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Mechanisms for Uranium and Technetium Reduction in *Geobacter sulfurreducens*

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Abstract

The aim of this project was to characterize the mechanisms of electron transfer to U(VI) and Tc(VII) in a model dissimilatory Fe(III)-reducing bacterium. *Geobacter sulfurreducens* was chosen for initial studies because: 1) this organism is closely related to the predominant organisms that emerge when dissimilatory metal reduction is stimulated in subsurface environments by the addition of various electron donors and/or electron shuttling compounds; 2) the genome sequence of this organism was soon be completed; and 3) a genetic systems for this organism was available.

The protein hypothesized to reduce U(VI) in vivo, a periplasmic tri-heme cytochrome c_7 designated CycP, was purified to homogeneity and characterized in detail. In common with an analogous 13,000 kDa c-type cytochrome of closely related *Desulfovibrio* species, cytochrome c_7 reduced U(VI) in vitro. A mutant, prepared by PCR-mediated disruption of the *cycP* gene, was constructed and was compromised in its ability to couple acetate oxidation to the reduction of U(VI) and other metals. When *cycP* was expressed *in trans* the full capacity for metal reduction with acetate was restored. However, the mutant was able to couple hydrogen oxidation to U(VI) reduction at the same rate as the wild type strain, suggesting that the 9.6 kDa cytochrome, although involved in electron flow from acetate to U(VI), may not be the terminal reductase for the radionuclide. An outer membrane cytochrome of molecular mass 41 kDa was also studied in detail. This protein was active against U(VI) in vitro, and was localized to the surface of cells using a range of biochemical techniques, consistent with a role in reductive precipitation of extracellular insoluble U(IV). However, selective removal of the cytochrome from the surface of whole cells by protease treatment had no effect on U(VI) reduction, suggesting that this protein is not involved in U(VI) reduction. Reduction of Fe(III) oxide was abolished by protease treatment suggesting a role for the 41 kDa cytochrome in Fe(III) reduction, and confirming that Fe(III) and U(VI) are reduced via different mechanisms in *G. sulfurreducens*.

Several potentially important mechanisms of Tc(VII) reduction were also characterized. Whole cells of *G. sulfurreducens* coupled the oxidation of hydrogen to Tc(VII) reduction, resulting in precipitation of Tc(IV) in the periplasm. A periplasmic Ni/Fe hydrogenase was implicated as the Tc(VII) reductase by CO profiling,. An alternative, indirect mechanism for Tc(VII) reduction was also demonstrated. Fe(II)-bearing magnetite formed during the reduction of insoluble ferric oxide by *G. sulfurreducens* was able to abiotically transfer electrons to Tc(VII), leading to rapid and efficient precipitation of Tc(IV) on the surface of the mineral. Tc was removed to below the limit of detection by scintillation counting. Environmental relevance of this indirect mechanism was confirmed in enrichment cultures and sediment experiments. Uranium was also shown to function as an electron shuttle to Tc(VII), resulting in the capture of insoluble Tc(IV) by the extracellular U(IV) mineral phase formed. These results suggest

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that it may be possible to immobilize Tc(VII) through a direct enzymatic route, or by an indirect route via optimization of Fe(III) or U(VI) reduction in the subsurface.

Introduction

The use of Fe(III)-reducing bacteria for the remediation of radionuclide contaminated sediments. Dissimilatory metal-reducing bacteria utilize high valence metals as electron acceptors under anaerobic conditions. The most intensively studied metal-reducing bacteria have been those that reduce Fe(III) because dissimilatory Fe(III) reduction is likely to have been an early form of microbial respiration on Earth (44, 45) and remains one of the most geochemically significant processes in sedimentary environments (29, 43). Dissimilatory Fe(III)-reducing bacteria have also attracted considerable interest because they may prove useful for the bioremediation of contaminated environments (18, 20, 28). Many Fe(III)-reducing bacteria can also reduce metals other than Fe(III), including toxic metals and radionuclides such as uranium, technetium, chromate, cobalt, selenate, and arsenate (11, 13, 21, 30). Such metabolism can impact on the fate of these inorganic contaminants in the environment, and hence, may provide a mechanism for the bioremediation of metal- and radionuclide-contaminated waters and waste streams (18).

