A Comparative Study of Calf Thymus DNA Binding to Cr(III) and Cr(VI) Ions

EVIDENCE FOR THE GUANINE N-7-CHROMIUM-PHOSPHATE CHELATE FORMATION*

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Chromium(VI) salts are well known to be mutagens and carcinogens and to easily cross the cell membranes. Because they are powerful oxidizing agents, Cr(VI) reacts with intracellular materials to reduce to trivalent form, which binds DNA. This study was designed to investigate the interaction of calf thymus DNA with Cr(VI) and Cr(III) in aqueous solution at pH 6.5–7.5, using Cr(VI)/DNA(P) molar ratios (r) of 1:20 to 2:1 and Cr(III)/DNA(P) molar ratios (r) of 1:50 to 1:2. UV-visible and Fourier transform infrared (FTIR) difference spectroscopic methods were used to determine the metal ion-binding sites, binding constants, and the effect of cation complexation on DNA secondary structure. Spectroscopic results showed no interaction of Cr(VI) with DNA at low anion concentrations (r = 1:20 to 1:1), whereas some perturbations of DNA bases and backbone phosphate were observed at very high Cr(VI) contents (r > 1) with overall binding constant of $K = 508 \ M^{1}$. Cr(III) chelates DNA via guanine N-7 and the nearest PO$_2$ group with overall binding constant of $K = 3.15 \times 10^{5} \ M^{-1}$. Evidence for cation chelate formation comes from major shiftings and intensity variations of the guanine band at 1717 and the phosphate asymmetric stretching vibration at 1222 cm$^{-1}$. At low Cr(III) concentration (r = 1:40), the number of Cr(III) ions bound to DNA were 6–7 cations/500 base pairs, and this increased to 30–35 cations/500 base pairs at high metal ion content (r = 1:4). DNA condensation occurred at high cation concentration (r = 1:10). No major alteration of DNA conformation was observed, and the biopolymer remained in the B family structure upon chromium complexation.

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The abbreviations used are: FTIR, Fourier transform infrared.

Chromium(VI) salts are well known to be mutagens and carcinogens and to easily invade the insides of cells (1). Cr(VI) produced DNA cross-links in rat tissues in vivo (2) and in cultured cells in vitro (3, 4). Although Cr(VI) damaged nuclear DNA in whole cells, no reaction of Cr(VI) with isolated DNA occurred in vitro at physiological pH in the absence of a metabolizing system (5). The Cr(VI) that is taken up is considered to be reduced by glutathione, cysteine, or ascorbic acid into Cr(III) (6), and the resulting cation reacts with DNA to form Cr(III)-DNA adducts. Because Cr(III) is a final form of chromium within the cell, the interaction of Cr(III) with DNA may play crucial role in the carcinogenic action of Cr(VI) salts.

The conversion of B form into Z form in the purine-pyrimidine sequence of DNA has been considered to be a factor in the transcriptional activity of genes (7). Cr(III) is found to interact with the poly(dG-dC) at low concentration and change B form to Z form in the presence of ethanol (8). However, Cr(III) at high concentration causes DNA condensation, inhibiting the alteration of B to Z structure (8). Moreover, the study on the effect of Cr(III) on DNA replication with single-stranded DNA template and micromolar concentration of Cr(III) revealed that Cr(III) bound in a dose-dependent manner to the template DNA and prevents DNA replication (9). However, if the unbound chromium was removed from the system by gel filtration, the rate of DNA replication by polymerase I (Klenow fragment) on the chromium-bound template increased more than 6-fold relative to control. This increase was paralleled by as much as a 4-fold increase in processivity and a 2-fold decrease in replication fidelity. When the concentration of Cr(III) increased further, DNA-DNA cross-links occurred to inhibit the polymerase activity. Trivalent chromium can bind purified DNA and form lesions capable of obstructing DNA replication in vitro (10, 11). It has also been observed that intact Novikoff ascites hepatoma cells exposed to potassium chromate formed cross-linking of nuclear proteins to DNA (12). Recently, Cr(III) was shown to cause mutational spectrum in shuttle vector systems replicated in human cells (13). Thus, the interaction of Cr(III) with DNA may be responsible for carcinogenic activity of chromium.

