Hairpin-shaped DNA duplexes with disulfide bonds in sugar-phosphate backbone as potential DNA reagents for crosslinking with proteins

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Abstract Convenient approaches were described to incorporate -OP(=O)O$^-$-SS-O$^-$(O=)PO- bridges in hairpin-shaped DNA duplexes instead of regular phosphodiester linkages: (i) H_2O_2- or 2,2′-dipyridyldisulfide-mediated coupling of 3′- and 5′-thiophosphorylated oligonucleotides on complementary template and (ii) more selective template-guided autoligation of a preactivated oligonucleotide derivative with an oligomer carrying a terminal thiophosphoryl group. Dithiothreitol was found to cleave completely modified internucleotide linkage releasing starting oligonucleotides. The presence of complementary template as an intrinsic element of the molecule protects the hairpin DNA analog from spontaneous exchange of disulfide-linked oligomer fragments and makes it a good candidate for auto-crosslinking with cysteine-containing proteins.

Key words: Hairpin DNA duplex; Oligonucleotide 3′- or 5′-thiophosphate; Disulfide bond formation; Disulfide bond exchange

1. Introduction

Modified synthetic DNA and RNA molecules are known to be real tools in fundamental molecular biology research; they are also broadly applied in biotechnology (antisense and sense strategy). Recently a new class of preactive double-stranded DNA and RNA derivatives which are able to crosslink with proteins recognizing specific nucleic acid sequences was proposed in our laboratory [1,2]. Trisubstituted pyrophosphate [1–6] or acylphosphate [7] linkages incorporated at predetermined positions in double-stranded nucleic acids permit crosslinking with nucleophilic amino acids (Lys and His) right at the protein active site or binding region (at zero distance) without any influence from the outside. Sets of DNA or RNA duplexes with chemically active internucleotide linkages were successfully used for affinity modification and probing the nucleic acid binding regions of restriction-modification enzymes [2,8], RNA recognizing TAT peptide [4] and transcription factors NF-κB [3,5,9] and HNF1 [6].

In our study the synthetic method was developed to produce a new type of reactive DNA duplexes containing selectively integrated -OP(=O)O$^-$-SS-O$^-$(O=)PO- linkages instead of native phosphodiester ones. We believe these modified dsDNAs would be able to react with cysteine residues by thiol-disulfide interchange reaction within the specific dsDNA-protein complex. Earlier Chu and Orgel [10] developed a general method of combining any 2-pyridyldisulfide derivative of an oligonucleotide with a cysteine-containing peptide or protein. Directed disulfide bond formation between the active site cysteine and thiol-containing analog of guanine within the complex of methylguanine methyltransferase and modified oligonucleotide was described recently [11]. We propose to incorporate disulfide bridges into the sugar-phosphate backbone of regular and hairpin-shaped DNA duplexes (Fig. 1) by oxidation or by thiophosphoryl-disulfide interchange of two oligonucleotides, tandemly stacked on a complementary template, containing 3′- and 5′-thiophosphoryl groups (or their derivatives).

Hairpin-shaped DNA duplexes were chosen as the models for coupling procedures and decoys targeting essential nucleic acid binding proteins because of their increased thermal stability and nuclease resistance compared to regular double-stranded oligonucleotides formed by hybridizing two separate strands [12–14]. Evidence that coupling of 3′- and 5′-thiophosphoryl derivatives (under the action of $K_3Fe(CN)_6$ or KJ_3) can result in an oligonucleotide with disulfide internucleotide linker has been reported [15]. In our approach we suggest using the much more convenient and easily removable H_2O_2 as the oxidant. We also used preactivated thiophosphoryl derivatives of oligonucleotides, allowing template-guided autoligation of oligomers to proceed. Spontaneous and template-controlled exchange of oligonucleotide fragments joined by disulfide bond is also discussed.

