NMR investigations of duplex stability of phosphorothioate and phosphorodithioate DNA analogues modified in both strands

Jerzy W. Jaroszewski*, Vicki Clausen, Jack S. Cohen¹ and Otto Dahl²

Department of Medicinal Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, Denmark, ¹Department of Pharmacology, Georgetown University Medical Center, Washington, DC 20007, USA and ²Department of Chemistry, The H. C. Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark

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ABSTRACT

Duplex formation from the self-complementary 12mer d(CGCGAATTCGCG) (Dickerson dodecamer) in which all phosphodiester linkages were replaced by phosphorothioate or phosphorodithioate linkages was studied using variable-temperature ¹H and ³¹P NMR spectroscopy. Melting temperatures of the dodecamer, measured spectrophotometrically, showed significant decrease upon sulfur substitution (Tₘ 49°C for the phosphorothioate and 21°C for the phosphorodithioate, compared with 68°C for the unmodified oligomer, in 1 M salt). Hyperchromicity observed upon melting of the dithioate was surprisingly low. NOESY spectra of the monothioate showed a cross-peak pattern characteristic for a right-handed duplex. Imino proton resonances of the duplex, shown by the mono- and the dithioate, were similar to those of the parent compound. In spite of monophasic melting curves, temperature dependence of the imino proton resonances and phosphorus resonances of the phosphorodithioate indicated heterogeneity with respect to base-pairing, compatible with the presence of a hairpin loop. Relaxation times (T₁) of the imino protons in the phosphorothioate, determined by the saturation recovery method, were considerably shorter than in the unmodified oligomer. Base-pair lifetimes in the unmodified Dickerson dodecamer, determined by catalyst-dependent changes in relaxation rates of imino protons, were in the range of 2–30 ms at 20°C. Strongly reduced base-pair lifetimes were found in the phosphorothioate analogue.

INTRODUCTION

Synthetic DNA analogues with non-bridging oxygen atoms of the phosphodiester group replaced by sulfur (phosphorothioates and phosphorodithioates) are of great importance as biochemical and pharmacological tools (1–4). They have the important property of being resistant to most hydrolytic enzymes, and thus can persist in biological environment such as the interior of a cell. Phosphorothioates are the most easily prepared and widely used type of chemically modified DNA. Phosphorodithioates have been synthesized only recently (5–8), and their availability and use is still restricted. Previous studies have established the ability of both analogues to form stable duplexes with native DNA and RNA, and to inhibit RNA translation, although the heteroduplexes had somewhat diminished melting temperatures (9,10).

Although the formation of B-type duplexes between native DNA and the sulfur-containing analogues is widely assumed, only little is known about the effects of sulfur substitution on duplex stability and geometry (11–13), apart from what can be concluded from analysis of melting curves. A self-complementary decamer which forms a stable B-type duplex as unmodified DNA changed to a hairpin when one or two central phosphorodithioate sites were introduced, and A-type structures were observed (8,14,15). Theoretical investigations of phosphorothioates identified destabilizing interactions depending on the stereochemistry of the phosphate group (13). Thus, the Rᵢ configuration of the chiral phosphorothioate group, in which sulfur points into the major groove, is helix-destabilizing, whereas the Sᵢ configuration, in which sulfur points outwards, is not (13), in agreement with NMR detectable changes observed in B-DNA upon stereospecific substitution with sulfur at a single site (12). In this work, we applied various NMR techniques to obtain additional information about structure and stability of oligodeoxynucleotide duplexes modified in both strands. In particular, properties of the exchangeable hydrogens (imino protons) were of interest. The imino protons are especially suitable for studies of phosphorothioates, which are prepared and used as heterogeneous mixtures of Rᵢ and Sᵢ diastereomers, because the change of stereochemistry at phosphorus has relatively little effect on the chemical shifts of the imino protons. The Dickerson dodecamer (Fig. 1), a prototype B-DNA sequence, was used.

