Slowing of Cisplatin Aquation in the Presence of DNA but Not in the Presence of Phosphate: Improved Understanding of Sequence Selectivity and the Roles of Monoaquated and Diaquated Species in the Binding of Cisplatin to DNA

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^{1}H−^{15}N HSQC NMR spectroscopy is used to study the aquation reactions of cisplatin in 9 mM $NaClO_4$ and 9 mM phosphate (pH 6) solutions at 298 K. For the first time in a single reaction and, therefore, under a single set of reaction conditions, the amounts of all species formed are followed and the rates of aquation, diaquation, and related anation processes are determined in both media. Binding of phosphate to aquated Pt species is observed, but the initial rate of aquation is not affected by the presence of 9 mM phosphate. The reaction between cisplatin and the 14-base-pair self-complementary oligonucleotide 5′-d(AATT**GG**TACCAATT)-3′, having a GpG intrastrand binding site, is investigated. Various kinetic models for this reaction are evaluated and the most appropriate found to be that with a reversible aquation step and a single binding site for the self-complementary duplex. The rate constant for aquation is $(1.62 \pm 0.02) \times 10^{-5}$ s^{-1}, with the anation rate constant fixed at 4.6×10^{-3} M^{-1} s^{-1}, the value obtained from the aquation studies. The rate constants for monofunctional binding of *cis*-[PtCl($^{15}NH_3$)$_2$(OH_2)]$^+$ to the sequence were 0.48 ± 0.19 and 0.16 ± 0.06 M^{-1} s^{-1} for the 3′- and 5′-guanine bases, respectively. Closure rate constants for the monofunctional adducts are $(2.55 \pm 0.07) \times 10^{-5}$ and $(0.171 \pm 0.011) \times 10^{-5}$ s^{-1}, for the 3′- and 5′-guanines, respectively. The presence of DNA slows the aquation of cisplatin by 30−40% compared to that observed in 9 mM $NaClO_4$ or 9 mM phosphate, and there is some evidence that the degree of slowing is sequence dependent. The possibility that *cis*-[Pt(OH)(NH_3)$_2$(OH_2)]$^+$ contributes to the binding of cisplatin to DNA is investigated, and it is found that about 1% followed this route, the majority of the binding occurring via the monoaquated species *cis*-[PtCl(NH_3)$_2$(OH_2)]$^+$. Comparison of the rates of disappearance of cisplatin in reactions at single defined GpG, ApG, GpA, GpTpG and 1,2-interstrand GG binding sites shows that the adduct profile is determined at the level of monofunctional adduct formation.

Introduction

When the anticancer drug cisplatin (*cis*-[$PtCl_2(NH_3)_2$]) binds to DNA, a number of structurally different adducts are formed in varied amounts. The major adducts are the GpG and ApG intrastrand adducts that together account for 80−90% of the bound Pt.[1−7] It is not yet clear what determines the adduct profile, but Pt binding to nucleobases is kinetically controlled[8−11] and the bifunctional adducts are believed to be stable on the time scale of days to weeks. Consequently, the adduct profile should reflect the results of the initial interactions between Pt and DNA[12] although this has not been confirmed experimentally.

Following and determining the rates of the manifold reactions that occur in the formation of even a single adduct are difficult tasks. Lippard and co-workers[13] successfully used ^{195}Pt NMR spectroscopy to follow the binding of cisplatin to DNA, but their kinetic analysis did not separate the aquation process from monofunctional adduct formation. The use of ^{1}H−^{15}N HSQC NMR spectroscopy has permitted the quantification of all intermediate and product species that form. Kinetic analysis of the change in species concentrations over time has led to the determination of the rate constants, and the binding of ^{15}N-cisplatin and *cis*-[Pt($^{15}NH_3$)$_2$(OH_2)$_2$]$^{2+}$ at 5′-d(ATACAT**GG**TACATA)-3′•5′-d(TATGTACCATGTAT)-3′ (-***GG***-), 5′-d(AATT**AG**TACTAATT)-3′ (-**AG**-), and 5′-d(AATT**GA**TATCAATT)-3′ (-**GA**-) sequences has been studied.[14−17] Comparison of the

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results of these studies has revealed much about factors that lead to the observed adduct profile. The sequence types used for the study of binding to ApG and GpA sites[16,17] differed from that used for binding at GpG[14,15] in that they are self-complementary. To ensure the validity of these important comparisons, we have now undertaken a study of binding to an analogous, self-complementary, GpG-containing sequence, 5′-d(AATT**GG**TACCAATT)-3′ (-**GG**-). More importantly, approximations and assumptions in the kinetic modeling of these reactions have raised a number of issues with regard to the accuracy of the results. For example, in the previous analyses of the binding rates, the first step, aquation of cisplatin,[18,19] was assumed to be irreversible. This is not the case, but none of the numerous previously reported studies[20−28] of cisplatin aquation and anation employed conditions identical to those used in the kinetic analyses. In addition, our studies on DNA binding have been carried out in the presence of phosphate buffer. Phosphate is known to bind to cisplatin,[29−32] but the effect on rates of cisplatin aquation and on subsequent reactions with DNA are not known. Therefore, we have undertaken a detailed study of the aquation of cisplatin in the absence and presence of phosphate, the first study that has allowed the individual species to be monitored and their rates of formation to be determined.

Finally, we have assumed in previous modeling that it is only the singly aquated species, *cis*-$[PtCl(NH_3)_2(OH_2)]^+$, that binds to DNA.[16,17] Chottard, Kozelka, and co-workers[12,33,34] used HPLC-based separation and quantification of adducts to study the reaction of *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ with oligonucleotides containing GpG and ApG binding sites and recently suggested that this diaquated species, or its deprotonated form, might be the major contributor to DNA platination in vivo.[35] Likewise, unaquated cisplatin might also react directly with DNA. Therefore, we have used the study of this new GpG-containing sequence, and the information derived from the aquation studies, to more fully investigate these issues, fundamental to the understanding of interactions between cisplatin and DNA.

Experimental Section

Chemicals. *cis*-$[PtCl_2(^{15}NH_3)_2]$ was synthesized according to Kerrison and Sadler[36] and recrystallized from aqueous KCl. The sodium salt of the HPLC-purified oligonucleotide 5′-d(AATTGGTACCAATT)-3′ (-**GG**-) was purchased from OSWEL.

Sample Preparation. A stock solution of -**GG**- was prepared in 500 μL of 5% D_2O/95% H_2O. The concentration was estimated spectrophotometrically to be 5.10 mM[37] by using the absorption coefficient provided by OSWEL ($\epsilon_{260} = 286\,200\ M^{-1}\ cm^{-1}$ per duplex).

Aquation of Cisplatin in the Presence of 9 mM $NaClO_4$. ^{15}N-Cisplatin (1.77 mg, 5.86 μmol) was dissolved in 5% D_2O/95% H_2O (733 μL) by sonicating at 298 K for 10 min to give an 8.00 mM stock solution. An 82.5 μL aliquot of the stock solution was added to 340.4 μL of 5% D_2O/95% H_2O and 15.1 μL of a 263 mM $NaClO_4$ solution (*caution: perchlorate salts are explosive!)* that had been equilibrated at 298 K to give an initial ^{15}N-cisplatin concentration of 1.51 mM. The initial pH was 6.6, and after 40 h the solution pH was found to be 4.9.

Aquation of Cisplatin in the Presence of 9 mM Phosphate. ^{15}N-Cisplatin (1.25 mg, 4.14 μmol) was dissolved in 5% D_2O/95% H_2O (517.4 μL) by sonicating at 298 K for 2 min, giving an 8.00 mM stock solution. A 162.5 μL aliquot of this solution was added to a solution containing sodium phosphate buffer (29.3 μL of a 200 mM solution, pH 5.9), TSP (3 μL of a 13.3 mM solution), and 5% D_2O/95% H_2O (455.2 μL), to give initially 200 mM ^{15}N-cisplatin and 9.0 mM phosphate. There was no change in the solution color indicative of the "platinum blues".[30,32]

Reaction of Cisplatin with -GG-. Duplicate reactions were carried out as follows: Into a 5 mm Shigemi NMR tube were placed TSP (sodium 3-(trimethylsilyl)propionate-d_4) (2 μL, 13.3 mM in 5% D_2O/95% H_2O), sodium phosphate buffer (pH 5.9, 19.8 μL, 200 mM in 5% D_2O/95% H_2O), -**GG**- oligonucleotide stock solution (181.9 μL), and 5% D_2O/95% H_2O (153.8 μL). The solution was warmed to ∼325 K and allowed to cool slowly. The duplex nature of the oligonucleotide was confirmed from the imino region of the ^{1}H NMR spectrum. To this solution was added an 82.5 μL aliquot of a freshly prepared solution of ^{15}N-cisplatin (1.69 mg, 5.60 μmol) in 5% D_2O/95% H_2O (700 μL). ^{15}N-Cisplatin was dissolved by sonicating 8.0 mM solution for 2 min at room temperature until the solution was clear, and shaking the solution to ensure homogeneity. Aquation of cisplatin was minimized by rapidly adding this stock solution to the reaction mixture. The time from addition of solvent to cisplatin until addition of the cisplatin solution to the reaction mixture was 3 min. Confirmation of the initial lack of aquated species was obtained from the first of the 2D spectra where, in each case, the concentration of *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ was less than 0.5% of the total Pt. This gave initial reactant concentrations of 2.10 mM for the -**GG**- duplex[37] and 1.50 mM for ^{15}N-cisplatin and a phosphate buffer concentration of 9.0 mM. The kinetic runs were

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carried out at 298 K and covered the time periods 0−288 and 49−344 h to maximize the use of NMR time. Samples were maintained at 298 K when not in the spectrometer. Kinetic data were combined from both kinetic runs, during which spectra with identical reaction times were collected to confirm that the rates were identical.

