Linkage isomerization reaction of intrastrand cross-links in trans-diamminedichloroplatinum(II)-modified single-stranded oligonucleotides

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ABSTRACT

The stability of trans-[Pt(NH3)2[d(CGAG)-N7-G,N7-G]] adducts, resulting from cross-links between two guanine residues at d(CGAG) sites within single-stranded oligonucleotides by trans-diamminedichloroplatinum(II), has been studied under various conditions of temperature, salt and pH. The trans-[Pt(NH3)2[d(CGAG)-N7-G,N7-G]] cross-links rearrange into trans-[Pt(NH3)2[d(CGCG)-N3-C,N7-G]] cross-links. The rate of rearrangement is independent of pH, in the range 5–9, and of the nature and concentration of the salt (NaCl or NaClO4) in the range 10–400 mM. The reaction rate depends upon temperature, the t1/2 values for the disappearance of the (G,G) intrastrand cross-link ranging from 120 h at 30°C to 70 min at 80°C. The linkage isomerization reaction occurs in oligonucleotides as short as the platinated tetramer d(CGAG). Replacement of the intervening residue A by T has no major effect on the reaction. The C residue adjacent to the adduct on the 5' side plays a key role in the reaction; its replacement by a G, A or T residue prevents the reaction occurring. No rearrangement was observed with the C residue adjacent to the adduct on the 3' side. It is proposed that the linkage isomerization reaction results from a direct attack of the base residue on the platinum(II) square complex.

INTRODUCTION

cis-Diamminedichloroplatinum(II) (cis-DDP) is widely used as a chemotherapeutic agent in the clinical treatment of tumors. Several studies suggest that the covalent bifunctional adducts formed in the reaction between cis-DDP and cellular DNA are implicated in the cytotoxicity of the drug (general reviews, 1–4). trans-Dichlorodiammineplatinum(II) (trans-DDP), the stereoisomer of cis-DDP, is clinically ineffective, although it binds to DNA and forms bifunctional adducts (1–4). It is not yet explained why only cis-DDP is an antitumor drug.

In their reaction with DNA, the two isomers present similarities and differences. To illustrate in part these similarities and differences, one can consider the 1,3-intrastrand cross-links at the d(GNG) sites (the intervening N residue being any base). Both cis-DDP and trans-DDP react with the G residues at the d(GNG) sites within single-stranded DNA and yield, respectively, cis- or trans- [Pt(NH3)2[d(GNG)-N7-G,N7-G]] intrastrand cross-links (5–8). Both cis-DDP and trans-DDP bifunctional lesions are considered stable under physiological conditions (1–4). However, two exceptions have been reported. The first one deals with a trans-DDP-modified dodecamer in which the trans- [Pt(NH3)2[d(CGXG)-N7-G,N7-G]] intrastrand cross-link rearranges into the 1,4-trans- [Pt(NH3)2[d(CGCG)-N3-C,N7-G]] intrastrand cross-link (7). This reaction is sequence-specific, in that the rearrangement was reported not to occur in the related sequence d(CGAG) (7,8). The second one deals with oligonucleotides containing trans- [Pt(NH3)2[d(GXG)-N7-G,N7-G]] intrastrand cross-links (X being A, T or C), which rearrange into interstrand cross-links as soon as the platinated oligonucleotides are hybridized with their complementary ribonucleotide or deoxyribonucleotide strands (9).

Rearrangement of the (G,G) intrastrand cross-links into interstrand cross-links could be useful in the antisense strategy. It is well established that antisense oligonucleotides can control the stability and function of mRNA (general reviews, 10,11). Binding of the oligonucleotides to their targets is reversible, which in some cases decreases their efficiency. To get an irreversible binding, one can take advantage of conversion of the intrastrand cross-link into an interstrand cross-link promoted by DNA–RNA duplexes (9). This application requires that the intrastrand cross-links are stable within the single-stranded oligonucleotides, which led us to study the stability of trans-DDP intrastrand cross-links within single-stranded DNAs. In this paper we show that rearrangement of the trans- [Pt(NH3)2[d(CGCG)-N3-C,N7-G]] intrastrand cross-link into the 1,4-trans- [Pt(NH3)2[d(CGCG)-N3-C,N7-G]] intrastrand cross-link occurs in trans-DDP-modified single-stranded oligonucleotides at d(CGAG) or d(CGTO) sites. The dependence of the linkage isomerization reaction on several parameters (ionic strength, temperature, length and sequence of the oligonucleotide) has been studied.
Materials and Methods

