

An enzyme which specifically splits a covalent bond between picornaviral RNA and VPg

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An enzyme which specifically splits a covalent bond between VPg and picornaviral RNAs (tentatively designated Y-pUpN PDE) has been partially purified from mouse ascites Krebs II cells. Using substrates labelled in vitro with ^{125}I -Bolton-Hunter reagent and a new assay based on Kieselgel thin-layer chromatography, several biochemical characteristics of the enzyme have been determined, depending on the pH and on Mg^{2+} , K^+ , spermidine and PEG concentrations, etc. We found that the enzyme does not 'unlink' VPg from comoviral RNA. We suggest that Y-pUpN PDE represents a new class of enzymes.

RNA-protein complex; Picornavirus; Comovirus; Heteropolymeric covalent bond cleavage

1. INTRODUCTION

An enzyme, which specifically splits a heteropolymeric covalent bond between picornaviral RNA and VPg, was partially purified from mouse ascites Krebs II cells. Tentatively (until its complete substrate specificity was determined), we designated the enzyme as uridylpolynucleotide-(5'P→O)-tyrosine phosphodiesterase (briefly, Y-pUpN PDE) [1,2].

Earlier, similar 'unlinking' activity was detected in HeLa and L-cells, in reticulocyte and wheat germ cell extracts [3–6] using the polioviral RNA-VPg compound as substrate. The isolated enzyme was found to have a molecular mass of 27 kDa and a very low turnover number – $7 \times 10^{-3} \text{ min}^{-1}$ [4].

The enzyme preparations, purified by us according to an identical scheme from ascites cells, were enriched in the 24–27 kDa protein but were

found to be non-homogeneous after silver staining [1]. Thus, another minor protein in the preparations could be responsible for the unlinking activity.

Relevant studies were delayed largely due to the unavailability of substrates and laborious assays. The estimation of enzymatic activity by the phenol-extraction assay routinely used [1–6] was correct only with the purified enzyme. Crude cell extract proteolytic activity [4–6] (the activity also observed by us) rapidly converted free VPg or VPg-pU to water-soluble peptides, thus decreasing the quantity of unlinked phenol-extracted VPg.

Recently, we developed new assays of unlinking activity. More convenient substrates were also prepared and Y-pUpN PDE was purified by a modified procedure as in [2]. Enzyme activity was cochromatographed on a Superose-12 column (FPLC system, Pharmacia) with a protein of an apparent molecular mass of 100 kDa rather than 27 kDa [2].

In this paper some properties of Y-pUpN PDE purified from the ascites cell S-100 extract which was fractionated by ammonium sulphate precipitation, Sephadex G-100 gel filtration and DEAE-Sephadex chromatography, are described.

This article is the third of this series. For the two previous articles, see [1,2]

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2. MATERIALS AND METHODS

125 I-labelled K-peptide of the EMC or CPMV virus was produced from VPg-RNA by proteinase K treatment (2 mg/ml of proteinase K in 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EDTA; 0.2% SDS for 2 h at 37°C) with subsequent 125 I-Bolton-Hunter reagent labelling. A specific activity of 10^4 cpm/ μ g of K peptide-RNA was usually obtained.

Thin-layer chromatographic assay of the unlinking activity was carried out as described [2].

The reaction mixture and incubations with enzyme preparations were as in [2].

3. RESULTS AND DISCUSSION

The unlinking activity of Y-pUpN PDE was detected with the aid of an exogenous substrate, i.e. the picornaviral RNA-VPg compounds [1-5]. The true cellular substrates of the enzyme are yet unknown.

In the previous paper [2] the focus was on new and more convenient substrates for the unlinking enzyme. We used successfully VPg-RNA, K peptide-RNA and K peptide-small fragment RNA compounds labelled in vitro with 125 I-Bolton-Hunter reagent instead of [3 H]Tyr-VPg-RNA and its derivatives. Also, we developed new effective and specific assay of Y-pUpN PDE activity by thin-layer chromatography on Kieselgel or PEI-cellulose plates. The assay is highly specific for the cleavage of the heteropolymetric phosphodiester bond between the proteic and the nucleic constituents of the substrate even by crude enzymic preparations [2].