2. Materials and methods

Oligonucleotides with 3′- or 5′-thiophosphoryl groups were prepared as described [16] and were kept in boiled water to avoid their oxidation to disulfide-linked dimers by solved oxygen. Small amounts of disulfide-containing oligomers generated by atmospheric oxygen can be reduced with 0.05 M DTT (1 h, room temperature) to form oligonucleotide phosphorothioates. After this procedure oligonucleotide phosphorothioates were isolated by ethanol precipitation and washed with ethanol to eliminate the DTT traces.

2.1. Template-directed coupling of oligonucleotide 3′- and 5′-phosphorothioates by H_2O_2 and 2,2′-dipyridyldisulfide

Duplex-forming oligonucleotide phosphorothioates were mixed in 0.05 M MES adjusted with triethylamine to pH 7.0, 0.02 M $MgCl_2$ (buffer 1) to give about 1 mM concentration (per monomer). Shorter oligomer was added in 1.2-fold excess (unless otherwise noted) relative to the dangling-ended hairpin oligomer (systems **II**–**IV** on Fig. 1). In three-component duplex **I** the ratio of oligomer (1) to oligomer (2) and to template (3) was 1:1.3:1.5. Oligomer mixtures were heated to

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Abbreviations: CDI, 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide; MES, 2-morpholinoethane sulfonate; DTT, dithiothreitol; Sp(i) or (i)pS, 5′- or 3′-thiophosphoryl oligonucleotide number *i*; (i)pSSp(j) (heterodimer), oligonucleotides number *i* and *j* linked by a (3′-5′)-OP(=O)O$^-$-SS-O$^-$(O=)PO- bridge; (i)pSSp(i) (homodimer), bis(oligonucleotide number *i*) (3′-3′)- or (5′-5′)-diphosphoryldisulfide

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75°C and slowly annealed to 0°C. Then cold 0.3% H_2O_2 stock solution was added to a final concentration of 0.03% and the reaction was left to proceed for 1 h at 0°C.

When 2,2′-dipyridyldisulfide was used as a coupling reagent, its saturated aqueous solution was added in a 1:3 ratio (v/v) to the annealed mixture of thiophosphorylated oligonucleotides on the complementary template (1 mM per nucleotide residue) in buffer 1; the reaction mixture was incubated overnight at 0°C. The resulting oligonucleotides were isolated by ethanol precipitation and analyzed by 20% denaturing polyacrylamide gel electrophoresis with registration of bands by UV shadow or by ion-exchange HPLC on the SCOUT WP PEI column (5 μm, 4.6×50 mm, J.T. Barker) using a potassium phosphate (pH 7.0) gradient (0.005–0.5 M) in 25% acetonitrile and a flow rate of 0.5 ml/min. Reaction product yields were determined from HPLC data. After extraction from gel slices with 10 mM Tris-HCl, pH 7.6, 0.3 M NaCl, 1 mM EDTA overnight at room temperature, condensation products were isolated by ethanol precipitation.

2.2. *Template-directed autoligation of two oligonucleotides to disulfide-containing dimer by thiophosphoryl-disulfide interchange*

Oligonucleotide 3′- (or 5′-) phosphorothioates (1–5 A_{260}) were converted with 95% yield to 2-pyridyl- or 3-carboxy-4-nitrophenyldisulfide derivatives by treatment with 0.03 M 2,2′-dipyridyldisulfide or Ellman's reagent [17] in 2.5 mM Tris-HCl, pH 7.5, containing 50% DMF for 3 h at room temperature and were isolated from the reaction mixture by ethanol precipitation. Then each was mixed with other duplex-forming component(s) in buffer 1. The mixture was heated to 75–80°C, slowly annealed to 0°C and incubated overnight at 0°C.

Dimers with disulfide bonds were converted back to the original oligonucleotide phosphorothioates by 0.05 M DTT treatment (1 h, room temperature). Cleavage products were desalted (ethanol precipitation) and quantitatively analyzed by ion-pair HPLC (Waters) in an acetonitrile gradient (5–40%) in 48 mM potassium phosphate buffer, pH 7.0, containing 2 mM tetrabutyl ammonium dihydrophosphate as in [18]. Elution rate 1 ml/min, 45°C.

