Characterization of the Vaccinia Virus RNA 5'-Triphosphatase and Nucleoside Triphosphate Phosphohydrolase Activities

DEMONSTRATION THAT BOTH ACTIVITIES ARE CARRIED OUT AT THE SAME ACTIVE SITE*

(Received for publication, January 17, 1996)

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DIR1–545, an active subdomain of the large subunit of vaccinia virus mRNA capping enzyme possessing ATPase, RNA 5'-triphosphatase, and guanylyltransferase activities, was expressed in Escherichia coli and shown to be functionally equivalent to the heterodimeric enzyme (Myette, J. R., and Niles, E. G. (1996) J. Biol. Chem. 271, 11936–11944). A detailed characterization of the phosphohydrolytic activities of DIR1–545 demonstrates that, in addition to ATPase and RNA 5'-triphosphatase activities, the capping enzyme also possesses a general nucleoside triphosphate phosphohydrolase activity that lacks a preference for the nucleoside base or sugar. Nucleoside triphosphate and mRNA saturation kinetics are markedly different, with RNA exhibiting a $K_m$ and turnover number 100- and 10-fold less, respectively, than those values measured for any NTP. The linear competitive inhibition of RNA 5'-triphosphatase activity by ATP, and the relative manner by which both ATPase and RNA 5'-triphosphatase activities are inhibited by specific oligonucleotides, kinetically demonstrate that each activity is carried out at a common active site. Direct UV photo-cross-linking of either $^{32}$P-radiolabeled ATP or 23-mer triphosphorylated RNA, followed by cyanogen bromide cleavage of the photo-linked enzyme, localizes the major binding site for both ATP and mRNA to a region between amino acids 1 and 221. The inability of ATP to competitively inhibit either E-GMP formation or the transfer of GMP to RNA kinetically differentiates the phosphohydrolase active site from the guanylyltransferase active site.

The vaccinia virus mRNA capping enzyme catalyzes three of the four reactions required for cap I formation, including RNA 5'-triphosphatase (1, 2), guanylyltransferase, and (guanine-7-)methyltransferase activities (3–5). The RNA 5'-triphosphatase, nucleoside triphosphate phosphohydrolase, and guanylyltransferase active sites have been mapped to a 60-kDa NH$_2$-terminal domain of the DIR subunit (7, 8, 10), while the methyltransferase resides in an independent domain comprised of the carboxyl terminus of DIR together with D12L (9–11). In an accompanying report (12), kinetic analyses demonstrated that the DIR1–545 subdomain expressed in Escherichia coli is functionally equivalent to the full-size capping enzyme with respect to RNA 5'-triphosphatase, ATPase, and guanylyltransferase activities, indicating that the methyltransferase domain exerts little influence on catalysis carried out at the active sites within DIR1–545. In addition to triphosphorylated RNA, the mRNA capping enzyme employs ATP and GTP as substrates for phosphohydrolysis (1, 6, 23, 24), raising the question whether both the nucleoside triphosphate phosphohydrolase activity and the RNA 5'-triphosphatase activity are carried out at the same active site.

In this report, we describe the kinetic properties of the RNA 5'-triphosphatase and nucleoside triphosphate phosphohydrolyase activities of the vaccinia virus mRNA capping enzyme. Through competitive inhibition analyses, we demonstrate that both activities are carried out at a single active site. We also present UV photo-cross-linking mapping data which locates the phosphohydrolysis active site to the 25-kDa NH$_2$-terminal region of DIR (amino acids 1–221). A kinetic argument is made for the separation of this active site from the guanylyltransferase active site, supporting a three-active site model for the mRNA capping enzyme.

EXPERIMENTAL PROCEDURES

RNA Synthesis

Triphosphorylated RNA substrate was synthesized by in vitro transcription of linearized plasmid DNA templates as described (12). Linearization of either pGEM3Zf(+) or pGEM9zf(+) plasmids at different restriction sites enabled the synthesis of RNA of discrete lengths.

