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Addition of DNA to Cr^{VI} and Cytochrome *b*₅ Containing Proteoliposomes Leads to Generation of DNA Strand Breaks and Cr^{III} Complexes

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Abstract

Chromium (Cr) is a cytotoxic metal that can be associated with a variety of types of DNA damage, including Cr-DNA adducts and strand breaks. Prior studies with purified human cytochrome *b*₅ and NADPH :P450 reductase in reconstituted proteoliposomes (PLs) demonstrated rapid reduction of Cr^{VI} (hexavalent chromium, as CrO₄²⁻), and the generation of Cr^V, superoxide (O₂⁻), and hydroxyl radical (HO[•]). Studies reported here examined the potential for the species produced by this system to interact with DNA. Strand breaks of purified plasmid DNA increased over time aerobically, but were not observed in the absence of O₂. Cr^V is formed under both conditions, so the breaks are not mediated directly by Cr^V. The aerobic strand breaks were significantly prevented by catalase and EtOH, but not by the metal chelator diethylenetriaminepentaacetic acid (DTPA), suggesting that they are largely due to HO[•] from Cr-mediated redox cycling. EPR was used to assess the formation of Cr-DNA complexes. Following a 10-min incubation of PLs, CrO₄²⁻, and plasmid DNA, intense EPR signals at *g* = 5.7 and *g* = 5.0 were observed. These signals are attributed to specific Cr^{III} complexes with large zero field splitting (ZFS). Without DNA, the signals in the *g* = 5 region were weak. The large ZFS signals were not seen, when Cr^{III}Cl₃ was incubated with DNA, suggesting that the Cr^{III}-DNA interactions are different when generated by the PLs. After 24 h, a broad signal at *g* = 2 is attributed to Cr^{III} complexes with a small ZFS. This *g* = 2 signal was observed without DNA, but it was different from that seen with plasmid. It is concluded that EPR can detect specific Cr^{III} complexes that depend on the presence of plasmid DNA and the manner in which the Cr^{III} is formed.

Introduction

While small amounts of chromium (Cr) are naturally present in many environments, the major human exposures are from industrial uses and release. Greater than 10⁵ tons of Cr are released annually into the environment, and Cr is a significant contaminant at hundreds of sites (*Superfund* and other) across the USA [1–3].

Cr^{VI} and Cr^{III} are the predominant stable oxidation states. Cr^{III} Species are generally insoluble and do not easily cross cell membranes [4]. Several Cr^{VI} compounds, *e.g.*, chromates ($\text{Cr}^{\text{VI}}\text{O}_4^{2-}$), are soluble and, given their similarity to sulfate, can be rapidly transported into cells *via* an anion carrier [5]. While some industrially used chromates are water-insoluble, they are still implicated in toxicity. Cr^{VI} Exposure is often considered a predisposing factor to Cr-induced toxicity [6–10].

Once internalized, cells rapidly reduce Cr^{VI} by a variety of possible reductants including ascorbate, cysteine, glutathione, glutathione reductase, microsomal enzymes, and others [11–18]. These reductants mediate either one- or two-electron transfers in biological systems, so the reduction of Cr^{VI} to Cr^{III} results in the generation of the intermediates Cr^{V} and/or Cr^{IV} . These intermediates have high reactivity and likely contribute to toxicity resulting from Cr^{VI} exposure [9] [19–23]. Different intracellular Cr^{VI} reductants could result in the generation of distinct types of reactive species. In human systems, NADPH-dependent lung and liver microsomal enzymes generate Cr^{V} , Cr^{IV} , and Cr^{III} from $\text{Cr}^{\text{VI}}\text{O}_4^{2-}$ [24]. In particular, human cytochrome *b*₅ plus P450 reductase have prominent Cr^{VI} -reducing activity [18] [25]. The nature of the resulting Cr^{V} and Cr^{III} species are not known with certainty, but various Cr-carbohydrate, Cr-NAD(P)H, and other species might result from ligand exchange reactions [26].

In vitro, the reduction of Cr^{VI} by at least some reductants can lead to various types of DNA damage including DNA strand breaks [27–29]. Similarly, exposure of animal and human cells to Cr^{VI} results in DNA strand breaks [30–33]. While Cr^{VI} does not significantly react with or bind DNA [34], both Cr^{V} and Cr^{III} have known effects on DNA. For example, a Cr^{V} complex can directly mediate strand breaks [35].

The reduction of Cr^{VI} by ascorbate yields Cr^{III} -DNA adducts, a portion of which can cause polymerase arrest [36]. Cr^{III} can also bind DNA [34], and at least a subset of the resulting Cr^{III} -DNA adducts can interfere with DNA replication [37] [38].

Furthermore, Cr^{V} , Cr^{IV} , and Cr^{III} are all capable of reacting with H_2O_2 to generate $\text{HO}^\cdot$ *via* Cr-mediated *Fenton*-like reactions. Of these, Cr^{III} is the slowest, whereas Cr^{V} and Cr^{IV} are highly active [14] [39–43]. In accord, oxidation of Cr^{III} picolinate by H_2O_2 can result in the regeneration of higher oxidation states such as Cr^{VI} [44]. In the presence of low micromolar concentrations of Cr^{VI} , the human microsomal enzymes cytochrome *b*₅ plus P450 reductase generate superoxide ($\text{O}_2^{\cdot -}$) and a stoichiometric excess of $\text{HO}^\cdot$ *via* the redox cycling of Cr^{V} [18]. This system generates its own H_2O_2 to support this process. These microsomal enzymes are located in the endoplasmic reticulum and nuclear membrane so the resulting reactive oxygen species could damage the nuclear membrane and potentially even the DNA.

