The reaction of platinum(II) complexes with DNA. Kinetics of intrastrand crosslink formation *in vitro*

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ABSTRACT

The kinetics of the formation of bifunctional DNA platinum(II) adducts (DNA-crosslinks) have been investigated by endonuclease digestion and subsequent HPLC analysis of the soluble nucleotides and nucleotide platinum(II) adducts. The results indicate two waves of crosslinking [rate constants (0.2 – 0.3) min^{-1} and (0.015 – 0.025) min^{-1}] that correlate with changes in ultra violet absorbance and ethidium bromide dependent fluorescence intensity, previously interpreted in terms of two consecutive, local conformational rearrangements of platinum-DNA (Schaller, W., Reisner, H., and Holler, E. (1987) *Biochemistry 26*, 943 – 950). The formation of crosslinks at sequences d(GpG) and d(GpNpG) follows identical kinetics. A minimal reaction mechanism is proposed for the binding of *cis*-diamminedichloroplatinum(II) to DNA under *in vitro* conditions. The approximately 3-fold higher rate for *meso*-[1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]diaquaplatinum(II) in comparison to the rate for *cis*-diamminediaquaplatinum(II) indicates that crosslink formation is affected by the nature of the non-leaving platinum ligand(s).

INTRODUCTION

Cis-diamminedichloroplatinum(II) ($cPtCl_2$) is employed in the chemotherapy of a variety of human tumors (1,2). Its cytotoxic effect is largely due to binding with cellular DNA by forming monofunctional as well as bifunctional adducts with neighboring or more distant guanine and occasionally adenine bases (3 – 7). These adducts lead to intrastrand crosslinks at guanine or guanine/adenine residues in the sequences d(GpG), d(ApG) or, less frequently, d(GpNpG) and d(ApNpG), and between dGuo in opposite strands.

Evidence has been accumulated by chromatographic analysis of either endonucleolytic or chemical cleavage of platinum(II) complex treated DNA (8 – 12) and by sequence gel analysis of DNA synthesized at platinated DNA templates (13) that $cPtCl_2$ forms primarily N7-guanine monoadduct intermediates. These coordinate bifunctionally with a second guanine or adenine residue of the DNA yielding the crosslinks. A detailed reaction path has been proposed that was based on results from a kinetic investigation employing ultra violet absorbance, ethidium bromide dependent fluorescence, circular dichroism, [^{14}C]-KCN labelling and inhibition of template-primer properties for DNA synthesis (14). This kinetic mechanism was subsequently confirmed and extended by (i) HPLC measurement of nucleotide platinum adducts after exonucleolytic digestion of platinum DNA with *Escherichia coli* DNA polymerase I associated 3′-5′ exonuclease (15), (ii) by spectroscopic techniques (16,17), (iii) by preparing bifunctional DNA adducts from metastable, mixed DNA/ethidium bromide adducts (18), (iv) by pulse polarography (19) and (v) by a ^{195}Pt NMR measurement of the products (20).

The formation of monofunctional and crosslinking adducts from DNA and $cPtCl_2$ proceeds in two, solvent assisted reactions in sequence. The exchange of the chloro groups with water is rate-limiting in both the attack and the crosslinking of DNA. The kinetics seen for *cis*-diamminechloroaquaplatinum(II) (cPtClaq) reflect the attack of DNA; but the kinetics of the elementary reactions in crosslink formation are still obscured by the slow release of the second chloro ligand. The attack of DNA by *cis*-diamminediaquaplatinum(II) ($cPtaq_2$) is very rapid, and, since there is no second chloro group to be exchanged by water, the elementary reactions of crosslink formation are accessible (15).

However, the kinetics by which the bifunctional adducts are formed, are still under debate (14 – 16): The bimolecular attack of DNA by $cPtaq_2$ is followed by two consecutive first-order reactions ('conformational rearrangements'), which have been characterized by a hyperchromic ultra violet absorbance change at 272 nm wavelength, a decrease in fluorescence intensity of DNA bound ethidium bromide and by a first-order rate constant of 0.22 min^{-1} for the fast and 0.022 min^{-1} for the slow rearrangement. These are candidates for being the crosslinking reactions. Previous results comparing the kinetics for [^{14}C]CN^- chemical labelling of the DNA monoadducts with the kinetics measured by ultra violet absorbance, circular dichroism and enhancement of terbium(III) fluorescence suggest that the slow rearrangement is involved in crosslink formation (14 – 16). More recent results, however, indicate that *Escherichia coli* DNA polymerase I inherent 3′-5′exonuclease activity is arrested only at crosslinks, and that the time dependence of crosslink formation is the same as that for the fast rearrangement (15). This suggests that crosslinks are formed at an earlier stage than thought before.

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The evidence was indirect, and a recognition of the types of crosslinks has not been possible.

We now report a reinvestigation indicating crosslink formation during both the fast and the slow phases of the absorbance and fluorescence intensity changes. The important new points in our investigation are (i) the arrest of the platinum reaction by an alkaline pH-shift avoiding erroneous conclusions that could arise from uncontrolled reaction prolongation, (ii) an endonucleolytic digestion that allows the recognition of different types of crosslinks, (iii) a digestion at alkaline pH avoiding a reinitiation of platinum reactions, and (iv) an identification and quantitation of adducts by HPLC analysis.