There are many agents that are specific for guanine alkylation in the O-6, N-7, or C-8 position. Several of these are highly active carcinogens, such as N-acetoxy-N-2-acetylaminofluorene, which alkylates in the C-8 position, and nitrosoamines, nitrogen mustards, nitrourca, and aflatoxin, which alkylate on the N-7 position (14). The action of certain carcinogens, e.g. modification of guanine by N-7 methylation or by alkylation at C-8 with N-acetoxy-N-2-acetylaminofluorene facilitated the B to Z transition of poly(dG-dC) (15–17). On the contrary, modification of poly(dG-dC) with the antitumor drug cis-diaminedichloplatinum (II) (a cross-linking agent) prevented the B to Z conversion (18, 19).

It was found that Cr(III) preferentially binds guanine-containing DNAs (5, 20). The results of a study on the DNA replication system showed that most of Cr(III) binding to the single-stranded template DNA is electrostatic because 40% or more of bound cation could be displaced by high salt wash, whereas only 20% or less chromium is chelatable by EDTA (9).

In the present study, we have investigated the complexation of Cr(III) and Cr(VI) with calf thymus DNA using UV-visible and FTIR difference spectroscopy at pH 6.5–7.5 with Cr(III)/ Cr(VI)
DNA(P) of 1:80 to 1:2 and Cr(VI)/DNA(P) of 1:20 to 2:1. Structural analysis regarding the chromium-binding sites, binding constants, and the alteration of DNA secondary structure are reported here. This is a first infrared spectroscopic study regarding the Cr(III)-DNA chelate formation and should help to elucidate the nature of the carcinogenic potential of chromium.

EXPERIMENTAL PROCEDURES

Materials—Highly polymerized type I calf thymus DNA sodium salt (7% sodium content) was purchased from Sigma and was deproteinated by the addition of CHCl3 and isooamyl alcohol in NaCl solution. Crystalline CrCl3·(H2O)6 and K2CrO4, salts were of reagent grade.

Preparation of Stock Solutions—DNA was dissolved to 1% w/w (25 mM DNA(phosphate)) in 50 mM NaCl and 1 mM sodium cacodylate (pH 7.30) at 5°C for 24 h with occasional stirring to ensure the formation of a homogeneous solution. The appropriate amount of CrCl3·(H2O)6 and K2CrO4 (0.3 to 10 mM) was prepared in distilled water and added dropwise to DNA solution to attain desired Cr(III)/DNA(P) molar ratios of 1:80, 1:40, 1:20, 1:10, 1:4, and 1:2 and Cr(VI)/DNA(P) molar ratios of 1:20, 1:10, 1:5, 1:1, and 2:1 at a final DNA concentration of 0.5% w/w or 12.5 mM DNA(phosphate). The pH values of solutions were adjusted to 6.5–7.5, using NaOH solution. The infrared spectra were recorded 2 h after mixing of chromium salt and DNA solutions. The infrared spectra of Cr(III)-DNA complexes with r = 1/2 could not be recorded as solution because of solid gel formation. When UV absorption spectra were recorded, solutions containing DNA at a final concentration of 6.25 mM phosphate and 50 mM NaCl were used.

Separation of Cr(III)-DNA Complexes—The mixtures containing calf thymus DNA (6.25 mM of phosphate) and Cr(III) at molar ratios of 1:40 to 1:4 in 50 mM NaCl were applied to Sephadex G-25 (0.8 × 4.5 cm) column equilibrated in water, and 20 fractions were collected. The concentrations of Cr(III) and DNA in each fraction were determined by atomic absorption spectroscopy at 357.9 nm and by UV at 260 nm. The concentrations of Cr(III)-DNA complexes in each mixture were analyzed from regions where elution patterns of Cr(III) and DNA overlapped.

