# Evaluation of Class II Chromate Reductases and their Bioremediation Potential

Claudio Gonzalez[§], David Ackerley[§], Mimi Keyhan, ***A. Matin*** a.matin@stanford.edu (Stanford University, California, USA), and Robert Blake II (Xavier University of Louisiana, New Orleans, USA). [§] These authors contributed equally to this manuscript

**ABSTRACT:** Microbial bio-reduction to Cr(III) is a promising strategy for detoxification of chromate, a prevalent anthropogenic pollutant. We propose that this activity can be enhanced in bacteria through engineering of soluble enzymes, such as NfsA, the model Class II chromate reductase of *Escherichia coli*. We show here that *nfsA* is induced by chromate, and that it reduces this compound by a "semi-tight" reaction mechanism, involving asynchronous transfer of electrons from its FMN cofactor to chromium. This mechanism produces a reactive Cr(V) intermediate; and consequently generates higher levels of reactive oxygen species (ROS) than the "tight" (simultaneous two-electron transferring) Class I chromate reducer YieF. Strengthening the activity of NfsA and other Class II enzymes by directed evolution would likely enhance chromate transformation by a host cell; but our data to date indicate that the Class I enzymes are superior candidates for such studies.

**INTRODUCTION.** Hexavalent chromium [Cr(VI); most commonly chromate] is a widespread environmental pollutant, arising as a by-product of numerous industrial processes. Chromate is highly toxic, mutagenic and carcinogenic, and because of its solubility in water, chromate contamination can be difficult to contain (Singh et al, 1998). Bacteria can reduce Cr(VI) to Cr(III), offering promise for an environmentally friendly and affordable solution to chromate pollution. However, *in situ* remediation is limited by the toxicity of chromate and co-contaminants to remediating cells, and chromate has been projected to persist at dangerous levels at waste sites for over 1000 years (Okrent and Xing, 1993). To provide a potential solution to this problem we have proposed molecular engineering of cells and enzymes to decrease chromate toxicity to bacteria and increase levels of chromate transformation (Keyhan et al, 2004). Identification of suitable soluble chromate-reducing enzymes will enable directed evolution studies aimed at enhancing the activity of these enzymes.

Previously we have described the biochemical purification, cloning and characterization of ChrR, a class I chromate-reducing flavoenzyme from *Pseudomonas putida* (Park et al, 2000; Ackerley et al, 2004a). Shortly after this work was published, Okazaki and coworkers released the sequence of a further chromate-reducing flavoenzyme from *Pseudomonas ambigua* that they had earlier characterized (Suzuki et al, 1992). These two FMN-binding enzymes share no homology with one another, but each belongs to a family that is highly conserved in bacteria and has homologs with greater than 30% sequence identity in over 60 genome-sequenced strains. We have cloned and characterized three homologs of each enzyme, and to distinguish the two families we refer to the ChrR homologs as Class I enzymes, and the *P. ambigua* enzyme

