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Separation and Characterization of Products Resulting from the Reaction of *cis*-Diamminedichloroplatinum(II) with Deoxyribonucleosides†

Alan Eastman

ABSTRACT: The cancer chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) was reacted with deoxyribonucleosides, and the products were separated by high-pressure liquid chromatography and characterized by ^{1}H nuclear magnetic resonance. Monofunctional platination occurred at N(3) of cytidine, N(7) of guanosine, N(1) of adenosine, and N(7) of adenosine. Bifunctional platination occurred with guanosine N(7) to N(7), adenosine N(7) to N(7), and adenosine N(1) to N(7). In addition, mixed bifunctional products N(7) of guanosine–N(1) of adenosine and N(7) of guanosine–N(7) of adenosine were obtained. No bifunctional adducts were obtained that contained cytidine, presumably due to steric hindrance as *trans*-DDP did cross-link two cytidine residues. Similarly, no adenosine N(1) to N(1) was detected with *cis*-DDP. The only other products detected were obtained at high levels of *cis*-DDP and probably represented polymeric forms in which platinum chelates to itself.

During the past decade *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] has been the subject of intensive investigation because of its effectiveness in treating a variety of human tumors. Much evidence supports DNA as the target molecule and that the bifunctionality of the drug is essential [reviewed in Roberts & Thomson, (1979)]. The trans isomer is chemotherapeutically ineffective even though it can also react extensively with DNA (Pascoe & Roberts, 1974; Eastman, 1982). The distance between the two chloride ligands is therefore critical and probably reflects a particular site in DNA that is accessible to the cis but not the trans compound.

Controversy still surrounds both the structure of the platinated DNA and which of the platinations are potentially most lethal to a cell. Guanine is the preferred site of reaction although a slower reaction also occurs with adenine and cytosine (Robins, 1973a,b). Various forms of bidentate chelation to a single base have been suggested as the trans isomer would be excluded from such a reaction (Mansy et al., 1973). Other evidence implicates cross-linking of adjacent guanines within the same strand of DNA as important to the mechanism of action (Kelman et al., 1972). Interstrand cross-links are also known to occur, and their formation often correlates with observed toxicity in various cell systems (Zwelling et al., 1979, 1981). However, in recent work from this laboratory, murine leukemia L1210 cells with acquired resistance to the cytotoxic action of *cis*-DDP were shown to be tolerant of much higher levels of interstrand cross-links than their sensitive parent cell line (Strandberg et al., 1982).

In all the studies so far, no satisfactory system has been reported that separates the various platinum adducts of DNA; rather, characterization has been attempted in complex mixtures. This if of particular concern in the case of deoxyadenosine where at least two positions can be platinated that potentially give rise to three bifunctional adducts. The presence of minor platinum products would also be obscured in unpurified reaction mixtures. This paper describes a system for the separation of deoxyribonucleoside-bound *cis*-DDP and their subsequent characterization as an approach to better understanding the mechanism of action of this drug.

Materials and Methods

cis-DDP and *trans*-DDP (Alfa Ventron, Danvers, MA) were dissolved in 0.02 M $NaClO_4$, pH 5.5, overnight at 37 °C. Deoxyribonucleosides (Sigma Chemical Co., St. Louis, MO) were also dissolved in 0.02 M $NaClO_4$, pH 5.5. In most experiments, *cis*-DDP at concentrations of 0.2, 1, or 5 mM was incubated at 37 °C with 1 mM deoxyribonucleoside for various time periods. The reaction products (10 μL) were then separated by HPLC on an Altex Ultrasphere ODS column (5-μm particle size; 25 × 0.4 cm) attached to a Varian Model 5000 HPLC. Elution was performed with a linear gradient of 0–30% methanol in 0.1 M aqueous ammonium acetate, pH 5.5, over 30 min at 1 mL/min and monitored with an ultraviolet (A_{254}) detector.

Reaction mixtures were scaled up in size to facilitate ^{1}H NMR. Equimolar nucleoside and *cis*-DDP (5 mM) were incubated for 24 h in the case of deoxyguanosine and deoxy-

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance.

