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Reaction mechanism of mRNA guanylyltransferase from rat liver: Isolation and characterization of a guanylyl–enzyme intermediate

(capping mechanism/GTP-PP_i exchange/covalent catalysis/phosphoamide linkage)

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ABSTRACT **Rat liver RNA guanylyltransferase catalyzes a GTP-PP_i exchange reaction in the absence of acceptor RNA [Mizumoto, K. & Lipmann, F. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4961–4965] suggesting that the reaction proceeds through the formation of a covalent guanylylated intermediate. We now present more direct evidence for the existence of the enzyme–GMP intermediate: (*i*) the enzyme–[^{32}P]GMP intermediate was formed on incubation of rat liver guanylyltransferase with [α-^{32}P]GTP and migrated as a single radioactive band with M_r 69,000 on NaDodSO$_4$/polyacrylamide gel electrophoresis, and (*ii*) the intermediate isolated on gel filtration can transfer its GMP moiety to ppGpCpC-poly(A_2,U_2,G) to form the capped RNA molecule or it can react with PP_i to regenerate GTP. The formation of the intermediate was dependent on Mg^{2+} and was strongly inhibited by PP_i. The addition of pyrophosphatase markedly increased the amount of the intermediate complex. On blue dextran-Sepharose affinity column chromatography, the activity of guanylyltransferase to form an enzyme–[^{32}P]GMP intermediate comigrated with activities of cap formation and GTP-PP_i exchange. A phosphoamide type linkage between GMP and enzyme is suggested by its acid-labile and alkali-stable nature and also by the susceptibility to acidic hydroxylamine. These results indicate that the reaction catalyzed by rat liver guanylyltransferase occurs through the following two partial steps: (*i*) E + GTP $\rightleftharpoons$ E-pG + PP_i; and (*ii*) E-pG + ppN ····· → GpppN ····· + E.**

Most viral and cellular mRNAs in eukaryotes contain the "cap" structure—i.e., m^{7}G(5′)ppp(5′)N^m—at their 5′ termini (see refs. 1–3 for reviews). The mechanism of biosynthesis of the cap structure has been studied mostly with virion-associated enzymes (4–8). A multifunctional capping enzyme complex containing activities of guanine-7-methyltransferase and guanylyltransferase with a M_r of 120,000–127,000 has been purified to apparent homogeneity from vaccinia virus (9, 10).

Methyltransferase and guanylyltransferase were also purified from HeLa cells (11, 12) and rat liver nuclei (13). In contrast to the viral enzyme, two activities were readily separated at the early stage of purification. Methyltransferase and guanylyltransferase partially purified from rat liver had M_rs of 130,000 and 65,000, respectively (13). From biochemical studies on these two enzymes, capping of the cellular mRNA was found to occur by the following mechanism:

$$(i)\quad \underset{\text{pppG}}{\overset{\gamma\beta\alpha}{}} + \underset{\text{pp N-}}{\overset{\beta'\alpha'}{}} \xrightarrow{\text{guanylyl-transferase}} \underset{\text{Gpp pN-}}{\overset{\alpha\beta'\alpha'}{}} + \underset{\text{PP}_i}{\overset{\beta\gamma}{}}$$

$$(ii)\quad \text{GpppN-} + \text{AdoMet} \xrightarrow[\text{transferase}]{\text{guanine-7-methyl-}} \text{m}^7\text{GpppN-} + \text{AdoHcy}.$$

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By studying the mechanism of the first reaction catalyzed by guanylyltransferase in detail, we found that a GTP-PP_i exchange reaction occurred in the absence of acceptor RNA and suggested that the guanylylation reaction may involve an enzyme–GMP complex as an intermediate (13). A similar finding was also made by Shuman *et al.* (10) with the capping enzyme from vaccinia virus. Furthermore, Shuman and Hurwitz (14) have recently succeeded in isolating a covalent enzyme–GMP intermediate in which GMP is linked to the M_r 95,000 subunit of the enzyme complex.

In this paper, we report isolation and characterization of an enzyme–GMP complex as a functional intermediate of the cellular guanylyltransferase reaction.