The goal of these studies was to better understand some of the consequences of Cr^{VI} reduction by the human microsomal enzymes cytochrome *b*₅ plus P450 reductase. One goal was to determine if this reduction was capable of causing DNA strand breaks *in vitro*, and if these strand breaks were due to Cr^{V} or to reactive oxygen species. Another goal was to determine if specific Cr^{V} and/or Cr^{III} EPR signals could be detected that would be indicative of Cr–DNA interactions or complexes.

Results and Discussion

DNA Strand Breaks

Various chemical reductants of Cr^{VI} can generate species that result in DNA damage including strand breaks. These strand breaks could be caused by the reactive Cr intermediates Cr^{V} and/or Cr^{IV} , or by $\text{HO}^\cdot$ if H_2O_2 is included [28] [35] [43] [45] [46].

Previous studies with proteoliposomes (PLs) demonstrated that they reduce $\text{Cr}^{\text{VI}}\text{O}_4^{2-}$ in an NADPH-dependent manner, generating the reactive intermediate Cr^{V} , and $\text{HO}^\bullet$ under aerobic conditions *via* the redox cycling of Cr [18]. Since both have the potential to damage DNA, the generation of DNA breaks as a consequence of Cr^{VI} reduction by this system was examined using a plasmid nicking assay. Plasmid DNA represents natural double-stranded DNA with sequence variety. Its circular nature facilitates the assay. The majority of isolated plasmid is supercoiled (SC), which migrates faster than plasmid with DNA strand breaks, which can either represent nicked (relaxed circular) DNA that is due to single-strand breaks, or the linear form that is due to double-strand breaks [47]. An example of the nicking assay is shown in Fig. 1,a. The vast majority of the untreated DNA ('uncut') was supercoiled. As a positive control, digestion with the restriction enzyme *XhoI* converted all of the DNA to the linear form as expected. As a positive control for $\text{HO}^\bullet$ generation, $\text{Fe}^{\text{II}}+\text{H}_2\text{O}_2$ resulted in a large decrease in the supercoiled form and a corresponding increase in the nicked form (Fig. 1,a). With this particular plasmid and gel conditions, the linear and nicked forms migrated similarly, but both are indicative of strand breaks. Other reports demonstrated that Fe^{II} plus H_2O_2 generates mostly nicked DNA (single-strand breaks). The increase in strand breaks can be seen in Fig. 1,a. The Cr-induced strand breaks were not affected by the metal chelator diethylenetriaminepentaacetic acid (DTPA), but they were significantly diminished by catalase indicating dependence on H_2O_2 (Fig. 1,a). In accord with this, DTPA only inhibits a minority of the $\text{HO}^\bullet$ generated by this system [18]. Exogenous H_2O_2 was not included in these experiments because the PLs generate sufficient H_2O_2 under aerobic conditions to support $\text{HO}^\bullet$ generation [18]. Single-strand breaks increased over time aerobically, and were enhanced by Cr^{VI} (Fig. 1,b). In the presence of Cr, the percentage of nicked DNA at 20–40 min was greater than that at 10 min ($P<0.001$), and the nicks were greater in the presence of Cr than in its absence ($P<0.05$). In contrast to aerobic results, DNA nicks were not observed under anaerobic conditions (Fig. 1, c). Aerobically, the PLs generate both Cr^{V} and $\text{HO}^\bullet$, but anaerobically they generate Cr^{V} but not $\text{HO}^\bullet$ [18]. This indicates that Cr^{V} does not have a significant direct role in DNA strand breaks in this system but that $\text{HO}^\bullet$ may have a significant role.

The effects of various chelators and potential antioxidants on the Cr^{VI} -mediated DNA strand breaks were examined in triplicate experiments under aerobic (Fig. 2, a) and anaerobic conditions (Fig. 2,b). The chelator DTPA did not decrease the strand breaks. However, both catalase (degrades H_2O_2) and EtOH (an $\text{HO}^\bullet$ scavenger) markedly inhibited the nicks (Fig. 2, a). These data imply that H_2O_2 -dependent $\text{HO}^\bullet$ generation is largely responsible for the aerobic DNA nicks. Both formate and DMSO, which can also scavenge $\text{HO}^\bullet$, caused a partial decline in the DNA strand breaks, but the declines were not statistically significant (Fig. 2,a). It is possible that they were not as effective at scavenging $\text{HO}^\bullet$ in this system, or that the resulting radicals ($\text{CO}_2^{\bullet-}$ and $\text{H}_3\text{C}^\bullet$, resp.) caused some strand breaks. Deferoxamine (DFX) caused a partial but insignificant decline in strand breaks (Fig. 2,a). In these PLs, it has been shown that Cr^{V} chelation by DFX can result in decreased $\text{HO}^\bullet$ generation [18] which could explain the partial decline in DNA nicks. Given the low level of nicks under anaerobic conditions, none of these chelators or antioxidants had a significant effect in anaerobic experiments (Fig. 2,b).

