Communication

Limited Tryptic Digestion of Messenger RNA Capping Enzyme from Artemia salina

ISOLATION OF DOMAINS FOR GUANYLYLTRANSFERASE AND RNA 5'-TRIPHOSPHATASE*

(Received for publication, December 29, 1983)
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The partially purified preparation of messenger RNA guanylyltransferase from Artemia salina contains, as in the case of the rat liver enzyme (Yagi, Y., Mizumo, K., and Kaziro, Y. (1983) EMBO J. 2, 611–615), the RNA 5'-triphosphatase activity which specifically removes the γ-phosphoryl group from the 5'-triphosphoryl end of the newly synthesized mRNA molecule. The enzyme consists of a single polypeptide chain of Mr = 73,000 and forms a covalent enzyme-GMP complex as an intermediate for the guanylyltransferase reaction. Upon limited hydrolysis with trypsin, the enzyme-[32P]GMP complex is converted to a smaller 32P-containing fragment of Mr = 44,000. When the free enzyme, not complexed with GMP, is digested with trypsin under the same conditions as above, the digests retain almost all activities of both guanylyltransferase and RNA 5'-triphosphatase and can form an enzyme-[32P]GMP complex of the size of Mr = 44,000 on incubation with [α-32P]GTP. Functional domains harboring the activities of guanylyltransferase and RNA 5'-triphosphatase are separated by gel filtration on a Sephacryl S-200 column at positions corresponding to Mr = 44,000 and 20,000, respectively. They can be separated completely from each other by CM-Sephadex column chromatography. While the native, undigested enzyme can transfer the GMP moiety to mRNA molecules with either triphosphoryl (pppN-) or diphosphoryl (ppN-) 5'-terminal, the purified Mr = 44,000 domain under the guanylyltransferase activity can utilize only the latter as an acceptor.

The capping enzyme (mRNA guanylyltransferase) has been purified from vaccinia virus (1) and various cellular sources, including rat liver (2), HeLa cells (3-5), wheat germ (6), calf thymus (7), and mouse myeloma cells (5). Previous studies have revealed that the capping reaction occurs on the 5'-diphosphoryl end of the nascent mRNA as shown in the following equation (2).

γδαβ + ppG + γδαβ′ → γδαβ′ + GppN + δPP

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Therefore, γ-phosphoryl group of the primary transcript has to be removed before transguanylylation takes place.

pppN → RNA triphosphatase → ppN + P

The presence of an RNA 5'-triphosphatase activity has been reported in purified vaccinia virus capping enzyme complex (M, = 127,000) (8, 9). More recently, we have shown that the guanylyltransferase purified from rat liver, consisting of a single polypeptide chain of Mr = 69,000, contains an RNA 5'-triphosphatase activity which specifically removes the γ-phosphoryl group from the triphosphate-terminated polyribonucleotide (10).

In this paper, we report that guanylyltransferase from Artemia salina is also a multifunctional enzyme possessing both guanylyltransferase and RNA 5'-triphosphatase activities in a single polypeptide chain. Furthermore, the two domains retaining the catalytic activities are separated by limited proteolysis with trypsin and purified by ion exchange column chromatographies.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]GTP and sodium [32P]pyrophosphate were purchased from Amersham Japan and New England Nuclear, respectively. Bacterial alkaline phosphatase and nuclease P1 were obtained from Worthington Biochemical Co. and Yamasa Shoyu, Choshi, respectively. Bovine pancreas trypsin and soybean trypsin inhibitor were the products of Sigma. A. salina eggs (encysted embryos) were obtained from Aquarium Stock Co., New York.

RNA Substrates—5'-[γ-32P]ATP-terminated poly(A) was prepared as previously described (10) with a slight modification as follows. Cordicemin (3'-deoxyadenosine)triphosphate was added to the reaction mixture at a concentration of 10 μM (ATP/cordicemin triphosphate, 60:1) to decrease the chain length of poly(A) and to increase the incorporation of [γ-32P]ATP.