MATERIALS AND METHODS

The unmodified dodecamer (I) was synthesized by standard cyanoethylphosphoramidite method on an Applied Biosystems
Figure 1. Dodecamers studied; pS and p2S indicate phosphorothioate and phosphorodithioate linkages respectively.

Model 394 DNA synthesizer, purified as the tritylated derivative by reverse-phase HPLC on a PRP-1 column eluted with a 0.1 M triethylammonium acetate buffer with an acetonitrile gradient, detritylated, and precipitated with ethanol as the sodium salt. The phosphorothioate (II) was obtained using 3H-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile as the sulfurization reagent (16) and purified similarly (the material obtained consisted of a mixture of stereoisomers with respect to the chiral phosphorothioate groups). The dithioate (III) was obtained by the thiosphoramide method and purified as previously described (6); according to a 31P NMR spectrum the preparation contained 8% of monothioates.

NMR spectra were obtained on a Bruker AMX 400 WB spectrometer in 5 mm tubes. Concentrations of (I), (II) and (III) were 2.5, 1.9 and 0.9 mM respectively (single strand), in 10 mM phosphate buffer containing 0.1 or 1 M NaCl and 1 mM EDTA, pH ∼7. The solvent was either D2O or H2O/D2O 9:1. In the latter solvent, exchangeable protons were observed by means of a 13T pulse (17), for the relaxation rate measurements preceded by a low-power selective saturation pulse and a variable delay. 1H NMR spectra were standardized by means of sodium 3-(trimethylsilyl)propanesulfonate. 31P NMR spectra were obtained with WALTZ16 decoupling of protons and standardized to external conc. phosphoric acid. NOESY spectra were obtained with sweep widths in both dimensions of 4000 Hz and a data matrix of 1024 × 512 points, zero-filled to 1k × 1k points prior to transformation. Measurements of pH were carried out with a microelectrode directly in the NMR tubes.

Concentrations of oligonucleotides were measured spectrophotometrically with a Shimadzu-265 spectrophotometer at 256–258 nm, assuming standard values of absorptivity for purines and pyrimidines (respectively 14 000 and 7000 l×mol−1×cm−1). Melting curves were determined at 1 M ionic strength (10 mM phosphate buffer, 1 M NaCl, 1 mM EDTA, pH 7.2), after initial heating to 90°C and cooling to 0–20°C with a Gilford Response II spectrophotometer, using temperature intervals of 0.5°C; the concentrations of oligodeoxynucleotides were 8–11 µM (single strand).

RESULTS AND DISCUSSION

Melting temperatures (Tm) of (I), (II) and (III) were determined spectrophotometrically in 1 M salt as 68 (18), 49 and 21°C respectively (Fig. 2). The melting of (III) was also followed by temperature dependence of NMR chemical shifts of non-exchangeable protons (Fig. 2). The Tm values indicated a steady decrease of the duplex stability from (I) to (III), in agreement with previous results for monothioates (9) and for oligomers containing one dithioate strand (6,10). The optical hyperchromicity observed upon melting of (III) was much lower not only compared with (II) (Fig. 2), but also to the previously studied (6,10) heteroduplexes. As described below, this behaviour is presumably due to the presence of multiple structures in the solution of (III).

Figure 3 shows a fragment of a NOESY spectrum of the phosphorothioate (II) showing cross-peaks between the base protons (H6 in pyrimidines or H8 in purines) and anomeric protons, which are diagnostic for the right-handed helix, and, for
comparison, the corresponding region of the spectrum of the unmodified oligomer (I) (19). Because of the stereochemical heterogeneity of (II) many of its resonances were spread out over a range of chemical shifts. However, the connectivities within the central AATT stretch could be readily assigned, and the cross-peaks of the CG base-pairs were also apparent (Fig. 3). The remaining regions of the NOESY spectrum of (II) were also closely similar to the spectrum of (I) except for the presence of broadened or multiple resonances. This provided evidence for the presence of a B-like structure formed by (II). NOESY spectra of the phosphorodithioate (III), recorded at 0, 15 or 20°C, failed to detect interresidual NOEs, although intraresidual NOEs were apparent.