When DNA was instead added 60 min after Cr^{VI} , at which point nearly all Cr^{VI} was reduced to Cr^{III} , DNA strand breaks were not observed (not shown). This implies that Cr^{III} is not involved in the generation of these breaks. This is consistent with the observation that Cr^{III} acetate hydroxide, $(\text{AcO})_7\text{Cr}_3(\text{OH})_2$, and products of Cr^{III} acetate hydroxide do not cause DNA strand breaks in human lymphocytes whereas Cr^{VI} does [30].

A significant role for $\text{HO}^\bullet$ in generating Cr-mediated DNA strand breaks is in agreement with that for some other systems. Cr^{VI} Reduction by the NADPH-dependent enzyme glutathione reductase generates $\text{HO}^\bullet$ and DNA strand breaks, and the strand breaks are inhibited by catalase consistent with a role for $\text{HO}^\bullet$ from Cr-mediated *Fenton*-type chemistry [48]. Catalase similarly

inhibited strand breaks in our PLs (Fig. 2). 18 mM H_2O_2 plus 1.8 mM Cr^{VI} causes DNA strand breaks presumably due to $\text{HO}^\cdot$, but Cr^{VI} plus GSH does not [29]. GSH plus Cr^{VI} yields Cr^{V} but does not result in strand breaks, whereas extensive strand breaks are seen when H_2O_2 is included, implicating $\text{HO}^\cdot$ as a significant mediator of DNA breaks [27] [46]. In mice given dichromate, the resulting DNA breaks were likely due to $\text{HO}^\cdot$ and not Cr^{V} [49].

We noted an O_2 -dependence of the PL-mediated strand breaks (Fig. 1) which corresponds to the conditions under which $\text{HO}^\cdot$ is generated by this system [18]. In some systems, there is an O_2 -dependence that does not necessarily involve $\text{HO}^\cdot$ -mediated strand breaks. For example, strand breaks mediated by a Cr^{V} complex with 2-hydroxycarboxylato ligands causes strand breaks in an O_2 -dependent manner, but there was no diminution of the breaks by catalase [50]. In contrast, a defined Cr^{IV} complex, Cr^{IV} quinate where quinate is (1*R*,3*R*,4*R*,5*R*)-1,3,4,5-tetrahydroxycyclohexane carboxylate(2-), can directly cleave DNA independent of O_2 [50]. Since we did not observe significant strand breaks anaerobically, it implies that there is not a significant role for Cr^{IV} in PL-mediated strand breaks.

In other cases, Cr-mediated strand breaks may not directly involve either Cr^{V} or $\text{HO}^\cdot$. For example, with ascorbate-mediated Cr^{VI} reduction, the DNA strand breaks are not apparently related to Cr^{V} or $\text{HO}^\cdot$, but rather to ascorbyl and carbon radicals [28]. Furthermore, not all Cr^{VI} reductants result in DNA strand breaks. Cysteine plus Cr^{VI} can generate Cr^{III} -DNA complexes, but fails to generate significant strand breaks [51]. Interestingly, our PLs result in both DNA strand breaks and in Cr^{III} -DNA complexes (see EPR results below).

EPR for Cr^{V}

A Cr^{V} signal at $g = 1.979$ was previously reported for the PLs [18], and this signal was observed both in the presence and absence of plasmid DNA (Fig. 3, a). The signal from Cr^{V} is readily detectable after 10 min and remains for at least 24 h (Fig. 3, a and b). At 30 min and 24 h, the intensity of the Cr^{V} signal was 1.7- and 2.5-fold greater, respectively, in the presence of plasmid DNA (Fig. 3, a and b). It is unknown if the DNA facilitates stabilization of the Cr^{V} species, or facilitates continued generation over time. Consistent with the latter possibility, Cr^{VI} reduction (*i.e.*, the net decline in Cr^{VI}) was greater in the presence of DNA than in its absence (Fig. 3, c). The reason(s) for the effect of DNA on net Cr^{VI} reduction are not known, but both in the presence and absence of DNA, the net decline in Cr^{VI} was most rapid early and slowed considerably between the 30 min and 24 h interval (Fig. 3, c). This slowdown is not due to Cr^{VI} limitation because the Cr^{VI} concentration remains well above the K_m reported for human microsomal enzymes [52]. It is also not due to NADPH depletion, because the NADPH-generating system maintains a constant level of NADPH over time. A possible explanation for the slowdown is that the enzymes may be potentially damaged by reactive species such as $\text{HO}^\cdot$ over time, and that the presence of plasmid affords some protection for the enzymes by reacting with some of the $\text{HO}^\cdot$. The $\text{HO}^\cdot$ -dependent generation of DNA strand breaks (Fig. 1 and Fig. 2) demonstrates that the plasmid DNA does in fact react with some of the $\text{HO}^\cdot$.

