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Effects of Neutral, Cationic, and Anionic Chromium Ascorbate Complexes on Isolated Human Mitochondrial and Genomic DNA

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The relative activities of neutral, cationic, and anionic chromium ascorbate complexes toward isolated human mitochondrial and genomic DNA were investigated at physiologically relevant conditions by agarose gel electrophoresis. A direct relationship between the charge of the Cr(III) species and their DNA-damaging properties was found. The cationic species were found to be fully capable of DNA-cleavage, even in short incubation periods. Incubations were also performed in the presence of amino acids. No apparent effect was observed under the applied experimental conditions to facilitate or prevent damage through the ternary amino acid-Cr-DNA adduct formation or binary chromium-amino acid complex formation.

Keywords: Ascorbate, Chromium, DNA-cleavage

Introduction

The studies that were performed on chromium genotoxicity were mainly focused on hexavalent Cr. At normal physiological pH values, the predominant form of hexavalent chromium is the CrO_4^{2-} anion; its reduction potential (E = 0.52V) is sufficiently positive for the oxidation of a range of biological molecules. While chromate alone does not react with DNA *in vitro*, the intracellular reduction by cell constituents leads to the formation of hypervalent chromium species, which cause DNA lesions. These lesions include Cr-DNA adducts, DNA-DNA cross-links, DNA-protein cross-links, strand breaks, alkali-labile sites, and oxidative damage (Cieslak-Golonka, 1995; Wetterhahn and Dudek, 1996; Codd *et al*., 2001). The interaction of Cr(VI) with a series of low molecular weight cellular reductants has been examined by several workers (Connett and Wetterhahn, 1985; Goodgame and Joy, 1987; Adach *et al*., 1995). With some amino acids, such as serine, methionine, and carbohydrates, this interaction is negligible when compared with the more powerful reductants (e.g. glutathione, ascorbate, vitamin E, or catechols). Ascorbate was reported to be the major reductant of Cr(VI) in rat liver and kidney ultrafiltrates and may be the major non-enzymatic reductant *in vivo* (Standeven and Wetterhahn, 1992). Reduction of Cr(VI) to Cr(III) is a three-electron process, whereas ascorbate acts as a two-electron donor, so the reaction with Cr(VI) will necessarily pass through Cr(V/IV) intermediates. Long-lived Cr(V) and Cr(IV), carbon-based radicals and final Cr(III) that are formed during chromate reduction by ascorbate, are considered the actual genotoxic species (Stearns and Wetterhahn, 1994; Sugiyama *et al*., 1991; Luo *et al*., 1996; Tsou *et al*., 1999; Zhitkovich *et al*., 2001). Trivalent chromium is a stable end product of the Cr(VI) reduction. There are conflicting reports on the toxicity of Cr(III). Even though the compounds of Cr(III) are weakly carcinogenic (IARC, 1990), DNA-Cr(III)-protein and DNA-Cr(III)-DNA cross-links have been well documented in both *in vitro* and *in vivo* systems (Fornace *et al*., 1981; Beyersmann and Köster, 1987; Kortenkamp *et al*., 1992; Stearns *et al*., 1995; Zhitkovich *et al*., 1996; Singh and Snow, 1998), or in combination with agents that cause the generation of reactive oxygen species (Qi *et al*., 2000). Recently, a comparison of the *in vitro* genotoxicity of tri- and hexavalent chromium in the absence and presence of vitamin C was reported (Blasiak and Kowalik, 2001), indicating that both ions are able to damage DNA but through different mechanisms.

While there are many studies on chromium genotoxicity *via* chromate reduction by cellular components, the interaction of isolated products with DNA has not been extensively explored so. Model complexes of Cr(V) and Cr(IV) possess DNA