MATERIALS AND METHODS

Materials. [β-^{32}P]GTP was prepared as described (15) and purified through a column of DEAE-Sephadex. ppGpCpC-poly(A_2,U_2,G) (average chain length, 40–80) was synthesized as described (13). The synthesis of ppGpC was carried out as described for the synthesis of ppGpCpC (13) but with cytidine instead of CpC. Blue dextran-Sepharose 4B was prepared according to the reported procedure (16). All other materials were obtained from described sources (13).

Capping Enzymes. Rat liver guanylyltransferase was purified from isolated nuclei up to the step of CM-Sephadex column as described (13). Crude capping enzyme of vaccinia virus (first DEAE-cellulose fraction) was prepared as described by Martin *et al.* (9) from the particles of Lister strain which was kindly supplied by T. Urushibara (Kitasato University, Tokyo).

Assay for Guanylyltransferase. Transguanylylation from [α-^{32}P]GTP to the 5′-end of ppGpCpC-poly(A_2,U_2,G) was assayed essentially as described (13). After incubation for 60 min at 30°C, RNA was digested with nuclease P1 and alkaline phosphatase. The digests were electrophoresed on Whatman DE81 paper at pH 3.4 (13).

GTP-PP_i Exchange Assay. The GTP-PP_i exchange activity was measured by the incorporation of [^{32}P]PP_i into the acid-soluble and Norit A-adsorbable material (13).

Enzyme–GMP Complex Formation. The standard 50-μl reaction mixture contained 50 mM Tris·HCl (pH 7.9), 5 mM $Mg(OAc)_2$, 10 mM dithiothreitol, 10 μg of bovine serum albumin, 2–5 μM [α-^{32}P]GTP (4–18 × 10^4 cpm/pmol), and enzyme fraction. Incubation was for 10 min at 30°C. The reaction mixtures received 3 μl of 0.2 M EDTA at the end of the incubation and were processed as follows. For NaDodSO$_4$/polyacrylamide gel electrophoresis, proteins were precipitated with 5% trichloroacetic acid, washed with ether, and denatured in 62 mM Tris·HCl, pH 6.9/2.3% NaDodSO$_4$/5% 2-mercaptoethanol/30% (vol/vol) glycerol for 3 min at 100°C. Electrophoresis was essentially as described by Laemmli (17) in 10% gel.

*Abbreviation: $\overset{*}{p}$, ^{32}P label.

When the native enzyme–GMP was to be prepared, the reaction mixture was passed through a column of Sephadex G-50 (see legend to Fig. 3 for details).

RESULTS

Formation of a Covalent Enzyme–GMP Complex by Rat Liver Guanylyltransferase. Rat liver guanylyltransferase purified up to the step of CM-Sephadex column chromatography (13) was further fractionated on a blue dextran-Sepharose column. The cap-structure-forming activity was eluted as a single peak at 0.25 M KCl and coincided with the GTP-PP_i exchange activity (Fig. 1*A*). To demonstrate the formation of an enzyme–GMP intermediate which was predicted by the occurrence of GTP–PP_i exchange in the absence of an acceptor RNA (13), active fractions were incubated with [α-^{32}P]GTP and the radioactivity bound to protein was precipitated with cold 5% trichloroacetic acid, denatured in $NaDodSO_4$ and subjected to