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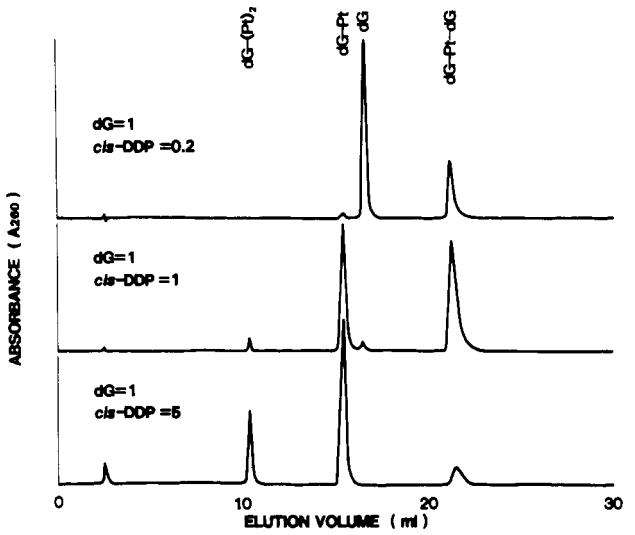

FIGURE 1: HPLC separation of products resulting from the reaction of deoxyguanosine and *cis*-DDP at the molar ratios indicated.

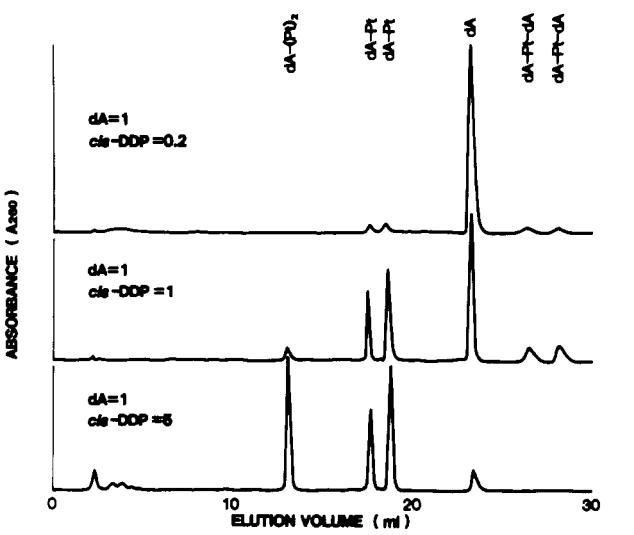

FIGURE 2: HPLC separation of products resulting from the reaction of deoxyadenosine and *cis*-DDP at the molar ratios indicated.

adenosine and 3 days for deoxycytidine. Aliquots (500 μL) were then injected onto the HPLC and 0.5-mL fractions collected. The required fractions from repeated preparative chromatograms were pooled, solvent and buffer were removed by lyophilization, and the residue was dissolved in D_2O. This was lyophilized again and redissolved in 0.1 M NaCl–D_2O. ^1H NMR was performed in a Bruker 250-MHz instrument in the FT mode with chemical shifts recorded in ppm from the trimethylsilane reference peak. Atomic absorption was performed on a Perkin-Elmer instrument equipped with a graphite atomizer.

Results

cis-DDP Binding to Deoxyribonucleosides. The four normal deoxyribonucleosides were incubated with *cis*-DDP at various molar ratios, and the products were analyzed by HPLC. In the case of deoxyguanosine under reaction conditions with excess nucleoside, the major product detected should represent dG–Pt–dG (Figure 1). Reaction with excess *cis*-DDP resulted in a more hydrophilic product, presumably dG–Pt. Another product that was even more hydrophilic was also detected and was tentatively designated dG–Pt_2. The ratios of dG to Pt in these products were confirmed by comparing ultraviolet absorption (A_{260}), a measure of the quantity of nucleoside present, with the quantity of Pt as assayed by atomic absorption spectroscopy. The actual values obtained for the ratio by dG:Pt were 2.04, 1.10, and 0.52, respectively. The peak at the beginning of the chromatogram was residual *cis*-DDP.