FTIR Spectra—Infrared spectra were recorded on a Bomem DA3–0.02 FTIR spectrometer equipped with a nitrogen cooled HgCdTe detector and KnBr beam splitter. The solution spectra were taken using AgBr windows with resolution of 2–4 cm⁻¹ and 100–500 scans. Each set of infrared spectra were taken (three times) on three identical samples with the same DNA and metal ion concentrations. The water subtraction was carried out with 0.1 M NaCl solution used as a reference at pH 6.5–7.5 (21). A good water subtraction was achieved as shown by a flat baseline around 2200 cm⁻¹ where the water combination mode is located. This method is a rough estimate but removes the water content in a satisfactory way. The difference spectra ((DNA solution + chromium solution) − (DNA solution)) were produced, using a sharp DNA band at 968 cm⁻¹ as internal reference. This band, because of deoxyribose C-C stretching vibrations, exhibits no spectral changes (shifting or intensity variations) on Cr-DNA complexation, and it was cancelled upon spectral subtraction. The spectra were smoothed with a Savitzky-Golay procedure (21). The intensity ratios of several DNA in-plane vibrations related to A-T and G-C base pairs and the PO2 stretches were measured (with respect to the reference band at 968 cm⁻¹) as a function of chromium concentration with an error of ±3%. These intensity ratios were used to calculate binding constants of Cr(III) with DNA bases and the backbone phosphate group.

Absorption Spectra—UV absorption spectra were recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer. The chromium concentrations were determined using atomic absorption Perkin-Elmer Aanalyst 100 Spectrometer.

RESULTS AND DISCUSSIONS

Cr(VI)-DNA Complexes—At r = 1:20 to 1, Cr(VI) does not bind to DNA in aqueous solution. Evidence for this comes from the lack of major spectral changes (intensity or shifting) of the prominent DNA in-plane vibrations (21–28) at 1717 (mainly guanine, 1663 (mainly thymine), 1609 (mainly adenine), 1492 (mainly cytosine), and 1222 cm⁻¹ (asymmetric PO2 stretch) (Figs. 1B and 2). However, at high chromium concentration (r > 1), some perturbations of DNA vibrations were observed. The guanine band at 1717 cm⁻¹ shifted toward a lower frequency at 1715, whereas the thymine band at 1663 shifted toward a higher frequency at 1665 cm⁻¹ (Fig. 1A). Similarly, the adenine band at 1609 cm⁻¹ was observed at 1614 cm⁻¹ in the presence of CR3 of 1:80 to 1:2 and Cr(VI)/DNA(P) of 1:20 to 2:1. The phosphate asymmetric stretching vibration at 1222 cm⁻¹ of the uncomplexed DNA was shifted toward a lower frequency at 1220 cm⁻¹ as Cr(VI) concentration increased (Fig. 1A, r = 2). Positive peaks centered at 1653 cm⁻¹ and 1215 cm⁻¹ in the difference spectra of chromate-DNA complexes is coming from minor increases in the intensity of DNA vibrations (guanine, thymine, and adenine) bases and backbone phosphate) (Fig. 1B). The observed spectral changes are due to indirect anion-DNA interaction
though water molecules of hydration on the DNA and hydrogen donor site of the bases. The two negative charges associated with the chromate anion and the presence of a negative charge on the backbone PO2 group are the major factors for a weak chromate-DNA complexation. It should be noted that the presence of a strong band at 893 cm⁻¹ in the infrared spectra, which appears at 877 cm⁻¹ in the difference spectra of the chromate-DNA complexes are due to the CrO₄²⁻ vibrations (29) (Fig. 1, A and B, r = 2). Our observations are consistent with other spectroscopic studies that showed no major Cr(VI)-DNA interaction in vitro in the absence of reducing agents (5, 8, 30).

