METHOD OF DETERMINING THE POSITION OF GUANOSINE RESIDUES IN OLIGODEOXYRIBONUCLEOTIDES

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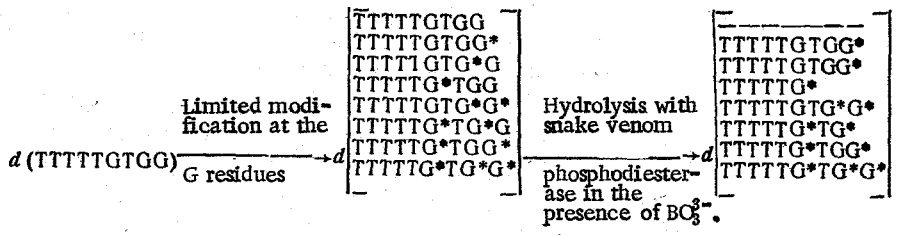
A method is proposed for determining the position of guanosine residues in oligodeoxyribonucleotides. After limited modification of the oligonucleotide with glyoxal, a mixture of molecules of the initial oligonucleotide with different degrees of modification is formed. In the presence of borate ions, the glyoxal-modified guanosine residues form negatively charged borate complexes which inhibit the action of snake venom phosphodiesterase. The treatment of such a complex with the enzyme forms a mixture of fragments of the initial oligonucleotide the 5'-terminal sequences of which are identical while at the 3'-end there are modified guanosine residues. The determination of the length of these fragments provides the necessary information on the positions of the guanosine residues in the chain of the initial oligonucleotides. The positions of the guanosine residues in eight synthetic oligodeoxyribonucleotides have been confirmed with the aid of the method developed.

Various methods are used to analyze the primary structures of oligonucleotides [1-7]. In the case of synthetic oligodeoxyribonucleotides, apparently, it is desirable to use those of which are based on the specific chemical modification of various functional groups of the heterocyclic bases. The use of such methods can give information on the possible changes in the structure of the heterocycles during the synthesis of an oligonucleotide and serve as a check on its purity.

A method proposed by Sverdlov et al. [5] is based on a lag in the action of exonucleases at specifically modified units of the oligonucleotide chains. It has been used to determine the positions of cytidine [5] and thymidine [6] residues. In addition, the basic possibility has been shown of using this modification by glyoxal of guanine residues [8, 9]. In the present work, the method of modifying guanine has been made the basis of determining the position of guanosine residues in oligodeoxyribonucleotides by the following scheme (G* represents a guanosine residue modified by glyoxal):



After limited modification of the oligonucleotide with glyoxal, a mixture of molecules of the initial oligonucleotide with different degrees of modification is formed. In the presence of borate ions, the glyoxal-modified guanosine residues form negatively charged borate complexes which inhibit the action of snake venum phosphodiesterase. The treatment of such a complex with snake venum phosphodiesterase forms a mixture of fragments of the initial oligonucleotide the 5'-terminal sequences of which are identical while at the 3'-ends there are modified guanosine residues. The determination of the lengths of these frag-

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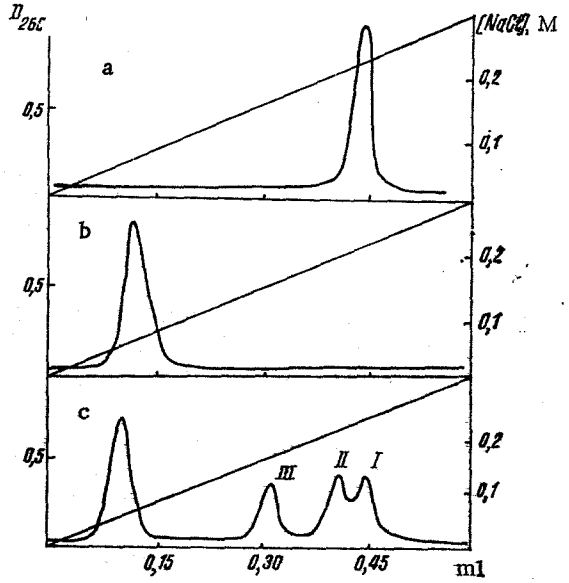

Fig. 1. Chromatography of the initial d(TTTTTGTGG) (a) and of the hydrolysis products of the unmodified d(TTTTTGTGG) with snake venom phosphosphodiesterase (b), and of the products of the modified d(TTTTTGTGG)* with snake venom phosphodiesterase (c).

ments gives the necessary information on the positions of the guanosine residues in the chain of the initial oligonucleotide.

Thus, the performance of the analysis includes three basic items: a) limited chemical modification; b) enzymatic hydrolysis; c) determination of the lengths of the products of enzymatic hydrolysis.

Investigations have shown that when oligonucleotides are modified with a 1% solution of glyoxal in borate or phosphate buffer the optimum conditions for the purpose of analysis are 20°C and pH 8. Under these conditions the necessary degree of modification is achieved in 10-20 min.

The formation of a borate complex with the vicinal grouping of the glyoxal derivatives of guanosine has been shown with the aid of potentiometric titration and by electrophoresis (see the Experimental part). It has been found that the complex formed is stronger than the corresponding complex with the vicinal grouping of the ribose ring and has a pK_a value of about 7.6. When hydrolysis is performed in a medium with pH 9 and a borate concentration of 0.2 M, practically all the modified residues are present in the form of negatively charged borate complexes. It has been shown that these conditions ensure the complete suppression of the action of the enzyme at the modified residues in an oligodeoxyribonucleotide.

It is known [10] that areneboronic acids (we used benzeneboronic and o-aminomethylbenzeneboronic acids) form with vicinal groupings complexes that are stronger than those formed by borates. The complete suppression of the action of snake venom phosphodiesterase is achieved in this case at pH 8 and 0.02-0.05 M areneboronate.

