Design and synthesis of double-stranded oligonucleotides containing reactive acylphosphate internucleotide groups

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Abstract DNA duplex and dumbbells containing chemically active acylphosphate internucleotide groups were synthesized. To obtain these compounds the chemical ligation method was used. The acylphosphate group was inserted into a DNA duplex and dumbbells as a result of template-directed condensation of 5′-phosphate and especially introduced 3′-carboxy groups of oligonucleotides. 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC) was used as a condensing agent. Oligonucleotides containing a carboxy group were obtained by the interaction of their 3′-phosphate with glycine methyl ester under the action of EDC, followed by ester hydrolysis. The yields of acylphosphate-containing double-stranded oligonucleotides achieved 15–25% depending on the structure of their precursors. It was shown that these compounds are acylating agents and are efficiently cleaved in near-physiological conditions under the action of ethylenediamine or *N*-methylimidazole. These results indicate that double-stranded oligonucleotides carrying acylphosphate internucleotide groups could constitute new crosslinking reagents for affinity modification of DNA recognizing proteins.

Key words: Reactive oligonucleotide synthesis; DNA duplex; DNA dumbbell; Template-induced chemical ligation; Sugar phosphate backbone; Acylphosphate internucleotide group

1. Introduction

Reactive double-stranded (ds) DNAs capable of crosslinking to DNA binding proteins are extensively used to study nucleic acid-protein interactions. Photochemical crosslinking with participation of thio-, halogeno-, or azidonucleotide-containing DNAs as well as crosslinking using Pt(II) complexes have been successfully applied to explore the topography of protein active centers and mechanisms of molecular recognition [1–3].

Recently, we suggested a novel method for inserting active groups in ds DNAs to permit crosslinking with proteins [4,5]. Our studies revealed that DNAs carrying substituted pyrophosphate internucleotide (SPI) groups react in near-physiological conditions with different nucleophiles including nucleophilic amino acids. Modified DNA duplexes containing these reactive groups in the recognition site of DNA binding proteins interact with amino acids close to the DNA interface. This approach has been successfully employed for the affinity modification of some enzymes participating in DNA recognition, such as *Eco*RI and *Rsr*I restriction and modification enzymes [6], *Eco*RII and *Sso*II restriction endonucleases [7,8] and for affinity modification of HNF1 transcription factor [9] and NF-κB p50 subunit [10]. In order to continue our investigations into the design and synthesis of reactive ds DNAs, we propose new reagents – ds oligonucleotides containing acylphosphate groups.

Acylphosphates are known to be intermediates in many biochemical processes catalyzed by enzymes. They are formed, for example, during peptide bond biosynthesis as a result of the initial activation of the carboxyl function by ATP. ATP assists in this process by donating its γ-phosphoryl group [11,12]. Thus, acylphosphates are reactive acylating agents involved in the formation of a stable covalent bond as a result of the interaction with a nucleophilic amino group. We proposed that this property could be kept in nucleic acids. Here, we describe synthesis and properties of DNA duplex and dumbbells containing acylphosphate internucleotide groups.

2. Materials and methods

2.1. Oligonucleotides

Oligodeoxyribonucleotides were synthesized by a standard amidophosphite method in an Applied Biosystems 380 B DNA synthesizer [13]. Oligonucleotides carrying a 3′-terminal phosphate group were obtained by the phosphoramidite method using the 5′-phosphorylation reagent as previously described [6]. 5′-End labeling of oligonucleotides was carried out with T4 polynucleotide kinase and [γ-^{32}P]ATP following standard procedures [14]. The concentrations of oligonucleotides were determined spectrophotometrically.

2.2. Synthesis of oligonucleotides containing 3′-carboxy groups

Oligonucleotides with 3′-glycine residue were synthesized by the interaction of their 3′-phosphate with glycine methyl ester under the action of 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC), followed by ester hydrolysis as described in [15].

