Published in final edited form as:

J Inorg Biochem. 2008 April ; 102(4): 809–823. doi:10.1016/j.jinorgbio.2007.12.001.

The Uptake and Fate of Vanadyl Ion in Ascidian Blood Cells and A Detailed Hypothesis for the Mechanism and Location of Biological Vanadium Reduction: A Visible and X-Ray Absorption Spectroscopic Study

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Abstract

Vanadium K-edge x-ray absorption spectroscopy (XAS) has been used to track the uptake and fate of VO²⁺ ion in blood cells from *Ascidia ceratodes*, following exposure to dithiothreitol (DTT) or to DTT plus VO²⁺. The full range of endogenous vanadium was queried by fitting the XAS of blood cells with the XAS spectra of model vanadium complexes. In cells exposed only to DTT, ~0.4% of a new V(III) species was found in a site similar to Na[V(edta)(H₂O)]. With exposure to DTT and VO²⁺, average intracellular [VO(aq)]²⁺ increased from 3% to 5%, and 6% of a new complexed form of vanadyl ion appeared evidencing a ligand array similar to [VO(edta)]²⁻. At the same time, the relative ratio of blood cell [V(H₂O)₆]³⁺ increased at the expense of [V(H₂O)₅(SO₄)]⁺ in a manner consistent with a significant increase in endogenous acidity. In new UV/visible experiments, VO²⁺ could be reduced to 7-coordinate [V(nda)(H₂O)₃] or [V(nda)(ida)]²⁻ with cysteine methyl ester in pH 6.5 solution. Ascorbate reduced [VO(edta)]²⁻ to 7-coordinate [V(edta)(H₂O)]⁻, while [VO(trdta)]²⁻ was unreactive. These results corroborate the finding that the reductive EMF of VO²⁺ is increased by the availability of a 7-coordinate V(III) product. Finally a new and complete hypothesis is proposed for an ascidian vanadate reductase. The structure of the enzyme active site, the vanadate-vanadyl-vanadic reduction mechanism, the cellular locale, and elements of the regulatory machinery governing the biological reduction of vanadate and vanadyl ion by ascidians are all predicted. Together these constitute the new field of vanadium redox enzymology.

Phlebobranch ascidians (tunicates) are vanadium-concentrating, filter-feeding marine Urochordates that have a continuous evolutionary history reaching back into the upper Cambrian [1–4]. Although vanadium in ascidian blood cells has been under active investigation for almost 100 years [5–7], the mechanisms of uptake, transport, and reduction of oceanic

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vanadate remain largely unknown. The biologically unique highly acidic intracellular vacuolar aqua vanadium (III, IV) solutions, concentrated primarily in so-called signet ring blood cells, has been successfully described [8–22]. Biologically exotic intracellular V(III) and V(IV) complexes have also been detected in high concentrations with blood cells from *Phallusia nigra* [19], as well as possibly V(III,IV) micro-granules within the blood cells of *Ascidia sydneiensis samea* [23]. Reviews discussing various aspects of ascidian blood cell chemistry are available [20,24,25].

Exposure of the blood cells of *P. nigra* to the aliphatic dimercaptan dithiothreitol (DTT) was shown to induce a new intracellular V(III) environment similar to 7-coordinate [V(edta)(H₂O)][−], possibly reflecting a metalloprotein site [19]. This new V(III) was likely reductively produced at the expense of a significant fraction of the antegenous (i.e., pre-originant) intracellular aqua vanadyl ion.

Here we report results from experiments designed to explore whether exposure to DTT also reorganizes the intracellular vanadium in whole blood from the tunicate *Ascidia ceratodes*. A preliminary report of some of this work has been given [26,27]. Unlike the case for *P. nigra* [19,28], however, aquated vanadyl ion is typically scarce in the blood cells of *A. ceratodes* [15,22,29–31]. Nevertheless, tunicate blood cells can directly take up vanadium [32–34]. Thus, isolated *A. ceratodes* whole blood cells were incubated with DTT either alone or along with added inorganic vanadyl ion. The uptake and fate of vanadium was tracked using vanadium K-edge XAS. New UV/visible spectroscopic experiments testing the generality of the mild aqueous-phase reductive chemistry of VO²⁺ [35] are also reported. Finally, on the basis of these and previous results, a new molecular-level hypothesis for the enzymatic reduction of vanadate and vanadyl to V(III) in ascidians is herein proposed. On the basis of this hypothesis, the structure of the enzyme active site, the mechanism of vanadium reduction, and the sub-cellular locale where vanadium reduction occurs in ascidians are all predicted.

