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FULL PAPER

Glutathione readily replaces the thioether on platinum in the reaction with [Pt(dien)(GSMe)]$^{2+}$ (GSMe = S-methylated glutathione); a model study for cisplatin–protein interactions

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As models for *cis*-[$PtCl_2(NH_3)_2$] (cisplatin)–protein interactions, the reactions of [Pt(dien)Cl]$^+$ with the tripeptides GSMe (S-methylated glutathione) and GSH (glutathione, γ-glutamylcysteinylglycine) have been studied. The substitution reaction of the platinum–methionine model adduct [Pt(dien)(GSMe)]$^{2+}$ with GSH has been investigated using ^{1}H and ^{195}Pt NMR. It was found that GSH substitutes GSMe in [Pt(dien)(GSMe)]$^{2+}$, readily forming [{Pt(dien)}$_2$GS]$^{3+}$. At pD ≥ 7.0 the intermediate [Pt(dien)(GS)]$^+$ was observed. Kinetic and thermodynamic parameters of this reaction were determined at pD 3.2: $k = 1.1 \times 10^{-3}$ M^{-1} s^{-1}, $t_{1/2} = 24.7$ h, $\Delta H^{\ddagger} = 1.5 \pm 0.3$ kJ mol^{-1}, $\Delta S^{\ddagger} = 5 \pm 1$ J K^{-1} mol^{-1} at 298 K; and $k = 28.5 \times 10^{-3}$ M^{-1} s^{-1}, $t_{1/2} = 0.97$ h, $\Delta H^{\ddagger} = 1.5 \pm 0.3$ kJ mol^{-1}, $\Delta S^{\ddagger} = 4 \pm 1$ J K^{-1} mol^{-1} at 316 K. At alkaline pH the substitution reaction occurs within 5 min, illustrating the dramatic influence of the pH on this reaction. These parameters are discussed in relation to the competition between GSMe and the N7 atom of guanosine monophosphate. The intermolecular substitution of GSMe by GSH is much faster than the substitution by guanine N7, even under acidic conditions. This platinum–thioether to –thiolate substitution can play a significant role in the cellular processing of platinum–protein adducts, and is an important mechanism in the circumvention of cisplatin induced toxicity by thiol-containing protective agents.

Introduction

The detailed pathway in which the anticancer drug cisplatin *cis*-[$PtCl_2(NH_3)_2$] reaches DNA, its ultimate target in the cell, remains unknown. The therapeutic effects of platinum complexes are related to co-ordination of platinum to, preferentially, the N7 positions of the guanines in DNA. This co-ordination to two neighboring guanines causes a distortion of the double helix, which, in turn, is thought to trigger cell death by binding of DNA recognizing proteins like the HMG (High Mobility Group) proteins.[1–3] DNA, however, is not the sole cellular component that platinum complexes may co-ordinate to inside the cell. Proteins and peptides containing the amino acids methionine or cysteine are known readily to react with platinum through their sulfur donor atoms. Complexes with methionine have been detected in the urine of patients treated with cisplatin[4] and carboplatin,[5] *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II). Platinum–sulfur interactions have traditionally been associated with undesired phenomena such as resistance[6] and toxicity (*e.g.* nephrotoxicity).[7] However, these Pt–S interactions may also be used to our profit. Co-administrating thiol-containing molecules (so-called protective agents) in cisplatin therapy[8] alleviates the toxicity of platinum antitumor drugs.

There has been much interest in studying the interaction of platinum complexes with sulfur-containing proteins and peptides, in order to clarify their role in platinum metabolism.[8] In an early study the reactivities of [Pt(dien)Cl]$^+$ towards glutathione, γ-glutamylcysteinylglycine (GSH), *S*-methylglutathione (GSMe) and guanosine 5′-monophosphate (5′-GMP) were determined.[9] For reactions of platinum(II) compounds with the sulfur atom a direct substitution of the chloride ligand without prior aquation was proposed as a mechanism. More recently these studies have focussed on the possibility for platinum–sulfur adducts to serve as intermediates towards DNA platination.[9–14] In these studies 5′-GMP and GpG were employed as models for DNA, and methionine[9–11] or GSMe[13] as models for methionine-containing proteins; GSH[13] was used to model cysteine-containing proteins. The N7 atom of guanine was shown to be able to substitute the sulfur atom in a platinum–thioether adduct using Pt(dien)$^{2+}$ model compounds. This indirect DNA platination, *via* a sulfur intermediate, was shown to be limited to thioethers as platinum–thiolate adducts were shown to be unreactive towards the N7 atom of guanine.[13]