Figures 4 and 5 show temperature-dependent $^{31}$P-$^1$H NMR spectra and $^1$H NMR spectra of the imino protons (the spectra of the unmodified oligomer were investigated previously, 18,20–25). Downfield shift of the phosphorus resonances observed upon heating is characteristic for transition from double-stranded to single-stranded DNA, as the result of changes of the torsion angles of the phosphate bonds. The downfield shift of the resonances of (III) was approximately twice as large as that for (I) (20) or (II) (Fig. 4). For (III) it was characteristic, that only well above the melting temperature of $\sim$20°C the $^{31}$P resonances were fully shifted downfield. At 0°C, the spectrum of (III) contained a low-field shoulder, presumably corresponding to a single-stranded structure, which upon heating to 30°C gradually increased to $\sim$50% of total signal intensity with only little change of chemical shift. The high-field components of the spectrum disappeared only $>40^\circ$C (Fig. 4). By contrast, the $^{31}$P chemical shifts of (I) changed gradually over the whole temperature range, and no high-field components (corresponding to the double strand) were observed above the spectrophotometric $T_m$ (data not shown). The temperature-induced changes observed with (II) represented an intermediate case (Fig. 4). It appears, therefore, that both (II) and (III) contain non-helical structures below $T_m$.

That (III) exhibits a much complex behaviour in solution compared with (I) was further demonstrated by temperature-dependent spectra of the imino protons of base-pairs (Fig. 5). At 0°C, all three compounds exhibited well-defined resonances corresponding to the six base-pairs. The imino resonances of (I) were assigned previously (18); the assignment for (II) and (III) followed from comparison with (I) and from the observed sequential melting effects (Fig. 5). For (II), the resonances represented envelopes due to the presence of diastereomers; the imino resonance of the terminal CG pair of (II) exhibited two distinct chemical shifts, presumably reflecting $R_p$ and $S_p$ stereochemistry at this site. While the imino resonances of (I) (18)
and (II) showed strong exchange broadening already below their spectrophotometric melting temperatures, the signals of (III) were perfectly sharp around 20°C (Fig. 5). Thus, distinct imino resonances of the central CGAATTTCG fragment of (III) were still observed at a temperature 15°C above the $T_m$; at this temperature the spectrum of (III) was similar to that of (I) around 60°C, i.e., 5–10°C below its $T_m$ (Fig. 5). The imino resonances observed with (III) cannot originate from the phosphorothioate impurity present in the material, not only because its amount was small, but also because the behaviour of the imino protons was closely paralleled by the behaviour of the $^{31}$P NMR dithioate signals, which appear in a spectral region different from that of the monothioate groups.

It appears, therefore, that base-paired species prevail in the solution of (III) at temperatures above the apparent $T_m$ (cf. Figs 2 and 5). By contrast, the NMR results for (I) and (II) were fully consistent with the spectrophotometric $T_m$. The discrepancy between the spectrophotometric $T_m$ (Fig. 2) and the behaviour of the $^{31}$P NMR spectra (Fig. 4) and imino proton spectra (Fig. 5) of (III) cannot be explained by the differences in the oligomer concentrations used, because the temperature dependence of the chemical shifts of thymine methyl groups was closely similar to that of the UV absorption, giving $T_m \sim 20$°C (Fig. 2). Moreover, the differences in concentration between solutions used for UV and NMR were smaller for (III) than for (I) and (II).