Preparation of Diphosphorylated RNA

Diphosphorylated RNA was generated in vitro through the RNA 5'-triphosphatase active site of the capping enzyme using triphosphorylated 23-mer RNA as substrate. Diphosphorylated RNA (100 pmol, specific activity 50 pmol of $P_i$/min/pmol of enzyme) was added to a 2000-cpm/pmol) in the reaction and assaying for the release of $^{32}$P by PEI-cellulose thin layer chromatography as described for the RNA 5'-triphosphatase assay (12). The reaction was quenched and extracted by adding an equal volume of 24:24:1 phenol:chloroform:isoamyl alcohol. RNA was precipitated with 3 volumes of 95% ethanol, washed, dried and resuspended in 300 µl of diethyl pyrocarbonate-treated water. The sample was further desalted and concentrated by ultrafiltration in a Micron 3 concentrator (Amicon).

Enzyme Assays

ATPase, RNA 5'-triphosphatase, and guanylyltransferase assays were conducted as described elsewhere (12).

Inhibition of ATPase by Oligonucleotides—DIR1–545 ATPase activity was measured in a standard assay containing 10 mM MgCl$_2$ in the presence of varying concentrations of oligonucleotides (see below). In the measurement of triphosphorylated 23-mer RNA inhibition of ATPase activity, a 2-min reaction time was employed to ensure that less than 20% of the mRNA was diphosphorylated.

Nucleoside Triphosphate Phosphohydrolase Assay—Nucleoside triphosphate phosphohydrolase activity was measured by a colorimetric phosphate assay as described elsewhere (13). A standard assay contained 50 mM Tris-HCl, pH 8.0, 20 mM MgCl$_2$, 10 mM $\beta$-mercaptoetha-
nol, 10% glycerol, 5 mM nucleoside triphosphate (U. S. Biochemical Corp., ultrapure grade), and 100 mM enzyme, in a 50-μl reaction volume. Enzyme was added last to the chilled assay mixture, and the reaction was transferred to 37°C for 5 min. Reactions were quenched by the addition of 5 μl of 0.5 mM EDTA and assayed for the release of phosphate using a molybdate-based colorimetric assay. Sterols were omitted from the color development reagent. Color development reagent (800 μl, malachite green:ammonium molybdate 3:1) was added to the enzyme assay mix at room temperature, gently vortexed, and developed for 1 min at room temperature. Development was quenched by the addition of 100 μl of 34% sodium citrate (w/v), pH 8.5. Moles of phosphate were determined from the absorbance at 660 nm by use of a KH2PO4 standard curve. Absorbances were corrected for background phosphate generated by the nonenzymatic hydrolysis of nucleoside triphosphates. Absorbances were linear up to 1 mM phosphate and did not vary over the time course of the assay. The assay was linear for 15 min at an enzyme concentration between 20 nM and 150 nM.

**UV Photo-cross-linking**

Photo-cross-linking of ATP to D1R1-545. The standard photo-cross-linking reactions were carried out in a 96-well microtiter dish floating in an ice water slurry. Direct UV photo-linkage was achieved using a single GE G15T8 15-watt germicidal ultraviolet lamp held at a distance of 10 cm for 45 min. A standard reaction mixture of 30 μl contained 50 pmol of D1R1-545[232,235,238,241], 50 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 10% glycerol (w/v), 50 μM cold ATP, and 10–20 μl of [α-32P]ATP (DuPONT NEN, 800 Ci/mmol). The reaction was quenched by adding 10 μl of 4 × SDS sample buffer, and the samples were boiled for 5 min and separated by SDS-PAGE on 12% acrylamide gels. Modified enzyme was visualized by autoradiography of dried gels and incorpo-rated radiolabel quantified by Cerenkov counting. Approximately 1% direct photo-cross-linking product was achieved at saturating ATP concentrations.