Using the new assay, we found that both the 125 I-VPg and 125 I-K peptides are protected from complete proteolysis by the bound RNA. The 125 I-K peptide linked to pU is hydrolysed by proteinase K more extensively than the [125 I]-peptide bound to the high-molecular mass RNA (fig.1, lanes 3,4). We also found that in the Mg^{2+} concentration range from 0.5 to 5 mM the enzyme activity was the same. An increase in the Mg^{2+} concentration to 10 mM reduced the activity partially (fig.2A, lanes 4-8). This concentration of Mg^{2+} may apparently change the substrate conformation. We were not able to eliminate Mg^{2+} from the reaction mixture in full, since the enzyme was isolated in 1.5 mM $MgCl_2$ solution. However, the addition of EDTA to 50 mM inhibited the investigated enzymatic activity completely (fig.2B). 1 mM spermidine inhibits the enzyme slightly, whereas at 10 mM it

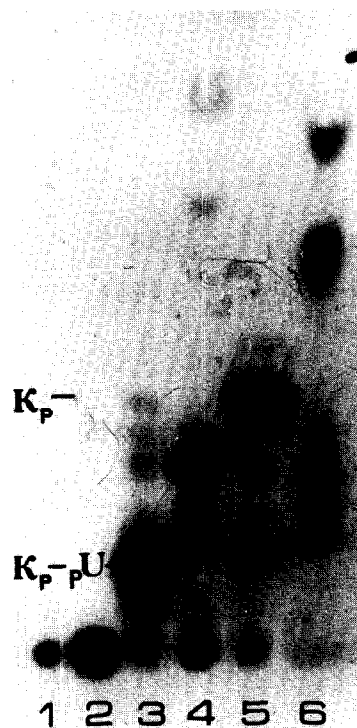


Fig.1. Protection of the bound 125 I-K-peptides (Kp-RNA) by encephalomyocarditis RNA (Kieselgel thin-layer chromatography). Lanes: (1) control, Kp-RNA incubated in water (37°C, 1 h); (2) Kp-RNA treated repeatedly with proteinase K; (3) Kp-RNA P_1 nuclease-treated; (4) Kp-RNA treated with P_1 nuclease and subsequently with proteinase K; (5) successive treatments of Kp-RNA with P_1 nuclease and snake venom phosphodiesterase (SVE); (6) as in (5) but subsequently treated with proteinase K. Thin-layer chromatography on Kieselgel plates in butanol/acetic acid/water (3.75:1:1).

arrests the reaction (fig.2C). As demonstrated earlier [1,2], the Y-pUpN PDE was completely inactivated by heat treatment at 55°C for 15 min.

The activity of the purified enzyme was evidently dependent on pH (it was active in the pH range 5.7-7.5; see fig.2A, lanes 9-15), on the potassium chloride concentration (the activity maximum was between 50 and 200 mM; fig.2C, lanes 1-4). Addition of up to 5% PEG 6000 to the reaction mixture increased the yield of cleavage products (fig.2B, lanes 5,6) probably because of both the substrate and the enzyme concentrating effect.

It is easy to see that the cleavage of EMC viral substrates under standard assay conditions rarely