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cleavage properties under a wide range of reaction conditions (Bose *et al*., 1998; Levina *et al*., 1999). Rao *et al.* studied the biological activities of Cr(III) compounds with biogenic ligands, such as saccharides (Rao *et al*., 1994; Kaiwar *et al*., 1995; Sreedhara *et al*., 1997) and SH-containing molecules (Kaiwar *et al*., 1996). The *in vitro* interactions of these products with plasmid DNA have indicated alterations in the DNA structure. To the best of our knowledge, only one chromium ascorbate complex, $K_3[Cr_2(\mu\text{-}OH)_2(L\text{-}asc)_4] \cdot H_2O$, where "asc" is the ascorbate monoanion ($C_6H_7O_6^-$), has as yet been tested for its DNA reactivity and found to cause substantial nicking in single-stranded DNA (Sreedhara *et al*., 1997; Susa *et al*., 1997). The present *in vitro* study's aim is to investigate the effects of the nature of the synthetically-prepared chromium ascorbate complexes and related aqua species on human mitochondrial and genomic DNA. Because of its small-sized and well-documented primary structure and nucleotide sequences, the 1.2 kb PCR product of the control region of human mitochondrial DNA and also human bulk DNA were chosen to show the relative activities of the neutral, cationic, and anionic chromium ascorbate complexes at physiologically relevant conditions in a comparative manner using agarose gel electrophoresis.

Materials and Methods

Chemicals K_2CrO_4 (Carlo Erba) was used as the Cr(VI) source. $Cr_2(SO_4)_3 \cdot 15H_2O$ was used as the Cr(III) source. The solutions were prepared in doubly distilled, deionized water. The neutral complex, $[Cr_2(\mu\text{-}OH)_2(H_2O)(asc)_3(OH)] \cdot 4H_2O$ (**1**), was synthesized according to the following procedure: Solid $Ba(OH)_2 \cdot 8H_2O$ (20 mmol) was added to 80 mL aqueous solution of L-ascorbic acid (44 mmol). Into this solution was then added 80 mL aqueous solution of $Cr_2(SO_4)_3 \cdot 15H_2O$ (20 mmol). The precipitated $BaSO_4$ was removed and the green solution was concentrated until dryness in a vacuum system, after aging for about three months. Found (**1**): C, 27.78; H, 4.67; Cr, 13.47. Calculated for $Cr_2(H_2O)(C_6H_7O_6)_3(OH)_3 \cdot 4H_2O$ (MW 770 g mol^{-1}): C, 28.05; H, 4.41; Cr, 13.51 (Zümreoglu-Karan, to be published). The cationic complex, $[Cr_2(\mu\text{-}OH)(\mu\text{-}SO_4)(H_2O)_2(asc)]SO_4 \cdot 2H_2O$ (**2**) was synthesized similarly as described elsewhere (Zümreoglu-Karan *et al*., 2002a). Complexes of anionic type: $K[Cr(H_2O)_2(asc)_2(OH)_2] \cdot 5H_2O$ (**3**) and $K_2[Cr(H_2O)(asc)_2(OH)_2] \cdot 2H_2O$ (**4**) were synthesized *via* chromate reduction in THF (Zümreoglu-Karan *et al*., 2002b).

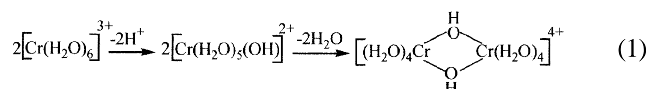
DNA interaction studies The reactivity of complexes **1**-**4** with DNA samples was studied by agarose-gel electrophoresis. A 1.2 kb fragment of 16 kb of human mitochondrial DNA (mt-DNA) was amplified by a polymerase chain reaction (PCR) using a proper primer set (Mergen *et al*., 2000). Genomic DNA (gen-DNA) was isolated from white blood cells according to the method of Poncz *et. al.* (Poncz *et al*., 1982). The DNA samples were treated individually with aqueous solutions of $Cr_2(SO_4)_3$ and K_2CrO_4, **1**, **2**, **3** and **4** alone or in the presence of amino acids : glycine (Merck) and histidine (Merck). The reactions were carried out in 10 × TE buffer (10 mM tris and 5 mM EDTA, pH = 8.0) employing biologically realistic concentrations (Capellmann and Bolt, 1992; Horning, 1975) at 37°C. Each reaction mixture contained 1 μL of the buffer, 3 μL of the DNA sample (PCR product of mt-DNA or gen-DNA of 0.2 μg mL^{-1}), 6 μL of aqueous chromium species for which the final concentrations varied between 0.16-12.3 mM in a total volume of 10 μL. The experiments were also performed by adding 1 μL of the amino acid solution to a typical reaction mixture for which the final concentrations were in the range of 0.24-3.0 mM. The mixtures were incubated for 10-30 min, then electrophoresed in 1% agarose at 50 V for 90-180 min. The gels were stained in ethidium bromide (5 μg mL^{-1}) for 10 min and photographed under ultraviolet transillumination.

