RIBOPOLYNUCLEOTIDES MODIFIED AT PYRIMIDINE RESIDUES ARE CLEAVED SELECTIVELY BY T_2 RIBONUCLEASE AT PURINE RESIDUES

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1. Introduction

We have found recently [1] that T_2 ribonuclease does not hydrolyze phosphodiester bonds between cytidine residues modified with methoxyamine-bisulfite mixture [2] and their 3′-neighbouring residues in ribopolynucleotides. Restriction of the hydrolytic action of T_2 RNase is presumably due to the SO_3^--group present at position 6 of the cytosine residues, because ribopolynucleotides modified with methoxyamine alone and containing N^4-methoxycytidine instead of cytidine residues are easily cleaved by the enzyme. Therefore it was anticipated that modification of uridine residues with bisulfite resulting in formation of an SO_3^--group-containing adduct [3,4] would also render the 3′-adjacent phosphodiester bonds stable to T_2 RNase.

In the course of the present studies we succeeded in finding conditions for complete simultaneous modification of C and U residues in ribopolynucleotides with methoxyamine-bisulfite mixture. Action of T_2 RNase upon ribopolynucleotides modified in such a way affords oligomers of the general formula $(Pyr)_n$-Pur, where $n \geqslant 1$. Thus, a method of selective cleavage of polyribonucleotides at purine residues was elaborated, and T_2 RNase became 'puryl-ribonuclease'.

2. Experimental

2.1. *Materials*

Ribosomal RNA of *E. coli* was isolated from 50S particles generously given by Dr I. N. Shatsky (Moscow State University). Poly C was obtained from Special Bureau for Design and Technology of Biologically Active Compounds (Novosibirsk) of the Main Management of Microbiological Industries. Poly U – from Reanal (Hungary) T_2 Ribonuclease (EC 2.7.7.17) was purchased from Sankyo (Japan), alkaline phosphomonoesterase of *E. coli* – from Worthington (USA) FND-cellulose – from Filtrack (DDR), Sephadex G-10 – from Pharmacia, (Sweden).

2.2. *Methods*

Thin-layer chromatography was performed on silica plates (16 × 16 cm) coated with cellulose (3.5 g). To scan the chromatograms and to measure spectra we used an Opton (FRG) scanning spectrophotometer. Absorbance of solutions at 260 and 280 nm was measured with a Specord UV–VIS spectrophotometer (Carl Zeiss, Jena). Thin-layer chromatography was performed in Solvents: (1) *n*-butanol, saturated with water, and (2) *iso*-butyric acid – 0.5 M ammonia (10:6, v/v), pH 3.7.

2.3. *Modification*

Modification of poly C and poly U was done in 0.1 ml reaction mixtures containing 10 A_{260} units of polymer, 1.5 M $NH_2OCH_3 \cdot HCl$ and 1–2 M $Na_2S_2O_5$ at pH 5 or 7 at 37°C; 0.01 ml aliquots were removed at time intervals, diluted to 2 ml with water, and the absorbance measured at 260 and 280 nm.

Modification of only the cytidine residues was done with 1.5 M $NH_2OCH_3 \cdot HCl$ and 1 M $Na_2S_2O_5$ at pH 5 for 4 h at 37°C, the polymer desalted by gel-filtration on Sephadex G-10 (80 ml column, 20 ml/h) and

partially formed 5,6-dihydro-6-sulfouridine residues were demodified by 12 h incubation in 0.1 M ammonia at 25°C. No demodification of $\overset{*}{C}$-residues† takes place [2].