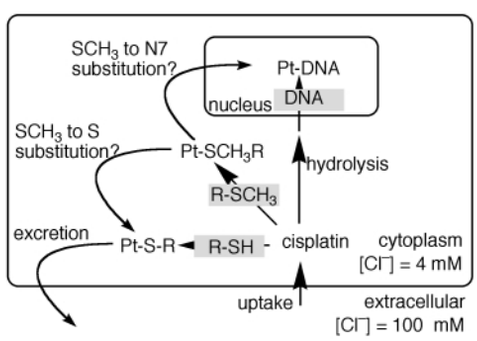
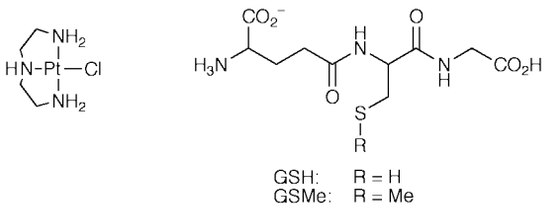


Fig. 1 Possible reaction pathways for cisplatin in the cell.

The kinetics and thermodynamic parameters of this substitution reaction using 5′-GMP and [Pt(dien)(GSMe)]$^{2+}$ have been determined in detail.[13] Recent work showed that indirect platination *via* platinum–thioethers is also possible for large oligonucleotides.[14] The rates of these reactions ($t_{1/2} = 147$ h for d(ATATGCATAT), 84 h for d(ATTACCGGTAAT), and 21 h for d(ATCCTATTTTTTTTAGGAT)), however, suggest that DNA platination *via* platinum–thioether intermediates will probably not contribute significantly to the mechanism of action of cisplatin. The possible pathways for cisplatin inside the cell are illustrated in Fig. 1.

The present paper deals with the substitution of a thioether by a thiolate in the reaction of [Pt(dien)(GSMe)]$^{2+}$ with GSH. This reaction is strongly pH dependent and the exact rates have

been determined at pD 3.2 and compared with the kinetic and thermodynamic parameters determined[13] for the reaction of $[Pt(dien)(GSMe)]^{2+}$ with 5′-GMP. The relevance of reactions of this type is discussed in relation to the mechanism of action of cisplatin. In addition the role of Pt–SCH_3 to Pt–S substitution in the mechanism of action of thiol-containing rescue agents will be discussed.



Results and discussion

Preparation of $[Pt(dien)(GSMe)]^{2+}$

The reaction of GSMe with one equivalent of [Pt(dien)Cl]Cl led to the formation of $[Pt(dien)(GSMe)]^{2+}$. This complex has been well characterized and the co-ordination at the sulfur atom is observed without detectable side products.[9] In the ^1H NMR spectrum the Pt-co-ordinated S–CH_3 group resonates at δ 2.59, no methyl signal corresponding to the non-platinated GSMe (at δ 2.12) being detected. In the ^{195}Pt NMR spectrum $[Pt(dien)(GSMe)]^{2+}$ gives rise to a signal at δ −3357, no peak corresponding to unchanged $[Pt(dien)Cl]^+$ resonating at δ −2727 being detected.