Results and Discussion

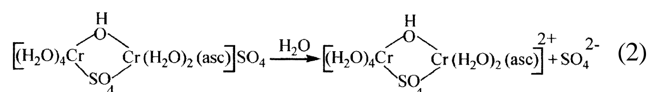
The interaction of synthetic chromium ascorbate complexes with DNA varied with the charge of the complex, ligand lability, and interaction conditions.

Effect of the nature of the chromium species on human mitochondrial and genomic DNA Although most of the Cr(III) complexes were reportedly non-mutagenic, there are studies demonstrating that Cr(III) can directly bind to DNA (Cohen *et al*., 1993; Levina *et al*., 1999). During the chromate reduction, a number of cations and DNA-derived radicals were formed where Cr(III) was suggested to be the ultimate carcinogenic form of Cr. Cr(III) salts, such as $CrCl_3$ or $Cr(NO_3)_3$, bind to DNA *via* strong interactions of the positively-charged $[Cr(H_2O)_6]^{3+}$ and $[Cr(H_2O)_4Cl_2]^+$ aqua complexes in neutral media (Beyersmann and Köster, 1987).

At neutral pH, the Cr(III) ions that were supplied from $Cr_2(SO_4)_3 \cdot 15H_2O$, which were used as the Cr(III) source in this study, exist as $[Cr(H_2O)_6]^{3+}$ aqua ions. Aqua ligands deprotonate to form hydroxo bridges, and then polymerize to cationic oligomers through a reaction called "olation" (1).



Aqueous solution of the cationic complex (**2**) also provides cationic Cr(III) species (2):



In the absence of any coordinating groups, these aquated cations are stable (λ_{max} of $Cr(H_2O)_6^{3+}$ is 555 nm and that of **2** is 575 nm). However, the H_2O and SO_4^{2-} ligands are very good leaving groups. In the presence of DNA, they can be readily displaced by the potential binding sites of the DNA polymer. The kinetically labile, positively-charged complexes may attack the proteins or negatively-charged phosphate backbone of DNA (Kortenkamp *et al*., 1992; Sugden and Wetterhahn, 1996). Evidence comes from the detailed mechanistic and spectroscopic studies that were performed recently. During the

oxidation of a synthetic oligonucleotide with an anionic oxochromium (V) model compound, Bose and co-workers characterized a Cr(III)-orthophosphate complex by EPR and ^{31}P NMR studies (Bose *et al*., 1998). On the other hand, a luminescence spectroscopy-capillary electrophoresis study demonstrated that the stable cationic Cr(III) complexes, e.g. $Cr(diimine)_3^{3+}$, interact electrostatically with the negatively-charged phosphate groups on DNA (Wattson *et al*., 1999). UV-visible and FTIR spectroscopic results showed that Cr(III) in an aqueous solution but not aqueous Cr(VI), chelates calf thymus DNA *via* guanine-N-7 and the nearest PO_2 group (Arakawa *et al*., 2000). A recent study that compared the genotoxicity of tri- and hexa-valent chromium has pronounced the ultimate damaging effect of Cr(III) (Blasiak and Kowalik, 2001). The lymphocytes that were treated with Cr(III) showed no measurable DNA repair, even in the presence of vitamin C or catalase, the anti-oxidant reagents that prevent the extent of DNA migration. Although our results with cationic Cr(III) species are in accord with the literature outlined previously, $[Cr(L\text{-}his)_2]NO_3 \cdot H_2O$ reportedly displays lower binding (Hneihen *et al*., 1993), while a negatively charged Cr(III)-saccharide complex cleaved the PSV neoplasmid (Rao *et al*., 1994). Obviously, further research is needed to clarify the mechanism of DNA damage. Also, factors other than electrostatic interactions should be considered.

Figures 1 and 2 show that the remaining neutral (**1**) and anionic (**3**,**4**) complexes were inert towards DNA. We observed no interaction of Cr(VI) (as $K_2CrO_{4(aq)}$) with mitochondrial DNA and genomic DNA (data not shown), which supports the previous reports that Cr(VI) does not react with DNA unless organic reducing agents are present (Kortenkamp *et al*., 1991). The DNA damaging effect of Cr(VI) is dose-dependent, and no interaction with DNA has been observed at low concentrations (Arakawa *et al*., 2000).