We conclude that in spite of the apparently monophasic melting curves multiple forms are present in the solution of (III). The formation of a hairpin loop by (III) was suggested both by the presence of the low-field shoulder in the $^{31}$P NMR spectrum and by integrals of the imino resonances observed at 0°C. Thus, while the integral ratio between the imino protons of AT and CG base-pairs (theoretically two) was as expected for (I) and (II), a 25% excess of the intensity of the CG signals was observed with (III) (Fig. 5), even though a partial exchange of the CG1 imino proton might already have occurred. The excess of the CG signals is expected when structures with an AATT loop are formed. The observed peak intensities would correspond to a duplex to hairpin ratio of two. Moreover, multiple thymine methyl resonances (26–28) were observed with (III). However, no resonances of unpaired imino protons could be detected around 11 p.p.m., presumably because of exchange broadening (29,30). The existence of multiple structures explains the failure to obtain satisfactory NOESY spectra with the solution of (III). The presence of partly unstacked structures already at low temperatures accounts for the low hyperchromicity exhibited by (III) (Fig. 2). Hairpin formation at low concentration and low ionic strength has been observed with (I) (31,32).

In order to assess the effect of sulfur substitution on duplex dynamics, longitudinal relaxation times ($T_1$) were measured for all imino protons of (I) and (II) using the saturation recovery

![Image]

Figure 5. 400 MHz $^1$H NMR spectra of imino protons in (I)–(III) as a function of temperature (ionic strength 1 M, pH 7.2, H$_2$O/D$_2$O 9:1). The spectra were acquired using the $\hbar$ pulse adjusted to excitation maximum centered at 13 p.p.m.
method (Table 1). Because of the stereochemical heterogeneity of (II), the values represent ‘average’ relaxation times (Table 1). Examples of such experiments are shown in Figure 6. The obtained $T_1$ relaxation times of (I) and their variations along the strand and upon heating were closely similar to those observed previously (33,34). The data in Table 1 show that the $T_1$ values for (II) are both shorter and diminish more sharply as the temperature rises. This change is presumably due to increased imino proton exchange with bulk solvent, i.e., to the differences in the base-pair lifetimes between (I) and (II).

Table 1. Observed longitudinal ($T_1$) relaxation times (ms) of imino protons in (I) and (II) from saturation recoveries ($\text{H}_2\text{O}/\text{D}_2\text{O}$ 9:1, 1 M NaCl, 10 mM phosphate buffer, 1 mM EDTA, pH 6.7).

<table>
<thead>
<tr>
<th>Compound</th>
<th>CG2</th>
<th>CG3</th>
<th>CG4</th>
<th>AT5</th>
<th>AT6</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I), 20°C</td>
<td>218 ± 10</td>
<td>289 ± 12</td>
<td>281 ± 14</td>
<td>317 ± 13</td>
<td>414 ± 17</td>
</tr>
<tr>
<td>(II), 20°C</td>
<td>2 ± 7</td>
<td>227 ± 14*</td>
<td>200 ± 6</td>
<td>224 ± 11</td>
<td></td>
</tr>
<tr>
<td>(I), 40°C</td>
<td>12 ± 2</td>
<td>119 ± 3</td>
<td>283 ± 5</td>
<td>88 ± 2</td>
<td>148 ± 3</td>
</tr>
<tr>
<td>(II), 40°C</td>
<td>–</td>
<td>26 ± 2</td>
<td>85 ± 3</td>
<td>29 ± 2</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

*aClosely spaced resonances of CG3 and CG4 saturated together.

Base-pair lifetimes can be obtained by measuring the effect of concentration of an exchange catalyst on the relaxation rates, either for $T_1$ or for $T_2$ (saturation recovery method or line-width method) (35,36). Thus, in addition to the intrinsic, magnetic contributions $T_1^0$ and $T_2^0$ (relaxation times in the absence of exchange catalyst), the observed relaxation rates have a component due to exchange with water, which depend on the concentration of added catalyst. Assuming that the imino proton exchange can only take place from the open form of a base-pair and predominantly by virtue of a base-catalyzed process, and that the equilibrium between the open and the closed form is far towards the latter, the observed relaxation rates as a function of added catalyst are limited by the base-pair opening rate. Thus, relaxation time measurements at different catalyst concentrations enable evaluation of base-pair lifetimes (35,36).

Although the base-pair lifetimes of (I) have already been measured several times (21,23–25) the reported values vary by more than one order of magnitude (Table 2). Thus, we decided to obtain the data for (I) as well as for (II) using the same pH, base catalyst, temperature and ionic strength.