However, in the presence of metabolizing system, where Cr(VI) is reduced to Cr(III), major Cr(III)-DNA complexation has been observed (5, 8, 10, 11).

Cr(III)-DNA Complexes—At low cation concentrations (r = 1:80 to 1:20), Cr(III) binds DNA through guanine and the backbone PO2 group. Evidence for this comes from major spectral shiftings of the bands at 1717 cm⁻¹ (mainly guanine) and at 1222 cm⁻¹ (PO2 asymmetric stretch) (21–28). The guanine band at 1717 cm⁻¹ shifted toward a lower frequency at 1709 cm⁻¹ and the phosphate band at 1222 cm⁻¹ was observed at 1216 cm⁻¹, upon Cr(III) complexation (Fig. 1A). In addition, a major increase in the intensity of guanine band at 1717 cm⁻¹ (40%) and PO2 band at 1222 cm⁻¹ (30%) was observed as Cr(III) concentration increased to r = 1:20 (Fig. 3). The weak positive peaks at 1710 (G), 1660 (T), 1611 (A), 1215, and 1088 cm⁻¹ (phosphate) in the difference spectrum of the Cr-DNA complexes are due to a major increase of the intensity of DNA bases and the PO2 vibrations at low Cr(III) concentration (r = 1:80) (Fig. 1B). However, as cation concentration increases to r = 1:20, the positive features in the difference spectrum of Cr-DNA complexes grow in intensity, particularly for the guanine band at 1709 and the PO2 band at 1214 cm⁻¹ (Fig. 1B, r = 1:20). In addition, the relative intensity ratios of the ν₈ PO2 (1088 cm⁻¹) and ν₃ PO2 (1222 cm⁻¹) have changed from 1.7 (DNA-free) to 1.5 (Cr-DNA complexes). The observed spectral shiftings (Δν = 8 to 6 cm⁻¹) and the major intensity variations for the guanine band at 1717 cm⁻¹ (40%) and the PO2 vibration at 1222 cm⁻¹ (30%) are due to the cation chelation via guanine N-7 and the backbone phosphate group (Figs. 1B and 3, r = 1:20). It has been suggested that the infrared spectral changes related to the DNA in-plane vibrations at 1720–1600 cm⁻¹ are due to the metal interaction through guanine N-7 site (21, 22). It should be noted that although a minor intensity variations were observed for the thymine band at 1663 (10%) and adenine band at 1609 cm⁻¹ (5%), no major spectral shiftings for this vibration occurred upon Cr(III) complexation (Figs. 1A and 3). Thus, the amount of chromium that directly binds to the A-T base pair is negligible, whereas an indirect cation binding (via H₂O) to the A-T bases cannot be excluded.

The calculation of the overall binding constants were carried out using UV spectroscopy as reported (31). If the equilibrium for chromium cation and DNA is established as in Equation 1, then the binding constants of chromium cation complexes with DNA can be described as in Equation 2.

\[
\text{DNA + Chromium cation} \rightarrow \text{DNA:Chromium} \quad (\text{Eq. 1})
\]

\[
K = \frac{[\text{DNA:Chromium}]}{[\text{DNA}][\text{Chromium}]} \quad (\text{Eq. 2})
\]

The double reciprocal plot of 1/[complexed chromium] versus 1/[free cation] is linear and the binding constant (K) is calculated from the ratio of the intercept on the vertical coordinate axis to the slope (31). Concentrations of complexed chromium were determined by subtracting absorbance of uncomplexed DNA at 280 nm from those of complexed DNA. Concentrations of free cation were determined by subtracting those of complexed chromium from total chromium used for the experiment. Our data of 1/[complexed Cr] almost proportionally increased as a function of 1/[free Cr], and overall binding constants for Cr(VI)-DNA and Cr(III)-DNA were estimated to be \( K = 508 \text{ M}^{-1} \) and \( K = 3.15 \times 10^3 \text{ M}^{-1} \), respectively.