Inhibition of ATP Photo-cross-linking by Oligonucleotides—ATP photo-cross-linking was carried out in the presence of varying concentrations of oligonucleotide and nucleotide competitors. Oligonucleotide competitors included triphosphorylated RNA, dihydrophosphorylated RNA, 5′-OHH RNA (Oligos, Etc.), and DNA 23-mers, all of the sequence GGGC-GAAUUAUUAAUUUGAAU (with uracil replaced by thymidine in DNA). Nucleotide competitors included ADP, AMP, adenosine, and the dinucleotides ApU and GppA (a methyl acceptor in the methyltransferase reaction). In a parallel experiment, the inhibition of ATP hydrolysis by these same reagents was also examined. ATPase activity was measured as described previously (12) except a subsaturating concentration of 50 nM ATP and 10 mM MgCl2 were employed. UV Photo-cross-linking of RNA to D1R1-545—Direct UV photo-cross-linking of triphosphorylated 23-mer RNA to D1R1-545 was conducted as described (12) except 200 pmol of D1R1-545 were employed in a 200-μl reaction volume.

Cyanogen Bromide Cleavage of D1R1-545—D1R1-545 (200 pmol) was UV photo-cross-linked with 50 μM ATP or 2 μM 23-mer triphosphorylated RNA as described above. Cross-linking reactions were scaled up to a 200-μl reaction volume and split into four microtiter wells containing 50 μl each. Following cross-linking, samples were pooled and precipitated with ice-cold 10% trichloroacetic acid. Pellets were washed twice with 200 μl of acetone, dried, and resuspended in 200 μl of 70% formic acid containing 20 mg/ml CNBr (Pierce) (17). The digested were carried out for 18–24 h at room temperature while under nitrogen. Samples were then dried three times under a stream of nitrogen, each time resuspending in 200 μl of water. The final wash and concentration step was by Speed-Vac desiccation, after which samples were resuspended in 20 μl of 1X peptide sample buffer and boiled, and the pH was adjusted when necessary by addition of 1–2 μl of 1M Tris-HCl, pH 8.5, 0.1% SDS. Samples were resolved on Tricine gels (14), stained with Coomassie Brilliant Blue (Sigma), and dried. Radiolabeled peptides were visualized by autoradiography. To prepare cleavage products for mass sequencing, 600 pmol of D1R1-545 were directly digested with CNBr. The dried cleavage products were resuspended in 45 μl of 1× peptide sample buffer, and the peptides were resolved by electrophoresis on a Tricine gel pre-run at a low voltage in the presence of 100 μM thiorphanic acid as recommended (Probe-Design Peptide Separation System Technical Manual, Promega, 1992). Approximately 200 pmol of protein were loaded per lane. The peptides were transferred to a Transblot (Bio-Rad) polyvinylidene difluoride membrane and the pop-1

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

**Fig. 1.** Magnesium concentration dependence of ATPase and RNA 5′-triphosphatase activities. The ATPase and RNA 5′-triphosphatase activities in D1R1-545 were measured at saturating ATP (5 mM) and RNA (20 μM) substrate concentrations, 100 mM (ATPase) and 20 mM (RNA 5′-triphosphatase) enzyme concentrations, and in the presence of varying concentrations of MgCl2. Additional conditions for both assays are as described under “Experimental Procedures.” ATPase (●); RNA 5′-triphosphatase (○).

**Fig. 2.** The effect of RNA chain length on RNA 5′-triphosphatase substrate saturation kinetics. γ-32P-Labeled RNA of defined lengths was synthesized by in vitro transcription of linearized plasmid DNA templates and employed as substrates in a standard RNA 5′-triphosphatase assay at 20 nM enzyme and 2 mM MgCl2. Substrate saturation kinetics were measured for the full-size capping enzyme. Kinetic values are obtained from initial velocities measured at varying concentrations of triphosphorylated RNA. The kinetic values for ATP are as measured in parallel under standard ATPase assay conditions (100 mM enzyme, 20 mM MgCl2), are plotted as length = 1 nucleotide.
gesting that ATP binds to the RNA 5'-triphosphatase was assessed. ATP was shown to be a linear competitive inhibitor of RNA 5'-triphosphatase, Fig. 3, strongly suggesting that this activity is not affected by the methyltransferase domain (12).