Instrumentation. $^1H\{^{15}N\}$ NMR spectra were recorded at 599.924 and 400.13 MHz on Varian UNITY-INOVA 600 MHz and Varian UNITY 400 MHz spectrometers, respectively, fitted with pulsed field gradient modules and 5 mm triple-resonance probeheads. The ^1H spectra were acquired with water suppression using the WATERGATE sequence.[39] Both the 1D ^{15}N-edited ^1H NMR spectra and 2D $^1H-^{15}N$ HSQC NMR spectra (optimized for $^1J(^{15}N-^1H) = 72$ Hz) were recorded using the sequence of Stonehouse et al.[40] Samples were not spun during the acquisition of data. All samples (including buffers, acids, etc.) were prepared so that there was a 95% H_2O/5% D_2O proportion of concentrations (for a deuterium lock but with minimal loss of signal as a result of deuterium exchange).

^1H chemical shifts were referenced to internal 1,4-dioxane for the cisplatin aquation studies and to TSP for the cisplatin + **-GG-** reactions. ^{15}N chemical shifts were calibrated externally against $^{15}NH_4Cl$ (1.0 M in 1.0 M HCl in 5% D_2O/95% H_2O). The ^{15}N signals were decoupled by irradiating with the GARP-1 sequence at a field strength of 1 kHz during the acquisition time.

Typically, for 1D ^1H spectra, 64 or 128 transients were acquired using a spectral width of 12 kHz, a relaxation delay of 1.5 s, and a line broadening of 1.0 Hz. For kinetics studies using $^1H-^{15}N$ HSQC NMR spectroscopy, between 4 and 12 transients were collected for 16−64 increments of t_1 (allowing spectra to be recorded on a suitable time scale for the observed reaction), with an acquisition time of 0.152 s and spectral widths of 4 kHz in f_2 (^1H) and 1.823 kHz in f_1 (^{15}N). 2D spectra were completed in 4−30 min.

The 2D spectra were processed using Gaussian weighting functions in both dimensions and zero-filling by ×2 in the f_1 dimension. The pHs of the solutions were measured on a Shindengen pH Boy-P2-(su19A) pH meter and calibrated against pH 6.9 and 4.0 buffers. In each case, a portion 5 μL of the solution was placed on the electrode surface and the pH recorded. These aliquots were not returned to the bulk solution (as the electrode leaches Cl^-). Adjustments in pH were made with 1.0 M HNO_3 in 10% D_2O/90% H_2O or 1.0 M NaOH in 10% D_2O/90% H_2O.

Data Analysis. The kinetic analysis of the reactions was undertaken by measuring the peak volumes in the $^1H-^{15}N$ HSQC NMR spectra using the Varian VNMR software and calculating the relative concentration of *cis*-$\{Pt(^{15}NH_3)_2\}$ at each time point. For a given reaction, peak volumes were determined using an identical vertical scale and threshold value. It was assumed that all peaks have ^{195}Pt satellites; only those of cisplatin are visible in the spectra (600 MHz), but are additionally seen for *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ in the 400 MHz spectra, and their absence for other species is presumably a consequence of relaxation through chemical shift anisotropy.[41] All species, other than cisplatin and *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$, give rise to two peaks, the inequivalence of the NH_3 groups being due to the ligands trans to them. In some cases, overlap of peaks is significant, but in these instances, it was only one of the pair of the peaks that was overlapped. Thus, reliable intensities were obtained from the second of the peaks. The peak volumes were normalized, and the concentration of each species was determined for each spectrum. The data were then subjected to kinetic analysis. The appropriate differential equations[42] were integrated numerically and rate constants determined by a nonlinear optimization procedure using the program SCIENTIST (Version 2.01, Micromath Inc.). The errors quoted represent one standard deviation. In all cases, the data were fit using appropriate first- and second-order rate equations. Minima were found by using simulations and by gradually optimizing the input parameters, fixing some while others were refined, and then finally allowing all parameters to be refined simultaneously.

Kinetic Models. The cisplatin + **-GG-** kinetic data were treated with a number of kinetic models. These can be broadly categorized into four sets:

(1) Irreversible Aquation. The aquation of cisplatin was treated as a first-order irreversible reaction with the concentration of binding sites treated as (i) twice the concentration of the **-GG-** duplex (model 1), (ii) equal to the concentration of the duplex (model 2), or (iii) twice the concentration of the duplex but with the loss of both binding sites upon the binding of Pt (model 3).

(2) Reversible Aquation, with a Variable Aquation (Forward) Rate Constant and a Fixed Anation Rate Constant. The aquation process was treated as fully reversible with the anation rate constant fixed to the value determined for the aquation of cisplatin in the absence of DNA (vide infra) and the forward (aquation) rate constant varied. The treatment of the concentration of binding sites was the same as described in models 1−3, giving models 4−6, respectively.

Alternatively, the aquation process was treated as reversible, the forward (aquation) rate constant fixed and the reverse (anation) rate constant varied and the concentration of binding sites was the same as in model 2 (model 7).

(3) Reversible Aquation with Aquation and Anation Rate Constants Fixed to the Values Found in the Absence of DNA. The forward (aquation) and reverse (anation) rate constants were fixed at the values obtained from the study of the aquation of cisplatin in the absence of DNA, and the concentration of binding sites was the same as in model 2 (model 8).

(4) Incorporation of the Second Aquation Process (Model 9). The values for all rate parameters from model 5 were used as input parameters, and the values from the second aquation step in aqueous perchlorate were fixed (Table 2). Rate constants for the binding of *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) to the 5′- and 3′-guanines ($k_{5'A}$ and $k_{3'A}$ in Scheme 2) were fixed to the values (18 and 15 M^{-1} s^{-1}, respectively) determined for a similar GpG sequence by Legendre, Kozelka, and Chottard at 293 K.[34] Rates of closure of the monofunctional aqua adducts to form *cis*-[Pt(G5-*N7*)(G6-*N7*)$(^{15}NH_3)_2$] (**10**) ($k_{5'ch} = 0.18 \times 10^{-3}$ s^{-1} and $k_{3'ch} = 1.9 \times 10^{-3}$ s^{-1}) were taken from the same source.[34] Contributions to binding via the different routes were calculated by artificially designating their products as different species and fixing all rate constants. Allowance for the difference in temperature between the reactions carried out by Legendre et al.[34] and those reported here was made by doubling their rate constants.

The initial concentration of *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) was varied from 0 to 0.000 15 M for all the models to determine whether prior aquation significantly affected the rate constants. In all the kinetic models, the time-dependent behavior of the total of the bifunctional species (**10a** + **10b**) was used rather than those of the separate conformational forms. This was necessary because the forms interconvert during the reaction and no information regarding the kinetics of the interconversion under these conditions is available. Model 5 was also used to re-examine the kinetic data for the previously reported reactions between cisplatin and **-AG-** and **-GA-**.[16] The kinetic models used for both aquation reactions and the various models for binding of cisplatin to **-GG-** are provided as Supporting Information.

Cisplatin Half-Life Calculations. The half-life times of cisplatin with a variety of sequences were determined using the formula

$$t_{1/2} = \ln(0.5)/k_H$$

The values of k_H were taken from Table 3 for reactions between cisplatin and **-GG-**, **-AG-**, and **-GA-**. Those for reactions between cisplatin and duplexes containing an interstrand 1,2-GG binding site and an intrastrand 1,3-GTG binding site were from unpublished work.[43,44] The value for the reaction with ***-GG-*** was taken from Berners-Price et al.[14]

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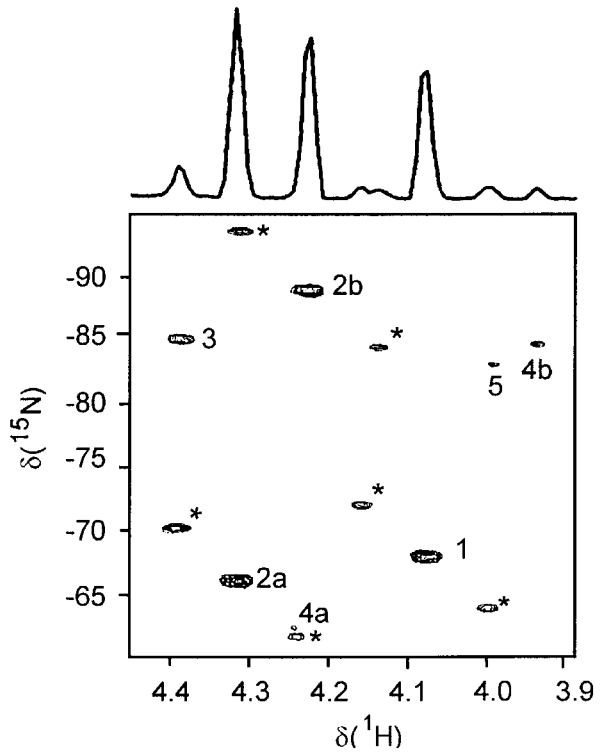

Figure 1. $^{1}H-^{15}N$ HSQC NMR spectrum (400 MHz) at 298 K of ^{15}N-cisplatin in 9 mM $NaClO_4$ after 40 h. Asterisks = ^{195}Pt satellites; peaks are assigned to $^{15}NH_3$ in structures **1**−**5**, defined in Scheme 1. Assignments are based on data in refs 45 and 46.

