Facile Formation of a *cis*-Platin-Nonapeptide Complex of Human DNA Polymerase-α Origin

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cis-DDP readily forms a complex with a nonapeptide, E R F K C P C P T, selected from near the DNA binding domain of human DNA polymerase-α, through the two cysteine residues; the reaction is more than an order of magnitude faster than that for the *cis*-DDP and DNA reaction.

It is commonly believed that DNA is the cellular target for platinum based antitumour drugs.[1–3] However, mechanisms of antineoplastic activities primarily based on platinum-protein or platinum–protein–DNA cross binding cannot be excluded based on available data.[2,4] In fact, we have recently shown[5] that *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and dichloro(ethylenediamine)platinum(II) (*cis*-DEDP) complexes arrest the DNA synthesis by direct binding to DNA-polymerase-α, a key replication enzyme. The specific binding sites are not known at this point. However, the reaction of this replication enzyme is accompanied by the release of zinc from the DNA binding Zn-finger motif. Here we report the facile formation of a Pt-nonapeptide complex with Acetyl-ERFKCPCPT-CO-NH_2, a peptide selected from the DNA binding domain of Human DNA Polymerase-α[6] covering the amino acid residues 1277 to 1285. Results presented here unequivocally establish that platination takes place exclusively through the two cysteine sites and that the reaction with the peptide is much faster than DNA and dGMP.

The peptide was prepared by solid state synthesis, purified by HPLC by using 0.1% trifluoroacetic acid in acetonitrile as a mobile phase, and characterized by electrospray mass spectrometry† and two dimensional NMR spectroscopy such as COSY, DQF-COSY, and TOCSY.‡[7] The DQF-COSY experiments aid us in identifying the methyl singlet for the acetyl group, and establishing connectivities among protons bonded to the adjacent carbons. The TOCSY spectra in H_2O reveal the connectivity between amide and α-protons along with protons within the extended spin network. The ROSEY spectra reveal connectivity between alpha and amide protons of i and $i + 1$ amino acids, except for proline which lacks an amide proton, indicating a 'random coil' structure for the peptide.§

The antitumor drug, *cis*-DDP readily reacts with the peptide to form a yellow complex¶ which exhibits a single resonance at −3730 ppm in the Pt-195 spectrum with respect to potassium hexachloroplatinate(IV). This chemical shift is taken to be indicative of PtN_2S_2 coordination environment reflecting a covalent bonding to two cysteine residues.[10,11] The chemical shift datum indicates that two ammonia molecules are retained in the platinum coordination sphere upon reaction with the peptide.‖ The structure of the platinum complex bonded through the two cysteines is shown below.

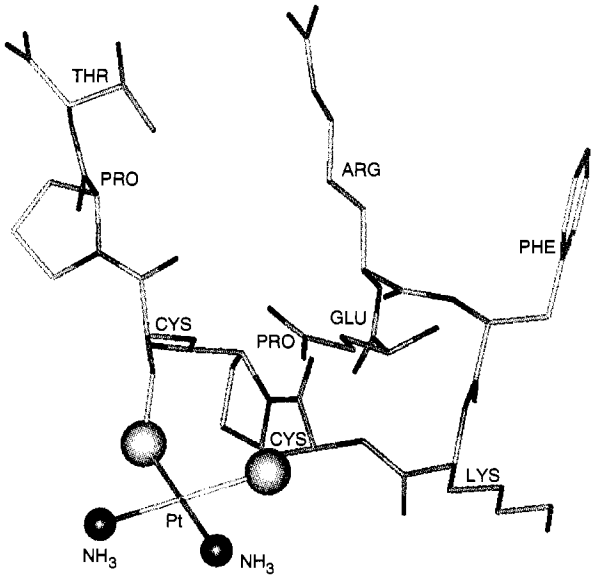

Fig. 1 Structure of *cis*-$Pt(NH_3)_2$(pep) complex exhibiting coordination through the thiol of two cysteine residues intervened by a proline

The reaction between the nonapeptide and *cis*-DDP exhibited a complex kinetic profile. The rate profile can be adequately described by eqns. (1), (2), (3) and (4),** where pep represents the nonapeptide (ammonia is omitted).