The fact that the mRNA capping enzyme is a general nucleoside triphosphate phosphohydrolase, additional nucleoside triphosphatases were tested as potential substrates. A broad substrate specificity was observed, Table I. The kinetic properties were comparable for each substrate, exhibiting a range of $K_m$ values from 0.8 to 1.3 mm and turnover numbers from approximately 500 to 800 min$^{-1}$. This enzyme is able to hydrolyze equally well both purine and pyrimidine nucleoside triphosphates, containing either ribose or 2'-deoxyribose. However, the capping enzyme does not possess a general phosphohydrolase activity, since phosphohydrolysis is specific for the 5'-triphosphorylated RNA. 5'-triphosphorylated RNA, the product of the RNA 5'-triphosphatase and ATPase, activity by 50% at a concentration of approximately 35 μM, roughly 1000-fold lower than the $K_m$ measured for ATP. Moreover, RNA turnover is considerably slower, 50 min$^{-1}$ for RNA as compared to 500–600 min$^{-1}$ for ATP. RNA substrates of defined lengths from 9 to 42 nucleotides exhibit similar kinetic properties which demonstrates that beyond 9 bases, RNA chain length has little influence on RNA 5'-triphosphatase activity.

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The Number of Active Sites—The fact that the mRNA capping enzyme possesses both RNA 5'-triphosphatase and general nucleoside triphosphate phosphohydrolase activities raises the question of whether the catalysis of each reaction is carried out at a common active site or at two independent sites. Two approaches were taken to investigate this issue. In the first, the ability of ATP to competitively inhibit RNA 5'-triphosphatase was assessed. ATP was shown to be a linear competitive inhibitor of RNA 5'-triphosphatase, Fig. 3, strongly suggesting that ATP binds to the RNA 5'-triphosphatase active site and prohibits the binding of the RNA 5' terminus. The apparent $K_i$ for this ATP inhibition was calculated from a Dixon plot to be approximately 1.4 mm. This value is comparable to the $K_m$ for ATPase activity (12) as would be predicted if both substrates are competing for the same active site. The minor variation in the $K_m$ and $K_i$ values is a result of the different MgCl$_2$ concentrations (Fig. 1) employed in the two measurements.

In a second approach, the ability of various oligonucleotides to inhibit ATPase activity was assessed, Fig. 4. Inhibition was measured at 5 mm ATP while varying the concentration of the oligonucleotide present in the assay. The oligonucleotides employed were all 23 nucleotides in length and possessed the same nucleotide sequence but differed in the nature of their 5' terminus, and in the case of single-stranded DNA, the substitution of thymidine for uracil. As expected, triphosphorylated RNA 23-mer was an effective inhibitor of ATPase, inhibiting activity by 50% at a concentration of approximately 35 μM. Diphosphorylated RNA, the product of the RNA 5'-triphosphatase activity, also inhibited the hydrolysis of ATP, although to a lesser extent in comparison to the inhibition by triphosphorylated RNA. 5'-OH RNA was a relatively weak inhibitor of ATPase activity while single-stranded DNA did not significantly inhibit ATP hydrolysis. The inhibition of ATP hydrolysis by these oligonucleotides is consistent with their relative inhibition of RNA 5'-triphosphatase activity (12). Although RNA 5'-triphosphatase and ATPase differed in the relative extent to which their activities were inhibited by these reagents, both activities were inhibited in a similarly graded fashion by the same oligonucleotides. The most effective inhibition of either ATPase or RNA 5'-triphosphatase activity was observed with a nucleic acid possessing either a triphosphorylated or diphosphorylated 5' terminus. Taken together, these kinetic competition data argue for a common active site for RNA 5'-triphosphatase and ATPase.

Relationship of Phosphohydrolase and Guanylyltransferase
we employed direct UV photo-cross-linking of ATP followed by enzyme. The extent of ATP photo-cross-linking to D1R1–545 was evaluated.

