Volume 269, number 1, 101–104 FEBS 08735 August 1990

Porphyrin-linked oligonucleotides

Synthesis and sequence-specific modification of ssDNA

E.I. Frolova, E.M. Ivanova, V.F. Zarytova, T.V. Abramova and V.V. Vlassov

Institute of Bioorganic Chemistry, Siberian Division of the USSR Academy of Sciences, Prospekt Lavrentieva 8, Novosibirsk 630090, USSR

Received 15 June 1990

Oligonucleotide derivatives bearing hemin and deuterohemin groups were synthesized. The derivatives efficiently react with the complementary nucleotide sequence in ssDNA forming covalent adducts and piperidine-labile sites. In the case of the deuterohemin derivative, some direct cleavage of the target DNA occurs.

Antisense oligonucleotide; DNA affinity modification

1. INTRODUCTION

Coupling of the nucleic acid-damaging reactive groups to antisense oligonucleotides should improve their ability to inhibit functions of the target nucleic acids [1]. Oligonucleotides bearing the nucleic acid-cleaving groups can be used as 'artificial restriction enzymes' for mapping DNA [2]. Among the reactive groups tested, the most promising are metal complexes such as phenanthroline-Cu(II) [3], EDTA-Fe(III) [2,4–7] and porphyrin-Fe(III) [1,8–10] which produce active oxygen species causing degradation of nucleic acids. It is believed that under certain conditions, groups of this type may function very efficiently, in a catalyst-like manner, since in theory they can be repeatedly activated in the presence of a reducing agent and oxygen, and each group can cause damage to several target nucleic acid molecules.

In this report we describe the synthesis of new hemin- and deuterohemin-linked oligodeoxyribonucleotides and results of the investigation of their reaction with single-stranded DNA, possessing a complementary nucleotide sequence. The derivatives were found to react efficiently and in a sequence-specific manner with the DNA. Both reagents covalently bind to the DNA and introduce piperidine-sensitive lesions. Reaction with the deuterohemin derivative results in a specific, although weakly efficient, cleavage of the DNA.

Correspondence address: E.I. Frolova, Institute of Bioorganic Chemistry, Siberian Division of the USSR Academy of Sciences, Prospekt Lavrentieva 8, Novosibirsk 630090, USSR

Abbreviations: $(pN)_{16}$, oligonucleotide d(pTGACCCTCTTCCCATT); 5'Hem$(pN)_{16}$ and 3'Hem$(pN)_{16}$, oligonucleotide $(pN)_{16}$ bearing hemin residue at the 5'- and at the 3'-end, respectively; 5'DHem$(pN)_{16}$ and 3'DHem$(pN)_{16}$, similar derivatives bearing the deuterohemin group

2. MATERIALS AND METHODS

Target DNA, a 3'-terminally labeled single-stranded DNA fragment 303 nucleotides long, was prepared as described earlier [11]. Hexadecadeoxyribonucleotide d(pTGACCCTCTTCCCATT), $(pN)_{16}$, complementary to nucleotide sequence 259–274 of this fragment was synthesized according to the published method [12]. Attachment of an amino-linker to the oligonucleotide terminal phosphate was accomplished by coupling of aminoethanol (protected at the amino group with the trifluoroacetic residue) under conditions of the phosphotriester oligonucleotide synthesis. Hemin was from Sigma, deuterohemin was synthesized by Dr. S.A. Grachev (Institute of Molecular Biology, Academy of Sciences of the USSR) according to the published procedure [13]. To prepare the internal anhydride of the hemins, the compounds (150 mg) were boiled in 4 ml of acetic anhydride for 30 min. The acetic anhydride was removed by vacuum distillation at room temperature. The synthesized products were homogeneous according to the TLC analysis (methanol : chloroform). Their structure was proved by the infrared spectra which possess bands of 1790 and 1810 cm^{-1} characteristic of the anhydrides. To couple porphyrins to the oligonucleotide, the oligonucleotide bearing the aminoethanol spacer either at the 3' or 5' terminus (1 equiv. cetyltrimethylammonium salt) was dissolved in absolute DMSO and added to the anhydride of hemin or deuterohemin (20 equiv.) and 1-hydroxybenzotriazole (20 equiv.). The mixture was incubated at 37°C for 24 h. The derivatized oligonucleotide was isolated by HPLC on a Lichrosorb RP-18 column using gradient elution with acetonitrile (0–80%) in 0.05 M aqueous $LiClO_4$. A typical yield was 40–60%. The derivatives have UV-absorbance spectra with maxima characteristic of oligonucleotides (260 nm) and of hemins (400 nm).