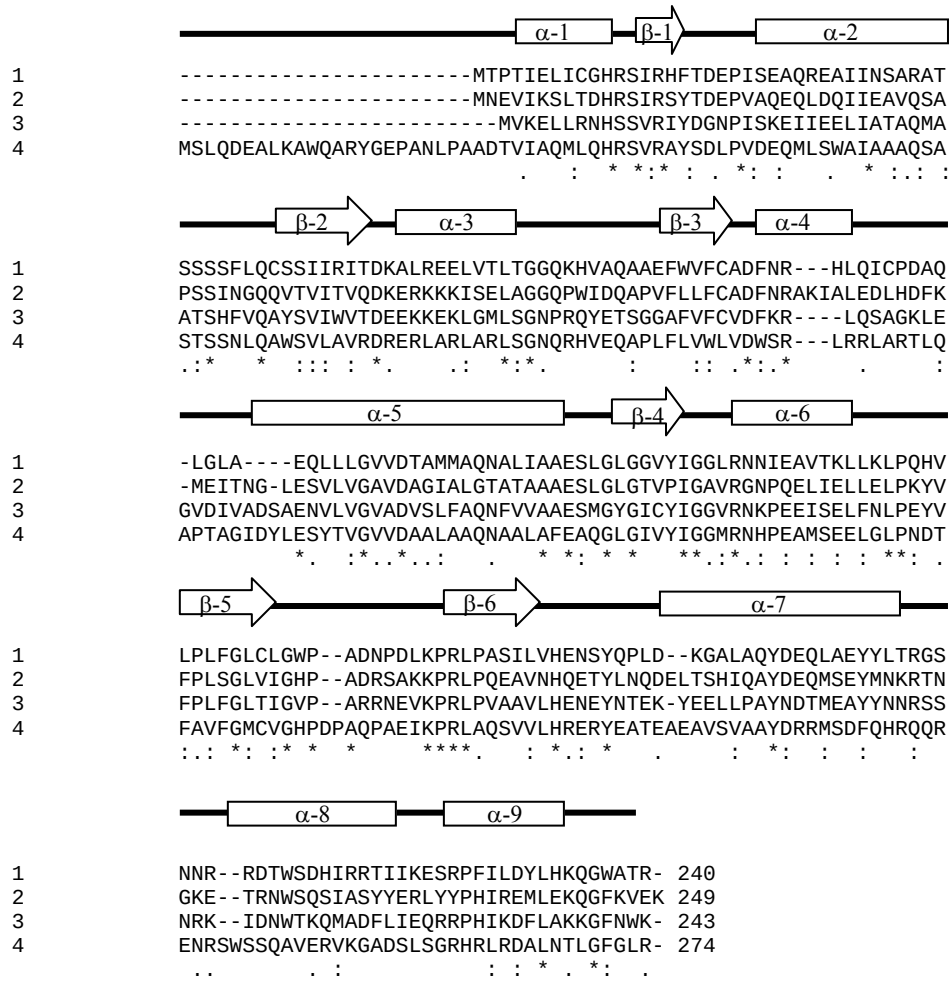


**Figure 1.** Alignment and secondary structural prediction for Class II enzymes.

| | | |
|---|---|---|
| ▭ | = α-helix | 1 = NfsA (*E. coli*) |
| ⇨ | = β-sheet | 2 = YcnD (*Bacillus subtilis*) |
| * | = fully conserved amino acid | 3 = Suzuki enzyme *(P. ambigua*) |
| : | = highly similar amino acid | 4 = EcdA (*P. putida*) |
| . | = similar amino acid | |

homologs as Class II (Park et al, 2002). All of the Class I enzymes that we have examined are efficient chromate and quinone reducers, but have no activity with nitrocompounds; while the Class II enzymes all reduce quinones and nitrocompounds effectively, but vary in their ability to transform chromate (Park et al, 2002). The most effective Class II chromate reducer that we identified, NfsA of *Escherichia coli*, had previously been well characterized in a nitroreduction context (e.g. Whiteway et al, 1998; Liochev et al, 1999). We have shown that in contrast with *chrR* (Ackerley et al, 2004a), an *nfsA* mutant is unaffected in viability and rate of chromate transformation in chromate-amended media; but that a strain overexpressing *nfsA* at high levels gives an increased rate of chromate reduction in cell suspensions (Ackerley et al, 2004b). Here we expand upon our earlier characterization of NfsA as a model Class II chromate

reductase, and consider whether the Class I or Class II enzymes are better suited to directed evolution studies aimed at generating an enhanced chromate-transforming strain.

## RESULTS AND DISCUSSION.

**The Class II Enzymes are Conserved in Secondary Structure.** The high degree of sequence conservation and wide distribution of the Class II enzyme family suggests a conservation of function also. This hypothesis would be strengthened if the proteins share structural similarity. To test this we compared the predicted secondary structures of the Class II enzymes that we had previously purified and characterized against that of the Suzuki et al (1992) enzyme (Fig 1). Strong similarities were found, suggesting that the Suzuki enzyme is likely also a nitro- and quinone reductase, as well as a chromate reducer.

