A NOVEL APPROACH TO STRUCTURAL ANALYSIS OF OLIGONUCLEOTIDES

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1. Introduction

Unlike structural analysis of ribonucleic acids, determination of the primary structure of deoxyribonucleic acids and even of deoxyribo-oligonucleotides still remains a difficult problem because of the lack of nucleoside-specific endonucleases. For this reason it seems timely to look for methods that do not involve the application of such enzymes.

The present communication describes a new approach to oligonucleotide structural analysis based upon nucleoside-specific chemical modification which stops the action of an exonuclease. The approach is illustrated by determination of the positions of deoxycytidine residues in a model oligonucleotide dpC–C–A–C–G. The chemical modification was performed by combined action of *O*-methylhydroxylamine and bisulfate transforming the cytidine residues (C) into negatively charged N^4-methoxy-5,6-dihydrocytidine-6-sulfonate (C*) residues [1].

2. Materials and methods

Bisulfite and *O*-methylhydroxylamine were prepared as described earlier [1]. The preparation of *O*-[^{3}H]methylhydroxylamine hydrochloride (Isotop, USSR) had a specific radioactivity 133 Ci/mole; to prepare its stock solution, 5 mCi (3.2 mg) was dissolved in 65 μl 0.3 N NaOH.

The pentanucleotide dpC–C–A–C–G was synthesized in the laboratory of Prof. M.N. Kolosov in the Institute for Chemistry of Natural Products. It was homogeneous in ion-exchange chromatography in neutral and acidic media.

Chick erythrocyte DNA was a preparation of Reanal, Hungary. Pancreatic DNAase (EC 3.1.4.5) and alkaline phosphatase of *E. coli* were obtained from the Special Bureau of Design and Technology of Biologically-Active Substances (Novosibirsk). *Vipera lebetina* (gurza) venom phosphodiesterase (EC 3.1.4.1) having no phosphomonoesterase or endonuclease activity was isolated according to Nikolskaya et al. [2]. The stock solution of phosphatase in 0.05 M Tris-HCl pH 8.5, 1 M NaCl had an activity of 25 standard units per ml, that of phosphodiesterase in 0.06 M triethylammonium bicarbonate pH 8.2, 0.01 M $MgCl_2$, 4 standard units per ml. The hydrolysis with phosphatase was performed in Buffer A (0.1 M Tris-HCl, pH 9), with phosphodiesterase in Buffer B (0.2 M Tris-HCl, pH 8.2, 0.1 M $MgCl_2$).

Ion-exchange chromatography was performed using the micro-column techniques elaborated in the Institute of Organic Chemistry (Novosibirsk) [3, 4]. 50 μl, 10 cm long columns with DEAE-cellulose (for TLC, Serva, BRD) were employed. The absorbancy of the effluents at the desired sets of wavelengths was recorded using the microspectrophotometer designed in the Institute of Organic Chemistry (Novosibirsk); the volume of the flow cell of this instrument is 2 μl. The desalting of oligonucleotides was performed by gel-filtration on a 600 μl, 20 cm long Sephadex G-10 (Pharmacia, Sweden) columns in bidistilled water.

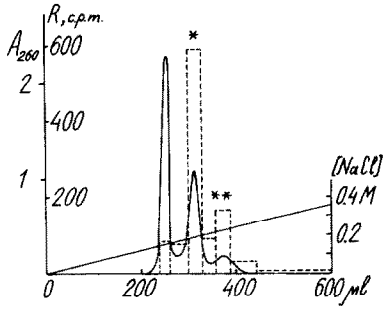

Fig. 1. Separation according to the extent of modification of the products obtained by treatment of oligonucleotide dpC–C–A–C–G with *O*-methylhydroxylamine-bisulfite mixture. Chromatography on a 50 μl DEAE-cellulose column, linear gradient of NaCl 0 to 0.36 M in 0.1 M NaOAc, 7 M urea, pH 5.5; 25°; 75 μl/hr. (– – –) Radioactivity R, cpm; (——) A_{260}.

The radioactivity was measured by means of a Nuclear-Chicago Mark II scintillation counter in Bray's dioxane scintillation fluid.

2.1. *Hydrolysis of DNA with pancreatic DNAase*

10 mg DNA were dissolved with heating in 2 ml 0.02 M Tris-HCl, pH 7.7, 10^{-3} M $MgSO_4$, 0.05 ml of DNAase solution (2 mg/ml) in the same buffer was added, the mixture incubated for 4 hr at 37° and subsequently kept for 20 min at 100° to destroy the enzyme.

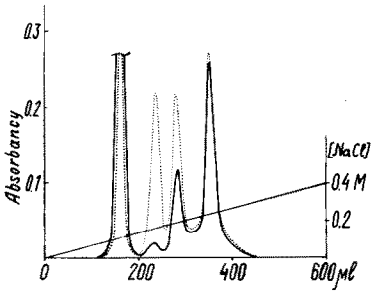

Fig. 2. Chromatography of the products of digestion of mono-modified dpC–C–A–C–G with snake venom phosphodiesterase. 50 μl DEAE-cellulose column, linear gradient of NaCl 0 to 0.4 M in 0.01 M Tris-HCl, 7 M urea, pH 7.0; 25°; 300 μl/hr. (——) A_{260}; (·····) A_{230}.

