GG versus AG Platination: A Kinetic Study on Hairpin-Stabilized Duplex Oligonucleotides

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The kinetics of the reactions between the diaqua form of the antitumor drug cisplatin, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, and two hairpin-stabilized duplex oligonucleotides, d(TAT*GG*TATTTTTATACCATA) (**I**) and d(TAT*AG*TATTTTATACTATA) (**II**), were investigated. Oligonucleotides **I** and **II** were used as models for GG and AG sequences within duplex DNA, which are known as the major sites of platinum binding. The two GG guanines of **I** are shown to react with similar rates ($k_{5'} = 18 \pm 2$ and $k_{3'} = 15 \pm 1$ M^{-1} s^{-1}), roughly twice as fast as the AG guanine of **II** ($k_{3'} = 9 \pm 1$ M^{-1} s^{-1}). Platination of the AG adenine of **II** was also observed to a minor extent ($k_{5'} = 1.5 \pm 0.3$ M^{-1} s^{-1}), whereas no other adenine of **I** or **II** was platinated to a detectable extent. The overall platination rate of **I** is approximately three times larger than that of **II**. The 3′-monoadduct of **I** undergoes chelation to the GG intrastrand adduct with a rate 10.5 times larger than the 5′-monoadduct ($k_{3'c} = (1.9 \pm 0.1) \times 10^{-3}$ s^{-1} and $k_{5'c} = (0.18 \pm 0.05) \times 10^{-3}$ s^{-1}). For **II**, the chelation rate constants of the guanine- and adenine-bound monoadducts are $k_{5'c} = 0.3 \pm 0.1$ and $k_{3'c} = 0.08 \pm 0.01$ s^{-1}, respectively. These results are discussed in relation to the platination kinetics determined for other model systems.

Introduction

The antitumor drug *cis*-$[PtCl_2(NH_3)_2]$ ("cisplatin") binds selectively to GG sequences of DNA, forming 60−65% of N*7*(G)−N*7*(G) chelates; the second major adduct is the N*7*(A)−N*7*(G) chelate, accounting for ∼20% of the platinum bound to DNA.[1−4] These two major adducts have been found to display different biological activities. For instance, DNA polymerases are inhibited more severely by *cis*-$[Pt(NH_3)_2$\{d(GpG)-N*7*(1),N*7*(2)\}] than by *cis*-$[Pt(NH_3)_2$\{d(ApG)-N*7*(1),N*7*(2)\}] chelates.[5] In bacteria, the AG chelates are repaired more efficiently [6,7] and are more mutagenic.[8,9] It is likely that in humans, too, the effects of the individual adducts are not identical. On the basis of the different mutagenic activities of the AG and GG adducts, Bradley et al. have suggested that it may prove useful to try to design platinum complexes with smaller propensity than cisplatin to form AG adducts.[10] Such a rational drug design requires an understanding of the effects that are at the origin of the GG/AG selectivity.

The reaction between cisplatin (more exactly, its hydrolyzed forms, *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$) and DNA is known to be under kinetic control. From the following statistical consideration, it can be deduced that guanines implicated in GG and AG sequences do not react with the same rates. In the DNA which has been used for the quantitation of adducts (having 20.5% of G[2]), the probability that a guanine has another guanine as a neighbor is 37%, whereas AGX sequences (X = C, T, A) account for 23% of all guanines. This would yield a GG/AG ratio of 1.6, if all the guanines were equireactive and the platination of the AG adenine were negligible. The actual ratio of ≥3 suggests that guanines implicated in GG sequences are more reactive toward the hydrolyzed forms of cisplatin than those in AGX sequences. However, it is not a priori clear whether the reactivity of only one of the GG guanines, or that of both, is enhanced.

In an effort to contribute to the understanding of this preferential platinum binding to GG sequences, we have developed an HPLC-based method for kinetic studies on oligonucleotides as models for DNA.[11] Using this method, we have determined the rate constants for the reaction between the duplex $d(TTGGCCAA)_2$ and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, the diaqua form of cisplatin, and have shown that, in this particular sequence, the 5′-guanine is platinated 12 times faster than the 3′-guanine.[12]

Our next aim is to compare the platination rate constants for guanines implicated in different sequences, e.g., TGGT, TAGT, TGAT, TGT, or TGCT, to investigate the influence of the nearest neighbors on the guanine reactivity. To keep the variations in local structure at a minimum, we have placed each of the above sequences in the identical environment of a hairpin-stabilized duplex. We report here the results for the two first members of this series, the hairpins d(TAT*GG*TATTTTTATACCATA) (**I**) and d(TAT*AG*TATTTTTATACTATA) (**II**) (Figure 1).