Purification of Guanylyltransferase—Guanylyltransferase from A. salina was partially purified from encysted embryos. Detailed purification procedure will be described elsewhere. Crude extracts (100,000 × g supernatant fraction) were prepared as described (11) and fractionated with ammonium sulfate between 40 and 80% saturation. After dialysis against 20 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 mM Mg(OAc)2 and 20% glycerol (Buffer A) containing 50 mM KCl, the proteins were chromatographed on a DEAE-Sephadex column with a linear gradient of KCl (60–300 mM) in Buffer A. The guanylyltransferase activity was eluted at about 0.22 M KCl as a single peak. The active fraction was concentrated with ammonium sulfate and chromatographed on a DEAE-Sephadex column with a linear gradient of KCl (50–300 mM) in Buffer A. Approximately 170-fold purification was obtained with a recovery of 12% starting from the crude extracts. The preparation did not contain any GpppG synthetase activity (12).

Guanylyltrasferases from rat liver nuclei (2) and Saccharomyces cerevisiae (13) were purified up to the step of CM-Sephadex column as described previously. Vaccinia virus capping enzyme (1st DEAE-cellulose fraction) was prepared according to the reported procedure (1) from the particles of Lister strain which was kindly supplied by T. Urushibara (Kitasato University, Tokyo).

Assay for Guanylyltransferase—Three different assays, i.e. formation of the cap [32P]GpppG structure, GTP-[32P]PP exchange, and formation of enzyme-[32P]GMP, were carried out as described (2, 14). The capping reaction was assayed using the synthetic acceptor RNAs, i.e. ppGpCpC-(An,Un,G), (2) or pppA(pA). Assay for RNA 5'-Triphosphatase—The release of inorganic [32P] phosphate from [γ-32P]pppA(pA) was measured as described previously (10) by chromatography on polyethyleneimine-cellulose thin layer plates.

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RESULTS

Association of RNA 5'-Triphosphatase with Guanylyltransferase—When the ammonium sulfate fraction (40–80% saturation) of the A. salina crude extract was chromatographed on a CM-Sephadex column with a linear gradient of KCl, the activities of guanylyltransferase and RNA 5'-triphosphatase were co-eluted at 0.22 M KCl and well separated from the activity of RNA (guanine-7-)methyltransferase which was eluted at 0.18 M KCl (data not shown). The active fractions were combined and further purified on a DEAE-Sephadex column. As shown in Fig. 1, RNA 5'-triphosphatase activity was eluted again in parallel to the activities of guanylyltransferase assayed by formation of the enzyme-GMP complex and GTP-PP, exchange.

The molecular weight of the A. salina guanylyltransferase-[32P]GMP complex was estimated to be 73,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Fig. 2A, lane 1). Since the molecular weight of the native enzyme was 74,000 as determined by gel filtration (see Fig. 3A), we concluded that A. salina guanylyltransferase is also a multifunctional enzyme consisting of a single polypeptide chain and catalyzing two different reactions as in the case of rat liver guanylyltransferase (10).

Limited Proteolysis of Guanylyltransferase—The guanylyltransferase-[32P]GMP complex was incubated with trypsin or chymotrypsin (protein/protease, 45:1 (w/w)) for 30 min at 0°C and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 2A, lane 1, the 73-kDa enzyme-GMP complex was quantitatively converted to a single band of 44 kDa by digestion with trypsin (lane 2). On the other hand, chymotrypsin was less active than trypsin and the enzyme was partially converted to a fragment of 45 kDa (lane 3). Fig. 2B shows the time course of trypsin digestion of the guanylyltransferase.
Digestion of the enzyme-[^32]P]GMP complex. Almost all the complex was converted to a 44-kDa fragment after incubation for 10 min at a protein/trypsin ratio of 60.

To examine whether or not the 44-kDa fragment generated by limited proteolysis is catalytically active, we first treated guanylyltransferase with trypsin for various times at a protein/trypsin ratio of 70 and then analyzed for the activity of the digested enzyme to form a protein-[^32]P]GMP complex. As shown in Fig. 2C, a 44-kDa fragment-[^32]P]GMP complex was formed when the material which had been treated with trypsin for 5 min was incubated with [α-[^32]P]GTP (lane 2). It was also found that the RNA 5'-triphosphatase activity was not impaired, but rather stimulated to about 1.7-fold (data not shown).