Results

$^{1}H-^{15}N$ HSQC NMR spectroscopy has been used to examine the aquation of cisplatin in aqueous perchlorate media (unbuffered) and in aqueous phosphate buffer (pH 6) and the reaction between cisplatin and a self-complementary 14-base-pair oligonucleotide with an intrastrand GpG binding site (-**GG**-).

Aquation of Cisplatin. The aquation of a 1.51 mM solution of cisplatin in 9 mM $NaClO_4$ was monitored for 40 h at 298 K. The reaction commences with no significant concentration of any species other than cisplatin. *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) is the first new species to be observed, comprising 8% of the total Pt within 2 h. The first indication of *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) occurs after 3.5 h, and the bridged dinuclear Pt complexes $[\{cis\text{-}Pt(^{15}NH_3)_2\}_2(\mu\text{-}Cl)(\mu\text{-}OH)]^{2+}$ (**4**) and $[\{cis\text{-}Pt(^{15}NH_3)_2(OH_2)\}_2(\mu\text{-}OH)]^{3+}$ (**5**), appear at 10.5 and 26 h, respectively. The dinuclear species were assigned on the basis of their ^{15}N shifts, which are in good agreement with those reported by Appleton and co-workers.[30,45] The dinuclear Pt complexes account for a total of ∼5% of the total Pt after 40 h. A typical $^{1}H-^{15}N$ HSQC NMR spectrum of the reaction mixture is shown in Figure 1, and the time-dependent behavior of the species is shown in Figure 2, along with curves from kinetic fits to the reaction model depicted in Scheme 1. Chemical shifts and assignments of the resonances are given in Table 1, and the rate constants are listed in Table 2.

The pH of the solution was monitored throughout the course of the aquation reaction via the ^{1}H chemical shifts of the *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ and *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ complexes[46] and was experimentally measured at the commencement of the reaction and after the establishment of equilibrium. A decrease in pH from 6.6 to 4.9 was observed. No consideration of pH was incorporated into the kinetic fit (vide infra). The aquation and anation rate constants for both the first and second steps are listed in Table 2. The formation of the minor products $[\{cis\text{-}Pt(^{15}NH_3)_2\}_2(\mu\text{-}Cl)(\mu\text{-}OH)]^{2+}$ (**4**) and $[\{cis\text{-}Pt(^{15}NH_3)_2(OH_2)\}_2(\mu\text{-}OH)]^{3+}$ (**5**) was assumed to arise from the combination of two separate *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) and cis-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) ions, as depicted in Scheme 1.

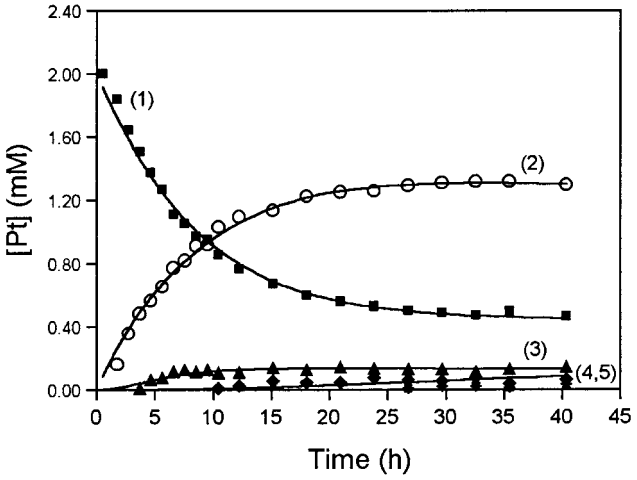

Figure 2. Plots of the relative concentrations of species observed during the aquation of cisplatin in 9 mM $NaClO_4$ at 298 K: ■, **1**; ○, **2**, ▲ **3**, ♦, **4**, ∗, **5**. The curves are computer best fits for the rate constants listed in Table 2.

After 40 h, cisplatin accounted for 23% of the total Pt in solution, the major species being *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ (**2**), which comprised ∼65% of the total. The remainder consisted of *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ (**3**) (7%) and the various dimeric species (∼5%). Ignoring the dinuclear species, these values give equilibrium constants of $pK_1 = 2.07$ and $pK_2 = 3.49$.

Aquation of Cisplatin in the Presence of Phosphate. The aquation of cisplatin in the presence of a 4.5-fold excess of phosphate (pH 5.9) was monitored for 24 h at 298 K. After this time, it became apparent that the reactions which ensue between the phosphate and the various aquated forms of cisplatin are complex. The rate of the initial aquation step (Table 2) was the same as that observed in the perchlorate solution. However, within 3.5 h, new peaks indicative of species not observed in the reaction in sodium perchlorate solution became visible, and, after 24 h of reaction, a total of 19 resonances were observed in the $^{1}H-^{15}N$ HSQC NMR spectrum (Figure 3). The chemical shifts of these species are listed in Table 1, and tentative assignments, based on identification pairs and on those of Appleton and co-workers,[30,45] are given where possible. The time dependencies of the major species in solution are shown in Figure 4, along with fits to the reactions giving the rate constants defined in Scheme 1 and listed in Table 2.

Reaction between Cisplatin and -GG-. $^{1}H-^{15}N$ HSQC NMR spectroscopy has been used to examine the rate of reaction between cisplatin and the self-complementary 14-mer duplex 5′-d(AATTGGTACCAATT)-3′ (-**GG**-) in an aqueous phosphate-buffered solution at pH 6 at 298 K. ^{1}H NMR spectra were concurrently acquired to monitor both binding via changes in the chemical shifts of the aromatic protons ($\delta = 6.5-9.2$ ppm) and oligonucleotide base pairing through examination of the imino resonances ($\delta = 10-15$ ppm). The reaction conditions were selected to allow direct comparison with recently published studies of the reactions with the non-self-complementary -***GG***-[14] and with -**AG**- and -**GA**-.[16,17]

(45) Appleton, T. G.; Hall, J. R.; Ralph, S. F.; Thompson, C. S. M. *Inorg. Chem.* **1989**, *28*, 1989−1993.
(46) Berners-Price, S. J.; Frenkiel, T. A.; Frey, U.; Ranford, J. D.; Sadler, P. J. *J. Chem. Soc., Chem. Commun.* **1992**, 789−791.

Scheme 1. Aquation Processes of Cisplatin in Aqueous Perchlorate and Phosphate Solution.

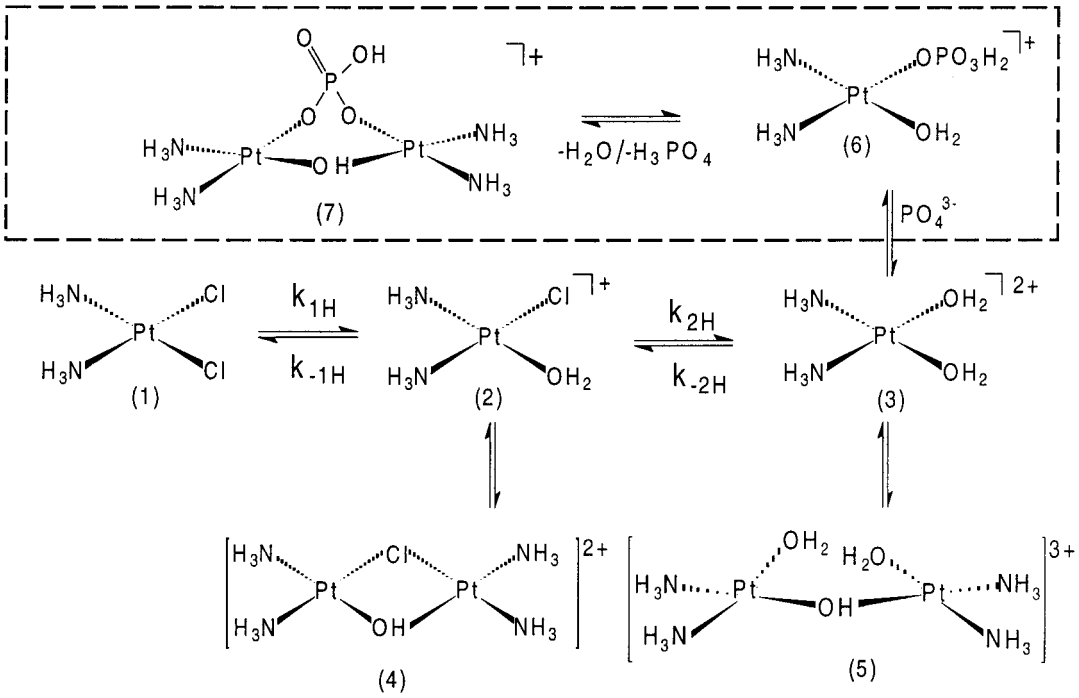

Table 1. ^{1}H and ^{15}N NMR Chemical Shifts and Assignments for Aquation Products of ^{15}N-Cisplatin in 9 mM Phosphate Solution (pH 5.9) at 298 K[a]

