Identification of the ATP Binding Domain of Recombinant Human 40-kDa 2',5'-Oligoadenylate Synthetase by Photoaffinity Labeling with 8-Azido-α-[32P]ATP*

(Ning Kon‡ and Robert J. Suhadolnik‡§)

From the ‡Department of Biochemistry and the §Fox Chase Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Three isoforms of the interferon-inducible 2',5'-oligoadenylate (2-5A) synthetase that require double-stranded RNA have been isolated and cloned. However, identification of the amino acid(s) of 2-5A synthetase directly interacting with ATP is crucial to the elucidation of the mechanism of the enzymatic conversion of ATP to 2',5'-oligoadenylates by 2-5A synthetase. Recombinant human 40-kDa 2-5A synthetase has been expressed as a glutathione S-transferase fusion protein in E. coli and purified to near homogeneity in milligram quantities. The azido photoprobe, 8-azido-α-[32P]ATP, has been used to identify the ATP binding domain of the recombinant human 40-kDa 2-5A synthetase. Specific covalent photoincorporation of 8-azido-α-[32P]ATP into the 2-5A synthetase, tryptic digestion of the covalently labeled enzyme, isolation of the photolabeled phoshopeptide by metal (Al3+) chelate chromatography, and high pressure liquid chromatography identified a photopeptide, which has been assigned to the ATP binding domain of 2-5A synthetase. The radioactive pentapeptide has the sequence D196FLKQ200 in which the binding domain of 2-5A synthetase. The radioactive pentapeptide has the sequence D196FLKQ200 in which the binding domain of 2-5A synthetase. Theradioactive pentapeptide by metal (Al3+) chelate chromatography, and high pressure liquid chromatography identified a photopeptide, which has been assigned to the ATP binding domain of 2-5A synthetase. The radioactive pentapeptide has the sequence D196FLKQ200 in which the binding domain of 2-5A synthetase.

Interferon (IFN)-α and -β induce two double-stranded RNA-dependent binding proteins, 2-5A synthetase and PKR; both of these enzymes inhibit viral protein synthesis (1–3). Following the association of the cytoplasmic, membrane-associated IFN-α receptor-Jak1 and IFN-β1 receptor-Tyk2 subunits about an IFN-α or -β ligand, a series of phosphorylation events leading to the formation of a Stat1-Stat2 complex ensues (4, 5). These active heterodimers migrate into the nucleus and complex with p48 to form the ISGF3α transcriptional complex. A series of IFN-inducible genes that contain an interferon-stimulatory response element consensus sequence in their promoters such as 2-5A synthetase, PKR, interferon regulatory factor 1, and possibly the dsRNA-dependent adenosine deaminase serve as targets for enhanced transcriptional activity (6). 2-5A synthetase (EC 2.7.7.7) converts ATP to 2',5'-oligoadenylates (2-5A, p3A), which in turn activate a latent endoribonuclease (RNase L, EC 3.1.2.7) to hydrolyze single-stranded RNA. The dsRNA-activated PKR inhibits translation by phosphorylation of eIF-2α. Three major isoforms of human 2-5A synthetase, 40–46 kDa, 69–71 kDa, and 100 kDa, have been reported (7, 8). The 40–46 kDa and 69–71 kDa human 2-5A synthetases have been cloned (9). The isoforms of 2-5A synthetase are located in the cell membrane, cytoplasm, and nucleus with various cellular functions (9).