Model studies have demonstrated that Fe(III)-reducing bacteria have the potential to remediate uranium contamination in the subsurface via reductive precipitation of the radionuclide (6, 13, 31). This is of considerable relevance to the DOE because uranium has been highlighted as a primary radioactive metal contaminating DOE sites (35). The oxidized form of uranium, U(VI), is water soluble and mobile in most environments, and dominates uranium waste speciation. When reduced to U(IV) the radionuclide is highly insoluble and precipitates from most natural waters (10). Microbial reduction and precipitation of uranium may therefore prevent further migration of uranium contamination and could be used to concentrate uranium *in situ* for subsequent extraction. Natural analogs for this type of process include the concentration of uranium in sandstone or roll-type uranium deposits; the accumulation of uranium and other redox sensitive metals in "reduction spots"; and the reductive precipitation of uranium in anaerobic marine sediments (27, 30, 32). The potential application of Fe(III)-reducing bacteria to treat uranium contamination in the subsurface was illustrated using flow-through columns packed with material from a typical sand and gravel aquifer (6). U(VI) was completely removed as the mineral uraninite from contaminated groundwater in columns inoculated with the Fe(III)-reducing bacterium *Geobacter metallireducens*. In comparison, U(VI) passed through the aquifer material in uninoculated control columns. In another study, U(VI)-reducing microorganisms were able to reduce and precipitate U from contaminated groundwater from the DOE Hanford site (31). More recent studies have demonstrated that Fe(III)-reducing bacteria indigenous to the Shiprock aquifer are also able to remove U(VI) from solution via enzymatic reduction (4).

Technetium is another problematic radioactive contaminant at DOE sites (35) and it may also be immobilized via microbial activity (20). The oxidized form of technetium, Tc(VII), is highly soluble and mobile in the environment (47) but several reduced forms of Tc are insoluble (9). Recent studies have confirmed that Tc(VII), like U(VI), can be reduced and precipitated as Tc(IV) by Fe(III)-reducing bacteria (13, 21, 24, 46). Similar activities have also been detected in the enteric bacterium *Escherichia coli* (14), and sulfate-reducing bacteria (19, 22, 23). Furthermore, flow-through bioreactors containing cells of the enteric bacterium *Escherichia coli* (15), and the sulfate-reducing bacterium

Desulfovibrio desulfuricans (23, 25), have been used successfully to treat Tc(VII)-contaminated water.

Although U(VI) and Tc(VII) have been highlighted as the principal radioactive contaminants at DOE facilities, the mechanisms of electron transfer to these radionuclides remain poorly defined in the Fe(III)-reducing bacteria that will dominate in the subsurface in zones of metal reduction. In response to these requirements, a preliminary 2-year study was funded through the Biomolecular Sciences and Engineering Element of NABIR to determine the mechanisms of U(VI) and Tc(VII) reduction in *Geobacter sulfurreducens*.

Results

Choice of model system Initial studies focused on the mechanisms of electron transfer to U(VI) and Tc(VII) in *Geobacter sulfurreducens*. The primary reason for this choice was that molecular analysis of both field and laboratory studies had demonstrated that microorganisms closely related to *G. sulfurreducens* are the predominant Fe(III)-reducing bacteria present in a diversity of aquifers when dissimilatory metal reduction is stimulated (8, 38, 39, 41, 42). This was true whether metal reduction was enhanced with electron shuttling compounds such as humic acids or the humics analog, anthraquinone-2,6-disulfonate, or when any of a variety of electron donors for metal reduction was added. These results suggest that commonly considered strategies for stimulating the activity of metal-reducing microorganisms in the subsurface are most likely to stimulate the growth and activity of species closely related to *G. sulfurreducens*. This organism was also selected as the species of choice because it is able to reduce both U(VI) and Tc(VII) (13), the genome of this bacterium had recently been sequenced (available at http://www.tigr.org), and a genetic system was also available to make deletion mutants for physiological studies (3). Finally, we had extensive experience of culturing this organism for biochemical studies, a factor that helped lead to rapid progress towards the project goals.

