

Bioreduction of Uranium: Environmental Implications of a Pentavalent Intermediate

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The release of uranium and other transuranics into the environment, and their subsequent mobility, are subjects of intense public concern. Uranium dominates the inventory of most medium- and low-level radioactive waste sites and under oxic conditions is highly mobile as U(VI), the soluble uranyl dioxocation $\{\text{UO}_2\}^{2+}$. Specialist anaerobic bacteria are, however, able to reduce U(VI) to insoluble U(IV), offering a strategy for the bioremediation of uranium-contaminated groundwater and a potential mechanism for the biodeposition of uranium ores. Despite the environmental importance of U(VI) bioreduction, there is little information on the mechanism of this transformation. In the course of this study we used X-ray absorption spectroscopy (XAS) to show that the subsurface metal-reducing bacterium *Geobacter sulfurreducens* reduces U(VI) by a one-electron reduction, forming an unstable $\{\text{UO}_2\}^+$ species. The final, insoluble U(IV) product could be formed either through further reduction of U(V) or through its disproportionation. When *G. sulfurreducens* was challenged with the chemically analogous $\{\text{NpO}_2\}^+$, which is stable with respect to disproportionation, it was not reduced, suggesting that it is disproportionation of U(V) which leads to the U(IV) product. This surprising discrimination between U and Np illustrates the need for mechanistic understanding and care in devising in situ bioremediation strategies for complex wastes containing other redox-active actinides, including plutonium.

Introduction

The nuclear weapons and nuclear energy generation programs of the past 60 years have created a legacy of waste and contamination worldwide. Three of the most problematic radioactive contaminants are the actinide elements uranium, neptunium, and plutonium. All three pose considerable long-

term environmental risks. The most stable environmental oxidation states of uranium and neptunium are VI and V, respectively, as the dioxo cations $\{\text{UO}_2\}^{2+}$ and $\{\text{NpO}_2\}^+$; both are highly soluble and so are relatively mobile and biologically available in the environment (1–4). Plutonium is expected to exist mainly as Pu(IV), which forms a highly insoluble hydrous oxide and is strongly bound by mineral surfaces and humic substances, although it may also be environmentally stable in the more soluble III, V, and VI oxidation states (3, 5, 6).

The bioreduction of U(VI) by anaerobic subsurface microorganisms has been the focus of much recent interest (7–10). Both Fe(III)- and sulfate-reducing bacteria have been shown to reduce U(VI), with *c*-type cytochromes involved in electron transfer to the actinide (11–13). Periplasmic cytochrome c_3 has been implicated in electron transfer to U(VI) in sulfate-reducing bacteria (12–14), while analogous periplasmic cytochromes, including PpcA (cytochrome c_7) and homologues, may play a similar role in *Geobacter* species (15). The uranium is precipitated within the periplasm and outside the cell. However, the mechanism of reduction is not clear. It is possible that U(VI) is reduced directly to U(IV) by a two-electron transfer, or alternatively, the mechanism may involve a one-electron transfer, forming a U(V) intermediate. This disproportionates readily $[2 \text{ U(V)} \rightarrow \text{U(VI)} + \text{U(IV)}]$, particularly at pH values below neutral, but has been characterized in aqueous solution and shown to form an actinyl ion, $\{\text{UO}_2\}^+$, as might be expected by analogy with Np(V) or Pu(V) (16, 17). The mechanism of U(VI) reduction also has important implications for the potential microbial reduction of transuranic elements with environmentally stable lower oxidation states. Reduction of mobile $^{237}\text{Np(V)}$, another long half-life α -emitting radionuclide, to Np(IV) and subsequent precipitation may be advantageous, while remobilization of strongly sorbed Pu(IV) by reduction to more soluble Pu(III) species could have important environmental implications. Conversely, selective reduction might allow targeting of particular radionuclide species. Moreover, the reduction of pentavalent actinides per se remains to be tested rigorously, despite the environmental relevance of Np(V) and Pu(V) (5, 18).