species	no.	$\delta(^1H)$	$\delta(^{15}N)$	trans ligand
cis-[$PtCl_2(^{15}NH_3)_2$]	**1**	4.08	−68.0	Cl
cis-[$PtCl(^{15}NH_3)_2(OH/H)]^+$	**2a**[b]	4.26	−66.3	Cl
cis-[$PtCl(^{15}NH_3)_2(OH/H)]^+$	**2b**[b]	4.07	−86.9	OH_2
cis-[$Pt(^{15}NH_3)_2(OH/H)_2]^{2+}$	**3**[b]	4.10	−83.6	OH_2
[{cis-$Pt(^{15}NH_3)_2$}$_2$(μ-Cl)(μ-OH)]$^{2+}$	**4a**	4.24	−62.5	Cl
[{cis-$Pt(^{15}NH_3)_2$}$_2$(μ-Cl)(μ-OH)]$^{2+}$	**4b**	3.94	−84.7	OH
[{cis-$Pt(^{15}NH_3)_2(OH_2)$}$_2$(μ-OH)]$^{3+}$	**5**[c]	4.00	−83.1	OH
cis-[$Pt(OPO_3H_2)(^{15}NH_3)_2(OH/H)]^+$	**6a**	4.18	−85.3	OH_2/OPO_3H_2
cis-[$Pt(OPO_3H_2)(^{15}NH_3)_2(OH/H)]^+$	**6b**	4.26	−83.8	OH_2/OPO_3H_2
[{$Pt(^{15}NH_3)_2$}$_2$(μ-OH)(μ-OPO_3)]$^+$	**7a**	3.94	−79.6	OH
[{$Pt(^{15}NH_3)_2$}$_2$(μ-OH)(μ-OPO_3)]$^+$	**7b**	4.08	−84.1	OPO_3H

[a] Additional unassigned ^{1}H/^{15}N peaks occur at δ 4.37/−65.7, 4.26/−84.8, 4.19/−83.6, 4.12/−80.5, 4.09/−81.4, 3.84/−79.4 and 3.93/−87.4 (see Figure 3). [b] The ^{1}H and ^{15}N shifts are strongly dependent on pH.[46] [c] The partner peak for [{cis-$Pt(^{15}NH_3)_2(OH_2)$}$_2$(μ-OH)]$^{3+}$ (**5**) is unassigned but is expected at δ (^{15}N) ∼ −83 ppm,[30,45] where there are several possible peaks (Figure 3).

The reaction was followed for a total of 350 h at 298 K. A typical ^{1}H−^{15}N HSQC NMR spectrum is shown as Figure 5. Peak assignments and chemical shifts are provided as Supporting Information. Assignments are based on the time dependent behavior of the peaks and are almost identical to those reported for ***-GG-***,[14] a different sequence but one maintaining the same TGGT binding site region. The time-dependent behavior of species in the reaction is shown in Figure 6. The kinetic model used to fit the data (model 5) gave the rate constants that are defined in Scheme 2 and listed in Table 3.

Under the experimental conditions, *cis*-[$PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) never accumulates to more than 1.1% of the total Pt and its concentration decreases steadily until it becomes undetectable after 20 h of the reaction. None of the resonances observed during aquation in the phosphate buffer reaction, other than those of cisplatin and *cis*-[$PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**), are seen here, nor is that of *cis*-[$Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**). Two monofunctional DNA adducts, *cis*-[PtCl(G6-*N7*)($^{15}NH_3$)$_2$] (**8**) and *cis*-[PtCl(G5-*N7*)($^{15}NH_3$)$_2$] (**9**), are formed and exhibit different time-dependent behaviors similar to those of the analogous adducts with ***-GG-***.[14] The more rapidly formed monofunctional chloro adduct, that of the 3′-guanine, *cis*-[PtCl(G6-*N7*)($^{15}NH_3$)$_2$] (**8**), also ring-closes to form bifunctional platinated species more rapidly than the 5′-guanine adduct, *cis*-[PtCl(G5-*N7*)($^{15}NH_3$)$_2$] (**9**).[14,47] The monofunctional adducts ring-close to form the bifunctional species *cis*-[Pt(G5-*N7*)(G6-*N7*))($^{15}NH_3$)$_2$] (**10**), which exists as two conformers (**10a**,**b**). The resonances of these species also bear a strong resemblance to those found with ***-GG-***,[14] where it was shown that the bifunctional adduct exists as two conformers in a pH- and temperature-dependent conformational equilibrium.[48] It is reasonable to suppose that a similar conformational equilibrium exists with **-GG-**. Small amounts of a minor product species are also observed (δ(^{1}H/^{15}N): 4.54/−67.4); although unassigned, this species accounts for only ∼5% of the final product. There is no evidence for aquation of either of the monofunctional chloro species preceding ring closure, and this is despite the slow closure step for *cis*-[PtCl(G5-*N7*)($^{15}NH_3$)$_2$] (**9**), where there is ample time for the approach and substitution by water to occur. The time-dependent behaviors of all species in this reaction were similar to those of the analogous species in the reaction between cisplatin and ***-GG-***.[14]

Discussion

Aquation of Cisplatin. An understanding of the aquation of cisplatin is important because it is believed to be the means by which the drug is activated prior to binding to DNA in vivo.[49] In recent years, the aquation of cisplatin has become the focus

(47) Assignment of the two monofunctional adducts is based on the similarity of their chemical shifts and time dependencies to those found with ***-GG-***,[14] the assignments of which were later confirmed by enzymatic digestion.[15]

(48) Berners-Price, S. J.; Corazza, A.; Guo, Z.; Barnham, K. J.; Sadler, P. J.; Ohyama, Y.; Leng, M.; Locker, D. *Eur. J. Biochem.* **1997**, *243*, 782−791.

(49) Johnson, N. P.; Hoeschele, J. D.; Rahn, R. O. *Chem. Biol. Interact.* **1980**, *30*, 151−169.

Table 2. Aquation and Anation Rate Constants for ^{15}N-Cisplatin in 9 mM $NaClO_4$ and 9 mM Phosphate (pH 5.9) at 298 K[a]

parameter	ClO_4^-	phosphate	parameter	ClO_4^-	phosphate
k_{1H} (10^{-5} s^{-1})	2.38 ± 0.04	2.37 ± 0.04	k_{2H} (10^{-5} s^{-1})	1.4 ± 0.3	2.2 ± 0.4
k_{-1H} (10^{-3} M^{-1} s^{-1})	4.6 ± 0.3	4.5 ± 1.2	k_{-2H} (M^{-1} s^{-1})	0.081 ± 0.022	0.08 ± 0.08

[a] Rate constants for the formation of dinuclear and phosphate-bound species are not included.

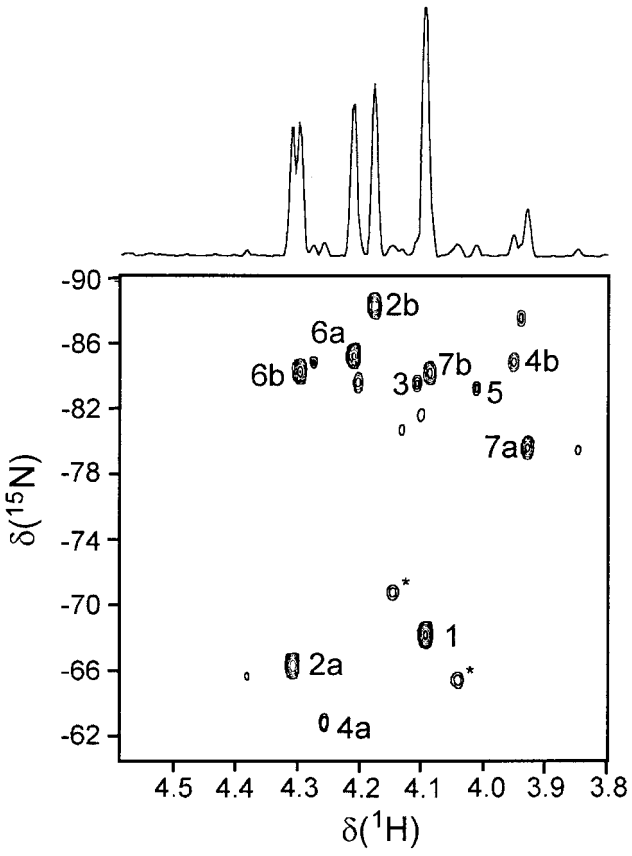

Figure 3. ^{1}H−^{15}N HSQC NMR spectrum (600 MHz) at 298 K of ^{15}N-cisplatin and its hydrolysates in 9 mM sodium phosphate (pH 5.9) after 24 h. Asterisks = ^{195}Pt satellites; peaks are assigned to $^{15}NH_3$ in structures **1**−**7**, defined in Scheme 1.