Similar experiments with deoxyadenosine demonstrated the formation of two monofunctional adducts and two bifunctional adducts (Figure 2). A more hydrophilic peak was again observed at high *cis*-DDP levels and represented dA–Pt_2. The ratios of dA:Pt as assayed by A_{260} and atomic absorption were 0.55, 1.03, 1.08, 1.89, and 1.97. This confirmed the designations shown in Figure 2.

The reaction of *cis*-DDP with deoxycytidine resulted in only a single detectable adduct (Figure 3). No bifunctional adduct was detected even after long incubations. The ratio of dC:Pt was calculated to be 0.92. No evidence was obtained for any reaction between thymidine and *cis*-DDP.

All of the products so far discussed were mixed and chromatographed together (Figure 4). Complete resolution of all the peaks was obtained. This profile could be markedly altered as a function of the salt in the eluting buffer. Optimum separations were obtained with 0.1 M ammonium acetate.

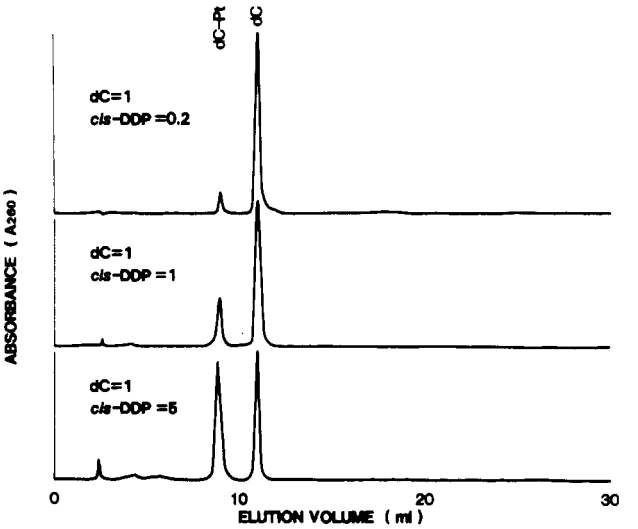

FIGURE 3: HPLC separation of products resulting from the reaction of deoxycytidine and *cis*-DDP at the molar ratios indicated.

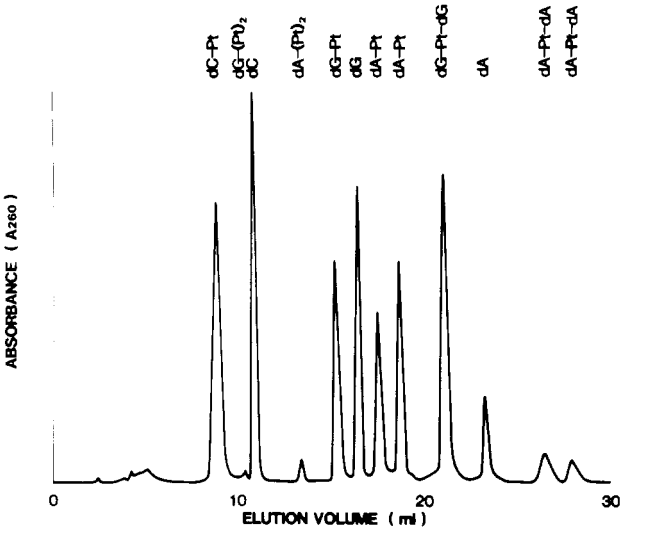

FIGURE 4: HPLC separation of a mixture of products derived from the reactions of *cis*-DDP with equimolar deoxyguanosine, deoxyadenosine, and deoxycytidine. Additional deoxyguanosine was added prior to the separation.

Lower salt concentrations resulted in much later elution and peak broadening of the adducts but had no effect on elution

Table I: ^{1}H NMR Spectral Data

	ppm			
	H(2)	H(5)	H(6)	H(8)
dG				8.08
dG-Pt				8.49
dA	8.12			8.25
dA-Pt[a]	8.68, 8.73			8.40
dA-Pt[b]	8.35			8.89, 8.90
dC		6.03, 6.07	7.82, 7.85	
dC-Pt		6.10, 6.13	7.86, 7.90	

[a] The earlier eluting dA-Pt product, dA N^1-Pt. [b] The later eluting dA-Pt product, dA N^7-Pt.

of the unmodified nucleosides. This is characteristic of ion-suppression chromatography in which the positive charges on the complexes are suppressed by providing sufficient competing cations. The effect could also be observed with different concentrations of $NaClO_4$ or NaCl as eluant. In both cases, 0.1 M was optimum but did not give improved separations over ammonium acetate.

