (91 μCi/μmol) and without (lane 1) or with addition of ATP at 3.3, 5.0, 6.6, and 8.0 mM (lanes 2–5, respectively) or GTP at 3.3, 5.0, and 8.0 mM (lanes 6–8, respectively). Samples were UV-irradiated and analyzed on 8% SDS–PAGE as described under Materials and Methods. The control represents 100% photolabeling.

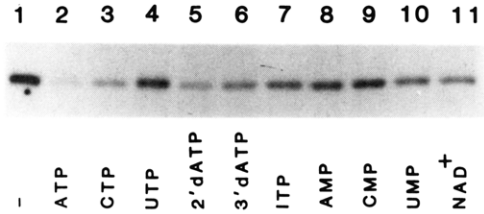

FIGURE 5: Inhibition of 8-N_3ATP photoincorporation into 2-5A synthetase by nucleotides. The 2-5A synthetase photolabeling mixtures were incubated with 0.15 mM [α-^{32}P]8-N_3ATP (136 μCi/μmol) and without (lane 1) or with the addition of 8.0 mM ATP (lane 2), CTP (lane 3), UTP (lane 4), 2′dATP (lane 5), 3′dATP (lane 6), ITP (lane 7), AMP (lane 8), CMP (lane 9), UMP (lane 10), or NAD^+ (lane 11). Samples were UV-irradiated and analyzed on 8% SDS–PAGE as described under Materials and Methods. The control experiment (lane 1) is referred to as 100%.

mM with 8-N_3ATP. The data obtained by saturation photolabeling were subjected to Scatchard analysis (Figure 3A,C) as described by Julin and Lehman (1987). Computer analysis of the curvilinear Scatchard plots suggests two classes of binding sites on the 2-5A synthetase. Dissociation constants of 5 and 380 μM were calculated for 2-N_3ATP and of 9 and 1000 μM for 8-N_3ATP. Photolyzed products of 8-N_3ATP do not inhibit 2-5A synthesis.

Photoincorporation of [α-^{32}P]8-AzidoATP into 2-5A Synthetase in the Presence of ATP or GTP. The addition of either ATP or GTP to incubation mixtures resulted in concentration-dependent protection against photoaffinity labeling of 2-5A synthetase by [α-^{32}P]8-N_3ATP. With ATP as a competitor at concentrations of 3.3, 5.0, 6.6, and 8.0 mM, the photoinsertion of [α-^{32}P]8-N_3ATP was decreased by 50%, 75%, 90%, and 90%, respectively (Figure 4, lanes 2–5). With GTP as a competitor at concentrations of 3.3, 5.0, and 8.0 mM, the photoinsertion of the azido probe was decreased by 20%, 50%, and 75%, respectively (Figure 4, lanes 6–8). The photolabeling was also inhibited by nucleoside 5′-monophosphates and 5′-triphosphates and NAD^+ (Figure 5). The order of competition of the nucleotides for photoinsertion of [α-^{32}P]8-N_3ATP into the synthetase was ATP > 2′dATP = 3′dATP > CTP > ITP > AMP > NAD^+ > UTP > UMP > CMP (Figures 4 and 5). To demonstrate that the high concentrations (8 mM) of the nucleotides used in the competition experiments did not interfere with photolabeling, control experiments were done in which 8-N_3ATP and 8 mM nucleotide were mixed and irradiated for 1 min. When this mixture was combined with 2-5A synthetase and further irradiated, no photolabeling of the synthetase was observed. Therefore, the 8-N_3ATP was completely photolyzed in the presence of 8 mM nucleotides. Furthermore, photolysis of 8-N_3ATP in the presence of 8 mM ATP showed a complete disappearance of 8-N_3ATP as determined by HPLC.

