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Cis-dichlorodiammineplatinum(II) has a Binding Specificity for Adjoining Guanine Bases

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Abstract

Self complementary deoxyribooctanucleotides, [5′-d(GpGpTpCpGpApCpC)-3′]$_2$ and [5′-d(CpGpGpApTpCpCpG)-3′]$_2$, were allowed to react with *cis*-$PtCl_2(NH_3)_2$, and the reaction mixture was treated with exonuclease (snake venom phosphodiesterase and/or calf spleen phosphodiesterase). Semi-quantitative analysis by HPLC showed that the adjoining guanine bases have a specific binding affinity to *cis*-$PtCl_2(NH_3)_2$.

Introduction

It is generally thought that a bifunctional attack of the anti-tumor active platinum complex, *cis*-$PtCl_2(NH_3)_2$, on DNA is responsible for its biological activities. For this reason, the interaction of *cis*-$PtCl_2(NH_3)_2$ with DNA and its constituents has been widely investigated. It has been generally accepted that a guanine base has a high selectivity for binding with platinum and that its N(7) is the kinetically favored site [1, 2]. Recently, considerable evidence has indicated that the intrastrand crosslink between adjacent guanine bases on the same strand of DNA is the plausible bifunctional platinum binding mode, especially at low levels of platinum modification of DNA [3–17]. The purpose of our study is to make clear whether or not *cis*–DDP has a binding specificity to adjacent guanine bases. Do adjoining guanine bases have a binding specificity beyond the monofunctional binding specificity of the *cis*-DDP to N(7) site of the guanine base? In the study on the interaction of *cis*-DDP with 5′-GMP, Reedijk *et al.* have reported that the initial product of the reaction is [*cis*-Pt-$(NH_3)_2$(GMP)Cl] and/or [*cis*-Pt$(NH_3)_2$(GMP)-(H_2O)]$^+$ and that the binding of the bifunctional platinum compound to DNA probably occurs in two stages [18, 19]. Assuming that the binding specificity of *cis*-DDP is governed by the first attack on the N(7) site of the guanine base and that all guanine bases in DNA and the oligonucleotide are equivalent upon the first platinum binding (monofunctional intermediate), the binding specificity of *cis*-DDP may be explained by nucleophilicity and statistical probability of the N(7) site of the guanine base. Alternatively, adjoining guanine bases may have some additional factor other than the nucleophilicity of the N(7) site of the guanine base. In the present work we used octanucleotide including three guanine bases, of which two are adjoining guanine bases, and examined whether the binding of *cis*-DDP is specific for the adjoining guanine bases.

The second purpose of our study was to indicate how substrates like Pt–modified-oligonucleotide and -DNA are digested by enzymes. Up to now, the determination of the platinum binding base in DNA has been carried out on the basis of several enzymatic digestion techniques of Pt–modified DNA [11–16]. But the capability of the enzymes to digest the Pt–modified substrates remains uncertain. Therefore we have considered the enzymatic digestion of the Pt–modified substrate. An experiment was carried out using a Pt–modified oligonucleotide having a known sequence.

The third purpose of our study was to develop an analytical method to evaluate the amount of *cis*-Pt$(NH_3)_2$ chelated GpG in Pt–modified DNA. It has been reported that the intrastrand crosslink may be responsible for the inactivation produced by *cis*-DDP in pharge λ DNA [20]. Quantitative evaluation of the intrastrand crosslink is thought to be necessary for elucidating the correlation between frequency of intrastrand crosslink and such a biological activity.

Experimental

Self complementary deoxyribonucleotides, [5′-d(GpGpTpCpGpApCpC)-3′]$_2$ and [5′-d(CpGpGpApTpCpCpG)-3′]$_2$, were obtained from P–L Biochemicals. All enzymes, snake venom phosphodiesterase (VPD), calf spleen phosphodiesterase (SPD), alkaline phosphatase, and deoxyribonuclease I (DNase I), were purchased from Worthington Co. Ltd. Alkaline phosphatase may contain deaminase

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as an impurity, probably adenosine deaminase. Therefore, a long incubation time with alkaline phosphatase sometimes leads to the disappearance of the peak of deoxyadenosine.