$$PtCl_2 \xrightarrow{k_1 = 1.2 \times 10^{-4}\ s^{-1}} Pt(H_2O)Cl^+ + Cl^- \quad (1)$$

$$Pt(H_2O)Cl^+ + pep \xrightarrow{k_2 > 10\ dm^3\ mol^{-1}\ s^{-1}} Pt(pep)Cl \quad (2)$$

$$PtCl_2 + pep \xrightarrow{k_3 = 0.20\ dm^3\ mol^{-1}\ s^{-1}} Pt(pep)Cl + Cl^- \quad (3)$$

$$Pt(pep)Cl \xrightarrow{k_4 = 8.5 \times 10^{-5}\ s^{-1}} Pt(pep) + Cl^- \quad (4)$$

An examination of the rate data reveals that *cis*-DDP-peptide complexation takes place significantly through the direct reaction with the dichloro complex [eqn. (3)], while the DNA reacts predominantly through the aquated pathway [eqns (1) and (2)].[12–14] The latter pathway is common to both the nucleotide and peptide complexation. Although the direct reaction with *cis*-DDP is of importance, specific rates with the aquated complex need to be addressed as well, since the concentration of the enzyme in the cell is small.[15] Therefore, it is instructive to compare the rates of formation of the peptide and nucleotide complexes through the reaction with the aquachloroplatinum complex. Our kinetic data demand that the bimolecular rate constant for the aqua-chloroplatinum complex should be > 10 $dm^3\ mol^{-1}$. Although *cis*-$Pt(NH_3)_2Cl(H_2O)^+$ has been prepared to almost pure form,[16] some diaqua– complex coexists with it. In order to avoid kinetic complications due to parallel reactions of the diaqua and chloro-aqua complexes, we have examined the reaction of the completely hydrolyzed *cis*-DDP with the peptide near the physiological pH. At this pH the hydrolysed complex is deprotonated and mainly exists as a mixture of *cis*-$Pt(NH_3)_2(H_2O)(OH)^+$ and *cis*-$Pt(NH_3)_2(OH)_2$. The reaction of the hydrolysed complex with the nonapeptide proceeds in two distinct phases. The initial phase is a second order process (k_1 = 7.9 $dm^3\ mol^{-1}\ s^{-1}$) that leads to the formation of an intermediate in which one cysteine is coordinated to the platinum ion. The intermediate is converted to a product in which both cysteines are coordinated through a first order process ($k_2 = 6.2 \times 10^{-3}\ s^{-1}$).

The second or rate constant for the initial peptide complexation is an order of magnitude higher than that for GpA complexation with the same platinum complex.[17] Likewise, the second step of the reaction is more than an order of magnitude faster than that observed for a dinucleotide coordination with GpA or ApG. These data imply that in the cell where the aqua-platinum species is generated due to reduced chloride concentration, the aqua species should exhibit preference toward the

protein, provided that the reactivity of the enzyme is comparable to the model peptide used here. In fact, when the reaction of *cis*-DDP with a mixture of the peptide and GMP (up to two fold excess over the peptide) was examined by proton NMR spectroscopy, insignificant GMP complexation (< 5%) was observed. The GMP complexation was judged by the appearance of a new H8 resonance of platinum(II)-GMP unit which is accompanied by 0.4–0.8 ppm positive coordination chemical of the H8 proton.[13]

Platinum drugs can readily react with the replication enzyme and arrest the DNA synthesis.[5] The platination is accompanied by the release of zinc implying that Zn-finger domain is involved in binding platinum. Wang *et. al.*[6] pointed out that four cysteine residues are coordinated to Zn in the DNA binding domain. Although the Zn-finger motif contains about 20 cysteine residues, two or more of such sites may coordinate with the platinum centres. If more than two sites are involved for a given platinum centre, release of ammonia is expected. The release of ammonia from the platinum coordination sphere was observed for the reaction of *cis*-DDP with matellothionein,[18] a detoxification enzyme extremely rich in cysteine.[19] Cysteine residues that are separated by several amino acids might bind platinum as well, depending on the tertiary structure of the enzyme which is currently unknown.