For simultaneous complete modification of C and U residues, 20 A_{260} units of ribosomal RNA were incubated in 0.05 ml of 1.5 M $NH_2OCH_3 \cdot HCl$–2 M $Na_2S_2O_5$, pH 7.0, for 4 h at 37°C. The mixture was 50 times diluted with water, pH adjusted to 4.5 with sodium acetate buffer and hydrolysed for 2 h at 37°C with 2 activity units of T_2 RNase. The digest was passed through CM-cellulose to remove T_2 RNase, evaporated to a small volume and desalted on a Sephadex G-10 column. For dephosphorylation, 1 A_{260} unit of oligonucleotide was incubated for 2 h at 37°C in 0.1 ml of 0.05 NH_4HCO_3 pH 8 with 6 μg of phosphomonoesterase.

3. Results

3.1. *Modification of poly C and poly U with NH_2OCH_3 and $Na_2S_2O_5$*

Modification of cytidine residues with NH_2OCH_3 and $Na_2S_2O_5$ affords 5,6-dihydro-6-sulfo-N^4-methoxycytidine [2]. The maximum absorbance of this compound is at 230 nm; it does not absorb at 260 nm [2]. The rate of the modification of polycytidylic acid with 1.5 M NH_2OCH_3 and 1–2 M $Na_2S_2O_5$ was measured by monitoring the absorbance at 280 nm. The modification of poly C at pH 5.0 and 7.0 was complete in 1 h at both 1 M and 2 M $Na_2S_2O_5$.

Modification of uridine residues with 2–3 M bisulfite affords 5,6-dihydro-6-sulfouridine which does not absorb at 260 nm [3,4]. We decided to use NH_2OCH_3 mixture to modify both the pyrimidine nucleoside residues in ribopolynucleotides. It is seen in fig.1 that complete modification of poly U takes place in 1.5 M NH_2OCH_3–2 M $Na_2S_2O_5$ at pH 7.0. The reaction is complete in 3 h as revealed by disappearance of absorption at 260 nm. The $\overset{*}{U}$ residue is unstable at alkaline pHs [3,4]. Treatment of the product with 0.1 M NH_4OH for 12 h at 25°C results in complete

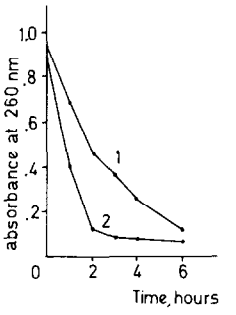

Fig.1. Kinetics of poly U modification at pH 5.0 with 1.5 M NH_2OCH_3–1 M $Na_2S_2O_5$ (1) and at pH 7.0 with 1.5 M NH_2OCH_3–2 M $Na_2S_2O_5$ (2).

transformation of $\overset{*}{U}$ into starting uridine. Demodification of $\overset{**}{C}$ does not take place under such conditions. Thus, one may modify either both U and C residues or C residues alone, if $\overset{*}{U}$ is subjected subsequently to demodification.

The results obtained with poly U and poly C suggested that long ribopolynucleotides or RNA could be completely modified under the above conditions.

3.2. *Action of T_2 RNase upon modified rRNA*

For simultaneous modification of C and U, rRNA was incubated for 4 h at 37°C in 1.5 M NH_2OCH_3–2 M $Na_2S_2O_5$, pH 7.0. Desalting after the reaction was omitted because it could result in partial demodification of $\overset{*}{U}$. The reaction mixture was diluted 50 times with water, its pH adjusted to 4.5, and T_2 RNase added. It was shown by preliminary experiments that unmodified rRNA is completely hydrolyzed to mononucleotides in this medium. The digest was passed through a column with CM-cellulose to remove the enzyme, the solution concentrated by evaporation and the oligonucleotides obtained were dephosphorylated.

Thin-layer chromatography of the digest in Solvent 1 revealed only two nucleosides G and A (fig.2A); Solvent 1 only removes nucleosides from the starting point. Development along a second direction with Solvent 2 showed that $\overset{*}{U}$ and $\overset{**}{C}$ were not present in the mixture. Hence, T_2 RNase digested modified rRNA as a 'puryl-ribonuclease'.