***nfsA* is Induced by Chromate.** Despite the broad distribution and high degree of conservation of these enzymes, the biological role of even the best studied family member, NfsA of *E. coli*, remains unclear. No clearly beneficial role for the nitroreductase activity of NfsA has been identified, and it has been speculated that this may be a promiscuous activity, with quinone metabolism being more physiologically relevant (Bryant and DeLuca, 1991). Likewise, chromate reduction seems likely to be a vicarious property of this enzyme, given that chromate is a relatively recent environmental pollutant. Nevertheless, an *nfsA::lacZ* single copy transcriptional fusion was induced by chromate (Fig 2). Since *nfsA* is also induced by superoxide anions (Liochev et al., 1999), this induction may be a reflection of ROS generation within the cell in the presence of chromate.

**Mechanism of Chromate Reduction by NfsA.** Working with the Class I flavoenzymes ChrR (*P. putida*) and YieF (*E. coli*) we found that chromate reduction is unavoidably associated with ROS production (Ackerley et al, 2004a). The transfer of two electrons from the enzyme-associated FMN can either occur simultaneously ("tight"; as with YieF) or non-simultaneously ("semi-tight"; as with ChrR). In the former case there is the problem of four electrons from a dimeric enzyme being utilized in a three-electron reduction [Cr(VI) → Cr(III)], and we found that the fourth electron was consumed in stoicheometric production of ROS (Ackerley et al, 2004a). With non-simultaneous electron transfer, however, the problem is that chromate reduction appears to proceed via a Cr(V) intermediate; this species is highly prone to

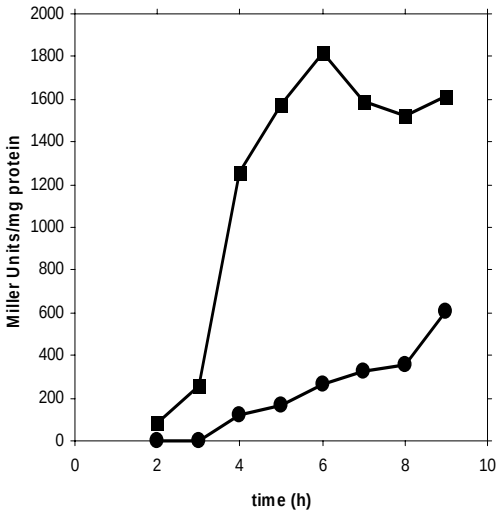


**Figure 2.** Expression of a single copy *nfsA::lacZ* fusion in *E. coli* K12.
■ *nfsA::lacZ* fusion expression + chromate
● *nfsA::lacZ* fusion expression - chromate