Kinetics of Reaction. Reaction times were varied, and the areas under each peak were integrated. At equimolar ratios of nucleoside and *cis*-DDP the $T_{1/2}$ (50% disappearance of unmodified nucleoside) for deoxyguanosine was 3 h with almost complete reaction by 24 h. The $T_{1/2}$ for deoxyadenosine was 20 h and for deoxycytidine was 3 days.

Characterization of Reaction Products. The monomeric reaction products were analyzed by ^{1}H NMR, and the spectral data are shown in Table I. The H(8) proton of deoxyguanosine shifted downfield by 0.4 ppm as a result of platination, which is consistent with chelation at N(7) of the guanine base (Inagaki & Kidani, 1979).

It has been reported that the preferred site for platination of deoxycytidine is N(3) (Mansy et al., 1973), and this is supported by a 0.05-ppm downfield shift of both H(5) and H(6) doublets, which is attributed to platinum interaction at a distant site. The two deoxyadenosine–platinum adducts resulted in a downfield shift of both H(2) and H(8) protons, but in each case, the magnitude of the shift was much greater for a particular proton. The proton closest to the site of platination also produced a split signal. It can be concluded that the earlier eluting adduct on HPLC resulted from chelation at N(1) of adenosine and the second adduct represented modification at N(7) of adenosine. The latter identities were also corroborated by their different ultraviolet absorption spectra. The deoxyadenosine-N^7–platinum adduct exhibited an absorption maximum at 267 nm, the same as that for authentic N^7-methyladenine, while deoxyadenosine-N^1–platinum absorbed maximally at 261 nm, compared to a value of 263 nm for N^1-methyladenine. Other absorption maxima recorded were deoxyadenosine, 260 nm, and N^3-methyladenine, 272 nm.

Analysis of Mixed Bifunctional Adducts. In DNA *cis*-DDP has the potential to cross-link different deoxyribonucleosides. Their formation was therefore studied at the nucleoside level. Two methods were used. First, the reaction with equimolar nucleoside and *cis*-DDP was allowed to proceed for 24 h at which time an alternate nucleoside was added. This gave complex mixtures of products because the second nucleoside was able to react with residual *cis*-DDP as well as the monofunctional adducts of the first nucleoside. An example of this type of analysis is shown in Figure 5. Deoxyadenosine was incubated with *cis*-DDP for 24 h at which time deoxyguanosine was added and the incubation continued for a further 24 h. Two dA–Pt–dG adducts were detected, one of

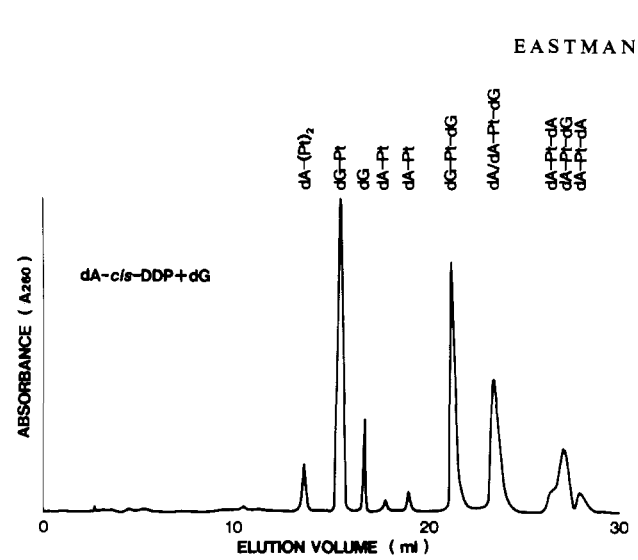

FIGURE 5: HPLC separation of heterogeneous bifunctional *cis*-DDP reaction products. Equimolar deoxyadenosine and *cis*-DDP were incubated for 24 h; then equimolar deoxyguanosine was added for an additional 24 h.

which cochromatographed with deoxyadenosine and the other in the region of the dA–Pt–dA adducts.