Materials and Methods

Used as received were 99+% disodiumdihydrogenethylenediamine-N,N,N',N'-tetraacetic acid (Na₂H₂edta), 99% trisodium nitrilotriacetic acid monohydrate (nta), 98% iminodiacetic acid (ida), 98% cysteine methyl ester hydrochloride, 97% VCl₃, and 99.99+% VOSO₄·3H₂O, all obtained from Aldrich Chemical Co. 1,3-Diaminopropane-N,N,N',N'-tetraacetic acid (trdta) was obtained from Fluka Chemicals, and 2-[N-morpholino]ethanesulfonic acid (MES) was obtained from Sigma Chemicals. Deionized water, and aqueous 100 mM pH 6.5 MES buffer, were made anaerobic by three freeze-pump-thaw cycles under an argon atmosphere. All pH measurements were made using an Orion Model 720 pH meter and a Ross combination electrode.

The chemical reduction experiments described below were carried out in a PlasLabs anaerobic glove box, under a dinitrogen atmosphere with a typical measured dioxygen level of 200 ppm. Solid VCl₃ was weighed out on a Mettler SAG 204 microbalance under a dinitrogen atmosphere within a Vacuum Atmospheres glove box operating at ~1 ppm dioxygen. Aqueous solutions of VCl₃ with various chelating ligands were prepared in the PlasLabs glove box, and these solutions were passed through a 0.2μ nylon filter prior to use.

Unless otherwise noted, UV/visible spectra were measured over the range 190 nm – 820 nm using a Hewlett-Packard 8452A diode array spectrophotometer. Fresh blank scans were recorded before each measurement. Reaction solutions were sealed in anaerobic Spectrosil quartz cuvettes of 10 mm path length using silicone-greased 14/35 ground glass caps. These cuvettes are capable of maintaining N,N-dimethylformamide solutions of iron(II)-tetraethanethiolate (E_{1/2} = ~−1V vs. NHE) overnight in air without detectable oxidation [36].

Except as described below, anaerobic reduction experiments were carried out by appropriate dilution of ~100 mM stock solutions to a final volume of 1.00 mL. Stock solutions of $[\text{VO}(\text{edta})]^{2-}$, $[\text{VO}(\text{trdta})]^{2-}$, of all the chelating reagents, of cysteine methyl ester, and of ascorbic acid were prepared in 100 mM 2-[N-morpholino]ethanesulfonic acid (MES) pH 6.5 buffer. MES buffer was chosen because it shows no discernable affinity for $[\text{Cu}(\text{aq})]^{2+}$ [37] and thus likely none for $[\text{VO}(\text{aq})]^{2+}$. The ligand H_4trdta was neutralized using powdered solid KOH, and made to a stock solution of 0.10 M $\text{K}_{2.35}\text{H}_{1.65}\text{trdta}$ in water. Solutions of cysteine methyl ester in pH 6.5 MES were prepared from the hydrochloride and discarded after two days. Stock vanadyl sulfate was 100.7 mM in deionized water. Where necessary, solution pH was adjusted to 6.5 in the glove box using powdered solid KOH and the pH was monitored using colorpHast® strips of pH 5.5–10 range. The color strip response was validated within the PlasLabs glove box using anaerobic buffers of known pH. Typical reaction conditions were 10 mM $[\text{VO}(\text{edta})]^{2-}$, 3 mM free VO^{2+} and 20 mM cysteine methyl ester, all in 100 mM pH 6.5 MES buffer. When used as reductant, ascorbic acid was 10 mM to keep the number of electron equivalents equal to two. These reactions were carried out sealed in the above anaerobic cuvettes and monitored by means of UV/visible spectra.

For the experiments shown in Figure 8, reduction of $[\text{VO}(\text{edta})]^{2-}$ using cysteine methyl ester in the presence of excess vanadyl ion [35], was carried out in duplicate, in either 10.00 mL or 6.00 mL total volume in MES buffer, pH 6.5. One mL aliquots were removed from the stirred solutions at intervals and passed through a 0.2 μ nylon filter before measuring the UV/visible spectrum. This filtration removed the suspension of hydrous vanadyl oxide that otherwise obscured these data. Filtration was not necessary for the experiments using the NTA/IDA ligand mixture, or for the experiments that employed ascorbate as the reductant. All other reductive model experiments reported here were carried out at least in duplicate. Solutions were quantitatively transferred using Hamilton gas-tight micro-syringes.