The oligodeoxyribonucleotides, purchased from Institut Pasteur, were purified as previously described (12). Their sequences and abbreviations are given in Figure 1. The oligonucleotides have different lengths, but they have in common d(GAG) or d(GTG) as the central sequence. Endonuclease P1 and alkaline phosphatase were from Boehringer Mannheim, T4 DNA polymerase and T4 polynucleotide kinase from Biolabs. The radioactive products were from Amersham, trans-DDP from Johnson Matthey (Reading, UK) and the other chemicals from Merck.

Platination

The oligonucleotides with one equivalent of trans-DDP in 10 mM NaClO₄, pH 3.2, adjusted with metal-free nitric acid, were incubated at 37°C for 24 h (7) and then for 10 min at 37°C in the presence of thiourea (10 mM) to remove the monofunctional adducts (7,13). The oligonucleotides containing a single trans-[Pt(NH₃)₂(d(GXG)-N7-G,N7-G)] intrastrand cross-link were purified by FPLC with a 0.2-0.7 M NaCl gradient. In the case of the platinated oligonucleotides containing the sequences d(GGTTG) or d(AGAG), they were further purified by C₁₈ reverse-phase HPLC in a 0-30% acetonitrile gradient, in the presence of 0.13 M ammonium acetate, pH 5.9. The HPLC apparatus was a Hitachi 655 with a Lichrosopher C₁₈ column.

Formation of the trans-[Pt(NH₃)₂(d(GXG)-N7-G,N7-G)] intrastrand cross-links was verified by two techniques: (a) reaction with dimethylsulfate (DMS) (14); the N7 of the G residues are no longer reactive with DMS once they are modified by trans-DDP (5-7); (b) analysis by reverse-phase HPLC of the digests after enzymatic hydrolysis of the platinated oligonucleotides by endonuclease P1 and alkaline phosphatase (15-17). Standard trans-[Pt(NH₃)₂(dGuo)₂]⁺⁺ was prepared as described (13).

Linkage Isomerization Reaction

The oligonucleotides containing the trans-[P(NH₃)₂(d(GXG)-N7-G,N7-G)] intrastrand cross-link (concentration 1-100 μM) were incubated in 10 mM NaClO₄, 0.1 mM EDTA plus 5 mM buffer (phosphate, Tris-HCl or acetate buffer). At various times, aliquots were withdrawn and analyzed by three techniques; (a) reverse-phase HPLC; (b) gel electrophoresis under denaturing conditions (24% polyacrylamide-8 M urea); (c) gel electrophoresis under denaturing conditions (24% polyacrylamide-8 M urea). The radioactive products were from Amersham, trans-DDP from Johnson Matthey (Reading, UK) and the other chemicals from Merck.

Results

Stability of the trans-[Pt(NH₃)₂(d(CGAG)-N7-G,N7-G)] intrastrand cross-link

The ³²P-end-labeled platinated oligonucleotide d₁₂(CG*AG*) containing a single trans-[Pt(NH₃)₂(d(CGAG)-N7-G,N7-G)] intrastrand cross-link was incubated at 60°C in 10 mM NaClO₄, 5 mM phosphate buffer, pH 7, 0.1 mM EDTA (see Fig. 1 for the complete sequence of the dodecanucleotide; * indicates the platinated residues). At various times, aliquots were withdrawn and analyzed by gel electrophoresis, before or after treatment with T4 DNA polymerase, and by HPLC. As shown in Figure 2, the intrastrand cross-link is unstable.