The reaction of the oligonucleotide derivatives and the DNA fragment was performed in 10 μl of 50 mM Tris-HCl, pH 7.6, containing 0.1 M NaCl and $1 \cdot 10^{-5}$ M poly(A) (per nucleotide residue). Concentration of the 3'-[^{32}P] labeled DNA fragment was $1 \cdot 10^{-8}$ M. Concentrations of the oligonucleotide derivatives varied in the range of $1 \cdot 10^{-9}$–$1 \cdot 10^{-5}$ M. The reaction was started either by adding H_2O_2 to a final concentration of $1 \cdot 10^{-3}$ M (followed by incubation for 1 h at 25°C) or by adding a reducing agent (β-mercaptoethanol, dithiothreitol, glutathion, NADH) to a final concentration of $1 \cdot 10^{-3}$ M (followed by incubation for 16 h at 25°C). After the incubation, the reaction was stopped by precipitation of the DNA with 2 vols of ethanol. To cleave the DNA at positions of the modified residues, the DNA was dissolved in 1 M piperidine, incubated for 30 min at 90°C and precipitated by 2% $LiClO_4$ solution in acetone. The products

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

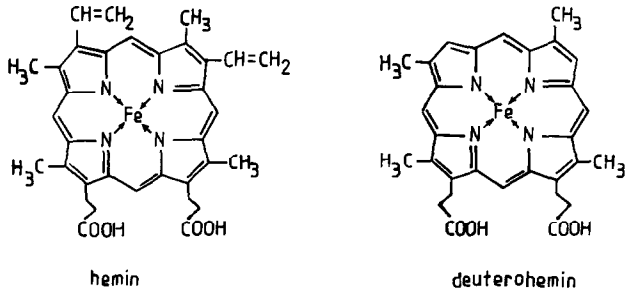

Fig. 1. Hemin and deuterohemin which were coupled to the oligonucleotide $(pN)_{16}$.

were analyzed by electrophoresis in a denaturing 10% polyacrylamide–8 M urea gel and quantitized by scanning the autoradiographs using an Ultrascan laser densitometer (LKB, Sweden).

3. RESULTS AND DISCUSSION

Coupling of hemin groups (Fig. 1) to oligonucleotides was accomplished by acylation of the aminospacers of the oligonucleotides by internal anhydrides of hemins in the presence of 1-hydroxybenzotriazole. The products were isolated by HPLC (Fig. 2). The oligonucleotide derivatives were used for modification of a DNA fragment which possesses a complementary nucleotide sequence (Fig. 3). Treatment of the DNA with 5′Hem(pN_{16}) in the presence of hydrogen peroxide results in the formation of a slowly migrating product (Fig. 4, lane 5), apparently the DNA with the covalently coupled oligonucleotide. Piperidine treatment of the modified DNA results in a specific cleavage of the DNA at positions of guanosines G275 and G276 of the fragment and complete disappearance of the crosslinking products (Fig. 4, lane 6). In addition to this reaction, some modification within the oligoguanylic region of the DNA (G17–G34) occurs, apparently due to imperfect complex formation of the oligonucleotide in accordance with the previous observations with the same DNA and an alkylating derivative of the same oligonucleotide [11]. The total yield of the cleavage reaction