(1) Eastman, A. *Biochemistry* **1986**, *25*, 3912−3915.
(2) Fichtinger-Schepman, A. M. J.; Van der Veer, J. L.; Den Hartog, J. H. J.; Lohman, P. H. M.; Reedijk, J. *Biochemistry* **1985**, *24*, 707−713.
(3) Fichtinger-Schepman, A. M. J.; Van Oosterom, A. T.; Lohman, P. H. M.; Berends, F. *Cancer Res.* **1987**, *47*, 3000−3004.
(4) Reed, E.; Ostchega, Y.; Steinberg, S. M.; Yuspa, S. H.; Young, R. C.; Ozols, R. F.; Poirier, M. C. *Cancer Res.* **1990**, *50*, 2256−2260.
(5) Comess, K. M.; Burstyn, J. N.; Essigmann, J. M.; Lippard, S. J. *Biochemistry* **1992**, *31*, 3975−3990.
(6) This has been demonstrated for AG and GG adducts formed by the cisplatin derivative $[PtCl_2(dach)]$.[7]
(7) Page, J. D.; Husain, I.; Sancar, A.; Chaney, S. G. *Biochemistry* **1990**, *29*, 1016−1024.
(8) Burnouf, D.; Gauthier, C.; Chottard, J. C.; Fuchs, R. P. P. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6087−6091.
(9) Yarema, K. J.; Lippard, S. J.; Essigmann, J. M. *Nucleic Acids Res.* **1995**, *23*, 4066−4072.
(10) Bradley, L. J. N.; Yarema, K. J.; Lippard, S. J.; Essigman, J. M. *Biochemistry* **1993**, *32*, 982−988.
(11) Gonnet, F.; Kozelka, J.; Chottard, J. C. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1483−1485.
(12) Reeder, F.; Gonnet, F.; Kozelka, J.; Chottard, J.-C. *Chem. Eur. J.* **1996**, *2*, 1068−1076.

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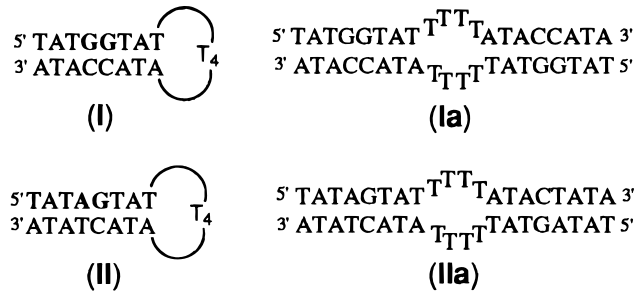

Figure 1. Hairpin oligonucleotides **I** and **II** studied in this work. **Ia** and **IIa** are hypothetical bimolecular associates of **I** and **II**, respectively.

Materials and Methods

Starting Materials. The oligonucleotides were synthesized as their ammonium salts. Their purity was checked by capillary electophoresis and HPLC by the group of T. Huynh Dinh (Institut Pasteur, Paris). Approximate concentrations were evaluated photometrically by means of the molar absorption coefficient ϵ_{260} of 8000 M^{-1} cm^{-1} per base. Cisplatin was provided by Johnson-Matthey. Solutions of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ were prepared by dissolving *cis*-$[Pt(NO_3)_2(NH_3)_2]$[13,14] in water. Perchloric acid and all salts were purchased from Merck. The 3′-exonuclease venom phosphodiesterase (VPD) was obtained from Boehringer-Mannheim.