The platinum bound polymerase-α did not recognize the template DNA. This lack of recognition may be due to severe structural distortion caused by the platinum drug or simply due to an irreversible substitution of Zn^{II} by Pt^{II}, the former is a required metal for the enzymatic activities.[20] By selecting the model nonapeptide, we have demonstrated that platinum can readily coordinate with two cysteine sites which are intervened by a proline and that such a coordination is much faster than the DNA. Although a change in reactivity of *cis*-DDP with the polymerase compared to that of the peptide might be anticipated, available kinetic data suggest that the reaction with the enzyme may be even faster. For example, a rate acceleration for the reaction between *cis*-DDP and metallothionein compared to that for the *cis*-DDP and glutathione has been observed.[18]

It is accepted that platinum(II) binds preferentially to sulfhydryl groups over DNA in the cellular milieu. Some of the platinum(II)-thiol complexes [such as Pt(II)-glutathione] are escorted out by an ATP-dependent glutathione-S-conjugate export pump.[21] However, the fate of the platinum(II)-protein complexes and their roles in cytotoxicity are unknown. Two recent communications[22,23] in this journal point to a possibility of a "drug reservoir" mechanism in which initially formed platinum(II)-protein complexes are slowly converted to platinum-DNA adducts. Since cell apoptosis triggered by *cis*-DDP is believed to be at the G2 phase of the cell cycle,[2] secondary reactions leading to platinum-DNA adduct formation must not be too slow compared to the cell cycle time. The present work is a model study of kinetics and binding sites of cysteine rich region of DNA polymerase-α to *cis*-DDP and its implication to antineoplastic activity.

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Footnotes

† Molecular weight of the peptide was determined by Mass Spectrometry (JOEL, AX505HA) utilizing an electrospray ionization source (Analytica Branford). The peptide solution (10 mol dm^{-3} in equal volumes of methanol and 2% acetic acid in water) was injected at a flow rate of 0.5 dm^3 min^{-1}. Electro-spray mass spectrum reveals a molecular mass of 1122.2 compared to a calculated average mass 1122.34. The calculated molecular mass was based on H_2N-Glu-Arg-Phe-Lys-Cys-Pro-Cys-Pro-Thr-$CONH_2$.

‡ NMR experiments were performed on Varian 500 MHz and Bruker 400 MHz instruments. The TOCSY, phase sensitive NOESY, and ROSEY experiments were performed following the pulse sequences described elsewhere.[7]

§ Initial structures of the peptide and platinum-complex were generated by 'QUANTACHARMm' software after energy minimization by ABNR method.[8] The peptide structure was then refined based on complete relaxation matrix analysis by 'CORMA' and 'MARDIGRAS' programs[9] (kindly supplied by Professor T. L. James of University of California, San Francisco) utilizing experimental NOE intensities.

¶ The platinum-peptide complex was prepared by mixing *cis*-DDP and the peptide in 1 : 1 mole ratio in aqueous solution. The platinum complex was precipitated by concentrating the sample on a rotary evaporator.

‖ A thiolato bridged dinuclear or polynuclear complexes can be ruled out based on the observed NOE data. These bridged complexes are expected to exhibit intraligand NOEs which were not observed.

** Kinetic experiments were performed at pH 7.0 in Bis-tris buffer maintaining a constant ionic strength at 0.5 mol dm^{-3} by $NaClO_4$. Reactions were followed at 310 for *cis*-DDP and 280 nm for the aqua-complex. The concentration of platinum complexes were in 0.2–0.5 mol dm^{-3} range in order to avoid hydroxo-bridged oligomer formation. The absorbance-time traces were solved by using a 'Kin-Sim' computer program kindly provided by Professor Frieden of the University of Washington, St. Louis. The program generates the kinetic profiles identical to those observed by varying the rate constants and molar absorptivity of the intermediate (160 dm^3 mol^{-1}) indicated in the reaction sequences 1 through 5. The molar absorptivities of the *cis*-DDP, (102 dm^3 mol^{-1}), nonapeptide (308 dm^3 mol^{-1}) and the platinum(II)-peptide complex (2.4×10^3 dm^3 mol^{-1}) were independently determined and kept invariant.

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