2.3. *CDI-induced chemical ligation*

CDI-induced chemical ligation of 3′- and 5′-phosphorylated oligonucleotides in duplexes **I**–**IV** was performed in 0.05 M MES adjusted with triethylamine to pH 6.0, 0.02 M $MgCl_2$. The final concentration of oligomers was 1 mM (per monomer residue), that of CDI was 0.2 M. The reaction products were precipitated by adding 2% $LiClO_4$ in acetone and analyzed by ion-exchange HPLC.

3. Results and discussion

One of the limitations in the use of oligonucleotide derivatives as potential drugs is their nuclease susceptibility. However, oligonucleotides with hairpin structures at the 3′ end have shown increased nuclease resistance compare to single-stranded oligonucleotides and regular oligomeric duplexes [13,14]. In addition, nicked hairpin DNA duplexes seem to be very promising constructs for the template-directed chemical coupling of oligonucleotides because of their increased thermostability. In the present investigation hairpin-forming sequences were used to study the introduction of reactive disulfide bonds in the sugar-phosphate backbone of DNA duplexes. One of the ligating fragments folds upon itself to form an intramolecular hairpin with an AAA base loop and single-stranded dangling end (see Fig. 1), the stability of this structure being concentration independent. It was shown that the trinucleotide loop AAA helps to form an extraordinarily stable DNA mini-hairpin because of the good stacking of the loop adenosine base on the closing A·A sheared pair [19]. The intramolecular DNA hairpin serves simultaneously as a template and the reactive oligomer. A shorter DNA fragment tandemly stacks to the complementary hairpin due to formation of H-bonds with dangling ends. The junction point is the site to introduce any sugar-phosphate backbone modifications. A series of nicked hairpin-shaped duplexes **II**–**IV** differing in sequence and loop position were synthesized together with the regular DNA duplex **I** (Fig. 1), the base pair sequence of duplex **I** being identical to that in the stem regions of hairpin-shaped duplexes **II** and **III**. Duplex **IV** contains the recognition site for DNA human transcription factor NF-κB whose Cys-59 was shown by X-ray analysis [20] to interact with the internucleotide phosphate group of the DNA substrate. Duplex-forming oligomers contain 3′- or 5′-thiophosphoryl groups which come together in a double-stranded complex. Preliminary evaluation of the vicinity of interactive groups was performed on analogous systems where the phos-

