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A bacterial flavin reductase system reduces chromate to a soluble chromium(III)–NAD^+ complex

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Abstract

Biological reduction of carcinogenic chromate has been extensively studied in eukaryotic cells partly because the reduction produces stable chromium(III)-DNA adducts, which are mutagenic. Microbial reduction of chromate has been studied for bioremediation purposes, but little is known about the reduction mechanism. In eukaryotic cells chromate is mainly reduced non-enzymatically by ascorbate, which is usually absent in bacterial cells. We have characterized the reduction of chromate by a flavin reductase (Fre) from *Escherichia coli* with flavins. The Fre-flavin system rapidly reduced chromate, whereas chemical reduction by NADH and glutathione was very slow. Thus, enzymatic chromate reduction is likely the dominant mechanism in bacterial cells. Furthermore, the end-product was a soluble and stable Cr(III)–NAD^+ complex, instead of Cr(III) precipitate. Since intracellularly generated Cr(III) forms adducts with DNA, protein, glutathione, and ascorbate in eukaryotic cells, we suggest that the produced Cr(III) is primarily complexed to NAD^+, DNA, and other cellular components inside bacteria.

Keywords: Chromate; Reduction; Flavin reductase; NAD(P)H:flavin oxidoreductase; Cr(III)–NAD^+ complex

Chromium(VI) [Cr(VI)] has been released into the environment in large quantities from a broad range of industrial applications, posing a severe environmental problem [1]. Cr(VI) exposure leads to cancer and hereditary disease [2]. Under typical environmental conditions, Cr(VI) forms oxyanions, primarily as chromate, which is highly soluble [3].

Chromate reduction in eukaryotic cells has been extensively studied because the reduction process generates reactive intermediates, Cr(V) and Cr(IV), and leads to the incorporation of Cr(III) into DNA molecules. Recent studies have demonstrated the role of Cr(V) in chromate-induced DNA damage, cancer, and apoptosis [4,5]. The biologically generated Cr(III) has also been shown to be incorporated into DNA, causing DNA–DNA, DNA–peptide, and DNA–amino acid cross-links that are mutagenic [6–10]. Chromate can readily cross the membranes of both prokaryotic and eukaryotic cells [11] and can be reduced to Cr(III) inside eukaryotic cells primarily by cellular reducing agents, such as ascorbate, glutathione, and cysteine [12], of which ascorbate is shown to be the principal reductant of chromate [13].

Bacterial reduction of chromate to Cr(III) has been studied mainly for the purpose of bioremediation, which is based on the concept that Cr(III) has negligible toxicity and minimal solubility compared with Cr(VI) [14]. Many bacteria can reduce chromate under both aerobic and anaerobic conditions, including *Escherichia coli* [15]. Anaerobic bacteria may use chromate as a terminal electron acceptor [16] or reduce chromate in the periplasmic space by hydrogenase [17] or reduced cytochrome *c* [17]. Aerobic bacteria can reduce chromate by cellular reducing agents and NADH-dependant chromate reductases [15,18–20]. Since ascorbate is usually absent in bacterial cells, the primary reductant is glutathione that reduces chromate slowly [21]. Thus, it is unknown whether enzymatic or non-enzymatic reduc-

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tion of chromate is dominant in bacterial cells under aerobic conditions. Furthermore, a significant issue that has not been vigorously demonstrated is the species and solubility of Cr(III) produced by microbial reduction of chromate. It also remains unsolved whether the NADH-dependant reductases are specific chromate reductases. In this paper, we address these three important questions.

NAD(P)H:flavin oxidoreductases (flavin reductases) catalyze the reduction of flavin by NAD(P)H. One of the functions of flavin reductases is to reduce ferrisiderophores [22] and Cob(III)alamin [23]. Despite this known function of flavin reductases for metal reduction, the role of flavin reductases in chromate reduction has not yet been documented. Here, we report that a previously characterized *E. coli* flavin reductase [24] with flavins rapidly reduced chromate to a soluble and stable Cr(III)–NAD^+ complex, which is especially surprising as it is generally assumed that the produced Cr(III) forms precipitate, usually $Cr(OH)_3$ or Cr_2O_3 [25].

