Formation of the Amino Acid−DNA Complexes by Hexavalent and Trivalent Chromium *in Vitro:* Importance of Trivalent Chromium and the Phosphate Group[†]

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ABSTRACT: We have recently shown that a substantial fraction of all Cr−DNA adducts in chromate-exposed cells are represented by ternary complexes involving amino acids or glutathione bridged by Cr(III) to DNA. The tridentate amino acids such as cysteine, glutamic acid, and histidine were predominantly found cross-linked to DNA. The mechanism by which Cr can cross-link these amino acids to DNA has been modeled by reacting DNA and trivalent and hexavalent chromium with cysteine and histidine. The formation of a Cr(III)−amino acid binary complex was required before Cr(III) reacted with DNA to yield a ternary complex. Cr(III)-pretreated DNA did not bind cysteine or histidine even after prolonged incubations. Reduction of Cr(VI) in the presence of DNA gave rise to an extensive cross-linking of cysteine and histidine. Addition of DNA to Cr(VI) mixtures at the start of reduction or after the reduction was complete had little effect on the level of ternary complexes indicating that Cr(III)−amino acid binary complexes were DNA-attacking species. In order to identify DNA groups involved in the ternary complex formation, pre-formed Cr(III)−histidine complexes were reacted with nucleosides and nucleotide monophosphates followed by separation and analysis of the products. The incubation of the Cr(III)−histidine complexes with nucleotide monophosphates but not with nucleosides gave rise to ternary complexes that contained both histidine and Cr, showing the primary importance of the phosphate group in this reaction. All four DNA nucleotides were capable of the ternary complex formation with Cr(III) and histidine. No apparent base preference in the amino acid cross-linking was also found in the reaction of Cr(III)/cysteine and Cr(VI)/cysteine mixtures with oligonucleotides of base-specific composition.

Numerous epidemiological studies have established that hexavalent Cr compounds are potent human carcinogens (IARC, 1990; Langardt, 1990). Cr(VI)-containing compounds are strong clastogens (Sen & Costa, 1986; Wise et al., 1994) and have been found to be mutagenic in both bacterial and mammalian test systems (Snow, 1992; Yang et al., 1992; Chen & Thilly, 1994). In contrast, water-soluble Cr(III) compounds are generally inactive in cellular assays and are not considered carcinogenic (IARC, 1990). The observed inactivity has been attributed to the inability of the hydrated octahedral Cr(III) complexes to cross plasma membranes (Snow, 1992). A dominant chemical form of Cr(VI) in solution at physiological pH is chromate ion which is isostructural with phosphate and sulfate. Chromate readily enters cells through the general anion channel leading to a rapid accumulation of Cr intracellularly hundreds-fold over the concentration in the media (Buttner & Beyersmann, 1985). The Cr(VI) form is not reactive toward DNA, and it requires a reductive activation in order to inflict DNA damage (DeFlora & Wetterhahn, 1989). Inside the cells Cr(VI) undergoes a reductive metabolism leading to the formation of stable Cr(III) and accompanied by production of several reactive species including intermediate Cr(V) and Cr(IV) forms and radical species (Bose et al., 1992; Shi et al., 1994; Stearns & Wetterhahn, 1994). Ascorbate and other low molecular weight intracellular reductants such as cysteine, glutathione, and NADH are thought to be largely responsible for the Cr(VI) metabolism inside cells (Connett & Wetterhahn, 1985; Snow, 1992). Depending on the particular reductant and the ratio of Cr(VI) to reductant, the levels of Cr(V) and Cr(IV) during the reduction reaction can vary dramatically (Goodgame & Joy, 1987; Stearns et al., 1995).

It has long been known that chromate-exposed cells contained abundant Cr(III)−DNA adducts although their chemical nature and mutagenic potential remained unknown (Salnikow et al., 1992; Tsapakos et al., 1983). We have recently found that the treatment of cells with chromate leads to the formation of stable ternary Cr−DNA adducts involving amino acid ligands (Voitkun et al., 1994; Zhitkovich et al., 1995). Cysteine, histidine, glutamic acid, and glutathione were the predominant ligands bridged by Cr(III) to cellular DNA. At biologically relevant chromate doses the ternary complexes of Cr(III) with DNA and amino acids/glutathione were estimated to represent up to 50% of all Cr−DNA adducts (Zhitkovich et al., 1995). A very low lability of Cr(III) complexes with tridentate amino acid and peptide ligands as compared to those formed with the bidentate amino acids is likely to account for the high level of histidine, cysteine, glutamic acid, and glutathione cross-links with DNA found in chromate-treated cells. Interestingly, studies analyzing amino acid groups involved in Cr-mediated DNA−protein cross-linking have concluded that cysteine and histidine residues are the most likely ligands responsible for the Cr-dependent protein−DNA linkage (Lin et al., 1992; Salnikow et al., 1993).

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In the present study the mechanism of formation of amino acid−Cr(III)−DNA complexes *in vitro* as well as the base specificity of the adduct formation were studied. Cr-mediated cysteine and histidine cross-linking was analyzed with both Cr(III) and Cr(VI) compounds. The results point to the importance of DNA phosphate groups as the site of Cr binding and show a lack of any base specificity in Cr-dependent complexing of amino acids to DNA.