Ascidia ceratodes were collected from beneath the floating docks of the Spud Point Marina, Bodega Bay, California, USA. Specimens were again noted to be typically larger than their conspecifics at the Monterey Yacht harbor, 200 km to the south. Individual *A. ceratodes* exhibiting a firm integument, a strong heart beat, and an immediate response of the siphons to gentle stimulus were judged to be healthy. Whole blood, used for the experiments described below, was obtained by cardiac puncture using 5 cc sterile plastic syringes equipped with 1-inch 25-gauge Luer-lock needles. Blood taken from several specimens was pooled in closed sterile plastic tubes, which were kept on ice. No discoloration or untoward cell lysis were observed in pooled blood cell samples, and, as noted previously, mixed cells from several *A. ceratodes* specimens exhibited viability in the Trypan Blue assay after hours of maintenance on ice [22].

The incubation experiments were carried out in triplicate. Preliminary tests showed that the blood cells remained viable under the described protocol (see the Results section). Three individual blood cell samples were prepared from three separate cohorts of individuals. Each pooled blood cell sample was suspended and then further divided into three equi-volume fractions, each of which should have reflected the entire sample of cells from the apposite cohort of specimens. Each of the three sample sets consisted of an untreated control, blood cells incubated with 25 mM DTT, and blood cells incubated with a combination of 25 mM DTT plus 25 mM VOSO_4 . All reagent solutions were prepared in ice-cold PBS buffer, which consisted of a pH 6.6 solution of 0.5 M NaCl and 25 mM sodium phosphate. Blood cells were also kept on ice during incubation. Series 1 samples consisted of the combined blood cells from 15 specimens and were incubated for 40 min. Series 2 samples included whole blood from 9 specimens and a 35 min. incubation time. Series 3 sample included whole blood from 14 specimens and 30 min. incubation. In all cases the total volume of blood obtained from the chosen *A. ceratodes* specimens determined the number of individuals used. Each series was

thus composed to be internally self-consistent, so that any x-ray spectroscopic changes observed within a given series should reflect the conditions of incubation rather than an artifact of differential blood cell populations.

After the above-described incubation, each blood cell sample was washed by centrifugation at 100×g/5 min., followed by re-suspension in ice-cold PBS buffer and a second similar centrifugation. The pellet from the second centrifugation step was re-suspended in 150 µL of 0.5 M NaCl in 30% aqueous ethylene glycol. Previous work showed blood cells to be viable in this solution [16]. The thick suspension of blood cells was then loaded into a Lexan XAS sample cell and frozen in liquid nitrogen until XAS measurement. Post-incubation preparations from all nine XAS blood cell samples were examined microscopically. In every case, the cells appeared completely normal and healthy. Images of all nine XAS samples were obtained at 400× or 600× magnification using a Nikon Eclipse TE 300 photomicroscope (Supplemental Figure 1).

Blood cell vanadium K-edge XAS spectra were measured on SSRL SPEAR-2 beam line 7-3 under dedicated operating conditions of 3 GeV, a wiggler field of 17 kG, and 70–98 mA of current. The x-ray beam was energy resolved using a Si[220] double crystal monochromator detuned 50% at 5861 eV (end-of-scan) to minimize harmonic contamination. Vanadium K-edge fluorescence excitation spectra were measured using an Ar-filled ionization chamber detector (Stern-Heald-Lytle detector), with a Ti filter and Soller slits set at 90° from the incident X-ray beam.

Vanadium foil calibrations were measured as transmission spectra after every four data scans, using an in-line nitrogen-filled ionization chamber as detector. All XAS samples were held at 10 K using an Oxford Instruments CF1208 continuous flow liquid helium cryostat.

The raw vanadium XAS spectra were reduced to analyzable XAS spectra using the program Process within the general EXAFSPAK software package, written by Prof. Graham George, University of Saskatchewan, and available free of charge at the SSRL web-site: <http://www-ssrl.slac.stanford.edu/exafspak.html>. A 2nd order polynomial was fit to the vanadium K-edge XAS data over the range 5550–5861 eV and subtracted from the data. The spectra were then normalized to an edge jump of 1.00. The blood cell XAS spectra were calibrated with respect to the first inflection at 5464.0 eV on the rising edge of a vanadium foil K-edge spectrum, measured concurrently. Each final spectrum represents the average of 16 to 20 individual scans. The vanadium K-edge XAS spectra of all the solid and solution models used herein have been reported previously [15,22,26,31,38].