In an attempt to localize the site(s) of ATP photo-linkage, D1R1–545 was photolabeled with ATP in the presence or absence of triphosphorylated RNA 23-mer, was cleaved with CNBr and deacylated products were resolved by electrophoresis on Tricine gels, Fig. 8. The products obtained were assigned to the expected RNA, ApU, and GpApG which are poor inhibitors of ATPase activity were also poor inhibitors of UV photo-linkage. In contrast, compounds such as single-stranded DNA, ApU, and GpApG which are poor inhibitors of UV photo-linkage. In comparison to 5′ triphosphorylated 23-mer RNA, 5′ OH RNA was a relatively poor inhibitor of ATPase activity and also a less effective inhibitor of the UV photo-linkage of ATP to D1R1–545.

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As expected, triphosphorylated RNA 23-mer was a good inhibitor of both ATPase and photo-cross-linking activities. In contrast, compounds such as single-stranded DNA, ApU, and GpApG which are poor inhibitors of ATPase activity were also poor inhibitors of UV photo-linkage. In comparison to 5′ triphosphorylated 23-mer RNA, 5′ OH RNA was a relatively poor inhibitor of ATPase activity and also a less effective inhibitor of the UV photo-linkage of ATP to D1R1–545.

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D1R\textsuperscript{1–545} primary sequence, complete digestion by CNBr should yield six fragments whose respective molecular weights and location are depicted in Fig. 8A. The typical cleavage products, including some partial digestion fragments, are represented by the Coomassie-stained Tricine gel in Fig. 6B. \textit{NH}_2-terminal amino acid sequence analysis of the 15-kDa fragment demonstrates that this peptide is a partial cleavage product (denoted by the P\textsubscript{2} in Fig. 8B) corresponding to the 10.0- and 5.2-kDa fragments. On the basis of size, we also conclude that P\textsubscript{1} is a partial cleavage product comprised of the 25.1 and 2.1 fragments. The persistence of these partial cleavage products indicate that the respective methionines at positions 221 and 410 in the primary sequence are less efficiently cleaved by CNBr.

A low cross-linking efficiency (less than 1\% ATP cross-linked to D1R\textsuperscript{1–545} at saturating ATP) prohibited the isolation of a specific radiolabeled peptide in sufficient amounts for sequencing. However, an analysis of the distribution of radiolabel in the CNBr cleavage products of ATP photo-linked enzyme was informative, Fig. 8C, lanes 4 and 5. Greater than 75\% of the radiolabeled nucleotide cross-linked to the amino-terminal 25.1-kDa peptide corresponding to amino acids 1–221 (Fig. 8C, lane 4) with additional radiolabeled peptides also resolved by gel electrophoresis. These additional cleavage products always represented a minor portion of the total radioactivity. The labeling of each peptide was competed by including 10 \mu M triphosphorylated RNA in the cross-linking reaction prior to cleavage by CNBr (Fig. 8C, lane 5). Inclusion of RNA in the reaction diminished the extent of ATP cross-linking and not the pattern of radiolabeled cleavage products obtained. From these analyses, we conclude that the major ATP binding site resides within the first 221 amino acids of D1R.

In an attempt to localize the RNA binding site in D1R\textsuperscript{1–545}, \textit{32P}-radiolabeled RNA was UV photo-cross-linked to D1R\textsuperscript{1–545}, the modified enzyme was treated with RNase A and was then subject to CNBr cleavage. The resulting cleavage products were resolved by electrophoresis on Tricine gels, Fig. 9. As was the case for ATP, RNA was cross-linked primarily to the 25.1-kDa \textit{NH}_2 terminus of D1R, Fig. 9B, lanes 4 and 5. Additional radiolabeled peptides of lower molecular weight (not evident in Fig. 9B) were also detected in longer autoradiographic exposures. The percentage of radioactivity present in these fragments somewhat varied between experiments. However, these bands always represented less than 25\% of the total radiolabeled peptide present. Prominent photo-linkage to the 25.1-kDa region of D1R was observed whether the RNA employed as a cross-linking reagent was radiolabeled at internal guanosines located primarily in the 5’ region (Fig. 9, A and B, lane 4) or exclusively in the γ phosphate at the 5’ terminus, Fig. 9B, lane 5. This latter result demonstrates that RNA binding to this region of D1R is specific to the 5’ terminus of RNA and presumably at the phosphohydrolisis active site.