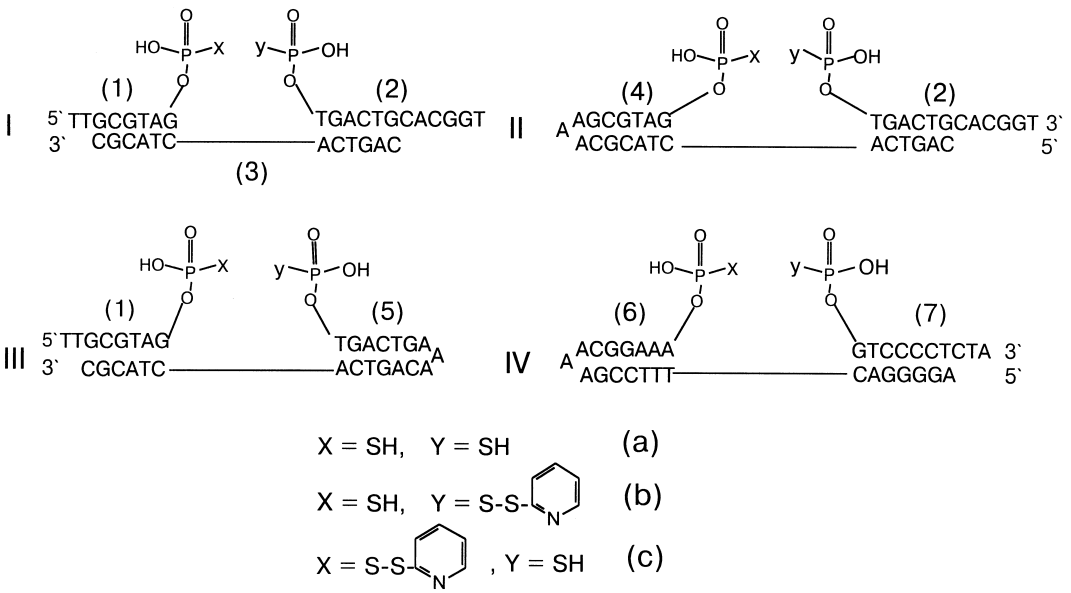

Fig. 1. Sequences of nicked DNA duplexes. The number of oligonucleotides is given in parentheses. The couples of reactive groups facing the nick (a, b and c) are indicated under the duplex structures.

phorothioate moieties were substituted for phosphate groups. These systems serve as an assay to confirm duplex structure and stability under chemical ligation conditions. 100% yield of coupling product with pyrophosphate linkage was achieved under the action of CDI on the nicked duplexes **II**–**IV** containing internal hairpins, where X and Y=OH (see, for example, Fig. 2). A slightly lower coupling efficiency (85%) was observed for three-component duplex **I**, where X and Y=OH. These results confirm the stable duplex formation for all systems under investigation.

Duplex **I** consisting of three oligomers was used to elucidate the role of template (3) for producing oligonucleotide ligation via disulfide bond formation. Mild oxidation of 3′-thiophosphoryl oligonucleotide (1) and 5′-thiophosphoryl oligonucleotide (2) with H_2O_2 provides a fast (1 h), one-step and convenient way to link oligonucleotides by disulfide bridges and gives yields ranging from 70 to 80% for the dimeric derivative (1)pSSp(2) (Fig. 3, lane 1); the reaction was terminated by adding ethanol. In the absence of a template, an essentially less effective coupling reaction takes place and homodimers (1)pSSp(1) and (2)pSSp(2) are obtained as major byproducts together with the desired heterodimer (1)pSSp(2) (Fig. 3, lane 2). The accumulation of homodimers in the reaction mixture occurs during the oxidation reaction also in the case of an excess of one of the thiophosphorylated oligonucleotide components (see below). In order to increase the selectivity of template-controlled oligonucleotide coupling, phosphorothioate-disulfide interchange was used instead of direct oxidation. Earlier it was shown that *N*-[2-(2-pyridyldisulfanyl)-ethyl]phosphoramidate or phenyldisulfide derivatives of oligonucleotide readily reacted with the free thiol group of peptide cysteines [10] or with a thiol function which was

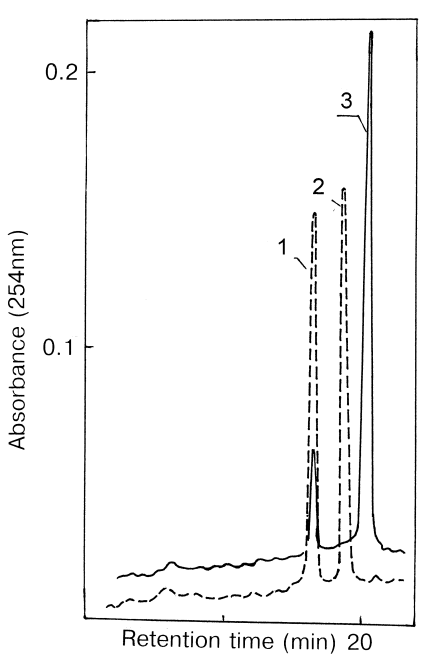

Fig. 2. Ion-exchange HPLC analysis of CDI-induced ligation in hairpin DNA duplex **II**, where X and Y=OH. Initial mixture (dashed line) of duplex forming 5′-phosphorylated oligonucleotide (2) (peak 1) and 3′-phosphorylated oligonucleotide (4) (peak 2) in ratio 1.2:1. Post-ligation mixture (solid line) containing pyrophosphate-linked reaction product (peak 3). For reaction conditions see Section 2.