Fits to the vanadium K-edge spectra were carried out using the program DATFIT4, which is part of the EXAFSPAK software package mentioned above. The blood cell vanadium K-edge spectra were first further processed within the program Kaleidagraph (Synergy Software, Reading PA) to remove extraneous curvature before carrying out the fits. The EXAFSPAK-processed vanadium K-edge spectra were deglitched by linear interpolation, and any steps in the data were removed by vertical re-alignment of the sections of data across the step. The baselines of the deglitched spectra were fully zeroed by fitting a polynomial through the pre-edge region over the energy range 5139 eV to 5453 eV, and then forward extrapolating this polynomial over the rest of the data range, i.e., from 5453 eV through to 5861 eV, using a zero-slope line. The polynomial plus extrapolation was then subtracted from the data. A 3rd order polynomial was fit through the pre-edge subtracted data over the range 5500 eV to 5861 eV, and extrapolated back to the beginning of the XAS spectrum at 5139 eV. The pre-edge subtracted data were finally re-normalized by division with this polynomial. The resulting vanadium K-edge spectra exhibited zero-intensity pre-edges and horizontally normalized EXAFS energy regions. This uniformity among the vanadium K-edge spectra of the blood cell

samples assured that the results from the several fits were internally comparable. The component percents obtained from the DATFT4 fits were finally normalized to 100%, and the normalized percents represent the values reported in this work. The reported fractional errors are the statistical errors calculated by the fit, except for the fractions of V(III) complex aqua ions. The reported fractional values of the complex ions were calculated from the fitted fraction of the model solution, and the equilibrium fractional concentrations of each ion in that solution as determined using the program Medusa [39] and well-established equilibrium constants [40]. The combined fractionally weighted r.m.s. errors of the fitted contributory solution models are reported for each of these complex ions.

Results

Preliminary experiments tested the resistance of isolated whole blood cells to the experimental incubation conditions. Two mL of whole blood combined from two *A. ceratodes* specimens were diluted to 6.0 mL using pH 6.6 PBS buffer (see the Materials and Methods section). The 6 mL blood cell suspension was divided into three 2 mL samples, consisting of an untreated control, a sample made 25 mM in dithiothreitol (DTT), and a third sample made 25 mM each in DTT and vanadyl sulfate (VO_2SO_4). The suspended cells were allowed to incubate on ice for up to 2 hr. Periodically, a sample was taken from each suspension of blood cells and diluted 1:100 directly into a solution of 0.04% Trypan Blue. Exclusion of Trypan Blue by blood cells was taken to indicate viability [41]. Blood cells were examined in a grid under a microscope, and 100 cells were assayed each time. The results are shown in Table 1.

Blood cell stability was further tested against the experimental washing procedure, which initially consisted of three $500\times g/5$ min. centrifugations interspersed with re-suspension in ice-cold PBS buffer. After incubation in ice for 2 hr. while suspended in PBS buffer with either DTT or $\text{DTT}/\text{VO}_2^{2+}$, whole blood cells appeared visually healthy. However, progressive lysis was observed to follow the second and third washing steps. In contrast, fresh blood cells exposed to the same washing sequence showed no sign of lysis. On the basis of these results, the revised experimental protocol used for the results described below employed incubation times of 30–40 minutes, rather than 2 hr., followed by centrifugation at $100\times g$ with only one re-suspension in fresh PBS buffer, and finishing with a final centrifugation at $100\times g$. Microscopic examination of re-suspended blood cells revealed no lysis after this treatment, and blood cells were visually identical to healthy living untreated blood cells (Supplemental Figure 1). These results lend confidence that the following XAS results reflect the response of healthy living blood cells to exogenous vanadyl ion and DTT.

XAS Studies

Figure 1a,b shows the vanadium K-edge XAS absorption and first derivative spectra of the control samples of the three independent preparations of pooled whole blood cells used in the incubation experiments. These represent the starting conditions of all three sets of blood cells, and are consistent with the nearly pure V(III) sulfate in sulfuric acid solution that has been found to predominate in *A. ceratodes* [10,16,22,29,42]. Also typically, the pre-edge regions of the XAS spectra, where quadrupole-allowed $1s\rightarrow 3d$ transitions occur, show small variations in intensity at 5466 eV and 5469 eV (Figure 1a,b insets), consistent with differences in average vacuolar acidity or concentrations of sulfate and/or vanadyl ion, respectively [31]. Variations of intracellular V(III) among these biochemical themes have epitomized *A. ceratodes* blood cells over the entire organismal range, comprising single animals, multiple animals, and two geographically separated populations.

Although vanadyl ion forms an inner-sphere complex with DTT in alkaline solution [43], under the pH 6.6 condition of incubation, vanadium K-edge XAS spectra showed that vanadyl ion was overwhelmingly present as an aqua complex (Supplemental Figure 2). Therefore, any new

vanadyl ion appearing in the blood cells following incubation should have been taken up without chelation-assisted artifactual diffusion across cell membranes. However, the incubation medium included native blood plasma. Thus an unknown plasma vanadium transporter [10] could have been present and could have facilitated biogenic vanadyl ion uptake. The questions addressed below concern the intracellular appearance and fate of vanadyl ion but not the uptake mechanism itself, and so any unknown biological uptake variables leave the results unaffected.