2.3. Synthesis of DNA duplex and dumbbells bearing acylphosphate groups

Equimolar mixtures of oligodeoxyribonucleotides consisting of nicked DNA duplex I or nicked dumbbells II and III (oligonucleotide concentration per monomer was 10^{-3} M) were incubated in 0.05 M MES buffer, pH 6.0, 0.02 M $MgCl_2$ at 95°C for 2 min and slowly cooled for several hours. Then EDC was added to a concentration of 0.2 M. The reaction was carried out in the dark at 10 or 20°C (for DNA duplex and dumbbells, respectively) for 12 h. Ligation products were isolated by electrophoresis on a 20% or 12% denaturing polyacrylamide gel for linear duplex and dumbbells, respectively, followed by elution with 2 M $LiClO_4$ and precipitation with 5 volumes of acetone.

2.4. Treatment with N-methylimidazole (MeIm) and ethylenediamine (EDA)

DNA duplex I′ and dumbbells II′, III′ (0.1–0.5 nmol) were treated with a 0.4 M aqueous solution of MeIm, pH 8.0 or a 0.5 M aqueous solution of EDA, pH 8.0 for 16 h at 37, 50 or 90°C, respectively. The oligonucleotide material was precipitated with 2% $LiClO_4$ in acetone,

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Abbreviations: EDC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide; MeIm, *N*-methylimidazole; EDA, ethylenediamine; SPI groups, substituted pyrophosphate internucleotide groups; ds, double-stranded

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reprecipitated twice and analyzed by 12% PAGE for dumbbells or by 20% PAGE for linear duplex.

3. Results and discussion

3.1. Design of initial ds oligonucleotides

Nicked DNA duplex I and nicked dumbbells II and III were chosen as the initial systems for the introduction of acylphosphate groups:

ACCTA**CCx pTGG**TGGT
TGGAT**GG**—**ACC**ACCA (I)

AACACCTA**CCx pTGG**TGGTGTAA
ATGTGGAT**GG**—**ACC**ACCACAA (II)

AGCGT**GGx pTTAATGATCTACAG**TGCGA (III)
GCGCA**CC**—**AATTACTAGATGTC**ACGC

x = –O–P(O⁻)–N(H)–CH₂–C(=O)–OH p = –O–P(O⁻)–O–

The compounds containing an acylphosphate linkage (x-p) are named by the addition of a prime to the names of the respective initial systems, i.e. I′, II′ and III′.

Nicked DNA duplex I and nicked DNA dumbbell II contain the recognition sites of *Eco*RII, *Sso*II and *Mva*I restriction/modification enzymes ($CC^T/_AGG$). Nicked DNA dumbbell III contains the 15 bp pseudopalindromic binding site of HNF1 transcription factor (marked in bold). Earlier, we successfully applied these sequences containing substituted pyrophosphate internucleotide (SPI) groups between nucleotides adjacent to the nick for affinity modification of the above proteins [7–9]. The glycine residue connected to the 3′-phosphate of oligonucleotide was chosen as a carboxy-containing group. This was because of its accessibility of glycine and by the well-developed and rather simple method of its introduction into oligonucleotides [15]. DNA dumbbells were taken because they provide some advantages over linear DNAs due to the absence of strand separation and end-fraying effects [16]. Moreover, dumbbells have been found to possess an increased stability towards nucleolytic degradation [17]. This is important for their use, especially in vivo.

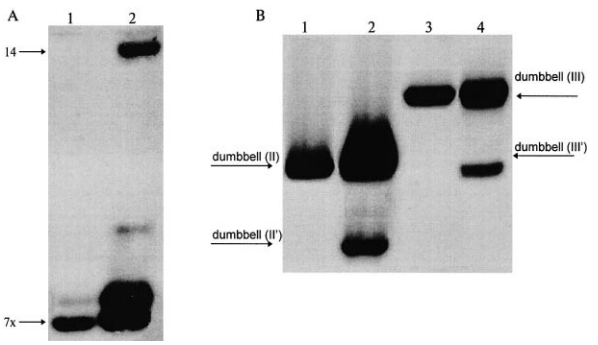
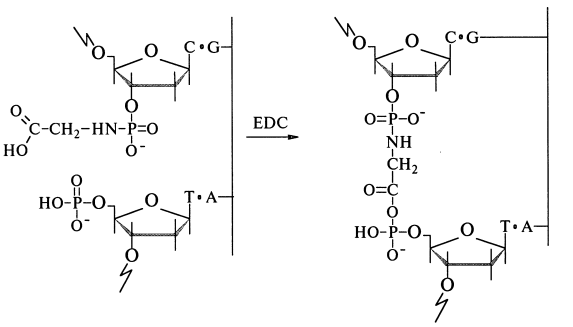


