Reactions of the Double-Stranded Oligonucleotide d(TTGGCCAA)$_2$ with *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ and [Pt(NH$_3$)$_3$(H$_2$O)]$^{2+}$**

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Abstract: The kinetics of the reactions between the GG-containing double-stranded oligonucleotide d(TTGGCCAA)$_2$ (**II**) and the platinum complexes *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ (**1**) and [Pt(NH$_3$)$_3$(H$_2$O)]$^{2+}$ (**2**) were studied and compared with those already determined for the reactions of the single-stranded octanucleotide d(CTGGCTCA) (**I**).[1] The results were as follows: i) Complex **1** reacted faster than **2** with both **I** and **II**. ii) Both complexes **1** and **2** reacted faster with **II** than with **I**. This acceleration was greater for **1** (×13) than for **2** (×4) and only due to the increase of the platination rate of the 5′-G of the GG sequence. iii) For both **I** and **II**, the first platination by **1** and **2** was faster on the 5′-G than on the 3′-G. This difference was more significant for the platination of **II** ($k_{5'}/k_{3'} = 12$ for **1** and 5 for **2**) than of **I** ($k_{5'}/k_{3'} \leq 2$). iv) The cyclization reaction of the monoadducts (G*) of **1** to yield the GG *cis*-Pt(NH$_3$)$_2^{2+}$ chelate (G*G*) was considerably slowed down in the duplex. This rate decrease was significantly larger for the chelation of the 5′-G* (factor of 16) than of the 3′-G* (factor of 4) monoadducts. v) The intrastrand chelation of the 3′-G* monoadducts ($k_{3'c}$) was faster than that of the 5′-G* monoadducts ($k_{5'c}$), both for **I** and **II** ($k_{3'c}/k_{5'c} = 3$ and 13, respectively). vi) In addition to the intrastrand G*G* crosslink, we also observed the interstrand crosslink d(GG*CC)-d(GG*CC) between the two 3′-Gs of the central tetranucleotide. The rate constant for the interstrand crosslinking ($k_{3'i}$) was half that of the intrastrand chelation ($k_{3'c}$). vii) The 5′ monoadduct, which was formed faster ($k_{5'} > k_{3'}$) and was chelated more slowly ($k_{5'c} \ll k_{3'i} < k_{3'c}$), exhibited a half-life of 3.2 h under our experimental conditions.

Keywords
antitumor agents · DNA · kinetics · platinum complexes

Introduction

A number of results suggest that the antitumor activity of cisplatin is related to the biological processing of its major DNA intrastrand diadducts, the GG- and AG *cis*-Pt(NH$_3$)$_2$(*N7,N7*) chelates.[1-5] Monoadducts and interstrand diadducts, whose proportions might have been initially underestimated in in vitro and cell culture experiments,[6,7] have also been invoked as possible initiators of antitumor activity.[8-10] Recent studies of DNA platination that used enzymatic methods to identify DNA–platinum adducts revealed the presence of minor adducts on GA, GC, and TCAT sequences whose biological activities have not yet been investigated.[11,12]

The selective binding of cisplatin to (dG)$_n$ sequences of DNA, the competition between various intra- and interstrand chelation steps, and also DNA–protein crosslinking reactions are not yet fully understood. For the platination step, a contributing factor could be the mutual enhancement of negative electrostatic potential exhibited by adjacent guanines.[13,14] However, as we showed in Part I of this study (devoted to the kinetics of reactions of the complexes *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ (**1**) and [Pt(NH$_3$)$_3$(H$_2$O)]$^{2+}$ (**2**) with the single-stranded GG-containing oligonucleotides d(GG), d(TGG), d(TTGG) and d(CTGGCTCA) (**I**)), the ligands of platinum do affect the platination rate constant.[15] This observation suggested that other factors than the electrostatic potential play a role in the platination step, at least for single-stranded oligonucleotides. In the present part of our study, devoted to the reaction of the double-stranded oligonucleotide d(TTGGCCAA)$_2$ (**II**) with **1** and **2**, we show that the previously observed preferential binding to the 5′-G of a GG-sequence, as well as its dependence on the ligands of platinum, are considerably enhanced. Moreover, the duplex structure induces large differences between the chelation rates of the 5′- and 3′ monoadducts. For reactions run in nearly stoichiometric conditions, the calculation of the platination (for **1** and **2**) and chelation (for **1**) rate constants was based on the HPLC determination of the concentrations of all the species of the reaction mixture as a function of time, after KCl or KBr quenching of the reactive aqua monoadducts. This method has already proven its reliability[15-17] and turned out to be perfectly applicable to reactions of double-stranded oligonucleotides.

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[**] Kinetic Analysis of the Reactions Between GG-Containing Oligonucleotides and Platinum Complexes, Part II. Part I: Reactions of Single-Stranded Oligonucleotides with *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ and [Pt(NH$_3$)$_3$(H$_2$O)]$^{2+}$, ref. [15].

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Experimental Section

Starting materials and sample preparation: The self-complementary octanucleotide d(TTGGCCAA) was synthesized as its ammonium salt on a 100 μmol scale, based on phosphoramidite chemistry on solid support (Milligen 8800) [18]; the purity was checked by capillary electrophoresis and analytical HPLC by the group of J. Igolen and T. Huynh Dinh (Institut Pasteur, Paris, France). Approximate concentrations of $d(TTGGCCAA)_2$ were evaluated photometrically by means of the molar absorption coefficient ε_{260} ca. 128000 $M^{-1}cm^{-1}$. *Cis*-$[PtCl_2(NH_3)_2]$ was kindly provided by Johnson Matthey. Solutions of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and $[Pt(NH_3)_3(H_2O)]^{2+}$ were prepared from solid *cis*-$[Pt(NO_3)_2(NH_3)_2]$ and $[Pt(NO_3)(NH_3)_3](NO_3)$ as described elsewhere [15,17]. Perchloric acid and all salts were purchased from Merck. The exonuclease venom phosphodiesterase (VPD) was obtained from Boehringer-Mannheim.

The reactions were initiated by mixing a solution containing 1.2×10^{-7} mol of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**1**) or $[Pt(NH_3)_3(H_2O)]^{2+}$ (**2**) with a solution containing 1.5×10^{-7} mol of $d(TTGGCCAA)_2$. The initial concentrations were 10^{-4} M for $d(TTGGCCAA)_2$ and 0.8 or 1.6×10^{-4} M for **1** and **2** in 1.5 mL of 0.1 M $NaClO_4$ at 20±0.2 °C. The pH value was adjusted to and maintained at 4.4 by addition of $HClO_4$ by means of a pHstat (Metrohm) with 0.1 M $LiClO_4$ as internal electrolyte of the electrode. Aliquots were collected at several time intervals and quenched by addition of saturated KBr solution. After stirring for 1.75 min at 20 °C to allow exchange of the aqua ligands by halide [16], the samples were stored at liquid nitrogen temperature until they were analyzed.