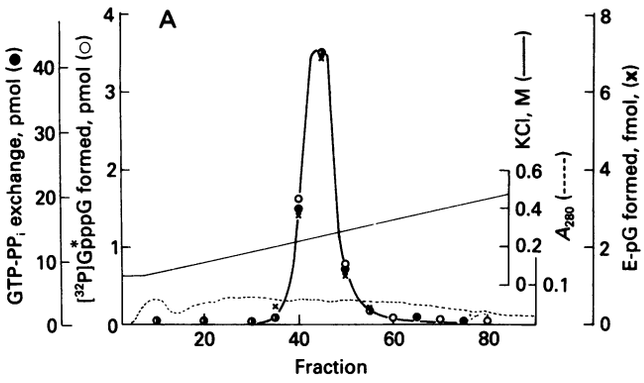

FIG. 1. (*A*) Blue dextran-Sepharose column chromatography of rat liver guanylyltransferase. Approximately 0.2 mg of the enzyme from the CM-Sephadex column was applied to a column of blue dextran-Sepharose 4B (0.5 × 13 cm) previously equilibrated with buffer A (20 mM Tris·HCl, pH 7.9/5 mM dithiothreitol/0.1 mM EDTA/20% glycerol) containing 50 mM KCl. The proteins were eluted with 60 ml of a 50–500 mM KCl gradient in buffer A. Fractions (0.7 ml) were collected, and 10 μl of each fraction was assayed for the capping activity (○), GTP-PP_i exchange (●), and formation of the protein-[^{32}P]GMP complex (x). The amount of the protein-[^{32}P]GMP complex was determined by measuring the radioactivity of the M_r 69,000 bands in *B*. (*B*) Demonstration of the enzyme-[^{32}P]GMP complex on $NaDodSO_4$/polyacrylamide gel electrophoresis. Enzyme fractions were incubated with [α-^{32}P]GTP to generate the enzyme–[^{32}P]GMP complex under the standard assay conditions in the presence of inorganic pyrophosphatase. Lanes 1, 0.6 μg of rat liver enzyme preparation purified through CM-Sephadex; 2–6, 10-μl samples of blue dextran-Sepharose column fractions 35, 40, 45, 50, and 55; 7, 2 μg of vaccinia capping enzyme; M, a mixture of ^{14}C-labeled marker proteins (CFA 626, Amersham, Japan). *p, ^{32}P label. M_r are shown × 10^{-3}.

polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. A single radioactive band that migrated with an apparent M_r of 69,000 was found in peak fractions of guanylyltransferase activity (Fig. 1*B*, lanes 2–6). The radioactivity incorporated into the enzyme–GMP complex was found to be proportional to the catalytic activity of the corresponding fractions (Fig. 1*A*). The preparation before the blue dextran-Sepharose column chromatography gave also a single radioactive band (lane 1, Fig. 1*B*), indicating that no other enzyme that produces the covalent protein–GMP complex was present at this stage of purification. The incubation of the capping enzyme complex from vaccinia virus with [α-^{32}P]GTP yielded a protein–GMP complex whose M_r was estimated to be 95,000 (lane 7, Fig. 1*B*), thus confirming the results of Shuman and Hurwitz (14) that the large capping enzyme subunit is guanylylated.

As expected, the ^{32}P radioactivity bound to the protein was stable during the above isolation procedure—i.e., precipitation with cold 5% trichloroacetic acid and electrophoresis in a buffer containing 0.2% $NaDodSO_4$. It was not released from the protein by treatment with nuclease P1, alkaline phosphatase, nucleotide pyrophosphatase, or phosphodiesterase, but digestion with proteinase K converted all the ^{32}P radioactivity to an acid-soluble form.

Formation of the enzyme–[^{32}P]GMP complex was dependent on the presence of the enzyme and Mg^{2+} and was completely inhibited by 50 μM PP_i (Fig. 2*A*, lanes 1–4). It was stimulated severalfold by the addition of inorganic pyrophosphatase either in the presence or absence of 50 μM PP_i (lanes 5 and 6) but inorganic pyrophosphatase by itself did not form any radioactive derivative (lane 7). These results indicate that formation of the enzyme–GMP complex proceeds via a reversible reaction: enzyme + GTP $\rightleftharpoons$ enzyme–GMP + PP_i. The overall guanylylation reaction was also highly sensitive to PP_i, about 80% inhibition being observed in the presence of 25 μM PP_i.

Formation of the covalent intermediate was specific to guanine nucleotides. Addition of 100-fold molar excess of unlabeled ATP, UTP, or CTP did not affect the incorporation of [α-^{32}P]GTP into the intermediate (Fig. 2*B*, lanes 1–4). Only unlabeled dGTP diminished formation of the labeled intermediate to about 50% (lane 5). An unusual guanine nucleotide, diguanosine tetraphosphate, which was isolated from the *Artemia salina* embryo (19), did not show any effect on the reaction (lane 7). The reaction was rapid, reaching completion within a short period of time at 0°C (lane 9). No radioactivity was incorporated when [α-^{32}P]GTP was replaced by [β-^{32}P]GTP (lane 8), indicating that the GMP moiety of the GTP molecule is transferred to the enzyme with the displacement of PP_i. Furthermore, incorporation of the ^{3}H radioactivity from [8-^{3}H]GTP was nearly stoichiometric with that of the ^{32}P from [α-^{32}P]GTP (lanes 1 and 10); about 30 fmol of ^{3}H and 31 fmol of ^{32}P radioactivities were found per μg of the protein.