Gel electrophoresis of the entire oligonucleotide. As a function of the incubation time, the intensity of the band corresponding to the starting product decreases, while a new band appears (Fig. 2A). The new product migrates slower than the unplatinated oligonucleotide, which shows that the oligonucleotide is still platinated. Its migration was unchanged if before the electrophoresis the samples were incubated in the presence of thiourea (10 mM). This excludes the possibility that the new product contains a monofunctional adduct.

Figure 1. Sequences of the oligonucleotides and their abbreviations. In the text, the trans-DDP-modified bases are marked with the symbol *.

Figure 2. Instability of the trans-[Pt(NH₃)₂(d(CGAG)-N7-G,N7-G)] cross-link in d₁₂(CG*AG*). (A) Autoradiograms of denaturing 24% polyacrylamide gels of 5'-end-labeled d₁₂(CG*AG*). The samples were incubated at 60°C in 10 mM NaClO₄, 5 mM phosphate buffer, pH 7, 0.1 mM EDTA. Incubation times in hours are indicated above the lanes. (B) The samples were digested with T4 DNA polymerase prior to electrophoresis. Lanes S, migration of molecular weight markers: unplatinated d(CCTCCTCCTC), d₁₂(CGAG) and d(CCTCCTCCTCCTG). (C) HPLC profile of d₁₂(CG*AG*) after 7 h incubation. The gradient was 0-80% buffer B over 40 min. Buffer A was 2% acetonitrile, 0.13 M ammonium acetate, pH 5.9, and buffer B was 40% acetonitrile, 0.13 M ammonium acetate, pH 5.9. The flow rate was 1 ml/min.
Gel electrophoresis of the oligonucleotide treated with T4 DNA polymerase. After incubation, the samples were treated with T4 DNA polymerase and then analyzed by gel electrophoresis. [We found (unpublished results) that trans-DDP intrastrand cross-links at the d(GTG) and d(GTGG) sites stopped the 3'→5' exonuclease activity of T4 DNA polymerase.] As shown in Figure 2B, only one band is detected at time 0 of incubation. As a function of incubation time, the intensity of the initial band decreases, while a new band appears.

HPLC. The reaction was followed by HPLC analysis of the entire oligonucleotides. The reverse-phase HPLC elution profile after 7 h incubation is shown in Figure 2C. There are only two peaks and one corresponds to platinated d12(CG*AG*).

In the three experiments, only one new product is detected. The results were independent of the d12(CG*AG*) (unpublished Figure 2B, only exonuclease activity, HPLC. The electrophoresis of Gel links polymerase DNA function oligonucleotides). The oligonucleotides were digested with endonuclease P1 and alkaline phosphatase. The gradient was 0-20% buffer B over 40 min (the buffer composition is given in the legend to Fig. 2), the flow rate was 1 ml/min. Peaks C, G, T and A are 2'-deoxycytidine, 2'-deoxyguanosine, 2'-deoxythymidine and 2'-deoxyadenosine, respectively. Peak 1 co-elutes with trans-[Pt(NH3)2{d(Guo)2}(d(CpG))]; peak 2 with trans-[Pt(NH3)2{d(Guo)}(d(CpG))]++.

Characterization of the cross-links. These results resemble those reported by Comess et al. (7) on the trans-DDP-modified dodecamer d(TCTAC*GCG*TTCT). It is likely that we are dealing with a similar linkage isomerization reaction, transforming the trans-[Pt(NH3)2[d(CGAG)-N7-G,N7-G]] intrastrand cross-link into the trans-[Pt(NH3)2[d(CGAG)-N3-C,N7-G]] intrastrand cross-link. This was already suggested by the analysis of the fragments generated by the 3'→5' exonuclease activity of T4 DNA polymerase and confirmed as follows.