EXPERIMENTAL PROCEDURES

Materials. Deoxynucleosides, deoxynucleotides monophosphates, L-histidine and ascorbic acid were obtained from Sigma (St. Louis, MO). L-cysteine was from Calbiochem (La Jolla, CA). The DNA substrate was the double-stranded ϕX174 plasmid (form II) purchased from New England Biolabs (Beverly, MA). The plasmid DNA was purified from EDTA by ethanol precipitation and dissolved in distilled water. Purified oligonucleotides of defined composition were obtained from National Biosciences (Plymouth, MN) and were used without further purification. Sephadex G-50 columns were from Boehringer Mannheim (Indianapolis, IN). The Cr(VI) source was K_2CrO_4 (Fisher Scientific, Springfield, NJ), and the Cr(III) source was $CrCl_3 \cdot 6H_2O$ (Sigma). Radiolabeled L-[^{35}S]cysteine, $^{51}CrCl_3$ and $K_2{}^{51}CrO_4$ were obtained from NEN (Boston, MA); L-[^{3}H]histidine was from Amersham (Arlington Heights, IL). All reagent solutions except MES buffer were freshly prepared before each experiment. In order to prevent the formation of insoluble Cr(III) hydroxospecies, all experiments were conducted at pH 6.1. This pH value is in the range of acidity observed in tissue culture media of actively growing cells.

Reaction of Amino Acids with Cr(III)-Pretreated DNA. ϕX174 plasmid DNA (0.5 μg) was treated with 5 μM $CrCl_3 \cdot 6H_2O$ in 50 μL of 10 mM MES buffer (pH 6.1) for 30 min at 37 °C. In preliminary time course studies, it was found that during the 30 min incubation period Cr(III) binding to DNA was complete. Cr(III)−DNA was purified from unbound Cr(III) by passing through Sephadex G-50 columns. Commercially prepared Sephadex columns such as those used here are typically equilibrated in EDTA-containing buffers. EDTA chelates Cr(III) with an extremely high affinity, and, therefore, any material eluted from Sephadex columns requires further purification to remove EDTA. We have found that precipitation of DNA with 2 volumes of 100% ethanol in the presence of 200 mM NaCl followed by two washings of the DNA pellet with 70% ethanol effectively eliminated trace amounts of EDTA from the DNA preparations. The Cr(III)−DNA samples were resuspended in 10 mM MES buffer and were immediately used to study amino acids cross-linking. The amount of DNA-bound Cr(III) was calculated by using radiolabeled $^{51}CrCl_3$ in a parallel set of tubes. Cross-linking of amino acids was performed by reacting 0.5 μg of Cr(III)−DNA and 50 μM cysteine or histidine in 50 μL of 10 mM MES buffer (pH 6.1) for 0.5, 2, 5, and 20 h at 37 °C. Radiolabeled [^{35}S]cysteine or [^{3}H]-histidine (20 μCi/mL) was included in the reaction mixtures in order to quantify DNA binding of the respective amino acids. DNA was separated from unreacted amino acids on G-50 columns, and the radioactivity was quantified in an Ecolume scintillation cocktail with a scintillation counter. Background amino acids or chromium radioactivity was determined in mixtures containing all the components except DNA. We have found that commercial [^{3}H]histidine preparations were not radiochemically pure based on the HPLC comparison of the authentic standard and the freshly obtained [^{3}H]histidine batches. The purity of [^{3}H]histidine batches was taken into account for DNA binding calculations.

Reaction of Cr(III)−Amino Acids Complexes with DNA. Cr(III)−amino acid complexes were formed by incubation of 50 μM $CrCl_3 \cdot 6H_2O$ and 500 μM cysteine or histidine in 10 mM MES buffer (pH 6.1) either at 37 °C for 2 h or at 50 °C for 5 h. Radiolabeled [^{35}S]cysteine (20 μCi/mL), [^{3}H]histidine (20 μCi/mL), or $^{51}CrCl_3$ (10 μCi/mL) was also added to the reaction mixtures in order to quantify the DNA binding. DNA binding was determined by incubation of the prereacted Cr(III)−cysteine or Cr(III)histidine mixtures with 0.5 μg of plasmid DNA at 37 °C for 0.5, 2, or 20 h. DNA was separated from unreacted components on G-50 columns.

DNA Binding of Amino Acids in the Course of Cr(VI) Reduction. DNA binding of cysteine or histidine in the course of Cr(VI) reduction was carried out in 10 mM MES, pH 6.1, at 37 °C for 2 h. DNA (0.5 μg) was added to Cr(VI)/reductant/amino acid mixtures at the start of the reduction and at later time points. The binding reaction was performed by incubating DNA with 0.1 mM K_2CrO_4, 1 mM cysteine, and 20 μCi/mL [^{35}S]cysteine. Cross-linking of histidine was carried out by reacting DNA with 50 μM $CrCl_3 \cdot 6H_2O$, 500 μM histidine, 300 μM ascorbate, and 20 μCi/mL [^{3}H]histidine. Parallel sets of samples contained $K_2{}^{51}CrO_4$ (10 μCi/mL) in place of radiolabeled amino acids in order to determine Cr binding. DNA-associated radioactivities were measured after the Sephadex G-50 chromatography. In all experiments reduction of Cr(VI) was monitored by the disappearance of UV absorbance at 372 nm and the concomitant appearance of absorbance for Cr(III) at 550 nm.