Sample Preparation for the HPLC Studies. The reactions were carried out in 0.1 M $NaClO_4$ at 20 °C, pH 4.4−4.8 adjusted by $HClO_4$; the initial concentrations were (1.2−1.5) × 10^{-4} M for the oligonucleotides and (0.8−1.0) × 10^{-4} M for the platinum complex. Aliquots were collected at several time points, quenched by addition of saturated KBr or KCl solutions, and stored at liquid nitrogen temperature until they were analyzed. These quenching conditions have been shown to trap all the aqua intermediates within 2 min (after this time, no change in the HPLC profile is observed) and to avoid any evolution of the reaction.[11,15]

HPLC Analysis. HPLC analysis of the reaction aliquots was performed using a Beckman 126 pump coupled to a Beckman 168 diode array detector and a System Gold V810 integrator. The system was connected to a Rheodyn 7125 valve. A Nucleosil C8 (150 × 4.6 mm i.d., 5 μm, 300 Å) stainless steel column (Colochrom, Gagny, France) was used for the separation of the reaction products of **I**, and a POROS RS/H (100 × 4.6 mm i.d., 10 μm) column (PerSeptive Biosystems GmbH) was employed for the analysis of the quenched reaction aliquots of **II**. Operating conditions are described in the caption for Figure 4. Relative concentrations were determined from the ratios of the peak areas. The detection wavelength of 245 nm was chosen to be close to the quasi-isosbestic point of the reactions. The reaction intermediates were identified as previously described, by enzymatic digestion of the products followed by MALDI mass spectroscopy analysis of the isolated fragments.[16] The rate constants were calculated by numerical integration of the kinetic equations using the program ITERAT.[17]

Spectroscopic Studies. The melting profiles of the duplex oligonucleotides were recorded using an Uvikon 941 spectrophotometer. The NMR spectra were recorded on a Bruker 500 MHz spectrometer using a 1−3−3−1 pulse sequence to suppress the H_2O signal.[18]

Results and Discussion

Stabilization of the Short Duplex Structure by Means of a T4 Hairpin Loop. The two oligonucleotides **I** and **II** studied here could form, in principle, either monomolecular hairpins or bimolecular duplex-loop structures (Figure 1). The fact that their melting temperatures recorded in 0.1 M $NaClO_4$ solution were concentration-independent indicates that both structures are monomolecular in the investigated concentration range (10^{-5}−10^{-4} M). This result is in agreement with previous findings,[19,20] which have shown that T_4 hairpin loops are preferred over dimeric structures. The melting temperatures (T_m) were 55 and 46.5 °C for **I** and **II**, respectively. The former value can be compared with the melting temperatures for non-hairpin octamer duplexes containing two GC pairs, determined in our laboratory under the same conditions: $d(ATGTACAT)_2$, 27.5 °C; $d(TTAGCTAA)_2$, 26 °C; $d(TATGCATA)_2$, 28 °C. Thus, the T_4 loop increases the melting temperature by about 30 °C. A similar stabilization was reported by Durand et al.[20]

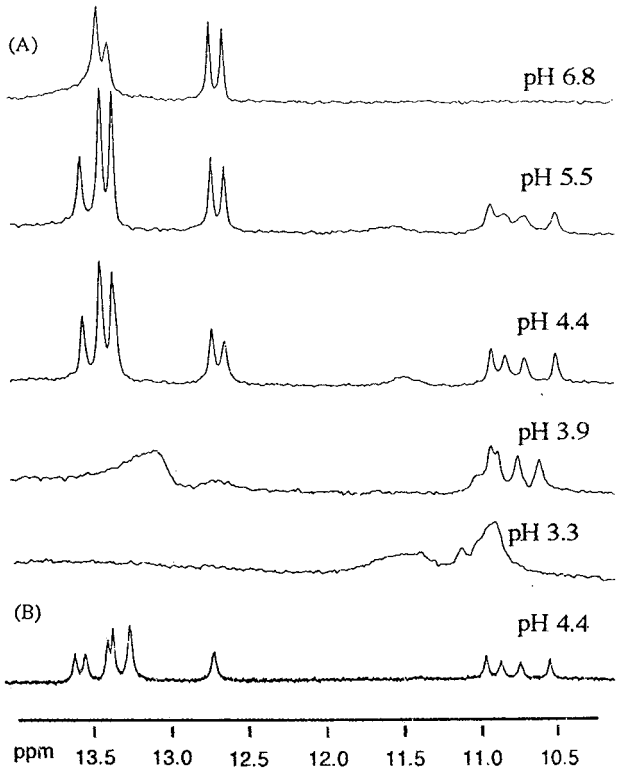

Figure 2. Imino proton region of the ^1H NMR spectum of 0.4 mM H_2O/D_2O (9:1) solutions of **I** (A) and **II** (B) in 0.1 M $NaClO_4$, T = 20 °C.