Enzyme–GMP Complex as a Functional Reaction Intermediate. We isolated a substantial amount of the enzyme–GMP complex in the undenatured form to test whether the complex is active as an intermediate of the guanylylation reaction. After incubation of the enzyme with [α-^{32}P]GTP, the enzyme–[α-^{32}P]GMP complex was isolated by gel filtration on a small column of Sephadex G-50. Approximately 50% of the radioactivity recovered in the void fraction was precipitable with cold 5% trichloroacetic acid (Fig. 3). On analysis by paper electrophoresis, the acid-soluble ^{32}P radioactivity was identified as a mixture of GTP (10%), GDP (88%), and a trace of GMP. We do not know why GDP is preferentially associated with this fraction. This could be due to some other enzymes present in this fraction as contaminants because the radioactivity of acid-soluble nucleotides were largely eliminated by inclusion of unlabeled ATP

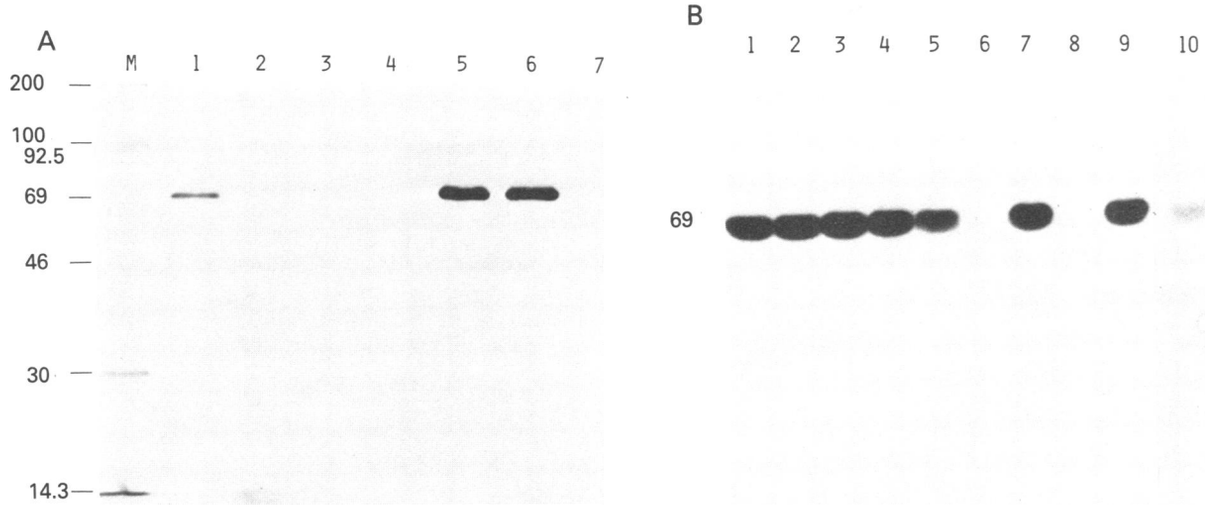

FIG. 2. Autoradiograph of $NaDodSO_4$/polyacrylamide gel electrophoresis of the acid-precipitable fraction, showing requirements for formation of the enzyme–[^{32}P]GMP complex. (*A*) The reaction mixture included the standard components as described in *Materials and Methods* with 5 μM [α-^{32}P]GTP (38,500 cpm/pmol) and 1.25 μg of rat liver guanylyltransferase (CM-Sephadex enzyme preparation). Incubation was for 15 min at 30°C. Lanes: 1, complete system; 2, without $Mg(OAc)_2$; 3, without guanylyltransferase; 4, plus 50 μM PP_i; 5, plus 0.1 μg of inorganic pyrophosphatase; 6, plus 50 μM PP_i and 0.1 μg of inorganic pyrophosphatase; 7, without guanylyltransferase but plus inorganic pyrophosphatase; M, ^{14}C-labeled marker proteins. (*B*) The complete system (lane 1) contained 0.37 μg of rat liver guanylyltransferase (CM-Sephadex enzyme preparation) and 2 μM [α-^{32}P]GTP (86,000 cpm/pmol). Lanes: 2, plus 200 μM ATP; 3, plus 200 μM UTP; 4, plus 200 μM CTP; 5, plus 200 μM dGTP; 6, plus 200 μM GTP; 7, plus 200 μM GppppG; 8, plus 2 μM [β-^{32}P]GTP (128,000 cpm/pmol) instead of [α-^{32}P]GTP; 9, incubation at 0°C instead of 37°C; 10, with 2.5 μg of guanylyltransferase and 9 μM [^{3}H]GTP (12,000 cpm/pmol). The gel was fluorographed by using Na salicylate as a scintillant (18).