HPLC analysis: The reactions were analyzed by HPLC. Two HPLC systems were used: i) a Spectra Physics SP8800 pump with a Spectra 100 UV detector and a Chromjet integrator (Spectra Physics, Thermo Separation Products, Les Ulis, France); ii) two Shimadzu LC-6A pumps, with a SPD-6A UV detector, and a C-R3A integrator (Shimadzu, Touzart & Matignon, Vitry-sur-Seine, France); both systems were equipped with a Rheodyne 7125 valve with a 20 μL sample loop. The chromatographic conditions were optimized for each reaction on a Kromasil C18 (250 × 4.6 mm ID, 5 μm, 100 Å) stainless steel column (Colochrom, Gagny, France). Operating conditions are described in the figure captions. Relative concentrations were determined from the ratio of the peak areas. The detection wavelength was 255 nm, chosen to be close to the quasi-isosbestic point of the reactions. At this wavelength, the absorbance of the reaction mixture did not vary appreciably upon the reaction of $d(TTGGCCAA)_2$ with the two complexes.

The nature of the oligonucleotides actually detected and quantified by HPLC during the platination of $d(TTGGCCAA)_2$ was determined as follows. The melting profile of the oligonucleotide dissolved in the HPLC eluent (KBr 0.4 M, $NH_4CH_3CO_2$ buffer 0.01 M, pH = 4.7, acetonitrile (93:7 v/v) or urea 1 M, NH_4Cl 0.1 M, $NH_4CH_3CO_2$ buffer 0.01 M, pH = 4.7, acetonitrile (94:6 v/v)) was recorded, first, directly after dissolution in this eluent and, second, immediately after collection of the oligonucleotide fraction at the outlet of the column. The first melting curve had the typical sigmoidal profile of $d(TTGGCCAA)_2$ (Fig. 1(1)), with a T_m of about 40 °C. After HPLC elution no such profile was found (Fig. 1(2)). The A/A_{70} value recorded corresponded to the single-stranded species showing that the duplex is denatured in the column. Thus the peaks analyzed in the chromatogram correspond to single-stranded species. The narrowness of the peaks is in agreement with elution of a single species; an equilibrium between single- and double-stranded forms would cause smearing of the peaks. The elution of single-stranded species is not surprising, taking into account the working temperature between 38 and 55 °C according to the selected analytical conditions.

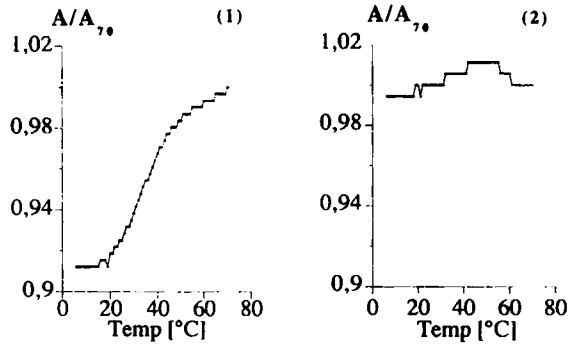

Fig. 1. 1) Melting profile of $d(TTGGCCAA)_2$ (9.8×10^{-5} M) dissolved in the HPLC eluent. 2) Melting profile of $d(TTGGCCAA)_2$ recorded immediately after collection of the oligonucleotide fraction at the outlet of the column after HPLC elution (for compositions see caption of Fig. 4a).

Enzymatic digestion of the platinated oligonucleotides: The quenched monoadducts were identified by enzymatic digestion with the 3'-exonuclease venom phosphodiesterase VPD (one enzymatic unit in 0.5 mL; Boehringer), as previously described [19,20]. The monoadducts of each reaction, quenched as bromo derivatives at the time of their maximum concentration in the reaction mixture (after about 15 min for **1**) were collected at the outlet of the column, digested by 10 μL of VPD preparation without addition of buffer at room temperature, and the digestion fragments were identified by coelution with reference samples. VPD digests the single strands from the 3'-end and stops at a platinated base. The digestion products of the monoadducts formed between **2** and the duplex **II** were identified, apart from mononucleotides, as d(TTG*) and d(TTGG*) (G* indicating the $Pt(NH_3)_3$ platinated base) by HPLC comparison with the authentic samples synthesized for this purpose. The 5' monoadduct was found to be the major one. In the same way, the major 5' monoadduct formed between **1** and **II** was identified after bromide quenching by digestion and HPLC by coelution with d(TTG*) (G* indicating the *cis*-$Pt(NH_3)_2Br$ platinated base) available from Part I of this study [15]. The amount of the purified minor monoadduct being very small (ca. 3% of the mixture), it had to be reconcentrated by lyophilization before VPD digestion. HPLC analysis of the reconcentrated sample showed, however, that most of this intermediate was readily converted into the GG chelate. This precluded its identification after enzymatic digestion. Nevertheless, the observed conversion to the intrastrand chelate was considered a sufficient proof that this intermediate was the 3' monoadduct.

Spectroscopic studies: The concentration and the melting profile of **II** were determined on a Uvikon 820 spectrophotometer. The CD spectra were recorded on a Jobin Yvon Mark IV spectrometer. The ESI/MS spectra were recorded on a Bio-Q triple quadrupole mass spectrometer (VG Fisons, Manchester, UK) with a mass/charge range of 10–4000. The extraction cone voltage was set to 50 V and the source ionization mode from $m/z = 300$ to $m/z = 1500$ in 12 s. The maximum entropy program MASS LYNX (Windows) was used to reconstitute a real mass scale spectrum.

Abstract in French: *Une étude cinétique fondée sur la chromatographie liquide à haute pression a permis d'analyser les réactions de platination du duplex $d(TTGGCCAA)_2$ (II) par les complexes cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (1) (dérivé diaqua du cisplatine) et $[Pt(NH_3)_3(H_2O)]^{2+}$ (2) et de les comparer à celles précédemment étudiées avec le simple brin d(CTGGCTCA) (I). Les résultats sont les suivants: i) le complexe 1 réagit plus vite que 2 avec I et II. ii) Les deux complexes 1 et 2 réagissent plus vite avec II qu'avec I (respectivement par un facteur × 13 et × 4) et cette accélération n'affecte que la platination de la guanine 5' (5'-G). iii) Pour 1 et 2, la platination de 5'-G est plus rapide que celle de 3'-G et cette différence est plus prononcée sur II ($k_{5'}/k_{3'} = 12$ pour 1 et 5 pour 2) que sur I ($k_{5'}/k_{3'} \leq 2$). iv) La cyclisation des monoadduits issus de 1 (G*), qui conduit au chélate (G*G*) est très ralentie sur le duplex, comparée à celle sur le simple brin. Ce ralentissement est de 16 pour le 5'-G* comparé à 4 pour le 3'-G*. v) La chélation intrabrin des monoadduits est plus rapide pour le 3'-G* que pour le 5'-G*, sur I et sur II (respectivement $k_{3'c}/k_{5'c} = 3$ et 13). vi) La séquence choisie met en compétition la chélation intrabrin et le pontage interbrin pour le monoadduit 3'-G*. La constante de vitesse de ce dernier ($k_{3'i}$) n'est que la moitié de celle de la chélation intrabrin ($k_{3'c}$). vii) Le monoadduit 5'-G* formé le plus vite ($k_{5'} > k_{3'}$) est chélaté le plus lentement ($k_{5'c} \ll k_{3'i} < k_{3'c}$) et sa durée de demi-vie est de 3.2 h dans nos conditions.*