Cysteine Binding to Oligonucleotides of Base-Specific Composition. All oligos contained 39 nucleotides placed in random order. ATC oligo contained 13 nucleotides of A, T, and C; ATG oligo contained 13 nucleotides of A, T, and G; TC oligo contained 19 T and 20 C. In all experiments, the concentration of chromium was 50 μM and cysteine = 500 μM. Oligos and Cr compounds were incubated for 2 h at 37 °C. Recovery of the oligos from G-25 columns was measured by UV absorbance at 260 nm before and after chromatography (recovery of ATC, 65%; AGT, 90%; TC, 86%).

Binding of Cr(III)−Histidine Complexes to 2′-Deoxynucleosides and 2′-Deoxynucleotides 5′-Monophosphates. A solution containing 10 mM histidine, 2 mM $CrCl_3 \cdot 6H_2O$, [^{3}H]histidine (4.5 μCi/mL), or $^{51}CrCl_3$ (10 μCi/mL) in 25 mM MES buffer, pH 6.1, was incubated at 50 °C for 5 h. The nucleotide or nucleoside was added to the Cr(III)−histidine solution and the mixture was incubated overnight at 37 °C. The products were analyzed on a Waters HPLC system with a reversed-phase Adsorbosphere Nucleotide-Nucleoside column (Altech, Avondale, PA), utilizing a gradient system: solvent A, 60 mM KH_2PO_4 and 5 mM tetrabutylammonium phosphate (TBAP), pH 5.0; solvent B, 100% methanol and 5 mM TBAP. The best resolution was achieved by applying an isocratic flow of solvent A for 10 min, followed by ramping in solvent B from 0 to 35% over 35 min. The overall flow rate was maintained at 1 mL/min throughout the analysis. All HPLC solvents were purchased from Fisher Scientific (Springfield, NJ). The peaks were detected by their UV absorbance at 254 nm. To determine

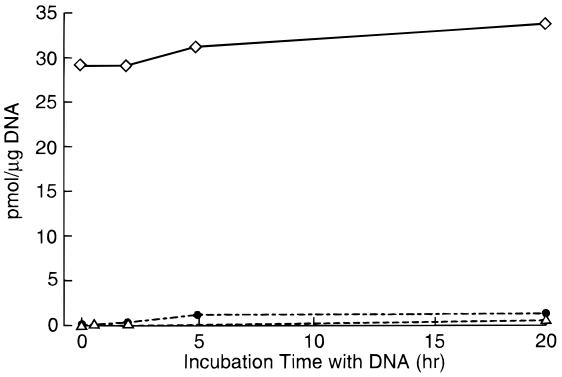

FIGURE 1: Binding of amino acids to Cr(III)−pretreated DNA. DNA (0.5 μg) was treated in 10 mM MES (pH 6.1) with 5 μM $CrCl_3 \cdot 6H_2O$ for 30 min at 37 °C. The Cr(III)-treated DNA was purified through G-50 columns, ethanol precipitated, and dissolved in 10 mM MES buffer. Cr(III)−DNA and 50 μM cysteine or histidine were incubated for 0.5, 2, 5, and 20 h at 37 °C. DNA was separated from unbound amino acids on G-50 columns. (●) Cysteine binding; (△) Histidine binding; (◇) Cr binding.

the presence of a nucleotide in separate HPLC peaks, the individual fractions were collected, treated with 0.2 N NaOH for 2 h at 37 °C, and analyzed by HPLC following neutralization with HCl.

RESULTS

DNA-Bound Cr(III) Does Not React with Histidine or Cysteine. The adduction of amino acids to DNA was first studied with trivalent chromium. Two pathways can be envisioned as to how Cr(III) can link amino acids to DNA. Amino acid can react with Cr(III) first, and then this complex will attack DNA forming a ternary adduct. Another scenario invokes initial Cr(III)−DNA complexing followed by capturing of amino acid. In order to distinguish between these two possibilities of the cross-link formation, Cr(III) as $CrCl_3 \cdot 6H_2O$ was first reacted with DNA, and then cross-linking of added amino acids was studied (Figure 1). Cr(III)−DNA was purified from unbound Cr(III) by the Sephadex G-50/ethanol precipitation procedure and then was immediately reacted with histidine or cysteine over different time intervals. Essentially no complexing of these amino acids with DNA was observed even after prolonged incubations.

Incubation of Prereacted Cr(III)−Cysteine or Cr(III)−Histidine with DNA Results in the Formation of Ternary DNA Adducts. Substitution reactions of Cr(III) coordinated by H_2O ligands are known to be quite slow, and therefore, different DNA reactivities might be observed depending on the extent of displacement of the initial ligands in the Cr(III) coordination sphere by the amino acid groups. DNA reactivities of Cr(III)−amino acid complexes formed under two different conditions were studied. Reaction of DNA with Cr(III)−cysteine preincubated for 2 h at 37 °C led to a significant DNA−cysteine cross-linking with the ratio of Cr(III) to cysteine approximately 2.5 to 1 (Figure 2A). Incubation of DNA with Cr(III)−cysteine pre-formed for 5 h at 50 °C gave rise to the equmolar DNA binding of Cr(III) and cysteine (Figure 2B). A similar pattern of cross-linking was observed when DNA reactivity of preincubated mixtures of Cr(III)−histidine was studied (Figure 3A,B). Cr(III) coordination to histidine, however, seemed to be somewhat slower and/or more labile than that to cysteine as evidenced by a higher ratio of DNA-bound Cr(III) to amino