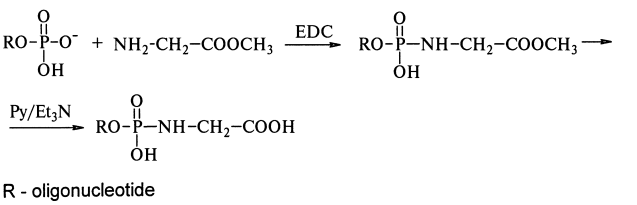
Fig. 1. Electrophoretic analysis of the reaction mixtures after synthesis of DNA duplex I′ (A) and DNA dumbbells II′ and III′ (B). A: lane 1: ^{32}P-ACCTACCx; lane 2: reaction mixtures after synthesis of I′. B: lanes 1, 3: ^{32}P-labeled nicked dumbells II and III, respectively; lanes 2, 4: reaction mixtures after synthesis of II′ and III′, respectively. For structures see Section 3.1, for conditions see Section 2.

3.2. Introduction of acylphosphate groups into the sugar phosphate backbone of ds oligonucleotides

In order to introduce the acylphosphate group into DNA duplex I we performed template-induced chemical ligation of a heptanucleotide carrying a 5′-phosphate group to another heptanucleotide bearing a 3′-carboxy group. EDC was used as a condensing agent. The reaction was performed according to the following scheme:



The heptanucleotide containing the carboxy group was obtained by the interaction of its 3′-phosphate with an excess of glycine methyl ester under the action of EDC, followed by ester hydrolysis:



This reaction was carried out with a quantitative yield. It is in agreement with data obtained earlier [15].

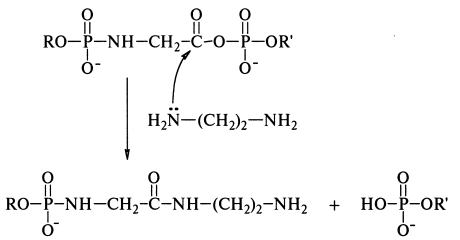
Fig. 1A shows electrophoretic analysis of the reaction mixture after synthesis of DNA duplex I′. Ligation efficiency giving rise to the modified tetradecanucleotide was 25%.

DNA dumbbells II′ and III′ with acylphosphate groups were synthesized in the same way during condensation in nicked dumbbells II and III. In order to introduce the carboxyl group, initial 3′-phosphorylated nicked dumbbells were modified with an excess of glycine methyl ester, followed by ester hydrolysis as described above. Then nicked DNA dumbbells containing carboxy groups were 5′-phosphorylated using T4 polynucleotide kinase. Fig. 1B shows electrophoretic analysis of the reaction mixtures after synthesis of DNA dumbbells II′ and III′ bearing acylphosphate groups. Ligation efficiency was 15% for DNA dumbbell II′ and 20% for dumbbell III′. It is worth noting that ligation efficiencies observed for analogous DNA duplexes and dumbbells containing SPI groups were the same [18]. DNA dumbbells that are formed during ligation have greater electrophoretic mobility than that observed for the nicked ones. The increased electrophoretic mobility of dumbbells can be explained by their more compact structure in comparison to their precursors. This result also correlates with data obtained for DNA dumbbells containing SPI groups [18].