2.2. *Hydrolysis of DNA to pyrimidine oligonucleotides*

Was performed according to Burton and Petersen [5]. To dephosphorylate the oligonucleotides, 5 μl of the ether-extracted hydrolysate (1.3 A_{260} units) were diluted with 20 μl of Buffer A and 80 μl of water, 10 μl of phosphatase added and the mixture incubated for 2 hr at 37°.

2.3. *Modification of dpC–C–A–C–G with the unlabeled reagents*

10 μl (0.5–0.75 A_{260} units) of the pentanucleotide in aqueous solution was evaporated to dryness in a teflon cup. The residue was dissolved in 1 M sodium metabisulfite, 1 M *O*-methylhydroxylamine, pH 6 [1]. The thoroughly mixed solution was kept for 10

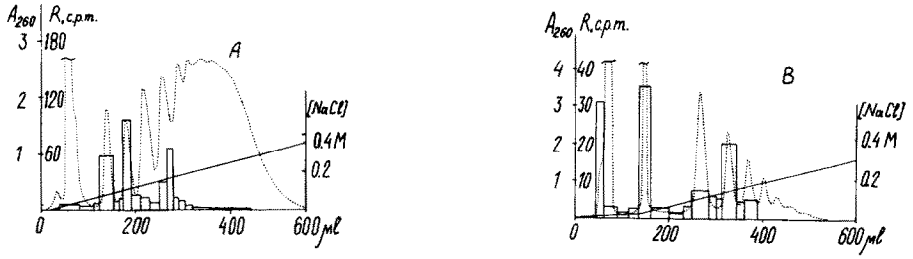

Fig. 3. Determination of the charge of the products of phosphodiesterase digestion of the mono-modified oligonucleotide mixture. A) Co-chromatography of the products of digestion (^{3}H-labeled at the modified cytidine residue) with DNAase hydrolysate of DNA. 50 μl DEAE-cellulose column, linear gradient of NaCl 0 to 0.36 M in 0.01 M Tris-HCl, 7 M urea, pH 7.6; 65°; 150 μl/hr. B) Co-chromatography of the ^{3}H-labeled dephosphorylated products of phosphodiesterase digestion with dephosphorylated Burton–Petersen DNA digest. 50 μl DEAE-cellulose column, two linear NaCl gradients, first 140 μl 0 to 0.02 M, second 460 μl 0.02 to 0.32 M, in 0.01 M Tris-HCl, 7 M urea, pH 7.6; 65°; 150 μl/hr. (——) Radioactivity R, cpm; (·····) A_{260}.

min at 25° and desalted by gel-filtration. The desalted modified oligonucleotide was applied to a DEAE-cellulose micro-column and fractionated under the conditions indicated in the legend to fig. 1 to obtain the mono-modified product (second peak). The mono-modified oligonucleotide was rechromatographed on a DEAE-cellulose micro-column at pH 7 (0 to 0.4 M NaCl in 7 M urea, 0.01 M Tris-HCl, pH 7, 600 μl) and desalted by gel-filtration. The yield of the mono-modified fraction was usually about 30–40%.

2.4. *Modification of dpC–C–A–C–G with bisulfite – O-[^{3}H]methylhydroxylamine*

4 μl of *O*-[^{3}H]methylhydroxylamine stock solution was added to 2 μl of the pentanucleotide aqueous solution (0.5–0.75 A_{260} units) followed by 2 μl of 5 M unlabeled *O*-methylhydroxylamine, pH 6, and finally by 6.2 μl of 2 M sodium metabisulfite pH 6.0. The mixture was kept for 10 min at 25°. The subsequent treatment was the same as that of the reaction mixture with unlabeled *O*-methylhydroxylamine. The total radioactivity of the finally isolated mono-modified fraction was about 6×10^4 cpm. The fractionation pattern of a small aliquot of the desalted reaction mixture is shown in fig. 1; it is seen that the first radioactive peak is the second peak of absorbancy.

2.5. *Phosphodiesterase digestion of the unlabeled mono-modified pentanucleotide*

20 μl of Buffer B and 15 μl of phosphodiesterase solution were added to 45 μl (0.1 A_{260} units) of the mono-modified pentanucleotide. In 1.2 hr, a 15 μl aliquot was withdrawn, diluted with water (100 μl) and applied to a DEAE-cellulose micro-column. The fractionation pattern is shown in fig. 2. It remains practically the same for 3 hr of phosphodiesterase digestion.

2.6. *Determination of the positions of C-residues in the oligonucleotide dpC–C–A–C–G*

2.6.1. Without phosphatase digestion

10 μl of Buffer B and 10 μl of phosphodiesterase were added to 10 μl (7000 cpm) of the labeled mono-modified pentanucleotide solution. The mixture was incubated for 2 hr at 37°, diluted with 200 μl of water and kept for 15 min at 90° to destroy the enzyme. 5 μl (0.5 A_{260} units) of the DNAase digest of chick erythrocyte DNA and 5 μl (0.05 A_{260} units) of dpC were added, and the whole mixture applied to a DEAE-cellulose micro-column. The fractionation pattern is shown in fig. 3A.