A solution of *cis*-DDP (2.0×10^{-4} M) was prepared by dissolving *cis*-DDP in deionized water just before use. The octanucleotides were dissolved in 0.05 M phosphate buffer (pH = 7.2) to make a stock solution of 8.11×10^{-5} M. The concentration was determined by using HPLC after digesting the stock solution by VPD.

To each microtest tube (100 μl), 20 μl of the stock solution of the octanucleotide and 0–7 μl of *cis*-DDP (2×10^{-4} M) were added, and the total volume was then made up to 27 μl by adding deionized water. The reaction was performed at 37 °C for 24 h. 3 μl of 1.0 M Tris-HCl buffer solution (pH = 8.9) was added to the reaction solution and each sample solution was then treated with 0.2 unit of VPD at 37 °C for 2 h. Usually, 1 h incubation was enough for complete digestion.

For the purpose of the quantitative determination of unplatinated nucleotides, aliquots (5 μl) of the solution were injected onto a Partisil-10 SAX column (strong anion exchange column) attached to a Hitachi Model 655 HPLC. The parameters of the run are as follows: column size, 4 × 300 mm; mobile phase, 0.05 M potassium phosphate buffer (pH = 3.5); flow rate, 0.7 ml/min. The absorption of the eluted materials was monitored continuously using two UV detectors operating in this experiment at both 260 and 280 nm. For the purpose of the quantitative determination of *cis*-$Pt(NH_3)_2^{2+}$ modified dinucleotide or trinucleotide, resulting from digesting the *cis*-Pt-$(NH_3)_2^{2+}$ modified octanucleotide by VPD, aliquots (5 μl) of the sample solution were injected on to TSK-Gel CM-2SW (weak cation exchange column) attached to a Shimadzu LC-3A (HPLC). The parameters of the run are as follows: column size, 4.6 × 500 mm; mobile phase, 0.05 M potassium phosphate (pH = 4.5); flow rate, 0.7 ml/min. Elution was monitored at 260 nm using a UV detector.

Treatment with alkaline phosphatase was carried out by adding 10 units of alkaline phosphatase to the sample solution. Usually, an incubation time of 20 min. was enough for complete removal of the phosphate group. In the case of SPD digestion, the pH of the sample solution obtained after VPD digestion was adjusted to pH 4.5–6.0 by adding 1.0 M acetic acid solution; 3 μl of SPD solution was then added to the solution. Incubation was performed at 37 °C for 24 h.

Results

Octanucleotide, $[5'\text{-d(GpGpTpCpGpApCpC)-}3']_2$, was allowed to react with various amounts of *cis*-$Pt(NH_3)_2Cl_2$ in 0.037 M phosphate buffer solution (pH 7.3). All experiments were conducted at *r*-values of less than 1.0 (r = [Pt]/[octanucleotide]). The separation of the enzymatic digestion products is shown in Fig.1, which is the chromatogram obtained by injection of an aliquot of the products resulting from digesting the platinum modified octanucleotide by VPD. The peak arising from the Pt-adduct appeared at t_R = 5.8 min (t_R = retention time) and increased with increasing r. The peak height of dG (t_R = 6.2 min) decreased with increasing Pt-adduct, suggesting that the guanine base at the 5′-OH end (G(1)) should participate in Pt-binding. The peak height of 5′-dGMP (t_R = 14.4 min) also decreased with increasing r, but the height of the other peaks (5′-deoxymononucleotides) was almost independent of change in r, especially in the case of low r-values. Because the separation on the column is based on charge difference, the Pt-adduct is thought to be a positively charged compound. This was confirmed by an experiment using HPLC with a weak cation exchange column.