The oligonucleotides d12(CGAG), d12(CG*AG*) and d12(C*GAG*) were exhaustively digested with nuclease P1 and alkaline phosphatase. The digests were analyzed by HPLC. The HPLC profile of d12(CG*AG*) digests as compared with that of d12(CGAG) shows the disappearance of the peak corresponding to 2'-deoxyguanosine, while a new peak is observed, which co-elutes with the standard trans-[Pt(NH3)2{d(Guo)2}]++ (Fig. 4). In the profile of the d12(C*GAG*) peak, the peak corresponding to trans-[Pt(NH3)2{d(Guo)2}]++ disappears and another peak appears, which co-elutes with trans-[Pt(NH3)2{d(Guo)}(d(CpG))]++,

\[ \text{d}_{12}(\text{CGAG})_t \Rightarrow \text{d}_{12}(\text{CG*AG*})_t \]

\[ \text{d}_{12}(\text{CG*AG*})_t \leftrightarrow \text{d}_{12}(\text{C*GAG*})_t \]

a compound prepared by enzymatic hydrolysis of the platinated dodecamer d(TCTAC*GCG*TTCT) as described by Comess et al. (7).

To complete the identification, the oligonucleotides were reacted with DMS. The results (not shown) confirmed that the probe reacted with G(5) and G(7) in d12(CGAG), but not in d12(CG*AG*). On the other hand, the probe reacted with G(5) and not with G(7) in d12(C*GAG*).

Factors influencing the linkage isomerization reaction

Temperature. To determine the kinetic activation parameters of the linkage isomerization reaction, the stability of the trans-[Pt(NH3)2{d(CGAG)-N7-G,N7-G}] intrastrand cross-link within d12(CGAG) was studied as a function of temperature in the range 30–80°C. The results were analyzed by following the procedure of Comess et al. (7) with minor modifications.

The isomerization can be written

\[ k_+ \] and \( k_- \), the apparent reaction rate constants, are given by

\[ k_+ = \frac{k}{1 + K} \]

\[ k_- = \frac{1}{1 + K} \]
At 50, 60 and 80°C, the reaction was followed until equilibrium. The percent of d12(CG*AG*) was then found to be 6 ± 1%, the equilibrium constant being 16 ± 3. These values were assumed to be identical at 30 and 39°C. At each temperature, ln[(100 - Peq)/(P - Peq)] was represented as a function of time for Peq = 5 and Peq = 7. The k values were the average of the slopes of the corresponding lines. ka and ks were deduced from equations (1) and (2). The values of the rate constants are summarized in Table 1.

Table 1. Reaction rates for the isomerization of d12(CG*AG*) as a function of temperature

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>t1/2 (h)</th>
<th>10^6 k (s^-1)</th>
<th>10^6 ka (s^-1)</th>
<th>10^6 ks (s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>108</td>
<td>2.04 ± 0.05</td>
<td>1.91 ± 0.05</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>39</td>
<td>38</td>
<td>6.51 ± 0.05</td>
<td>6.1 ± 0.6</td>
<td>0.40 ± 0.08</td>
</tr>
<tr>
<td>50</td>
<td>11</td>
<td>22 ± 3</td>
<td>21 ± 3</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>60</td>
<td>4</td>
<td>54 ± 2</td>
<td>50 ± 2</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>80</td>
<td>1.1</td>
<td>220 ± 40</td>
<td>206 ± 38</td>
<td>14 ± 5</td>
</tr>
</tbody>
</table>

An Eyring plot of temperature dependence of ks was used to obtain kinetic activation parameters according to the following relation

\[ \ln(k_a/T) = \ln(k_B/h) + \Delta S^R/T - \Delta H^R/(R.T) \]

where kB is Boltzmann’s constant and h is Planck’s constant. The kinetic activation parameters are: \( \Delta H^R = 79 ± 5 \) kJ mol\(^{-1}\) and \( \Delta S^R = -91 ± 16 \) J mol\(^{-1}\) K\(^{-1}\). Comess et al., in the case of the d(TCTACGCGTTCT) oligonucleotide, found \( \Delta H^R = 91 ± 2 \) kJ mol\(^{-1}\) and \( \Delta S^R = -58 ± 8 \) J mol\(^{-1}\) K\(^{-1}\) (7).

pH and salt. The oligonucleotide d12(CG*AG*) was incubated at 60°C in 10 mM NaClO₄, 5 mM buffer, 0.1 mM EDTA with various buffers covering the pH range 5–9 (above the pH of cytosine residues). As a first approximation, the rate of the reaction was unchanged between pH 5 and 9. At neutral pH the reaction rate was unchanged in NaCl or NaClO₄ and in the salt concentration range 10–400 mM.