MATERIALS AND METHODS

Materials

The platinum(II) compounds $cPtCl_2$ and *trans*- diamminedichloroplatinum(II) ($tPtCl_2$) were gifts of Degussa (Frankfurt). *Meso*-[1,2-bis(2,6-dichloro-4- hydroxyphenyl)ethylenediamine-]diaquaplatinum(II) (meso-6-Pt) was a gift of Dr. Schönenberger/ Regensburg. Salmon testis DNA was purchased from Sigma (Munich), oligonucleotides from Pharmacia (Freiburg), radioactively labelled nucleotides from Amersham (Braunschweig), nuclease P1 (300 unit/mg), DNase I (2,000 unit(mg) and alkaline phosphatase (1,500 unit/mg) from Boehringer (Mannheim), BENZON-nuclease (1×10^6 unit/mg) from Merck (Darmstadt). All other chemicals were of highest available purity from Merck (Darmstadt). Solutions of cPtClaq, $cPtaq_2$ and *trans*-diamminechloroaquaplatinum(II) (tPtClaq) were prepared as described (14,15). Meso-6-Pt was obtained directly by dissolving the sulfato complex. The sulfato ligand is immediately exchanged by water (4, and references therein). Gapped salmon testis DNA ('activated' DNA) was obtained by limited digestion with DNase I according to (15), the amount of acid soluble product corresponding to 10% of the absorbance at 260 nm wavelength after complete digestion. Activated DNA 3'-labelled with [^{3}H]dGMP (14 Ci/mmol) was prepared as described (15).

Reaction of platinum(II) complexes with DNA

Radioactively labelled DNA (0.05−1 mM in terms of nucleotides) was reacted with platinum(II) complexes at 37°C in the dark (1 mM MES buffer pH 5.5). The platinum complexes were applied at a ratio of 0.1 mole platinum per mole DNA nucleotide. Aliquots of 1 nmol DNA were drawn at various times and immediately added to prepared solutions for the digestion with endonucleases. These solutions (10−70 μL) contained 66 mM glycin/NaOH buffer pH 8.3, 100 mM KCl, 6 mM $MgCl_2$, 2 mM $ZnCl_2$, 0.1 mM EDTA, (0.25−0.5) unit/μL BENZON-nuclease, and (0.06−0.12) unit/μL nuclease P1. The reactions of platinum(II) with DNA was immediately interrupted by the shift to pH 8.3 following the addition of the sample to the digestion mixture. Evidence for the interruption is the immediate arrest of the increase in ultra violet absorbance change (280−295 nm wavelength) or of the decrease in intensity of ethidium bromide dependent fluorescence [see also (15)]. Due to the presence of high concentrations of chloride ions in the digestion mixture, platinum complexes eventually converted to their chloro forms.

The digestion was allowed for 2 h at 37°C. Alkaline phosphatase (1.5 unit/sample) was added, and the incubation continued for 15−30 min at 37°C. Under this condition, digestion had reached a plateau. A prolongation did not result in a further increase of nucleosides and adducts as judged by HPLC analysis. The digests were finally stored at −60°C until analysed by HPLC.

HPLC of digested samples

Separation of nucleotides and adducts was performed at room temperature on Waters RPC_{18} in the above buffer employing a 0.3−30% methanol gradient [see also (15) and the legend to Figure 1]. Ultra violet absorbance was recorded at 254 nm wavelength. Radioactivity counts were corrected for methanol-dependent counting efficiency. The HPLC apparatus and the detection devices were those described previously (15). HPLC-eluting material was identified on the basis of the known retention times of marker compounds as described (15). The preparation of markers for the reaction of meso-6-Pt will be published elsewhere. The commercial sample of alkaline phosphatase contained amounts of an enzymatic activity that degraded dAMP to an unknown material, presumably adenine, eluting as a single compound in the position of 'A' in Figure 1. The molar absorbance of the compound was identical with that of dAdo, and the amount of dAdo was computed from the combined absorbances of the fractions in the positions of 'A' and dAdo.

Kinetic analysis

Single exponential time dependences were evaluated on the basis of equation [1], where symbols denote A = peak area in relative absorbance units 'AU' at 254 nm wavelength, A_o = peak area at zero reaction time ('reaction amplitude'), and k_{obs} = rate constant.

$$[1] \qquad A = A_o \cdot \exp(-k_{obs} \cdot t)$$

Biphasic kinetics were treated as a superposition of two single exponential time dependences following equation [2]. The symbols A_o(slow), A_o(fast) refer to the reaction amplitudes and k(slow), k(fast) to the rate constants of the slow and the fast dependence, respectively.

$$[2]\ A = A_o(\text{fast}) \cdot \exp[-k(\text{fast}) \cdot t] + A_o(\text{slow}) \exp[-k(\text{slow}) \cdot t]$$

The kinetics of the formation and the consumption of monofunctional DNA platinum(II) adducts were analysed on the basis of equation [3], accounting for the rapid bimolecular formation of monofunctional DNA adducts as intermediates [pseudo first-order rate constant k(ps.)] and by a biphasic conversion of the intermediates to the crosslinking adducts. This biphasic reaction is regarded as a superposition of a fast [k(fast), α(fast)] and a slow first-order reaction [k(slow), α(slow)]. Symbols α(slow) and α(fast) refer to relative reaction amplitudes [α(slow) + α(fast) = 1]. The bimolecular attack of DNA by platinum(II) complexes was treated in terms of a pseudo first-order reaction since DNA (in terms of nucleotides) was in excess of platinum(II) complexes.

$$[3] \quad A = A_o \cdot k(\text{ps.})/[k(\text{fast}) - k(\text{ps.})] \cdot \{\exp[-k(\text{ps.}) \cdot t] - \alpha(\text{fast}) \cdot \exp[-k(\text{fast}) \cdot t] - \alpha(\text{slow}) \cdot \exp[-k(\text{slow}) \cdot t]\}$$

Evaluation of kinetics required the equilibrium absorbances (concentrations) of nucleotides at 'infinite' times. These were measured after >20 h reaction periods (> 10 reaction half-lifes). The kinetic data were approached by curves obtained with different sets of values for the kinetic parameters and the equations [1]−[3]. The one giving the best fit to the experimental data

(visual inspection) is reported in the results. Two-exponential kinetics were also analysed as has been described (15,16). The rate constants were identical with those obtained by curve-fitting.