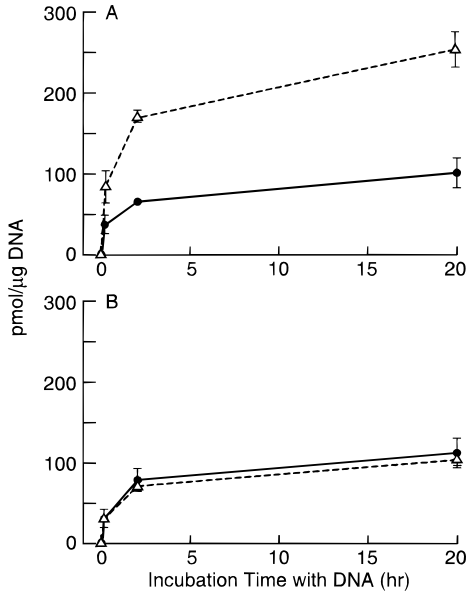

FIGURE 2: Binding of Cr(III)−cysteine complexes to DNA. DNA and pre-formed Cr(III)−cysteine complexes were reacted at 37 °C for different time intervals. DNA was purified from unbound components by passing through G-50 columns. (A) 50 μM $CrCl_3 \cdot 6H_2O$ and 500 μM cysteine were pre-incubated in 10 mM MES buffer (pH 6.1) for 2 h at 37 °C; (B) $CrCl_3 \cdot 6H_2O$ and cysteine were pre-incubated for 5 h at 50 °C. Shown are means ± SD. Where error bars are not seen, they were smaller than the line marker. (●) Cysteine binding; (△) Cr binding.

acid in the case of histidine at both preincubation conditions. In all experiments binding of amino acids to DNA plateaued after a 2 h incubation period. Quantitative comparison of the amino acids cross-linking by 5 μM $CrCl_3 \cdot 6H_2O$ showed that DNA binding of histidine and cysteine to Cr(III)−DNA was only about 3% and 6% of that with prereacted Cr−histidine and Cr−cysteine, respectively. Simultaneous incubation of $CrCl_3 \cdot 6H_2O$ at 20μM and below with DNA and cysteine or histidine resulted in an extensive Cr binding to DNA with only weak amino acid cross-linking (data not shown). Higher Cr(III) concentrations led to a severe loss of DNA during purification on G-50 columns because of DNA aggregation caused by Cr(III)−mediated DNA−DNA cross-linking. Thus, *in vitro* DNA cross-linking of amino acids by Cr(III) can only be successfully achieved by reacting DNA with pre-formed Cr(III)−amino acid complexes. Cr(III) has much greater affinity for DNA than for histidine or cysteine, and its binding to DNA virtually precludes subsequent reactions with amino acid ligands.

DNA Cross-Linking of Cysteine by Chromium(VI). In addition to binding Cr(III), cysteine is also capable of the reductive conversion of Cr(VI) to Cr(III). Figure 4A illustrates the kinetics of Cr(VI) reduction by 10-fold molar excess of cysteine. The reduction was complete after 2 h based on the disappearance of chromate absorbance at 372 nm and the concomitant appearance of absorbance for Cr(III) at 550 nm. The reduction kinetics of Cr(VI) was not altered by the presence of DNA (data not shown). Addition of DNA at different time intervals after the initiation of the Cr(VI) reduction resulted in significant cysteine−DNA cross-linking, and the level of the adduct formation was not affected by the time of DNA addition (Figure 4B). The unchanged cysteine−DNA cross-linking activity long after the completion of Cr(VI) to Cr(III) reduction strongly indicates that it is Cr(III)−cysteine complexes but not Cr-

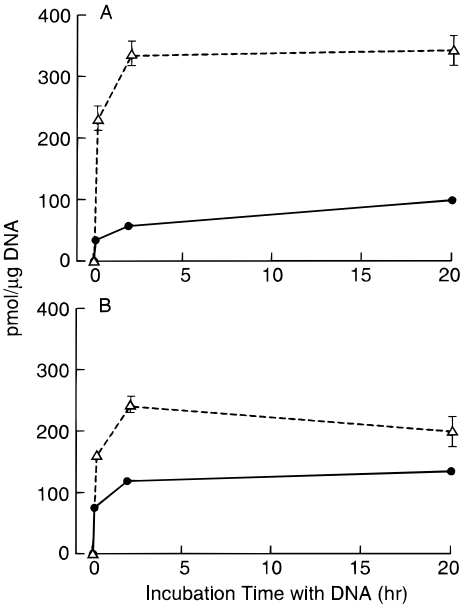

FIGURE 3: Reaction of pre-formed Cr(III)−histidine complexes with DNA. DNA and pre-reacted Cr-histidine mixtures were incubated at 37 °C for different time intervals. (A) 50 μM $CrCl_3 \cdot 6H_2O$ and 500 μM histidine were pre-incubated in 10 mM MES buffer (pH 6.1) for 2 h at 37 °C; (B) $CrCl_3 \cdot 6H_2O$ and histidine were pre-incubated for 5 h at 50 °C. (●) Histidine binding; (△) Cr binding.