Length of the oligonucleotide. Three platinated oligonucleotides d3(CG*AG*) (z is equal to 4, 6 or 12) having in common the central sequence d(CGAG) were incubated at 60°C in 10 mM NaClO₄, 5 mM phosphate buffer, pH 7, 0.1 mM EDTA. Aliquots were withdrawn at various times and analyzed by HPLC. As a function of the incubation time, two peaks were detected, corresponding to the starting product and to a new one, respectively. In a second set of experiments, the oligonucleotides were \(^32\)P 5’-end-labeled prior to incubation and then the reaction was followed by gel electrophoresis. For the three oligonucleotides, the appearance of a new product was noticed.

In Figure 5 are plotted, as a function of time, the relative concentrations of d3(CG*AG*) (5’-end-phosphorylated or not) deduced from the two experiments (gel electrophoresis, HPLC). The t₁/2 values are in the range 4–12 h for the dodecamer, the hexamer and the 5’-end-phosphorylated tetramer and significantly higher (~50 h) for the 5’-end-dephosphorylated tetramer. The phosphate group is not essential for the isomerization, but favors it.

It has been verified by HPLC analysis of the enzymatic digests that after incubation the adducts in the tetramer and hexamer were 1,4-trans-[Pt(NH₃)₂[d(CGAG)-N₃-C,N₇-G]] intrastrand cross-links.

Sequence. The rearrangement of the (G,G) intrastrand cross-link into the (C,G) intrastrand cross-link occurs in two oligonucleotides with the central sequence either d(CGCG) or d(CGAG). The possible role of the intervening residue in the rearrangement was further investigated by the study of d22(CG*AG*). The platinated oligonucleotide was incubated under the conditions described in the legend to Figure 2. At various times, aliquots were withdrawn, digested with T4 DNA polymerase and then analyzed by gel electrophoresis. In addition to the band corresponding to the starting product, only one new band was detected. It was verified by the DMS reaction and by HPLC analysis of the enzymatic digests of the new product (without treatment with T4 DNA polymerase) that the new product was d22(C*GTG*) containing the 1,4-intrastrand cross-link. The equilibrium constant for the rearrangement is about 6, in favor of the 1,4-intrastrand cross-link. The t₁/2 for disappearance of the (G,G) intrastrand cross-link is ~8 h at 60°C. As a first approximation, we conclude that rearrangement of the (G,G) intrastrand cross-link into the (C,G) intrastrand cross-link does not depend upon the nature of the intervening base.

We also wanted to know whether the linkage isomerization reaction could occur in sequences in which the C residue adjacent to the adduct on the 5’ side was replaced by A, G or T. The behavior of the three oligonucleotides d20(AAG*AG*), d20(GG*TG*) and d12(GA*ACG*) (in d12(GAGC) the base residue adjacent to the adduct on the 5’ side is a T) has been studied at 60°C and at neutral pH. The results can be summarized as follows. The (G,G) intrastrand cross-links within d20(AAG*AG*) and d12(GA*ACG*) were stable. No new products were detected. In the case of d20(GG*TG*), after 24 h incubation, a new band of very weak intensity (~10% of the intensity of the band corresponding to the starting material) was detected by gel electrophoresis after treatment with T4 DNA polymerase. Because of the small amount of available material, the new product was not further characterized and thus it was not proven that the 1,4-intrastrand cross-link was formed. Even if the rearrangement occurred, the yield of the reaction was so low that...
as a first approximation we conclude that replacement of the C residue adjacent to the (G,G) intrastrand cross-link on its 5' side by an A, T or G residue prevents rearrangement of the (G,G) intrastrand cross-link into a (C,G) intrastrand cross-link. Moreover, no rearrangement occurs when the C residue is adjacent to the adduct on its 3' side.