Reaction of $[Pt(dien)(GSMe)]^{2+}$ with GSH

The reaction of $[Pt(dien)(GSMe)]^{2+}$ with GSH (one equivalent) involved a rapid displacement in which the thioether of the platinum bound GSMe was replaced by the thiolate of glutathione. This competition in platinum co-ordination between GSH and GSMe was studied under acidic (pD 3.2), neutral (pD 7.0), and basic conditions (pD 8.5). Reactions were monitored at 289, 304, 310 and 318 K for each pD value. The stable end product was found to be $[\{Pt(dien)\}_2GS]^{3+}$ for all conditions used. The initial substitution reaction, and the subsequent rearrangement reactions leading to $[\{Pt(dien)\}_2GS]^{3+}$, are shown in Scheme 1. The main product has been well

$$GSH + [Pt(dien)(GSMe)]^{2+} \xrightarrow[-GSMe,\ -H^+]{k_1} [Pt(dien)(GS)]^+ \quad (1)$$

$$2\,[Pt(dien)(GS)]^+ \xrightarrow[-GS^-]{k_2} [\{Pt(dien)\}_2GS]^{3+} \quad (2)$$

$$[Pt(dien)(GS)]^+ + [Pt(dien)(GSMe)]^{2+} \xrightarrow[-GSMe]{k_3} [\{Pt(dien)\}_2GS]^{3+} \quad (3)$$

Scheme 1

characterized in a previous study on the direct reactions of GSH with $[Pt(dien)Cl]^+$.[18] An intermediate 1:1 and the final 1:2 complex of GS^- with $[Pt(dien)]^{2+}$ were found to be formed in that study. The thiolate in $[\{Pt(dien)\}_2GS]^{3+}$ was shown to act as a bridging ligand between the two identical Pt atoms. These Pt atoms give rise to one single peak at δ −3185.[18] In the present study the course of the reaction of $[Pt(dien)(GSMe)]^{2+}$ with GSH could be monitored by appearance of the methyl peak at δ 2.12 in the ^1H NMR spectrum, corresponding to the release of free GSMe. Owing to the overlap of peaks corresponding to the other protons in GSMe and GSH, this methyl peak appeared to be the only peak that provided accurate information on the course of the reaction, and this information is limited to the leaving ligand. Accurate information on platinum co-ordination could be obtained from ^{195}Pt NMR.

At pD ≥ 7.0 the reaction of $[Pt(dien)(GSMe)]^{2+}$ with GSH proceeds rapidly. Fig. 2 shows plots of the relative percentages of the platinum species formed in the reaction under neutral conditions. At 318 K the first step in the reaction occurs within 2 h. The thioether in GSMe is substituted on the platinum atom by the thiolate in GS^- to form the intermediate $[Pt(dien)(GS)]^+$, which gives rise to a peak at δ −3154. This intermediate is then slowly converted into $[\{Pt(dien)\}_2GS]^{3+}$, resonating at δ −3185. Although k_1 is larger than k_2 and k_3, these constants are all in the same order of magnitude and could not be determined separately. At pD 8.5 the initial reaction step occurred even faster; after 5 min (at 318 K) the proton spectrum showed that most GSMe was released from Pt(dien). Subsequent rearrangement $[\{Pt(dien)\}_2GS]^{3+}$ occurred at the same rate as for pD 7.0. This illustrates the great pH dependence of the first substitution reaction.

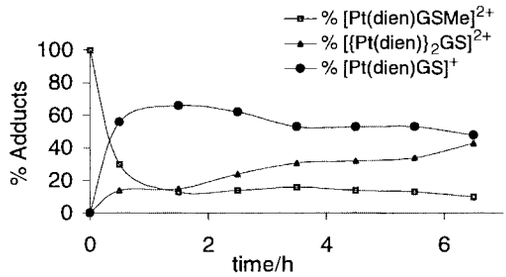


Fig. 2 Plot of the relative intensity of ^{195}Pt signals *versus* time in the reaction of $[Pt(dien)(GSMe)]^{2+}$ (10 mM) with 1 equivalent of GSH at pD 7.0, 316 K. The starting complex $[Pt(dien)(GSMe)]^{2+}$ reacts with GSH to form $[Pt(dien)(GSH)]^+$ within 2 h, this then slowly reacts to $[\{Pt(dien)\}_2GS]^{3+}$.