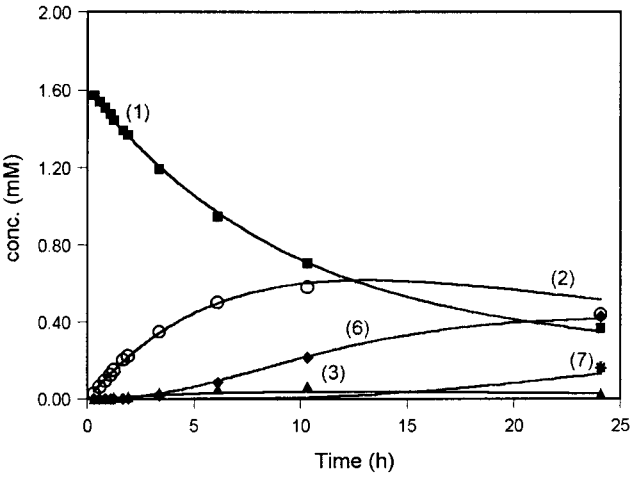

Figure 4. Plots of the relative concentration of species observed during the aquation of ^{15}N-cisplatin in 9 mM sodium phosphate solution (pH 5.9) at 298 K: ■, **1**; ○, **2**; ▲, **3**; ♦, **6**; *, 7. The curves are computer best fits for the rate constants listed in Table 2.

of numerous studies.[20,21,27,28,46,50,51] House and co-workers[22−27] have reported extensive studies on the aquation of cisplatin and

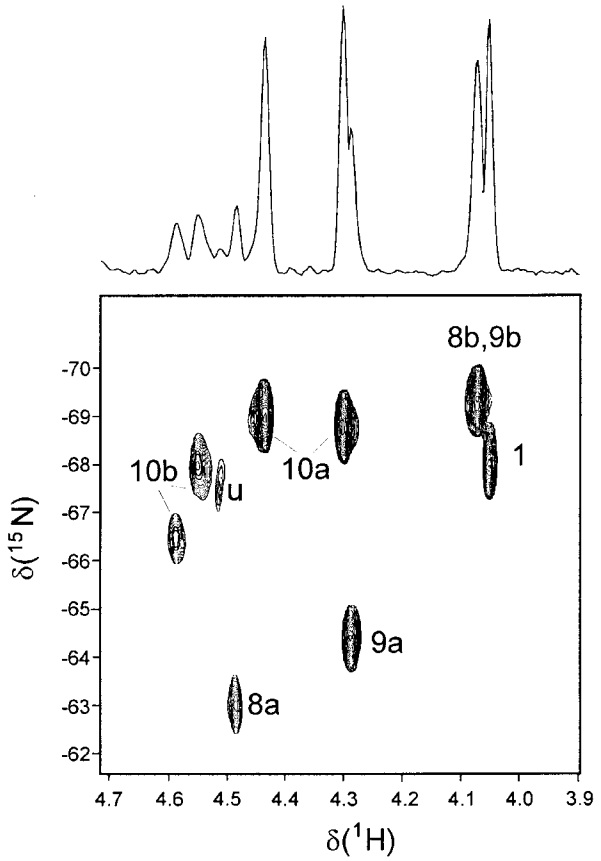

Figure 5. ^{1}H−^{15}N HSQC NMR spectrum (600 MHz) at 298 K of ^{15}N-cisplatin after reaction with -**GG**- for 31 h. Asterisks = ^{195}Pt satellites; peaks are assigned to $^{15}NH_3$ in structures **1**, **8**, **9**, and **10**. Minor peak "u" is unassigned (see text).

related complexes and the formation kinetics and properties of the aquated species under a wide range of experimental conditions, but in no case under the experimental conditions employed in our work. ^{1}H−^{15}N HSQC NMR spectroscopy has been used to characterize the hydrolysis products of cisplatin and to determine the pK_a values of the coordinated aqua ligands, as well the equilibrium constants for the aquation reaction, but the reaction kinetics were not followed.[46] The ^{1}H−^{15}N HQSC NMR technique is particularly well suited to studies of this type because all of the platinum species in solution can be identified and their time dependencies examined independently. This is not achievable with more conventional UV/vis absorption or electropotential techniques, by which usually only one species is examined, or with HPLC techniques, where reaction quenching renders discrimination between aqua and chloro ligands impossible.

The ^{1}H−^{15}N HSQC NMR spectra reveal that the aquation of cisplatin is dominated by the formation of the *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ (**2**) species. After 30 h at 298 K, 23% of the cisplatin remains and 65% has been converted to *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ (**2**). The diaqua species *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) becomes

(50) Lee, K. W.; Martin, D. S. *Inorg. Chim. Acta* **1976**, *17*, 105−110.

(51) Berners-Price, S. J.; Appleton, T. G. The chemistry of cisplatin in aqueous solution. In *Platinum-based drugs in cancer therapy*; Kelland, L. R., Farrell, N., Eds.; Humana Press: Totowa, NJ, 2000.

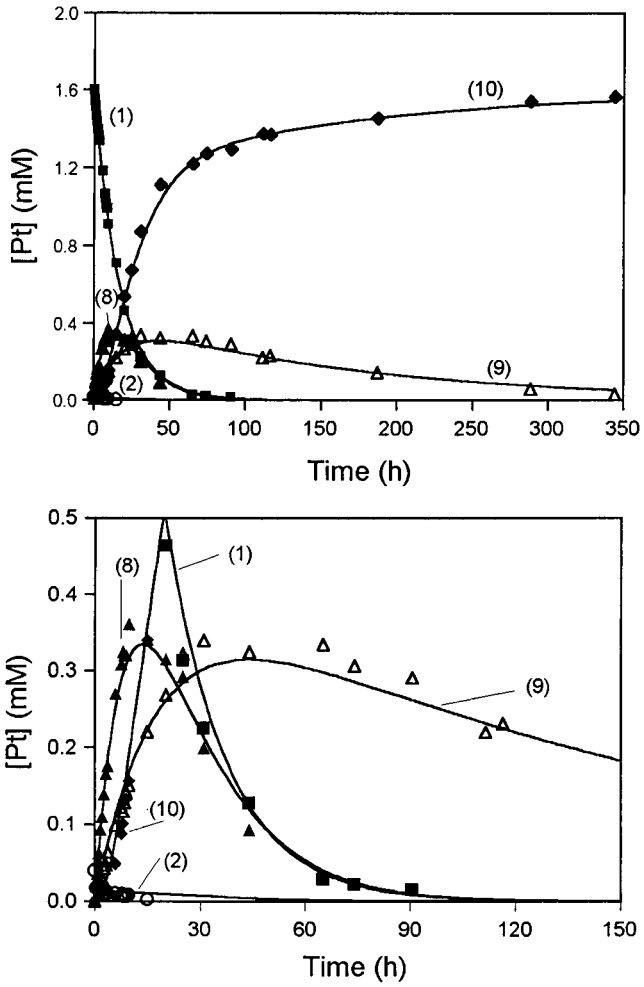

Figure 6. Plots of the relative concentrations of species observed during reaction at 298 K of ^{15}N-cisplatin and **-GG-** in 9 mM sodium phosphate buffer (pH 6): ■, **1**; ○, **2**; ▲, **8**; Δ, **9**; ♦, **10**. The curves are computer best fits for the rate constants derived from model 5 and listed in Table 3.

evident after 3.5 h and never accounts for more than 7% of the Pt. Bridged species appear at later stages of the reaction and after 40 h account for less than 5% of the Pt. It is not clear whether equilibrium with respect to dimer formation has been reached at this point. The calculated rate constants for aquation and anation are all within the range of values reported previously; for instance, Reishus and Martin[20] reported a value for k_{1H} of 2.5×10^{-5} s^{-1} at 298 K. Detailed comparison of other values is complicated by the effects of pH; for example the rate constants reported by House and co-workers[27] for aquation are higher than those determined here, but the experimental conditions they used were different (e.g., the solutions were 0.1 M $HClO_4$). In the present study the reactions were not buffered and therefore the pH varied, whereas in previous studies the pH was often set low or high.[22−26]