Results

The aim of this work was to determine the rate constants for the platination of both guanines of the duplex $d(TTGGCCAA)_2$ (**II**) by the complexes *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**1**) and $[Pt(NH_3)_3(H_2O)]^{2+}$ (**2**) and, for the former, to measure the rate constants of the two intrastrand chelation reactions of the 5' and 3' monoadducts. In addition, the central d(GGCC)–d(GGCC) sequence is predisposed to 3'-G/3'-G interstrand crosslinking,[7] thus allowing the direct evaluation of the competition between intrastrand and interstrand chelations (Scheme 1).

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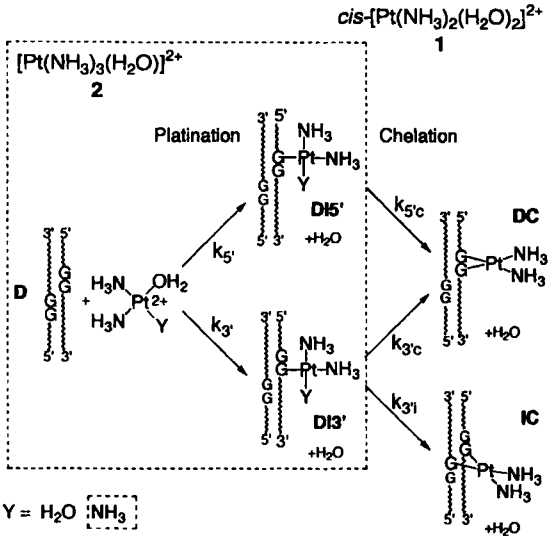

Scheme 1. Kinetic scheme for binding of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (Y = H_2O) and $[Pt(NH_3)_3(H_2O)]^{2+}$ (Y = NH_3) to a GG sequence of the double-stranded oligonucleotide $d(TTGGCCAA)_2$.

Duplex structure: To obtain the platination and chelation rate constants characteristic of a duplex structure, the following points had to be ascertained.

i) Stability of II: The melting profile of **II** was recorded under the conditions of the kinetic study (10^{-4} M, pH = 4.4, 0.1 M $NaClO_4$, 20 °C). Figure 2 reveals a typical sigmoidal curve with T_m = 43 °C, showing that at 20 °C **II** is almost entirely in the duplex form.

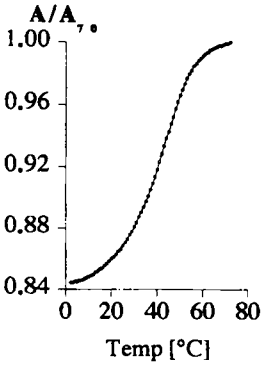

Fig. 2. Melting profile of $d(TTGGCCAA)_2$ (9.3×10^{-5} M), under the conditions of the kinetic experiment: 0.1 M $NaClO_4$, pH = 4.4.

ii) Duplex structure of the monoadducts and of the intrastrand diadduct of II: The duplex structure of the monoadducts was checked by a comparison of the CD spectra of **I** and **II** (recorded at pH 4.4 in 0.1 M $NaClO_4$ at 20 °C) with those of their monoadducts formed with **2**. Figure 3 shows that for the pure oligonucleotides, only **II** presents an excitonic signal characteristic of a duplex structure (Fig. 3, top, a). This signal is not altered after binding of the $Pt(NH_3)_3^{2+}$ moiety from **2** (b), which demonstrates the retention of the duplex structure. The final diadduct of **1** with **II** (Fig. 3, bottom, b) also presents the signal characteristic of a B-type DNA, although the ellipticity at 280 nm is slightly decreased. The *cis*-$[Pt(NH_3)_2d(GG)]^{2+}$ chelate usually slightly increases the ellipticity of duplex oligonucleotides and DNA at around 280 nm.[21-25] The small decrease observed here could result from the fraying of the AT ends of our short kinked duplex, altering base-pair stacking. Such a destabilization of GG-diadducts of *cis*-$[Pt(NH_3)_2]^{2+}$ has been observed previously.[24, 26, 27] The alternative explanation would be that the duplex platinum chelate dissociates completely, whereby the unplatinated self-complementary strands reassociate and form 0.5 equiv of free **II**. We started with 10^{-4} M **II** and 0.8×10^{-4} M **1**. After complete reaction, the dissociation

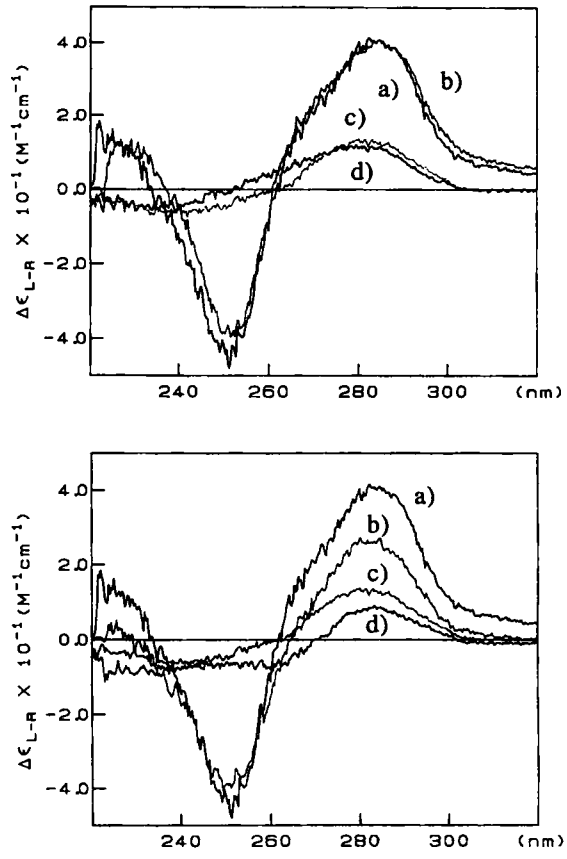

Fig. 3. Circular dichroism spectra (in 0.1 M $NaClO_4$ at pH = 4.4 and 20 °C) of: top, a) $d(TTGGCCAA)_2$: 10^{-4} M; b) $d(TTGGCCAA)_2$–$Pt(NH_3)_3$ (unresolved mixture of both adducts): 10^{-4} M; c) d(CTGGCTCA): 2×10^{-4} M; d) d(CTGGCTCA)–$Pt(NH_3)_3$ (unresolved mixture of both adducts): 2×10^{-4} M; bottom, a) $d(TTGGCCAA)_2$: 10^{-4} M; b) $d(TTGGCCAA)_2$–*cis*$Pt(NH_3)_2$ (final chelate): 10^{-4} M; c) d(CTGGCTCA): 2×10^{-4} M; d) d(CTGGCTCA)–*cis*$Pt(NH_3)_2$ (final chelate): 2×10^{-4} M.

of the duplex **II**–*cis*-$Pt(NH_3)_2$ should give 0.8×10^{-4} M of platinated single strand together with 0.6×10^{-4} M unplatinated duplex **II**. If we try to reconstitute the CD spectrum of such a mixture from the spectra of **II** (Fig. 3, bottom, a) and the GG-chelate of **I** (d), we do not obtain an acceptable fit in the 250 nm region. Complete dissociation of the diadduct of **II** and reassociation of the unplatinated strands are therefore unlikely to have occurred under our experimental conditions.