**DISCUSSION**

We have investigated the RNA 5’-triphosphatase and nucleotide triphosphate phosphohydrolase activities of the mRNA capping enzyme. In the previous report (12), we demonstrated that both activities, along with the guanylyltitransferase, reside entirely within the fully active D1R\textsuperscript{1–545} subdomain. We report here that the kinetic properties of the two phosphohydrolytic
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Fig. 8. Localization of the ATP binding site in DIR1$^{1-545}$. α$^{32}$P]ATP was photo-linked to DIR1$^{1-545}$ and the ATP-labeled enzyme subject to chemical cleavage by cyanogen bromide. UV photo-cross-linking reactions were scaled up to a 200-μl reaction volume containing 50 μM ATP, 60 μCi of [α$^{32}$P]ATP (800 Ci/mmol), and 200 pmol of DIR1$^{1-545}$. Following cross-linking, the photo-linked product was precipitated with 10% trichloroacetic acid and subject to CNBr cleavage (20 mg/ml CNBr in 70% formic acid) for 18–20 h at room temperature. CNBr cleavage products were resolved by electrophoresis on Tricine gels, and the gels were stained with Coomassie Brilliant Blue R-250. The radiolabeled peptides were visualized by autoradiography of the dried gel. A, CNBr cleavage map for DIR1$^{1-545}$ indicating the size and location of the expected products from a complete cleavage by CNBr at the indicated methionine residues. B, representative Coomassie-stained Tricine gel of DIR1$^{1-545}$ CNBr cleavage products. The identification of each cleavage product (based on NH$_2$-terminal amino acid sequencing of the isolated peptides) is indicated on the left. Partial cleavage products are denoted by the letter P. C, CNBr cleavage of ATP photo-linked DIR1$^{1-545}$. Lanes 1 and 2, Coomassie-stained Tricine gel of uncleaved E–ATP (100 pmol), lane 1, or CNBr cleavage products (200 pmols), lane 2. Lanes 3–5, autoradiograph of 32P-radio labeled CNBr cleavage peptides; lane 3, uncleaved radiolabeled DIR1$^{1-545}$; lane 4, radiolabeled cleavage products; lane 5, same as lane 4, except 10 μM triphosphorylated 23-mer RNA was included in the photo-cross-linking reaction prior to CNBr cleavage. Arrow indicates the most prominent radiolabeled cleavage product obtained, corresponding to the 25.1 NH$_2$-terminal fragment shown in A. Results in panels B and C represent two separate experiments.

activities vary markedly. First, the two activities differ in the MgCl$_2$ concentration at which maximal catalysis is achieved: 2 mM MgCl$_2$ versus 20 mM MgCl$_2$ for RNA 5'-triphosphatase and ATPase, respectively. Second, the capping enzyme exhibits an approximately 1000-fold lower Km for RNA compared to the Km for ATP and an approximately 10-fold slower turnover of RNA compared to ATP, indicating substantive binding differences for the two substrates. Catalytically relevant binding of RNA at the RNA 5'-triphosphatase active site must occur in a region of the RNA downstream from the 5' terminus. The ability of a 5'-OH RNA oligonucleotide to inhibit RNA 5'-triphosphatase activity (12) reflects this fact. Based on the influence of RNA chain length on RNA 5'-triphosphatase kinetics, this binding site resides within 9 nucleotides from the 5' terminus.

The ability of the mRNA capping enzyme to employ ATP, GTP, and triphosphorylated RNA as a substrate for phosphoryl transfer raises questions related both to substrate specificity and to the presence of a single or multiple phosphoryl transfer active site(s). In regard to the question of substrate specificity, the capping enzyme exhibits comparable substrate saturation kinetics for a broad range of nucleoside triphosphates. The ability of each NTP to serve equally as substrates indicates that the enzyme exhibits little specificity based on sugar or base. In regard to the number of phosphohydrolase active sites, we conclude, based on two kinetic observations, that a common active site exists. First, ATP is a linear competitive inhibitor of RNA 5'-triphosphatase activity. The calculated K$_i$ of 1.4 mM is comparable to the K$_m$ measured for ATPase activity (12). Second, ATPase and RNA 5'-triphosphatase activities (12) are inhibited in a similarly graded manner by the same set of oligonucleotides. The degree of competition of ATPase activity by these oligonucleotides reflects the need for either a tri- or diphosphorylated 5' RNA terminus.

