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High-performance liquid chromatography of the photoproducts of nucleic acid components

III☆. Detection of the secondary structure differences in sequence isomeric self-complementary oligonucleotides

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to detect differences in the secondary structures of two self-complementary oligodeoxyribonucleotides. The $[d(CCTTTAAAGG)]_2$ duplex assumes an ordinary B-conformation in aqueous solution, while $[d(GGAAATTTCC)]_2$ is known to contain in its central part a stretch of a more rigid B′-form conformation with significantly lowered fluctuational mobility of base pairs. The latter factor causes a marked difference in the amounts of thymine cyclobutane photodimers formed under UV irradiation of corresponding duplexes as revealed by chromatography of two single-stranded oligonucleotides. Increasing the temperature below the duplex melting temperature (stimulation of the B′–B structural transition) results in an increase in photodimer formation that was inhibited in the B′-form. Thus, we demonstrate the usefulness of RP-HPLC for duplex DNA structural studies.

INTRODUCTION

HPLC has been successfully used in preparative and analytical separations of oligonucleotides for more than a decade [1,2]. While many efforts were directed at enhancing selectivity and optimizing separation of nucleic acid constituents, the potential of HPLC for structural studies has not been fully exploited. Except for purity control, a limited number of analytical HPLC applications have been reported. HPLC has been used for the separation of oligonucleotides from their 5′-phosphorylated derivatives [3], sequence isomers of oligonucleotides [4], various tRNAs [5], polynucleotides according to their base composition [6] and single- and double-stranded DNA [7]. Solid-phase hybridization of complementary oligonucleotides on an ion-exchange resin made it possible to follow an equilibrium between single-stranded and duplex forms of short DNAs [8]. Several groups have applied RP-HPLC to photochemical studies of oligonucleotides [9–15]. It has been shown that the formation of pyrimidine photohydrates [10] and photodimers [9,11–15] in oligonucleotides significantly changes their retention on a reversed phase. Modified oligonucleotides can be separated according to the type and sequence

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☆ For Parts I and II, see refs. 9 and 10, respectively.

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position of the pyrimidine photoproduct they contain.

We became interested in whether this last property can be applied to studies of the secondary structures of oligonucleotides. If nucleobase photoreactivity depends on the local structure of the nucleic acid fragment, then the high resolving power of HPLC can be used to locate specific photoproducts and hence the parts of the molecule with different conformations. Studies over the last several years have clearly shown that the duplex form of DNA can contain distinctive local structures that depend on the sequence of the bases. For instance, the $(dA)_n$·$(dT)_n$ stretches can adopt the B′-form, which is different from the standard B-form [16–26]. This difference is manifested in the helical repeat [27,28], the helical pitch [18] and the width of the major groove [19,29–31]. The ability of short $d(A_nT_n)$ regions to adopt the B′-form depends on the order of bases: 5′-AT-3′ junction (AT step) is well accommodated in the B′-form geometry, while the TA step has a disrupting effect on the B′-form, promoting instead the B-form [17,32,33].

We studied two self-complementary oligonucleotides that contain the same bases but in different sequences. One of them, $[d(CCTTTAAAGG)]_2$ (Fig. 1), is known to assume the standard solution DNA conformation (B-form [16]) with the characteristic fluctuational mobility of constituent bases [17]. The second, $[d(GGAAATTTCC)]_2$, has a mixed structure: several terminal base pairs (bp) have the B-form geometry and at least four central AT bp are included in a stretch of the B′-form [17]. We UV-irradiated double-stranded oligonucleotides under conditions in which $[d(CCTTTAAAGG)]_2$ is in the B-form and $[d(GGAAATTTCC)]_2$ contains the B′-form, and separated the resulting products as single strands. The reduced mobility of bases and hence their reduced probability of adopting a disposition necessary for photoreaction results in a decrease in thymine photodimer yield in the B′-form sequence, as compared with the B-form. Elevated temperature destabilizes the B′-form and shifts the structure to the B-form. This results in an increase in the inner TT photodimer formation that was inhibited in the B′-form.

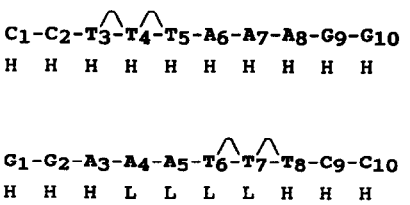

Fig. 1. Sequences of oligodeoxyribonucleotides studied showing high (H) and low (L) probabilities of fluctuational opening of base pairs in the duplexes. The positions of probable thymine dimers are indicated above the sequences by the ∧ symbol.