The results described in i) and ii) strongly suggest that the chelation of the monoadducts of **1** actually occurs on the duplex structure in agreement with Scheme 1, and that the final intrastrand adduct on **II** is stable and does not release the unplatinated strand.

HPLC analysis of the reactions: The chromatograms in Figure 4 show the excellent separation obtained for both reactions between **II** and complexes **1** and **2**; this allowed accurate measurement of concentration ratios of the various single-stranded species (see Experimental Section). The 5′ and 3′ monoadducts, together with the GG-chelate, were identified by VPD digestion (Experimental Section). This revealed that the major adduct formed by **2** was the 5′-G complex. For the reaction of **1**, the major monoadduct trapped by bromide was also the 5′-G intermediate I5′, which was much more abundant than the 3′-G intermediate I3′. Both I5′ and I3′ were converted into the GG-chelate C. Moreover, a small peak at considerably longer retention time (labeled IC), which is not digested by VPD, is also apparent in Figure 4a.

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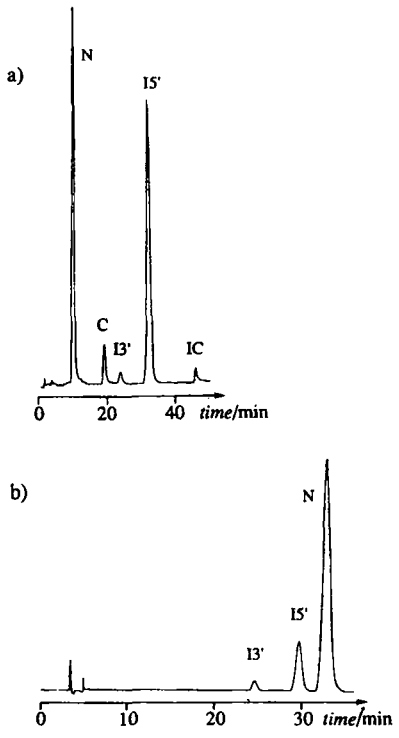

Fig. 4. Typical chromatogram for reaction mixtures quenched after 15 minutes by addition of an excess of KBr. Reaction conditions: 0.1 M $NaClO_4$, pH = 4.4, T = 20 °C, $d(TTGGCCAA)_2$ 10^{-4} M, **1** or **2** 0.8×10^{-4} M. (N = d(TTGGCCAA); I5' = 5' monoadduct; I3' = 3' monoadduct; C = intrastrand chelate; IC = interstrand chelate). a) *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with $d(TTGGCCAA)_2$, Kromasil C18 column; mobile phase: potassium bromide 0.4 M, ammonium acetate buffer 0.01 M, pH = 4.7, acetonitrile gradient: 7% for 30 min; 7 to 9% from 30 to 50 min, 9% from 50 to 60 min; flow rate 0.8 mL min^{-1}; T = 38 °C. b) $[Pt(NH_3)_3(H_2O)]^{2+}$ with $d(TTGGCCAA)_2$, Kromasil C18 column; mobile phase: urea 1 M, ammonium chloride 0.1 M, ammonium acetate buffer 0.01 M, pH = 4.7, acetonitrile (94:6 v/v); flow rate 0.8 mL min^{-1}; T = 55 °C.

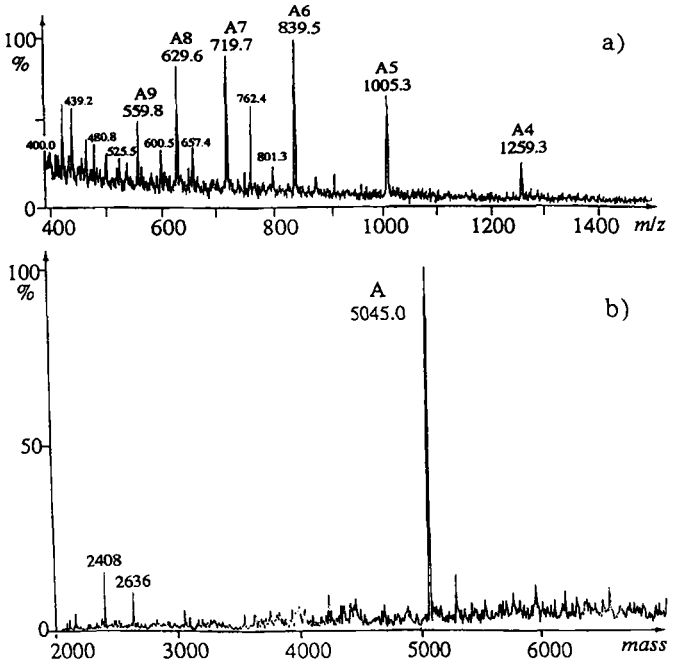

Fig. 5. a) Experimental and b) transformed ESI/MS spectra of the interstrand chelate, d(TTGG*CCAA)-d(TTGG*CCAA). In order to record the spectrum, the platination reaction was run to completion and the mixture purified by HPLC with aqueous acetonitrile (1:1) and 10^{-3} M triethylammonium acetate (Merck). A sample of the last peak was collected from the HPLC outlet, lyophilized, and rediluted to a concentration of approximately 20 pmol μL^{-1} in aqueous acetonitrile (1:1) containing 0.5% triethylamine.

Identification of an interstrand chelate: Whereas in reactions of **I** with **1** three major reaction products (two intermediates and one final product) were observed, the reaction of **II** with **1** gave four reaction products. Two of them were intermediate species (Fig. 4a). The three major products had similar retention times. The fourth product (IC), which is a final adduct, was eluted after a considerably longer time and only upon use of an acetonitrile gradient. It was collected at the outlet of the column and analyzed by ESI/MS. Experimental and reconstructed spectra are shown in Figure 5. The peaks at m/z = 1259.3, 1007.3, 839.5, 719.7, 629.6, and 559.8 are assigned to a major product of molecular weight M = 5045 Da. This molecular weight corresponds to an interstrand chelate, in which two octanucleotides are crosslinked by a $Pt(NH_3)_2$ moiety, with a theoretical molecular weight of 5049 Da. For such a species, the theoretical multicharged ions should cause peaks at m/z = 1261.2, 1008.8, 840.5, 720.3, 630.1, and 560.0. We checked that under these analytical conditions the unplatinated duplex did not give any peaks due to nonspecific oligonucleotide di- or oligomerization.