The oligonucleotides were eluted from the starting point and hydrolyzed with 0.3 M KOH for 18 h at 37°C

†*Designations* used: $\overset{*}{C}$ - N^4-methoxycytidine residues; $\overset{*}{U}$ - 5,6-dihydro-6-sulfouridine residues; $\overset{**}{C}$ - 5,6-dihydro-6-sulfo-N^4-methoxycytidine residues.

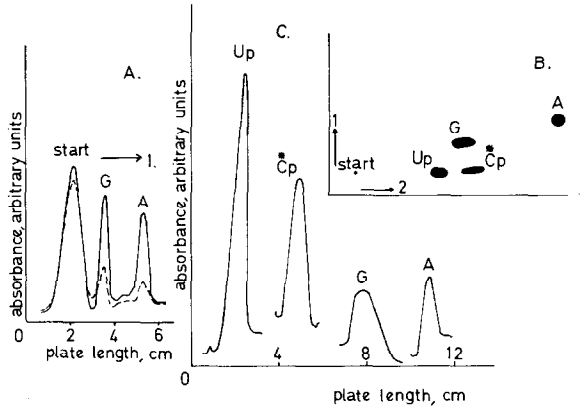

Fig.2. Thin-layer chromatogram scans: (A) Separation in Solvent 1; product of digestion of rRNA ($\overset{*}{U},\overset{**}{C}$) with T_2 RNase and phosphomonoesterase. (B) Two-dimensional chromatography in Solvents 1 and 2 of the substance from the start of fig.2A hydrolysed with 0.3 M KOH. (C) Scan of the spots of fig.2B. (———) A_{260}; (– – – –) A_{230}.

Guanosine and adenosine were the only two nucleosides present in this hydrolysate as revealed by chromatography in the same Solvents (fig.2B). This finding indicated that T_2 RNase does not cleave $\overset{**}{C}p$-X and $\overset{*}{U}p$-X bonds (X=A,G,$\overset{**}{C}$, $\overset{*}{U}$). Dephosphorylation of the mononucleotides which remained at the starting point after chromatography of the alkaline hydrolysate afforded only N^4-methoxycytidine and uridine (fig.2B). It was shown earlier [1] that treatment of 5,6-dihydro-6-sulfo-N^4-methoxycytidine with 0.3 M KOH leads to its transformation to N^4-methoxycytidine. As for $\overset{*}{U}$ residues, it was mentioned above that they are demodified by alkali to give uridine residues. The fact that Gp and Ap were not found at the starting point after hydrolysis with KOH indicated that T_2 RNase acted upon the modified substrate highly selectively.

4. Discussion

Hence, it was found in the course of the present studies that simultaneous modification of C and U in rRNA with NH_2OCH_3 and $Na_2S_2O_5$ renders $\overset{**}{C}p$-X and $\overset{*}{U}p$-X bonds (X=$\overset{**}{C}$,$\overset{*}{U}$,A,G) stable to attack by T_2 RNase and restricts the action of the enzyme to bonds adjacent to adenosine and guanosine residues. T_2 RNase behaves as a 'purylribonuclease' with the modified substrate.

It was mentioned above, that $\overset{*}{U}$ residues may be demodified under conditions which leave $\overset{**}{C}$ residues intact. We made use of this fact in experiments which are not documented here; modified rRNA ($\overset{**}{C}$,$\overset{*}{U}$) was digested with T_1 RNase, $\overset{*}{U}$-residues were demodified with alkali, and the polypyrimidine tracts obtained were cleaved at uridine residues with T_2 RNase.

It was shown earlier that T_2 RNase does not cleave bonds adjacent to guanosine residues modified with kethoxal [5], whereas pancreatic RNase cleaves $\overset{**}{C}$-containing polynucleotides only at uridine residues [1]. Taking into account these data and evidence obtained in the present studies, it is seen that it is now possible to cleave ribopolynucleotides selectively by action of T_2 and pancreatic RNases upon modified substrates. Such 'restriction' methods combined with classic cleavage procedures may help in RNA sequencing.

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