Materials and methods

Chromate reduction by the Fre system. The production and purification of Fre were done as previously reported [26]. One unit of Fre was defined as the amount of Fre required to catalyze the consumption of 1 nmol of NADH per min with 10 μM FMN. The specific activity of the purified Fre was 49,600 U mg^{-1} of protein. Chromate reduction was assayed under both aerobic and anaerobic conditions. Reaction mixtures contained 38 U Fre, 400 μM NADH, and 250 μM chromate in 1 ml of 40 mM KPi buffer (pH 7.0) with 10 μM FMN, FAD, or riboflavin. Reaction was started by the addition of Fre. The reaction was stopped by mixing 100 μl sample with 5 μl of 2 M Na_2CO_3, and chromate was measured. Anaerobic chromate reduction was done identically, but inside an anaerobic chamber (Coy Laboratory Product, Grass Lake, MI) under a N_2 atmosphere with 2–3% H_2. All assays were done at 24 °C.

Chromium detection. Chromate was measured by a reported method [27] using K_2CrO_4 (Sigma, St. Louis, MO) as the standard and using ChromaVer powder pillows (HACH, Loveland, CO) containing diphenylcarbazide. Soluble Cr(III) species were detected by absorption spectra (Ultrospec 4000, Amersham Pharmacia, Piscataway, NJ). Because Cr(III) precipitates out in aqueous solutions at neutral pH, the spectrum of $Cr(NO_3)_3$ in distilled water (pH < 3) was used as a control. Total soluble chromium was determined using an inductively coupled plasma/mass spectrophotometer (ICP/MS) (HP 4500 Plus Series, Agilent Technologies, Palo Alto, CA). The size of the Cr(III)–NAD^+ complex was estimated by high performance liquid chromatography (HPLC) equipped with a size-exclusion BioSep SEC 3000 column (300 × 7.8 mm, Phenomenex, Torrance, CA) and a photodiode array detector (Waters, Milford, MA). The running condition was 0.5 ml min^{-1} of 50 mM KPi (pH 7.0).

NADH consumption. NADH consumption was monitored by the absorbance at 340 nm (A_{340}, $\varepsilon = 6220\,M^{-1}\,cm^{-1}$). The reaction contained 400 μM NADH, 10 μM flavin, and 38 U Fre in 1 ml of 40 mM KPi buffer (pH 7.0) with or without 250 μM Cr(VI). Anaerobic experiments were assembled with identical reagents but in rubber-stopper sealed cuvettes in the anaerobic chamber. Reactions were initiated by adding Fre. Michaelis–Menten kinetic parameters were determined with 1, 2, 3, 4, or 5 μM FMN in triplicate, and averages were used to calculate the kinetic parameters.

Electron paramagnetic resonance spectroscopy (EPR). The conversion of Cr(VI) to Cr(III) by the Fre system was further analyzed by EPR. EPR spectra were obtained using a Bruker 200tt EPR spectrometer (Bruker Instruments, Billerica, MA) in a GFS-300 transfer tube. All experiments were carried out at 70 K with the following EPR settings: microwave frequency = 9.428 GHz, microwave power = 6.32 mW, gain = 6.3×10^5, time constant = 500 ms, modulation amplitude = 2 mT, center field = 330 mT, sweep width = 200 mT, and sweep time = 2 min.

Dialysis. A sample (0.5 ml) of enzymatically reduced Cr(III) mixture with 23.7 mM chromium was transferred into dialysis tubing having a molecular weight cutoff of 3500 (Spectrum, Houston, TX) and dialyzed against 500 ml of 40 mM KPi buffer (pH 7.0) for 12 h. The dialysis was then continued in 500 ml of fresh 40 mM KPi buffer for another 12 h. The first and second dialysis solutions as well as the dialyzed sample inside the tubing were analyzed for chromium by ICP/MS. NAD^+ was estimated by absorbance at 260 nm ($\varepsilon = 18{,}000\,M^{-1}\,cm^{-1}$).

