Study on the Reaction Product of *cis*-$Pt(NH_3)_2Cl_2$ with d(GpCpGpC) by UV Spectroscopy and Enzymatic Digestion Method

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It is generally accepted that the bifunctional attack of the antitumor active platinum complex, *cis*-$Pt(NH_3)_2Cl_2$, on DNA is related to the mechanism of action. Among the various bifunctional binding modes proposed, attention has recently been focused on the intrastrand crosslink between two guanine bases [1]. Considerable evidence indicates that the instrastrand crosslink between two adjacent guanine bases is the plausible platinum binding mode, especially at low level of platinum modification of DNA [1–5]. On the other hand, the intrastrand crosslink between guanine bases separated by a third base (–GpApG) and –GpCpG–) has also been proposed on the basis of studies on base pair substitution mutation induced by *cis*-$Pt(NH_3)_2Cl_2$ in the lac I gene of *E. Coli* [6, 7]. The possibility of the formation of such an intrastrand crosslink was confirmed by a NMR study of the main product upon the reaction of *cis*-$Pt(NH_3)_2Cl_2$ with d(GpCpG) [8, 9]. The purpose of the present paper is to verify such an intrastrand crosslink by means of UV spectral analysis and is to make clear the enzymatic action for such a platinum modified substrate. In the present work, the reaction product of *cis*-$Pt(NH_3)_2Cl_2$ with 5′-d(GpCpGpC)-3′ was investigated. In the previous paper, we used enzymatic digestion of platinum modified oligonucleotides to determine the platinum binding base in oligonucleotides [10–12]. Cleavage of the phosphodiester bond of platinum modified oligonucleotides either by snake venom phosphodiesterase (VPD) or calf spleen phosphodiesterase (SPD) was found to stop at the platinum binding base [10–12]. Our interest in this work is to know whether the third base being interposed by two guanine bases can be removed or retained during enzymatic digestion.

A 200 μl of 0.175 m*M* d(GpCpGpC) was allowed to react with 8.5 μl of 4 m*M* *cis*-$Pt(NH_3)_2Cl_2$ at 37 °C in an aqueous solution. The reaction was almost complete within 20 hours, being followed by the measurement of the unreacted d(GpCpGpC) using HPLC with TSK-Gel IEX-530 K (weak cation exchange column). However, the reaction products could not be separated well by the use of the IEX-530 K column. Figure 1-a shows the chromatogram of the reaction mixture obtained using Partisil 10 SAX column (strong anion exchange column). The reaction gave five reaction products shown in Fig. 1-a, and one of them was a predominant species. The unplatinated d(GpCpGpC) was tightly retained on the SAX column and could not be eluted under the conditions used. The peak area of the main product occupies 75% of all the peak area, being monitored at 260 nm. The main product was fractionated by using the same column, and the chromatogram of the fractionated solution is shown in Fig. 1-b. The experiment described below was carried out by using the fractionated solution.

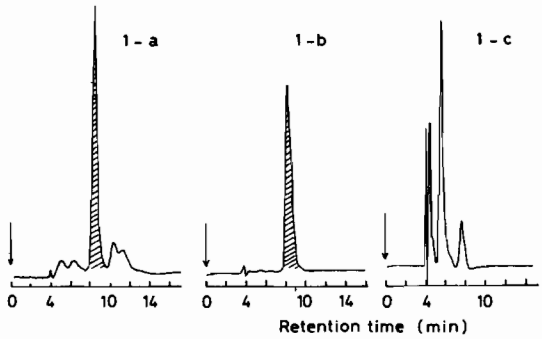

Fig. 1. HPLC chromatograms of the reaction product of *cis*-$Pt(NH_3)_2Cl_2$ with d(GpCpGpC) and enzymatically digested products. 1-a: Reaction mixture. 1-b: Main product fractionated. 1-c: Enzymatically digested products. HPLC conditions: column, Partisil 10-SAX (strong anion exchange column); detector, UV at 260 nm; eluant, 0.05 *M* KH_2PO_4 (1.0 ml/min).

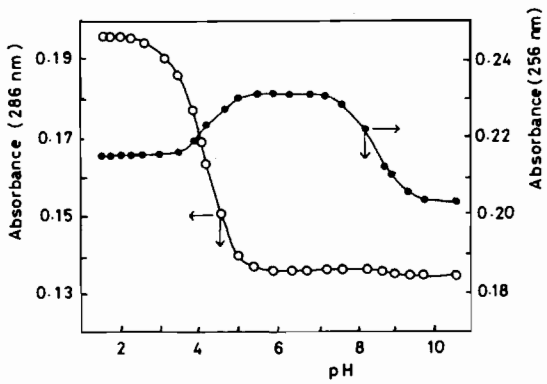

Fig. 2. Absorption change of the main product as a function of pH.