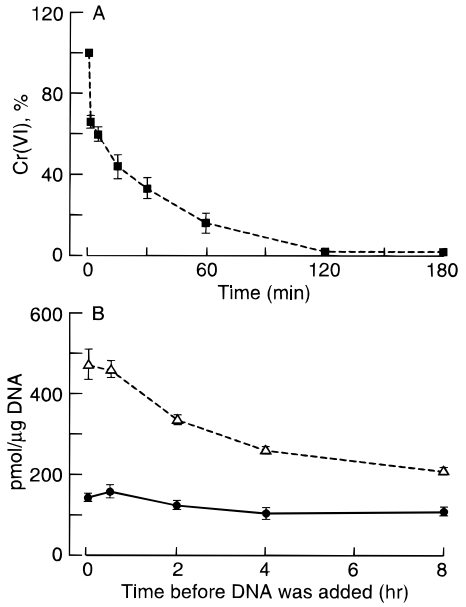

FIGURE 4: Kinetics of Cr(VI) reduction by cysteine and DNA binding of Cr and cysteine during Cr(VI) reduction. (A) 100 μM K_2CrO_4 was mixed with 1 mM cysteine in 10 mM MES buffer (pH 6.1) and incubated at 37 °C. Reduction of Cr(VI) was monitored by UV absorbance at 372 nm. (B) 100 μM K_2CrO_4 was mixed with 1 mM cysteine and incubated at 37 °C for different periods of time followed by addition of DNA. The mixture was incubated for additional 2 h at 37 °C and then passed through G-50 columns. (△) Cr binding; (●) cysteine binding.

(V/IV)−cysteine attack DNA leading to the formation of ternary DNA adducts. Total Cr binding to DNA was decreasing over the reduction and postreduction periods that can be attributed to a more complete complexation of the newly formed Cr(III) by cysteine diminishing the activity of Cr(III) to bind DNA.

DNA Cross-Linking of Histidine in the Presence of Cr(VI) and Ascorbate. Ascorbate is considered to be the major

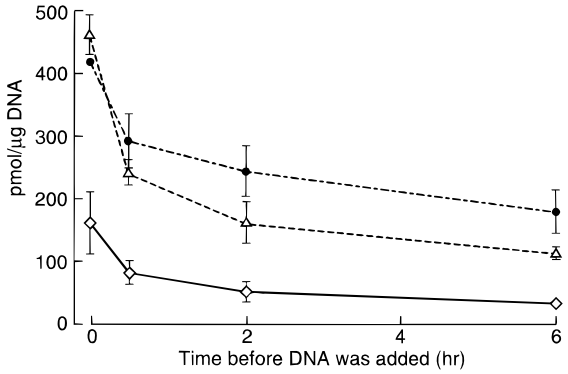

FIGURE 5: Chromium and histidine binding to DNA during reduction of Cr(VI) by ascorbate. 100 μM K_2CrO_4 was mixed with 300 μM ascorbate in 10 mM MES buffer (pH 6.1) and incubated at 37 °C. DNA was added immediately and at different time intervals after the reduction was initiated. Unreacted components were separated from DNA on G-50 columns. (●) Cr association with DNA during Cr(VI) reduction by ascorbate; (△) Cr association with DNA during Cr(VI) reduction by ascorbate in the presence of 1 mM histidine; (◇) Binding of histidine to DNA during Cr(VI) reduction by ascorbate.

Cr(VI) reductant in animal tissues that are targets of chromium genotoxicity (Standeven & Wetterhahn, 1991; Suzuki & Fukuda, 1990). In order to model a Cr(VI)-dependent DNA cross-linking of histidine, ascorbate was therefore chosen to reduce Cr(VI) and study DNA binding of Cr and histidine. The reduction of Cr(VI) by ascorbate gave rise to a substantial Cr binding to DNA, the level of which decreased when DNA was added at later time intervals following the initiation of the reduction (Figure 5). The inclusion of histidine in the reaction mixture led to a somewhat lower Cr binding to DNA that is most likely to be due to a kinetic inertness of some Cr(III) complexes with this amino acid (Hneihen et al., 1993). Parallel measurements of Cr(VI) reduction kinetics by 3-fold molar excess of ascorbate showed that the reduction was complete after 30 min, and the presence of histidine did not influence this reaction (not shown). The reduction of Cr(VI) by ascorbate has also led to the formation of histidine−DNA cross-links with levels of the histidine cross-links declining as DNA was added at later time points. One possibility explaining the observed decrease in the adduct yield when DNA was added to prereacted Cr(VI)−ascorbate mixtures could be due to chelation of Cr(III) by ascorbate. In order to test a possible sequestering effect of ascorbate, Cr(III) and ascorbate were preincubated for different time periods before DNA was added (Figure 6). As mentioned earlier, at this concentration range simultaneous addition of Cr(III) and DNA led to DNA precipitation, and therefore, this time point was omitted from the analysis. The results obtained with the preincubation of Cr(III) with ascorbate confirmed that complexation with ascorbate significantly decreases the ability of Cr(III) to react with DNA. Stearns et al. (1995) studied adduction of DNA in the process of Cr(VI) reduction by ascorbate, and they did not find any cross-linking of ascorbate to DNA. In conclusion, the active formation of binary and ternary Cr−DNA complexes after completion of the Cr(VI) reduction, as well as similarity in the kinetics of the adduct formation by Cr(III) and Cr(VI) in the presence of ascorbate, again indicates that it is trivalent form of chromium that is responsible for the formation of DNA reactive species. In addition, participation of Cr(V) in the histidine adduction to

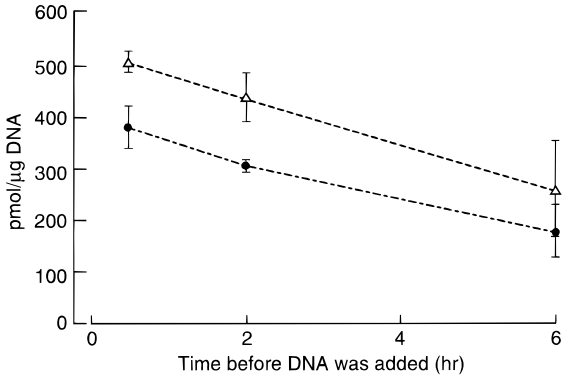

FIGURE 6: Cr(III) binding to DNA in the presence of ascorbate and histidine. 100 μM $CrCl_3 \cdot 6H_2O$ and 300 μM ascorbate were pre-incubated in 10 mM MES buffer (pH 6.1) for 0.5, 2, or 6 h and then reacted with DNA for additional 2 h. (●) Cr binding to DNA in the presence of ascorbate; (△) Cr binding in the presence of ascorbate and 1 mM histidine.