2-5A synthetase is a nonprocessive enzyme, as evidenced by the formation of a series of 2',5'-oligoadenylates of the formula, 2',5'-A(p3A)m, in which m ≥ 1 (10, 11). Two nucleotide binding sites on 2-5A synthetase have been suggested as requirements for formation of 2-5A, i.e., an acceptor site and a donor site. The donor site binds ATP, 2-5A, NAD+, or other nucleotides with AMP moieties (10–12). The acceptor site binds ATP, thereby providing the substrate for 2'-adenylation (10, 11). Kinetic studies suggest a high affinity (Kd = 9 μM) and a lower affinity (Kd = 1000 μM) binding site (12). Therefore, identification of one or both of these nucleotide binding sites would enhance our understanding of the nonprocessive mechanism by which 2-5A synthetase, as a nucleotidyldtransferase, converts ATP to 2-5A. To elucidate the catalytic mechanism of 2-5A synthetase, it is essential to understand the interactions between 2-5A synthetase and ATP and to identify the amino acid(s) at the ATP binding domain. The study described here has focused on the identification of ATP binding domain(s) of 2-5A synthetase by photoaffinity labeling technology using 8-azido-ATP as a substrate analog of ATP to bind to the ATP binding domain of the 40-kDa 2-5A synthetase. The covalent photoincorption of 8-azido-α-[32P]ATP into 2-5A synthetase is highly specific and saturable, which provides the basis for the use of this photoprobe to identify the amino acids in the ATP binding domain of 2-5A synthetase, which are covalently modified by the azido acrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.
photoprobes. Azido photoprobes of purine and pyrimidine nucleotides covalently cross-link following UV irradiation such that photobleached peptide fragments and/or specific amino acids have been identified (13–20). Upon UV irradiation, a reactive nucelophile nitrene is produced, which forms a covalent bond with an electrophilic group in juxtaposition for covalent bonding (13, 16).

Expression and Purification of Recombinant Human 40-kDa 2-5A Synthetase—Plasmid pTLE-1 containing human-40 kDa 2-5A synthetase cDNA was isolated from pTLE-1 by restriction digestion and used to transform E. coli (17). The plasmid DNA was isolated and linearized with the restriction enzyme SmaI. Following ligation, the construct was transformed into Competent E. coli cells and selecting for ampicillin resistance. The E. coli cells were cultured in 500 ml of LB medium with ampicillin (50 μg/ml). The culture was incubated at 37 °C, and the absorbance at 600 nm was 0.8, isopropyl-1-thio-β-d-galactopyranoside was added to 0.3 mM. The culture was incubated with shaking for an additional 3 h before harvesting (23, 24).

**EXPERIMENTAL PROCEDURES**

Materials—Restriction enzymes and Taq polymerase were purchased from Boehringer Mannheim. T7 DNA polymerase and T4 DNA ligase were from U. S. Biochemical Corp. pGEX 2T, poly(I)-poly(C) agarose, glutathione-Sepharose 4B (GS-Sepharose) and S-Sepharose were from Pharmacia Biotech Inc. [α-32P]ATP (400 Ci/mmol) was purchased from Amersham Corp. 8-azido-[α-32P]ATP was generously supplied by Dr. B. E. Haley (University of Kentucky). Human thrombin was provided by Dr. Andrei Budzynski (Department of Biochemistry, Temple University School of Medicine).

Expression of Recombinant Human 40-kDa 2-5A Synthetase—Plasmid pTLE-1 containing human-40 ka 2-5A synthetase cDNA was provided by Dr. Judith Chebath (Weizmann Institute of Science). The DNA fragment containing the coding region of the human 40-kDa 2-5A synthetase cDNA was isolated from pTLE-1 by restriction digestion with XbaI and EcoRI and subcloned into M13 mp18 digested with the same enzymes. The recombinant plasmid was used to prepare radiolabeled single-stranded RNA, which was used as the template for in vitro mutagenesis using T7 DNA polymerase (U. S. Biochemical Corp.) (22). The primer, 5′-GATGAGGGGTAACTACATGATGATGCTCAAG-3′, was synthesized to generate a NdeI site at the first ATG codon of the 40-kDa 2-5A synthetase coding region. After the identification of the recombinant M13 phage with the NdeI site mutation, the human 40-kDa 2-5A synthetase cDNA insert was released by restriction digestion with NdeI and EcoRI, filled in with Klenow fragment, and subcloned into the expression vector pGEX 2T linearized with Smal to generate pMK14. The correct reading frame was confirmed by restriction digestion, and the coding sequence of 40-kDa 2-5A synthetase was confirmed by dideoxynucleotide sequencing using the Sequenase 2.0 kit (U. S. Biochemical Corp.).