The concentrations of nucleotides and adducts were measured in terms of their absorbances at 254 nm wavelength. In order to compare reaction amplitudes of nucleosides and corresponding platinum(II) adducts, the absorbance values had to be corrected for the hypochromic effect by platinum(II) at this wavelength. Correction factors were determined by comparison of the radioactivity and the absorbance peak area of guanosine adducts. It was found that the molar absorbance (254 nm) for the free nucleoside was 1.2 fold higher than for the guanosine adducts of cis-diammineplatinum(II), and 1.5 fold higher than for the guanosine adducts of meso-6-Pt.

RESULTS AND DISCUSSION

Digestion of 3'-labelled salmon testis DNA, after a 5 min reaction with $cPtaq_2$, gave rise to a product mixture consisting of the four nucleosides as well as of monofunctional and bifunctional adducts of dGuo (Figure 1). These were dGuo-Pt-Cl, dGuo-Pt-dGuo, and d(GpG)=Pt. The adduct dGuo-Pt-Cl was derived from dGuo-Pt-H_2O, the digestion product of the monofunctional adducts of DNA, by reaction with chloride in the digestion mixture. In the following we shall refer to this adduct in terms of dGuo-Pt-H_2O. The bifunctional adduct d(GpG)=Pt was the digestion product of DNA crosslinks between neighboring guanine residues. The bifunctional adduct dGuo-Pt-dGuo was derived from DNA containing crosslinks in d(GpNpG) sequences and, to a minor extend, from interstrand crosslinks between dGuo residues in opposite strands. The adducts derived from DNA crosslinks containing Ado had retention times > 20 min. They were poorly resolved and thus not examined. Radioactively labelled dGuo and its adducts were also observed. While unlabelled nucleosides and adducts refer to interior and 5'-segments of DNA, the labelled ones originate from 3'-segments. The results for dGuo and its adducts from both kinds of segments were the same (not shown) suggesting similar degrees of reactivity in these segments. From the amount of total radioactivity in the HPLC eluate, the recovery of dGuo and its adducts was calculated.

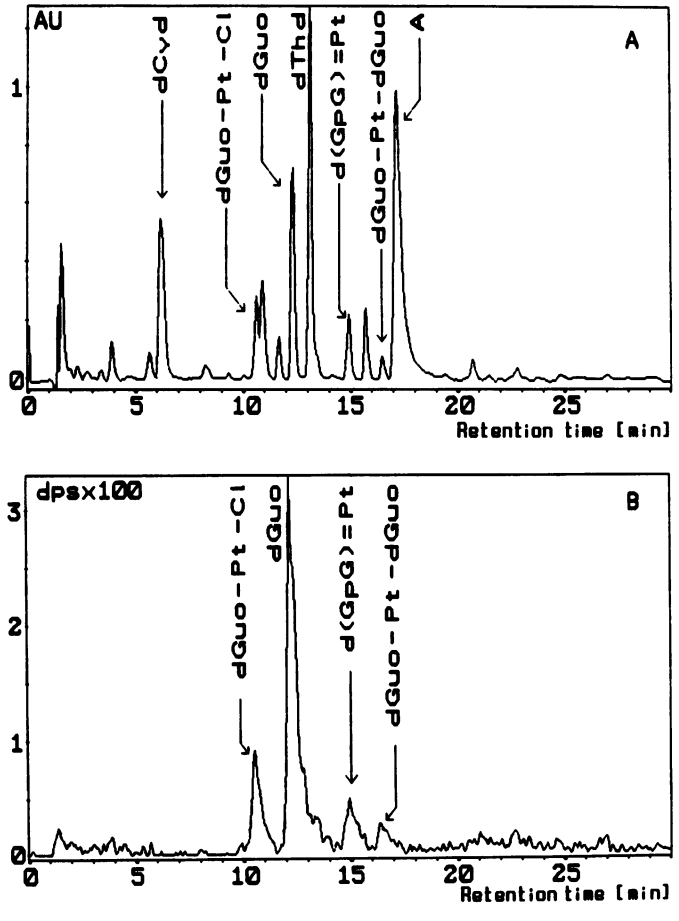

Figure 1. HPLC elution profile of endonuclease-digested platinum(II) DNA (0.1 mM). DNA was reacted for 5 min at 37°C with $cPtaq_2$ (0.01 mM). Waters reversed phase RPC_{18} was employed with 100 mM $(NH_4)_2HPO_4$ buffer (pH 5.3) containing 3 min 0.3%, 30 min 0.3–30%, 32 min 30% methanol. Panel A: AU = relative absorbance units at 254 nm wavelength. Panel B: Radioactivity in decompositions per minute.