3.3. Chemical properties of oligonucleotides bearing acylphosphate internucleotide groups

Modified DNA duplex I′ and dumbbells II′ and III′ proved to be stable in aqueous media for at least 72 h. They were also

incubated under conditions optimal for digestion with R.*Mva*I, R.*Eco*RII and RNase H. It was found that modified DNA duplex and dumbbells were stable under *Mva*I or *Eco*R-II digestion conditions (10–40 mM Tris-HCl, pH 8.5, 5–10 mM $MgCl_2$, 37°C) and under RNase H treatment conditions (0.2 M Tris-HCl buffer, pH 7.9, 0.1 M $MgCl_2$, 25°C) for at least 12 h. Also the modified DNAs could be cleaved in the aqueous solution MeIm or primary amines (see Figs. 2 and 3). Fig. 3 shows electrophoretic analysis of the reaction mixtures after treatment of DNA duplex I′ with 0.5 M EDA (pH 8.0). The ^{32}P label was introduced into the 5′-end of the carboxy group-containing heptanucleotide (A) or into the 5′-end of the proximate heptanucleotide (B). As shown in Fig. 3A, the aminolysis of modified tetradecanucleotide ^{32}P-ACCTACCx-pTGGTGGT results in a product with lower electrophoretic mobility than the initial carboxy group-containing heptanucleotide. This product proved to be an aminoethylamide of this oligonucleotide, i.e. ^{32}P-ACCTACCx-NH$(CH_2)NH_2$. This was proved by the other way of its synthesis using the reaction between ^{32}P-ACCTACCx and $NH_2(CH_2)NH_2$ under the action of EDC [15], followed by a comparison of the HPLC retention times of the compounds obtained. At the same time, after EDA treatment of ACCTACCx-^{32}P-TGGTGGT we observed only initial ^{32}P-TGGTGGT (Fig. 3B). Therefore, the aminolysis of acylphosphate group-containing oligonucleotides follows the scheme below:



R, R' - oligonucleotides

Thus, acylphosphate-modified DNAs are effective acylating agents in aqueous media in contrast to SPI-modified DNAs which are phosphorylating agents [4,18]. Our results indicate that the compounds proposed could be used as crosslinking reagents for affinity modification of DNA binding proteins. They required no additional activators such as light or heat. Also, the linkage formed between the nucleophile and a part of the DNA is stable in acidic medium which is usually used for isolation and analysis of nucleic acid-protein complexes (for example, Edman protein degradation). Therefore, DNAs with acylphosphate groups could be advantageous as compared with SPI-containing ones in studies on the probing of protein binding sites with determination of amino acids involved in DNA-protein interaction. The reactive DNA dumbbells have some advantages in comparison with similar linear DNA duplexes because of their greater DNA binding affinities and sequence selectivities.

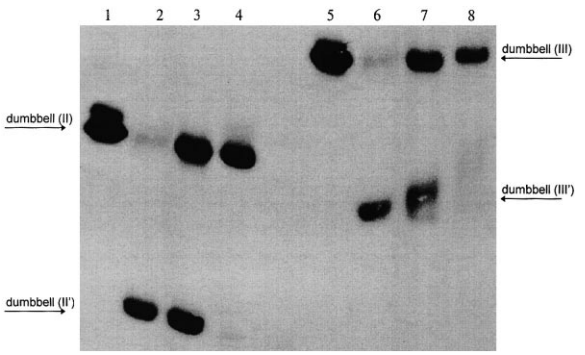


Fig. 2. Electrophoretic analysis of the reaction mixtures after treatment of DNA dumbbells II′ and III′ with MeIm. Lanes 1, 5: ^{32}P-phosphorylated nicked DNA dumbbells II and III respectively; lanes 2, 6: DNA dumbbells II′ and III′ containing internal ^{32}P label into acylphosphate group; lanes 3, 4: reaction mixtures after treatment of DNA dumbbell II′ with MeIm at 50 and 90°C, respectively; lanes 7, 8: reaction mixtures after treatment of dumbbell III′ with MeIm at 50 and 90°C, respectively. For conditions see Section 2.