The presence of glyoxal in a concentration of 1% does not appreciably affect the occurrence of enzymatic hydrolysis. Since the necessary degree of modification is achieved fairly rapidly, and the conditions of performing the modification and enzymatic hydrolysis are similar, the enzyme can be added to the sample almost immediately after the addition of the glyoxal. The concentration of enzyme is selected in such a way that complete hydrolysis of the unmodified oligonucleotide takes place in 30 min. All this permits a considerable simplification of the analysis and an increase in its sensitivity.

The determination of the lengths of the products of enzymatic hydrolysis is performed with the aid of microcolumn chromatography on DEAE-cellulose by a standard method [11]. In the chromatographic process, the borate complexes are decomposed, which facilitates the interpretation of the results.

As an example, Fig. 1 shows the results of the chromatographic separation of a phosphodiesterase hydrolysate of the nonanucleotide d(TTTTTGTGG) modified with glyoxal. In accordance with the Scheme (see above), in addition to the mononucleotides, oligonucleotide fragments are detected — d(TTTTTGTGG) (I), d(TTTTTGTG) (II), and d(TTTTTG) (III). These facts show that the sixth, eighth, and ninth residues in the oligonucleotide analyzed are quanosine residues.

Using the method developed, the positions of the guanosine residues in eight synthetic oligodeoxyribonucleotides have been determined: d(TCGTGG), d(TCGTGGT), d(pTAGT), d(pTCATG), d(T_5GTGG), d(TATAG), d(TG_6), d(TGCACATG).

The performance of the analysis requires 0.03-0.05 o.u. of oligonucleotide material; the whole analysis takes about three hours. The proposed method is simple and does not require the preliminary introduction of a radioactive label into the oligodeoxyribonucleotide under investigation.

EXPERIMENTAL

The analysis was performed on oligodeoxyribonucleotides synthesized in our laboratory. The benzeneboronic and aminomethylbenzeneboronic acids were kindly given to us by I. I. Kolodkina and A. M. Yurkevich [All-Union Scientific-Research Institute of Vitamins].

Ion-exchange chromatography was performed on columns of DEAE-cellulose (Serva, GFR) using an MKSFP-4 instrument [11] in a linear concentration gradient of NaCl in tris-HCl buffer, pH 7.5, or 0.05 M acetate buffer, pH 5.5, in 7 M urea. The rate of elution was 360 µl/h.

Modification of the Guanine Residues with Glyoxal. The optimum conditions for performing the modification reaction were selected on dG, dpG, and d(pG-G). For modification we used a 1% solution of glyoxal in 0.03 M phosphate or 0.05 M borate buffer (pH 7.5, 8.05, 8.35, 8.5). The completeness of the reaction was determined by using the difference in the spectrophotometric properties of the initial and the modified compounds.

Measurement of the Stability Constant and pK Value of the Complex of Boric Acid, the Glyoxal Adduct, and dpG. To 0.001 mole of dpG was added 15 ml of 5% glyoxal in 0.03 M phosphate buffer (pH 7.1). The mixture was kept at room temperature for a day. The resulting solution of the adduct of deoxygluanosine 5'-phosphate with glyoxal, dpG*, was made up to 100 ml (0.01 M solution). A 0.01 M solution of unmodified dpG in phosphate buffer of approximately the same concentration and pH was used as control.

A series of 0.05 M borate buffers with approximate pH values of 7.0, 8.0. 9.0, 10, 11, 12, and 12.4 was prepared. In the determinations of the depression of the pH, 0.05 ml of borate buffer was taken and its pH was measured accurately, and then 2.5 ml of a 0.01 M solution of dpG* (equimolar amounts) was added to it and the pH was measured again. The error of the experiment was taken into account by performing similar measurements for the depression of the pH in the case of the ummodified dpG. The stability constants pKa of the complex were calculated as described previously [9] from the values of the pH depression (ΔpH) obtained.

Method of Performing the Analysis for Synthetic Oligonucleotides. 1. To 0.03-0.05 o.u. λ_{260} of oligonucleotide was added 20 µl of a 1% solution of glyoxal in borate buffer and the mixture was kept at room temperature, after which 18 µl of 0.5 M borate buffer (pH 9), 5 µl of a 0.01 M solution of $MgCl_2$, and 0.75 µg of bovine serum albumin and snake venom phosphodiesterase in an amount sufficient for the complete cleavage of 0.05 o.u. λ_{260} were added (total volume 50 µl). The mixture was incubated for 30 min. Then 200-300 µl of double-distilled water was added and the mixture was heated at 100°C for 10 min to activate the enzyme.

The solution so obtained was chromatographed on DEAE-cellulose.

2. To 0.1 o.u. λ_{260} of oligonucleotide was added 20 µl of the solution of glyoxal in phosphate buffer and the mixture was kept at room temperature for 5-10 min, after which 25 µl of a 0.1 M solution of a aminomethylbenzene boronic acid or benzeneboronic acid (pH 8.5), 5 µl of 0.01 M MgCl solution, and 0.75 µg of bovine serum albumin and the enzyme in the amount necessary for the complete hydrolysis of 0.1 o.u. λ_{260} of the unmodified oligonucleotide were added. (Total volume 65-70 µl.) The mixture was incubated at 37°C for 30 min and was then heated at 100°C for 10 min to activate the enzyme. Before chromatography, the solution was diluted to a volume of 250-300 µl. After the deposition of the mixture on the column, the column was washed with 150-200 µl of 7 M urea in 0.01 M tris-HCl (pH 7.5) to eliminate the arylboronic acid.

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