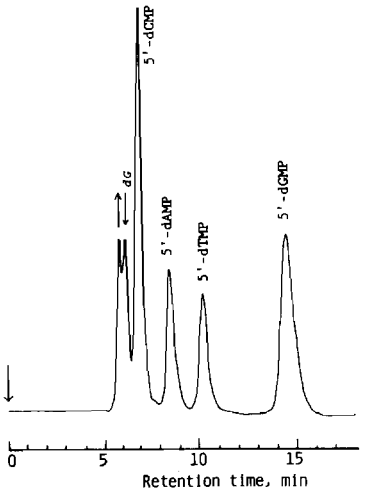

Fig. 1. HPLC elution pattern of the VPD digestion products of *cis*-$Pt(NH_3)_2^{2+}$ modified $[5'\text{-d(GpGpTpCpGpApCpC)-}3']_2$ at a molar ratio of 0.492 Pt/octanucleotide; strong anion exchange column; monitored at 260 nm.

The peak area of the 5′-deoxynucleotides in Fig. 1 was employed for the determination of unplatinated nucleotides, and the results are shown in Fig. 2. The 5′-dGMP shows a sharp decrease with increasing r, but the other 5′-deoxymononucleotides do not change, especially when $r < 0.7$. The result strongly suggests that the guanine bases selectively participate in platinum binding. The enzyme, VPD, degrades oligonucleotide exonucleolytically from the 3′-OH end. In platinum modified oligonucleotides, a cutting of the phosphodiester bond by VPD just stops at the platinum binding base, as reported in our previous paper [17]. Assuming that G(5) in 5′-d(GpGpTpCp-

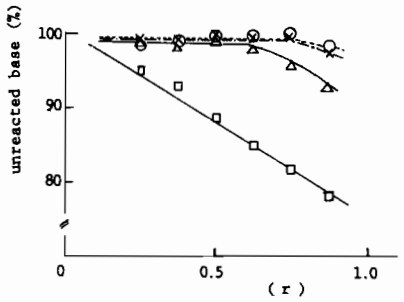

Fig. 2. Analytical results of unplatinated nucleotides as a function of r (r = Pt/octanucleotide): —○—, 5′-dCMP; —×—, 5′-dTMP; —△—, 5′-dAMP; —□—, 5′-dGMP.

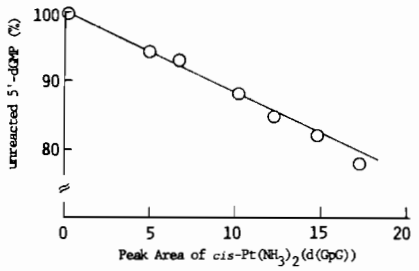

Fig. 3. Correlation between unplatinated 5′-dGMP and *cis*-$Pt(NH_3)_2$(d(GpG)): Y = −1.28X + 100.9, correlation coefficient = −0.995; 5′-dGMP and *cis*-$Pt(NH_3)_2$(d(GpG)) were quantified using HPLC with a strong anion exchange column and weak cation exchange column, respectively.

GpApCpC)-3′ binds to *cis*-$[Pt(NH_3)_2]^{2+}$, a decrease of 5′-dGMP should be accompanied by a decrease of 5′-dTMP and 5′-dCMP because the degradation by VPD just stops at G(5). This is not the case in Fig. 2. The result obtained in Fig. 2 may be explained only if G(2) participates in binding with platinum. As mentioned above, G(1) also participates in binding with platinum. Therefore, it is concluded that the binding of *cis*-$Pt(NH_3)_2Cl_2$ to the octanucleotide occurs at the adjacent guanine bases, G(1) and G(2). Actually, the peak of *cis*-$Pt(NH_3)_2$(d(GpG)) was identified by HPLC with a weak cation exchange column. In order to verify this conclusion, the correlation between unplatinated 5′-dGMP and *cis*-$Pt(NH_3)_2$(d(GpG)) was examined, and is shown in Fig. 3. A correlation coefficient of 0.995 was found. This means that *cis*-$[Pt(NH_3)_2]^{2+}$ binds to the adjacent guanine bases G(1) and G(2) much more selectively than the guanine base G(5). That is, the complex with the intrastrand crosslink between G(1) and G(2) is almost quantitatively produced at $r < 0.7$. Eastmann has reported that the Pt-complex with an intrastrand crosslink between two adjacent guanine bases occupies more than 50% of all the products in Pt-modified DNA, especially at a low level of platination [16]. Our result shows that there