There are a few peaks which have not been assigned in the presented mass spectrum. For one of them, m/z = 762.4, we cannot propose an assignment. However, we can identify the fragmentation (1), which leads to species with molecular weights of M = 2639 and 2409.9 Da. This fragmentation is

cis-$[Pt(NH_3)_2d(TTGGCCAA)d(TTGGCCAA)] \longrightarrow$
cis-$[Pt(NH_3)_2d(TTGGCCAA)] + d(TTGGCCAA)$ (1)

based on the interpretation of the peaks at m/z = 657.4, 525.0, and 439.2, which are assigned to a species with molecular weight of M = 2639 Da with charge states of 4, 5, and 6, respectively, and of the peaks at m/z = 801.3, 600.5, 480.8, and 400.0, which are assigned to a species with molecular weight of M = 2409.9 Da with charge states of 3, 4, 5, and 6, respectively. For the species with molecular weight M = 2639 Da, the theoretical multicharged ions should be m/z = 658.8, 526.8, and 438.8, and for the species with molecular weight of M = 2409.9 Da, they should be m/z = 802.3, 601.5, 481.0, and 400.7.

Possibility of double platination: As we have recently found, the monoadducts of **I** with complex **2** can easily react with a second equivalent of **2**, yielding a species with each guanine coordinated to one $Pt(NH_3)_3^{2+}$ unit.[17] With the bifunctional complex **1**, no such diplatinated species were observed, presumably because intrastrand chelation effectively competes with the bimolecular reaction with a second platinum complex. In the duplex **II**, there are four guanines which, in principle, could coordinate a platinum center.

We have investigated the reaction between **II** and **2** with an approximately twofold molar excess of **2** and found that, indeed, more than 50% of the single-stranded species (detected by HPLC in the denaturing conditions used) were platinated, indicating that a part of the duplex reacted with more than 1 equiv of **2**. On the other hand, we did not detect any new peak attributable to a diadduct of the same strand. An observation characteristic of the use of **2** in excess was an increased ratio of the 5' adduct to the 3' adduct. Since, in a monoplatinated duplex, the 3'-G of the unplatinated complementary strand is closer to the platinum residue, its reaction with a second platinum could be slightly more inhibited than that of the 5'-G, which would favor the platination of the 5'-G towards the end of the reaction. Importantly, this time dependence of the ratio between

the 5′ and 3′ (mono)adducts detected by HPLC was not observed if the initial molar ratio between **II** and **2** was ≤ 0.8. We conclude that if the platinum complex is not in excess, the diplatination is negligible. Pseudo-first-order conditions with the platinum complex in excess, which are frequently used in kinetic measurements, are particularly prone to cumulative platination. In order to minimize the risk of the coordination of more than one platinum atom by the same oligonucleotide, we ran all the experiments with a platinum/duplex ratio of ≤ 0.8.

Optimized rate constants: The kinetic scheme for the reactions of the double-stranded octanucleotide **II** with complexes **1** and **2** is similar to that for the reactions of the single-stranded oligonucleotide **I**[15] and d(TGG),[16] except for two modifications: i) the possibility of interstrand crosslinking; ii) the possibility of a second platination on the complementary strand to that bearing a mono- or diadduct. The occurrence of interstrand crosslinking has been established (vide supra) and was included in the kinetic scheme (Scheme 1).

The relative percentages of the single-stranded species detected by HPLC (N, I5′, I3′, and C for the reaction with **1**: for abbreviations see Fig. 4), were converted into those corresponding to the double-stranded species (D, DI5′, DI3′ and DC) actually involved in the reaction. At 255 nm the extinction coefficient ε of the interstrand chelate (IC) was assumed to be twice that of the single-stranded monoadducts, that is, $\varepsilon N = \varepsilon I5' = \varepsilon I3' = \varepsilon C = {}^1/_2\varepsilon IC$. This conversion is expressed by equations (i)–(iii).

$$D(\%) = (N(\%) - 50 + 0.5\,IC(\%)) \times 2 \quad \text{(i)}$$

$$DI(\%) = I(\%) \times 2 \quad \text{(ii)}$$

$$DC(\%) = C(\%) \times 2 \quad \text{(iii)}$$

Differential equations used for the optimization of the rate constants:

a) reaction **II** + **1**

$$\frac{d[DI5']}{dt} = k_{5'}[D][Pt] - k_{5'c}[DI5']$$

$$\frac{d[DI3']}{dt} = k_{3'}[D][Pt] - (k_{3'c} + k_{3'i})[DI3']$$

$$\frac{d[DC]}{dt} = k_{3'c}[DI3'] + k_{5'c}[DI5']$$

$$\frac{d[IC]}{dt} = k_{3'i}[DI3']$$

$$[D] = [D]_0 - ([DI5'] + [DI3'] + [DC] + [IC])$$

b) reaction **II** + **2**

$$\frac{d[DI5']}{dt} = k_{5'}[D][Pt]$$

$$\frac{d[DI3']}{dt} = k_{3'}[D][Pt]$$

$$[D] = [D]_0 - ([DI5'] + [DI3'])$$

The differential equations above corresponding to the reactions shown in Scheme 1 were integrated numerically and the rate constants iterated by means of the program ITERAT until the best fit with the experimental points was obtained.[28] Representative fits of experimental concentration curves with those calculated from optimized rate constants are shown in Figures 6a and 6b. The averaged rate constants calculated are presented in Table 1, together with values determined previously for the reactions of **I**.[15]

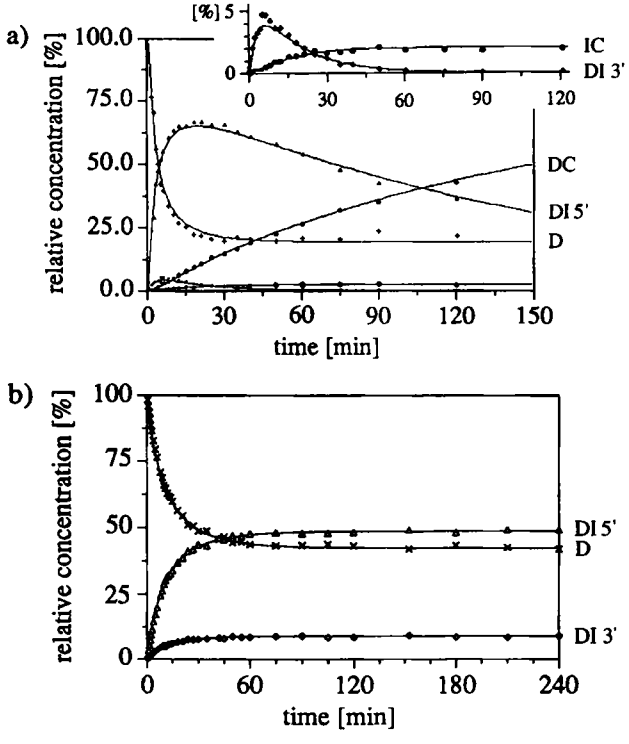

Fig. 6. Calculated curves and experimental points for the reaction between d(TTGGCCAA)$_2$ and a) *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (+: unreacted d(TTGGCCAA)$_2$ (D); ▲: 5′ monoadduct (DI5′); ·: intrastrand chelate (DC); ◆: 3′ monoadduct (DI3′); ●: interstrand chelate (IC)). b) $[Pt(NH_3)_3(H_2O)]^{2+}$ (×: unreacted d(TTGGCCAA)$_2$ (D); △: 5′ adduct (DI5′); ◇: 3′ adduct (DI3′)).