Expression and Purification of Recombinant Human 40-kDa 2-5A Synthetase—pMK14, which fuses the 40-kDa 2-5A synthetase cDNA to the 3′ terminus of glutathione S-transferase cDNA, was transformed into Escherichia coli HMS174 cells (Navagen). The E. coli cell was streaked on an LB 15% agar plate containing 50 μg/ml ampicillin. A single colony was inoculated into 50 ml of LB medium containing 50 μg/ml ampicillin followed by incubation at 37 °C with shaking at 200 rpm/min, overnight (New Brunswick shaker). The culture was inoculated into 500 ml of LB medium with ampicillin (50 μg/ml). The absorbance of the culture at 600 nm was 0.8, isopropyl-1-thio-β-d-galactopyranoside was added to 0.3 mM. The culture was incubated with shaking for an additional 3 h before harvesting (23, 24). Unless otherwise specified, all protein purification procedures were performed at 4 °C. The E. coli cells were collected by centrifugation (6000 × g, 5 min) and suspended in 10 volumes of PBS containing 0.1 mM phenylmethylsulfonyl fluoride and 10 mM β-mercaptoethanol. The cells were broken in a French pressure cell (500 psi, twice). The cell lysate was centrifuged (25000 × g, 1 h).

After centrifugation, the supernatant (40 mg protein/ml, 100 ml) was loaded onto a 2 ml GSH-Sepharose column, flow rate at 0.2 ml/min. The column was washed with 20 ml of PBS. The GSH-Sepharose resin was resuspended in 2 ml of PBS containing human thrombin (1 unit/ml). The GSH-Sepharose suspension was incubated with constant mixing at 25 °C, 4 h. The recombinant human 40-kDa 2-5A synthetase was released into PBS buffer by centrifugation (12000 × g, 5 min). The supernatant was removed and stored at −70 °C. To remove glutathione S-transferase (GST) and glutathione, the recombinant human 40-kDa 2-5A synthetase was replaced with 8-azido-[α-32P]ATP and 8-azido-ATP replacing [α-32P]ATP and ATP.

Time-dependent Covalent Photoaffinity Labeling of Recombinant Human 40-kDa 2-5A Synthetase—Photolabeling assays (10 μl) were performed in 20 mM Tris-HCl, pH 7.5, 20 μM Mg(OAc)2, 0.1 μM of protein, 2 μl ATP, 2 μCi of [α-32P]ATP and 50 μg/ml poly(I)-poly(C). The reaction mixture was incubated at 30 °C for 2 h. The reaction mixture was incubated with poly(I)poly(C) and the reaction mixture was incubated with 5 μl of each reaction mixture were applied to a polyethyleneimine thin layer chromatogram (TLC) (Brinkmann). The TLC was developed in 1 M acetic acid. After autoradiography, the saturation photolabeling was determined at 5, 10, 20, 40, and 60 s. Reaction products were quenched by Cerenkov radiation. The yield was about 20% with a variability of ±2% 2-5A synthetase assays with 8-azido-ATP as the substrate used the above procedure with 8-azido-[α-32P]ATP and 8-azido-ATP replacing [α-32P]ATP and ATP.

Photolabeling Reactions—Photolabeling reactions in the absence of poly(I)poly(C). The reaction mixture was incubated on ice for 30 min before photolabeling for 30 s. To increase the amount of 8-azido-[α-32P]ATP that was covalently bound, the photolabeling was repeated twice by adding excess nonradioactive 0.1 mM 8-azido-ATP, which saturates the ATP binding site under the same conditions described. Photolabeled 2-5A synthetase was precipitated by adding 20% cold perchloric acid to a final concentration of 8%, incubation on ice for 20 min, and centrifugation (12000 × g, 10 min, 4 °C).
protein pellet was washed with 1 ml absolute methanol to remove
the unreacted 8-azido-[α-32P]ATP. The methanol was decanted.

Trypsin Digestion of the Photolabeled Recombinant Human 40-kDa
2-5A Synthetase—The precipitated 32P-photolabeled protein was dis-
solved in 80 μl of 8 M urea. To the protein solution was added 1100 μl
of 1% ammonium acetate, pH 7.9, and 100 μl of TPCK-treated trypsin
solution (1 mg/ml in 1% ammonium hydroxide, pH 7.9). The pH of the
digestion mixture was adjusted to 7.9 with 1 M ammonium hydroxide,
and the mixture was incubated at 37 °C for 12 h. An additional 100 μl
of trypsin solution was added, and the mixture was incubated for
150 min at 37 °C.