2.6.2. With phosphatase digestion

5 μl of Buffer B, 5 μl of phosphatase solution and 5 μl of phosphodiesterase were added to 5 μl of the labeled mono-modified pentanucleotide (3500 cpm). The mixture was incubated for 15 min at 90° to inactivate phosphodiesterase, 1.3 A_{260} units of the dephosphorylated mixture of pyrimidine oligonucleotides added and the solution applied to a DEAE-cellulose micro-column. The fractionation pattern is shown in fig. 3B.

3. Results and discussion

The approach proposed involves three stages:

i) Incomplete modification of the oligonucleotide that: (a) changes the charge of the oligonucleotide residue; (b) stops the action of an exonuclease at the modified residue; (c) is selective to nucleoside residues whose position is to be determined.

ii) Isolation of the mono-modified oligonucleotides fraction; it is simple to separate the mono-modified fraction from the unmodified oligonucleotide as well as from the more extensively modified fractions because the modification changes the charge of the nucleoside residue.

iii) Digestion of the mono-modified oligonucleotide mixture with an exonuclease and determination of the number of monomer units in each of the products of complete digestion. Obviously, the mono-modified oligonucleotides are isomers differing from each other by the position of the modified nucleoside residue (see scheme 1). Hence, complete digestion of each of the mono-modified oligonucleotides will result in an oligonucleotide with modified terminal nucleoside as the only product (besides mono-nucleotides). For this reason, the numbers of monomer units in the digestion products will be the same as the positions of the monomer units of given type, counting from the corresponding terminus.

It is seen in fig. 1 that 30% of the oligonucleotide dpC–C–A–C–G is transformed into a mixture of mono-modified oligonucleotides, each containing a single modified cytidine residue, after 10 min treat-

Scheme 1
The mixture of mono-modified oligonucleotides and the products of their complete digestion with two exonucleases.

X*pZ X*pYpXpZ X*pXpYpXpZ	Spleen phospho-diesterase ←	XpXpYpX*pZ XpX*pYpXpZ X*pXpYpXpZ Mixture of mono-modified oligonucleo-tides	Snake venom phospho-diesterase →	XpXpYpX* XpX* X*

ment with *O*-methylhydroxylamine-bisulfite mixture. Fig. 2 shows the chromatographic pattern of the products of digestion of the mono-modified oligonucleotides mixture with snake venom phosphodiesterase. It is seen, that in accord with scheme 1 only three products are formed (besides mononucleotides). It was found that 3 hr instead of 1 hr treatment with phosphodiesterase does not result in a change in the ratios of the digestion products.

In order to find the number of monomer units (the charge) of the digestion products, they were co-chromatographed with the oligonucleotide mixture obtained by action of DNAase upon DNA. The modified oligonucleotide in these experiments was obtained by action of radioactive *O*-methylhydroxylamine. It was found that the positions of the radioactive peaks corresponding to oligonucleotides containing negatively charged modified cytidine residues did not coincide with those of the peaks of absorbancy corresponding to oligonucleotides of the same negative charge; this was the case when chromatography was performed at 25°. The disadvanatge was overcome by increasing the temperature to 65°. It is seen in fig. 3A that at this temperature the positions of the radioactive peaks coincide with those of dpNpN, dpNpNpN and dpNpNpNpNpN. Thus, the mono-modified oligonucleotides digestion products have 3, 4 and 6 negative charges, respectively, as expected for dpC*, dpCpC* and dpCpCpApC* (see scheme 1). Hence, if the structure of the starting oligonucleotide dpC–C–A–C–G were unknown, we could conclude on the basis of the above evidence that its cytidine residues occupy the positions 1, 2 and 4, counting from the 5′-terminus.

It is well known that removal of the phosphate groups considerably improves the resolution of longer oligonucleotides in ion-exchange chromatography and thus simplifies the exact determination of their net charge. Bearing this in mind, we also investigated an alternative procedure involving the digestion of the mono-modified oligonucleotide mixture with phosphatase. In these experiments, the pyrimidine oligonucleotides mixture obtained according to Burton and Petersen [5] and digested with phosphatase was employed as carrier in co-chromatography. It is seen in fig. 3B that the positions of the radioactive peaks correspond to 1, 2 and 4 negative charges, indicating that the peaks belong to C*, CpC* and CpCpApC*.

Obviously, other modifying reagents which satisfy the requirements outlined above can be employed. It is known that exonucleases stop the digestion at nucleoside residues modified with *N*-cyclohexyl,*N*′-β(4-methylmorpholinium)ethylcarbodiimide [6]. Our preliminary data indicate that this reagent could be applied to determine the positions of guanosine, thymidine and uridine residues. A more detailed account of these results will be published elsewhere.

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