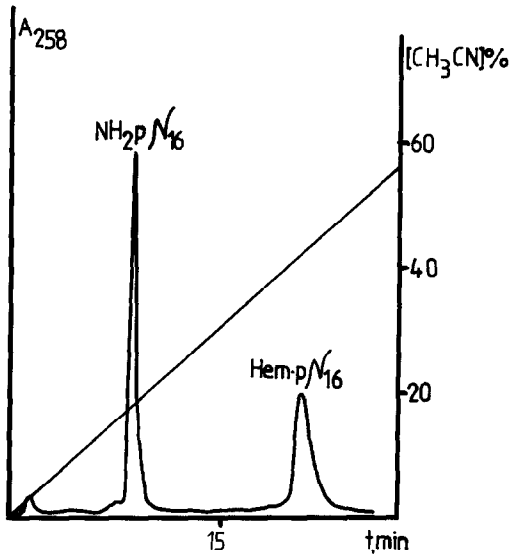

Fig. 2. Reverse phase chromatography of the products of the 5′Hem$(pN)_{16}$ synthesis.

(at position G275, G276 and within the sequence G17–G34) was 52% under the conditions used. The reaction of the DNA with 3′Hem(pN_{16}) yields the crosslinking products which are cleaved by the piperidine treatment at the position of G258 and guanosines within the sequence G17–G34. In this case, similarly to the earlier finding with the alkylating derivative of the oligonucleotide and the same DNA [11], some reaction occurs also with G179 which was ascribed to the effect of the tertiary folding of the DNA.

It is known that deuterohemin is more stable than hemin and it is a more efficient catalyst of the oxidative-reductive processes [14]. Therefore we attempted to synthesize 3′- and 5′-deuterohemin derivatives of the same oligonucleotides. However the HPLC separation of the reaction mixtures (Fig. 5) yielded two products in both cases (3′DHem$^1(pN_{16})$ and 3′DHem$^2(pN_{16})$; 5′DHem$^1(pN_{16})$ and 5′DHem$^2(pN_{16})$). In both cases, the two products have similar UV absorption spectra

```
                                                                    50
GATCC GTCGA CCTGC AGGGG GGGGG GGGGG GGGGT TGCTC AGGGT GAGGC
                  .... ..... ..... .....                          100
GGAAA AGAGT CGACC CAACC TTCCG CCGGC CGTCA CTGGC ACAGG CTGGA
                                                                   150
CAGCA AAAGG GCAGA TCACA GTGCT GGACA TGCAC CCAGG CTCTG GGAAG
                                    ↓                              200
ACCCA CAGAG TCCTC CCGGA GCTCA TTCGC CAATG CATTG ACAGA CGCCT
                                                                   250
AAGGA CATTG GTGTT GGCCC CAACC CGTGT GGTGC TTAAG GAAAT GGAGC
                                                                   300
GTGCC TTGAA TGGGA AGAGG GTCAG GTTCC ATTCT CCTGC AGGCT CGACG GAT*
```

Fig. 3. The target 3′-end labeled single-stranded DNA fragment. The sequence complementary to the oligonucleotide derivative is underlined. Dotted line shows the oligoguanylic sequence; the arrow indicates guanosine G179 (see text).