or UTP in the reaction mixture for generation of the enzyme–[α-^{32}P]GMP complex.

When the isolated enzyme–GMP complex was incubated with an acceptor ppG-RNA, the enzyme-bound GMP moiety was quantitatively transferred to the acceptor RNA molecule to yield G$\overset{*}{p}$pppG-RNA ($\overset{*}{p}$ is the ^{32}P label). This was demonstrated by isolation of G$\overset{*}{p}$pppG on paper electrophoresis after digestion of the capped RNA (purified by phenol extraction and gel filtration) with nuclease P1 and alkaline phosphatase (Fig. 4*A*, lanes 1–3). The quantitative transfer of the enzyme-bound GMP to the acceptor RNA was more clearly seen in the experiments

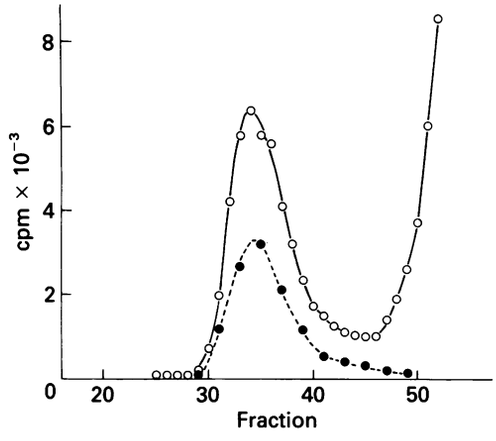

FIG. 3. Isolation of the native enzyme–GMP complex through a Sephadex G-50 column. Approximately 10.6 μg of rat liver guanylyltransferase (CM-Sephadex enzyme preparation) was incubated with [α-^{32}P]GTP (5.7×10^4 cpm/pmol) in the presence of 0.1 μg of inorganic pyrophosphatase in a 50-μl reaction mixture under the standard condition. The reaction was terminated by the addition of 10 mM EDTA, and the reaction mixture was immediately applied to a column of Sephadex G-50 (0.6 × 29 cm) that had been equilibrated with buffer A containing 50 mM KCl and 0.1% Triton X-100. Fractions (65 μl) were collected and assayed for total (○) and trichloroacetic acid-precipitable (●) radioactivity by Cerenkov spectrometry.

of Fig. 4*A* (lanes 4 and 5) in which the products of the transfer reaction were directly treated with nuclease P1 and alkaline phosphatase without prior purification by phenol extraction and gel filtration. The ^{32}P radioactivity covalently bound to the enzyme protein remained in the origin in the sample incubated without acceptor RNA but was converted completely to G$\overset{*}{p}$pppG when incubated with acceptor RNA. The ^{32}P radioactivity in the enzyme–[α-^{32}P]GMP complex was released when it was incubated with both Mg^{2+} and PP_i, to yield GTP almost quantitatively (Fig. 4*B*). The weak radioactivity in the spot of GTP in the absence of either PP_i (lane 1) or Mg^{2+} (lane 2) was due to contamination of the complex by free [α-^{32}P]GTP.