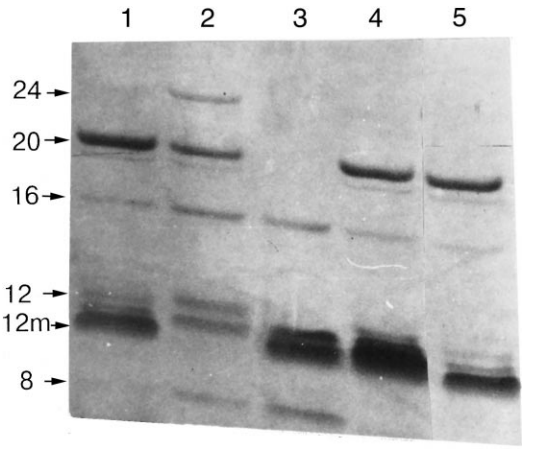

Fig. 3. Denaturing PAGE analysis of disulfide bond formation between oligonucleotides (1) and (2). H_2O_2-mediated coupling reaction in the presence (lane 1) and absence (lane 2) of template oligomer (3) (reactive groups are indicated in Fig. 1a). Template-controlled autoligation of 5′-(2-pyridyl)disulfide adduct of oligomer (2) with (1)pS (lane 4), and 3′-(2-pyridyl)disulfide adduct of oligomer (1) with Sp(2) (lane 5). For comparison, the coupling reaction between the 3′-(2-pyridyl)disulfide adduct of oligomer (1) and 5′-phosphorylated oligomer (2) is shown in lane 3. The reaction conditions are given in Section 2. The chain length of oligonucleotides is indicated. 12m corresponds to 12-mer template which is not changed during the reaction.

placed on an aliphatic linker attached to the sugar fragment of other oligonucleotide [21] to give the desired disulfide. We expanded this approach to perform a phosphorothioate-disulfide interchange reaction between the thiophosphoryl group of one oligonucleotide and the 2-pyridyldisulfide adduct of the neighboring (due to complementary interaction with template) oligonucleotide. These adducts were obtained by treatment of oligonucleotide-3′- or 5′-phosphorothioates with 2,2′-dipyridyldisulfide. They are stable under chromatographic and electrophoretic conditions and can be preserved without decomposition in aqueous solutions at −10°C for at least 2 weeks. Template-controlled coupling reactions were performed between (1)pS and 5′-(2-pyridyl)disulfide adduct of oligomer (2) and vice versa, between 3′-(2-pyridyl)disulfide adduct of (1) and Sp(2) (see Fig. 1b,c, respectively). Autoligation of these oligomers was initiated by duplex formation and proceeded overnight at 0°C to give 80% yield of (1)pSSp(2) (Fig. 3, lanes 4 and 5). As one can see from Fig. 3, the electrophoretic mobility of heterodimeric coupling products was identical for all reaction mixtures and ligation efficiency did not depend on the position (3′ or 5′) of the 2-pyridyldisulfide group. No dimeric product appeared if 5′-phosphorylated oligomer was used as a reaction component instead of a 5′-phosphorothioate derivative (Fig. 3, lane 3).