Our results demonstrate one more important HPLC application. RP-HPLC is able to reveal not only differences in the sequences of nucleic acid fragments (primary structure) but also, combined with photomodification, differences in geometry of spatial arrangement of bases (secondary structure).

EXPERIMENTAL

Materials

Oligonucleotides d(CCTTTAAAGG) and d(GGAAATTTCC) were synthesized by a modified phosphoramidite approach. They were purified by electrophoresis and RP-HPLC, desalted and sequenced. Snake venom phosphodiesterase I (PDE I) was from Pharmacia (Piscataway, NJ, USA). All other chemicals of analytical reagent grade were from various commercial sources.

UV irradiation

Oligonucleotides (25 μM strand concentration, in 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0, without or in the presence of 10 mM acetophenone as a photosensitizer) were irradiated at 302 nm in 1-mm quartz cuvettes in a thermostated cell holder by a TM-36 UV transilluminator (UVP, San Gabriel, CA, USA) at a distance of 2 cm for various periods of time. This irradiation wavelength was chosen to exclude photoreversal of cyclobutadithymines.

Chromatographic conditions

Chromatography was performed on a Jasco LC-800 instrument (Jasco, Tokyo, Japan) using

an 875 UV detector and an 860 CO column oven. Analysis of photomodified samples was accomplished on a Merck LiChrosorb RP-18 (250 × 4 mm) column at 60°C with a 0–30% B gradient, where eluent A was 0.1 *M* ammonium acetate (pH 6.8) and eluent B was 50% methanol in water. The detector signal at 254 nm was fed to a microcomputer to calculate peak areas.

Localization of photodimers

After UV irradiation, oligonucleotides were separated as described and peaks of interest were collected in Eppendorf test tubes. Samples were dried in water and methanol several times in a Speed Vac evaporator to eliminate residual ammonium acetate. The dried material was reconstituted in 40 μl of enzyme reaction buffer (100 m*M* Tris–HCl, pH 8.9, 100 m*M* NaCl, 14 m*M* $MgCl_2$). A 10-μl aliquot of PDE I (0.1 units in the same buffer) was added and the resulting mixture was vortexed and incubated at 37°C for 30 min, after which time it was placed in boiling water for 5 min to inactivate the enzyme. Samples were spun in an Eppendorf microcentrifuge for 10 min and 10- to 40-μl aliquots were separated on a DuPont PEP-RP1 (80 × 6 mm) column at room temperature with a 20 min 0–20% B linear gradient (A, 50 m*M* potassium phosphate, pH 4.9, 5 m*M* tetrabutylammonium dihydrogen sulphate; B, 50% methanol in water). The presence of particular nucleoside-5′-monophosphates cleaved by PDE I from the 3′-end of the oligonucleotides until the enzyme stopped at a photodimer position [9,34] was used to locate photodimers in the sequences (Table I). Cyclobutane types of thymine photodimers were confirmed by acetophenone photosensitization, which promotes the formation of pyrimidine dimers exclusively [34,35].

RESULTS

Experimental doses of UV irradiation were such that no more than one photodimer per duplex was formed (10-20% diminution of the intact oligonucleotide amount). Otherwise, the duplex-destabilizing effect of photodimers and hence the change in oligomer photoreactivity should be considered. Moreover, we expected to obtain largely cyclobutane photodimers, because the yield of non-cyclobutane (6–4) dimers at low doses is several-fold lower than of the cyclobutane dimers [36]. In order to avoid artifacts in analysis of the photoproducts due to probable elution off the column of double- and single-stranded oligonucleotides, chromato-

TABLE I

CLEAVAGE OF NUCLEOSIDE-5′-MONOPHOSPHATES BY PDE I INCUBATED WITH MODIFIED AND UNMODIFIED OLIGONUCLEOTIDES

Aliquots of 10 μl of PDE I (0.1 units in the reaction buffer: 100 m*M* Tris–HCl, pH 8.9, 100 m*M* NaCl, 14 m*M* $MgCl_2$) were incubated at 37°C for 30 min with chromatographically isolated oligonucleotides in a 40-μl total reaction volume. Aliquots of the reaction mixture were separated on a DuPont PEP-RP1 (6 × 80 mm) column at room temperature with a 20 min 0–20% B linear gradient (A, 50 m*M* potassium phosphate, pH 4.9, 5 m*M* tetrabutylammonium dihydrogensulphate; B, 50% methanol in water). Nucleoside-5′-monophosphates were cleaved by PDE I from the 3′-end of the oligonucleotides until the enzyme stopped at a photodimer position. The ∧ symbol indicates positions of dimer formation between two adjacent thymines.