DNA seems unlikely because at the 3:1 ratio of ascorbate to Cr(VI) used in our experiments the presence of Cr(V) is not detectable (Goodgame & Joy, 1987; Stearns et al., 1995).

Chromium(III)-Histidine Reacts with 2′-Deoxynucleotide 5′-Monophosphates but not with 2′-Deoxynucleosides. In order to identify chemical groups on DNA involved in the formation of chromate-induced DNA−amino acid complexes, products of the reaction of Cr(III) with individual amino acids in the presence of 2′-deoxynucleosides and 2′-deoxynucleotide monophosphates were characterized. Cr(III) and histidine were chosen for these studies; because unlike Cr(VI)/reductant/amino acid mixtures, this system was expected to give less complex HPLC profiles due to the absence of oxidative reactions. Cr(III), histidine, and mononucleotides reacted simultaneously resulted in the appearance of an insoluble material shortly after mixing of the ingredients. The precipitate consisted of Cr(III) and mononucleotides with all the unreacted histidine in the supernatant. In all subsequent experiments, $CrCl_3 \cdot 6H_2O$ and a 5-fold molar excess of histidine were allowed to form a binary complex for several hours prior to the addition of nucleotides. No insoluble material was observed utilizing this approach allowing separation of all products by reversed-phase HPLC. The formation of products was monitored by UV absorbance and radioactivity (^{51}Cr and [^{3}H]histidine). Figure 7 shows a representative HPLC profile of the reaction of dGMP with Cr(III)−histidine. Several UV-absorbing peaks, in addition to dGMP, were detected between 2 and 10 min (Figure 7A). The presence of ^{51}Cr and ^{3}[H]histidine radioactivities in these peaks (Figure 7B,C) suggested that they were formed by a direct involvement of Cr(III)−histidine. The first peak (<5 min elution) contained a very high amount of ^{51}Cr and [^{3}H]-histidine radioactivity but relatively low UV absorbance. The individual HPLC fractions were collected and treated with 0.2 N NaOH in order to dissociate the complexes, and the presence of histidine and dGMP was estimated by HPLC. This analysis revealed that peak 1 consisted only of chromium and histidine, while the remaining peaks contained ternary complexes of histidine, Cr(III), and dGMP. Subsequent studies of the reaction products between dTMP or dAMP and Cr(III)−histidine resulted in HPLC profiles similar to those observed for dGMP. UV spectra of five major individual HPLC fractions containing histidine−Cr(III)−dNMP complexes (where dNMP is dGMP, dAMP, or dTMP) were not different from those of corresponding unmodified mononucleotides. Cr(III)−histidine also formed complexes with dCMP, but due to a short retention time for dCMP in the HPLC column used, the product peaks were not easily resolved (data not shown).

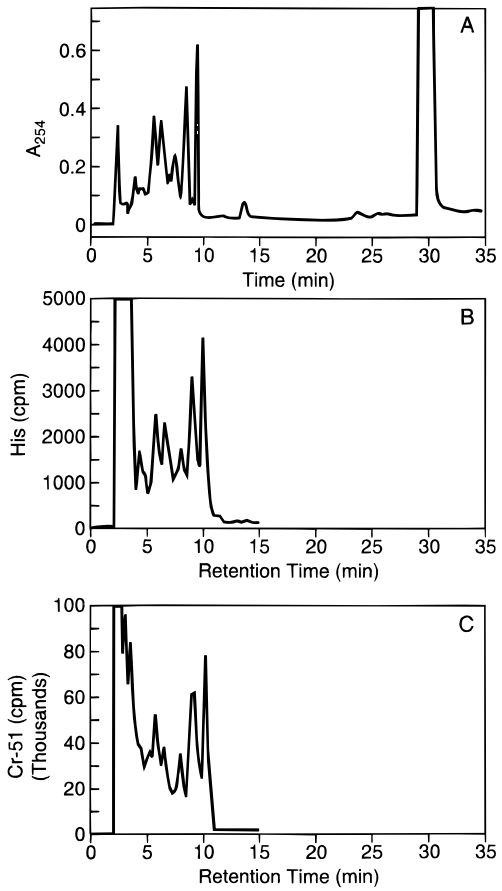

FIGURE 7: Reaction of Cr(III)-histidine with 2′-deoxyguanosine-5′-monophosphate. A mixture of 10 mM histidine and 2 mM $CrCl_3 \cdot 6H_2O$ in 25 mM MES buffer, pH 6.1, were preincubated for 5 h at 50 °C followed by addition of 2 mM dGMP and an additional incubation of the resultant solution for 16−18 h at the same temperature. The components of the reaction mixture were resolved on reverse-phase Adsorbosphere nucleotide-nucleoside column (Waters). The products were analyzed for UV absorbance (A) and [^{3}H]histidine (B) or ^{51}Cr radioactivity (C).