More definitive evidence for the character of a particular bifunctional peak was obtained by purifying the monofunctional adducts by HPLC and then reacting them again with another deoxynucleoside. The first preparative separation was performed with 0.1 M $NaClO_4$, pH 5.5, in the eluant, instead of ammonium acetate to exclude buffers from the subsequent reactions. This technique confirmed the formation of two dA–Pt–dG products as described above. However, no bifunctional adducts could be obtained on further reaction of dC–Pt.

Purified dA–Pt was also reacted with excess deoxyadenosine to determine which of the bifunctional products were derived from each monofunctional adduct. The later eluting monofunctional adenosine product gave rise to both bifunctional adducts whereas the earlier eluting monofunctional adduct only gave the earlier of the bifunctional adducts. After characterization as described above, it became evident that these monofunctional adducts represented N(1) of adenosine and N(7) of adenosine and that bifunctional adducts formed between N(1) of adenosine and N(7) of adenosine and between N(7) of two adenosines. It appeared that *cis*-DDP was unable to cross-link two adenosines both through their N(1) positions.

trans-DDP Binding to Deoxyribonucleosides. A comparative study was performed with *trans*-DDP. Equimolar deoxyribonucleoside and *trans*-DDP were incubated at 37 °C for 24 h and the products separated by HPLC (Figure 6). The adducts appeared to be more hydrophilic (eluted early) than their *cis*-DDP counterparts. Both deoxycytidine and deoxyguanosine gave rise to one mono- and one bifunctional adduct each. The identitiy of the latter was confirmed by its prevalence when excess deoxynucleoside was added. Deoxyadenosine gave rise to two monofunctional adducts that were unstable in excess deoxyadenosine, and no evidence of bifunctional adducts was obtained. The rate or reaction calculated as $T_{1/2}$ was 5 h for deoxyguanosine and about 24 h for both deoxyadenosine and deoxycytidine.

Discussion

The interaction of *cis*-DDP with DNA has been the subject of many investigations (Roberts & Thomson, 1979). At the macromolecular level, it has been relatively easy to assess the formation of DNA interstrand cross-links and DNA–protein cross-links. However, these lesions probably represent the

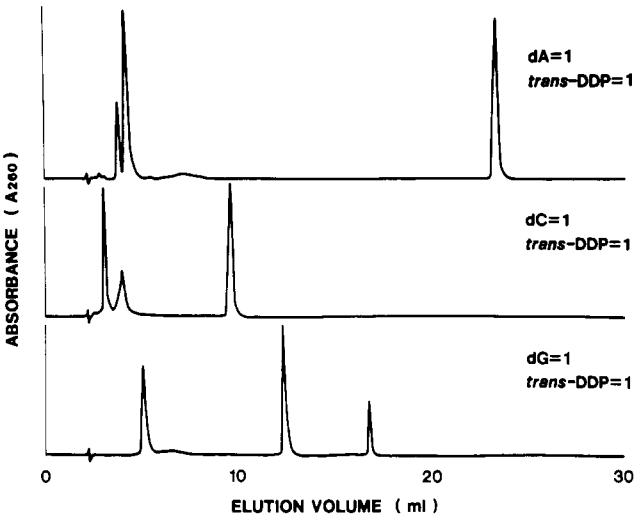

FIGURE 6: HPLC separation of products resulting from a 24-h incubation of *trans*-DDP with equimolar deoxyribonucleosides.