Results

Reduction of chromate by a Fre–flavin system

The ability of Fre, an *E. coli* general flavin reductase [24], to reduce chromate was tested. The recombinant Fre overproduced in *E. coli* was purified as previously reported [26]. Fre, together with riboflavin, FMN, or FAD rapidly reduced chromate under both aerobic and anaerobic conditions (Fig. 1). Chromate reduction was faster under anaerobic conditions than under aerobic conditions. Chromate reduction by NADH without the enzyme or without flavins was not apparent (Fig. 1). When NADH was replaced by glutathione, chromate reduction was observed but was much slower than that catalyzed by Fre (Fig. 1). Oxygen did not affect chromate reduction by either glutathione or NADH. The initial rates of chromate reduction under aerobic conditions were about 1/3 of those observed under anaerobic conditions (Table 1).

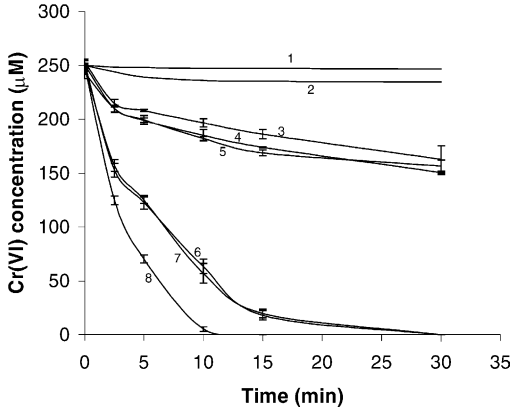

Fig. 1. Time course of chromate reduction. Controls contained only NADH (1) or glutathione (2). Chromate reduced by Fre with FAD (3), FMN (4), or riboflavin (5) under aerobic conditions. Chromate reduced by Fre with FAD (6), FMN (7), or riboflavin (8) under anaerobic conditions.

Table 1
Rates of chromate reduction by the Fre-flavin system[a]

Flavin	Aerobic Cr(VI) reduction (μmol mg^{-1} min^{-1})	Anaerobic Cr(VI) reduction (μmol mg^{-1} min^{-1})
FAD	26.0 ± 1.7	76.7 ± 0.6
FMN	27.1 ± 1.9	71.3 ± 1.1
Riboflavin	33.3 ± 0.5	96.5 ± 6.4

[a] The rates were determined after incubating for 1 min. Results were averages of triplicate measurements with standard deviations.

NADH consumption and kinetic analysis

The effects of chromate on NADH consumption by Fre were studied. Under aerobic conditions, the NADH consumption rates increased in the presence of chromate (Table 2). Under anaerobic conditions, NADH was oxidized only in the presence of chromate, and the rates were faster than those observed under aerobic conditions (Table 2). The apparent K_M and k_{cat} values of Fre (molecular weight of 26,110) for FMN were $1.2 \pm 0.18\,\mu M$ and $27.6 \pm 1.4\,s^{-1}$ when oxygen was the sole electron acceptor. When $250\,\mu M$ chromate was the sole electron acceptor, the apparent K_M and k_{cat} values were $1.0 \pm 0.16\,\mu M$ and $41.3 \pm 1.5\,s^{-1}$, respectively.

Stoichiometry

The amount of NADH consumed for chromate reduction was measured under anaerobic conditions. NADH was analyzed before reaction initiation and after reaction completion. Corrections to account for changes in NADH due to residual oxygen were accomplished by using samples without chromate as controls. The average amount of NADH consumed for the complete reduction of 1000 nmol of chromate in the presence of a specific flavin was as follows: FMN, 1633 ± 126 nmol; FAD, 1475 ± 81 nmol; and Riboflavin, 1650 ± 79 nmol. Thus, approximately 1.5 NADH was consumed for each chromate reduced, indicating that Cr(III) was the end-product.