The dominance of *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ (**2**) over *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ (**3**) is due to a combination of factors. Most importantly, the rate of aquation of *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) to *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) is almost a factor of 2 slower than is the rate of aquation of cisplatin to *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) and the rate of anation of *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) is an order of magnitude faster than is anation of *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**). Using the concentrations of cisplatin, *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$, and *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ after 40 h at 298 K, the equilibrium constants $pK_1 = 2.07$ and $pK_2 = 3.49$ are obtained. These values are in good agreement with the literature values of $pK_1 = 2.00$,[22] 2.2,[26,27] 2.44,[50] 2.72[46] and $pK_2 = 3.57$[23] and 3.53.[27]

Effect of Phosphate on the Aquation of Cisplatin. Of particular interest and importance to the series of studies on the reaction kinetics of cisplatin and defined oligonucleotides with specific binding sites is the effect that phosphate buffer has on the aquation process. The influence of phosphate on the aquation of cisplatin and its aquated derivatives has been examined in several independent studies.[28−32] Balch and co-workers[29] using ^{195}Pt and ^{31}P NMR spectroscopy determined the major product at pH 6 to be a dinuclear Pt complex with bridging phosphate and hydroxo ligands. Bose et al.[31,32] examined the rates of reaction between $[PtCl_2(en)]$ and $[PtCl(dien)]^+$ with ortho-, pyro-, and triphosphates and concluded that monodentate Pt complexes formed, that rates of reaction were pseudo first order, and that PO_4^{3-} exerted a greater chloride-labilizing effect on $[PtCl_2(en)]$ than on cisplatin. They also believed that phosphate bound to *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ (**2**) on the basis that increases in the concentration of the phosphate species resulted in increased rates of reaction. The results of the present study show that binding of phosphate to *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ (**2**) is not the major pathway, as no species with ^{1}H−^{15}N HSQC NMR peaks indicating ligation trans to one Cl and one OPO_3 grouping are apparent until the final spectrum is recorded (24 h) and this species is minor. No directly comparable kinetic data were provided in ref 32. Knox and co-workers[28] determined a first-order aquation rate constant of 8×10^{-5} s^{-1} at 310 K in 100 mM phosphate buffer with 10% dmso at pH 7.0, and this correlated with a cisplatin half-life of 2.4 h. Appleton and co-workers[30] examined the reactions between *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) and a number of O-donor ligands including phosphate, by ^{195}Pt, ^{15}N, and ^{31}P NMR spectroscopy. They identified some of the products formed with phosphate and found that the product distribution was highly sensitive to pH.

It has been suggested that phosphate slows the rate of aquation of cisplatin.[28] It is clear from the values for the first aquation rate constants (Table 2) that phosphate buffer, at least at the 9 mM concentration used here, does not interfere with this initial aquation process. On the basis of the number of peaks in the ^{1}H−^{15}N HSQC spectrum (Figure 3) that have ^{15}N chemical shifts consistent with trans O-donor ligands ($\delta = -75$ to -90 ppm), only binding of phosphate to *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ is a significant contributing reaction. A lack of data in the time period 10−24 h precludes identification of, and determination of, accurate rate constants for the formation of phosphate-bound species. In none of the ^{1}H−^{15}N HSQC NMR studies of the reactions between cisplatin and DNA carried out to date, has there been any evidence for formation of either *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ (**3**) or any of these phosphate-bound species seen in the absence of DNA.[14,16,17] This is not unexpected because the nucleobases on DNA are superior nucleophiles and would be expected to react more rapidly and irreversibly with the platinum. The similarity of the rate constants determined in aqueous perchlorate solution and in phosphate buffer confirms that the presence of a 9 mM phosphate buffer does not significantly influence the initial rates of aquation of cisplatin and therefore should not influence either the reactions with DNA or the final adduct profile.

Development of Kinetic Models of DNA Binding. The previous treatments of reactions between cisplatin and 14-base-pair duplexes by us and others have employed an irreversible aquation step.[14,16,17] This was necessary because no information on the anation rate constant under identical experimental

Scheme 2. Reaction Scheme of the Binding of Cisplatin to -GG-

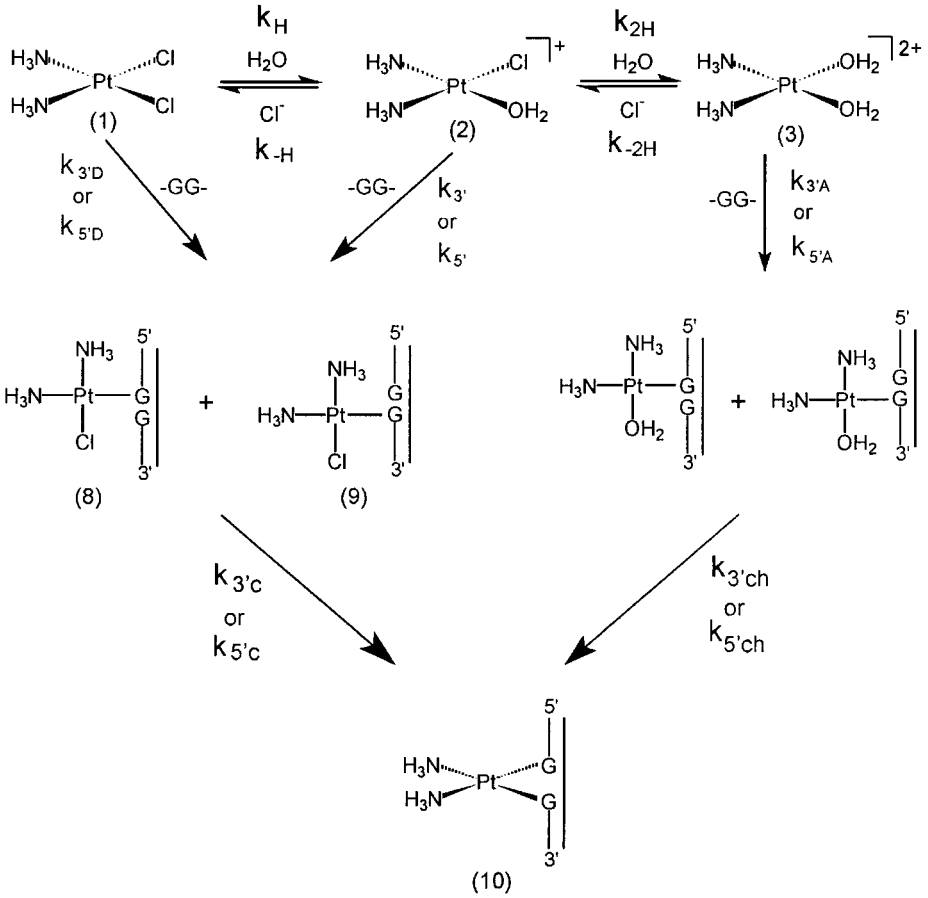

Table 3. Rate Constants for the Reaction of ^{15}N-Cisplatin and **-GG-** at 298 K and pH 6 and Comparisons to the Values Obtained with ***-GG-***, **-AG-**, and **-GA-**[a,b]

rate constant	**-GG-**	***-GG-***	**-AG-**	**-GA-**
k_H (10^{-5} s^{-1})	1.62 ± 0.02	1.83 ± 0.03	1.57 ± 0.02	1.23 ± 0.02
$k_{3'}$ (M^{-1} s^{-1})	0.48 ± 0.19	0.47 ± 0.04	0.37 ± 0.02	0.018 ± 0.002
$k_{3'}$ (M^{-1} s^{-1})	0.16 ± 0.06	0.15 ± 0.03	0.061 ± 0.007	0.046 ± 0.003
$k_{3'c}$ (10^{-5} s^{-1})	2.55 ± 0.07	3.2 ± 0.1	1.46 ± 0.05	2.5 ± 0.5
$k_{5'c}$ (10^{-5} s^{-1})	0.171 ± 0.011	0.24 ± 0.18	0.29 ± 0.09	0.07 ± 0.02

[a] Rate constants for **-GG-**, **-AG-**, and **-GA-** were obtained using model 5. Rate constants for ***-GG-*** were taken from ref 14. [b] k_H values for **-GG-**, **-AG-**, and **-GA-** are the aquation rate constants with the anation rate constant fixed at 4.6×10^{-3} M^{-1} s^{-1} (see Table 2).

conditions was available. Other workers who typically used the mono- and diaqua analogues of cisplatin[12,33−35] did not examine the aquation process either, primarily because the UV/vis absorption of the DNA was the phenomenon that they were probing. In a previous study by Lippard and co-workers[13] using ^{195}Pt NMR spectroscopy, cisplatin was reacted with chicken erythrocyte DNA at 310 K and pH 6.5. The authors used a pseudo-first-order rate constant to describe the rate of monofunctional adduct formation (encompassing the rate-limiting-step of aquation and the monofunctional adduct formation) and a first-order rate constant to describe the closure of monofunctional adducts to form bifunctional adducts. They found that the rate constant for monofunctional adduct formation did not vary with changes in the DNA:Pt ratio and that the rate of aquation was equal to the rate of adduct formation.