The above results clearly demonstrate that the enzyme–[α-^{32}P]GMP complex is a functional intermediate of the guanylyltransferase reaction. It is noteworthy that the transfer reaction with the enzyme–GMP complex as a substrate could be utilized to test the effect of chain length of an acceptor RNA, especially for the short oligonucleotides, on the capping reaction. The efficiency of [^{32}P]GMP transfer from the enzyme–GMP complex to the acceptor RNAs was found to be in the order ppGpCpC-poly(A_2,U_2,G) (average chain-length, 40) > ppGpCpC > ppGpC > ppG (Table 1). A dramatic increase of GMP transfer (50- to 100-fold) was observed upon the formation of the first phosphodiester bond; thereafter, a gradual increase in GMP transfer was seen with increasing chain length.

Chemical Nature of the Linkage Between Enzyme and GMP. The stability of rat liver guanylyltransferase–[^{32}P]GMP complex in alkaline or acidic solution is shown in Fig. 5*A*. Approximately 70% of the ^{32}P remained acid-precipitable even after heating for 5 min at 95°C in 0.1 M NaOH. On the other hand, the complex was cleaved almost completely in 1–2 min by exposure to 0.1 M HCl at 95°C. In 0.1 M HCl at 37°C, $t_{1/2}$ was estimated to be approximately 30 min by measuring the rate of appearance of [^{32}P]GMP by thin-layer chromatography on polyethyleneimine-cellulose. The acid lability and the alkali stability of the bond suggests that GMP may be linked to the enzyme through a phosphoamide bond (21). In agreement with this is the finding that in acidic hydroxylamine (pH 4.75) the

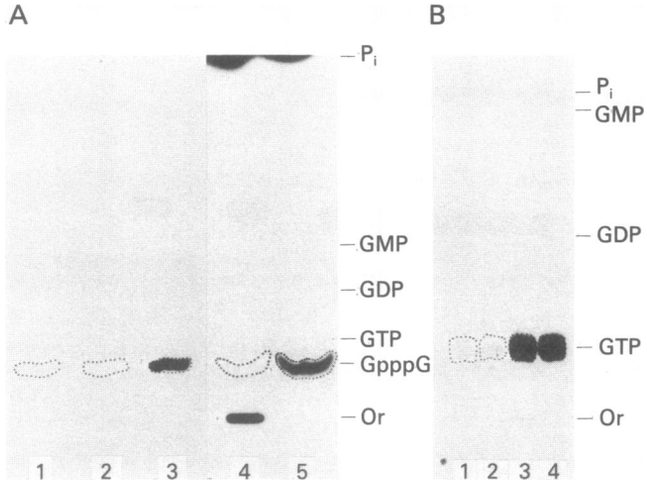

FIG. 4. Synthesis of capped RNA or GTP from the isolated enzyme–GMP intermediate. (*A*) Forward reaction. The 50-μl reaction mixture for the complete system (lanes 3 and 5) contained 40 mM Tris·HCl (pH 7.9), 10 mM dithiothreitol, 6 mM $Mg(OAc)_2$, 10 μg of bovine serum albumin, 200 pmol of ppGpCpC-poly(A_2,U_2,G), and approximately 7000 cpm of the enzyme–[^{32}P]GMP isolated in Fig. 3. Lanes: 1 and 4, without acceptor RNA; 2, without $Mg(OAc)_2$; 3 and 5, complete. Incubation was for 10 min at 30°C. For lanes 1–3, RNA was extracted with phenol, passed through a column of Sephadex G-25, treated with nuclease P1 and alkaline phosphatase, and then electrophoresed on Whatman DE81 paper at pH 3.4. For lanes 4 and 5, the reaction mixture after incubation was directly treated with nuclease P1 and alkaline phosphatase. The electropherogram was autoradiographed. (*B*) Reverse reaction. The reaction mixture was the same as in Fig. 4*A* except for the addition of 120 μM PP_i (lane 3) or 40 μM PP_i (lane 4) instead of acceptor RNA. Lane 1, without PP_i; lane 2, without $Mg(OAc)_2$. After incubation for 10 min at 30°C, the reaction was terminated by the addition of 75 μl of 10% trichloroacetic acid and the proteins were removed by centrifugation. The supernatant was lyophilized and electrophoresed on Whatman DE81 paper at pH 3.4. The radioactive material comigrating with marker GTP was eluted with 1 M triethylamine/HCO_3^- buffer (pH 8) and then analyzed by polyethyleneimine-cellulose chromatography with 1 M potassium phosphate buffer (pH 3.4). The autoradiogram of the thin-layer plate is shown. The positions of authentic nucleotides (GpppG and GTP) are marked with dotted circles.