Isolation of Domains for Guanylyltransferase and RNA 5'-Triphosphatase—When the enzyme was digested with trypsin and chromatographed on a Sephacryl S-200 column, the activities of guanylyltransferase and RNA 5'-triphosphatase were eluted at two separate peaks at positions corresponding to 44 and 20 kDa, respectively (Fig. 3B). On the other hand, in the case of the undigested enzyme, both activities were eluted at the position of 74 kDa (Fig. 3A).

Complete separation of two domains was achieved by ion exchange chromatography as shown in Fig. 4. When trypsin-digested guanylyltransferase was chromatographed on a CM-Sephadex column with a linear gradient of KCl, RNA 5'-triphosphatase and guanylyltransferase activities were separated completely and eluted at 0.05 and 0.18 M KCl, respectively. The fraction of RNA 5'-triphosphatase released [^32]P as inorganic phosphate from [γ-[^32]P]pppA(pA)n, but not from [γ-[^32]P]ATP, indicating that the RNA 5'-triphosphatase domain still retains the same substrate specificity as the native enzyme. The purified guanylyltransferase domain was active only when the ppG-terminated RNA, but not the ppG-terminated RNA, was used as “cap” acceptor molecules. This is in contrast to the native enzyme which can transfer GMP to either ppG- or ppG-terminated RNA to form “capped” GpppG-RNA. Only after incubation with RNA 5'-triphosphatase domain, triphosphate-terminated RNA (pppA-RNA) could serve as a substrate for the purified 44-kDa guanylyltransferase domain (Fig. 5).

**FIG. 4. Separation of guanylyltransferase and RNA triphosphatase domains by CM-Sephadex column chromatography.** The guanylyltransferase fractions from CM-Sephadex column (2.1 mg of protein) were incubated with trypsin (protein/trypsin, 70:1 (w/w)) for 10 min at 0°C and trypsin inhibitor was added to terminate the reaction (inhibitor/trypsin, 3:1). The digest was applied to a CM-Sephadex column (0.5 x 13 cm) and eluted with 100 ml of a linear gradient of 50-400 mM KCl in Buffer A. Fractions of 1 ml were collected and assayed for GTP-PPi exchange (■), capping activity (○), and RNA 5'-triphosphatase (△), using 15-, 15-, and 7-μl aliquots, respectively.

**FIG. 5. Requirement of two domains of guanylyltransferase for the capping of 5'-triphosphate-terminated RNA.** Lane 1, no enzyme. Lane 2, 0.2 μg of guanylyltransferase domain (Fraction 44 of Fig. 4) alone. Lane 3, both 0.2 μg of purified guanylyltransferase domain and 1.3 μg of purified RNA 5'-triphosphatase domain (Fraction 4 of Fig. 4). Lane 4, 1.6 μg of native guanylyltransferase. The reaction mixture contained (in 30 μl), 50 mM Tris-HCl (pH 7.9), 5 mM Mg(OAc)2, 10 mM dithiothreitol, 10 μg of bovine serum albumin, 24 pmol of pppA(pA)n, and 2.7 μM [α-[^32]P]GTP (100,000 cpm/pmol). Incubation was for 60 min at 30°C. For the experiment of lane 3, the RNA 5'-triphosphatase domain was first incubated with pppA(pA)n in the absence of Mg(OAc)2 (10) for 30 min at 30°C, and then the guanylyltransferase domain, [α-[^32]P]GTP, and Mg(OAc)2 were added to the reaction mixture and incubated an additional 60 min at 30°C. After incubation, RNA was treated with nuclease P1 and alkaline phosphatase and electrophoresed on Whatman DE81 paper at pH 5.4 as described (2).