U(VI) reduction Initial experiments focused on identifying the site of U(VI) reduction in whole cells of *G. sulfurreducens*. By analogy with previous results obtained using Fe(III)-reducing bacteria (7), it was expected that U(VI) would be reduced and precipitated outside the cell. This would be in keeping with the involvement of the surface-bound outer membrane cytochromes required for the transfer of electrons to insoluble Fe(III) oxides (5, 34, 37). By viewing thin sections of cells using transmission electron microscopy, however, we determined that, although some U(IV) was precipitated outside the cell, the primary site of U(IV) precipitation was within the periplasm (13). These results suggested that U(VI) is reduced within the periplasm by a mechanism that is distinct from the mechanism of Fe(III) reduction by *G. sulfurreducens*. This hypothesis was supported by the observation that cells treated with protease (which removed key redox active proteins on the surface of the cell including a *c*-type cytochrome of mass 40 kDa, a putative Fe(III) reductase (16)), were unable to reduce Fe(III) oxides but reduced U(VI) at a rate similar to that of untreated cells (13). These results confirmed that the mechanisms for Fe(III) reduction and U(VI) reduction are distinct in *G. sulfurreducens*. Insoluble Fe(III) oxides are reduced by an electron transfer

chain terminating at the cell surface (5, 16, 34, 37), while the terminal reductase for U(VI) is located in the periplasm (13).

Subsequent studies focused on the role of a 9.6 kDa *c*-type cytochrome in the reduction of U(VI) and other electron acceptors including Fe(III) and extracellular humics (12, 13, 16, 17). This protein was of interest because it is the major cytochrome present in the periplasm (12, 16, 40), identified as the site of U(IV) precipitation in *G. sulfurreducens* (13). It is also structurally related to the periplasmic cytochrome $c_3$of a number of sulfate-reducing bacteria of the Genus *Desulfovibrio*, shown previously to function as a U(VI) reductase in *D. vulgaris* (33). Initial experiments (12) confirmed that the protein was located in the periplasm, and was not secreted into the growth medium as proposed in a previous study (40). In addition, the protein did not function as an extracellular electron shuttle to insoluble Fe(III) oxides when added to cell cultures (12), as proposed previously (40). Given the contentious role of the 9.6 kDa *c*-type cytochrome in Fe(III) reduction, and its potential importance in U(VI) reduction, the protein was purified to homogeneity and characterized in detail. The purified protein was basic (pI = 9.5), contained three hemes, and had an N-terminal amino acid sequence closely related to the previously described tri-heme c_7 cytochromes of *Geobacter metallireducens* (1) and *Desulfuromonas acetoxidans* (2). The midpoint redox potential of the cytochrome c_7 was –170 mV as determined by cyclic voltammetry, consistent with the ability to transfer electrons to a range of metals including Fe(III), U(VI) and the humic analog menadione bisulfate *in vitro* (16). The gene encoding the c_7 cytochrome, designated *cycP*, was identified in the *G. sulfurreducens* genome from the N-terminal sequence of the mature protein. The gene encoded a basic, hydrophilic protein of 71 amino acids with three heme-binding domains, and an additional 20 amino acid N-terminal signal sequence peptide. The amino acid sequence deduced from *cycP* shared 49% identity with the cytochrome c_7 of *D. acetoxidans* (16). In order to determine the physiological role of the cytochrome c_7, a knockout mutant was prepared with a single-step recombination method (36).