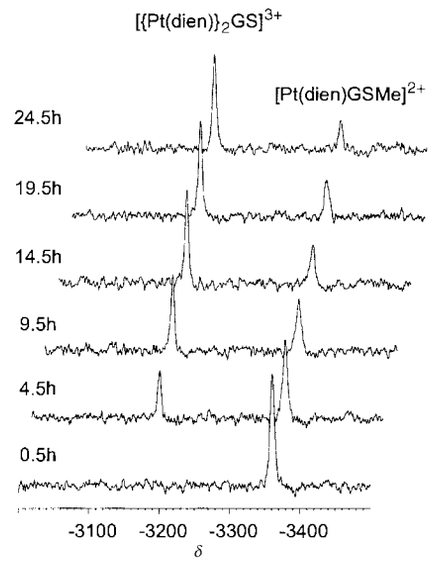


Fig. 3 Stacked ^{195}Pt NMR spectra of the reaction of $[Pt(dien)(GSMe)]^{2+}$ (10 mM) with 1 equivalent of GSH at pD 3.2, 304 K as a function of time. The peak at δ −3357 corresponds to $[Pt(dien)(GSMe)]^{2+}$, that at δ −3185 to the product $[\{Pt(dien)\}_2GS]^{3+}$. ^{195}Pt NMR spectra were calibrated using K_2PtCl_4 as an external reference at δ −1614 relative to K_2PtCl_6.

Under acidic conditions (pD 3.2) only the major product $[\{Pt(dien)\}_2GS]^{3+}$ could be observed, as seen from a single peak at δ −3185 in the ^{195}Pt NMR spectrum. The fact that the intermediate is not observed under acidic conditions can be explained by looking at the equations in Scheme 1. Reaction (2), leading to $[\{Pt(dien)\}_2GS]^{3+}$, is much faster at lower pD because it requires the release of glutathione. This release is greatly facilitated by deuteronation (addition of D^+) of the GS^- ligand. The fast second reaction under acidic conditions and the slow method of detection (a 80.000 scan ^{195}Pt NMR spectrum takes ≈50 min to record) prevent the detection of the intermediate. During the course of the reaction the intensity of the peak at δ −3357, corresponding to the starting complex, decreases

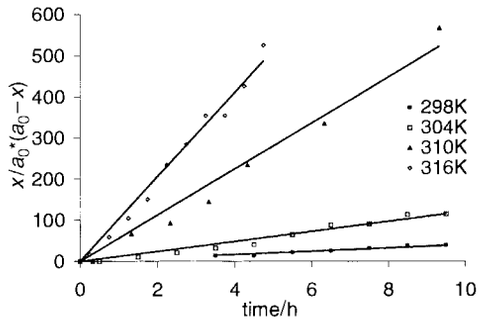


Fig. 4 Second-order Guggenheim plot of the reactions of [Pt(dien)(GSMe)]$^{2+}$ (10 mM) with 1 equivalent of GSH at pD 3.2 at 298, 304, 310 and 316 K.

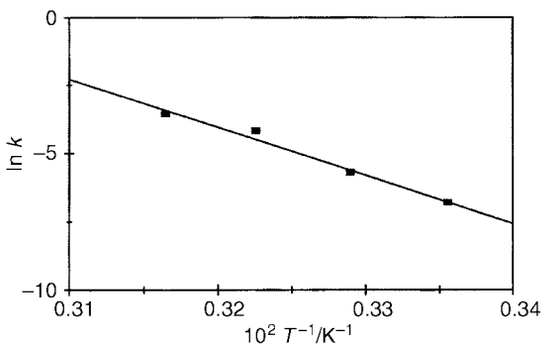


Fig. 5 Arrhenius plot for the reaction of [Pt(dien)(GSMe)]$^{2+}$ (10 mM) with 1 equivalent of GSH at pD 3.2.

while the new peak increases simultaneously, as clearly seen in Fig. 3. The reaction was found to be complete in 10 h at 318 K. At this pD the major part of glutathione is deuteronated and the first reaction (see Scheme 1) proceeds much slower than at higher pD. Relative to this first substitution step the subsequent rearrangement steps proceeded rapidly, hence no monoplatinated intermediate was observed.