The consumption of dGuo and dAdo

The kinetics of the reaction between DNA and $cPtaq_2$ were followed via the amounts of nucleosides in the HPLC eluate shown in Figure 2A. While the amounts of dThd and dCyd remained constant, those of dGuo and Ado decreased as a function of the reaction time. The dependences were biphasic. They were resolved on the basis of equation [2] in Methods. For dGuo, the fast phase depended on the concentration of DNA (10–200 μM nucleotide residues in a 10-fold molar excess over $cPtaq_2$, results not shown). The phase was attributed to the bimolecular attack of DNA by $cPtaq_2$. The bimolecular rate constant was calculated from the pseudo first-order rate constants to be k(bi.) = $(1.0\pm0.5)\times10^4$ $M^{-1}min^{-1}$. The value compares to 0.3×10^4 $M^{-1}min^{-1}$ (14) and 0.858×10^4 $M^{-1}min^{-1}$ (21) measured under slightly different conditions. The slow phase had the first-order rate constant k(slow) = (0.013 ± 0.003) min^{-1}. The reaction amplitude of the fast phase increased in parallel with the concentration of DNA. For 0.1 mM DNA, it corresponded to 80% of the overall reaction amplitude, which referred to 90% of the amount of dGuo before the reaction with $cPtaq_2$. For dAdo, both the fast and the slow phases followed first-order kinetics with rate constants k(fast) = (0.15 ± 0.05) min^{-1} and k(slow) = (0.01 ± 0.005) min^{-1}. The reaction amplitude of the fast phase was (65 ± 15)% of the overall reaction amplitude, which was 20% of the amount of Ado before the reaction with the platinum complex.

The formation and the consumption of the monofunctional DNA platinum(II) intermediate

The kinetics of formation/consumption of the guanosine $cPtaq_2$ adducts are shown for an example in Figure 2B. The amount of the monofunctional adduct dGuo-Pt-H_2O rises rapidly to a maximum and then decreases. The rise and the decrease are much faster than seen for the reaction of DNA with cPtClaq or tPtClaq (0.2–1.3 mM DNA in a 10-fold molar excess over the platinum(II) complexes, not shown). The kinetics of the rise depended on the concentration of DNA and were too rapid to be experimentally resolved in detail. Those of the decrease were concentration independent. The calculated best fit to the experimental points was obtained on the basis of equation [3] with the second-order rate constant k(bi.) = $(1-2)\times10^4$ $M^{-1}min^{-1}$, and the first-order rate constants k(fast) = (0.27 ± 0.05) min^{-1}, k(slow) = (0.025 ± 0.015) min^{-1}. The reaction amplitude α(slow) was 0.25–0.30.

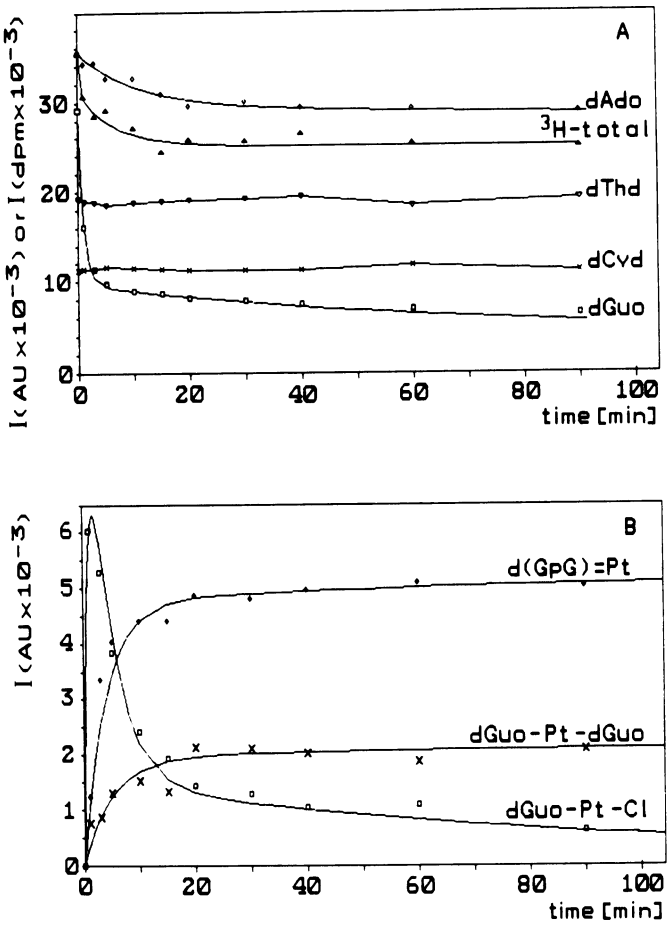

Figure 2. The time dependence of the reaction between DNA (0.1 mM) and $cPtaq_2$ (0.01 mM) at pH 5.5 (1 mM MES buffer) and 37°C. Digestion of Pt(II)-DNA was carried out with Benzon-nuclease, P1-nuclease and with alkaline phosphatase prior to HPLC. The digestion buffer (66 mM glycine/NaOH, pH 8.3) contained 100 mM potassium chloride that converted DNA-Pt-H_2O monofunctional adducts into DNA-Pt-Cl. After digestion, dGuo-Pt-Cl was obtained instead of dGuo-Pt-H_2O. In the text we refer to the term dGuo-Pt- H_2O only. Shown are values of the peak areas of relative absorbance units (same as in Figure 1) or of the total radioactivity in the eluate (in decompositions per minute). The drawn curves represent best fits to the experimental data, obtained for the kinetic parameters given below and in the text. Panel A: Time dependence of educts (in terms of nucleosides). Parameters not given in the text are: dAdo, AU(t = ∞)/AU(t=0) = 0.8; dGuo, AU(t=∞)/AU(t=0) = 0.14; ^{3}H-total, k(fast) = 1 min^{-1}, k(slow) = 0.2 min^{-1}, A_0(fast) = 50% of the overall reaction amplitude, AU(t=∞)/AU(t= 0) = 0.65; Panel B: The time dependence of the intermediate and of the products. Parameters not given in the text are: dGuo-Pt-Cl, A_0 = 1.1×10^4 AU, A_3 = 30% of the overall reaction amplitude, the concentration maximum corresponds to 20% of AU(t=0) for dGuo in panel A; d(GpG)=Pt, AU(t=∞) corresponds to 18% of AU(t=0) for dGuo; dGuo-Pt-dGuo, AU(t=∞) corresponds to 7% AU(t=0) for dGuo.