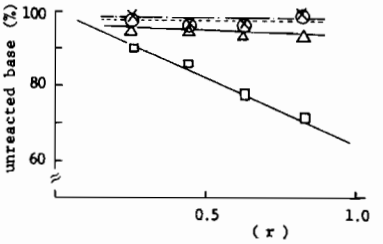

Fig. 4. Analytical results of unplatinated nucleotides resulting from the VPD digestion of *cis*-$Pt(NH_3)_2^{2+}$ modified [5′-d(CpGpGpApTpCpCpG)-3′]$_2$ (r = Pt/octanucleotide). —○—, 5′-dCMP; —×—, 5′-dTMP; —△—, 5′-dAMP; —□—, 5′-dGMP.

is higher sensitivity for the formation of this type of complex.

A similar experiment was carried out for the reaction mixture of *cis*-DDP with an octanucleotide ([5′-d(CpGpGpApTpCpCpG)-3′]$_2$), which has a guanine base at the 3′-OH end (G(8)) and adjacent guanine bases (G(2) and G(3)) within the sequence. In the experiment mentioned above, the adjacent guanine bases exist at the end of the sequence. In such a case, it is difficult to distinguish whether the binding specificity arises from the terminal guanine base or the adjacent guanine base pair. Assuming that *cis*-DDP has a binding specificity to a terminal guanine base, *i.e.*, G(8) in [5′-d(CpGpGpApTpCpCpG)]$_2$, Pt-modified 5′-d(CpGpGpApTpCpCpG)-3′ should not be digested by VPD because VPD cannot cut a phosphodiester bond when the platinum atom exists on the base of the side of the recognition site (3′-OH) even if its binding is a monofunctional one [17]. Figure 4 shows the analytical result of the 5′-deoxy-nucleotides, being produced by VPD digestion of the Pt-modified 5′-d(CpGpGpApTpCpCpG)-3′. The 5′-dGMP sharply decreases with increasing r. Moreover, the decrease in 5′-dGMP is not accompanied by a decrease in the other 5′-deoxynucleotides. This means that the G(8) base is not a preferential platinum binding site. This strongly suggests that the digestion by VPD just stops at G(3) or G(2).

Figure 5 shows a chromatogram of the VPD digestion products of Pt-modified 5′-d(CpGpGpApTpCpCpG)-3′ obtained using a weak cation exchange column. As is expected from its negative charge, unplatinated 5′-deoxynucleotide which appeared at t_R = 6–8 min, was hardly retained by the column. The peak at t_R = 15 min was identical with dC in retention time. This is thought to be the dC at the 5′-OH end, produced by VPD digestion of the unplatinated octanucleotide. The peak at t_R = 17 min, which increases with increasing r, is assigned to *cis*-$Pt(NH_3)_2$(d(CpGpG)). The peak of *cis*-$Pt(NH_3)_2$-(d(GpG)) was not observed at all. These results clearly indicate that VPD digestion just stopped at