Interaction of 2-5A Synthetase with 8-AzidoATP and dsRNA. The dependence of photoincorporation of 8-N_3ATP into 2-5A synthetase was studied by varing the concentrations

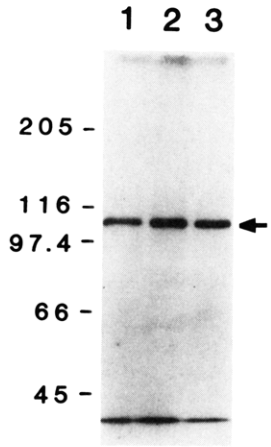

FIGURE 6: Effect of poly(rI)·poly(rC) on photoaffinity labeling of 2-5A synthetase by 8-N_3ATP. The 2-5A synthetase photolabeling mixtures were incubated without (lane 1) or with the addition of 50 and 250 μg/mL poly(rI)·poly(rC) (lanes 2 and 3, respectively) for 30 min at 30 °C. The samples were UV-irradiated and analyzed on 8% SDS–PAGE as described under Materials and Methods. The arrow indicates the position of the 2-5A synthetase. The size markers used were myosin (205 000), β-galactosidase (116 000), phosphorylase B (97 400), bovine serum albumin (66 000), and ovalbumin (45 000).

of poly(rI)·poly(rC) (Figure 6, lanes 1–3). In the absence of added dsRNA, 8-N_3ATP was photoinserted into 2-5A synthetase (Figure 6, lane 1); however, in the absence of added poly(rI)·poly(rC) the enzyme was *not* activated and could not convert ATP to 2-5A. In the presence of either 50 or 250 μg/mL poly(rI)·poly(rC), an increase in the photoincorporation of 8-N_3ATP was observed (Figure 6, lane 2). The activity of 2-5A synthetase was dsRNA dependent in that the enzymatic conversion of ATP to 2-5A was 0%, 18%, and 16% at 0, 50, and 250 μg/mL poly(rI)·poly(rC), respectively (Figure 6, lanes 2 and 3). We tested the possibility that the increased insertion of 8-N_3ATP into 2-5A synthetase in the presence of poly(rI)·poly(rC) (Figure 6, lanes 2 and 3) was simply due to the stabilization of 2-5A synthetase by dsRNA. When 2-5A synthetase was preincubated in either the presence or absence of poly(rI)·poly(rC) (50 μg, 30 min, 30 °C), the conversion of ATP to 2-5A was also 18%. Therefore, incubation of 2-5A synthetase in the absence of poly(rI)·poly(rC) for 30 min *did not decrease* the stability of the enzyme.

Discussion

In this study, we have utilized [^{32}P]2- and 8-azidoATP as photoaffinity probes to determine the nucleotide reactive binding site(s) of 2-5A synthetase. Several heretofore unknown aspects of 2-5A synthetase are presented with respect to (i) the kinetics and mechanism of the binding process, (ii) the structural requirements of nucleotides for binding to the enzyme, and (iii) the dsRNA requirement for the binding of ATP to the enzyme. In the preceding paper (Suhadolnik et al., 1988) we reported that 2- and 8-N_3ATP are converted to their corresponding 2- and 8-azido 2-5A trimer 5′-triphosphates by the 2-5A synthetase. These findings suggest that 2-N_3ATP and 8-N_3ATP bind to the same site(s) on the enzyme as does ATP. Therefore, 2- and 8-N_3ATP are structurally related photoactive nucleotides that can be used to identify the site(s) where ATP binds to 2-5A synthetase. 2-N_3ATP and 8-N_3ATP form 2- and 8-azido 2-5A trimer 5′-triphosphates in yields of 3% and 0.7%, respectively (Suhadolnik et al., 1988). Saturation binding analysis indicates that the nucleotide concentration required for 50% binding to 2-5A synthetase is 0.25 mM for 2-N_3ATP (Figure 3B) and 0.5 mM for 8-N_3ATP (Figure 3D). These results suggest that 2-N_3ATP is a better