Purification of Photolabeled Peptide by Immobilized Al3+- Affinity
Chromatography—Iminodiacetic acid-Sepharose 6B resin (1 ml) was
washed and equilibrated with 20 ml of glass-distilled water (20). A
column (0.75 × 4 cm) was prepared and equilibrated with 10 ml of 50
mM ACl3. The tryptic digestion mixture (1.5 ml) was mixed with 1.5 ml
of buffer A (1% ammonium acetate, pH 5.8) and 150 μl of 10% acetic
acid. The mixture was transferred onto the immobilized Al3+-
affinity column. Two-milliliter fractions were collected, and the column
was washed with buffer A (1 ml/min) until radioactivity in the eluant
decreased to background level (about 20 ml). One-milliliter fractions of
the 32P-photolabeled peptide were collected from the column with
100 μl of buffer A (pH 7.4, ammonium acetate) and eluted by their absorbance
at 214 nm. The 32P was determined by Cerenkov radiation. Fractions
20–25 containing radioactivity were pooled and lyophilized to 200–300
μl in a vacuum concentrator.

HPLC Analysis of the Photolabeled Tryptic Peptide from Al3+- Affinity
Chromatography by Reverse Phase HPLC—The photolabeled 32P-
peptide fractions were fractionated by Al3+- affinity chromatography
were further purified by HPLC using μC18 reverse phase column chroma-
tography (elution buffer A: 0.1% trifluoroacetic acid, pH 2; elution
buffer B: 80% acetonitrile, 0.1% trifluoroacetic acid, pH 1.5). The pep-
tides were displaced with the following gradient: 0–10 min, 0% B;
10–50 min, linear gradient to 40%B; 50–80 min, linear gradient to 75%
B; 80–85 min, linear gradient to 90% B; One ml of buffer A was added to
the lyophilized photolabeled 32P-peptide solution and it was injected
onto the column. One-milliliter fractions were collected (flow rate, 1 ml/min).
Absorption at 214 nm was monitored, and the radioactivity of an aliquot
of each fraction was measured by Cerenkov radiation. The radioactive
peptide peak was sequenced by the PTH-derivative method (14).

Site-directed Mutagenesis of Human 40-kDa 2-5A Synthetase—Four
oligodeoxynucleotides were designed and synthesized to mutate amino acid
Lys199 to Arg or His (primer 1, 5′-CAGTCGAGGATGAGCCAGCA-3′;
primer 2, 5′-AGCTTGATTGGGCGCTGATCGACCAAGTCTCT-CTG-3′; primer 3, 5′-CAGGGCCGACCAAGTCTCTG-3′; primer 4, 5′-TGG
CTCCAGGAGATGAC-3′). The first round of PCR was performed using
plasmid pNK14 as the template with primers 1 and 2 and primers
3 and 4, respectively. The cycles were (i) 95°C for 5 min; (ii) 95°C for 1
min, 52°C for 45 s, 72°C for 45 s (30 cycles); and (iii) 72°C for 10 min.
PCR products were purified by gel electrophoresis. The second round of PCR
was performed with the two PCR products, primers 1 and 4 using the cycles described above. The second round
PCR product was digested with restriction enzymes PstI and KpnI to generate
the 120-bp fragment, which was ligated with a 5000-bp fragment of
pNK14 digested with PstI and KpnI. The resulting clones were screened by
dideoxynucleotide sequencing for the mutation desired. Individual con-
structs were transformed into E. coli HMS 174 cells for expression
of mutant human 40-kDa 2-5A synthetases.

Expression and Purification of Recombinant Human 40-kDa
2-5A Synthetase—The GST fusion protein strategy increased
the yield of recombinant human 40-kDa 2-5A synthetase signi-
ficantly compared with that obtained by direct expression of 2-5A synthetase
in the absence of poly(I)-poly(C), and 0.1
mM trypsin solution was added to
the reaction products were dephosphorylated and separated by
copolyethyleneimine-cellulose TLC. An autoradiogram of the TLC
was shown. The positions of authentic 2-5A dimer and trimer cores and P,
are indicated.