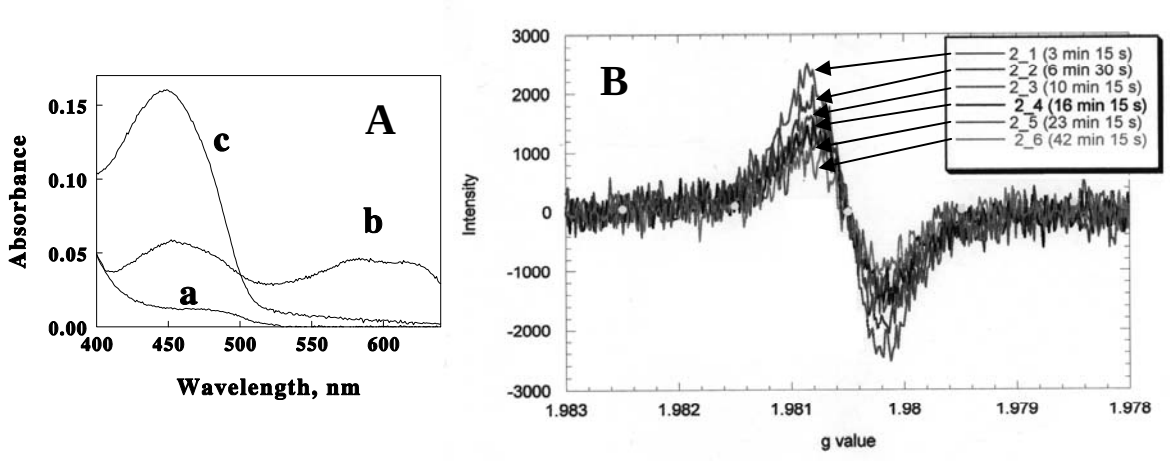


**Figure 3.** A flavin semiquinone and Cr(V) are intermediates of NfsA-catalyzed chromate reduction. **A.** Eigen spectra extracted from the data sets collected when 3.3 μM NfsA was rapidly mixed with limiting (10 μM) NADH and excess (40 μM) chromate. The spectra were obtained from a global fit of the absorbance versus wavelength versus time data to a biphasic kinetic mechanism. Spectra are identified as follows: a, reduced enzyme form; b, semiquinone form; c, oxidized form. **B.** ESR measurement of a Cr(V) intermediate; Cr(V) has a *g* value of *circa* 1.9805 at 3555 Gauss field.

redox cycling back to Cr(VI) and generating ROS in the process (Klein et al, 1998), and we found that ChrR consequently generates higher levels of ROS than YieF during chromate reduction (Ackerley et al, 2004a). To test whether or not NfsA is a tight or semi-tight chromate reductase we used a stopped-flow spectrophotometer to perform a rapid-scan kinetic analysis, measuring the change in absorption of the FMN cofactor of NfsA during electron transfer from the reduced enzyme to chromate. During this progression we observed three distinct species: a fully reduced flavin, whose disappearance was monitored at 403 nm; a fully oxidized flavin, whose appearance was recorded at 448 nm; and in between, a transitory flavin semiquinone whose brief appearance was noted at 580 nm (Ackerley et al, 2004b). Data presented here concerns scans taken across a 230 nm range every millisecond for 20 seconds. Figure 3A summarizes these data as a global fit of all of the absorbance versus time data to a biphasic kinetic mechanism. Spectrum a was that observed in the dead time of the instrument, and represents the fully reduced enzyme; spectrum c was the final absorbance recorded in the mixture after the reaction had ceased, and represents the oxidized enzyme; and spectrum b was the transient flavin semiquinone that formed and then disappeared during the overall oxidation reaction. The spectra of the semiquinone forms of NfsA and ChrR were slightly different; the ChrR semiquinone (Ackerley et al, 2004a) had lower absorbance at 450 nm than that of NfsA (Fig 3A, trace b).

The appearance of a one-electron flavin semiquinone during chromate reduction indicates a semi-tight reduction mechanism for NfsA, and implies formation of a Cr(V) intermediate. Appearance of Cr(V) was confirmed by electron spin resonance (ESR) studies (Fig 3B). In the ESR analysis NADPH was not limiting, so the reaction proceeded for a much longer duration, with Cr(V) levels progressively diminishing as all of the chromium was reduced to Cr(III) (Fig 3B).

**NfsA Generates Higher Levels of ROS Than Class I Enzymes.** The production of a Cr(V) intermediate during Cr(VI) reduction suggested that NfsA might generate higher levels of ROS than YieF, a tight chromate reducer. To test this we measured NAD(P)H utilization and $H_2O_2$ production during chromate reduction by YieF, ChrR, NfsA, and lipoyl dehydrogenase [LpDH; a one-electron chromate reducer (Shi and Dalal, 1990)]. We found that a higher percentage of electrons from NAD(P)H were consumed in $H_2O_2$ formation for NfsA than for either YieF or ChrR, but that NfsA generated lower levels of ROS than the one-electron reducer LpDH (Fig 4). These results are consistent with the stopped-flow and ESR data above and identify NfsA as a semi-tight chromate reducer; less tight than ChrR, but still more effective in reducing chromate per unit NAD(P)H consumed than the one-electron reducer LpDH.