Peaks	Oligomers	Liberated nucleotides			
		AMP	CMP	GMP	TMP
1	d(CCT∧T∧AAAGG)	+	−	+	+
2	d(CCT∧T∧AAAGG)	+	−	+	−
3	d(CCTTTA∧AAGG)	+	+	+	+
4	d(GGAAA∧TTTCC)	−	+	−	+
5	d(GGAAA∧TTTCC)	−	+	−	−
6	d(GGAAATTTCC)	+	+	+	+

graphic separations at temperature above the melting point were done. Elevated temperature was additionally justified as it has been found that using the same eluents and gradients it is possible to better resolve some peaks of photoproduct-containing oligonucleotides.

UV irradiation of oligonucleotide duplexes at low temperature resulted in the appearance of several photoproducts. Comparison of photoproducts (Fig. 2a and b) obtained during sensitized photomodification, which promotes formation of almost exclusively cyclobutane TT photodimers [35], with those obtained during direct photomodification (several additional minor peaks, data not shown) allowed us to conclude that in both chromatograms peaks 1 and 2 corresponded to oligonucleotides containing cyclobutane TT photodimers. Further, in all cases we used unsensitized direct photomodification of oligomers to exclude any possible influence of acetophenone on their conformations. The sequence positions of thymine dimers in photomodified oligomers were identified using an exhaustive snake venom phosphodiesterase digestion of oligonucleotides from the 3′-end, which stopped at the photodimer position [34] (Table I). Note that the closer the photodimer is to the 5′-end of oligonucleotide, the greater is the change in retention.

Structural differences between the B-form and mixed-form duplexes clearly manifest themselves in lower yields of photodimers obtained for $[d(GGAAATTTCC)]_2$, as compared with $[d(CCTTTAAAGG)]_2$. More important is the temperature dependence for the yield of the inner T_6T_7 photodimer in $[d(GGAAATTTCC)]_2$. In contrast to other photodimers (Fig. 3), its yield rises more than three-fold with temperature. At higher temperatures, when the duplexes are denatured, all TT photodimers have similar yields. As has been mentioned above, the corresponding T_6T_7 dinucleotide is included in that part of oligonucleotide that is capable of adopting the rigid B′-conformation. Therefore, distinct temperature dependence for the T_6T_7 photodimer at least partly reflects the B′–B form transition.

This assumption is confirmed by the spectrophotometric melting [37]. Both oligonucleo-

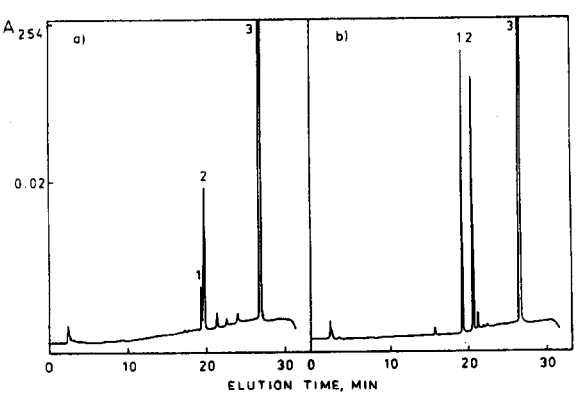

Fig. 2. Chromatograms of oligonucleotides UV-irradiated at 7°C for 10 min in the presence of 10 m*M* acetophenone under conditions in which the double-stranded structures exist. Column: LiChrosorb RP_{18} (250 × 4 mm). Eluents: A, 0.1 *M* ammonium acetate (pH 6.8); B, 50% methanol in water; 0–30% B linear gradient (30 min) at 60°C; flow-rate, 1 ml/min. (a) $[d(GGAAATTTCC)]_2$. Peaks: 1 = d(GGAAA$\widehat{TTT}$CC); 2 = d(GGAAA$\widehat{TTT}$CC); 3 = d(GGAAATTTCC). Unidentified peaks are those of minor photoproducts. (b) $[d(CCTTTAAAGG)]_2$. Peaks: 1 = d(CC$\widehat{TTT}$AAAGG); 2 = d(CC$\widehat{TTT}$AAAGG); 3 = d(CCTTTAAAGG).