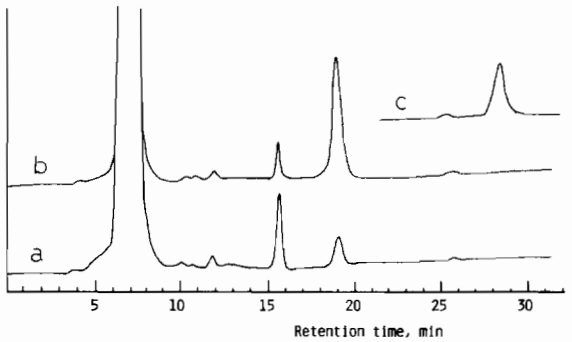

Fig. 5. HPLC elution patterns of the enzymatic digestion products of *cis*-$Pt(NH_3)_2^{2+}$–modified [5′-d(CpGpGpApTpCpCpG)-3′]$_2$; weak cation exchange column; monitored at 260 nm; (a) r = 0.26, (b) r = 0.81, (c) chromatogram shows the peak of *cis*-$Pt(NH_3)_2$(d(GpG)), obtained by treating with VPD, alkaline phosphatase and SPD of the *cis*-$Pt(NH_3)_2^{2+}$–modified [5′-d(CpGpGpApTpCpCpG)-3′]$_2$.

G(3). The result shown in Fig. 6 is thought to be pertinent evidence of such an assignment. That is, there is an excellent correlation between the peak areas of t_R = 15 and t_R = 17 min (r = 0.999). Moreover, the slope of the regression line is 0.267, the value of which is in good agreement with the ratio of the absorption coefficient of dG and dC, ϵ(dC)/(2ϵ(dG) + ϵ(dC)). It is important that *cis*-$Pt(NH_3)_2$(d(CpGpG)) is the only Pt-adduct observed in the chromatogram. In order to obtain further evidence, the VPD digestion products were treated with alkaline phosphatase and calf spleen phosphodiesterase. The peak at t_R = 17 min gradually decreases with incubation time, a new peak assigned to *cis*-$Pt(NH_3)_2$(d(GpG)) appearing at t_R = 28 min. These facts clearly indicate that *cis*-$Pt(NH_3)_2Cl_2$ selectively binds to the adjacent guanine bases in 5′-d(CpGpGpApTpCpCpG)-3′. That is, we can draw the same conclusion as before. The adjoining guanine bases in self complementary octanucleotide have a specific binding affinity for *cis*-$Pt(NH_3)_2^{2+}$.

Discussion

DNA-interstrand crosslinks and DNA-protein interstrand crosslinks induced by *cis*-$Pt(NH_3)_2^{2+}$ have been extensively studied by means of alkaline cesium chloride and alkaline elution techniques [21, 22]. It has been reported that such an interstrand crosslink correlates with cytotoxicity though there are certain exceptions. However, it should be noted that the techniques can detect an interstrand crosslink, but cannot detect an intrastrand crosslink. It has also been reported that the interstrand crosslink is a minor event in platinum binding with DNA. That is, a major part of the platinum bound to DNA seems to participate in an intrastrand crosslink.

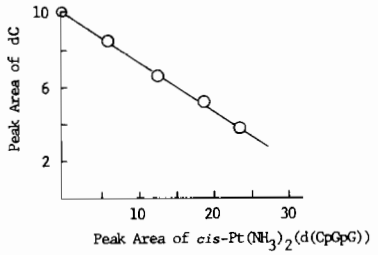

Fig. 6. Correlation between *cis*-$Pt(NH_3)_2$(d(CpGpG)) and dC; slope = 0.267, correlation coefficient = 0.999.