To examine the hyperfine structure of the Cr^{V} signal, experiments with PLs were repeated in which the modulation amplitude was reduced to 0.1 G. This greatly diminished the intensity of the Cr^{V} signal, so the number of scans was increased to 50. The hyperfine structure of the $g = 1.979$ Cr^{V} signal shows five or six resolved lines, but it also shows that the signals with or without plasmid DNA are not distinguishable (Fig. 4, a and b). The shape of the PLs signal is similar, but not identical, to that for Cr^{V} -NADPH (Fig. 4, c and d). The spectra suggest the influence of nearby H-atom in Cr^{V} -diol complexes [53]. However, the spectrum for NADPH- Cr^{V} was shifted *ca.* 1 G to the left, suggesting that the predominant signal in the PLs sample is not Cr^{V} -NADPH. The spectrum could instead largely result from a complex with glucose-6-phosphate which is present in 35-fold molar excess to NADPH. Several other Cr^{V} -diol complexes yield similar spectra including those due to NADH, FAD, and various sugars

(fructose, ribose, cellobiose, and others) [53]. While the PLs spectra are different from that for NADPH-Cr^V, they are not sufficient to provide evidence for Cr^V-DNA complexes. With ascorbate-mediated Cr^{VI} reduction *in vitro*, Cr^V may form the initial complexes with DNA, and Cr^{III}-DNA species are formed later by reaction or further reduction of the Cr^V-DNA [28]. This evidence was based on the observation that Cr was bound to DNA when the ascorbate/Cr ratio favored Cr^V formation, but not with ratios that favored Cr^{IV} and/or Cr^{III} [28]. It is possible that the Cr^V-DNA species were formed in our system, but that EPR was unable to resolve them from the other species. For example, Cr^V bound to the phosphates of DNA might also share similarities with that bound to glucose-6-phosphate. Furthermore, the molecular mass of the plasmid (1.85×10^6 Da) is too great to provide for effective averaging of the EPR parameters at room temperature. If a Cr^V-plasmid complex is formed, the immobilized signal with $g_{\parallel}$ and $g_{\perp}$ would be difficult to detect at room temperature, because it is less intense and superimposed on other signals. At lower temperatures where all the Cr^V signals are immobilized, two signals with a large ZFS were observed (see below) and are attributed to binding of Cr^{III} to plasmid.

While EPR did not detect Cr^V-DNA species, it is still possible that they were formed and served as precursors to the final Cr^{III} species. In support of this, $g = 5.7$ and 5.0 Cr^{III} signals were seen in the presence of plasmid DNA but not in its absence (see below). If we incubated Cr^{III}Cl₃ with the plasmid, we did not see these signals even after 24 h (not shown). Hence, they cannot be formed by the binding of free Cr^{III} to DNA. Thus, they might result from higher valence states of Cr bound to DNA. In agreement with this, Cr-DNA binding is not observed if Cr^{VI} is first reduced with ascorbate, and then, the DNA is added later, but binding is observed if the DNA is present during the Cr^{VI} reduction process [28]. The prolonged treatment of DNA with Cr^{III}Cl₃ will lead to some Cr-DNA binding [38], but cannot explain the $g = 5.7$ and $g = 5.0$ signals we observed with the PLs plus DNA (below).

EPR for Cr^{III}

Cr^{VI}O₄²⁻ shows little direct interaction with DNA [34], and Cr-DNA interactions are minimal with Cr^{VI} in the absence of reducing agents [54] [55]. However, Cr^{VI} reductants can result in the formation of Cr^{III}-DNA complexes [27] [28] [36] [54].

Unlike Cr^V species which are reactive and transient, Cr^{III} is stable and kinetically inert to ligand substitution [56]. Therefore, Cr^{III} complexes are much more stable and persistent. Cr^{III} EPR signals were examined at lower temperatures (6.3 K) (Fig. 5). In the absence of plasmid, a sharp Cr^V signal at $g = 1.98$ and a broader signal (710 G peak to peak) around this Cr^V signal were observed (Fig. 5,b). This broader signal (Fig. 5,b) increased in intensity over time and represents a Cr^{III} complex or complexes with a small ZFS consistent with near octahedral binding of O-atoms from solvent. This signal was even broader (940 G) in the presence of plasmid (Fig. 5, a), and a shoulder on the low-field side is apparent. The broader linewidth for the signal about $g = 2$ in the presence of DNA is attributed to Cr^{III} bound to phosphates from the plasmid DNA. Transitions from $S = +3/2$ to $+1/2$, $+1/2$ to $-1/2$, $-1/2$ to $-3/2$, and double quantum transitions are not resolved, suggesting that there are many overlapping non-specific EPR lines from several sites.

Low-field signals ($g_{(z),\text{eff}} = 5.7$ and 5.0 , $g_{\text{eff}} = 4.0$) from addition of plasmid DNA to chromate also were observed (Fig. 5,a). These signals are attributed to one or more Cr^{III} site(s) with a large ZFS. The largest value for $2(g_{\text{eff}}/g)$ from a rhombogram is 5.46 for the $S = +1/2$ transition [57]. The line with $g_{\text{eff}} = 5.7$ is assigned to a $S = +3/2$ transition. E/D (the ratio of E to D , which are the axial and rhombic zero-field splitting parameters, resp.) is 0.22 from the rhombogram indicating substantial distortion from cubic symmetry. The signal at $g = 4$ is due to a second species. These Cr signals are much like the signals observed for Co^{II}-substituted

horse liver alcohol dehydrogenase, another $S = 3/2$ spin system [58]. The concentration of the signal with the large ZFS is ca. $7 \mu\text{M}$ as determined by simulating the signal ($g_{(z)\text{eff}} = 5.0$, $g_{(y)\text{eff}} = 2.6$, $g_{(x)\text{eff}} = 1.7$, where $g_{(y)\text{eff}}$ and $g_{(x)\text{eff}}$ are too broad to detect or superimposed by other signals in the experimental spectrum and $g = 1.98$) and comparison of the simulated signal with the signal from a cupric standard (data not shown). Using this approximation, the ratio of Cr ($g_{(z)\text{eff}} = 5.0$) with a large ZFS to bp of plasmid is 1 Cr per 4.3 bp. Since the large ZFS signals ($g = 5.7$ and 5.0) appear early and do not increase appreciably over time, it is assumed that the binding is saturated. This is consistent with the observation that Cr^{III} preferentially binds to guanine residues [34], and 25% of the residues in *pGEM-7Zf(+)* are guanine.