Competition Experiments with the K199R Mutant—Competition pho-
toaffinity labeling of the K199R mutant were performed with 0.15 mM
8-azido-[α-32P]ATP (specific activity, 0.5 μCi/nmol) and increasing con-
centrations of ATP as described above for the wild type 2-5A synthetase.

Peptide Sequencing—Amino acid sequencing was completed by the Amino
Acid Sequencing Core Facility, Temple University School of Medicine and by the Protein Chemistry Laboratory, University of Penn-
sylvania School of Medicine.

Scintillation Spectrometry—Scintillation spectrometry was per-
formed with ScintiVerse I (Fisher) using a Tm Analytics model 6895
scintillation spectrometer (> 95% efficiency) or by Cerenkov radiation
(90% efficiency).

RESULTS

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copolyethyleneimine-cellulose TLC. An autoradiogram of the TLC
was shown. The positions of authentic 2-5A dimer and trimer cores and P,
are indicated.
Covalent Photoaffinity Labeling of 2-5A Synthetase by 8-Azido-ATP—In view of the utilization of 8-azido-[$\alpha$-$^{32}$P]ATP as a substrate for recombinant human 40-kDa 2-5A synthetase, 8-azido-[$\alpha$-$^{32}$P]ATP was used to bind to the ATP binding domain. The covalent photoinertion of 8-azido-[$\alpha$-$^{32}$P]ATP into recombinant human 40-kDa 2-5A synthetase specifically occurred upon activation by UV light. The incorporation of $^{32}$P was saturated after a 20-s exposure to UV light (Fig. 3, lane 4). No increase in the extent of photolabeling was observed at 40 or 60 s (Fig. 3, lanes 5 and 6). The enzymatic activity of the photolabeled 2-5A synthetase decreased with the increase of covalent photoinertion of 8-azido-[$\alpha$-$^{32}$P]ATP into 2-5A synthetase (Fig. 3B), which indicates that 8-azido-ATP competes for the ATP binding domain. UV irradiation of 2-5A synthetase in the absence of 8-azido-[$\alpha$-$^{32}$P]ATP had no effect on the activity of 2-5A synthetase (data not shown).

Saturation Photoaffinity Labeling of Recombinant Human 40-kDa 2-5A Synthetase—The photoaffinity labeling was saturable with increasing concentrations of 8-azido-[$\alpha$-$^{32}$P]ATP in the presence or absence of 10 $\mu$g/ml poly(I)-poly(C) (Fig. 4A). 2-5A synthetase activity decreased as the percentage of photolabeling increased; saturation of photolabeling was achieved at 0.3 mM 8-azido-[$\alpha$-$^{32}$P]ATP (Fig. 4B). In the presence of 10 $\mu$g/ml of poly(I)-poly(C), there was a decrease in the covalent photoinertion of 8-azido-ATP (Fig. 4B). This decrease is attributed to the shielding of UV absorption of the poly(I)-poly(C) (20). The stoichiometry of photolabeling was calculated to be 0.6 mol of 8-azido-ATP per mol of 2-5A synthetase.

Competition Experiments—Photoaffinity labeling of recombinant human 2-5A synthetase by 8-azido-[$\alpha$-$^{32}$P]ATP can be specifically inhibited by ATP (Fig. 5, A and B), indicating that photoaffinity labeling by 8-azido-ATP is saturable and highly specific for the ATP binding domain. Fifty percent saturation of photolabeling was achieved with 0.1 mM ATP in the absence of poly(I)-poly(C).