Unfortunately, a technique which is able to estimate such an intrastrand crosslink has not yet been developed. Recently, attention has been focused on the intrastrand crosslink between two adjacent guanine bases. Such a platinum binding mode has been proposed on the basis of the experimental results showing that the enzymatic digestion of Pt-modified DNA is selectively inhibited in the proximity of the sequences containing adjacent guanine bases [11–13]. Soon afterward, the platinum binding mode was confirmed by NMR studies of the Pt-modified oligonucleotides [3–10]. However, the method using H-NMR seems to be limited in making a direct identification of platinum binding base in macromolecules like DNA. On the other hand, the enzymatic digestion techniques seem to be useful for determination of platinum binding base in DNA. However, it is necessary to make clear how substrates like Pt–modified oligonucleotides and DNA are able to be digested by enzymes. The present study shows that VPD can digest the phosphodiester bond until just before the platinum binding base. But the phosphodiester bond interposed by *cis*-$Pt(NH_3)_2^{2+}$ chelated guanine bases cannot be digested. This was also verified by the experiment using the isolated *cis*-$Pt(NH_3)_2$(d(GpG)) in which *cis*-$Pt(NH_3)_2^{2+}$ is bound to both guanine bases via the N(7) sites. This is why *cis*-$Pt(NH_3)_2$(d(GpG)) can be identified by the VPD digestion of Pt-modified 5′-d(GpGpTpCpGpApCpC)-3′. On the other hand, the VPD digestion of *cis*-$Pt(NH_3)_2^{2+}$ modified 5′-d(CpGpGpApTpCpCpG)-3′ just stopped at G(3). In this case, VPD digestion could not give *cis*-$Pt(NH_3)_2$(dGpG)) as the digestion products because VPD could not digest the phosphodiester bond between C(1) and G(2). For the purpose of quantification of *cis*-$Pt(NH_3)_2$(d(GpG)), the products obtained after digesting with VPD must be further treated with SPD. This is important in the case of the quantification of *cis*-$Pt(NH_3)_2$(d(GpG)). Pt-modified DNA has usually been digested by an endonuclease like DNase I to give Pt-modified oligonucleotides. Does DNase I cut the phosphodiester bond adjacent to *cis*-$Pt(NH_3)_2^{2+}$ chelated GpG? According to our study [17], it is possible, but the rate of DNase I digestion seems to slow significantly, probably

because the intrastrand crosslink between the adjacent guanine bases distorts the structure in the vicinity of the platinum binding sites. Very recently, two research groups reported independently that the *cis*-$Pt(NH_3)_2^{2+}$ adduct with an intrastrand crosslink between two adjacent guanine bases in the oligonucleotides can form a duplex structure with the complementary oligonucleotide [9, 10]. But the vertical stacking interaction in the vicinity of the platinum binding bases is distorted. The effect of such a steric factor on enzymatic digestion remains uncertain. In our previous paper [17], the Pt-modified DNA obtained from the reaction of Pt(R,R-1,2-cyclohexanediamine)$^{2+}$ with calf thymus DNA, was treated with DNase I and VPD. It did not give the Pt-complex with intrastrand crosslink between two adjacent guanine bases, but subsequent treatment with alkaline phosphatase and SPD gave the Pt(R,R-1,2-cyclohexanediamine)d(GpG)). That is, the phosphodiester bond adjacent to the two guanine bases chelated by platinum appeared to resist cutting by DNase I. Similar behavior has also been observed in the digestion of *cis*-$Pt(NH_3)_2^{2+}$ modified DNA. SPD can digest the phosphodiester bond adjacent to the *cis*-$Pt(NH_3)^{2+}$ chelated (d(GpG)) in 5′-d(CpCpGpG)-3′, but the digestion rate between C(2) and G(3) is significantly slower than that between C(1) and C(2) (more than 10 times) [17]. As a result, we can see the partially digested product, *cis*-Pt-$(NH_3)_2$(d(CpGpG)).

In summary, the phosphodiester bond interposed by *cis*-$Pt(NH_3)_2$ chelated guanine bases is not cut by DNase I, VPD and SPD. The phosphodiester bond adjacent to *cis*-$Pt(NH_3)_2$ chelated guanine bases can be digested by either VPD or SPD, but its digestion rate is slow compared with that of unplatinated oligonucleotide. DNase I scarcely cut the phosphodiester bond. The bonds are thought to be caused by the steric distortion arising from bifunctional platinum binding to adjacent guanine bases. For the purpose of quantification of *cis*-$Pt(NH_3)_2$ chelated GpG, the fragments obtained after DNase I digestion must be further treated with VPD, alkaline phosphatase and SPD. Using these treatments, the quantification of *cis*-$Pt(NH_3)_2$ chelated GpG in Pt-modified DNA is thought to be possible.

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