Kinetics at pD 3.2

The initial reaction proved to be the rate-limiting step at pD 3.2. For the determination of rate constants the second-order rate law (4) was applied, where a_0 is the initial concentration of the

$$kt = x/[a_0(a_0 - x)] \quad (4)$$

reactant and x is the concentration of the product at time t. In the second-order Guggenheim plot shown in Fig. 4 the right side of this equation is plotted against time. Values of k_1 were obtained from the slope of the straight lines and are given in Table 1. The linear correlation obtained shows that this reaction can be treated as a second-order process, as is the case for the thioether to guanine-N7 substitution.[9–14] This implies a direct attack of the thiolate at the platinum atom in [Pt(dien)(GSMe)]$^{2+}$. Half-life times were determined from eqn. (5), and

$$t_{1/2} = 1/a_0k \quad (5)$$

are listed in Table 1. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ could be deduced from the Arrhenius equation (6). A plot (Fig. 5) of ln k *versus* $1/T$ shows

$$k = A\ e^{-E_a/RT} \quad (6)$$

a straight line. From the slope of this line, the value of $\Delta H^{\ddagger}$ was calculated (see Table 1).

Comparison of Pt–SCH_3 to –N7 substitution

In this paper it is shown that the thiolate of glutathione can easily substitute the thioether in a Pt–GSMe adduct. This substitution is pH dependent and occurs quite rapidly at pD ≥ 7.0. Under acidic conditions glutathione is deuteronated and the first step of the reaction occurs more slowly. Since the first step is rate-determining under these conditions, the values for k_1 could be determined at pH 3.2. Even under these, relatively unfavorable acidic conditions the Pt–SCH_3 to Pt–S substitution occurs much faster than substitution of the thioether in [Pt(dien)(GSMe)]$^{2+}$ by the N7 atoms of GMP and oligonucleotides.[13,14] Half-lives and thermodynamic parameters for Pt–SCH_3 to Pt–N7 (GMP) and Pt–SCH_3 to Pt–S substitution are compared in Table 2. It is obvious that the Pt–SCH_3 to Pt–S substitution studied here proceeds much faster. With this in mind it seems unlikely that intermediate platinum–thioether adducts contribute significantly to the anticancer activity of cisplatin by affording an additional pathway to DNA platination. It is more likely that they react with GSH in the manner described here, when it is kept in mind that GSH is the most abundant intracellular thiol with intracellular concentrations ranging from 0.5 to 10 mM. Substitution reactions of this type might serve to repair damage caused by co-ordination of platinum to methionines in proteins. The resulting platinum–GSH complex is known to be excreted by the cell, using the adenosine triphosphate (ATP) dependent glutathione S-export pump.[15] This can serve as a mechanism by which the cell repairs damage caused by platinum–thioether inactivated proteins. This substitution can also explain how and why certain thiol-containing protective agents referred to as "rescue agents" can react as GSH, and counteract the tendency of platinum complexes to co-ordinate to methionine containing proteins, thus reducing toxicity *e.g.* nephrotoxicity.[8]

In conclusion, the preference of Pt^{II} to co-ordinate to thiolates is illustrated, even though it is initially co-ordinated to thioether containing ligands. These results show that platinum–thioether adducts are more easily converted into thiolate adducts than to Pt–N7 adducts. This would suggest that it is unlikely that they play a significant role as intermediates towards DNA platination. Thioether to thiol substitution on platinum might be an important pathway by which the cell restores damage caused by inactivation of proteins due to platinum co-ordination to methionine. It is likely to play a role in the protective toxicity reducing effect that is achieved by co-administrating rescue agents in platinum anticancer therapy. The reactions of various rescue agents with platinum compounds are currently being studied.