Table 1. Optimized rate constants and their comparison for the reactions of **I** and **II** with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and $[Pt(NH_3)_3(H_2O)]^{2+}$ at pH 4.4, 20°C in 0.1 M $NaClO_4$ [a,b].

		With *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**1**)							
Oligonucleotide	No. of runs	$k_{5'}$ [$M^{-1}s^{-1}$]	$k_{3'}$ [$M^{-1}s^{-1}$]	$k_{5'}/k_{3'}$	$10^3 \times k_{5'c}$ [s^{-1}]	$10^3 \times k_{3'c}$ [s^{-1}]	$k_{3'c}/k_{5'c}$	$10^3 \times k_{3'i}$ [s^{-1}]	$k_{3'c}/k_{3'i}$
d(CTGGCTCA) (**I**)	4	4.2(6)	2.0(2)	**2.1**	1.0(3)	3.3(4)	**3.3**	–	–
d(TTGGCCAA)$_2$ (**II**)	4	54(7)	4.4(7)	**12**	0.06(4)	0.8(2)	**13**	0.4(1)	**2**
II/**I**:		**13**	**2**						
I/**II**:					**16**	**4**			

	With $[Pt(NH_3)_3(H_2O)]^{2+}$ (**2**)				1/2	
Oligonucleotide	No. of runs	$k_{5'}$ [$M^{-1}s^{-1}$]	$k_{3'}$ [$M^{-1}s^{-1}$]	$k_{5'}/k_{3'}$	$k_{5'}(1)/k_{5'}(2)$	$k_{3'}(1)/k_{3'}(2)$
d(CTGGCTCA) (**I**)	4	1.1(1)	0.49(5)	**2.2**	**I** **4**	**4**
d(TTGGCCAA)$_2$ (**II**)	4	4.5(5)	0.9(1)	**5**	**II** **12**	**5**
II/**I**		**4**	**1.8**			

[a] Labeling of the rate constants is defined in Scheme 1. [b] Esd's in parentheses.

0947-6539/96/0209-1072 $ 15.00+.25/0 Chem. Eur. J. 1996, 2, No. 9

Discussion

In Part I of this project,[15] we investigated the reactions between the platinum complexes *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**1**) and $[Pt(NH_3)_3(H_2O)]^{2+}$ (**2**) with single-stranded di- to octanucleotides containing the d(GG) sequence. The main observations were the following: i) **1** reacts faster than **2** with all the oligonucleotides studied; ii) both complexes show a slight preference for the 5′-G of the GG sequence; iii) the 3′ monoadduct of **1** forms the final chelate slightly faster than the 5′ monoadduct does. The rate constants listed in Table 1 indicate that for the reactions of the same complexes with the duplex octanucleotide **II**, all the above trends are maintained. Both studies were conducted at pH 4.4 in order to keep the complexes **1** and **2** in their aqua forms.[15, 29] The stability of the duplex structure in the conditions of the kinetic experiment (0.1 M $NaClO_4$ at 20 °C) was checked by CD.

The first finding of the present work is that a GG sequence is platinated considerably faster in a duplex than in a single-stranded oligonucleotide of the same length. The rate difference observed cannot be accounted for by the sole presence of two GG units in the duplex **II** compared with one in the single-strand **I**. Going from **I** to **II** the platination rate of the 5′-G within the GG sequence increases by a factor of 13 for complex **1** and 4 for complex **2**. Taking into account the presence of the two GG units in the duplex **II**, the acceleration factors are 6.5 and 2. For a reaction between positively charged platinum complexes and negatively charged oligonucleotides, one of the factors determining the different reaction rates could be the more negative charge of the double helix compared with that of the single strand. As the negative charges of the two strands add in a duplex, the electrostatic attraction towards cations should increase too.

The platination reaction is expected to proceed by the rapid formation of an outer-sphere complex followed by the rate-determining substitution of an aqua ligand by a guanine N7;[30] this step is irreversible under our experimental conditions. For example, for the reaction of complex **1** with the 5′-G of **II** to form the monoadduct, the kinetic scheme is that of Equation (2) ([D ··· **1**] represents the outer sphere complex; K_a = association equilibrium constant; $k_{5'}$ = substitution rate constant).

$$D + 1 \underset{k_{5'}}{\overset{K_a}{\rightleftharpoons}} [D\cdots 1] \xrightarrow{k_{5'}} D1_{5'} \qquad (2)$$

The value of the association equilibrium constant K_a is unknown. It is expected to be larger for **II** than for **I** in the reactions with both dicationic complexes, **1** and **2**. For hydrated Mg^{2+} ions interacting with poly(dG–dC)$_2$, poly(A)–poly(U), poly(I)–poly(C) and poly (G)–poly(C) at pH = 7, T = 20–25 °C and an ionic strength of 0.1 M, a K_a value of 1000–2000 M^{-1} has been determined.[31, 32] For our dicationic platinum complexes surrounded by four ammine and aqua ligands, also likely to interact with hydrogen-bond acceptor groups of the oligonucleotides, K_a values might be expected to range between 10^2 to 10^3 M^{-1}. The determination of this constant as a function of ionic strength is underway in our laboratory; this study should enable us to evaluate the contribution of K_a to the observed rate constants.