Strategies for the in situ bioremediation of uranium have focused on the stimulation of indigenous Fe(III)-reducing bacteria through simple amendments to the subsurface such as the addition of electron donor, e.g., acetate, for U(VI) reduction and precipitation (7). Under these conditions, *Geobacter* species predominate (19, 20), so for this reason we focused on the mechanism of U(VI) reduction by a model *Geobacter* species, *Geobacter sulfurreducens* (21), using X-ray absorption spectroscopy (XAS) to define U speciation in situ.

Experimental Section

Maintenance and Growth of Organism. *G. sulfurreducens* (ATCC 51573) was grown at 30 °C under anaerobic conditions in a modified freshwater medium as described previously (11). Acetate (20 mM) and fumarate (40 mM) were supplied as the electron donor and the electron acceptor, respectively. Cells were manipulated under an atmosphere of N_2 at all times.

Safety. Depleted uranium is radioactive and should be used with appropriate precautions. Neptunium-237 is a high specific activity α -emitter and should only be used in specialist facilities by suitably qualified and experienced personnel. The possession and use of radioactive materials is subject to statutory controls.

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Resting Cell Experiments. All manipulations of cells were carried out under an atmosphere of N_2 . Late-log-phase cultures were harvested by centrifugation and washed twice in $NaHCO_3$ buffer (30 mM, pH 7, degassed with N_2 - CO_2 80:20 mix) for uranyl experiments or with 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer (20 mM, pH 7, degassed with N_2) for neptunyl experiments. Aliquots of the washed cell suspensions were added to a final concentration of 0.5 mg mL^{-1} dry weight biomass, by use of a syringe fitted with a needle, to anaerobic bottles sealed with butyl rubber stoppers containing (i) uranyl(VI) acetate (5 mM) in $NaHCO_3$ buffer (30 mM, pH 7) and acetate (10 mM) as the electron donor, or (ii) neptunyl(V) (1 mM) in MOPS buffer (20 mM, pH 7), with acetate (10 mM) as the electron donor or no electron donor present. For the neptunyl experiments, bottles were also prepared with cells autoclaved prior to inoculation and without cells as controls.

Different buffers were used for the different radionuclides to prevent precipitation from solution. Bicarbonate buffer was used for the uranyl experiments to prevent hydrolysis of $\{UO_2\}^{2+}$ at pH 7. However, $\{NpO_2\}^+$ precipitates from solution at pH 7 in the presence of bicarbonate, necessitating the use of an alternative buffer (MOPS). All cultures were incubated at 30 °C in the dark under an atmosphere of N_2 , and samples of the cultures were taken at regular time intervals under an N_2 atmosphere. The supernatants were obtained by centrifugation (under an atmosphere of N_2) of the culture samples. Aliquots of both the culture and supernatant from the uranyl experiment were frozen in liquid N_2 and then analyzed by XAS (see below), alongside standards including U(V) model samples generated electrochemically (17). Supernatant samples from the neptunyl experiments were analyzed by γ -ray spectroscopy or α liquid scintillation counting to determine Np concentrations and by UV/vis/NIR spectroscopy to determine Np oxidation state (see below).

X-ray Absorption Spectroscopy. U L_{3} -edge X-ray absorption spectra were collected on station 16.5 at the CLRC Daresbury Synchrotron Radiation Source, operating at 2 GeV with a typical beam current of 150 mA. A double-crystal Si(220) monochromator and focusing optics were used, with the incident beam intensity detuned to 80% of maximum for harmonic rejection. Spectra were recorded at 80 K in fluorescence mode, by use of a 30-element Ge detector. Multiple (6–8) scans were collected for all samples to improve data quality. Samples containing U solids were also analyzed in fluorescence mode because there was insufficient material for measurement in transmission mode. As a result, although neighboring atom identities and interatomic distances will be correct for these samples, the occupancies will be lower than the true values.