The formation of DNA platinum(II) crosslinks at sequences d(GpG) and d(GpNpG)

During the reaction of DNA with $cPtaq_2$, the amount of the bifunctional adduct d(GpG)=Pt increased rapidly following a superposition of two exponential reactions with rate constants k(fast) = (0.24 ± 0.02) min^{-1} and k(slow) ≈0.01 min^{-1}. The slow phase had an amplitude, which corresponded to approximately 10% of the overall reaction amplitude.

The time dependence of the formation of the bifunctional adduct dGuo-Pt-dGuo was very similar to that for d(GpG)=Pt, the rate constants being k(fast) = (0.22 ± 0.03) min^{-1} and k(slow) 0.01 min^{-1}. The amplitude of the slow phase corresponded to 10–20% of the overall reaction amplitude.

Correlation of the reactions by their time dependences

The kinetics for the consumption of dGuo and for the formation of its adducts can be correlated as follows: (i) The rapid formation of the monofunctional adduct dGuo-Pt-H_2O corresponds to the fast decrease in dGuo concentration. (ii) The fall in dGuo-Pt-H_2O concentration parallels the formation of the bifunctional adducts d(GpG)=Pt and dGuo-Pt-dGuo. (iii) At the reaction equilibrium in Figure 2B, the relative amount of dGuo residue in d(GpG)=Pt is 18% and in dGuo-Pt-dGuo 7% with regard to dGuo in the starting DNA. These amounts compare with 20% of dGuo residue in dGuo-Pt-H_2O as the supposed intermediate in the formation of the bifunctional adducts. (iv) Amounts of both dGuo and dGuo-Pt-H_2O are consumed in slow reactions of rate constants (0.02 ± 0.01) min^{-1} concomitantly with the formation of the adducts d(GpG)=Pt and dGuo-Pt-dGuo.

The above correlation between the reactions is consistent with a kinetic mechanism, where monofunctional adducts are formed at dGuo residues of DNA [k(bi.) = $(1-2)\times10^4$ $M^{-1}min^{-1}$]. These primary adducts are the intermediates on the route to the bifunctional adducts d(GpG)=Pt, dGuo-Pt-dGuo [and probably d(ApG), d(ApNpG)]. The bifunctional adducts are formed during a first [k(fast) = $(0.22-0.24)$ min^{-1}] and a second wave [k(slow) = (0.02 ± 0.01) min^{-1}] of crosslink formation. The consumption of dAdo is also biphasic with rate constants of the sizes found for the crosslink formation of dGuo residues (see above). This suggests crosslinks at sequences d(GpG), d(GpNpG), d(ApG), and d(ApNpG) as has been described (8–12,13). These different types of crosslinks are formed at very similar rates.

Do changes in optical properties relate to the formation of DNA crosslinks?

The reaction between DNA and the diaqua complex of cis-platinum(II) is indicated by two consecutive changes in optical properties. The changes are increases in ultra violet absorbance at 272 nm wavelength or decreases in fluorescence intensity of DNA-bound ethidium bromide (15,16). The slower one is unimolecular [k(slow) = 0.022 min^{-1}] under all experimental conditions. The faster one depends on low DNA concentrations in a fashion compatible with a rate limitation by the bimolecular $cPtaq_2$ attack on DNA (16). At high DNA concentrations the attack is rapid, and an unimolecular reaction [k(fast) = 0.22 min^{-1}] becomes rate limiting. This reaction is the origin of the change in optical properties. Because of the unimolecular nature and the involvement of the optical properties, these reactions have been suggested to represent 'conformational rearrangements'. The slow one has been attributed to the local disruption of the DNA double helix that accompanies the reaction of cis-platinum(II) (14,16,22–29). The nature of the 'fast rearrangement' is a matter of speculations. It could reflect a rearrangement of the electrons of the purine-platinum(II)-purine system (30) following chemical bonding and/or changes in base ring stacking. Surprisingly, significant effects on ultra violet absorbance or ethidium bromide fluorescence are not observed during the formation of monofunctional DNA platinum(II) adducts (31). This is at variance with considerable absorbance changes seen in the