Acetate-dependent Fe(III) reduction was significantly inhibited in both growing cultures and cell suspensions of the *cycP* mutant (16). In contrast, the mutation had no impact on Fe(III) reduction when hydrogen was supplied as the electron donor. As predicted cell suspensions of the mutant were also unable to reduce U(VI) with acetate as the electron donor, but U(VI) reduction was not impaired when hydrogen was supplied (13, 16). Similar results were obtained with the humic analog AQDS. When *cycP* was expressed *in trans* the full capacity for metal reduction with acetate was restored (16). This is the first report of the targeted disruption of a gene involved in the reduction of Fe(III), U(VI) or extracellular quinones in *G. sulfurreducens*. These results also demonstrate that portions of the electron transport pathways for acetate-dependent Fe(III), U(VI) and extracellular quinone reduction are significantly different to those for hydrogen-dependent reduction of these electron acceptors. The *cycP* mutant was also able to reduce fumarate at the same rate as the parent strain with either acetate or hydrogen, demonstrating that fumarate is reduced via a mechanism distinct to those of Fe(III), U(VI) or extracellular quinone reduction. When coupled with previous studies on other electron transport proteins in *G. sulfurreducens,* the results suggest that cytochrome c_7 serves as an intermediary electron carrier involved in electron transport from acetate to terminal Fe(III) reductases in the outer membrane. The results also support our

hypothesis that cytochrome c_7 is able to function as a U(VI) reductase in the periplasm of *G. sulfurreducens*, and may also act as a humics reductase *in vivo*. Future experiments are required to determine if a hydrogenase in *G. sulfurreducens* can reduce these electron acceptors directly, or whether additional electron transfer proteins coupled to a hydrogenase are able to bypass the cytochrome c_7, and reduce metals or humics when hydrogen is supplied as an electron donor. Finally, homologues to the 9.6 kDa cytochrome were also detected in other members of the family *Geobacteraceae* tested, using an antibody raised against the purified protein from *G. sulfurreducens,* and also by PCR using primers designed against regions of homology in the cytochrome c_7 genes of *G. sulfurreducens* and *D acetoxidans*. The gene encoding the cytochrome c_7 may, therefore, be potentially useful as a functional probe for *Geobacter* species and related organisms that couple acetate oxidation to U(VI) reduction in the subsurface.

Finally, a successful collaboration was initiated with Dr Marianne Schiffer at the Argonne National Laboratory on the structure of cytochrome c_7. Dr Schiffer and colleagues have cloned and expressed *cycP* in *Escherichia coli* together with a cytochrome *c* maturation gene cluster (26). This clone is available for detailed studies on the reduction of U(VI) and other metals in recombinant *E. coli* cells. The recombinant protein has also been crystallized and a preliminary structure obtained. Dr Schiffer now plans to alter key residues in the protein using the tools of site directed mutagenesis, and these mutants will also be available for studies on electron transfer to metals and radionuclides in future collaborations.

Tc(VII) reduction Tc(VII) was reduced by an alternative mechanism catalyzed by a hydrogenase. Acetate, which is an excellent electron donor for the reduction of Fe(III) and U(VI) by *G. sulfurreducens*, was a poor electron donor for Tc(VII) reduction. Hydrogen was the only donor that supported the reduction of Tc(VII) to insoluble Tc(IV) (24). The Tc(IV) was precipitated within the periplasm (24), and a periplasmic Ni/Fe hydrogenase was implicated as the Tc(VII) reductase by CO profiling. An alternative, indirect mechanism for Tc(VII) reduction was also demonstrated in *G. sulfurreducens*. Fe(II)-bearing magnetite formed during the reduction of insoluble ferric oxide by *G. sulfurreducens* was able to transfer electrons abiotically to Tc(VII), leading to rapid and efficient precipitation of Tc(IV) on the surface of the mineral (24). Tc was removed to below the limit of detection by scintillation counting. Environmental relevance of this indirect mechanism was confirmed in enrichment cultures and sediment experiments (24). Uranium was also shown to function as an electron shuttle to Tc(VII), resulting in the capture of insoluble Tc(IV) by the extracellular U(IV) mineral phase formed (13). These results suggest that it may be possible to immobilize Tc(VII) through a direct enzymatic route, or by an indirect route through optimization of Fe(III) or U(VI) reduction in the subsurface.