Analogous experiments were conducted substituting commercially available calf thymus DNA for plasmid DNA. However, the Cr^{III} signals with a large ZFS that were observed with the plasmid DNA were not detected in the presence of calf thymus DNA (spectrum not shown). The reason(s) for the difference are not clear. However, plasmid DNA can bind several-fold more Cr^{III} than calf thymus DNA [34] [59], which could explain the absence of the Cr^{III} EPR signals with calf thymus DNA. The *pGEM-7Zf(+)* plasmid has a higher GC content (49.9%) than does calf thymus DNA (42%), which may explain a minority of the differences in Cr binding and EPR spectra. Other possible contributing factors to the different results with these DNAs include: a) the plasmid is circular, whereas the calf thymus is linear DNA; b) mutational analyses suggest the possibility of preferential sites for Cr^{III} -DNA adducts [59], and it is possible that calf thymus DNA has a lower percentage of such sites; c) the plasmid DNA has a much smaller mass than the thymus DNA which might contribute to different overall structure or conformation in solution; and d) eukaryotic DNA is typically associated with tightly associated proteins, which could have limited the ability of calf thymus DNA to interact with Cr. Overall, however, it is concluded that EPR can be used to detect various Cr^{III} species that are dependent on the presence of plasmid DNA. Further characterization of these DNA-dependent Cr^{III} signals may provide insights into the possible nature of the species, and if they are potentially detrimental to cells or DNA structure and function.

Experimental Part

General

Purified recombinant human cytochrome *b₅* and P450 reductase, and the restriction enzyme *XhoI* were purchased from *Invitrogen Corp.* (Carlsbad, CA). $\text{L-}\alpha$ -Phosphatidylcholine, hydrogenated, egg yolk (cat. no. 840059): *Avanti Polar Lipids* (Alabaster, AL); sodium cholate: *Calbiochem* (La Jolla, CA); (phenylmethyl)sulfonyl fluoride (PMSF) and *Tris*: *Research Organics* (Cleveland, OH). Sodium chromate (>99%) was the highest purity available from *Aldrich Chemical* (Milwaukee, WI). Chromates are known carcinogens and should be handled accordingly. Other chemicals obtained from *Aldrich* were activated aluminum oxide, activated carbon, and 1,5-diphenylcarbazide. Ferrous ammonium sulfate and H_2O_2 : *Mallinckrodt Chemicals* (Phillipsburg, NJ); H_2SO_4 : *J. T. Baker* (Phillipsburg, NJ); NaCl: *VWR Scientific* (West Chester, PA); EDTA: *Fisher Scientific* (Hampton, NH); catalase (cat. no. C100): *Sigma Chemical* (St. Louis, MO). This catalase has been shown to be free from inherent Cr^{VI} -reducing activity [18]. Other chemicals and reagents were purchased from *Sigma Chemical*. Plasmid DNA (*pGEM®-7Zf(+)*) was purchased from *Promega* (Madison, WI); seakem LE agarose was from *Cambrex*, and ethidium bromide was from *EMD Chemicals* (Germany).

Purification and Preparation of DNA

Escherichia coli JM109 containing *pGEM®-7ZF(+)* plasmid was grown overnight at 37° in *Luria-Bertani* medium pH 7.4 [60] supplemented with ampicillin ($100 \mu\text{g/ml}$). Plasmid DNA was purified from these cells using the *HiSpeed®* Plasmid Midi Kit from *Qiagen* (Valencia, CA). Before use, the purified DNA was treated with the chelator DTPA

(diethylenetriaminepentaacetic acid) to remove possible metal contaminants. DNA was dialyzed for 3 h at 4° against 1 mM DTPA in *Chelex* pre-treated 50 mM potassium phosphate buffer (pH 7.35) using *Slide-A-Lyzer*® mini dialysis units (Pierce, Rockford, IL). The DNA was then extensively dialyzed against DTPA-free buffer. DNA Concentration was determined from its UV absorbance at 260 nm, and DNA purity was determined from the ratio of absorbances at 260 and 280 nm.

Preparation of Proteoliposomes

Proteoliposomes (PLs) containing purified human P450 reductase plus cytochrome *b*₅ were reconstituted using a cholate dialysis procedure as described in [18]. They were stored at 4° and used as soon as possible (usually within 1 d) for the experiments. The ability of NADPH:P450 reductase to efficiently transfer electrons to cytochrome *b*₅ was confirmed by cytochrome spectral analysis as described in [18]. The amount of PLs used for each experiment was normalized to the *b*₅ content as determined from these spectra.

General Procedures

Experiments under anaerobic conditions were conducted in an anaerobic chamber (*Coy Laboratory Products*, Grass Lake, MI; 4 to 5% H₂, balance N₂) as described in [52]. Solns. were pre-incubated in the anaerobic chamber before use. Cr^{VI} reduction rates under these conditions were found to be indistinguishable from those in which the vials were made anaerobic by flushing with O₂-free N₂ [52]. Experiments under aerobic conditions were conducted in open-top vials under room air.