Recently, Elmroth and Lippard examined the platination of a phosphorothioate group by *cis*-$[PtCl(NH_3)(NH_2C_6H_5)(OH_2)]^+$.[29, 33] They found an approximately 40-fold rate increase for d(T_8p(S)T_8) compared with d(Tp(S)T), but no difference between platination kinetics of the single-stranded species d(T_8p(S)T_8) and that of the duplex [d(T_8p(S)T_8)–d(A_{16})]. Three factors could account for the discrepancy between their results and ours: i) The phosphorothioate sulfur is located on the phosphate "ridge", whereas the purine N7 atoms are situated in the major groove of the duplex. In the grooves, the electrostatic potentials of the single strands combine almost additively,[14] whereas a smaller mutual enhancement of the negative potentials is expected at the periphery of the helix. ii) *cis*-$[PtCl(NH_3)(NH_2C_6H_5)(OH_2)]^+$ is monocationic, whereas the complexes studied in our work are dications. The reactivity of the latter is expected to be more strongly dependent on the oligonucleotide electrostatic potential. iii) The enhancement of the rate constants for the platination of the duplex **II** compared with the single strand **I** could result from structure-specific binding interactions such as hydrogen bonds. This hypothesis is supported by the fact that the increase in reactivity of the GG sequence in the duplex is caused only by the 5′-G, the duplex structure inducing no acceleration of the platination of the 3′-G; Table 1 shows the accelerations by factors of 13 and 4 found for the 5′-G for complexes **1** and **2**, respectively. Calculation of the influence of the neighboring bases on the electrostatic potential at the N7 atom of a guanine within double-stranded DNA[13] yields a difference of 3.4 kJ mol^{-1} in favor of the central **G** of d(TGG) compared with that of d(GGC), which indicates that the 5′-G of **II** should be more nucleophilic than the 3′-G. On the other hand, the same type of calculations predict an increase in the electrostatic potential in the central part of a duplex structure compared with the ends, an effect which should favor the 3′-G in **II**.[14] It appears unlikely that the sum of these two compensating effects could account for one order of magnitude between $k_{5'}$ and $k_{3'}$. Finally, it has to be pointed out that the kinetic measurements by Elmroth and Lippard were made in a phosphate buffer.[29, 33] The buffer is expected to compete effectively with the oligonucleotide as nucleophile.[34] Therefore, it cannot be taken for granted that the observed pseudo-first-order rate constants reflect the net reaction of the phosphorothioate with *cis*-$[PtCl(NH_3)(NH_2C_6H_5)(OH_2)]^+$.

The second finding of this work is that complexes **1** and **2**, which are both dicationic, exhibit large differences in their platination rates and selectivities. **1** is four times more reactive than **2** towards the 5′- and 3′-Gs of the single strand **I**. The differences are still more pronounced for their reaction with the duplex **II** (Table 1). Neither the constant K_a for outer-sphere association with **II**, which is not expected to differ greatly for dicationic complexes **1** and **2**, nor the electrostatic potentials of the two guanines can account for the observed data. As we pointed out in Part I,[15] the different rate constants observed for **1** and **2** could result, in principle, from three effects: i) different electron-donating properties of H_2O and NH_3 modifying the electrophilic character of the two Pt atoms; ii) different *cis*-effects of H_2O and NH_3; iii) sequence- and structure-specific interactions between Pt ligands and DNA residues. The fact that on substitution of **II** for **I**, the rate constants change differently for **1** and **2**, strongly suggests that effect iii) is involved.

Part III of our work on this topic, devoted to *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ and *cis*-$[Pt(OH)(NH_3)_2(OH_2)]^+$ with chloro and hydroxo ligands in place of an ammine or aqua ligand, will yield relevant rate constants for different complexes reacting with the same duplex. This will allow an identification of the interactions that control the rate and selectivity of platination. Preliminary results from the reaction of *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ with **II** indicate similar rates of platination of the two guanines of the GG sequence.[36] P. J. Sadler and coworkers monitored the reaction of cisplatin with 10- and 14-mer single and double-stranded oligonucleotides containing a

  0947-6539/96/0209-1073 $ 15.00+.25/0

GG sequence by means of ^{1}H and ^{15}N NMR direct observation of $^{15}NH_3$ ligands; they found only a factor of approximately 4 between the platination rates of the two guanines.[37]

The third finding of this work is the large difference between the intrastrand chelation rates of the monoaqua monoadducts on **I** and **II**. Whereas the 3′ monoadduct is chelated about 3 times faster than the 5′ monoadduct on the single-strand **I**, this ratio rises to 13 on the duplex **II**. This actually results from the fact that the chelation of the 5′ monoadduct of **II** is 16 times slower than that of **I**, and the chelation of the 3′ monoadduct is only 4 times slower. The overall retardation is readily understandable, since the deformation of the DNA helix necessary to accomodate the intrastrand crosslink[26, 38, 39] is much more easily achieved in the single strand. The considerably slower chelation of the 5′ monoadduct than of the 3′ monoadduct follows from the distance between the bound Pt atom and the N7 atom of the adjacent guanine in a double helix, which is longer in the 5′ monoadduct, as already noted by Dewan.[40]

However, another factor contributing to the differences between the chelation rates could be the inherent reactivity of the two guanines. The large difference between the platination rate constants $k_{5'}$ and $k_{3'}$ and between the chelation rate constants could be caused, at least in part, by a more strongly nucleophilic character of the N7 atom of the 5′-guanine. In fact, $k_{5'}/k_{3'} \approx k_{3'c}/k_{5'c}$ for both **I** and **II** (Table 1), and one could interpret this as a result of both ratios originating from the inherently greater reactivity of the 5′-G. However, the different ratios $k_{5'}/k_{3'}$ measured for the platination of **II** with **1** and **2** (12 and 5, respectively) suggest that the ligands and their interactions with the oligonucleotide play a role in the discrimination between the two guanines as well. It appears therefore that the chelation rate constants are determined by a rather complicated combination of effects, which depend on the platinum complex and on the oligonucleotide structure. The fact that in this case intrastrand chelation competes with interstrand crosslinking sheds more light on the factors controlling the chelation step, as will be discussed in the following paragraph.

The fourth finding of this work is that GG interstrand crosslinking competes with GG-intrastrand chelation. There have been conflicting reports on the occurrence and biological significance of interstrand crosslinks in vivo.[41, 42] In this work we were able to determine simultaneously for the first time the rate constants for intra- and interstrand GG-crosslinking of the same monoadduct. The duplex **II** contains the central d(GGCC)-d(GGCC) sequence; the 3′ monoadduct, d(GG*CC)-d(GGCC) with *cis*-$[Pt(NH_3)_2(H_2O)]^{2+}$ bound to G* (Fig. 7), forms the interstrand crosslink d(GG*CC)-d(GG*CC) with a rate about half that of intrastrand chelation leading to d(G*G*CC)-d(GGCC). Our results are in agreement with the observation that cisplatin monoadducts of the d(AG*C)-d(GCT) sequence within a 22-mer yield only the interstrand crosslink d(AG*C)-d(G*CT).[7] Since adenine binds to platinum about one order of magnitude more slowly than guanine, extrapolation of our data to the d(AGC)-d(GCT) sequence gives an estimated ratio of ≥ 5 in favor of the interstrand GG- over the intrastrand AG-crosslink.

The fact that the rate constant for the interstrand chelation of the 3′ monoadduct ($k_{3'i}$) is of the same order of magnitude as that for the intrastrand chelation ($k_{3'c}$) (Table 1) indicates that the distance is not the only criterion: as an inspection of the model in Figure 7 shows, the two relevant Pt-N7 distances are very different (7.5 and 3.9 Å, respectively), whereas for the 5′ monoadduct the Pt-N7 distance for the (much slower) intrastrand chelation lies between the two values (5.5 Å, model not shown). The chelation rate will certainly depend on the strain energy in the transition state, which is obviously not simply related to the distance between the two atoms that have to be linked.