The spectral data were calibrated and background-subtracted by use of the Daresbury Laboratory programs EXCALIB and EXBACK. The isolated EXAFS data were analyzed with EXCURV98 (22), employing Rehr-Albers theory (23) and using full cluster multiple scattering calculations where necessary. Phase shifts were derived from ab initio calculations with Hedin-Lundqvist potentials and von Barth ground states (24). Theoretical fits were obtained by adding shells of backscattering atoms around the central absorber atom (U) and refining the Fermi energy, E_f , the absorber–scatterer distances, r , and the Debye–Waller factors, $2\sigma^2$, to minimize the sum of the squares of the residuals between the experiment and the theoretical fit. Initially, shell occupancies were fixed at the integral values that gave the best fit. Shells were only included in the analysis if they reduced the overall goodness of fit (R -factor) by $>5\%$. Further fitting was carried out for samples containing mixtures of U oxidation states that could not be fitted satisfactorily with integral shell occupancies. For these, the Debye–Waller

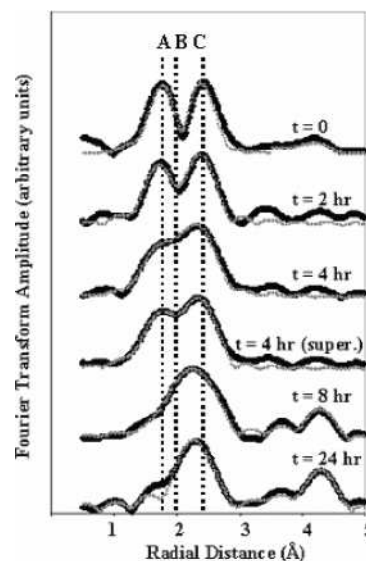


FIGURE 1. Progressive changes over time in Fourier transforms of U L_{3} -edge extended X-ray absorption fine structure spectra of (top to bottom) cell suspension at $t = 0$; cell suspension at $t = 2$ h; cell suspension at $t = 4$ h; supernatant from centrifuged cell suspension at $t = 4$ h; cell suspension at $t = 8$ h; and cell suspension at $t = 24$ h. Experimental data are denoted by solid black lines; fits, by broken gray lines. A, B, and C denote the positions of the principal features arising from U^{VI} –O (axial), U^V –O (axial), and $U^{V,VI}$ –O (equatorial), respectively.

factors were fixed at the values obtained from fits with integral occupancies and then the refinement was continued, with E_f , r , and $2\sigma^2$ allowed to vary. The different U species were then quantified; the occupancies of the shells at 1.76–1.81, 1.91–1.94, and 2.28–2.36 Å were used as measures of the U(VI), U(V), and U(IV) concentrations, respectively.

Np Analysis. Np(V) was determined by near-infrared spectroscopy with the characteristic absorption at 980 nm. Total Np concentrations were determined by γ -ray spectroscopy with a hyperpure Ge detector (50% relative efficiency) fitted with a 10 cm Pb shield, lined with Cu and Cd, to reduce background. For the determination of Np concentrations in solution, samples were centrifuged and the supernatant was analyzed by α liquid scintillation counting with an LKB Quantulus liquid scintillation counter, with pulse shape analysis for α - β discrimination.

Results and Discussion

The three uranium oxidation states of interest in this study can be distinguished clearly by extended X-ray absorption fine structure (EXAFS) spectroscopy (Figure 1; Table 1A). U(VI) and U(V) have diagnostic axial U–O distances of 1.80 and 1.94 Å, respectively, while hydrous UO_2 gives U–O distances of 2.37 Å and U–U distances of 3.87 Å. After 2 h of incubation, there was an indication of minor ($<5\%$) U(V), although it cannot be quantified accurately; by 4 h, $60\% \pm 10\%$ of the uranium was in oxidation state V (Table 1B), and by 8 h, the majority ($80\% \pm 10\%$) of the uranium was transformed into $UO_2 \cdot 2H_2O$ (Figure 2). After 24 h, all the uranium had been reduced. Analysis of the supernatant from a centrifuged sample after 4 h showed that the U(V) was not associated exclusively with the cells.