minority of platinations in DNA (Pera et al., 1981; Roberts & Friedlos, 1981). Characterization of the total platination of DNA would be facilitated by a system for separating the deoxyribonucleoside-bound adducts that can be obtained by enzyme digestion of DNA. As a preliminary to such an investigation, it is necessary to analyze the products obtained from reaction of deoxyribonucleosides with *cis*-DDP. Many communications have attempted characterization of products in unpurified reaction mixtures (Kelman et al., 1977; Mansy et al., 1973; Robins, 1973a,b; Roos et al., 1974). Although important information has been obtained, that direction is limited in its capability to analyze the digestion products of platinated DNA. The production, separation, and characterization of platinated deoxyribonucleosides reported here would appear to provide a convenient system for subsequent future analysis of platinated DNA. The present analysis is important because it can detect any minor products that occur. Further uses will also include assessing the capability of nucleases to digest modified DNA. A further advantage of this system is that it can be used to determine the stability of platinum adducts. An example of this is that during DNA purification by hydroxylapatite chromatography (Eastman et al., 1982) the lesions are exposed to a buffer containing 5 M urea–2 M NaCl–0.5 M sodium phosphate. Deoxyguanosine–platinum adducts incubated in this buffer have been found to be stable.

The reaction of deoxyribonucleosides with *cis*-DDP has demonstrated the production of four monofunctional platinations. These are platination at N(3) of cytidine, N(7) of guanosine, N(1) of adenosine, and N(7) of adenosine. The bifunctional adducts produced in the same reactions were identified as guanosine N(7) to N(7), adenosine N(7) to N(7), and adenosine N(1) to N(7). In addition, N(7) of guanosine was capable of forming bifunctional adducts with both adenosine N(1) and N(7). Perhaps the more striking observation was that no bifunctional adducts could be produced that contained deoxycytidine. This is supported by the lack of reaction of *cis*-DDP with cytidine in a guanosine–cytidine dinucleoside even though reaction occurred with guanosine (Inagaki & Kidani, 1979). The inability to detect an adenosine N(1) to N(1) adduct also receives support from a recent study with deoxyadenosine dinucleosides (Ingaki et al., 1982). It was considered probable that the formation of these bifunctional adducts was prevented as a result of steric hinderence. In this regard, *trans*-DDP adducts were synthesized as these should possess a less hindered character. The formation of a dC–*trans*-DDP bifunctional adduct did indeed occur in support of this hypothesis. Contrary to this was the inability to detect any bifunctional dA–*trans*-DDP adducts. However, this may be due to polymerization as both *trans*-DDP and deoxyadenosine have two sites for reaction.

The only other platinum products detected in this present study apparently contain two platinum moieties on each nucleoside. In the case of deoxyadenosine, this could be due to reaction of *cis*-DDP at both N(1) and N(7) of the same adenosine molecule. However, this appears unlikely in the case of deoxyguanosine. On purification, these products were unstable giving rise to monofunctional adducts. It seems most probable that they represent polymerization of platinum at the high concentrations used and do not represent forms that could arise in DNA in vivo.

No evidence was obtained for the formation of a deoxyguanosine N(7) to O(6) chelate although the O(6) bond would be weak and probably displaced by a convenient N(7) of an alternate guanine. The O(6) position has been implicated as important in the reaction of *cis*-DDP by comparison with the significance of O^6-alkylguanine in the reaction of simple alkylating agents. The involvement of the O(6) position of deoxyguanosine may still be important as a result of the ability of the amine hydrogen atoms to hydrogen bond with the guanosine ketone group. This was confirmed by the crystal structure of an analogous cation (Dehand & Jordanov, 1976), and this would be sufficient to induce cleavage of the labile platinum–ketone chelate, if indeed it ever forms.

Considerable attention has also been directed toward *cis*-DDP interaction with dinucleosides as a model for studying DNA intrastrand cross-links (Roos et al., 1974; Girault et al., 1982). In this regard, an N(7)–N(7) chelate of platinum with deoxyguanosine has been characterized (Girault et al., 1982). Such a lesion should result in a local denaturation of DNA. This is the lesion that is suspected of causing shrinkage in closed circular plasmid DNA (Macquet & Butour, 1978). Each platination produces only a relatively small distortion as they are not recognized by DNA single strand specific nuclease S_1 or by nitrocellulose filter binding (Eastman, 1982). Analysis of these lesions in DNA will be facilitated by the chromatographic technique presented here. Both the dinucleosides and their platinated derivatives are resolved by this system (preliminary observations).

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The efforts of Dr. David Brown and Sheri Duran, Department of Chemistry, in performing the NMR analysis are greatly appreciated. I am indebted to Dr. Edward Bresnick, Department of Biochemistry, for the use of his equipment.