The active chromium species, $[Cr(H_2O)_6]^{3+}$ and **2** destroyed DNA, even in shorter incubation periods. Over the course of a 10 min incubation time, the positively-charged chromium species were fully capable of DNA cleavage.

Effect of concentration Incubations were first performed with high concentrations of the chromium species (2400 ppm each). From these high dose phenomena (Figs. 1 and 2), it was deduced that Cr(VI), **1**, **3**, and **4** generated no observable damage above the biologically realistic levels. The following experiments were therefore continued with the active species ($Cr^{3+}_{(aq)}$ and **2**) toward physiological concentrations, considering the values that were applied in previous studies ranging from 0.1 mM to 3.0 mM (Luo *et al*., 1996; Tsou *et al*.,1999; Capellmann and Bolt, 1992; Hneihen *et al*., 1993; Standeven and Wetterhahn, 1991; Stearns and Wetterhahn, 1997). Ascorbate is also present in millimolar concentrations in cellular systems (Horning, 1975). Cr(III) destroyed DNA at each applied concentration (Fig. 3). On the other hand, the increase in the concentration of **2** progressively attenuated the DNA damage; complete destruction was observed after 1.2 mM (Fig. 4).

Effect of the presence of amino acids It has been suggested that if chromium(III) complexes with cellular peptides, amino acids, and proteins, then its reactivity toward DNA will be greatly diminished (Hneihen *et al*., 1993). On the other hand, Beyersman and Köster (Beyersmann and Köster, 1987) showed that $[Cr^{III}gly_3]$ caused a significant level

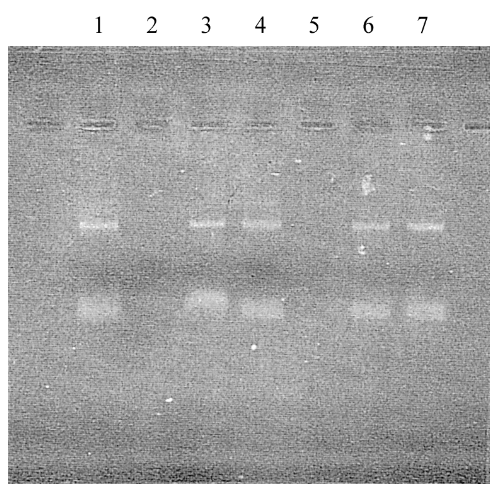


Fig. 1. Agarose gel showing the effects of chromium species on mt-DNA after a 30 min incubation at 37°C. Reaction solutions containing 3 μL of mt-DNA (1.2 kb PCR product), and 2400 ppm of chromium species buffered with $10 \times$ TE, pH = 8.0. Lane numbers: (1) DNA alone, (2) Cr(III), (3) Cr(VI), (4) **1**, (5) **2**, (6) **3**, (7) **4**.

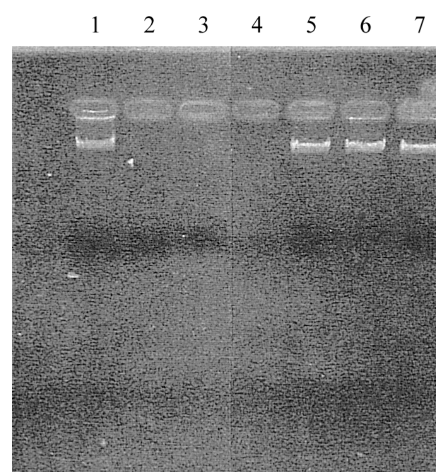


Fig. 2. Agarose gel showing the effect of chromium species on gen-DNA after a 30 min incubation at 37°C. Reaction solutions containing 3 μL of gen-DNA (0.2 μg mL^{-1}), buffered with $10 \times$ TE, pH = 8.0. Lane numbers: (1) DNA alone, (2) 3.6 mM Cr(III), (3) 7.6 mM Cr(III), (4) 3.8 mM **2**, (5) 3.1 mM **1**, (6) 4.3 mM **3**, (7) 3.5 mM **4**.