Photoaffinity Labeling of Recombinant Human 2-5A Synthetase—Identification of the Photolabeled Peptide and Identification of the Modified Amino Acid—Based on the covalent photoaffinity labeling of 2-5A synthetase, recombinant human 40-kDa 2-5A synthetase (300 $\mu$g of protein) was photolabeled with 0.1 mM 8-azido-[$\alpha$-$^{32}$P]ATP for 30 s and further saturated with 0.1 mM 8-azido-ATP. After perchloric acid precipitation and a methanol wash to remove unincorporated 8-azido-[$\alpha$-$^{32}$P]ATP, the protein was hydrolyzed with TPCK-treated trypsin. The photolabeled peptide was isolated and partially purified by immobilized Al$^{3+}$ chromatography, a metal ion chromatography procedure that has been successfully applied to the purification of phosphopeptides (20). Ninety percent of the $^{32}$P-containing peptide(s) retained by the Al$^{3+}$ column was displaced by 10 mM phosphate buffer in fractions 20–25 (Fig. 6A). The purified photolabeled peptide was rechromatographed by reverse phase HPLC. A single radioactive peptide peak was eluted in frac-
A 2-5A synthetase was incubated with 0.15 mM 8-azido-[α-32P]ATP and increasing concentrations of ATP (0, 1.9, 3.8, 7.5, and 15 mM) in the absence (A, lanes 1–5; B, ●) or presence (A, lanes 6–10; B, ▢) of 10 μg/ml poly(I)-poly(C). A, the autoradiogram of the dried SDS-PAGE gel is shown. B, the radioactive regions on the dried SDS-PAGE gel were excised and quantitated by scintillation spectrometry. The radioactivity (∙) of [32P]ATP is shown.

Fig. 6. Purification of tryptic [32P]-pentapeptide from photolabeled 40-kDa 2-5A synthetase by immobilized Al\(^{3+}\) affinity column and reverse phase HPLC. A, recombinant human 40-kDa 2-5A synthetase was photolabeled with 8-azido-[α-32P]ATP in the absence (●) or presence (○) of 10 μg/ml of poly(I)-poly(C) and was trypsinized. The photolabeled peptides were retained by Al\(^{3+}\) affinity chromatography purification and specifically displaced from the column by phosphate buffer. B, reverse phase HPLC profile of immobilized Al\(^{3+}\)-purified tryptic peptides from 40-kDa 2-5A synthetase photolabeled with 8-azido-[α-32P]ATP. The peptides were separated using H\(_2\)O and acetonitrile as the gradient system. UV absorbance at 214 nm (● – ○) and radioactivity (●) are plotted against fraction number.

Table I

| Cycle | PTH-derivatives (pmol)\(^a\) |
|-------|--------------------------|
|       | Exp. 1\(^b\) | Exp. 2\(^c\) | Exp. 3\(^d\) |
| 1     | Asp (6)  | Asp (50.8) | Asp (44.5) |
| 2     | Phe (6)  | Phe (3)    | Phe (3.8)  |
| 3     | Leu (4)  | Leu (3.4)  | Leu (2.3)  |
| 4     | X\(^e\)  | X\(^e\)    | X\(^e\)    |
| 5     | Gin (4.5)| ND\(^f\)   | Gin (2)    |

\(^a\) Picomole yields of PTH-derivatives are shown in parentheses.
\(^b\) Pentapeptide from experiment 1 in the absence of poly(I)-poly(C).
\(^c\) Pentapeptide from experiment 2 in the absence of poly(I)-poly(C).
\(^d\) Pentapeptide from experiment 3 in the presence of poly(I)-poly(C).
\(^e\) X is Lys-199 as deduced from the primary amino acid sequence of the 40-kDa 2-5A synthetase (7).
\(^f\) Not detectable.

Schematic Diagram of the Site-directed Mutagenesis of Lys-199 of Recombinant Human 40-kDa 2-5A Synthetase to Arg—His. The arginine mutant recombinant human 40-kDa 2-5A synthetase was constructed as shown in Fig. 7. Two rounds of PCR of the pNK14 plasmid produced a 120-bp PstI-KpnI DNA fragment, which was ligated with a 900-bp KpnI-PstI fragment and a 5000-bp PstI fragment. The second PstI site is within the ampicillin resistance gene. Therefore, the triple ligations could only occur in the 5' to 3' direction. The lysine 199 to arginine 199 mutation was inserted into human 40-kDa 2-5A synthetase cDNA. The sequence of the lysine 199 to arginine 199 mutant was confirmed by DNA deoxy sequencing (Fig. 8). The primer containing either C/A or T/C bases at the site of mutation allowed for the simultaneous generation of K199H and K199R mutants. Plasmid pNK16 containing the K199R mutant human 40-kDa 2-5A synthetase cDNA allows the expression of GST fusion protein. The purification of the K199R mutant 2-5A synthetase was performed as described under “Experimental Procedures.” The yield of the K199R 2-5A synthetase was the same as that obtained for the wild type 2-5A synthetase. Similar results were obtained with the K199H mutant (data not shown).