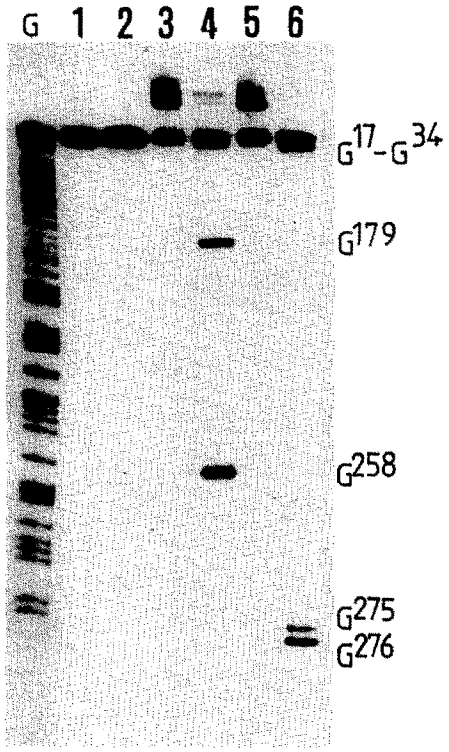

Fig. 4. ssDNA fragment reacted with 3'Hem(pN)$_{16}$ and 5'Hem(pN)$_{16}$, autoradiograph of the 10% polyacrylamide 8 M urea gel. The DNA was incubated for 1 h at 25°C, in 50 mM Tris-HCl, pH 7.6, containing 0.1 M NaCl, $1 \cdot 10^{-5}$ M poly(A) and 10^{-3} M H_2O_2. Lanes 1,2, no reagents were added; 3,4, the mixture contained $5 \cdot 10^{-6}$ M 3'Hem(pN)$_{16}$; 5,6, the mixture contained $5 \cdot 10^{-6}$ M 5'Hem(pN)$_{16}$. The reacted DNA in lanes 2,4,6 was subjected to the piperidine treatment.

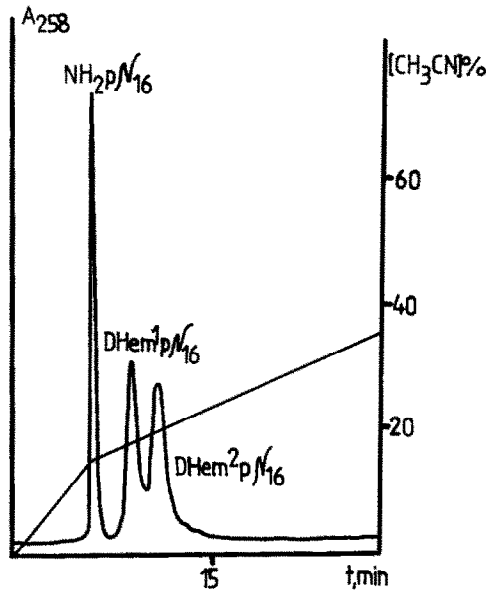

Fig. 5. Reverse phase chromatography of the products of the 5'DHem(pN)$_{16}$ synthesis.

and similar electrostatic charges, as could be judged from the data of the ion exchange chromatographic analysis. In the presence of H_2O_2, both the derivatives react with the target nucleotide sequence in the DNA fragment although sites of their attack are different (Fig. 6). Products 3'Hem^2(pN$_{16}$) and 5'Hem^2(pN$_{16}$) bring about modification of guanosines in the target sequence G258 and G275, G276, respectively (Fig. 6, lanes A5, A6, B3, B4). Similarly to the results obtained with the hemin derivatives, covalent coupling of the reagents to the DNA occurs and piperidine treatment cleaves the DNA at the positions of the modified guanosines. However, in the case of the deuterohemin derivatives, the reaction is more efficient (~60% under the conditions used) and some direct cleavage of the DNA by the reagents is observed (data not shown) although in a relatively low yield (~5%). A comparison of the reaction yields in the presence of 10 mM reducing agents: 2-mercaptoethanol, dithiothreitol, glutathione, and NADH, has revealed that the efficacy of the reducing agents decreases in the order they are mentioned.

Treatment of the DNA with products 3'Hem^1(pN$_{16}$) and 5'Hem^1(pN$_{16}$) resulted in a different modification pattern. No covalent coupling of the reagent to the DNA was observed and the cleavage of the target occurred at guanosines in a diffuse area covering the entire sequence complementary to the oligonucleotide carrying the reactive group (Fig. 6, lanes A3, A4, B1, B2). Although weakly efficient, the cleavage of the DNA was observed immediately after incubation with the reagent (Fig. 6, lanes A3, B1) and it was increased by the following piperidine treatment (Fig. 6, lanes A4, B2). The total yield of the products was up to 20%. Similarity of the DNA modification patterns observed in reactions with 3'Hem^1(pN$_{16}$) and 5'Hem^1(pN$_{16}$) suggested that the reactive groups in both the derivatives were located somewhere on the internal nucleotide units of the oligonucleotide. To check this possibility, the parent oligonucleotide without aminoethanol linker (pN$_{16}$) was treated with deuterohemin anhydride under standard conditions. The reaction yielded a product (yield 30%) which reacted with the DNA similarly to 3'Hem^1(pN$_{16}$) and 5'Hem^1(pN$_{16}$).