The UV spectrum of the main product showed an absorption maximum at 263 nm, and the molar extinction coefficient per platinum was found to be 35000, indicating that the binding ratio between platinum and d(GpCpGpC) is 1:1. The UV spectral changes in the main product as a function of pH were

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measured. In the solution of pH 6.0–1.5, an isosbestic point was observed at 262 nm, and the maximum change in absorbance appears at 286 nm. The protonated species of the main product showed the absorption maximum at 272 nm. The spectral characteristics agree with those arising from the protonation of the cytosine base [13]. Figure 2 shows changes in absorbance as a function of pH. The pK_a value calculated from the relationship between pH and absorbance was found to be 4.2, coinciding well with the pK_a at N(3) of the cytosine base [13]. These results strongly suggest that the N(3) of the cytosine bases in d(GpCpGpC) does not participate in binding with *cis*-$Pt(NH_3)_2^{2+}$. No UV spectral change has been observed at pH < 2.5, suggesting that N(7) of the guanine bases in d(GpCpGpC), the proton attachment site, was already occupied by *cis*-$Pt(NH_3)_2^{2+}$. This is also supported by the pK_a value of the N(1) of the guanine bases. The pK_a value of the main product calculated from Fig. 2 was 8.4, a significantly lower value than that of guanosine (pK_a = 9.6). Such a decrease of pK_a at the N(1) of the guanine base is characteristic of platinum binding to the N(7) site [13–15]. From these results, it is concluded that the main product is the complex in which *cis*-$Pt(NH_3)_2^{2+}$ binds to the N(7) site of the guanine bases, probably as an intrastrand crosslinked complex. The UV spectrum of the main product shows a decrease in absorbance at 256 nm with increasing pH. The UV spectral pattern obtained upon deprotonation is quite similar to the corresponding pattern of the N(7)-platinated guanosine derivatives reported in the previous paper [13, 14]. This also indicates that the N(7) site of the guanine bases participates in binding with *cis*-$Pt(NH_3)_2^{2+}$. There is no evidence for the existence of unplatinated guanine base in either the UV spectral characteristics or the pH dependence of the main product.

The platinum binding site in the main product was also studied by the enzymatic digestion method. 100 μl of the solution of the main product fractionated was treated with 0.1 unit of VPD after adding 20 μl of 1.0 *M* Tris-HCl buffer pH 8.9 [15]. The peak at t_R = 8.3 min (Fig. 1-b) decreased with increasing incubation time and completely disappeared after 2 hours' incubation. The decrease in the peak at t_R = 8.3 min was accompanied by the increase in two new peaks which appear at t_R = 5.4 and 7.6 min. The peak at t_R = 7.6 min was identical with 5′-dCMP in retention time. The peak arising from guanine base (5′-dGMP and dG) was not observed at all. This means that the G* base in d(GpCpG*pC) participates in binding with *cis*-$Pt(NH_3)_2^{2+}$ because a breaking of the phosphodiester bond by VPD just stops at the platinum binding base [10–12]. It is noteworthy that the ratio of the peak area of the enzymatic digestion products, Area (t_R = 7.6)/(Area(t_R = 5.4) + Area(t_R = 7.6)) = 0.19, is almost the same as the ratio of the absorption coefficient of dG and dC, $\epsilon(dC)/(2\epsilon(dC) + 2\epsilon(dG))$ = 0.21. This means that digestion by VPD produces just one mol of 5′-dCMP from the platinated d(GpCpGpC) and that the peak at t_R = 5.4 min is the platinated d(GpCpG). The solution of the main product was treated with SPD which degrades the oligonucleotide exonucleolytically from the 5′-OH end [10–12]. However, the peak at t_R = 8.3 min did not show any change upon treatment with SPD even when it was incubated for 2 days at 37 °C. If *cis*-$Pt(NH_3)_2^{2+}$ coordinates to dC* and dG* in d(GpC*pG*pC), digestion by SPD would be expected to give 3′-dGMP [10–12]. But this was not observed. It is therefore considered that the G* base in d(G*pCpGpC) participates in binding with *cis*-$Pt(NH_3)_2^{2+}$. From these results, it is concluded that the two dG* bases in d(G*pCpG*pC) bind to *cis*-$Pt(NH_3)_2^{2+}$. This coincides with the result of the UV spectral study described above.

In the previous paper, we reported that SPD and VPD could not break the phosphodiester bond between two adjacent guanine bases of the complexes such as *cis*-$Pt(NH_3)_2$(d(G*pG)), *cis*-$Pt(NH_3)_2$-(d(G*pG*pCpC)) and *cis*-Pt(d(CpCpG*pG*)) [11, 12]. In the case of monofunctional platinum binding, VPD could break the phosphodiester bond of the $(NH_3)_3$Pt(5′-G*pG-3′), but not the phosphodiester bond of the $(NH_3)_3$Pt(5′-GpG*-3′) [10]. This means that VPD can not break the phosphodiester bond when the monofunctional platinum complex, $(NH_3)_3$-Pt^{2+}, exists at the side of the recognition site (3′-OH end). On the other hand, SPD could break the phosphodiester bond of both complexes, $(NH_3)_3$-Pt(5′-G*pG-3′) and $(NH_3)_3$Pt(5′-GpG*-3′) [10]. That is, SPD could break the phosphodiester bond even when $(NH_3)_3Pt^{2+}$ exists at the side of the recognition site (5′-OH end). These results led us to inquire as to whether the third base being interposed by two guanine bases can be removed or retained during enzymatic digestion. Consequently, it was found that the phosphodiester bond between two guanines separated by a third base, in which the two guanine bases have bound to *cis*-$Pt(NH_3)_2^{2+}$, is retained during digestion of SPD and VPD.

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