The concentration at which triphosphorylated 23-mer RNA effectively inhibits ATPase (with 50% inhibition occurring at approximately 35 μM) is substantially higher than the approximately 1 μM K$_m$ measured for RNA 5'-triphosphatase activity (12). However, this inequivalence may be explained by an inhibition of RNA binding at the high MgCl$_2$ concentration present in the kinetic competition assay; at 10 mM MgCl$_2$, RNA 5'-triphosphatase activity is inhibited approximately 70% (Fig. 1). The apparent K$_m$ for 23-mer triphosphorylated RNA under such conditions is greater than 10-fold higher than the K$_m$ measured under optimal divergent cation conditions (data not shown). Nevertheless, the inhibition measured is both valid and specific. These competition data, taken together with the differential kinetic properties of the two activities, indicate that within this active site, triphosphorylated RNA preferentially binds. The strong substrate bias toward RNA reflects the fact that the primary function of this active site is the dephosphorylation of mRNA prerequisite to
mRNA cap formation.

A major goal in our study of the mRNA capping enzyme is to identify the amino acid residues present in the active sites catalyzing each of the three reactions in the cap formation pathway. The straightforward method of direct UV photo-cross-linking specific substrates to the methyltransferase domain has permitted the preliminary localization of the S-adenosylmethionine and GTP binding regions of the methyltransferase active site in the D1R1–545 subdomain. Specific UV photo-cross-linking of ATP to D1R1–545 was achieved as evidenced by several criteria. First, the extent of cross-linking was found to be dependent upon time and enzyme concentration, and was saturable by ATP. Second, the cross-linking of ATP to D1R1–545 was effectively inhibited by triphosphorylated 23-mer RNA in a manner which paralleled the inhibition of ATP hydrolysis by this RNA. In contrast, reagents such as single-stranded DNA, and the dinucleotides ApU and GpppA (a substrate for methyltransferase) which were poor inhibitors of ATPase activity were likewise poor inhibitors of ATP cross-linking. Direct photo-linkage of ATP to D1R1–545, coupled with the specific chemical cleavage of this subdomain by CNBr, has identified a major ATP binding site in a 25 kDa amino terminus of D1R from amino acids 1–221. The minor but specific labeling of other peptides indicate, however, that other regions in D1R1–545 interact with ATP. The observation of multiple radiolabeled cleavage products is not surprising given the approach taken. Direct photo-cross-linking of nucleotides to proteins is likely to result in a covalent linkage to any amino acids in juxtaposition to the nucleotide with a most efficient linkage to those residues that are most proximal to the adenine base.

Using this same experimental approach, the major RNA binding site was also localized to the same NH2-terminal 25-kDa region of D1R to which ATP binds. RNA cross-linking to this region represents specific binding to the RNA 5’-triphosphatase active site based on the following observations. First, photo-linkage to the same NH2-terminal 25-kDa fragment occurred when either α- or γ32P-radiolabeled 23-mer RNA was employed as a ligand. This result clearly indicates that D1R is binding to the 5’ terminus of RNA and protecting this region of RNA from RNase digestion. Second, kinetic competition data demonstrate that both ATPase and RNA 5’-triphosphatase activities share a common active site. Therefore, the observation that both ATP and RNA cross-link to the same region of D1R is consistent with both substrates binding at the same phosphohydrolysis site.