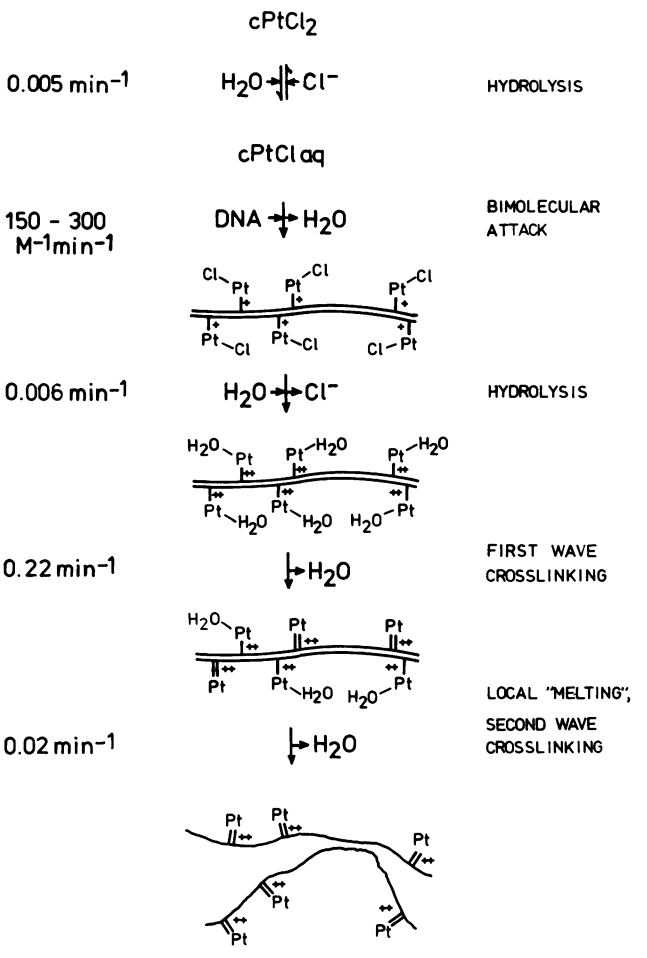

Figure 3. A proposed *in vitro* minimal reaction mechanism for the binding of $cPtCl_2$ to double-stranded DNA. DNA is symbolized by two parallel lines. Monofunctional adducts of DNA are indicated by single 'bonds' between DNA and platinum(II), bifunctional adducts irrespectively of their type of crosslink by double 'bonds'. Symbols '+' refer to unit positive charges. Ammine ligands are omitted from the graphs. The individual reactions are characterized at the right side of the figure. Rate constants are given at the left side and refer to conditions of pH 5–6 and 37°C. The result of the conformational change (local 'melting') at the end of the reaction scheme is exaggerated and may refer to unphysiologically high ratios (≥ 0.1) of mole platinum bound per mole DNA nucleotide. A preliminary form of this scheme has appeared elsewhere (15).

formation of monofunctional adducts with purine nucleosides and nucleotides (32,33). The discrepancy is not understood at present.

The 'fast rearrangement' (rate constant 0.22 min^{-1}) follows the same kinetics as the rapid formation of the bifunctional adducts d(GpG)=Pt and dGuo-Pt-dGuo from the intermediate dGuo-Pt-H_2O or the fast phase of the consumption of dAdo [rate constants = (0.22–0.24) min^{-1}]. The 'slow rearrangement' (rate constant 0.02 min^{-1}) exhibits kinetics comparable with those of the slow consumption of dGuo, dAdo and dGuo-Pt-H_2O, and is comparable with the slow formation of d(GpG)=Pt and dGuo-Pt-dGuo [rate constants (0.02 ± 0.01) min^{-1}]. The question at this point is whether the formation of crosslinks and the changes in the optical properties are coupled, or happen to occur just in parallel.

The rational of an experiment to answer this question was based on the observation that monofunctional DNA platinum(II) adducts, which were in coordination with the chloro instead of the aqua ligand, did not exhibit the 'rearrangement' reaction (14,16). A rapid conversion of large amounts of rearranged, not yet crosslinked adducts to their chloro forms (no coupling) should lead to a reversion of the 'rearrangement'. In the case of coupling, the fraction of not yet crosslinked adducts is small, and a change in the optical properties is not expected. The experiment was carried out by mixing DNA (0.1–1.0 mM) and $cPtaq_2$ (0.01–0.1 mM), and allow the 'fast rearrangement' to complete by 50% (half-life 3.1 min). A concentrated solution of NaCl was quickly added to the reaction mixture at a final concentration of 0.5 M. Given a bimolecular rate constant of 1 $M^{-1}min^{-1}$ (34), half of the platinum(II)-bound water was replaced by chloride within 1.4 min. Without coupling, this replacement would give rise to a measurable change in the optical properties. This was not observed indicating that the 'fast rearrangement' was strongly coupled to the irreversible formation of crosslinks.

Two mechanisms may account for the coupling: (i) The change in optical properties is the result of a direct rearrangement of the electrons of the purine-platinum(II)-purine system after coordination of the second purine residue. (ii) Either an energetically unfavourable conformational change renders a purine residue accessible for the attack by the monofunctional platinum or it is the formation of the crosslink that induces a conformation change.

An experimental evidences for a DNA conformational change is the large effect on circular dichroism during the reaction of double-stranded DNA with $cPtaq_2$ (31). The ellipticity at 280 nm wavelength decreases with a rate constant of 0.017 min^{-1} consistent with the 'slow rearrangement' (14). The 'fast rearrangement' is not indicated by a change in circular dichroism, and, therefore, does probably not involve large movements of base residues. The above dichroic effect is missing in the reaction with single-stranded DNA, which reveals only the fast hyperchromic effect (the 'fast rearrangement'). This indicates that the 'slow rearrangement' is a property of the reaction with double-stranded DNA (16). We assume that aquated, DNA-bound platinum(II) attacks a second purine residue without involving a large structural change in the DNA double helix (rate constant 0.22 min^{-1}). The reaction is accompanied by a hyperchromic effect mainly due to an electronic rearrangement in the purine ring(s). The crosslink thus formed imposes strain on its environment that is relieved by a local disruption of the double helix leading to the large change in circular dichroism (the 'slow rearrangemet') and to the well characterized, dearranged regions around the crosslinks [for a recent review see (7)].