Conversion of chromate to Cr(III)

Cr(III) precipitate was expected to be the end-product of chromate reduction. However, no Cr(III) precipitate was observed in any reactions, but addition of similar concentrations of $Cr(NO_3)_3$ to the same reaction mixture resulted in immediate precipitation. The nature of the chromium end-product was further tested. Anaerobic reactions containing 5 mM chromate, 10 mM NADH, and 76 U Fre in 1 ml of 40 mM KPi buffer (pH 7.0) were assembled in sealed cuvettes. Chromate alone in KPi buffer gave an absorption peak at 370 nm ($\varepsilon = 4700\,M^{-1}\,cm^{-1}$). After reduction by Fre for 90 min, Cr(VI) was completely reduced as confirmed by diphenylcarbazide analysis and by the lack of an absorption peak at 370 nm. The Cr(III) end-product was soluble with a greenish color. The absorption spectra of the end-product and 5 mM Cr(III) in distilled water gave peaks at a similar wavelength (Fig. 2) with a red shifting of the end-product peak, as reported for a Cr(III) tetramer [28]. The product was stable after one year at room temperature in a sterile container.

EPR was used to analyze the reaction intermediate and end-product. Samples frozen during the reduction of 5 mM chromate (3 min after reaction initiation) showed a distinctive, sharp EPR spectrum attributable to Cr(V), centered at $g = 1.97$ (342.5 mT) and line width of ca. 2 mT, and essentially identical to those observed previously [20]. Samples taken after completion of the reaction (3 h incubation) had no Cr(V) signal, but showed a broadened EPR spectrum (Fig. 3A) that was centered at $g = 1.94$ (347.3 mT) with a line width of approximately 50 mT, very similar to EPR spectra obtained for Cr(III) polymers [29]. Given the reaction

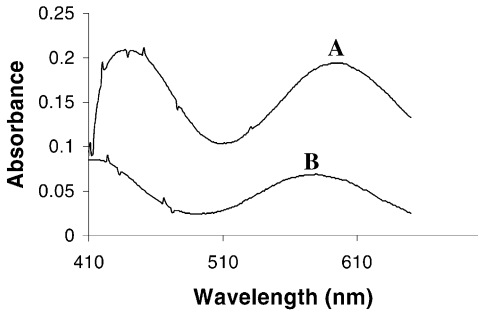

Fig. 2. Absorbance spectra of 5 mM Cr(III) samples. (A) Cr(III) produced by the Fre–FMN reduction of chromate with an absorption peak at 593 nm and (B) Cr(III) monomer obtained by dissolving $Cr(NO_3)_3$ in distilled H_2O with an absorption peak at 579 nm.

Table 2
NADH oxidation rates by the Fre-flavin system[a]

Flavin	O_2 only (μmol mg^{-1} min^{-1})	O_2 + Cr(VI) (μmol mg^{-1} min^{-1})	Cr(VI) − O_2 (μmol mg^{-1} min^{-1})
FAD	43.8 ± 0.5	58.9 ± 2.8	84.3 ± 1.0
FMN	49.6 ± 0.8	63.4 ± 2.7	85.9 ± 2.4
Riboflavin	78.0 ± 0.4	98.8 ± 4.4	124.6 ± 3.3

[a] The initial rates were determined by monitoring NADH oxidation at 340 nm. Results were averages of triplicate experiments with standard deviations.