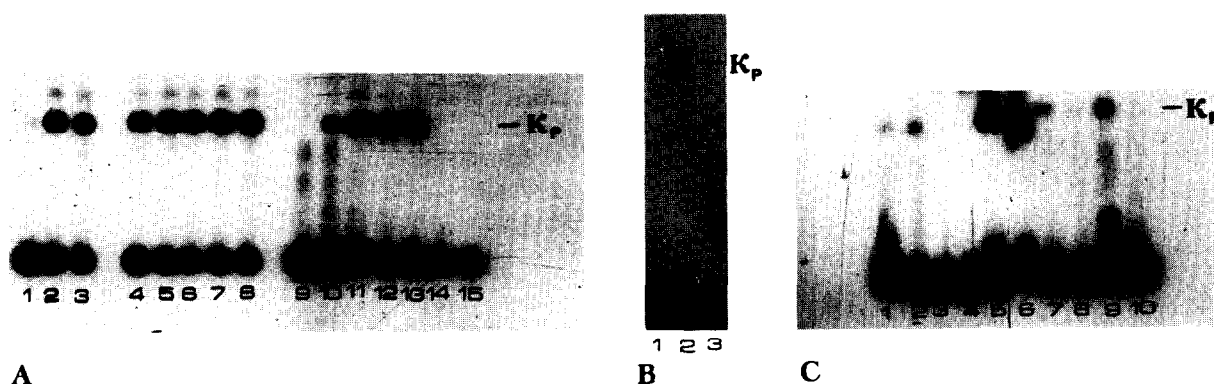


Fig.2. Dependence of Y-pUpN PDE activity on different incubation conditions using encephalomyocardial Kp-RNA as substrate. (A) Influence of Mg^{2+} and pH. Lanes: (1) control. Kp-RNA incubated in the assay mixture; (2) incubation of Kp-RNA with the enzyme under standard assay conditions; (3) as in (2), but at 2-fold greater enzyme concentration; (4–8) activity of Y-pUpN at different Mg^{2+} concentrations (10, 5, 1.5, 1.0 and 0.5 mM, respectively); (9–15) influence of pH on enzyme activity (pH 9.5, 8.5, 7.5, 6.5, 5.7, 4.5 and 3.5, respectively). (B) Inhibition of the enzyme by EDTA. Lanes: (1) control, incubation of the substrate in the assay cocktail; (2) standard assay conditions for enzymic activity determination; (3) as in (2), but in the presence of 50 mM EDTA. (C) Dependence of the enzymic activity on KCl concentrations and other additives. Lanes: (1–4) in the presence of 50, 200, 500 and 1000 mM KCl, respectively; (5,6) at 5 and 10% polyethylene glycol 6000, respectively, in the cocktail; (7,8) in the presence of 1 mM (7) or 10 mM (8) spermidine; (9) under standard assay conditions of the enzymic activity; (10) control, Kp-RNA incubation in the assay buffer.

exceeds 50%, irrespective of the enzyme activity assay used ([1,2] and fig.2A here). At the same time, 75–85% of the polioviral substrates were cleaved with the enzyme (fig.4A). We suggest that the Y-pUpN PDE recognizes the 5'-terminal secondary structure differences predicted for the EMC and polioviral RNAs [7,8].

As shown in [2], Y-pUpN PDE activity can be mimicked either by the combined actions of cellular endo- and exonucleases or by endonuclease followed by phosphatase. Nevertheless, several facts argue in favor of the specific activity of Y-pUpN PDE which we observed even in crude enzyme preparations. Firstly, in ascites cells the two described nucleolytic enzymes, exoI [9] and RNase D [10], have different properties compared with Y-pUpN PDE. For example, exoI is stable at 50°C for 10 min and active at pH 9.2 (Y-pUpN PDE is inactive under these conditions). ExoI was found in trough water by DEAE-cellulose chromatography, while most of the Y-pUpN PDE was eluted at 120–150 mM KCl. RNase D (like exoI) was not absorbed by DEAE-cellulose and was inhibited by the addition of single- or double-stranded nucleic acids, whereas the Y-pUpN PDE standard reaction mixture contained a 20-fold excess of tRNA over the substrate concentration.