The local 'opening' of double stranded into single stranded DNA can give rise to the second wave of crosslink formation [k(slow) = (0.02 ± 0.01) min^{-1}] for the following reasons. The formation of a crosslink requires a certain mobility of the attacking monofunctional adduct and/or the base in order to approach each other properly. The mobility depends on the strength of Watson-Crick base pairing and of stacking forces from neighboring bases. Furthermore, every bound $cPtqa_2$ introduces two positive charges into DNA leading to increasingly less attraction and eventually to a repulsion of further attacking positively charged $cPtaq_2$ molecules. The degree of this anticooperativity depends on the number and the distance of neighboring, already platinum-bound base residues. Local 'melting' can relief these constraints by increasing mobility and distances between bases. Work with lower molar ratios of platinum to DNA nucleotides and with sequencing gels is under way in order to substantiate this interpretation.

Effects of the conserved platinum(II) ligands

The chemical nature of the conserved platinum(II) ligand in 6-meso-Pt has an influence on the kinetics of the fast but not the slow crosslink formation. Rate constants are (0.6 ± 0.1) min^{-1} and (0.03 ± 0.01) min^{-1}, respectively [(16) and unpublished data]. This approximately 3-fold enhancement for the fast crosslinking may result from a more favorable orientation of the monofunctional adduct for the attack of the second purine residue.

The kinetics for cPtClaq and tPtClaq

The reaction of DNA with cPtClaq or tPtClaq was followed as above for $cPtaq_2$. Monofunctional DNA adducts were formed slower than for $cPtaqu_2$ at rate constants (118 ± 9) M^{-1} min^{-1} for cPtClaq and (250 ± 50) $M^{-1}min^{-1}$ for tPtClaq. The bisadducts d(GpG)=Pt and dGuo-Pt-dGuo of cPtClaq were formed from the monoadduct with rate constants $(5 \pm 2) \times 10^{-3}$ min^{-1}. For tPtClaq, the bisadduct dGuo-Pt-dGuo appeared with a rate constant of $(5 \pm 2) \times 10^{-3}$ min^{-1}. The adduct d(GpG)=Pt was not formed. The consumption of Ado was observed for both platinum compounds following rate constants of $(6 \pm 1) \times 10^{-3}$ min^{-1}, whereas the amounts of Cyd and Thd remained unchanged. These results confirmed previous findings by the 3'-5' exonuclease/HPLC technique indicating that the assumption of the exonuclease arrest at DNA platinum(II) crosslinks was correct.

Recovery of dGuo adducts in the HPLC eluate

Approximately half of the amounts of the total radioactivity in the HPLC eluate consisted of ^{3}H-dGuo and the $cPtaq_2$ or cPtClaq adducts of ^{3}H-dGuo, and was even less for reactions of meso-6-Pt. The remainder belonged to unidentifyable material in elution positions of oligonucleotide adducts. Neither an increase in endonuclease activity nor a prolongation of the digestion time could improve the yield. The recovery decreased during formation of bisadducts suggesting that the endonuclease digest of platinum(II) DNA was incomplete for heavily crosslinked DNA segments. Indeed, yields increased somewhat if the molar ratio of platinum to DNA nucleotides was lowered. It is considered unlikely that the incompleteness of the digestion grossly affects our conclusions. It might, however, have an influence on the relative degrees of crosslink formation during the 'fast' and the 'slow rearrangement' reactions.

Reaction scheme

When DNA reacts with $cPtCl_2$, $tPtCl_2$, cPtClaq, or tPtClaq, the formation of crosslinks and the changes in both the absorbance and fluorescence intensity follow first-order kinetics with rate constants $(3-7) \times 10^{-3}$ min^{-1} (14,15) [for a recent review see (7)]. The similarity of these rate constants with those for the exchange of the chloro ligands in $cPtCl_2$ or cPtClaq [$k = (5-6) \times 10^{-3}$ min^{-1}] suggests that the reactions are solvent assisted. The exchange of chloro ligands by water from either free $cPtCl_2$ or from the primary DNA-chloroplatinum(II) adducts is rate-limiting for the subsequent reaction path (14,15,19,20). We have recently proposed a reaction mechanism, which takes into account the limiting concentrations of DNA and millimolar concentrations of chloride ions in cell nuclei. The kinetic mechanism is outlined in Figure 3 including our present results on the kinetic mechanism of DNA crosslinking: In the initial part of the reaction, one of the chloro groups of $cPtCl_2$ is replaced by water. The half-life is of the order of 2 h. The replacement is followed by a second order attack of DNA. Second-order rate constants of $(100-300)$ $M^{-1}min^{-1}$ for cPtClaq have been determined by various methods (14,15, and data obtained of the present investigation). At an assumed 500 mM concentration of DNA in mammalian cell nuclei, the half-life of the attack would be 5 ms−12 ms. The attack is followed by the replacement of the chloro group still ligated to the monofunctional DNA platinum adduct with a half-life of 2 h. Next to this hydrolysis is a first wave of crosslink formation (half-life 3.5 min) and finally the slow conformational rearrangement accompanied by a second wave of crosslink formation (half-life 35 min). If the second wave of crosslink formation is only due to a relief of the electrostatic constraint (see above), it will probably not occur under *in vivo* conditions, where the molar ratio of platinum to DNA nucleotides is very low ($< 10^{-5}$). The schematic model does not take into account the protective binding of histones and other proteins to DNA.