These approaches were used and developed to introduce diphosphoryldisulfide bridges in hairpin-shaped duplexes **II**–**IV** (Fig. 1). H_2O_2-mediated oxidative coupling of tandemly stacked short oligonucleotide 3′- or 5′-monophosphorothioates and longer oligomers carrying 5′- or 3′-thiophosphoryl groups (Fig. 1a) occurs easily to give the desired heterodimers (2)pSSp(4), (1)pSSp(5) and (6)pSSp(7). If an excess of one duplex-forming oligomer occurs in the reaction mixture, the SS-linked homodimer of this component is formed together with the hairpin-shaped product, controlled by complementary base pairing. For example, heterodimer (6)pSSp(7) pre-

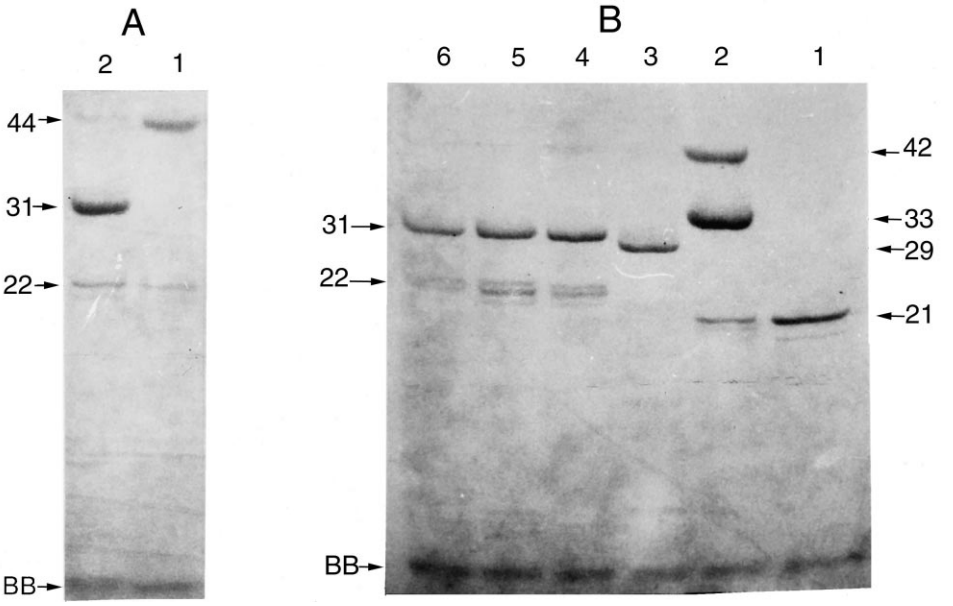

Fig. 4. Denaturing PAGE analysis of oligonucleotide coupling into hairpin-shaped DNA duplexes via diphosphoryldisulfide bridge formation. A: H_2O_2-mediated ligation of (6)pS and Sp(7) (lane 2); control (6)pS and its homodimer (6)pSSp(6) (lane 1). B: H_2O_2-mediated ligation of (4)pS and Sp(2) (lane 2); control (4)pS (lane 1). Autoligation of the 3′-(2-pyridyl)disulfide adduct of oligomer (1) with Sp(5) (lane 3). Autoligation of Sp(7) with the 3′-(2-pyridyl)disulfide adduct of oligomer (6) (lane 4) or with the 3′-(3-carboxy-4-nitrophenyl)disulfide adduct of oligomer (6) (lane 5). 2,2′-Dipyridyldisulfide-mediated ligation of (6)pS and Sp(7) (lane 6). The reaction conditions are given in Section 2. The chain length of oligonucleotides is indicated.

vails in the reaction mixture containing an about equimolar ratio of components (6) and (7) (Fig. 4A, lane 2), the product yield being over 90%. (6)pS and its disulfide-bonded dimer (6)pSSp(6) (lane 1) were used as oligomer size markers. In contrast, when components (2) and (4) were mixed in a molar ratio of 1:1.2 (duplex **II**), homodimer (4)pSSp(4) is accumulated together with the desired heterodimer product (Fig. 4B, lane 2). The use of 2,2′-dipyridyldisulfide instead of H_2O_2 showed that this reagent was capable of highly efficient one-step coupling of (6)pS and Sp(7) providing a 85–90% yield of heterodimer (6)pSSp(7) (Fig. 4B, lane 6). Obviously, in this case the coupling reaction proceeds via pyridyldisulfide adduct formation which reacts with the thiophosphate group of the neighboring oligomer in situ.