Secondly, the electrophoretic mobility of polio or EMC RNA incubated under assay conditions even with crude preparations of the Y-pUpN PDE changed insignificantly and the products of viral

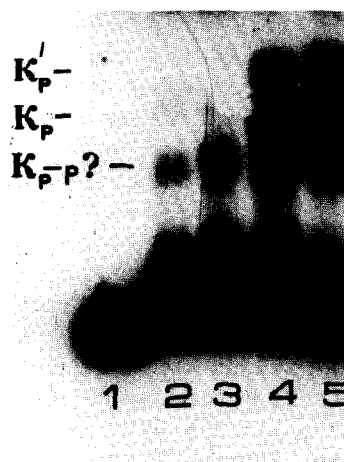


Fig.3. Y-pUpN PDE does not split the phosphodiester bond between K peptide and pUp. Lanes: (1) control incubation in the assay buffer; (2) EMC Kp-RNA digested by A, T₁ and T₂ RNases; (3) EMC Kp-RNA digested firstly by RNases A, T₁ and T₂ and then incubated with Y-pUpN PDE; (4) EMC Kp-RNA treated first with Y-pUpN PDE and then with RNases A, T₁ and T₂; (5) EMC Kp-RNA incubated with Y-pUpN PDE. K'p is one of the K peptides.

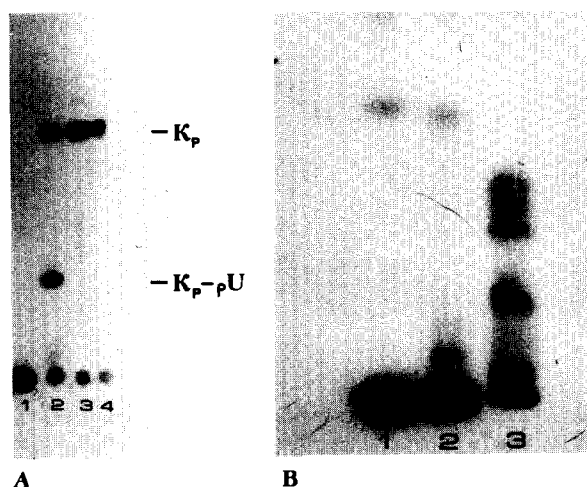


Fig.4. Y-pUpN PDE does split the picornaviral K peptide(s)-RNA substrate but not the comoviral one. (Also compare with the results on encephalomyocarditis Kp-RNA.) (A) Lanes: (1) control incubation of the polio Kp-RNA in the assay buffer; (2) Kp-RNA digested with nuclease P₁; (3) Kp-RNA treated with P₁ nuclease and snake venom phosphodiesterase, successively; (4) Kp-RNA treated with Y-pUpN PDE. (B) Comoviral (cow pea mosaic viral) Kp-RNA substrate. Lanes: (1) Kp-RNA incubated, no Y-pUpN PDE added; (2) Kp-RNA incubated in presence of the unlinking enzyme; (3) Kp-RNA treated with P₁ and snake venom phosphodiesterase.

RNA hydrolysis moved more slowly than did 16 S rRNA, while the K-peptide was split off from RNA very efficiently. This result cannot be explained by a non-specific hydrolysis of viral RNA.

Thirdly, the purified enzyme did not cleave K-peptide-pUp, but cleaved the high molecular mass precursor, i.e. K peptide-RNA (fig.3B).

Finally, we found that Y-pUpN PDE does not cleave a heteropolymeric phosphodiester bond between comoviral VPg and RNA of the cow pea mosaic virus (fig.4). We believe that the specificity of Y-pUpN PDE found in this case is explained by the difference in structure of the unit of linkage between VPg and picorna- [11-13] and como- [14,15] viral RNAs. The latter result is in agreement with the findings of De Varrenes et al. [16] on the unlinking activity of the reticulocyte crude extract.

Very recently, we found that some hydrolysing VPg proteolytic activity copurifies with Y-pUpN

PDE at all steps of the isolation procedure (possibly, this is an intrinsic property of the investigated enzyme). This finding will be described elsewhere.

Taking into consideration the enzymic properties described above, we have come to the conclusion that the Y-pUpN PDE is a representative of a new class of enzymes.

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