enzyme–GMP complex was rapidly degraded at 37°C (Fig. 5*B*), whereas the linkage was stable in neutral hydroxylamine (pH 7.5) or in acetate buffer at pH 4.75. Specific cleavage by acidic hydroxylamine suggests that a phosphoamide, rather than a phosphoester or a mixed anhydride bond, is formed between GMP and a lysine or histidine residue of the protein. For comparison, we measured also the stability of T4 DNA ligase–[^{32}P]AMP in which AMP is linked to the ε amino group of lysine (22). As shown in Fig. 5 *C* and *D*, almost identical results were obtained with T4 DNA ligase. These results imply that GMP may be bound to rat liver guanylyltransferase by a phosphoamide bond.

DISCUSSION

RNA guanylyltransferase partially purified from rat liver nuclei catalyzes the capping of an acceptor RNA through the following two-step reaction with a guanylylated enzyme as an intermediate:

$$(i)\ \text{enzyme} + \text{pppG} \xrightleftharpoons{Mg2+} \text{enzyme–pG} + PP_i$$

$$(ii)\ \text{enzyme–pG} + \text{ppG-RNA} \xrightarrow{Mg2+} \text{GpppG-RNA} + \text{enzyme}.$$

Several lines of evidence support this mechanism: (*i*) isolation of a covalent enzyme–GMP complex in denatured (Fig. 1) and catalytically active (Fig. 3) forms, (*ii*) occurrence of GTP-PP_i exchange in the absence of acceptor RNA (13), (*iii*) strong inhibition by PP_i of the enzyme–GMP formation (Fig. 2), and (*iv*) transfer of the α, but not β or γ, phosphate from [^{32}P]GTP into the cap structure to form [^{32}P]G$\overset{*}{p}$ppN-, as well as conservation of the β-^{32}P of [^{32}P]$\overset{*}{p}$pN-RNA in the formation of the cap structure [^{32}P]Gp$\overset{*}{p}$pN- (13).

Table 1. Effect of chain length of acceptor molecules on transfer of [^{32}P]GMP from enzyme–GMP complex

Acceptor	Concentration, μM	[^{32}P]G$\overset{*}{p}$ppG formed	
		cpm	%
ppG	5	201	10.4
	50	1353	64.4
	100	1850	88.1
ppGpC	0.02	76	3.6
	0.1	185	8.8
	1.0	1318	62.8
ppGpCpC	0.02	122	5.8
	0.1	521	24.8
	1.0	2027	96.5
ppGpCpC-poly(A_2,U_2,G)	0.02	970	46.2
	0.1	2086	99.3
	1.0	2028	96.6

The experimental conditions were the same as described in the legend to Fig. 4*A* except that the incubation was for 10 min at 20°C. Each reaction mixture contained 2100 cpm (acid-precipitable radioactivity) of the enzyme–[^{32}P]GMP complex isolated as in Fig. 3 and various acceptor molecules at the indicated concentrations. After paper electrophoresis, the radioactivity in GpppG spots was measured.

It has been reported that, in *Artemia salina* embryos, diguanosine triphosphate (GpppG) is synthesized by the guanylyltransfer reaction, GppppG + GDP → GTP + GpppG, catalyzed by GppppG synthetase (23). More recently, Smith and Furuichi (24) found that free GppppN is formed during the synthesis of mRNA by cytoplasmic polyhedrosis virus RNA polymerase. However, GppppG did not serve as guanylyl donor in the rat liver guanylyltransferase reaction (Fig. 2*B*) and the enzyme–GMP complex did not react with GTP to form GppppG (data not shown).

Oligonucleotides such as ppGpC and ppGpCpC were found to be active as guanylyl acceptors (Table 1). This may reflect the possibility that the capping of mRNA occurs at an early stage of transcription, possibly after formation of oligonucleotides at the 5′ termini of mRNA molecules. It was observed that the dinucleotide ppGpC functions as a guanylyl acceptor for the core-associated capping enzyme of reovirus (4) and for the purified vaccinia capping enzyme (6).