The formation of U(V) substantially before the first appearance of U(IV) suggests that biological transformation may proceed via one-electron reduction of U(VI), followed by disproportionation and hydrolysis to generate the final U(IV) product. This mechanism would not require a second enzymatic electron-transfer reaction to achieve the reductive precipitation of U(IV) and is consistent with the presence of insoluble $UO_2 \cdot 2H_2O$ both in the periplasm and outside the

TABLE 1. Fitting Parameters Obtained from Uranium L₃-Edge EXAFS Spectroscopy^a

sample	shell no.	occupancy, type	distance (Å)	2σ ² (Å ²)
(A) Model Samples Containing U(VI), U(V), and U(IV) and <i>G. sulfurreducens</i> Samples at t = 0 and t = 24 h				
[U ^{VI} O ₂ (CO ₃) ₃] ⁴⁻	1	2 O	1.80	0.008
	2	6 O	2.45	0.010
	3	3 C	2.88	0.007
	4	3 O	4.28	0.009
[U ^V O ₂ (CO ₃) ₃] ⁵⁻	1	2 O	1.94	0.012
	2	6 O	2.47	0.005
	3	3 C	2.90	0.010
[(U ^{VI} O ₂) ₃ (CO ₃) ₆] ¹⁶⁻	1	2 O	1.79	0.009
	2	6 O	2.45	0.015
	3	3 C	3.92	0.005
	4	2 U	5.07	0.013
	5	3 O	5.98	0.019
U ^{IV} O ₂ ·2H ₂ O*	1	1.5 O	2.37	0.013
	2	1.5 U	3.87	0.008
culture (t = 0)	1	2 O	1.81	0.010
	2	6 O	2.45	0.014
culture (t = 24 h)*	1	6 O	2.36	0.018
	2	5 U	3.85	0.011
(B) <i>G. sulfurreducens</i> Samples at t = 2, 4, and 8 h				
culture (t = 2 h)	1	2 O	1.80	0.016
	2	4 O	2.43	0.007
culture (t = 4 h)	1	0.6 O	1.76	0.012
	2	1.0 O	1.91	0.023
	3	5.0 O	2.43	0.026
culture (t = 4 h) (supernatant)	1	0.8 O	1.78	0.009
	2	0.8 O	1.92	0.007
	3	3.6 O	2.47	0.015
culture (t = 8 h)*	1	0.3 O	1.81	0.016
	2	3.0 O	2.28	0.012
	3	3.0 O	2.44	0.009
	4	3.0 U	3.84	0.014

^a Data for the U(V) and U(VI) models are consistent with results published elsewhere (17, 25). Uncertainty in interatomic distances is ±0.02 Å for well-resolved shells and ±0.06 Å for complex mixtures of species. Since all samples were analyzed in fluorescence mode, shell occupancies from data fitting will be underestimates in samples (marked with asterisks) where a significant proportion of U is present as dispersed, fine-particle U solid phases.

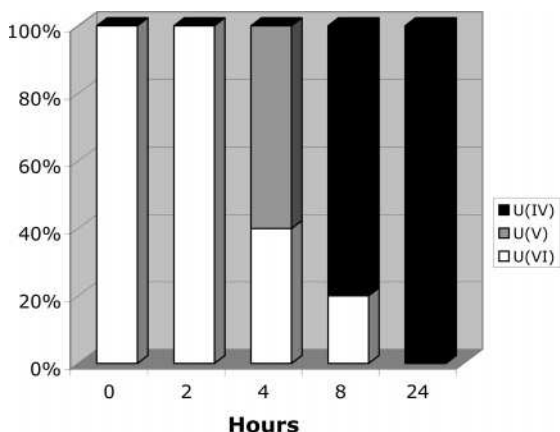


FIGURE 2. Reduction of U(VI) by washed cell suspensions of *G. sulfurreducens* and the detection of U(V) as an unstable intermediate. The three uranium oxidation states of interest in this study (VI, V, and IV) were distinguished by extended X-ray absorption fine structure (EXAFS) spectroscopy.