Dissemination of results

Results from the two-year study (1999-2001) were published in peer reviewed journals and also presented at conferences in poster form. In addition to the papers and poster presentations listed below, results from these studies were also presented in invited lectures at the 10th and 11th Annual Goldschmidt Meetings (2000 and 2001), the 223rd

ACS National Meeting (2002) and in seminars at several universities and National Laboratories in the US and Europe.

Publications

J. R. Lloyd, C. Leang, A.L. Hodges Myerson, M. Coppi, S. Cuifo, B. Methe, S. J. Sandler and D. R. Lovley (2002) Biochemical and genetic characterization of CytP, a small soluble cytochrome of *Geobacter sulfurreducens* Biochemical Journal (submitted)

Lloyd, J.R., Hodges Myerson, A.L., Yin, L. R.-S. and Lovley, D.R. (2002). Localization of c-type cytochromes in *Geobacter sulfurreducens* and the potential role of an outer-membrane cytochrome in Fe(III) oxide reduction. Geomicrobiology Journal. (submitted)

Lloyd, J.R. and Macaskie, L.E. (2002) The biochemical basis of radionuclide-microbe interactions. In "Microbiology and Radioactivity" Ed by F. R. Livens and M. Keith-Roach. Elsevier (in press)

Macaskie, L.E. and Lloyd, J.R. (2002) Microbial Interactions With Radioactive Wastes and Potential Applications In "Microbiology and Radioactivity" Ed by F. R. Livens and M. Keith-Roach. Elsevier (in press)

Lloyd, J.R., Chesnes, J., Glasauer S., Bunker, D.J., Livens, F.R. Lovley, D.R. (2002) Reduction of actinides and fission products by Fe(III)-reducing bacteria. Geomicrobiology Journal 19 103-120

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Lloyd, J.R., Sole, V.A., Van Praagh, C. V. G. and Lovley, D.R. (2000) Enzymatic and Fe(II)-mediated reduction of technetium by Fe(III)-reducing bacteria. Applied and Environmental Microbiology 66 3743-3749

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Poster presentations

Lloyd, J.R., Leang, C., Bunker, D., Livens, F., Glasauer, S, and Lovley, D.R (2001) Reduction of U(VI) by *Geobacter sulfurreducens*: physiological characterization and capture of technetium by insoluble U(IV). Poster Q-127 presented at 101st General meeting of the American Society for Microbiology, Florida May 20th-24th

Leang, C., Lloyd, J.R., Coppi, M., Sandler, S.J. and Lovley. D.R. (2001) Biochemical and genetic characterization of a small soluble cytochrome involved in Fe(III) reduction by *Geobacter sulfurreducens*. Poster Q-41 presented at 101st General meeting of the American Society for Microbiology, Florida May 20th-24th

Lloyd, J.R., Hodges Myerson, A.L., Methe, B. and Lovley, D.R. (2000) Localization of *c*-type cytochromes of *Geobacter sulfurreducens* and their role in Fe(III) reduction. Poster Q-257 presented at 100th General meeting of the American Society for Microbiology, Los Angeles May 21st-25th

Lloyd, J.R., Sole, V.A., Van Praagh, C. V. G. and Lovley, D.R. (2000) Direct and Fe(II)-mediated reduction of technetium by Fe(III)-reducing bacteria. Poster Q-323 presented at 100th General meeting of the American Society for Microbiology, Los Angeles May 21st-25th

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