To test base pairing within the hairpin, we monitored the guanine and thymine imino protons by NMR in H_2O solution. Figure 2 shows the low-field ^1H spectrum of the imino protons of **I** and **II** recorded at various pH values. The peaks due to the imino protons of the Watson−Crick (WC) GC pairs (12.5−12.9 ppm) and AT pairs (13.3−13.6 ppm) and to the four unpaired thymines of the loop (10.4−11 ppm) are clearly visible and are in agreement with a hairpin structure.[19] The detection of all the imino protons of the duplex stem within the region characteristic of WC base pairing at pH 4.4, 0.1 M $NaClO_4$, and T = 20 °C indicates that all the base pairs are closed in these conditions, under which all the kinetic measurements were carried out.[19,21,22]

(13) Lippert, B.; Lock, C. J. L.; Rosenberg, B.; Zvagulis, M. *Inorg. Chem.* **1977**, *16*, 1525−1529.
(14) King, H. J. S. *J. Chem. Soc.* **1938**, 1338−1346.
(15) Troujman, H.; Chottard, J. C. *Anal. Biochem.* **1997**, *252*, 177−185.
(16) Gonnet, F.; Kocher, F.; Blais, J. C.; Bolbach, G.; Tabet, J. C.; Chottard, J. C. *J. Mass Spectrom.* **1996**, *31*, 802−809.
(17) Zekany, L. *ITERAT Version 2*; Université de Lausanne: Lausanne, Switzerland, 1992.
(18) Hore, P. J. *J. Magn. Res.* **1983**, *54*, 539−543.
(19) Haasnoot, C. A. G.; de Bruin, S. H.; Berendsen, R. G.; Janssen, H. G. J. M.; Binnendijk, T. J. J.; Hilbers, C. W.; van der Marel, G. A.; van Boom, J. H. *J. Biomol. Struct. Dyn.* **1983**, *1*, 115−129.
(20) Durand, M.; Chevrie, K.; Chassignol, M.; Thuong, N. T.; Maurizot, J. C. *Nucleic Acids Res.* **1990**, *18*, 6353−6359.
(21) Gueron, M.; Kochoyan, M.; Leroy, J. L. *Nature* **1987**, *328*, 89−92.
(22) Nonin, S.; Leroy, J. L.; Guéron, M. *Nucleic Acids Res.* **1996**, *24*, 586−595.

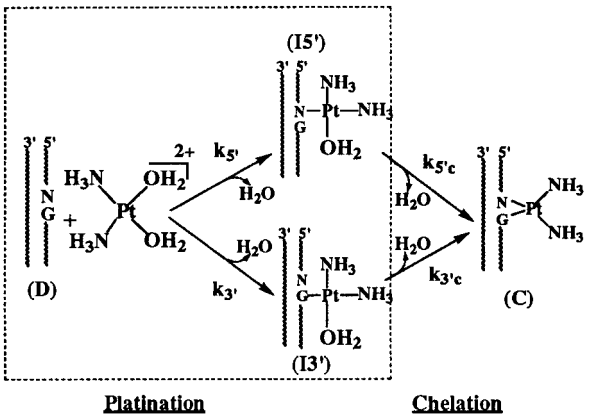

Figure 3. Kinetic scheme for the two-step reaction between *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and **I** (N = G) or **II** (N = A). Charges were omitted for clarity.