The reaction of dG with Cr(III)−histidine yielded only one additional UV-absorbing peak (Figure 8A) which did not contain any [^{3}H]histidine associated radioactivity (Figure 8B). The peak eluting at 2.5 min represents Cr(III)−histidine (Figure 8A). HPLC analysis of a reaction between Cr(III)−histidine and dA, dT, or dC yielded no histidine-containing adducts (data not shown). There was no reaction between deoxynucleosides and Cr(III)−histidine complexes under other conditions tested. The lack of any ternary adduct yield when nucleosides were used in place of mononucleotides strongly suggests that the phosphate group of mononucleotides acts as a primary reactive site in the ternary complex formation. High reactivity of Cr(III)−histidine to all four mononucleotides as well as the lack of changes in UV spectra of the ternary adducts formed also indicates that there is weak if any direct involvement of base groups in the adduct formation.

Reaction of Cr(III)−Cysteine with Oligonucleotides of Base-Specific Composition. Preferential coordination of chromium to guanine was previously suggested based on

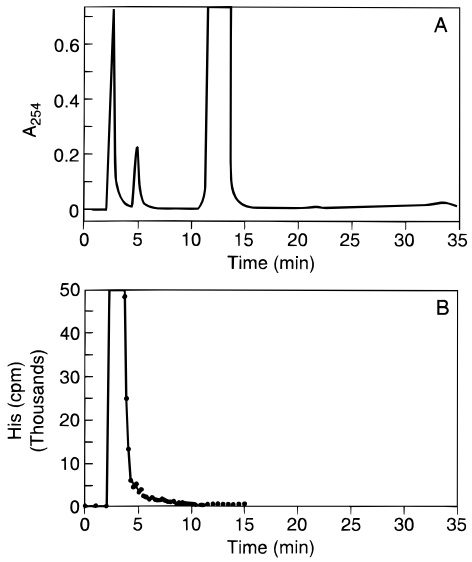

FIGURE 8: Analysis of the reaction between Cr(III)−histidine and 2′-deoxyguanosine. The formation of Cr(III)−His, reaction with dG and HPLC separation were performed as described in the legend of Figure 7. (A) UV absorbance; (B) [^{3}H]histidine radioactivity.

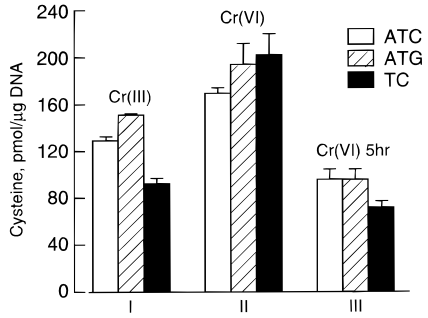

FIGURE 9: Binding of cysteine to oligonucleotides of base-specific composition. All oligos contained 39 nucleotides placed in random order. In all experiments concentration of chromium was 50 μM and cysteine was 500 μM. Oligos and Cr compounds were incubated for 2 h at 37 °C. DNA binding was corrected for different recoveries of the oligos from G-25 columns. (I) $CrCl_3 \cdot 6H_2O$ was preincubated with cysteine for 5 h at 50 °C before oligos were added. (II) K_2CrO_4, cysteine and DNA were mixed simultaneously. (III) K_2CrO_4 and cysteine were preincubated for 5 h at 37 °C before oligos were added.

some *in vitro* experiments and a known activity of N-7 nitrogen in metal binding (Spiro, 1980). Although our results with mononucleotides and nucleosides did not find any support for the base specificity in the ternary adduct formation, however, some preferential reactivity of purine-containing nucleotides might have been undetected. Another approach to study potential importance of purine bases in the reaction with chromium−amino acid complexes was to compare a number of the ternary adducts in oligonucleotides of defined composition. Three types of oligonucleotides lacking either A or G or both purines were reacted with Cr−cysteine, and the amount of oligonucleotide-bound amino acid was determined (Figure 9). Simultaneous reaction of Cr(VI), cysteine, and oligonucleotides resulted in almost equal cross-linking of the amino acid to all three substrates. Incubation of prereacted Cr(III)−cysteine or Cr(VI)−cysteine mixtures with the oligonucleotides gave rise to substantial adduct formation with all oligonucleotides tested. The level of cross-linked cysteine was only slightly lower in the TC-containing oligonucleotide as compared to the purine-containing substrates. Thus, there was no base preference in the formation of the ternary adducts using DNA-like oligonucleotide substrates and cysteine.

DISCUSSION

The continued formation of the ternary cysteine/histidine−Cr(III)−DNA cross-links by Cr(VI)-reducing mixtures long after Cr(VI) reduction to Cr(III) is complete strongly suggests that the final product of Cr(VI) metabolism, Cr(III) was responsible for the amino acid-DNA adducts formed. Cr(III) is involved in the production of DNA-attacking species and acts as a bridge linking amino acids to DNA. No evidence was found for the involvement of intermediate Cr forms in the adduct production with two biologically important reductants: ascorbate and cysteine. Previously, Cr(V) has been suggested to be the DNA-reactive form leading to Cr−DNA adduct formation. Recently, however, Cr(V) species generated in the presence of NADH were directly shown to be unreactive towards DNA, DNA bases, or mononucleotides (Molyneux & Davies, 1995). Summarizing our finding from this work, cross-linking of amino acids to DNA by carcinogenic Cr(VI) can be schematically described as following:

$$\text{Cr(VI)} + \text{reducer} \rightarrow \text{Cr(III)} + \text{oxidized reducer}$$

$$\text{Cr(III)} + \text{amino acid} \rightarrow \text{amino acid−Cr(III)} \quad \text{(binary complex)}$$

$$\text{amino acid−Cr(III)} + \text{DNA} \rightarrow \text{amino acid−Cr(III)−DNA (ternary complex)}$$