As a result of the determination of the aquation and anation rate constants (above) under the conditions of the DNA platination experiments, we are now in a position to more thoroughly analyze the processes taking place and to determine which intermediates play significant roles in these platination reactions. The kinetic data were evaluated by several models with both reversible and irreversible aquation steps, the latter to allow comparison with previously reported results. The **-GG-** sequence used here, like the **-AG-** and **-GA-** duplexes,[16,17] but unlike ***-GG-***,[14] is self-complementary and, therefore, contains two binding sites per duplex. Consequently, it is necessary to examine how the change in the number of binding sites changes the kinetic profile of the reaction and the rate constants for the processes undergone by the Pt reagent. With two potential GpG binding sites on the **-GG-** duplex, one might expect a statistical amount of duplex where both GpG binding sites are bound to Pt. The fact that the spectra are so similar to those where there is only one GpG site per duplex (***-GG-***[14]) indicates that the two GpG sites are not both being platinated on **-GG-** or that the diplatinated duplex exhibits identical chemical shifts. The latter would be highly unlikely, given the changes to the DNA conformation brought about by the binding of just one cisplatin molecule. Thus a question arises as to the effective concentration of platinum binding sites.

To address this question, kinetic models where the concentration of binding sites were treated differently were investigated. Three models were used, one in which the concentration of binding sites was treated as twice the concentration of the duplex (model 1) and one in which the concentration of binding sites was the same as the concentration of the duplex (model 2). A third model treated the initial concentration of binding sites as twice the concentration of duplex, but the concentration was then decreased so that when one binding site bound to a Pt the other was considered to be deactivated. Thus, the rate of loss of DNA binding sites upon binding to platinum is twice that

found from model 1. The rationale behind this third model was that the two GpG binding sites are sufficiently close to one another (separated by only two bases) that any distortion brought about by binding to one would be expected to affect the accessibility of the other site. The local DNA distortion caused by the binding of cisplatin has been investigated by examining a crystal structure[52,53] and by several NMR structural analyses.[54−57] Shifts in the thymine methyl and the imino ^{1}H resonances indicate that a change in the environment of the helix in the vicinity of the platination site occurs with monofunctional adduct formation.[14] Such a geometric change may restrict access of the solvent to the other GpG site.

The concentration of active sites has a significant effect on the rates of monofunctional binding; the rate constants for binding to the 3′-guanine vary from 0.22 ± 0.08 M^{-1} using model 1, to 0.30 ± 0.14 s^{-1} (model 3) and 0.45 ± 0.17 s^{-1} (model 2). The values for model 2, where the concentration of binding sites is the same as that of the duplex, are in good agreement with those found for the non-self-complementary duplex *-**GG**-*.[14] The rate constants from model 1, where the concentration of binding sites is twice that of the duplex, are approximately half of those from model 2. Those from model 3 have values that are intermediate between those of the first two. On the basis of the values determined from the various models and the similarity of the entire reaction profile to that found with *-**GG**-*,[14] the best treatment of the number of binding sites is that there is one binding site per duplex, as described in model 2. If the two GpG sites were farther apart along the strand, they may both be accessible to platination. The rate constants derived from models 1−6 are listed in the Supporting Information.

The first extension of the irreversible model used previously was to make the aquation process reversible. This was achieved by fixing the first anation rate constant to that obtained in the aquation study in perchlorate solution. The value of the aquation (forward) rate constant was approximately the same as the value obtained when the aquation process was treated as irreversible. This is partly explained by the fact that, at the early stages of the reaction, there is little chloride present in solution, so the contribution of the anation reaction to the net rate of aquation is small and by the fact that the rate of anation is much lower than the rates of monofunctional adduct formation.

Fixing the aquation rate constant to that obtained in the aquation study and allowing the anation rate constant to vary (model 7) were unsuccessful, giving unrealistically high anation rates. Fixing both the aquation and anation rate constants to the values determined from the aquation in aqueous perchlorate solution (given in Table 2) (model 8) and allowing the rates of binding to the oligonucleotide to vary also proved unsuccessful. In all attempts, the calculated rate of loss of cisplatin was substantially greater than the observed rate and adequate fits to the cisplatin and *cis*-[PtCl(^{15}NH$_3$)$_2$(OH$_2$)]$^+$ time dependence profiles could not be achieved. Thus, it is clear that, in the presence of DNA, the rate of aquation of cisplatin is slowed by 30−40% compared with that in aqueous perchlorate or phosphate buffer. This slowing could arise from the effect of increased ionic strength, but House [22,23,26] has shown that even very high ionic strengths (0.1−1.0 M) have a negligible effect on aquation rates. Alternatively, the slowing could arise if there was an association between unaquated cisplatin and DNA that reduced interactions between cisplatin and water. It was recently shown that DNA can substantially slow rates of reaction at Pt, but this was for positively charged complexes that intercalated between the bases of DNA.[58] However, weak hydrogen-bonding associations between cisplatin and DNA are feasible and might lead to the observed slowing of aquation. A net slowing of aquation could also arise from an increased rate of anation in the presence of DNA, but models in which the aquation rate constant was set to that observed in the absence of DNA and the anation rate constant was allowed to vary did not give a good fit to the observed data and returned an anation rate constant several orders of magnitude larger than that observed in the absence of DNA. The positively charged species *cis*-[PtCl(NH$_3$)$_2$(OH$_2$)]$^{2+}$ (**2**) would be expected to associate with DNA more strongly than would cisplatin, and so the anation rate might reasonably be expected to change. However, given that DNA is highly anionic, it is more likely that anation by the anionic chloride would be slowed rather than accelerated by any association with DNA. On this basis, the aquation rate constant is most likely an overestimate because we have assumed that the anation rate remains the same as that observed in the absence of DNA.

An extension of the model to include direct, irreversible binding of cisplatin to DNA as well as reversible aquation of cisplatin and irreversible binding of *cis*-[PtCl(^{15}NH$_3$)$_2$(OH$_2$)]$^+$ (**2**) was contemplated. Similar kinetic models for the binding of Pt(II) complexes to nucleophiles have been used since the 1950s[59−61] and necessitate repeating the reaction several times with different Pt:nucleophile ratios to separate the direct and indirect (via aquation) rate constants. Such models have not, to date, been applied to the binding of cisplatin to DNA and are not feasible for the present study because of the large amounts of oligonucleotide required for each experiment, the long periods of NMR time required to monitor each reaction, and the additional complication brought about by having more than one potential binding site. It should be noted that Lippard and coworkers[13] found that the overall rate of reaction was unaffected by changes to the ratio between the Pt drug and DNA, indicating the major pathway is that involving aquation.

An unknown relevant to all models was the starting concentration of *cis*-[PtCl(^{15}NH$_3$)$_2$(OH$_2$)]$^+$ (**2**). Despite rapid dissolution of cisplatin, there would inevitably be a small amount of aquation prior to mixing with the DNA. Therefore, the initial concentration of *cis*-[PtCl(^{15}NH$_3$)$_2$(OH$_2$)] $^+$ (**2**) was varied between 0 and 0.0015 M. The latter value is approximately 3 times the concentration that was determined from the first ^{1}H−^{15}N NMR spectrum. If a high initial concentration of *cis*-[PtCl(^{15}NH$_3$)$_2$(OH$_2$)]$^+$ was present, it would be expected to react quickly with the DNA and so affect the rate of formation of both monofunctional adducts and, to a lesser extent, the rate of loss of cisplatin. In all models, the best fits were obtained using an initial *cis*-[PtCl(^{15}NH$_3$)$_2$(OH$_2$)]$^+$ (**2**) concentration of 0. Nevertheless, fitting the time-dependent behavior of *cis*-[PtCl-

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$(^{15}NH_3)_2(OH_2)]^+$ (**2**) proved the most difficult treatment of any parameter in the kinetic scheme. In all cases, models gave slightly overestimated concentrations of this species. The low concentration of *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) observed in the reaction, never greater that 1.1% of the total platinum present, reduces the precision of the measured peak volumes, and this probably accounts for the discrepancies. Curiously, the rate constant for aquation found here, $(1.62 \pm 0.02) \times 10^{-5}$ s^{-1} was lower than that found with the non-self-complementary sequence -***GG***-, $(1.83 \pm 0.03) \times 10^{-5}$ s^{-1}.[14] This is possibly an artifact resulting from the method of dissolving the cisplatin and adding it to the duplex solution. In the reaction with -***GG***-,[14] *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) achieved a greater relative concentration than found here in the reaction with -**GG**-. Any change in the initial amount of *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) would affect the apparent aquation rate of cisplatin, and also the rate of monofunctional adduct formation with the DNA. Alternatively, the slowing of the aquation rate could be the result of a slightly lower pH in the present study, but this is unlikely because the data of Miller and House[25] show that the aquation rate of cisplatin increases with decreasing pH. Finally, the slowing of the aquation rate could be a sequence-dependent effect.