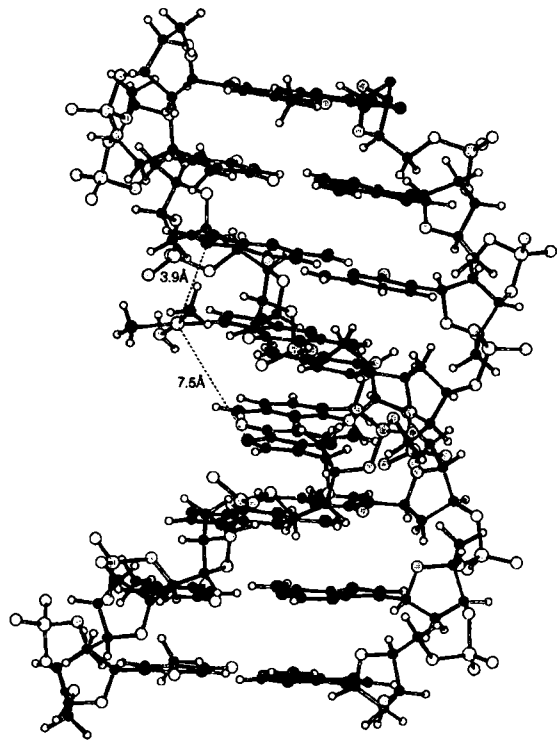

Fig. 7. Arnott B-DNA model [47] of the duplex d(TTGG*CCAA)-d(TTGGCCAA) with *cis*-$[Pt(NH_3)_2(H_2O)]^{2+}$ bound to the N7 atom of the G* guanine. The position of the platinum complex corresponds to a local energy minimum obtained by a belly minimization with the oligonucleotide atoms fixed, with force-field data described elsewhere [26].

The observation that interstrand 3′-G*/3′-G crosslinking competes with intrastrand 3′-G*/5′-G chelation is also noteworthy from another point of view: since the interstrand crosslinking involves the presumably less nucleophilic 3′-G of the complementary strand as entering ligand, we see once more that the relative nucleophilicities of the 5′- and 3′-G discussed above cannot be the only parameters controlling the chelation steps.

It is interesting that the small final yield of the interstrand chelate (5-7%) is not due to a small interstrand chelation constant (which is of the same order of magnitude as the faster intrastrand chelation constant, $k_{3'c}$) but to the smaller platination rate constant, $k_{3'}$, compared with $k_{5'}$. Since the interstrand chelate is formed only from the 3′ monoadduct, it is the selectivity of the platination step which disfavors the formation of the interstrand crosslink. A possible strategy towards the conception of platinum complexes yielding increased levels of interstrand crosslinks on DNA would therefore rely on a reversal of the selective 5′-platination of GG sequences to selective 3′-platination.

Another point to mention is that the faster-formed ($\times 10$) and more slowly chelated ($\times {}^{1}/_{10}$) 5′ monoadduct accumulates and persists in a significant concentration over several hours (Fig. 6a). This finding is important in view of the potential of such reactive intermediates to form covalent DNA-protein crosslinks (see next paragraph).

Relevance of this work to cisplatin-DNA interactions in vivo: In cells, the first step of the reaction between cisplatin and DNA is the hydrolysis of *cis*-$[PtCl_2(NH_3)_2]$ to *cis*-$[PtCl(NH_3)_2(H_2O)]^{+}$.[43] As we have discussed earlier,[44] *cis*-$[PtCl(NH_3)_2$-

0947-6539/96/0209-1074 $ 15.00+.25/0 Chem. Eur. J. 1996, 2, No. 9

$(OH_2)]^+$ can react with DNA either directly or by a second hydrolysis step to *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. The fairly complicated kinetic analysis of the reactions between *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ and an oligonucleotide will have to take into account both pathways, and the rate constants determined for the diaqua complex in this work will be a prerequisite for the calculation of the remaining unknown rate constants. The latter will include the rate constants for the hydrolysis of the chloro monoadducts. Our preliminary results[36] indicate that the chloro monoadduct on the 3′-G is hydrolyzed one order of magnitude faster than that on the 5′-G, with respective lifetimes ranging from several hours to three days at 20 °C. Compared with the lifetimes of approximately 30 min to approximately 3 h of the 3′-G and 5′-G aqua monoadducts (Fig. 6a), this shows that the aquation of the chloro monoadducts is the rate-determining step for the chelation of the latter. A similar conclusion has previously been drawn by Bancroft et al.,[45] who followed the reaction of *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ with chicken erythrocyte DNA by ^{195}Pt NMR and detected accumulating chloro monoadducts but no aqua monoadducts. Comparable results were recently obtained by Sadler and co-workers, who studied the platination of a decanucleotide duplex by ^1H and ^{15}N NMR. They detected no aqua monoadduct and found that one chloro monoadduct ring-closed much faster than the other.[37] The absence of accumulation of aqua monoadducts also indicates that *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ is not the predominant species reacting with DNA under these experimental conditions. In our opinion, it would be premature to extrapolate this result to in vivo conditions. What we can state, however, is that regardless of which pathway predominates, one sort of monoadduct (chloro or aqua) will accumulate for a period of minutes to hours at 37 °C. Evidence for persisting monoadducts in different organs of cisplatin-treated LOU/M rats emerges from the study of Fichtinger-Schepman et al.[46] The quantification method did not allow a distinction between chloro and aqua monoadducts, however. As we have recently demonstrated,[10] long-living monoadducts are excellent candidates for forming covalent crosslinks between DNA and recognition proteins.

Conclusion

By means of HPLC-based methodology, we have carried out a complete kinetic analysis of the reaction between the duplex $d(TTGGCCAA)_2$ **(II)** and the diaqua derivative of cisplatin *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ **(1)** at pH 4.4, 0.1 M ionic strength and 20 °C. We have determined the rate constants of the two platination reactions with the 5′- and 3′-guanines of the GG-sequence and the rate constants of the three crosslinking reactions of the monoadducts, two intrastrand chelations (of the 5′-G* and 3′-G* monoadducts), and one interstrand chelation of the 3′-G* monoadduct of one strand by the 3′-G of the other strand. The duplex structure introduces a strong differentiation between the two guanines. While the 5′-G* monoadduct is formed one order of magnitude faster than the 3′-G* monoadduct, it is chelated one order of magnitude more slowly. Selective binding to the 5′-G is also observed for $[Pt(NH_3)_3(H_2O)]^{2+}$ **(2)**, but to a much lesser extent than for **1**, pointing to the role of the metal ligands in the orientation of the reactions with DNA. The 3′-G* monoadduct of **1** also undergoes interstrand crosslinking at a rate half that of intrastrand chelation. Since the duplex structure accelerates the formation of the 5′-G* monoadduct and slows down its chelation, this monoadduct accumulates. Such a monoadduct on DNA, bearing a labile ligand, might crosslink DNA-binding proteins.

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