substrate for the enzymatic conversion of ATP to 2-5A by 2-5A synthetase than is 8-N_3ATP. Analysis of the Lineweaver–Burk plots reveals K_i values of 0.8 mM for 8-N_3ATP and of 1.9 mM for 2-N_3ATP. The activity of 2-5A synthetase was not decreased in the absence of 2- or 8-N_3ATP after 1 min of UV irradiation. However, the enzyme was inhibited about 85% when irradiated (1 min, 0 °C) in the presence of 0.15 mM 8-N_3ATP, providing evidence that the 8-N_3ATP was covalently photoinserted into the nucleotide binding site(s) of the enzyme (Figure 1). The covalent photoinsertion of [α-^{32}P]8-N_3ATP into 2-5A synthetase following only 10 s of UV irradiation (Figure 2) and almost complete inactivation of enzyme activity suggests that the photolabeling of the enzyme is highly specific and very efficient. Photolabeling of the 2-5A synthetase by 2-N_3ATP (Figure 3B) or 8-N_3ATP (Figure 3D) was concentration dependent and saturable. This suggests that the photoinsertion is a reflection of the nucleotide binding process. Fifty percent saturation of the photolabeling of 2-5A synthetase was achieved at 0.25 mM 2-N_3ATP, whereas 0.5 mM 8-N_3ATP was required. The difference in saturation kinetics may be due to the differing conformations of the 2- and 8-N_3ATP. 2-N_3ATP is in the natural anti conformation, whereas 8-N_3ATP is mostly in the syn conformation in solution (Czarnecki, 1984). The conformational difference between 2- and 8-N_3ATP has been suggested as one explanation for the 3% yield of the 2-azido 2-5A trimer 5′-triphosphate from 2-N_3ATP by 2-5A synthetase compared to the 0.7% yield from 8-N_3ATP (Suhadolnik et al., 1988).

Because 2- and 8-N_3ATP can be converted to 2′,5′ oligomers by 2-5A synthetase (Suhadolnik et al., 1988), these azido photoprobes of ATP should be able to bind to both the acceptor and the donor sites of the enzyme. This ability of 2- and 8-N_3ATP to bind to both sites of 2-5A synthetase will permit the further elucidation of the 2′-adenylation mechanism of the enzyme. Computer analysis of the curvilinear Scatchard plots obtained from photolabeling experiments of 2-5A synthetase by 2-N_3ATP (Figure 3A) or 8-N_3ATP (Figure 3C) suggests the presence of high- and low-affinity binding sites on 2-5A synthetase that may be the previously reported acceptor and donor sites of the enzyme (Ferbus et al., 1981). The dissociation constants for the high- and low-affinity binding sites of the 2-5A synthetase explain the inactivation of the enzyme by 8-N_3ATP while the enzyme is only partially saturated. For example, about 30% of the 2-5A synthetase is saturated by 0.15 mM 8-N_3ATP (Figure 3D); however, at this concentration, about 85% of the enzyme is inactivated (Figure 1). This suggests that the 8-N_3ATP at 0.15 mM preferentially photolabels the high-affinity binding site (K_d = 9 μM) (Figure 3C). An alternative explanation could be that only 30% of the enzyme molecules were labeled. The additional loss of enzyme activity over this 30% could have been due to the inhibitory activity of photolyzed 8-N_3ATP on 2-5A synthetase activity. However, we have established that the photolyzed product of 8-N_3ATP is *not* an inhibitor of the synthetase. The activity of the 2-5A synthetase is completely inhibited when the concentration of 8-N_3ATP is 0.6 mM or more (Figure 1) because the high-affinity binding site is completely saturated. However, there is a continued increase in photoinsertion of [α-^{32}P]8-N_3ATP as the concentration of 8-N_3ATP is increased to 2 mM (Figure 3D). At 2 mM 8-N_3ATP, the high-affinity (K_d = 9 μM) and low-affinity (K_d = 1000 μM) binding sites are saturated. Thus, the catalytic, saturation, and inhibition kinetic data strongly suggest that the ATP binding and 2′-adenylation processes of 2-5A synthetase are separate and distinguishable. Therefore, the characterization of the ATP binding and 2′-adenylation processes can be further explored as a means to understand the nonprocessive mechanism for 2-5A formation.