The identification of specific residues involved in nucleotide binding was not possible by the photo-cross-linking method as a low cross-linking efficiency prohibited the isolation of a modified peptide in amounts sufficient for sequencing. Moreover, an evaluation of the D1R amino acid sequence failed to reveal any consensus sequences in D1R1–545 for nucleotide binding. However a few sequences which show some homology to nucleotide binding sequences should be noted. In particular is the sequence GSGAQSKS located at position 167–174. This sequence falls within the 25-kDa amino-terminal CNBr peptide representing the major ATP (and RNA) cross-linking product and loosely fits the Walker A NTP binding motif GXXXXXGX- (S/T) (19). Another sequence in D1R which partially matches this motif is at position 434–443 and consists of the sequence GX3GXGK. This sequence lacks the typical S or T at the carboxyl terminus. However, following this sequence is a hydrophobic stretch of amino acids comprising a sequence motif reported for some herpesvirus proteins possessing ATPase functions (20). Other consensus sequences for nucleotide binding including the Walker B type, (R/K)X3GX3L-hydrophobic-D (19), and the GTP binding motif, NKXD (25), are not present in D1R1–545. This is not surprising given that the preferred substrate for phosphohydrolysis is RNA and not single nucleoside triphosphates.

An RNA 5’-triphosphatase activity for the West Nile virus has been reported (21). The authors have suggested the amino acids ILPRRW as comprising the putative RNA 5’-triphosphatase active site and note its homology to the vaccinia virus mRNA capping enzyme sequence LKPR starting at position 492. This sequence is based entirely on homology to other flaviviruses in a region of the viral genome to which no functional predictions had been previously made. No structure-function correspondence has actually been demonstrated.

By analysis of D1R carboxyl-terminal deletion mutations, both the guanylyltransferase and ATPase activities have been mapped to the same 60 kDa amino-terminal domain. This fact suggests a close structural linkage between the two activities (12). However, the inability of ATP to inhibit either E–GMP formation or the transfer of GMP to RNA clearly demonstrates that the two active sites do not overlap. This conclusion is noteworthy given that GTP is a substrate for both reactions. Based on the apparently rate-limiting kinetics for the transfer of GMP to RNA (2, 12), one would also expect some degree of inhibition by any nucleotide binding which interferes with this transfer. Clearly ATP does not inhibit this step. Two additional lines of evidence also argue for separate active sites. First, ATP does not effectively cross-link to the 14.2-kDa CNBr fragment containing the lysine 260 required for E–GMP formation. This result demonstrates that the major ATP binding site is not proximal to this lysine, at least not in the primary sequence. Second, the mutation of this lysine to methionine, while eliminating E–GMP formation (9, 16), had no effect on ATPase activity, indicating that this lysine is not required for phosphohydrolyase activity.

Employing a 23-mer triphosphorylated RNA substrate in vitro, D1R1–545 exhibits a 50-fold greater RNA 5’-triphosphatase than guanylyltransferase activity (12); 50 dephosphorylations occur for each nucleotidyl transfer event. This observation implies that the probability of dissociation of dephosphorylated RNA from the RNA 5’-triphosphatase active site is 50-fold greater than the transfer of GMP to the RNA 5’-terminus. Since guanylyl transfer measured in vitro is linear with respect to time at the earliest time points analyzed (data not shown), the dephosphorylated RNA must serve as an acceptor for this transfer without dissociating from the enzyme. The poor coupling of guanylyltransferase to RNA 5’-triphosphatase in vitro demonstrates, however, that the capping enzyme lacks the ability to effectively retain the nascent dephosphorylated RNA product and transfer it to the guanylyltransferase active site. It is believed that nascent RNA is capped in vivo without the release of the intermediate products. This presumed greater in vivo catalytic efficiency is likely to be due to the existence of a ternary complex containing the capping enzyme, nascent RNA, and the RNA polymerase (26, 27). A tethering of the nascent RNA to the RNA polymerase rather than to a site in the mRNA capping enzyme (12) would retain the intermediate products and ensure a proximity of substrate RNA to each capping enzyme active site. In turn, this favorable arrangement would increase the efficiency of catalysis by physically coupling each reaction in the cap formation pathway.

Acknowledgments—We thank Drs. Cecile Pickart and Dan Kosman for a critical evaluation of this manuscript.
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