Kinetics of the Reactions between Hairpins I and II and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. The kinetic scheme defining the platination and chelation rate constants is presented in Figure 3. Two typical HPLC chromatograms of the quenched reaction mixtures are shown in Figure 4. Figure 5 displays the experimental concentration curves and the calculated ones for two experiments. The optimized rate constants averaged over three experiments are listed in Table 1. They convey several interesting information:

First, the sum $k_{3'} + k_{5'}$ for **I** is about three times that for **II**; i.e., the GG sequence is three times more reactive toward *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ than is the AG sequence. This corresponds to the ratio between the GG and AG cross-links formed upon platination of DNA with *cis*-$[PtCl_2(NH_3)_2]$ in vitro[2] and in vivo.[3] This agreement is rather unexpected, since the species generally supposed to react with DNA in vivo is the monoaquated complex, *cis*-$[PtCl(NH_3)_2(H_2O)]^+$, and not the diaqua complex assayed here, and since these two complexes exhibit different sequence selectivity in model oligonucleotide reactions.[23] A detailed kinetic study of the reactions of *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ with **I** and **II**, respectively, is now underway in our laboratory and should give conclusive data about the actual contribution of the diaqua species, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, to the in vitro and in vivo DNA platination. It is interesting to note that the sum $k_{5'} + k_{3'}$ determined for **I**, 33 M^{-1} s^{-1}, is similar to that measured previously for the oligonucleotides d(TTGGCCCAA)$_2$ (29 M^{-1} s^{-1}, calculated per GG site)[12] and d(AACGGTTAACCGTTAATT)$_2$ (24 M^{-1} s^{-1}).[24] In these three duplexes, the platinated sequences are TGGT, TGGC, and CGGT, respectively. It seems therefore that, at least within the series XGGY, where X and Y are pyrimidines, the global rate of platination by *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ is insensitive to the nature of the bases surrounding the GG sequence.

Second, the guanine of **II** reacts more slowly than either guanine of **I**. This is in agreement with our earlier hypothesis[12,25] according to which the observed selective binding of cisplatin to GG sequences is based on an enhancement of the reactivity of either or both guanines of GG.

Third, the two guanines of **I** react with similar rate constants with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. This result is in contrast with the considerably different platination rate constants observed for the two guanines of the octanucleotide duplex d(TTGGCCAA)$_2$ studied previously.[12] This difference between d(TTGGCCAA)$_2$ and **I** can be due either to the effect of the nearest neighbors (TGGC versus TGGT) or to a global structural difference between the two oligonucleotides which might be related to the particular GGCC-GGCC sequence. The contribution of the nearest neighbors could be, at least in part, of electrostatic nature: it has been shown by means of quantum mechanical calculations[26] that the negative electrostatic potential at the site of the N7 lone pair of a guanine is dependent on the nature of both adjacent bases, and, in fact, a 3′-thymine enhances this negative potential with respect to a 3′-cytosine. It is thus possible that the clear preferential binding to the 5′-guanine observed for the TGGC sequence in d(TTGGCCAA)$_2$ is considerably reduced in the case of the TGGT sequence of **I** because of the replacement of the 3′-C by a 3′-T. Indeed, other TGGT-containing duplexes have been shown to be platinated by *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with similar rate constants at both the 5′-G and 3′-G.[23]