Comparison of the rate constants obtained in the reactions between ^{15}N-cisplatin and the two duplexes containing GpG binding sites (Table 3) shows that they are similar. A limitation of the data from the ^{15}N-cisplatin + -***GG***- reaction[14] is an absence of data points between 8 and 20 h as well as between 28 and 100 h of reaction. The first of these periods covers the time during which the 5′-G/Cl monofunctional adduct reaches its maximum relative concentration. The second covers the closure of both monofunctional adducts to form the bifunctional chelate species. The data in the current study of the ^{15}N-cisplatin/-**GG**- system cover the entire reaction more thoroughly than earlier investigations, and this is reflected in much more precise rate constants for monofunctional adduct closure. The rate constants for monofunctional adduct formation determined in the present study have higher errors than those reported previously,[14] a consequence of lower concentrations of *cis*-$[PtCl(^{15}NH_3)_2(OH_2)]^+$ (**2**) in the reaction mixture.

Recently, Chottard, Kozelka, and co-workers examined the rates of reaction between *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ (**3**) and defined oligonucleotides containing GpG and ApG binding sites using HPLC separation and quantification of the various adducts, after quenching with KCl or KBr and freezing.[33−35] On the basis of trends in rate constants, they proposed that *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ (**3**), or its deprotonated form, is the species that reacts with the DNA in vivo.[35] To test this hypothesis, we incorporated the second aquation process into the kinetic model using the rate constants for binding of *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ (**3**) to a GpG site on a hairpin duplex and the subsequent rates of closure of the monofunctional adducts to form the bifunctional species determined by Legendre, Kozelka, and Chottard.[34] To account for the difference in temperature between the reactions reported here (298 K) and those of Legendre[34] (293 K), we also investigated a further model where the rate constants obtained at 293 K were doubled. Using the rate constant for binding from model 5 as a starting point, we refit the data and found that the contribution to the pathway involving *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ was only 0.9−1.2% of the product resulting from binding of *cis*-$[PtCl(NH_3)_2(OH_2)]^+$. Taking into account the concentration of chloride within cancer cells (20−50 mM)[62] which would further suppress the formation of *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$ or $[PtOH(NH_3)_2(OH_2)]^+$, it can be reasonably concluded that the diaquated species makes a negligible contribution to the platination of DNA. This is in accord with the lack of evidence for *cis*-$[Pt(^{15}NH_3)_2(OH_2)_2]^{2+}$ or aquated monofunctional adducts, either in this investigation or in the study of binding of cisplatin with the -***GG***- sequence.[14]

Having established the most appropriate kinetic model (model 5) for the reaction between cisplatin and -**GG**-, we reanalyzed the reactions of cisplatin with -**AG**- and -**GA**- using the same model.[63] The revised rate constants are given in Table 3. The major change is in the rates of monofunctional adduct formation, which is not unexpected, given that we have shown above that these rates are highly dependent on the model employed. Conclusions based on comparisons of the rates of binding at -**GG**-, -**AG**-, and -**GA**- are not greatly different from those reported previously.[16] Rates of monofunctional binding at -**AG**- and -**GG**- are similar, with binding to the 3′-purine preferred over binding to the 5′-purine. Binding to the 5′-A of -**AG**- is about a factor of 2 slower than binding to the 5′-G of -**GG**-. Monofunctional binding at -**GA**- is much slower, with the 5′-G preferred over the 3′-A. Closure rate constants for the three sequences are remarkably similar, with closure from the 3′ side preferred by about an order of magnitude over closure from the 5′ side. This is largely irrespective of whether closure is to G or A, though closure to A is about half as fast as to the equivalent G. Interestingly, the aquation rate observed in the reaction with -**GA**- is slower than for the other sequences despite our use of a reversible aquation step in the kinetic modeling. It is not obvious why this is the case, but it may indicate differences in the associations between cisplatin and the -**GG**-, -**AG**-, and -**GA**- sequences. For instance, it may be that the aquation rate observed in the reaction with -**GA**- is closer to the inherent rate in the presence of DNA whereas the rates of aquation at -**GG**- and -**AG**- may be accelerated by the faster reactions at the GpG and ApG binding sites.

It is notable that the rates of monofunctional adduct formation at the -**GG**-, -**AG**-, and -**GA**- sequences are in broad agreement with the proportions of each adduct formed on salmon sperm or calf thymus DNA.[1,3] We recently studied rates of reactions at sequences that allow formation of the GG 1,2-interstrand and GTG 1,3-intrastrand adducts in order to obtain a more complete picture of the relationship between the rates of adduct formation and the final adduct profile.[43,44] The analysis has not yet been completed that would allow direct comparison of rate constants with those for the -**GG**-, -**AG**-, and -**GA**- sequences. However, the half-life of cisplatin in each of these reactions can be extracted readily. The rate at which cisplatin disappears reflects, in part, the rate of the initial reactions with DNA but is moderated by the fact that binding to DNA is preceded by aquation to produce *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ and this aquation is a reversible process, even though the anation rate is much lower (30−100-fold in the case of -**GG**-) than the rate of monofunctional binding. Consequently, variation in the rates of cisplatin disappearance is small. Despite this, as can be seen from the results shown in Table 4, the rates of disappearance do correlate with the amounts of adducts formed. This provides the first experimental evidence that is consistent with the adduct profile being determined at the level of monofunctional adduct

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(63) The concentrations of the -**AG**- and -**GA**- duplexes in the reaction mixtures reported previously, 1.85 and 1.59 mM, respectively[16] were recalculated using the extinction coefficients obtained from the model of Kallansrud and Ward[38] to be 2.56 and 2.15 mM.

Table 4. Cisplatin Half-Lives ($t_{1/2}$) for Reactions with Duplexes Having Specific Binding Sites and Percentages of Total Platinated DNA Adducts[a]

sequence	$t_{1/2}$ (h)	% total adducts[b]
5′-d(AATT**GG**TACCAATT)-3′ (-**GG**-)	11.89 ± 0.15	50
5′-d(ATACAT**GG**TACATA)-3′	10.52 ± 0.17	50
5′-d(TATGTACCATGTAT)-3′ (-***GG***-)[14]		
5′-d(AATT**AG**TACTAATT)-3′ (-**AG**-)[16]	12.26 ± 0.16	28
5′-d(AATTAT**GT****G**TATAT)-3′	12.46 ± 0.27	6−10
5′-d(ATATACACATAATT)-3′ (-**GTG**-)[44]		
5′-d(AATTAAC**G**TTAATT)-3′ (-C**G**-)[43c]	13.7 ± 0.4	<1
5′-d(AATTAT**G**CATAATT)-3′ (-**G**C-)[43c]	15.1 ± 0.3	1−4
5′-d(AATT**GA**TATCAATT)-3′ (-**GA**-)[16]	15.65 ± 0.26	<1

[a] The half-life of cisplatin in 9 mM ClO_4^- or phosphate is 8.09 ± 0.15 h (present study). [b] Adduct percentages are taken from ref 4. [c] Forms a 1,2-interstrand GG adduct.

formation, as is expected on the basis of Pt binding being kinetically controlled.[12]

With experimental confirmation that the adduct profiles are determined at the level of monofunctional adduct formation, the question arises as to what stage of the formation is the rate-determining step: outer-sphere recognition of the sequence or the transition state that leads to the monofunctional adducts. Lippard and colleagues[64,65] have shown that binding at sequences having a large number of sequential guanine bases is preferred over that at sequences having a small number. Monofunctional adduct formation is likely to be affected somewhat by the flanking bases but this is more consistent with outer-sphere recognition being the more important factor. Clearly, this is a question that deserves further attention because design of sequence-targeting complexes necessitates an understanding of the factors controlling the critical binding step.

Conclusions

The results reported herein and and others elsewhere[14,16,17] have provided important insights into the binding of cisplatin to DNA. It has long been known that aquation precedes the interaction between cisplatin and DNA,[49] and consequently the aquation process has been studied extensively. However, no prior aquation study has examined the time dependencies of all species formed. We have been able do so and have found that low concentrations of phosphate do not significantly influence the early stages of the aquation of cisplatin, evidently because phosphate reacts primarily with the diaquated species. In contrast, DNA does slow aquation, perhaps because of an association between cisplatin and DNA that limits solvent access to the Pt. This slowing may be sequence dependent. It has been widely assumed that the monoaquated complex, *cis*-$[PtCl(NH_3)_2(OH_2)]^+$, is largely responsible for the binding of cisplatin to DNA. We have been able to show that this is the case and that the diaquated species makes only a minor contribution to this binding process. This is because the diaquated species, even in the absence of DNA, only reaches significant levels after 2−3 h of aquation, by which time the reaction between the monoaquated species and DNA is well underway.

The binding of cisplatin is kinetically controlled, and therefore the initial reaction, formation of the monofunctional adducts, should, as suggested, determine the product profile. We have obtained the first experimental evidence that this is indeed the case from our studies of cisplatin binding at a series of defined Pt binding sites.

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Supporting Information Available: SCIENTIST models used for both the aquation studies and the [^{15}N]cisplatin/-**GG**- reactions, a listings of rate constants obtained from models 1−6, and a table of the chemical shifts and assignments for species formed in the reaction between [^{15}N]-cisplatin and -**GG**-. This material is available free of charge via the Internet at http://pubs.acs.org.

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