To remove polyvalent metal ion contaminants, solns. were pretreated with *Chelex-100* (5 g per 100 ml soln.) for at least 1 h before use.

The general reaction conditions for the proteoliposome experiments were as follows: the buffer (0.15M KCl, 2.5 mM potassium phosphate, pH 7.35), and NADPH-generating mix [52] were pre-incubated for 5 min at 37°. The PLs, Na₂CrO₄, and DNA (when included) were added, followed by incubation at 37° for the indicated times. The reactions were then analyzed by EPR or for Cr^{VI} as indicated below. Variables in each experiment (time, DNA, Cr concentration) are indicated in the results.

For quant. data, differences between multiple groups were assessed using one-way ANOVA and the *Tukey–Kramer* post test (InStat software, *GraphPad*). Significance was assumed at *P*<0.05.

Cr^{VI} Assay

In some experiments, a colorimetric assay was used to determine the Cr^{VI} concentration at fixed time points. The complete details of this 1,5-diphenylcarbazide colorimetric assay as applied to human enzymatic Cr^{VI} reduction were described in [17]. The only differences here were that the incubation buffer (0.15M KCl/2.5 mM potassium phosphate, pH 7.35) was treated with *Chelex-100* resin prior to use, and that PLs were substituted for microsomes. Volumes of all reagents were scaled down proportionally to accommodate smaller total reaction volumes.

DNA Strand Break Assay

The ability of the PL-mediated reduction of Cr^{VI} to generate DNA strand breaks was determined using the plasmid nicking assay as adapted from Fitzsimmons et al. [47]. *pGEM-7Zf(+)* Plasmid DNA was included in the reactions at the concentrations indicated in the results. The reactions were stopped by the addition of stop buffer (0.5% sodium dodecyl sulfate, 5 mM EDTA, 60% glycerol, 0.1% bromphenol blue). Aliquots of the reactions were then loaded onto a 0.6% agarose gel (in *Tris*/acetate/EDTA buffer) and electrophoresed for 40

min at 120 V. The DNA was visualized with the dye ethidium bromide (2 µg/ml). The gels were photographed on a UV transilluminator (302 nm) using a filter appropriate for ethidium bromide. The relative abundance of the supercoiled vs. nicked forms were determined by densitometric analysis of the gel images using UN-SCAN-IT software (Version 6.1, *Silk Scientific*, Orem, UT).

EPR for Cr^V and Cr^{III}

The reactions were stopped by immersion in liq. N₂ (77 K), and the samples were stored in quartz EPR tubes at 77 K, typically for less than one week. For some samples, spectra for Cr^{III} were first obtained at liq. He temp. (6.3 K) using a *Bruker E500 ELEXSYS* spectrometer (Silberstreifen, Germany) with an *Oxford Instruments ESR-9* He flow cryostat (Oxfordshire, UK) and a *Bruker DM0101* cavity. For Cr^V EPR, samples were quickly thawed, placed in a quartz flat cell, and ESR spectra were obtained without delay at r.t. using a *Bruker EMX* spectrometer. The spectra were stable during the ESR analysis time, with no noticeable changes in spectral intensity or pattern. Instrument settings are indicated in the results. ESR Spectra were confirmed in replicate experiments. The *g* values were determined by comparison to the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) which has a *g* value of 2.0036.

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REFERENCES

1. Environmental Protection Agency. Chromium, Integrated Risk Information System, Office of Health and Environmental Assessment, U.S. Washington, D. C: EPA; 1999.
2. Gadd GM, White C. *TIBTECH* 1993;11:353.
3. Zhitkovich A, Voitekun V, Kluz T, Costa M. *Environ. Health Perspect* 1998;106:969. [PubMed: 9703480]
4. Kortenkamp A, Beyersmann D, O'Brien P. *Toxicol. Environ. Chem* 1987;14:23.
5. Buttner B, Beyersmann D. *Xenobiotica* 1985;15:735. [PubMed: 4072261]
6. Cantoni O, Costa M. *Carcinogenesis* 1984;5:1207. [PubMed: 6467509]
7. Christie NT, Cantoni O, Evans RM, Meyn RE, Costa M. *Biochem. Pharmacol* 1984;33:1661. [PubMed: 6233980]
8. Levy LS, Venitt S. *Carcinogenesis* 1986;7:831. [PubMed: 3698209]
9. Tsapakos MJ, Hampton TH, Wetterhahn KE. *Cancer Res* 1983;43:5662. [PubMed: 6640521]
10. Whiting RF, Stich HF, Koropatnick DJ. *Chem.-Biol. Interact* 1979;26:267. [PubMed: 509690]
11. Standeven AM, Wetterhahn KE. *Carcinogenesis* 1992;13:1319. [PubMed: 1499083]
12. Mikalsen A, Alexander J, Ryberg D. *Chem.-Biol. Interact* 1989;69:175. [PubMed: 2495190]
13. Jennette KW. *J. Am. Chem. Soc* 1982;104:874.
14. Shi X, Dong Z, Dalal NS, Gannett PM. *Biochim. Biophys. Acta* 1994;1226:65. [PubMed: 8155741]
15. Shi XL, Dalal NS. *J. Inorg. Biochem* 1990;40:1. [PubMed: 2178178]
16. Suzuki Y, Fukuda K. *Arch. Toxicol* 1990;64:169. [PubMed: 2372230]
17. Pratt PF, Myers CR. *Carcinogenesis* 1993;14:2051. [PubMed: 8222053]
18. Borthiry GR, Antholine WE, Kalyanaraman B, Myers JM, Myers CR. *Free Radical Biol. Med* 2007;42:738. [PubMed: 17320757]
19. Standeven AM, Wetterhahn KE. *Chem. Res. Toxicol* 1991;4:616. [PubMed: 1807444]
20. Dillon CT, Lay PA, Bonin AM, Cholewa M, Legge GJF, Collins TJ, Kostka KL. *Chem. Res. Toxicol* 1998;11:119. [PubMed: 9511903]