The hydrolytic properties of rat liver guanylyltransferase–GMP complex (Fig. 5) strongly suggest that the GMP is bound to the enzyme by a phosphoamide linkage (21). Mixed anhydride (25, 26), phosphoester (21, 27), and thiol phosphate (28) bonds may be excluded because the stability of these linkages against acid, alkali, or hydroxylamine is considerably different from that of

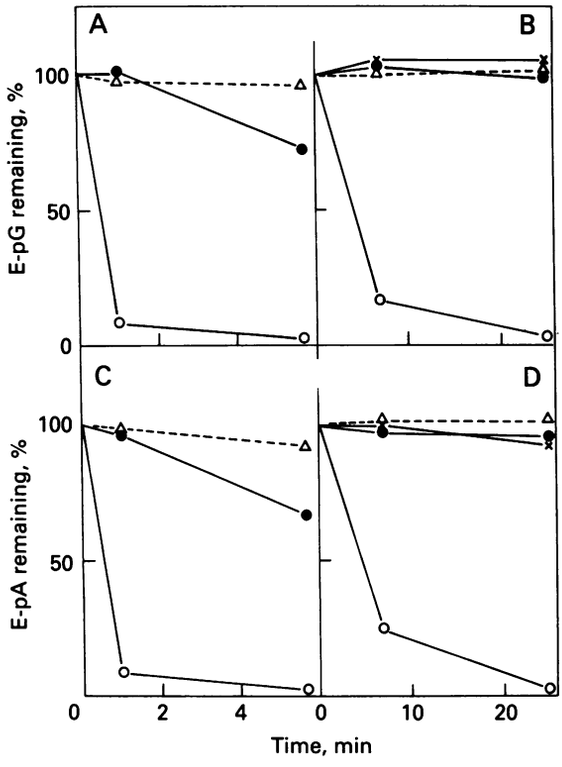

FIG. 5. Stability of the enzyme-nucleotide linkage. (*A* and *B*) Guanylyltransferase–[^{32}P]GMP complex isolated by gel filtration as in Fig. 3 was acid precipitated, washed with ether, and redissolved in 10 mM Tris·HCl, pH 7.9/1% $NaDodSO_4$. Aliquots (5 μl; about 1000 cpm) were incubated with 100 μl of various reagents for indicated times. The reaction was terminated by adding 30 μg of bovine serum albumin and 550 μl of 6% trichloracetic acid. The proteins were recovered by centrifugation and the radioactivity was measured by Cerenkov spectrometry. (*C* and *D*) Experiments were performed as in *A* and *B* but with DNA ligase–[^{32}P]AMP complex that had been isolated in a manner similar to that used for guanylyltransferase. T4 DNA ligase was purified according to the procedure of Tait *et al.* (20) through the step of hydroxylapatite column. Aliquots (5 μl; about 1300 cpm of the ligase–[^{32}P]AMP complex) were treated as above and the acid-precipitable radioactivity was determined. (A and C) Complex was incubated at 95°C with water (△), 0.1 M NaOH (●), or 0.1 M HCl (○). (*B* and *D*) Complex was incubated at 37°C with water (△), 3.86 M NH_2OH (pH 4.75) (○), 0.2 M NH_2OH (pH 7.5) (●), or 4 M sodium acetate (pH 4.75) (x).

the guanylyltransferase–GMP complex. Phosphoamide linkages in enzyme–nucleotide complexes have been reported for *Escherichia coli* and T4-induced DNA ligase and AMP (22), galactose-1-phosphate uridylyltransferase and UMP (29), and vaccinia capping enzyme and GMP (14).

Recently, we found that guanylyltransferase from HeLa cells, *A. salina* embryo, wheat germ, and yeast also formed a covalent enzyme–GMP complex (unpublished data). The protein–[^{32}P]GMP complexes have been isolated also from cytoplasmic polyhedrosis virus (M_r 124,000) and reovirus (one of λ polypeptides) (R. E. Smith and Y. Furuichi, personal communication). Thus, covalent catalysis seems to be the general feature of the capping reaction.

Note Added in Proof. Recently, the formation of a covalent enzyme–GMP complex was also found with guanylyltransferase from HeLa cells (30).

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