The proposed scheme implies that in case of cysteine the reduced form is involved in the adduct formation. Kwong and Pennington (1984) have reported that the reduction of Cr(VI) by L-cysteine led to the formation of Cr(III) complexes with the reduced form of this amino acid. It should also be noted that the isolated Cr(III) complexes were structurally similar to those formed by the reaction of Cr(III) with cysteine (De Meester et al., 1977). In both studies all three binding sites of cysteine were found to be coordinated to Cr(III).

Although no data were presented, binary amino acid−Cr(III) complex reacting with DNA is most likely to contain only one amino acid ligand. Hneihen et al. (1993) reported that Cr(III) coordination complexes containing two molecules of tridentate amino acid ligands did not react with DNA. In general, interactions with DNA should occur before all of Cr monodentate ligands initially present have been replaced by reactive sites of multidentate ligand. Cr(III) complexes with multidentate ligands are very stable and do not undergo exchange reactions under a variety of experimental conditions (Larkworty et al., 1988).

The lack of interaction between Cr(III)−histidine complexes and nucleosides, as well as the relative rapid reaction of Cr−histidine with mononucleotides, suggested that the phosphate groups were the primary binding sites on DNA for Cr-amino acid complexes. Efficient binding of cysteine by Cr(VI) and Cr(III) to oligonucleotides of base-specific composition further supported the conclusion that phosphate is a principal point of attachment. Previous studies have shown that aquo forms of Cr(III) bound to the phosphate group of mononucleotides (Terron & Moreno, 1983; Fiol et al., 1989). Moreover, Krishnamoorthy and Harris (1980)

reported that cytosine did not exhibit any readily detectable interactions with Cr(III). It was interesting to note that Campomar et al. (1986) observed the formation of reaction products between $Cr(urea)_6Cl_3 \cdot 3H_2O$ and nucleotides but not with the bases or nucleosides. In all cases coordination occurred between Cr(III) and the phosphate group. $Cr(urea)_6Cl_3 \cdot 3H_2O$ was considered as a model of Cr(III)−amino acid complexes. General considerations regarding Cr(III) coordination with oxygen-containing ligands also indicate that oxoanion groups are always preferred binding sites (Larkworthy et al., 1988; Nieboer & Jusys, 1988). Amino acid−Cr(III)−nucleotide complexes can offer several possibilities for interligand hydrogen binding particularly when water ligands are present in the metal coordination sphere. The hydrogen binding in complexes of transition metals with nucleotides frequently involves DNA base groups (Spiro, 1980), and it was also suggested to occur in Cr(III)−dAMP complexes (Terron & Moreno, 1983). The hydrogen interactions with base groups can additionally stabilize ternary Cr complexes, and, conceivably, it may even lead to some DNA sequence specificity in the adduct formation achieved by conformational effects. Differential Cr adduct formation on polynucleotides made of the same bases but placed in different order seems to support the importance of the conformational factor (Borges & Wetterhahn, 1989). Bulkiness of hexacoordinate Cr(III) complexes may impose certain steric requirements for binding on DNA and explain the inability of Cr(III)−histidine to react with nucleosides despite the presence of potential reactive sites.

The mutagenic potential of the Cr(III)−amino acid adducts is not yet known. Formation of methylphosphotriesters was not found to have mutagenic consequences (Horsfall et al., 1990). The fact that the DNA−Cr(III)−amino acid adducts are formed by the linkage to the phosphate group does not necessarily mean that these adducts are also not mutagenic. The ternary Cr(III) adducts are significantly larger than methylphosphotriesters, and, therefore, their presence may lead to more significant distortions in the phosphodiester bond in DNA. In fact, the conformation of the phosphodiester bond in DNA has recently been found to play a crucial role in the coding properties of certain promutagenic lesions (Tan et al., 1994). In addition, the amino acid−Cr(III) complexes may involve additional coordination to base groups that may have significant consequences with respect to base pairing.

Most of the current research in Cr(VI) toxicology has been focused on characterization of intermediate Cr forms and radical byproducts while intracellular reactions of the final Cr(III) form were relatively unexplored. Formation of Cr(III) was sometimes even considered as a detoxification event since Cr(III) was presumed to be unreactive. Although Cr(III) does not seem to readily participate in any redox reactions inside the cell, it can readily form coordination complexes with a number of intracellular molecules. Many Cr(III) complexes with multidentate intracellular ligands are likely to be unreactive while as shown in this work some reactive Cr(III) complexes can be quite persistent giving them a sufficient time in cells to reach nuclei and bind to DNA. Bridgewater et al. (1995) have found that the pattern of DNA polymerase arrests was identical on DNA templates treated with either Cr(III) or Cr(VI)/ascorbate. This suggested again that Cr(III) is likely to be the DNA-reactive form. Interestingly, water-insoluble Cr(III) compounds that apparently can be delivered into the cells by phagocytosis were positive in mutagenicity and cell transformation assays (Biedermann & Landolph, 1990; Ellis et al., 1986). The presence of Cr−DNA adducts, however, has not yet been studied in cells exposed to insoluble Cr(III).

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