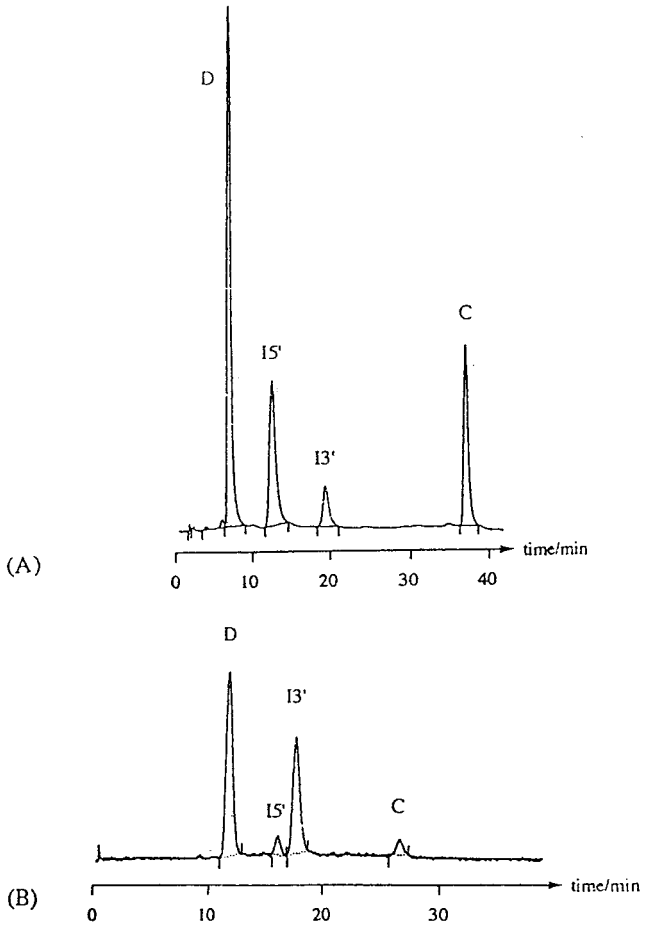

Figure 4. Typical chromatograms for reaction mixtures quenched by halide ions. Reaction conditions: 0.1 M $NaClO_4$; pH 4.4 ± 0.2; T = 20 °C. Detection wavelength: 245 nm. (A) Reaction between *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and **I** quenched at t = 15 min by excess KBr. Nucleosil C8 column: mobile phase, ammonium bromide 0.5 M, ammonium acetate buffer 0.02 M, pH = 4.7; acetonitrile gradient, 7% for 10 min; 7−10% from 10 to 50 min; flow rate 1 mL/min; column temperature, 50 °C. (**B**) Reaction between *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and **II** quenched at t = 45 min by excess KCl. POROS R2/H column: mobile phase, ammonium chloride 1 M, triethylammonium acetate 0.02 M, pH = 7; acetonitrile gradient, 3.5−10% from 0 to 40 min; flow rate 3 mL/min; column temperature, 25 °C. D, oligonucleotide; I5′, 5′ monoadduct; I3′, 3′ monoadduct; C, diadduct.

(23) Reeder, F.; Guo, Z.; Murdoch, S. P.; Corazza, A.; Hambley, T. W.; Berners-Price, S. J.; Chottard, J.-C.; Sadler, P. J. *Eur. J. Biochem.* **1997**, *249*, 370−382.
(24) Troujman, H.; Chottard, J.-C. Unpublished results.
(25) Laoui, A.; Kozelka, J.; Chottard, J. C. *Inorg. Chem.* **1988**, *27*, 2751−2753.
(26) Pullman, A.; Pullman, B. *Q. Rev. Biophys.* **1981**, *14*, 289−380.

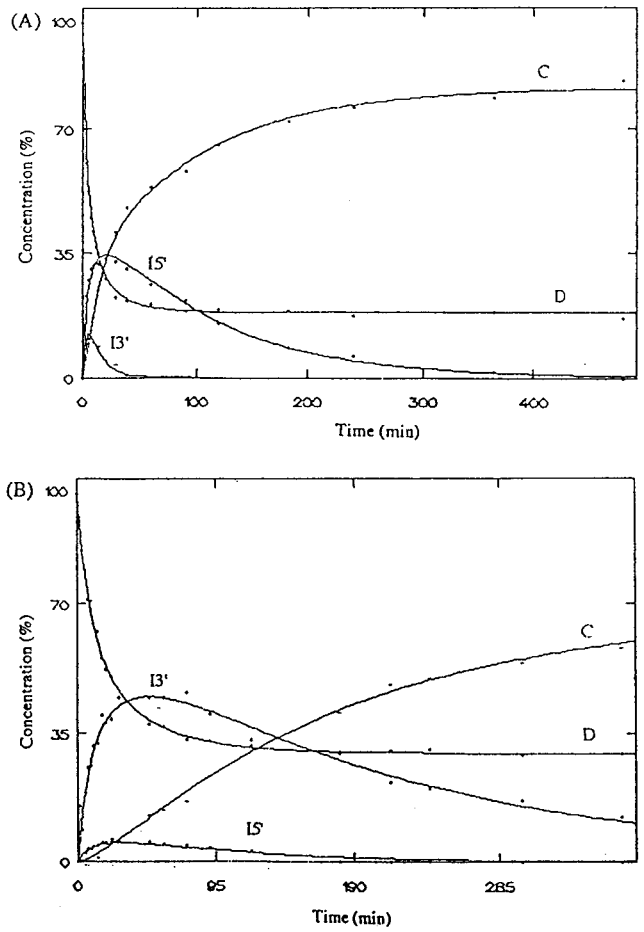

Figure 5. Experimental relative concentrations and calculated curves for two runs of the reactions (A) between **I** and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and (B) between **II** and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ D, oligonucleotide; I5′, 5′ monoadduct; I3′, 3′ monoadduct; C, diadduct.