cells of U(VI)-reducing bacteria (12, 15) since the U(V) precursor can diffuse into solution from the cellular site of

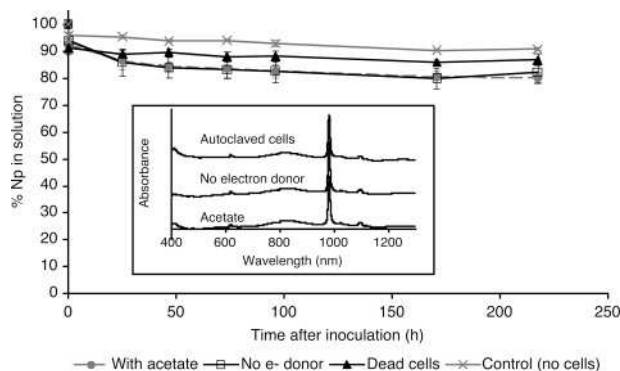


FIGURE 3. Interactions of *G. sulfurreducens* with Np(V), in the presence of different electron donors. Error bars show ±one standard deviation (n = 3). The inset shows UV/visible absorption spectra for supernatant samples from cultures containing {Np^VO₂}⁺ incubated for 217.5 h at 30 °C. The sharp peak at 980 nm is diagnostic of the presence of {Np^VO₂}⁺ in solution. There are no features in the spectra indicative of the presence of any other oxidation state of Np.

reduction, thought to be the periplasm (15, 26), before disproportionating.

While both U(V) and Pu(V) are unstable with respect to disproportionation, Np(V) is not. If U(V) is reduced via disproportionation and is not reduced by an enzyme-mediated electron-transfer step, then it might be expected that *G. sulfurreducens* would not reduce the Np(V) analogue. If enzymatic reduction of U(V) does occur, then thermodynamically, reduction of Np(V) should also be possible by this organism. To test this hypothesis, cells of *G. sulfurreducens* were challenged with 1 mM {Np^VO₂}⁺ and acetate (10 mM) as the electron donor under an atmosphere of N₂. Control cultures contained live cells with no electron donor or dead cells (autoclaved), both incubated with {Np^VO₂}⁺. Figure 3 shows the amount of Np in solution with time after inoculation. In all cultures, ~13% of Np was removed from solution within the first 24 h, with little more removed after longer periods (up to 218 h). This small loss of Np from solution is not dependent on the presence of electron donor, nor does it require viable cells, and therefore it is likely to be caused by biosorption to the cell rather than the reduction of Np(V) to Np(IV). UV/vis/NIR spectroscopy of the culture supernatants (Figure 3, inset) confirmed that the Np remaining in solution was still present as {Np^VO₂}⁺ after contact with the cells for 218 h, as only peaks attributed to {NpO₂}⁺ were observed, including the dominant 980 nm peak. Actively growing cells were also unable to reduce Np(V) added to the growth medium (data not shown). These results support the hypothesis that *G. sulfurreducens* is unable to reduce pentavalent actinides, despite the fact that, thermodynamically, an enzyme able to reduce Fe(III) should also be able to reduce Np(V) to Np(IV) (27). Therefore, it is most likely that the reduction of U(V) to U(IV) proceeds via disproportionation.

The selective reduction of U(VI) demonstrated by *G. sulfurreducens* has important implications both for the environmental behavior of actinides and for potential bioremediation processes. The most stable Np species in the environment is {Np^VO₂}⁺, leading to high environmental mobility. These results indicate that *G. sulfurreducens* alone is unlikely to be effective in reducing and immobilizing this form of Np. Environmental Pu is present in oxidation states V or IV (5, 18) and our results suggest that bioreduction of Pu(V) by *Geobacter* species would not occur. The potential for bioreduction of Pu(IV) to the more mobile Pu(III) is less clear, particularly since complexation can alter the redox properties substantially. To conclude, our results with *G. sulfurreducens* suggest that it will be very difficult to predict

the impact of microbial reduction on other actinides, including Pu, in the environment, and studies into the effect of microbes on Pu will require careful examination under field conditions.

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