Because the Cr(III)-DNA interaction mainly occurs through guanine N-7 and the backbone PO2 group, the binding constants of Cr(III) with guanine and backbone PO2 were determined from the intensity ratios of DNA in-plane vibrations related to guanine (1717 cm⁻¹) and backbone phosphate (1222 cm⁻¹) stretchings. Using the data from uncomplexed DNA and Cr(III)-DNA adduct formed at \( r = 1:10 \), the binding constants were estimated to be \( K(G) = 1.80 \times 10^3 \text{ M}^{-1} \) and \( K(P) = 1.25 \times 10^3 \text{ M}^{-1} \). The binding constants for thymine and adenine bases were much smaller than those of the guanine and phosphate group. This indicates that the interaction of Cr(III) with the A-T base pair is negligible. In a previous study, based on the intensity ratio variations of the infrared absorption bands, the binding constants of the chlorophyll-DNA complexes were determined, where Mg(II) was coordinated to the backbone PO2 group and the guanine N-7 sites (32).

The somewhat low stabilities (\( k = 10^3 \text{ M}^{-1} \)) of the cation chelate complexes are attributed mainly to the ionic nature of the Cr-O-P-O interaction. It has been suggested that most of Cr(III) binding to the single-stranded template DNA is electrostatic because 40% or more of bound cation could be displaced.
by high salt wash, whereas only 20% or less of the chromium is chelatable by EDTA (9). On the other hand, the overall binding constant for Cr(VI)-DNA was estimated to be 508 M⁻¹. Such a low stability of the complexes is indicative of no direct Cr(VI)-DNA interaction in aqueous solution.

Based on the data from UV absorption spectra, numbers of Cr(III) bound to DNA were estimated to be 9 cations/500 base pairs at r = 1:40 and 32 cations/500 base pairs at r = 1:4 (Fig. 4). To compare the numbers of Cr(III) bound to DNA by other method, the mixtures were subjected to Sephadex G-25 column (0.8 × 4.5 cm) equilibrated in aqueous solution, and concentrations of Cr(III)-DNA complexes were determined by atomic absorption and UV absorption spectroscopic methods. The number of Cr(III) bound to DNA were calculated to be 6 cations/500 base pairs at r = 1:40 and 30 cations/500 base pairs at 1:4. Another research group (13) showed that about 25 Cr(III) cations bound to 1000 nucleotides of a plasmid DNA at a low concentration of chromium(III) chloride in the presence of NaCl used in our DNA preparation with respect to plasmid DNA solution (10 mM potassium salt), which in contrast to our results (50 mM) as shown in Fig. 1, the DNA infrared marker bands at 836 and 1717 cm⁻¹ were shifted toward lower frequencies at 810 and 1700 cm⁻¹, respectively, whereas the PO₂ band at 1222 (phosphate) to 1216 cm⁻¹ in the spectra of the Cr(III)-DNA complexes are due to the direct Cr(III) coordination to both guanine N-7 and backbone phosphate group (Fig. 1A). Other spectroscopic studies also showed that chromium cations inhibit B to A or B to Z conformational changes upon DNA complexation (8). However, at low cation concentration, Cr(III) can induce B to Z conformational transition for synthetic poly deoxyribonucleotides in the presence of ethanol (8).

Concluding Remarks—On the basis of our spectroscopic results of calf thymus DNA in the presence of Cr(VI) and Cr(III) metal ions in aqueous solution, the following remarks can be made: (a) No direct Cr(VI)-DNA interaction was observed in vitro, whereas Cr(III) chelates DNA through guanine N-7 and the backbone phosphate group; (b) The somewhat low stability of the Cr(III)-DNA complex is mainly due to the ionic nature of the Cr(III)-phosphate interaction; (c) Cr(III) induces DNA condensation at high cation concentration; and (d) Cr(VI) and Cr(III) ions do not induce DNA conformational changes at low or high cation concentration.

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