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Poly(inosinic acid) Helices: Essential Chelation of Alkali Metal Ions in the Axial Channel†

Frank B. Howard* and H. Todd Miles*

ABSTRACT: Poly(I) forms ordered, helical, size-specific complexes with Na^+, K^+, Rb^+, and Cs^+. Under ordinary conditions, no complex is formed with Li^+. Formation of helical poly(I) is completely dependent on specific metal–polymer interactions, which are distinct from the usual metal–phosphate counterion association. Na^+ and K^+ each form a single complex with poly(I), and the reaction rates are very slow. Rb^+ and Cs^+ each give rise to two complexes with poly(I): a metastable form I in a relatively rapid reaction and a stable form II in a slow reaction. Ultraviolet titration curves of poly(I) with K^+, Rb^+, and Cs^+ are presented. Conditions of formation and regions of stability of the different complexes are given. Equilibrium dialysis experiments show specific binding of Rb^+ to poly(I) in the presence of an excess of Li^+. The binding ratio is approximately eight inosine residues per Rb^+. The IR spectra of the form I and form II Rb^+ (and Cs^+) complexes are distinct and provide a good tool for observing interconversions of the structures. The CD spectra of the stable (form II) Rb^+ and Cs^+ complexes are strikingly different from those of the metastable forms and from those of Na^+ and K^+. The familiar negative first extremum of the Na^+ and K^+ helices undergoes a reversal of sign in form II of both the Rb^+ and Cs^+ complexes. The CD spectra of the stable (form II) Rb^+ and Cs^+ complexes are inconsistent with exciton splitting of a single transition. They thus support assignment of the two long-wavelength extrema to distinct π–π^* transitions in all of the helical poly(I) spectra. The observations are interpreted in terms of a structural model in which the metal ion is specifically bound to inosine carbonyl oxygens at one of two distinct binding sites in the axial channel of a four-stranded helix. Na^+ is in the center of the planar array of four inosine residues with a M–O distance of ~2.3 Å and coordination number of four. K^+, Rb^+, and Cs^+ are on the helix axis midway between the planes of tetramers with a M–O distance of ~2.8–2.9 Å (for Cs^+, possibly somewhat greater) and coordination number of eight. Arguments and evidence for the model are presented and predictions of the model discussed.

The ordered form of poly(I) has been studied extensively by both fiber diffraction (Rich, 1959; Arnott et al., 1974; Zimmerman et al., 1975) and solution spectroscopic methods (Sarkar & Yang, 1965; Brahms & Sadron, 1966; Formoso & Tinoco, 1971; Thiele & Guschlbauer, 1973; Cech & Tinoco, 1976). The structure was first considered to be a three-stranded helix (Rich, 1959), but later diffraction studies and model building indicate that it is four stranded (Arnott et al., 1974; Zimmerman et al., 1975). Despite numerous investigations there has not been a satisfactory correlation of physical properties with the accepted structure, and reports of many measured properties have shown rather wide variability. A recent infrared study (Miles & Frazier, 1978) has shown that helix formation is strikingly dependent on the identity of the alkali metal cation present in solution. The results were interpreted in terms of a model based upon size-specific complexing of metal ions to one of two binding sites in the axial channel of the four-stranded helix (see Discussion).

The present paper has the objectives of examining further the alkali metal ion specificity of complex formation and determining more clearly the range of conditions under which helix formation may be expected. Whereas the infrared measurements were done on relatively concentrated polymer solutions (e.g., 0.02 M), most of the previous UV and CD studies had been done on quite dilute solutions (e.g., 10^{-4} M). In this report, we bring the observations into a common frame of reference and examine the new data for consistency with the model. Since poly(I) is the first and simplest polynucleotide shown to possess a marked alkali metal ion specificity, it is important to characterize the metal–polymer interactions in some detail as a base line for other more complex systems, such as poly(X).

Materials and Methods

Poly(I) purchased from P-L Biochemicals, Inc. (lot no. 200-15) was converted to the Li^+ salt and characterized as described previously (Howard & Miles, 1982).

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