2-Pyridyldisulfide adducts of oligonucleotides (1) or (7) readily participate in template-controlled thiophosphoryl-disulfide interchange with Sp(5) or (6)pS respectively (Fig. 1b,c) to give disulfide-linked heterodimers (1)pSSp(5) and (6)pSSp(7) with yields approaching 80–90% in 12 h (Fig. 4B, lanes 3 and 4). Replacing the 2-pyridyldisulfide groups with 3-carboxy-4-nitrophenyldisulfide (this adduct was prepared by the treatment of Sp(7) with Ellman's reagent) does

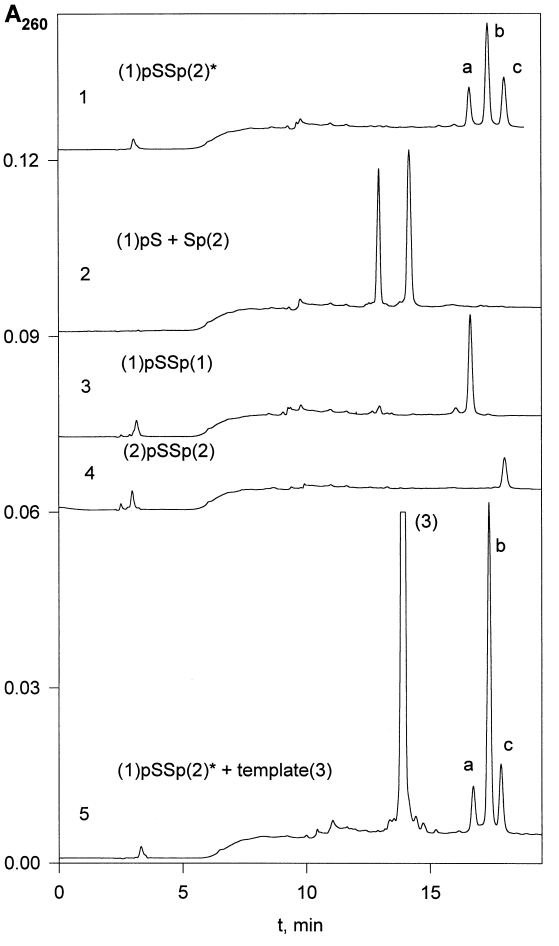

Fig. 5. Ion-pair HPLC of disulfide-linked oligonucleotides (1) and (2). Electrophoretically homogeneous coupling product obtained in duplex **I** (Fig. 1) before (profile 1) and after treatment with DTT (profile 2). The same product hybridized with complementary template (3) and kept overnight under conditions of duplex formation (profile 5). Homodimers (1)pSSp(1) (profile 3) and (2)pSSp(2) (profile 4). For chromatographic conditions see Section 2. Abbreviations of oligonucleotide derivatives are given above the profiles; (1)pSSp(2)* represents the products of disulfide bond exchange in (1)pSSp(2).

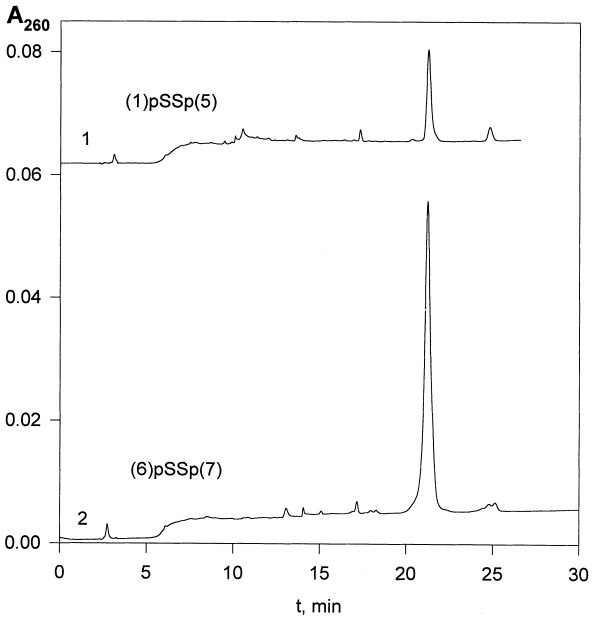

Fig. 6. Ion-pair HPLC of disulfide-linked coupling products obtained in duplex **III** (profile 1) and duplex **IV** (profile 2). Abbreviations of oligonucleotide heterodimers are given above the profiles. For chromatographic conditions see Section 2.