Table 2. Base-pair lifetimes (ms) in (I) and (II).

<table>
<thead>
<tr>
<th>Compound</th>
<th>CG2</th>
<th>CG3</th>
<th>CG4</th>
<th>AT5</th>
<th>AT6</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I), 25°C</td>
<td>5</td>
<td>&gt;85</td>
<td>1</td>
<td>10</td>
<td>ref. 21</td>
<td></td>
</tr>
<tr>
<td>(I), 25°C*</td>
<td>–</td>
<td>77</td>
<td>&lt;1</td>
<td>15</td>
<td>ref. 23</td>
<td></td>
</tr>
<tr>
<td>(I), 15°C</td>
<td>48</td>
<td>299</td>
<td>21</td>
<td>71</td>
<td>ref. 24</td>
<td></td>
</tr>
<tr>
<td>(I), 15°C*</td>
<td>5</td>
<td>25</td>
<td>8</td>
<td>26</td>
<td>ref. 25</td>
<td></td>
</tr>
<tr>
<td>(I), 20°C</td>
<td>2</td>
<td>18</td>
<td>30</td>
<td>14</td>
<td>22</td>
<td>this work</td>
</tr>
<tr>
<td>(I), 30°C</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>this work</td>
</tr>
<tr>
<td>(II), 20°C*</td>
<td>6*</td>
<td>1</td>
<td>1</td>
<td>this work</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Tris buffer, pH 7.2.
*Tris buffer, pH 7.1.
*Ammonia buffer, pH 9.0.
*Ammonia buffer, pH 8.8.
*Value obtained from the overlapped resonances of CG3 and CG4.

The measurements were carried out in 0.1 M NaCl using Tris (2-amino-2-hydroxymethyl-1,3-propanediol) as a base. Relaxation of imino protons in (I) and (II) was studied at pH 7.2, using seven different values of free base concentration in the range of 1–50 mM. For (I), which gives narrow lines (Fig. 5), it was convenient to use the line-broadening effect of the added base. For (II), the stereochemical heterogeneity gives a significant and variable contribution to the observed line-widths, and hence the saturation recovery method was used. The data were analyzed as described by Moe and Russu (24). Since the experiment depends critically on the ability to calculate free base concentration at a given pH from its $pK_a$, and the concentration acidity constants depend on the solution ionic strength, we determined the $pK_a$ of Tris in 0.1 M NaCl by $^{13}$C NMR titration as 8.38. The obtained base-pair lifetimes for (I) and (II) are shown in Table 2.

The values obtained for (I) at 20°C are in agreement with those reported in one of the recent studies at 15°C (25). The values obtained for the AT pairs at 30°C (Table 2) are similar to those determined at 25°C by Lyckell et al. (21). In contrast with the previous studies, the value for CG2 at 20°C could be obtained. In agreement with all earlier results, anomalously high lifetime for the central AT pair was found.

The values obtained for (II) were much shorter than the corresponding values for (I) (Table 2). Thus, the sulfur substitution not only decreases the thermodynamic stability of the duplex, but also alters strongly base-pair kinetics, possibly through...
geometric changes in the helix structure. Insight into these geometric alterations can at present be investigated only using theoretical models (13), since the stereochemical heterogeneity of phosphorothioates precludes quantitative interpretation of the NOE data (Fig. 3). However, progress in stereospecific methods of phosphorothioate synthesis (37) may make appropriate model compounds available soon.

The kinetics of base-pairs in oligodeoxynucleotides and their analogues is influenced by the duplex length, the alterations being observed three bases from each terminal of the duplex (25). Thus, investigation of sequence-specific variations of base-pair lifetimes compared with their non-bridged counterparts, apparently by removing duplex instability due to 'fraying' effects. Such oligodeoxynucleotides with non-nucleotide bridges may be appropriate for investigation of sequence specific structural and dynamic effects, and the effects of sulfur substitution thereupon.

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REFERENCES