Table 1 Reaction rates, half-lives, entropies and the activation energy as determined for the reaction of [Pt(dien)(GSMe)]$^{2+}$ (5 mM) with 1 equivalent GSH at pD 3.2

T/K	$k/10^{-3}$ M^{-1} s^{-1}	$t_{1/2}$/h	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J K^{-1} mol^{-1}
298	1.1 ± 0.1	24.7 ± 1.2	1.5 ± 0.3	5 ± 1
304	3.3 ± 0.2	8.3 ± 0.4	1.5 ± 0.3	5 ± 1
310	15.5 ± 0.7	1.8 ± 0.1	1.5 ± 0.3	5 ± 1
316	28.5 ± 1.4	0.97 ± 0.1	1.5 ± 0.3	4 ± 1

Experimental

Preparation of platinum compounds

GSMe and GSH were purchased from Sigma and used without purification, K_2PtCl_4 was obtained from Johnson Matthey and [Pt(dien)Cl]Cl was synthesized according to the literature.[17] [Pt(dien)(GSMe)]$^{2+}$ was prepared as a 10 mM solution in 1 ml D_2O at pD 3.2 by treating [Pt(dien)Cl]Cl with 1 equivalent of GSMe for 24 h in the dark. The presence and purity of the desired compounds were confirmed with ^{1}H and ^{195}Pt NMR,[18] and the resulting solutions were used without further purification.

Table 2 Thermodynamic and kinetic parameters of the substitution reactions of Pt–SCH_3 ⟶ Pt–S (this work) and Pt–SCH_3 ⟶ Pt–N7 (ref. 13)

Pt–SCH_3 ⟶ Pt–S			Pt–SCH_3 ⟶ Pt–N7		
$\Delta H^{\ddagger} = 1.5 \pm 0.3$ kJ mol^{-1}		$\Delta S^{\ddagger} = 5 \pm 1$ J K^{-1} mol^{-1}	$\Delta H^{\ddagger} = 90 \pm 5$ kJ mol^{-1}		$\Delta S^{\ddagger} = 16 \pm 17$ J K^{-1} mol^{-1}
T/K	$k/10^{-3}$ M^{-1} s^{-1}	$t_{1/2}$/h mol^{-1}	T/K	$k/10^{-4}$ M^{-1} s^{-1}	$t_{1/2}$/h
298	1.1	24.7	295	1.15	179
304	3.3	8.3	301	2.41	85.4
310	15.5	1.8	308	6.56	31.4
316	28.5	0.97	320	197	10.4

NMR spectroscopy

NMR spectra were recorded on a Bruker DPX 300 MHz spectrometer using a 5 mm multinucleus probe. A variable temperature unit was used to keep the temperature stable. ^{1}H chemical shifts in ppm were referenced to the HDO peak, which was calibrated to 4,4-dimethyl-4-silapenteane-1-sulfonate (DDS) at different temperatures. ^{195}Pt NMR spectra were calibrated using K_2PtCl_4 as an external reference at δ −1814 relative to K_2PtCl_6. A sweep width of 150 kHz was used and 80 000 scans were recorded for each spectrum. For kinetic measurements ^{1}H and ^{195}Pt NMR spectra were recorded subsequently every 30 min automatically.

pD measurements

All pD measurements were performed at 298 K. The pH meter was calibrated with Fischer-certified buffer solutions of pH 4.00, 7.00 and 11.00. Meter readings were corrected for the deuterium isotope effect by adding 0.4 unit to the display readout.

Reactions monitored with NMR

All reactions were carried out in NMR tubes (D_2O as solvent) at 10 mM concentrations of both starting compounds at three different pD values (3.2, 7.0, 8.5), and at 4 different temperatures (298, 304, 310 and 316 K). Reactions at pD 3.2 were not buffered, 50 mM phosphate buffer was used at pD 7.0 and 100 mM Na_2HPO_4 buffer was used for reactions at pD 8.5.

Determination of rate constants

Reaction rates and $t_{1/2}$ values were determined by integration of platinum signals (estimated error 5%). Second-order rate constants (k_2) were obtained from the slopes of a least-squares fit of a second-order Guggenheim plot[19] in which $x/a_0(a_0 - x)$ is plotted against time. For the determination of rate parameters only data collected over the first 40 h of the reaction were used.

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