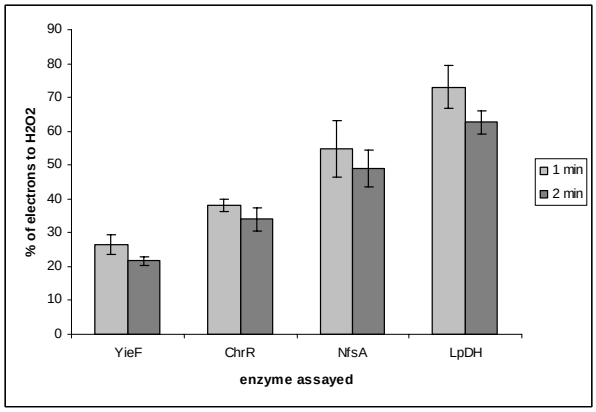


**Figure 4.** Bar graph showing the percentage of electrons from NAD(P)H that were consumed in $H_2O_2$ formation at one and two minute time points of a YieF-, ChrR-, NfsA-, or LpDH-catalyzed reduction of $K_2CrO_4$.

**CONCLUSIONS.** The Class I enzymes we have examined reduce chromate at a greater rate than the Class II enzymes (Park et al, 2002). That NfsA also generates higher levels of ROS than the Class I enzymes during this process suggests that the Class I enzymes are likely to be more effective in bio-engineering an improved chromate-transforming bacterial strain, and we are now proceeding with directed evolution studies focusing primarily on this Class. However, the great variety in chromate reducing capacity that we have observed with the different Class II enzymes (Park et al, 2002) offers promise that small changes in their primary sequence might have profound effects on their ability to reduce chromate, and the possibility remains that shuffling and evolving these enzymes will produce a highly effective chromate reducer.

**MATERIALS AND METHODS.**

**Bacterial Strains and Enzymes.** The single copy *nfsA::lacZ* fusion strain was generated by PCR amplifying the *nfsA* promoter region with the following primers: forward, (-319) 5'-ATCGAATTCAGACTGAAGGCTCACTTTGC-3'; reverse, (+180) 5'-ATCGCGGATCCACGTAACGCTTTGTCGGT-3' (Numbers in parentheses indicate position in relation to the translational start site; the forward primer binds approximately 40 bp upstream of the *nfsA* promoter, as determined by Paterson et al, 2002). The product was cloned into the fusion vector pRS415 (Simmons et al, 1987) using the *Eco* RI and *Bam* HI sites (underlined in primers), and the resulting plasmid was recombined in the phage RS45 (Simmons et al, 1987) and used to lysogenize *E. coli* K12 to obtain a single copy fusion strain.

Type I and II chromate reductases were purified as His-tagged protein from *E. coli* BL21 as described (YieF and ChrR, Ackerley et al, 2004a; NfsA, Ackerley et al, 2004b). LpDH from *Clostridium kluyveri* was purchased from Sigma.

**Computer Programs.** Homology searching for the Class I and II enzymes was performed using blastp at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. Sequences were aligned with Clustal W http://www.ebi.ac.uk/clustalw/, and secondary structural analysis was with J-PRED http://www.compbio.dundee.ac.uk/~www-jpred/ and PHD http://cubic.bioc.columbia.edu/predictprotein/.

**Assays.** To determine the induction pattern of the *nfsA::lacZ* fusion, overnight cultures grown in LB broth with 200 μM chromate were washed and used to inoculate LB with or without 200 μM chromate, and 250 μl aliquots were lysed using BugBuster solution (Novagen). β-galactosidase activity was assayed as described (Lomovskaya et al., 1995); the activity is expressed in Miller units. Rapid-scan kinetic analysis of NfsA was performed as described previously (Ackerley et al, 2004a). ESR spectra were obtained using a Burker EMX spectrometer, calibrated for the g value, as described (Palmer et al, 2002). Spectra were taken at room temperature. YieF, ChrR, NfsA, and LpDH were assayed under conditions described previously (Ackerley et al, 2004b). Chromate reduction was quantified by the diphenyl carbazide method (Greenberg et al, 1981), NAD(P)H utilization was measured spectrophotometrically at 340 nm, and $H_2O_2$ formation was quantified using the Amplex Red kit (Molecular Probes).