21. Shi X, Chiu A, Chen CT, Halliwell B, Castranova V, Vallyathan V. J. Toxicol. Environ. Health, Part B 1999;2:87.
22. Shi X, Dalal NS. Arch. Biochem. Biophys 1992;292:323. [PubMed: 1309299]
23. Shi X, Ding M, Ye J, Wang S, Leonard SS, Zang L, Castranova V, Vallyathan V, Chiu A, Dalal N, Liu K. J. Inorg. Biochem 1999;75:37. [PubMed: 10402675]
24. Myers CR, Myers JM, Carstens BP, Antholine WE. Toxic Subst. Mech 2000;19:25.
25. Jannetto PJ, Antholine WE, Myers CR. Toxicology 2001;159:119. [PubMed: 11223168]
26. Levina A, Zhang L, Lay PA. Inorg. Chem 2003;42:767. [PubMed: 12562191]
27. Aiyar J, Borges KM, Floyd RA, Wetterhahn KE. Toxicol. Environ. Chem 1989;22:135.
28. Stearns DM, Kennedy LJ, Courtney KD, Giangrande PH, Phieffer LS, Wetterhahn KE. Biochemistry 1995;34:910. [PubMed: 7827049]
29. Kawanishi S, Inoue S, Sano S. J. Biol. Chem 1986;261:5952. [PubMed: 3009436]
30. Gao M, Binks SP, Chipman JK, Levy LS, Braithwaite RA, Brown SS. Hum. Exp. Toxicol 1992;11:77. [PubMed: 1349223]
31. Sugiyama M. Environ. Health Perspect 1991;92:63. [PubMed: 1935852]
32. Cupo DY, Wetterhahn KE. Proc. Natl. Acad. Sci. U.S.A 1985;82:6755. [PubMed: 3863126]
33. Xu J, Wise JP, Patierno SR. Mutat. Res 1992;280:129. [PubMed: 1378537]
34. Arakawa H, Ahmad R, Naoui M, Tajmir-Riahi HA. J. Biol. Chem 2000;275:10150. [PubMed: 10744697]
35. Sugden KD. J. Inorg. Biochem 1999;77:177. [PubMed: 10643657]
36. Bridgewater LC, Manning FCR, Patierno SR. Carcinogenesis 1994;15:2421. [PubMed: 7955085]
37. Snow ET, Xu L-S. Biochemistry 1991;30:11238. [PubMed: 1958661]
38. Bridgewater LC, Manning FC, Woo ES, Patierno SR. Mol. Carcinog 1994;9:122. [PubMed: 8142016]
39. Shi X, Dalal NS. FEBS Lett 1990;276:189. [PubMed: 2176163]
40. Tsou T-C, Yang J-L. Chem.-Biol. Interact 1996;102:133. [PubMed: 9021167]
41. Tsou T-C, Chen C-L, Liu T-Y, Yang J-L. Carcinogenesis 1996;17:103. [PubMed: 8565117]
42. Shi X, Mao Y, Knapp AD, Ding M, Rojanasakul Y, Gannett PM, Dalal N, Liu K. Carcinogenesis 1994;15:2475. [PubMed: 7955094]
43. Luo H, Lu Y, Shi X, Mao Y, Dalal NS. Ann. Clin. Lab. Sci 1996;26:185. [PubMed: 8852428]
44. Weeks CL, Levina A, Dillon CT, Turner P, Fenton RR, Lay PA. Inorg. Chem 2004;43:7844. [PubMed: 15554650]
45. Molyneux MJ, Davies MJ. Carcinogenesis 1995;16:875. [PubMed: 7537182]
46. Aiyar J, Berkovits HJ, Floyd RA, Wetterhahn KE. Chem. Res. Toxicol 1990;3:595. [PubMed: 1966470]
47. Fitzsimmons SA, Lewis AD, Riley RJ, Workman P. Carcinogenesis 1994;15:1503. [PubMed: 8055626]
48. Leonard SS, Vallyathan V, Castranova V, Shi X. Mol. Cell. Biochem 2002;234–235:309.
49. Ueno S, Kashimoto T, Susa N, Furukawa Y, Ishii M, Yokoi K, Yasuno M, Sasaki YF, Ueda J, Nishimura Y, Sugiyama M. Toxicol. Appl. Pharmacol 2001;170:56. [PubMed: 11141356]
50. Levina A, Barr-David G, Codd R, Lay PA, Dixon NE, Hammershøi A, Hendry P. Chem. Res. Toxicol 1999;12:371. [PubMed: 10207127]
51. Zhitkovich A, Quievryn G, Messer J, Motylevich Z. Environ. Health Perspect 2002;110:729. [PubMed: 12426121]
52. Myers CR, Myers JM. Carcinogenesis 1998;19:1029. [PubMed: 9667741]
53. Shi X, Dalal NS. Arch. Biochem. Biophys 1990;277:342. [PubMed: 2155579]
54. Tsapakos MJ, Wetterhahn KE. Chem.-Biol. Interact 1983;46:265. [PubMed: 6627506]
55. Hneihen AS, Standeven AM, Wetterhahn KE. Carcinogenesis 1993;14:1795. [PubMed: 8403202]
56. Manning FCR, Xu J, Patierno SR. Mol. Carcinog 1992;9:122. [PubMed: 8142016]
57. Hagen WR. Adv. Inorg. Chem 1992;38:165.
58. Werth MT, Tang S-F, Formicka G, Zeppezauer M, Johnson MK. Inorg. Chem 1995;34:218.