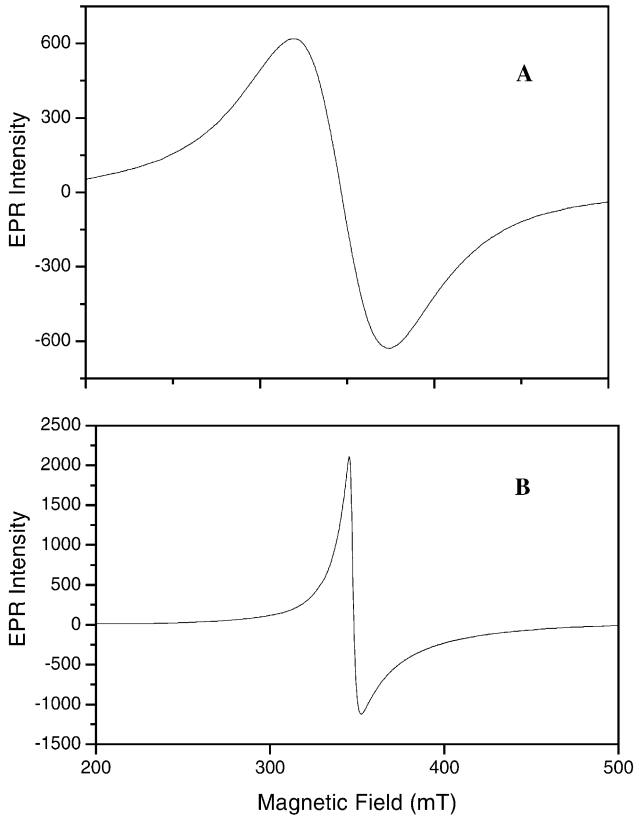

Fig. 3. EPR analysis of the reaction end product and standard Cr(III). (A) Enzymatic reaction end product produced from 5 mM chromate in 40 mM KPi and (B) standard 5 mM $Cr(NO_3)_3$ in distilled H_2O.

stoichiometry and the spectrum of the end-product, we deduced that the EPR spectrum represents a paramagnetic Cr(III) species. In contrast, a standard solution of 5 mM $Cr(NO_3)_3$ in H_2O gave a considerably narrower EPR spectrum, centered at $g = 1.94$ (347.3 mT) and line width of ca. 5 mT (Fig. 3B). These two signals represent two different Cr(III) states, where both peaks were centered at the same magnetic field. The broadening observed in the reaction product is likely due to the interaction of the unpaired electrons of Cr(III) in a complex. Double integrations of EPR spectra of the standard Cr(III) and end-product were within 3% of each other. This information indicates that the EPR relaxation properties of the two signals are reasonably similar, further supporting our assignment of the spectrum to Cr(III).

Characterization of the soluble Cr(III) product

The end-product was further analyzed by HPLC size-exclusion chromatography. The reaction product gave a retention time of 20.9 min with absorbance peaks at 260 and 590 nm, indicating the presence of NAD^+ and Cr(III). When compared to the protein standards run under the same conditions, the estimated size of the Cr(III)–NAD^+ complex was 12 kDa. To test the enzymatic reduction of chromate at high chromate concentrations, an anaerobic reaction containing 25 mM chromate, 50 mM NADH, and 78 U Fre per ml was done in 40 mM KPi. The reaction produced a green solution with neither detectable Cr(VI) nor $Cr(OH)_3$ precipitate. When the reaction product was dialyzed against 40 mM KPi, the distribution of chromium was 9406 nmol inside the tubing and 644 nmol in the dialysis solution. After continued dialysis in a fresh 40 mM KPi buffer, the chromium was 8898 nmol inside the tubing and 508 nmol in the buffer. The total loss of chromium through dialysis was only 11%. The NAD^+ content inside the tubing was estimated to be 16,372 nmol by absorption at 260 nm. The molar ratio of NAD^+ to Cr(III) was approximately 2 to 1 after dialysis.

Discussion

Chromate reduction in eukaryotic cells is primarily through non-enzymatic reduction by ascorbate [13]. Since bacterial cells normally do not contain ascorbate, glutathione becomes the major reductant [12]. However, chromate reduction by glutathione is much slower than by the Fre-flavin system (Fig. 1) [21], suggesting that chromate reduction is primarily enzymatic inside cells. Fre, a general flavin reductase in *E. coli*, does not contain bound flavins [24]. As shown here, the Fre system is highly effective in chromate reduction with the highest activity in the presence of riboflavin at 33.3 or 96.4 $\mu mol\,mg^{-1}\,min^{-1}$ at 24 °C under aerobic or anaerobic conditions (Table 1). The activities are significantly faster than those of two purified NAD(P)H-dependent chromate reductases previously reported. The soluble *Pseudomonas putida* chromate reductase has a specific activity of 0.533 $\mu mol\,mg^{-1}\,min^{-1}$ at 30 °C [19], and the soluble *Pseudomonas ambigua* G-1 chromate reductase (ChrR) has a specific activity of 0.025 $\mu mol\,mg^{-1}\,min^{-1}$ at 50 °C [20]. It has previously been suggested that the primary functions of these enzymes may not be for chromate reduction [19]. This is likely true as the gene sequences available from GenBank indicate that the *P. putida* chromate reductase is a quinone reductase (AF375641) that has a bound flavin and *P. ambigua* ChrR is a flavin reductase (D83142). The high rate of chromate reduction by the Fre-flavin system and the ubiquity of flavins and flavin reductases in cells suggest that flavin reductase systems may play a significant role in chromate reduction. Flavin reductases may also be used in bioremediation of Cr(VI) contamination.