ABBREVIATIONS

$cPtCl_2$ = *cis*-diamminedichloroplatinum(II)
cPtClaq = *cis*-diamminechloroaquaplatinum(II)
$cPtaq_2$ = *cis*-diamminediaquaplatinum(II)
tPtClaq = *trans*-diamminechloroaquaplatinum(II)
meso-6-Pt = *meso*-[1,2-bis(2,6-dichloro-4-hydroxyphenyl)-ethylene diamine]diaquaplatinum(II)
MES = 2-[N-morpholino]ethanesulfonic acid

REFERENCES

1. Rosenberg, B., Van Camp, L., Trosko, J. E., and Mansour, V. H. (1969) *Nature 222*, 385−386.
2. Loehrer, P. J., and Einhorn, L. H. (1984) *Ann. Intern. Med. 100*, 704−713.
3. Roberts, J. L., and Thomson, A. J. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 71−133.
4. Pinto, A. L., and Lippard, S. J. (1985) *Biochim. Biophys. Acta 780*, 167−180.
5. Reedijk, J. (1987) *Pure Appl. Chem. 59*, 181−192.
6. Von Angerer, E., Holler, E., Schönenberger, H., and Schönenberger, R. (1989). In Smith, D. F. (ed.), PCRC Handbook of Stereoisomers: Therapeutic Drugs, CRC Press, Boca Raton, pp. 247−284.
7. Lepre, C. A., and Lippard, S. J. (1990). In Eckstein, F. and Lilley, D. M. J. (eds.), *Nucleic Acids and Molecular Biology Vol 4.* Springer-Verlag, Heidelberg, pp. 9−38.
8. Eastman, A. (1982) *Biochemistry 21*, 6732−6745.
9. Eastman, A. (1983) *Biochemistry 22*, 3927−3933.
10. Eastman, A. (1986) *Biochemistry 25*, 3912−3915.
11. Fichtinger-Schepman, A. M. J., van der Veer, J. L., den Hartog, J. H. J., Lohman, P. H. M., and Reedijk, J. (1985) *Biochemistry 23*, 707−713.
12. Johnson, N. P., Mazard, J., Escalier, and Macquet, J. P. (1985) *J. Am. Chem. Soc. 107*, 6376−6380.
13. Pinto, A. L., and Lippard, S. J. (1985) *Proc. Natl. Acad. Sci. USA 82*, 4616−4619.
14. Schaller, W., Reisner, H., and Holler, E. (1987) *Biochemistry 26*, 943−950.
15. Bernges, F., Dörner, G., and Holler, E. (1990) *Eur. J. Biochem. 191*, 743−753.
16. Schaller, W., and Holler, E. (1988). In Nicolini, M. (ed.), *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy.* Martinus Nijhoff Publishing, Dordrecht, pp. 132−138.
17. Holler, E. (1988). In Nicolini, M. (ed.), *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy.* Martinus Nijhoff Puplishing, Dordrecht, pp. 83−88.
18. Malinge, J.-M., and Leng, M. (1988) *Nucleic Acids Res. 16*, 7663−7672.
19. Kleinwaechter, V., Vrana, O., Brabec, V., and Johnson, N. P. (1988). In Nicolini, M. (ed.), *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy.* Martinus Nijhoff Publishing, Dordrecht, pp. 102−109.
20. Bancroft, D. P., Lepre, C. A., and Lippard, S. J. (1990) *J. Am. Chem. Soc. 112*, 6860−6871.

21. Johnson, N. P., Hoeschele, J. D. and Rahn, R. O. (1980) *Chem. Biol. Interact. 30*, 151–169.
22. Mong, S., Daskal, Y., Prestayko, A. W., and Crooke, S. T. (1981) *Cancer Res. 41*, 4020–4026.
23. Scovell, W. M., and Capponi, V. J. (1982) *Biochem. Biophys. Res. Commun. 107*, 1138–1143.
24. Miller, K. J., Taylor, E. R., Basch, H., Krauss, M., and Stevens, W. J. (1985) *J. Biomol. Struct. Dyn. 2*, 1157–1170.
25. Kozelka, J., Archer, S., Petsko, G. A., and Lippard, S. J. (1987) *Biopolymers 26*, 1245–1271.
26. Lepre, C. A., Chassot, L., Costello, C. E., and Lippard S. J. (1990) *Biochemistry 29*, 811–823.
27. Kozelka, J., and Chottard, J.-C. (1990) *Biophysical Chemistry 35*, 165–178.
28. Schwartz, A., Marrot, L., and Leng, M. (1989) *Biochemistry 28*, 7975–7979.
29. Marrot, L., and Leng, M. (1989) *Biochemistry 28*, 1454–1461.
30. Inagaki, K., and Kidani, Y. (1980) *Inorg. Chim. Acta 46*, 35–39.
31. Macquet, J.-P., and Butour, J.-L. (1978) *Biochimie 60*, 901–914.
32. Scovell, W. M., and O'Connor, T. (1977) *J. Am. Chem. Soc. 99*, 120–126.
33. Bose, R. N., Cornelius, R. D., and Viola, R. E. (1986) *J. Am. Chem. Soc. 108*, 4403–4408.
34. Coley, R. F., and Martin, D. S. (1973) *Inorg. Chim. Acta 7*, 573–577.