The results obtained demonstrate that the oligonucleotides bearing hemin groups bring about efficient chemical modification of the target DNA. The reaction yields piperidine labile crosslinking products and some direct DNA cleavage. We believe that by choosing the appropriate porphyrin groups one can design oligonucleotide derivatives which will find applications as efficient antimessengers and tools for DNA mapping.

REFERENCES

[1] Knorre, D.G., Vlassov, V.V., Zarytova, V.F. and Lebedev, A.V. (1988) Sov. Sci. Rev. B. Chem. 13.
[2] Strobel, S.A., Moser, H.E. and Dervan, P.B. (1988) J. Am. Chem. Soc. 110, 7927–7929.
[3] Chen, C.H.B. and Sigman, D.S. (1986) Proc. Natl. Acad. Sci. USA 83, 7147–7151.
[4] Boutorin, A.S., Vlassov, V.V., Kazakov, S.A., Kutyavin, I.V. and Podyminogin, M.A. (1984) FEBS Lett. 172, 43–46.

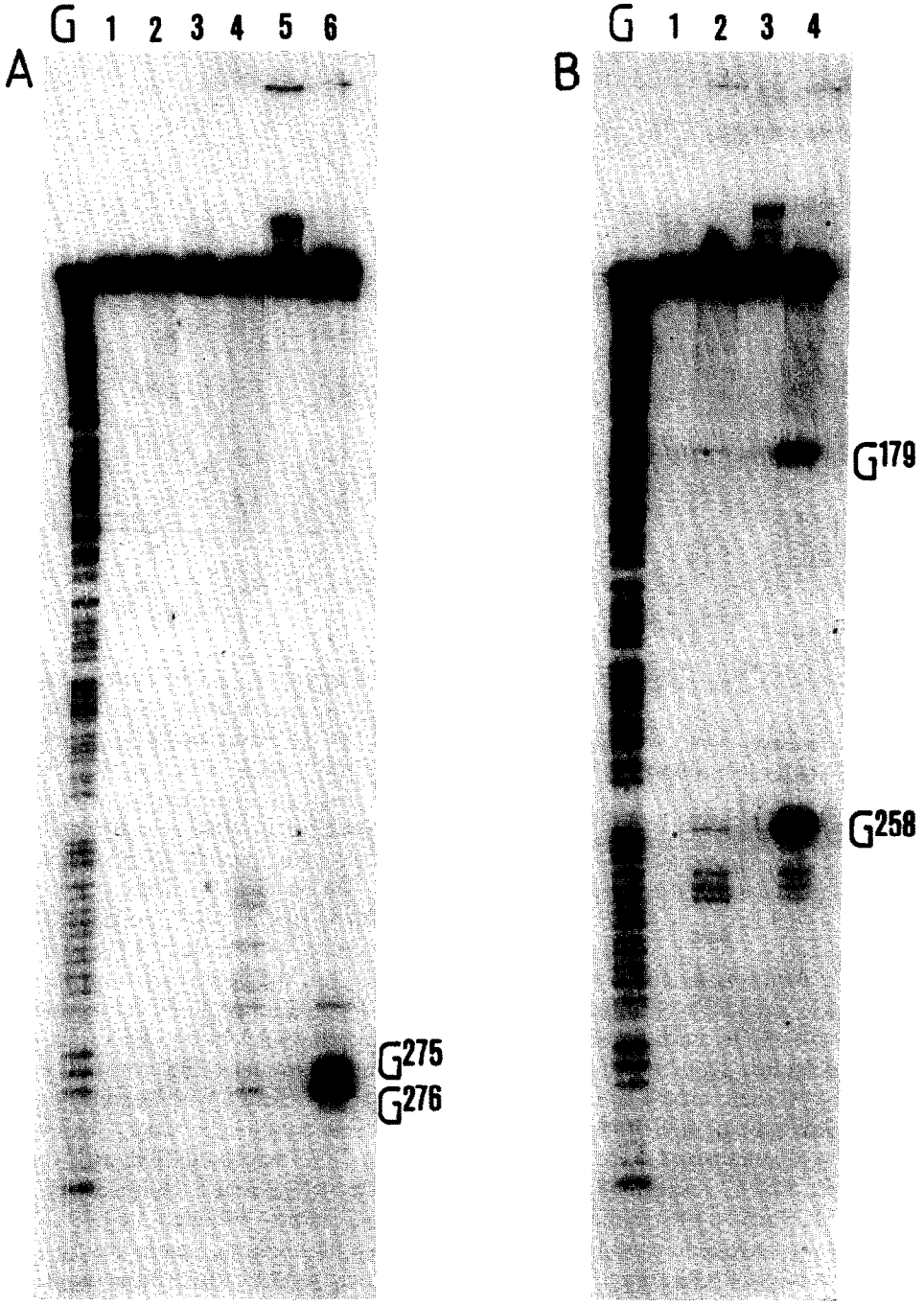

Fig. 6. ssDNA fragment reacted with 3′DHem(pN)$_{16}$ and 5′DHem(pN)$_{16}$, autoradiograph of the 10% polyacrylamide 8 M urea gel. For the reaction conditions see legend for Fig. 2. (A) Lanes 1,2, DNA incubated in the absence of the reagents; 4, reaction with $5 \cdot 10^{-6}$ M 5′DHem^1(pN)$_{16}$; 5, 6, reaction with $5 \cdot 10^{-6}$ M 5′DHem^2(pN)$_{16}$; lane