**REFERENCES.**


Ackerley, D. F., C. F. Gonzalez, C. H. Park, R. Blake, M. Keyhan, and A. Matin. 2004a. Chromate-reducing properties of soluble flavoproteins from *Pseudomonas putida* and *Escherichia coli*. *Appl Environ Microbiol 70*: 873-882.

Ackerley, D. F., C. F. Gonzalez, M. Keyhan, R. Blake, and A. Matin. 2004b. Mechanism of chromate reduction by the *Escherichia coli* protein, NfsA, and the role of different chromate reductases in minimizing oxidative stress during chromate reduction. *Environ Microbiol 6*: 851-860.

Bryant, C. and M. DeLuca. 1991. Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. *J Biol Chem 266*: 4119-4125.

Greenberg, A. E., Connors, J. J., Jenkins, D., and Franson, M. A. (ed.). 1981. Standard methods for the examination of water and wastewater, 15th ed., p.187–190. American Public Health Association, Washington, D.C.

Keyhan, M., D. F. Ackerley, and A. Matin. 2004. Targets of improvement in bacterial chromate bioremediation. *In: Remediation of Contaminated Sediments-2003.* M. Pellei and A. Porta (Eds), Batelle Press, Columbus, OH, ISBN 1-57477-143-4.

Klein, C., E. Snow, and K. Frenkel. 1998. Molecular mechanisms in metal carcinogenesis: role of oxidative stress (Chapter 4). *Molecular Biology of Free Radicals in Human Diseases*, ed. O. Aruoma, B. Halliwell: OICA International, p. 79-137.

Liochev, S. I., Hausladen, A., and Fridovich, I. 1999. Nitroreductase A is regulated as a member of the *soxRS* regulon of *Escherichia coli*. *Proc Natl Acad Sci USA 96*: 3537-3539.

Lomovskaya, O., Lewis, K., and Matin, A. 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. *J Bacteriol 177*: 2328-2344.

Okrent, D, and Xing, L. 1992. Future risk from a hypothesized RCRA site disposing of carcinogenic metals should a loss of societal memory occur. *J Hazard Mater 34*: 363-384.

Palmer, A.E., Quintanar, L., Severance, S., Wang, T.-P., Kosman, D. J., and Solomon, E. I. 2002. Spectroscopic characterization and $O_2$ reactivity of the trinuclear Cu cluster of mutants of the multicopper oxidase Fet3p. *Biochemistry 41*: 6438-6448.

Park, C. H., C. F. Gonzalez, D. F. Ackerley, M. Keyhan, and A. Matin. 2002. Molecular engineering of soluble bacterial proteins with chromate reductase activity. *Volume III of Proceedings of the First International Conference on Remediation of Contaminated Sediments*, Venice, Italy, Hinche et al. (ed.), Batelle Press, pp 103-111.

Park, C. H., Keyhan, M., Wielinga, B., Fendorf, S., and Matin, A. 2000. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl Environ Microbiol 66*: 1788-1795.

Shi, X. L., and Dalal, N. S. 1990. NADPH-dependent flavoenzymes catalyze one electron reduction of metal ions and molecular oxygen and generate hydroxyl radicals. *FEBS Lett 276*: 189-191.

Simmons, R. W., Houman, F., and Kleckner, N. 1987. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. *Gene 53*: 85-96.

Singh, J., Carlisle, D. L., Pritchard, D. E., and Patierno, S. R. 1998. Chromium-induced genotoxicity and apoptosis: relationship to chromium carcinogenesis (review). *Oncol Rep 5*: 1307-1318.

Suzuki, T., Miyata, N., Horitsu, H., Kawai, K., Takamizawa, K., Tai, Y., et al. 1992. NAD(P)H-dependent chromium (VI) reductase of *Pseudomonas ambigua* G-1: a Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). *J Bacteriol 174*: 5340-5534.

Whiteway, J., Koziarz, P., Veall, J., Sandhu, N., Kumar, P., Hoecher, B., et al. 1998. Oxygen-insensitive nitroreductases: analysis of the roles of *nfsA* and *nfsB* in

development of resistance to 5-nitrofuran derivatives in *Escherichia coli*. *J Bacteriol 180*: 5529-5539.