not significantly change the efficiency of (6)pSSp(7) formation (Fig. 4B, lane 5).

When the electrophoretically homogeneous reaction product (1)pSSp(2) (obtained in regular duplex I) was eluted from the gel and subjected to ion-pair HPLC analysis, it showed, unexpectedly, three peaks (Fig. 5, profile 1) on the chromatogram. That each component of this mixture contains disulfide-bonded oligomer fragments is demonstrated by the fact that the triplet completely transforms to two peaks corresponding to starting thiophosphorylated oligomers (1) and (2) after treatment with DTT (Fig. 5, profile 2). Peaks **a** and **c** were identified as homodimers (1)pSSp(1) and (2)pSSp(2), respectively, by comparing them with the chromatographic profiles of control homodimers (compare profiles 1, 3 and 4 in Fig. 5). Major peak **b** may be attributed to heterodimer (1)pSSp(2). Probably, a mixture of disulfide-linked homo- and heterodimers is formed as a result of spontaneous disulfide bond exchange upon denaturation of the double-stranded structure. Partial cleavage of the disulfide bond in the heterodimer results in release of the oligomers with terminal thiophosphoryl functions which react with the next heterodimer molecule displacing one oligonucleotide block for another due to formation of a new disulfide bond. The extent of the conversion is close to the statistical limit of 50% expected for a random distribution reaction. When the reaction product displaying as a triplet was hybridized with complementary template and kept overnight under conditions of duplex stability, the fraction of homodimers decreased significantly from 47 to 25% (compare profiles 1 and 5 in Fig. 5). According to these data, reversed disulfide bond exchange is controlled only by base pairing interactions of oligonucleotides with a template. This is an interesting example illustrating how the supramolecular interactions in nucleic acids catalyze and direct the covalent reactions. In lane, the only coupling product corresponding to heterodimer (1)pSSp(2) was detected by ion-pair HPLC in the full-component reaction mixture containing template (3) (data not shown).

If this assumption is correct, hairpin-shaped DNAs containing disulfide crosslinks into the stem region should be protected from spontaneous recombination of oligonucleotide blocks due to the presence of a complementary template as an intrinsic element of the molecule. Indeed, in contrast to singe-stranded unstructured oligonucleotides, hairpin-shaped DNAs with single incorporation of diphosphoryldisulfide linkages (2)pSSp(4), (1)pSSp(5) and (6)pSSp(7) demonstrate individual peaks on the HPLC chromatogram with only trace impurities of homodimers (see, for example, Fig. 6). Apparently, the extent of postreactional disulfide bond exchange in these oligonucleotide derivatives depends on the duplex stability: it is less if the duplex is more stable.

In general, chemically active hairpin-shaped DNA duplexes containing disulfide linkages at a predetermined position of the sugar-phosphate backbone can be easily prepared by one-step chemical coupling of 3′- and 5′-thiophosphoryl oligonucleotides or their derivatives. The modified moiety does not appear to alter the native conformation of the double helix and readily exchanges with free thiol groups of other molecules. Due to its specific secondary structure, selectively modified hairpin DNAs have a high thermostability and resistance to nuclease degradation and prevent recombination of disulfide-bonded oligomer fragments. We believe that these properties will make hairpin-shaped DNAs with disulfide bridges quite useful reagents for crosslinking with cysteine residues within the specific dsDNA-protein complex.

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