Chromate reduction by the Fre-flavin system is likely via reduced flavins, in a similar manner as the reduction of ferrisiderophore [22] and Cob(III)alamin [23] by Fre in *E. coli* and *Salmonella enterica*. Reduced flavins are highly reactive and can be reoxidized by either O_2 [26] or chromate. Since the oxidized forms of flavins are

continuously regenerated, flavin concentrations less than 10 μM (ca. 10 K_M) are required to support maximal activity for Fre as measured by NADH consumption [26]. However, the rates of NADH consumption were increased by replacing O_2 with chromate. Kinetic analysis suggests that the increased rate of NADH consumption with chromate as the oxidant is due to the increased V_{max}. The increased NADH consumption could be a result of the direct reaction of NADH with reactive intermediates Cr(IV) and Cr(V). Thus, the K_M for FMN remains the same, while V_{max} is the sum of enzymatic and chemical oxidation of NADH. The hypothesis is also in agreement with the moderate increase in NADH consumption rates by chromate under aerobic conditions due to an intermediate level of chromate reduction (Table 2).

Because most enzymatic chromate reduction has been studied at micromolar concentrations, the form of Cr(III) produced has not been carefully studied. Since Cr(III) precipitates in neutral solutions, the formation of Cr(III) precipitate is expected. To our surprise, no Cr(III) precipitate was formed even when 25 mM chromate was reduced. Instead, a soluble Cr(III)–NAD^+ complex was produced. The reaction stoichiometry and absorption spectrum (Fig. 2) indicate that the end-product is Cr(III). The EPR spectrum further demonstrated that Cr(III) is the end-product but in a complexed form (Fig. 3). Similar EPR spectra have been observed for Cr(III) species in ruby [30,31], where exchange-coupling of the two Cr(III) spin 3/2 systems results in a total spin 3 system. Similar conclusions can also be inferred from the spectra of Cr dimer, $Cr(III)_2O_6H_4$ [29]. The composition of the Cr(III) complex was composed of Cr(III) and NAD^+, at a molar ratio of 1 to 2. The complex may also contain some NADH. The soluble Cr(III) complex does not result from an association of the Cr(III) with the phosphate buffer because the soluble end-product was also formed in 40 mM Tris (pH 8.0), HEPES (pH 7.8), or MOPS (pH 7.2) buffer (data not shown). The Cr(III) complex was only produced by chromate reduction and not by mixing $Cr(NO_3)_3$ with NAD^+ in a KPi buffer. A Cr(III)–NAD^+ complex has not been reported to date.

The conditions used for the production of soluble Cr(III) complex are similar to physiological conditions inside cells, where sufficiently high concentrations of FMN, FAD, NADH, and NAD^+ are available. There are 88 μM FMN, 51 μM FAD, 16 μM NADH, and 790 μM NAD^+ in *Salmonella typhimurium*, a close relative of *E. coli* [32]. Thus, chromate reduction by soluble chromate reductases may result in soluble Cr(III) complexes inside cells. The complex could be leaked out of the cells or released after cell lysis. The stability of the complex in the environment is unknown, but the complex in the reaction solution was stable for over a year. Cr(III) is substitution-inert [33], which explains why the complex is not slowly converted to insoluble $Cr(OH)_3$. In addition, intracellularly produced Cr(III) is able to form adducts with DNA, protein, glutathione, and ascorbate as demonstrated in eukaryotic cells [6–10,34]. Thus, the Cr(III) produced intracellularly from chromate reduction is primarily bound to organic compounds including NAD^+, nucleic acids, and other cellular components. However, our results do not rule out the formation of Cr(III) precipitate outside the cytoplasm, but the production of soluble Cr(III), being mobile in groundwater, should be considered for bioremediation. Organically complexed Cr(III) species in wastewater have also been reported [35], but the exact structures and bioavailability of these complexes are unknown.

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