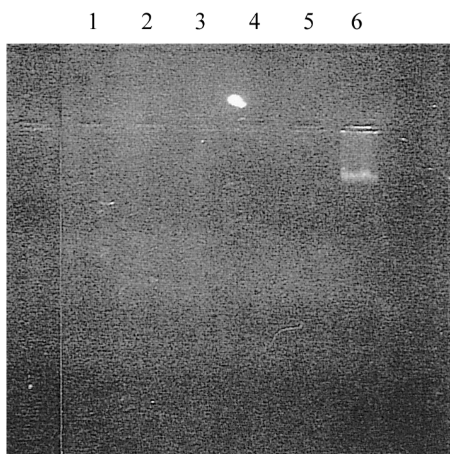


Fig. 3. Agarose gel showing the concentration effect of Cr(III) on gen-DNA after a 10 min incubation at 37°C. Reaction solutions containing 3 μL of gen-DNA (0.2 μg mL^{-1}), buffered with 10 × TE, pH = 8.0. Lane numbers: (1) 0.6 mM Cr(III), (2) 1.2 mM Cr(III), (3) 1.8 mM Cr(III), (4) 2.4 mM Cr(III), (5) 3.0 mM Cr(III), (6) gen-DNA alone.

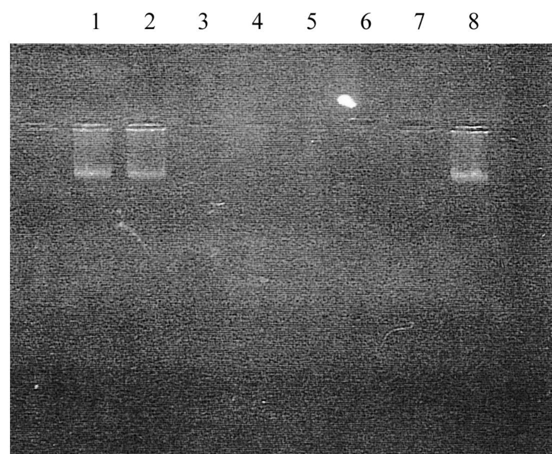


Fig. 4. Agarose gel showing the concentration effect of cationic complex (**2**) on gen-DNA after a 10 min incubation at 37°C. Reaction solutions containing 3 μL of gen-DNA (0.2 μg mL^{-1}), buffered with 10 × TE, pH = 8.0. Lane numbers: (1) 0.16 mM **2**, (2) 0.32 mM **2**, (3) 0.48 mM **2**, (4) 0.64 mM **2**, (5) 0.77 mM **2**, (6) 0.96 mM **2**, (7) 1.28 mM **2**, (8) gen-DNA alone.

of DNA-protein cross-links in isolated salmon sperm nuclei. Although arguments about the role of amino acids on DNA damage continue; in particular for the Cr-DNA-adduct formation, *in vitro* experiments have provided evidence for the requirement of the formation of a Cr(III)-amino acid binary complex before reacting with DNA (Zhitkovich *et al*., 1996). In order to determine the effect of amino acids in preventing or facilitating the DNA damage that is induced by chromium, incubations were performed with Cr(III) and (**2**) in the presence of glycine and histidine. Our results indicated that the amino acids that were applied failed to prevent destructive damage that is caused by the positively-charged species. Since most of the amino acids exist in the isoelectric dipolar form ($^{+}NH_3CHRCOO^{-}$) near pH~7.0, it is not surprising that they possess low complexing ability with the positively-charged chromium species (Lehninger, 1982).

Conclusions

The present study, though not aimed at a mechanistical approach, offers some key features towards understanding the role of the nature of the chromium species on chromium-induced DNA damage at the molecular level. We found a direct relationship between the charge of the Cr(III) species and their reactivity with DNA. The positively-charged complexes displayed ultimate DNA-breaking properties, while the neutral and negatively-charged complexes were almost inert. The results agree with the mechanism that positively-charged species interact electrostatically with the negatively-charged phosphate groups on DNA polymer, and therefore initiate DNA cleavage. However, many factors affect the actual cleavage mechanism and species that produce damage; the extent and type of damage will be dependent on the chosen experimental conditions. Further research is required to display the relative importance of these mechanistic pathways *in vivo*.

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