DISCUSSION

This work was devoted to the study of the stability of the trans-[Pt(NH3)2][d(GXG)-N7-G,N7-G]] intrastrand cross-links in trans-DDP-modified single-stranded oligonucleotides.

Comess et al. (7) were the first to report rearrangement of the trans-[Pt(NH3)2][d(CCGG)-N7-G,N7-G]] intrastrand cross-link into the 1,4-trans-[Pt(NH3)2][d(CCGG)-N3-C,N7-G]] intrastrand cross-link within a single-stranded oligonucleotide. We find that such a rearrangement also occurs in the sequences d(CGAG) and d(CGTA). The slight differences in the rates and the equilibrium constants of the rearrangement within the three sequences d(CGAG) (7), d(CGAG) and d(CGTA) show that the nature of the intervening base (A, T or C) does not play a major role in the reaction. Our results concerning the d(CGAG) sequence are in disagreement with those reported previously (7,8). At this point, we have no explanation for this disagreement. The residues on each side of the d(CGXG) sequence do not strongly interfere with the reaction, since it occurs even in the platinitated tetramer d(CGAG). Nevertheless, the reaction rate is faster in d(PCG*AG*) than in d(CG*AG*).

The reaction can be considered as direction-specific, in that it does not occur in the sequence d(TGAC), the C being adjacent to the 1,3-intrastrand cross-link on its 3' side. It can also be considered as sequence-specific, in that no rearrangement occurs if the 5' C residue adjacent to the (G,G) intrastrand cross-link is replaced by a G, T or A. Moreover, within d30(AGAG) no evidence was obtained for the formation of 1,5-intrastrand cross-links with the C residue on the 5' side of the d(AGAG) sequence.

A key question is to understand the role played by the 5' C residue adjacent to the (G,G) intrastrand cross-link in the linkage isomerization reaction. Considering the usual mechanism of labile ligand substitution in platinum(II) complexes (18), two extreme pathways can be proposed: (a) a two-step solvent-assisted process involving opening of the bifunctional cross-link to a monofunctional adduct and subsequent closure to a bifunctional cross-link; (b) a direct nucleophilic attack on the platinum residue by a base residue.

From the values of the activation parameters of the reaction, Comess et al. (7) tentatively propose that the isomerization occurs through an associative hydrolysis step. Although it is premature to draw conclusions, mechanism (b) could be relevant to explain our results, including a more negative entropy of activation and no trapping by chloride ions of monofunctional adducts [which are formed in the case of mechanism (a)]. Mechanism (b) requires that the platinitated oligonucleotides adopt a conformation allowing the nucleophilic N atom of the base residue adjacent to the adduct to attack the platinum residue in a well-defined direction. The reaction is faster in the platinitated tetramer d(PCG) than in the platinitated tetramer d(CGAG), which might suggest that the phosphate group stabilizes a conformation favoring the reaction. NMR studies are in progress to determine the conformation of the platinitated oligonucleotides.

Recently, we have shown that the trans-[Pt(NH3)2][d(GXG)-N7-G,N7-G]] intrastrand cross-link rearranges into interstrand cross-links as soon as the trans-DDP-modified oligonucleotides are hybridized with their complementary strands (9). [The double helix prevents intrastrand rearrangement of d(CG*AG*).] We have proposed that linkage isomerization results from a direct attack of the C residue complementary to the platinitated 5' G residue on the platinum residue. The instability of the (G,G) intrastrand cross-link, both in the single-stranded oligonucleotides and in the duplexes, could obey the same mechanism.

The instability of the trans-[Pt(NH3)2][d(GXG)-N7-G,N7-G]] cross-links within single-stranded oligonucleotides [X stands for C (7), A or T] is specific for the sequences d(CGXG) and is slow at 37°C, the half-life of the intrastrand cross-link being >38 h. This instability should not impede application of the double helix-promoted rearrangement of the trans- [Pt(NH3)2][d(GXG)-N7-G,N7-G]] intrastrand cross-link into interstrand cross-links in the context of the antisense strategy.

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