G, G-specific partial DNA cleavage. (B) Lanes 1,2, reaction with $5 \cdot 10^{-6}$ M 3′DHem^1(pN)$_{16}$; 3,4, reaction with $5 \cdot 10^{-6}$ M 3′DHem^2(pN)$_{16}$. The reacted DNA in lanes 2,4,6 was subjected to the piperidine treatment.

[5] Chu, B.C.F. and Orgel, L.E. (1985) Proc. Natl. Acad. Sci. USA 82, 963–967.
[6] Dreyer, G.B. and Dervan, P.B. (1985) Proc. Natl. Acad. Sci. USA 82, 968–972.
[7] Brosalina, E.B., Vlassov, V.V. and Kazakov, S.A. (1988) Bioorg. Khim. (USSR) 14, 125–128.
[8] Le Doan, T., Perrouault, L., Helene, C., Chassignol, M. and Thuong, N.T. (1986) Biochemistry 25, 6736–6739.
[9] Le Doan, T., Perrouault, L., Chassignol, M., Thuong, N.T. and Helene, C. (1987) Nucl. Acids Res. 15, 8643–8659.
[10] Ivanova, E.M., Mamaev, S.V., Fedorova, O.S. and Frolova, E.I. (1988) Bioorg. Khim. (USSR) 14, 551–554.
[11] Vlassov, V.V., Zarytova, V.F., Kutyavin, I.V., Mamaev, S.V. and Podyminogin, M.A. (1986) Nucl. Acids Res. 14, 4065–4076.
[12] Zarytova, V.F., Ivanova, E.M. and Romanenko, V.P. (1983) Bioorg. Khim. (USSR) 9, 516–521.
[13] Caughey, W.S., Alben, J.A., Fujimoto, W.Y. and York, J.L. (1966) J. Org. Chem. 31, 2631–2640.
[14] Portsmouth, D. and Beal, E.A. (1971) Eur. J. Biochem. 19, 479–487.