Competition experiments revealed that the nucleoside 5′-triphosphates, ATP, GTP, 3′dATP, ITP, CTP, and UTP, which have been reported to be converted to 2-5A analogues by 2-5A synthetase (Doetsch et al., 1981; Hughes et al., 1983), competed for the photoinsertion of 8-N_3ATP into the enzyme (Figures 4 and 5). Nucleoside 5′-monophosphates were significantly less efficient competitors of photolabeling than their corresponding 5′-triphosphates (Figure 5, compare lanes 8–10 with lanes 2–7). NAD^+, which was reported to be a primer of 2-5A synthetase (Ferbus et al., 1981), could compete out 50% of the [α-^{32}P]8-N_3ATP in photolabeling experiments (Figure 5, lane 11). Competition experiments with 2- and 8-N_3ATP and NAD^+ or other primers such as p_3A_2 will permit the mapping of the amino acids interacting in the ATP and the 2′-adenylation binding domains of 2-5A synthetase during the 2′-adenylation process. It is noteworthy that 2′dATP, which is not a substrate for 2-5A synthetase, inhibited the photoincorporation of 8-N_3ATP as efficiently as ATP (Figure 5, lanes 5 and 2, respectively). On the basis of the inverse relationship between cell proliferation and activity of the 2-5A/RNase L system (Jacobsen et al., 1983; Wells & Mallucci, 1985), 2′dATP may be a regulator of 2-5A synthetase under physiological conditions. The competition of 3′dATP for the photoinsertion of 8-N_3ATP would be expected because 3′dATP has been reported to be a substrate for 2-5A synthetase and is converted to the 2′,5′-cordycepin trimer and tetramer 5′-triphosphates (Doetsch et al., 1981; Suhadolnik et al., 1983). These 2′,5′-cordycepin 5′-triphosphates have been shown to be biologically active in poly(U) and rRNA cleavage assays (Karikō et al., 1987b; Nyilas et al., 1986).

Although the 2-5A synthetase from mammalian cell extracts requires dsRNA as an allosteric activator and dsRNA has been reported to increase the stability of the enzyme (Rovnak & Ranu, 1987; Wells et al., 1984), there have been no studies examining the binding of photoprobes of ATP to the enzyme in the presence or absence of poly(rI)·poly(rC). It is interesting to note that Chebath and co-workers recently reported that a 100-kDa isoenzyme of 2-5A synthetase from human cells does *not* require dsRNA for 2-5A formation from ATP (Chebath et al., 1987). Hovanessian et al. (1988) recently reported significant differences in enzyme activity between the 69- and 100-kDa 2-5A synthetases from interferon-treated human cells. At low concentrations of dsRNA, both the 69- and 100-kDa species synthesize mainly 2-5A dimer (i.e., p_3A_2); however, at optimum concentrations of dsRNA, the pattern of 2-5A oligomer synthesis is different.

The covalent photoincorporation of 8-N_3ATP into 2-5A synthetase can occur without the addition of dsRNA (Figure 6, lane 1). This finding suggests that the *binding* of ATP to 2-5A synthetase does *not* require dsRNA. However, addition of dsRNA to the 2-5A synthetase increased the amount of [α-^{32}P]8-N_3ATP photoinserted into the enzyme (Figure 6, compare lanes 1 and 2), supporting the conclusion that dsRNA increases the binding affinity of the enzyme for ATP. Alternatively, in the absence of dsRNA, it is possible that only one of the two nucleotide binding sites (donor or acceptor) may be available for binding 8-N_3ATP. Following the binding of dsRNA, both the donor and the acceptor sites would be exposed and available for binding, resulting in an increase in [α-^{32}P]8-N_3ATP photoincorporation. Under these conditions the allosteric modifier dsRNA activates 2-5A synthetase such that there is an 18% conversion of ATP to 2-5A. The stability

of the 2-5A synthetase was not altered when incubated either in the presence or absence of dsRNA for 30 min at 30 °C. Studies are currently under way with the cloned human 2-5A synthetases and [^{32}P]dsRNA photoprobes to characterize the amino acids in the dsRNA binding domain of 2-5A synthetase.

Although many structural modifications of ATP result in analogues that are substrates for 2-5A synthetase, these ATP analogues cannot be used to identify the amino acid residues located at the ATP binding sites of this enzyme. The aim of the study described here was to initiate investigations with the 2- and 8-N_3ATP as photoaffinity probes of 2-5A synthetase. Our eventual goal is to use these photoaffinity probes to define the geometry of the binding domain of the substrate (ATP) and the allosteric activator (dsRNA) of 2-5A synthetase as required for productive complex formation needed for the enzymatic synthesis of the 2′,5′-oligoadenylates.

Registry No. 2-N_3ATP, 117145-98-9; 8-N_3ATP, 117145-99-0; ATP, 56-65-5; 2-5A synthetase, 69106-44-1.

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