**DISCUSSION**

The activity to cap the nascent mRNA molecules was found in various viral and cellular sources. A capping enzyme complex purified from vaccinia virus consists of two subunits (95 and 31.4 kDa) and contains three enzymatic activities, i.e., RNA guanylyltransferase, RNA (guanine-7-)methyltransferase, and RNA 5'-triphosphatase (1, 8, 9). On the other hand, RNA guanylyltransferase purified from cellular sources consists of a single polypeptide chain of Mr = 65,000-69,000 and does not contain any RNA (guanine-7-)methyltransferase activity (2-5, 7).

In a previous paper (10), we have reported that RNA guanylyltransferase purified from rat liver nuclei contains the activity of RNA 5'-triphosphatase. In this paper, we present evidence that RNA guanylyltransferase purified from A. salina is also a multifunctional enzyme possessing both RNA guanylyltransferase and RNA 5'-triphosphatase activities in a single polypeptide chain. After tryptic digestion, both activities were fully retained but the enzyme was cleaved into two fragments which were separated on a Sephacryl S-200 column to yield a 44-kDa guanylyltransferase domain and 20-kDa RNA 5'-triphosphatase domain. Both domains were completely separated by CM-Sephadex column chromatography (Fig. 4).

Similar domain structures also seem to be present in rat liver capping enzyme, since the 69-kDa enzyme-[^32]P]GMP complex was converted to a 40-kDa complex by the partial digestion with trypsin. Itoh et al. (13) reported that the size of the yeast guanylyltransferase-[^32]P]GMP complex in sodium dodecyl sulfate-gel electrophoresis is 46 kDa, the value which is similar to that of the guanylyltransferase domain of A. salina and rat liver. The yeast enzyme-GMP complex was refractory to trypsin digestion. Since the yeast capping enzyme consists of two small (39-kDa) and two large (45-kDa)
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...subunits, we assume that, in yeast, guanylyltransferase and RNA 5'-triphosphatase may exist in two different subunits. From these observations it seems that the cellular guanylyltransferase activity resides in a 40- to 45-kDa polypeptide. On the other hand, vaccinia virus guanylyltransferase-[32P]GMP (95 kDa) was converted to a 60-kDa fragment by treatment with trypsin under similar conditions.

More recently, we succeeded in purifying both domains to almost homogeneous states. Studies to further characterize the two domain structures are now in progress.

Acknowledgments—We thank Catarina Darnfors for her collaboration in early stages of the purification of the enzyme and Reiko Toyama for helpful discussions.

REFERENCES
1. Martin, S. A., Paoletti, E., and Moss, B. (1975) J. Biol. Chem. 250, 9322-9329
2. Mizumoto, K., and Lipmann, F. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4961-4965
3. Venkatesan, S., Gershowitz, A., and Moss, B. (1980) J. Biol. Chem. 255, 2829-2834
4. Wang, D., Furuchi, Y., and Shatkin, A. J. (1982) Mol. Cell. Biol. 2, 993-1001
5. Shuman, S. (1982) J. Biol. Chem. 257, 7237-7245
6. Keith, J. M., Venkatesan, S., Gershowitz, A., and Moss, B. (1982) Biochemistry 21, 327-333
7. Nishikawa, Y., and Chambon, P. (1982) EMBO J. 1, 485-492
8. Venkatesan, S., Gershowitz, A., and Moss, B. (1980) J. Biol. Chem. 255, 903-908
9. Shuman, S., Surks, M., Furneaux, H., and Hurwitz, J. (1980) J. Biol. Chem. 255, 11588-11598
10. Yagi, Y., Mizumoto, K., and Kaziro, Y. (1983) EMBO J. 2, 611-615
11. Zasloff, M., and Ochoa, S. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 3059-3063
12. Warner, A. H., Beers, P. C., and Huang, F. L. (1974) Can. J. Biochem. 52, 231-240
13. Itoh, N., Mizumoto, K., and Kaziro, Y. (1983) FEBS Lett. 155, 161-166
14. Mizumoto, K., Kaziro, Y., and Lipmann, F. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1693-1697

1 N. Itoh and K. Mizumoto, unpublished observation.
Limited tryptic digestion of messenger RNA capping enzyme from Artemia salina. Isolation of domains for guanylyltransferase and RNA 5'-triphosphatase.
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J. Biol. Chem. 1984, 259:4695-4698.

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