Table 1. Optimized Rate Constants for the Reactions of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with **I** and **II** at 293 K, in 0.1 M $NaClO_4$, pH 4.6 ± 0.2 (Mean Values from Three Experiments with the Corresponding Standard Deviations in Parentheses)

	cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$					
	platination (M^{-1} s^{-1})			chelation (10^{-3} s^{-1})		
	$k_{5'}$	$k_{3'}$	$k_{5'}/k_{3'}$	$k_{5'c}$	$k_{3'c}$	$k_{3'c}/k_{5'c}$
I (GG)	18 (2)	15 (1)	1.2 (1)	0.18 (5)	1.9 (1)	10.5(1)
II (AG)	1.5 (3)	9 (1)	0.2 (1)	0.3 (1)	0.08 (1)	0.3 (1)

Fourth, we observe the formation of an adenine-bound monoadduct within the AG sequence. None of the previous platination studies on AG-containing oligonucleotides has revealed such a monoadduct, possibly due to the small concentration in which it accumulates (Figure 5B) in the course of reaction. No platination was observed on the other adenines present in **I** or **II**. This could be, again, related to the electrostatic potential, which has been calculated to be lower at the adenine-N7 of a TAG sequence than at adenines flanked by two pyrimidines.[26] Concurring with these calculated potentials, Table 1 shows that the adenine of the AG sequence of **II** is platinated only six times slower than the adjacent guanine, whereas the reactivity ratio between adenosine and guanosine, as determined by Arpalahti and Lippert[27] in reactions of nucleosides with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, is 14.

Fifth, the ratio of the chelation rate constants $k_{3'c}/k_{5'c} = 10.5$ observed for **I** is similar to those observed for the other GG-containing duplex oligonucleotides examined previously: d(T-TGGCCAA)$_2$, 13;[12] d(AACGGTTAACCGTTAATT)$_2$, 10;[24] d(ATACATGGTACATA)-d(TATGTACCATGTAT), 5.[23,28] Thus, the ratio between the chelation rate constants of the monoadducts of a GG sequence seems to be fairly sequence-independent.

Sixth, the ratio of the rate constants $k_{3'c}$ for the cyclization of the 3′-monoadducts of **I** and **II**, 24, reflects mainly the lower reactivity of A with respect to G[27] toward platinum binding.

Conclusion

We have prepared two octanucleotide duplexes stabilized by a T_4 hairpin loop, **I** and **II**, which contain respectively the sequences GG and AG in the middle of the duplex stem. We have shown that, in 0.1 M $NaClO_4$ solution at 20 °C and pH 4.4, both oligonucleotides are stable in the duplex-hairpin forms.

The two-step reaction of **I** and **II** with the diaquated form of cisplatin, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, was investigated, and the four rate constants were determined for both reaction systems. The comparison of these rate constants with results obtained from the platination of other model oligonucleotides and of DNA reveal several interesting points, the most surprising one being the fact that the ratio of the overall platination rates of **I** and **II** is the same as the ratio of the GG and AG adducts formed upon reaction of DNA with cisplatin, either in vitro or in vivo. Could *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ be the reactive species in the nucleus? Kinetic measurements with the monochloro species, *cis*-$[PtCl(NH_3)_2(H_2O)]^{+}$, currently underway in our laboratory, should give a more definitive answer. It is furthermore interesting that an adenine between two pyrimidines is not platinated, whereas an adenine in the 5′ position vs a guanine is, with a rate constant only six times inferior to that of the adjacent guanine but twelve times inferior to that of the 5′-guanine of a GG sequence in the same sequence environment.

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(27) Arpalahti, J.; Lippert, B. *Inorg. Chem.* **1990**, *29*, 104−110.
(28) This value is based on one single experiment in which the individual platination constants were not resolved.