Recombinant Mutant K199R Human 40-kDa 2-5A Synthetase—Binding of 8-Azido-ATP, Binding of Poly(I)-Poly(C) and 2-5A Synthetase Activity—To determine the role of lysine 199 in the enzymatic conversion of ATP to 2-5A synthetase, Lys-199 mutations were introduced into the 2-5A synthetase cDNA. The sequence of the lysine 199 to arginine 199 mutant 2-5A synthetase was not affected by a point mutation of Lys-199 to Arg. The K199R mutant 2-5A synthetase did not show enzyme activity (Fig. 9A), while the K199H mutant (data not shown).

Competition Experiments with Recombinant Human K199R Mutant 40-kDa 2-5A Synthetase by ATP—Although the K199R mutant 2-5A synthetase did not show enzyme activity (Fig. 9A),
the covalent photoinsertion of 8-azido-$\alpha^{32}$P-ATP into the K199R mutant was the same as observed for wild type 2-5A synthetase. Therefore, it was essential to determine if ATP could mimic the photoinsertion of 8-azido-$\alpha^{32}$P-ATP into the K199R mutant. The addition of ATP (0–15 mM) showed a similar competition of 8-azido-$\alpha^{32}$P-ATP (Fig. 10, A and B) to that observed with wild type 2-5A synthetase in the absence of poly(I)-poly(C) (Fig. 5, A and B). This strongly suggests that the photoinsertion of 8-azido-$\alpha^{32}$P-ATP occurred at the ATP binding domain of the K199R mutant recombinant human 40-kDa 2-5A synthetase.

**DISCUSSION**

2-5A synthetase is an important enzyme in the interferon-inducible antiviral and antiproliferative pathways. However, the identification of the ATP binding domain of 2-5A synthetase has not been reported. Previous studies from this laboratory have shown that the azido photoprobe, 8-azido-$\alpha^{32}$P-ATP was covalently linked by UV light to the highly purified rabbit reticulocyte 100-kDa 2-5A synthetase (12). However, insufficient quantities of 2-5A synthetase prevented identification of the amino acid(s) at the ATP binding domain. By fusion of the 40-kDa 2-5A synthetase cDNA to GST followed by transformation of the construct into E. coli, we have successfully purified milligram quantities of near homogeneously pure recombinant human 40-kDa 2-5A synthetase, which, following UV irradiation, 0 °C, 20 s in solution, resulted in the isolation of the $^{32}$P-pentapeptide, $^{199}$DFLKQ$^{200}$. Microsequencing of the amino acids in this pentapeptide revealed that lysine 199 was chemically modified by the azido photoprobe. Therefore, with the availability of sufficiently highly purified 2-5A synthetase, we...
have shown that the ATP photoprobe, 8-azido-ATP, is a substrate for 2-5A synthetase, which when irradiated decreases 2-5A synthetase activity with increased covalent photolabeling with 8-azido-[\alpha-^{32}P]ATP. The stoichiometry of photolabeling of 0.6 mol of poly(I)-8-azido-I) was photolabeled with 8-azido-[\alpha-^{32}P]ATP at 0°C for 30 min, followed by UV irradiation for 30 s. The photolabeled proteins were analyzed by SDS-PAGE. An image of the autoradiogram of the dried gel is shown. C, GST, wild-type 2-5A synthetase, or K199R mutant 2-5A synthetase was incubated in the absence of poly(I) at 0°C for 30 min, followed by washing the poly(I)-agarose gel. After washing the poly(I)-agarose gel, 8-azido-[\alpha-^{32}P]ATP was analyzed by SDS-PAGE. Protein markers are shown in lane 1. Wild type and K199R mutant 2-5A synthetase are indicated by arrows.