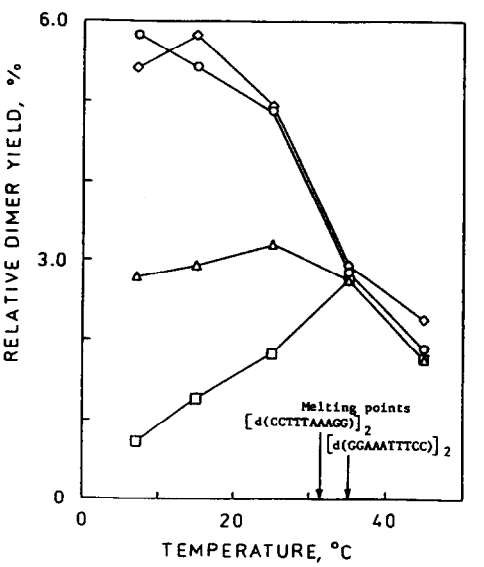

Fig. 3. Temperature dependencies of the photodimer yields (calculated relative to initial amounts of unmodified oligomers) for oligonucleotides in 150 m*M* sodium chloride, 15 m*M* sodium citrate, pH 7.0: □ = d(GGAAA$\widehat{TTT}$CC); △ = d(GGAAA$\widehat{TTT}$CC); ◇ = d(CC$\widehat{TTT}$AAAGG); ○ = d(CC$\widehat{TTT}$AAAGG). Melting points for the duplex form oligonucleotides are indicated by arrows.

tides undergo duplex-coil transitions at 31–35°C. $[d(GGAAATTTCC)]_2$ has also a broad "premelting" interval with a midpoint at about 15°C. Additional evidence of correlation between the B′–B transition and the yield of the T_6T_7 photodimer is provided by the other factor known to destabilize the B′-form but not the B-form, dimethyl sulphoxide (DMSO) [20,21]. DMSO 30% increases the yield of the T_6T_7 photodimer but affects the yields of other photodimers to a lesser extent (data not shown).

DISCUSSION

Several methods were used to study the B′-conformation in poly(dA)–poly(dT) and various oligonucleotides containing $(dA)_n$·$(dT)_n$ stretches. Among them were the UV absorbance, circular dichroism and NMR spectroscopies [17,20,22,33], calorimetry [33], structurally caused changes in electrophoretic mobility [32] and several methods of chemical [25,26] and photochemical [21] probing of nucleic acid duplexes. We used this relatively well-studied problem to ascertain the applicability of chromatographic methods to structural studies of oligonucleotides since we feel that to make the models of molecular structure most reliable, it is desirable to get as much independent evidence from various techniques as possible.

The suitability of liquid chromatography for the structural studies of biological oligomers has been illustrated by the investigations of peptides. Extensive work has been done to calculate the contribution of every constituent amino acid residue to the retention of peptides on a reversed phase [38–40]. Racemization of peptides at practically every peptide bond can be detected using RP mode [41]. Moreover, HPLC is able to reveal kinetic details of *cis–trans* isomerism in proline- and N-methylamino acid-containing peptides [42,43]. Recently, amphipathic α-helix content has been shown to influence peptide retention on RP supports [44].

Yet, application of HPLC to structural studies of nucleic acids is rarer. As far as the spatial structure is concerned, only separation of single- from double-stranded DNA [7], separation of supercoiled, open circular and linear plasmid DNA [45] and on-column hybridization of complementary oligonucleotides [8] can be mentioned. Our results show that it is possible to get more details of double-stranded nucleic acid structure using a combination of HPLC with some other suitable structurally dependent technique of nucleic acid modification (in our case, UV modification at characteristic sites). We have used this approach to study the influence of some cations, including alkali, earth alkali and transition metal ions, on the existence of the B′-form [37]. Since DNA conformations are not restricted to B- and B′-forms and a variety of different site modifications as well as modifying agents can be used, the presented method can be useful for other structural studies of nucleic acid fragments and their complexes with various ligands.

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