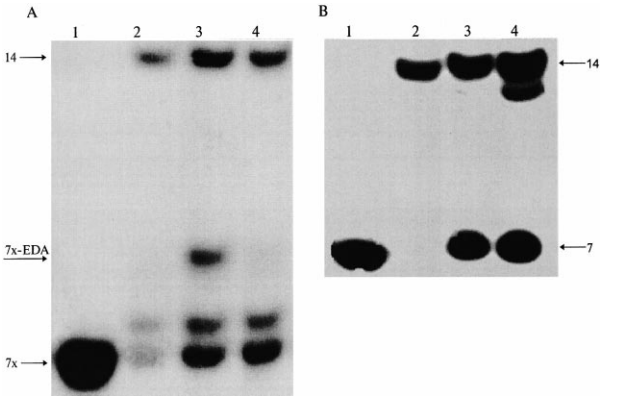


Fig. 3. Electrophoretic analysis of the reaction mixtures after treatment of DNA duplex I′ with EDA and MeIm. The ^{32}P label was introduced at the 5′-end of the carboxy group-containing heptanucleotide (A) or into the 5′-end of the proximate heptanucleotide (B). Lanes 1, 5: ^{32}P-ACCTACCx and ^{32}P-TGGTGGT. Lanes 2–4: ^{32}P-ACCTACCx-pTGGTGGT and its treatment with EDA and MeIm, respectively. Lanes 6–8: ACCTACCx-^{32}PTGGTGGT and its treatment with EDA and MeIm, respectively. For conditions see Section 2.

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References

[1] Chu, B.C. and Orgel, L.E. (1992) Nucleic Acids Res. 20, 2497–2502.
[2] Malkov, V.A. and Camerini-Otero, R.D. (1995) J. Biol. Chem. 270, 30230–30233.
[3] Mayer, A.N. and Barany, F. (1995) Gene 153, 1–8.
[4] Kuznetsova, S.A., Ivanovskaya, M.G. and Shabarova, Z.A. (1990) Bioorgan. Khim. (Russ.) 16, 219–225.
[5] Kuznetsova, S.A., Ivanovskaya, M.G. and Shabarova, Z.A. (1991) Bioorgan. Khim. (Russ.) 17, 1633–1639.
[6] Purmal, A.A., Shabarova, Z.A. and Gumport, R.I. (1992) Nucleic Acids Res. 20, 3713–3719.
[7] Sheflyan, G.Y., Kubareva, E.A., Kuznetsova, S.A., Karyagina, A.S., Nikolskaya, I.I., Gromova, E.S. and Shabarova, Z.A. (1996) FEBS Lett. 390, 307–310.
[8] Sheflyan, G.Y., Kubareva, E.A., Volkov, E.M., Oretskaya, T.S., Gromova, E.S. and Shabarova, Z.A. (1995) Gene 157, 187–190.
[9] Kuznetsova, S.A., Catherine, C., Edgardo, U., Elias, I., Vasseur, M., Blumenfeld, M. and Shabarova, Z.A. (1996) Nucleic Acids Res. 24, 4783–4790.
[10] Kozlov, I.A., Kubareva, E.A., Ivanovskaya, M.G. and Shabarova, Z.A. (1997) Antisense Nucleic Acid Drug Dev. 7, 279–289.

[11] Falk, P.J., Ervin, K.M., Volk, K.S. and Ho, H.-T. (1996) Biochemistry 35, 1417–1422.
[12] Millins, L.S., Zawadzke, L.E., Walsh, S.T. and Raushel, F.M. (1990) J. Biol. Chem. 265, 8993–8998.
[13] Matteucci, M.D. and Caruthers, M.H. (1981) J. Am. Chem. Soc. 103, 3185–3191.
[14] Ausubel, F.M., Bartent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York.
[15] Ivanovskaya, M.G., Gottikh, M.B. and Shabarova, Z.A. (1987) Nucleosides Nucleotides 6, 913–934.
[16] Doktycz, M.J., Goldstein, R.F., Paner, T.M., Gallo, F.J. and Benight, A.S. (1992) Biopolymers 32, 849–864.
[17] Chu, B.C. and Orgel, L.E. (1992) Nucleic Acids Res. 20, 2497–2502.
[18] Kuznetsova, S.A., Blumenfeld, M., Vasseur, M. and Shabarova, Z.A. (1996) Nucleosides Nucleotides 15, 1237–1251.