59. Tsou TC, Lin RJ, Yang JL. Chem. Res. Toxicol 1997;10:962. [PubMed: 9305577]
60. Sambrook, J.; Fritsch, EF.; Maniatis, T., editors. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N. Y: Cold Spring Harbor Laboratory; 1989.

Fig. 1. DNA Strand breaks in pGEM-7Zf(+) during NADPH-dependent Cr^{VI} reduction by the PLs

a) Representative agarose gel of DNA strand breaks resulting from aerobic Cr^{VI} reduction. FeII+H₂O₂ is a positive control for generating HO[•], and 'XhoI cut' is a positive control for linearizing DNA. b) and c) Densitometric analysis of the amount of supercoiled vs. nicked DNA over time in the presence or absence of 50 μM Cr^{VI} (as Na₂CrO₄) under aerobic (b) and anaerobic (c) conditions. Reactions (20 μl total volume) were incubated at 37° and included 50 μM Na₂CrO₄, PLs (P450 reductase and 0.0152 nmol cytochrome b₅), 0.25 μg pGEM-7Zf(+) plasmid DNA, and the NADPH-generating system in *Chelex-pretreated* buffer (0.15M KCl, 2.5 mM potassium phosphate pH 7.35). Results shown represent the mean ± S.D., n = 3.

Fig. 2. The effect of various chelators and potential antioxidants on Cr^{VI} reduction-mediated DNA nicks under aerobic (a) vs. anaerobic (b) conditions after a 20-min incubation

Reaction conditions were the same as those in Fig. 1 with the following additions: DTPA (0.1 mM), catalase (500 U), formate (0.25 M), DMSO (5%), EtOH (5%), or DFX (0.25 mM). Results shown represent the mean $\pm$ S.D., $n = 3$. In a), § $P \leq 0.001$ compared to Cr^{VI}.

Fig. 3.

a) EPR Spectra of Cr^{V} obtained during the NADPH-dependent Cr^{VI} reduction catalyzed by PLs (37° under room air) in the presence (left) vs. absence (right) of pGEM-7Zf(+) plasmid DNA. The Cr^{V} EPR signal intensity (b) and the decline in Cr^{VI} (c) in the presence vs. absence of DNA are shown. Reactions (0.3 ml total volume) were incubated at 37° and included 0.4 mM Na_2CrO_4 , PLs (P450 reductase and 0.0152 nmol cytochrome b_5), 7.5 μg plasmid DNA, and the NADPH-generating system in Chelex-treated buffer (0.15M KCl, 2.5 mM potassium phosphate pH 7.35). EPR Instrument settings were: 5 G modulation amplitude, 50 mW microwave power, 6.32×10^4 receiver gain, 40.96 ms time constant, 9.76 GHz microwave

frequency, sweep width 200 G, field set 3480 G, modulation frequency 100 kHz, scan time 42 s; number of scans 9.

Fig. 4. Hyperfine structure of the $g = 1.979$ Cr^{V} EPR signal

The spectrum for $\text{PLs} + \text{Cr}^{\text{VI}}$ (a) is compared to that with the inclusion of plasmid DNA (b). The reaction conditions were the same as for the previous figure, and the spectra were obtained following 30 min incubation at 37° . The spectrum for $\text{PLs} + \text{Cr}^{\text{VI}}$ (c), which is the same as a, is compared to that for $\text{NADPH} + \text{Cr}^{\text{VI}}$ (d) (30 min incubation of 5 mM NADPH plus 5 mM Na_2CrO_4 at 37° in potassium phosphate buffer, pH 7.4). Instrument settings: modulation amplitude 0.1 G, microwave power 50 mW, receiver gain 6.32×10^4 , ms time constant 327.68, microwave frequency 9.765 GHz, sweep width 20 G, field set 3516 G, modulation frequency 100 kHz, scan time 83.89 s, and conversion time 81.92 ms. The number of scans was 5 for the NADPH samples and 50 for the others. The y-axis scale for the intensity of each spectrum was adjusted so that the details of the spectra could be conveniently compared on the same plots.

Fig. 5. EPR Spectra for Cr^{III}

The reaction conditions were the same as those indicated in Fig. 3, with PLs incubated with the NADPH-generating system and Na₂CrO₄ for the indicated times in the presence (a) or absence (b) of plasmid DNA. Following incubation, samples were frozen in liquid N₂. Instrument settings: temp. 6.3 K, G modulation amplitude 10, microwave power 16 dB (5 mW), time constant 81 ms, microwave frequency 9.633 GHz, modulation frequency 100 kHz, scan time 83 s; number of scans, 5.