Fig. 9. Recombinant K199R mutant human 40-kDa 2-5A synthetase: binding of 8-azido-ATP, binding of poly(I)-poly(C), and 2-5A synthetase activity. A, wild type recombinant human 40-kDa 2-5A synthetase (lanes 1 and 2), or K199R mutant 2-5A synthetase (lanes 3 and 4) was incubated with 8-azido-[\alpha-^{32}P]ATP in the absence (lanes 1 and 3) or presence of 50 \mu{m}l poly(I)-poly(C) (lanes 2 and 4) at 30°C for 2 h. The 2-5A products were dephosphorylated and separated on polyethyleneimine-cellulose TLC. A scanned image of the autoradiogram of the dried gel is shown. B, wild type recombinant human 40-kDa 2-5A synthetase (lane 1) or K199R mutant 2-5A synthetase (lane 2) was photolabeled with 8-azido-[\alpha-^{32}P]ATP at 0°C for 30 min, followed by UV irradiation for 30 s. The photolabeled proteins were analyzed by SDS-PAGE. An image of the autoradiogram of the dried gel is shown. C, GST, wild-type 2-5A synthetase, or K199R mutant 2-5A synthetase was incubated in the absence of poly(I) at 0°C for 30 min, followed by washing the poly(I)-agarose gel. After washing the poly(I)-agarose gel, 8-azido-[\alpha-^{32}P]ATP was analyzed by SDS-PAGE. Protein markers are shown in lane 1. Wild type and K199R mutant 2-5A synthetase are indicated by arrows.

Fig. 10. Competition experiments with recombinant human L199R mutant 40-kDa 2-5A synthetase by ATP. A, K199R mutant 2-5A synthetase was incubated with 0.15 mM 8-azido-[\alpha-^{32}P]ATP and increasing concentrations of ATP (0, 1.9, 3.8, 7.5, and 15 \mu{m}) in the absence of poly(I)-poly(C) (lanes 1-5). The autoradiogram of the dried SDS-PAGE gel is shown. B, the radioactive regions on the dried SDS-PAGE gel were excised and quantitated by scintillation spectrometry. The [\alpha-^{32}P] incorporated into the K199R mutant 2-5A synthetase is plotted against increasing concentrations of ATP.
The data presented in this study can be compared with the report of Ghosh et al. (26). Using a nested set of deletion mutants from the carboxyl terminus of the murine 2-5A synthetase, clones encoding 320 and 304 nucleotides were produced. However, the encoded proteins bound to dsRNA but did not have enzymatic activity. These data indicate that the peptide region between 342 and 304 was essential for 2-5A synthetase activity. Furthermore, a lysine residue is located at position 333. Because many protein kinases have lysine residues that are essential for enzymatic activity at their ATP binding domains, lysine 333 was mutated to arginine 333. However, the K333R mutant 2-5A synthetase had the same enzyme activity as the wild type enzyme.

In summary, the data presented demonstrate that 8-azido[α-32P]ATP is covalently linked to the ATP binding domain in solution. X-ray crystallography studies with the recombinant human 40-kDa 2-5A synthetase have been initiated in this laboratory to provide three-dimensional data on the ATP binding domain in the solid state. Because of the 100-fold difference in the binding constants for the two putative binding sites for ATP (12), it will be necessary to identify the lower affinity ATP binding site on 2-5A synthetase by the x-ray crystallographic studies currently under way. RNase L and PKR have been reported to have tumor suppressor activity in dominant negative mutant studies (27,28). It is possible that the K199R mutant will have dominant negative effects on the wild type 40-kDa 2-5A synthetase upon overexpression in human cells. The K199R mutant may also have the same effect on the 69/71-kDa 2-5A synthetase because the same catalytic domain is present in these 2-5A synthetase isoforms. In vivo studies examining dominant negative activity of the mutant 2-5A synthetase will shed light on the cellular functions of antiviral and antiproliferative activities of the 2-5A synthetase/RNase L system.

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TABLE I

Sequence homology among the dodecapeptides from isoforms of 2-5A synthetases from different mammalian tissues

| Enzyme source | Dodecapeptide
|----------------|----------------|
| Human 2-5A synthetase (40–46 kDa) | LQRDFLKL*KQRPTK |
| Mouse 2-5A synthetase (40–46 kDa) | LQRDFLKL*KQRPTK |
| Rat 2-5A synthetase (358 amino acids) | LQRNFPLIKQRPTK |
| Mouse 2-5A synthetase (367 amino acids) | LQRNFPLIKQRPTK |
| Human 2-5A synthetase (69–71 kDa) | LQRNFPLIKSRPTK |

* The identified pentapeptide is underlined, the photolabeled Lys199 is indicated by an asterisk.