Regardless of incubation times, the nine blood cell XAS spectra stemming from all three experiments reflect the overwhelming dominance of intracellular aqua V(III) sulfate. In Figure 2 are shown the vanadium K-edge XAS spectra of the three whole blood cell samples after 40 minutes incubation in an ice-bath (the Series 1 experiment). Figure 3 shows the first derivatives of these XAS spectra. Incubation with DTT alone produced no changes that could be confidently resolved from the noise in the pre-edge energy region of the XAS spectrum. In contrast, incubation with DTT plus VO^{2+} produced a significant new feature at 5469 eV in the vanadium K-edge XAS. This result proved consistently true in each of the three experiments.

Both aqua vanadyl ion itself and sulfate ligation of V(III) produce intensity near 5469 eV in vanadium pre-K-edge XAS spectra [31]. However, the new 5469 eV feature stemming from the VO^{2+} -incubated blood cells is far too intense to reflect changes in intravacuolar c_{sulfate} , and thus indicates newly endocytosed vanadyl ion. The intensity of the new vanadyl K-edge XAS features across the three sets of samples did not follow the systematic variation in incubation time (see the Materials and Methods Section). In addition, the pre-edge energy position of the new intracellular vanadyl feature in all three VO^{2+} -incubated samples is shifted -0.3 eV from that of VOSO_4 in pH 1 H_2SO_4 solution, and -0.2 eV from that of VOCl_2 in 1 M HCl solution (Figure 3, inset b; the latter data are not shown). Thus, a significant proportion of uptaken vanadyl ion was shunted to an environment quite different from the pentaqua ion, $[\text{VO}(\text{H}_2\text{O})_5]^{2+}$, that is typically found as a minority species in the acidic vacuolar milieu of *A. ceratodes* signet ring cells (see Supplemental Figure 3) [10,22,31,44]. This finding was subjected to more detailed analysis using model-based curve-fitting of the blood cell XAS spectra in terms of known complexes, discussed below.

In previous work, it was shown that systematically fitting the vanadium K-edge XAS of whole blood cells with the vanadium K-edge XAS spectra of appropriate models could elucidate some of the details of blood cell vanadium chemistry [22,31]. That approach has been taken here, and Figure 4 shows the fit to the vanadium K-edge XAS spectrum of the Series 1 whole blood cells that had been incubated with VO^{2+} and DTT. In Figure 5 the first derivative of the blood cell XAS spectra of the data and the fit are compared, showing that the line shapes and the energy positions of the transition maxima of the data and the fit are nearly coincident.

Similarly good fits were found for all nine blood cell vanadium K-edge spectra representing the three series of incubation samples (Table 2; Table S1 and Supplemental Figures 4&5). From these fits, the blood cell vanadium K-edge XAS spectra can be rationalized in terms of a combination of chelated vanadium model complexes and of explicit solution complex ions derived from the equilibrium speciation of the V(III,IV)/X^{n-} ($\text{X} = \text{Cl}^-$ or SO_4^{2-}) model solutions used in the fits [22,31], as shown in Table 2. The array of vanadium complexes and complex ions represents a deductive hypothesis from structural chemistry applied to the vanadium content of whole blood cells.

The bar graph in Figure 6 shows the vanadium composition for whole blood cells averaged from all nine fits and separated according to incubation conditions. From Table 2 and Figure 6, V(III) in dilute sulfuric acid solution dominated the blood cell vanadium metallobiochemistry for all whole blood samples, regardless of incubation conditions. In native

and DTT-incubated blood cells, the very small vanadyl ion content was distributed between the pentaqua and bis-catecholate-like environments, consistent with past experiments [22,26,30,31].

In previous incubation experiments with whole blood cells from *P. nigra*, the fit to the vanadium K-edge XAS spectrum of DTT-treated cells uniquely required inclusion of the XAS spectrum of $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ [19], which accounted for about 8% of the total intracellular vanadium. This new EDTA-like V(III) complex was presumed to arise from the biological reduction of antigenous vanadyl ion utilizing uptaken DTT. However, in *P. nigra* blood cells about 30% of the resident vanadium is aqua vanadyl ion, whereas only ~3% $[\text{VO}(\text{aq})]^{2+}$ is usually found in the blood cells of *A. ceratodes* [22,31]. Therefore, any EDTA-like V(III) complex following from reduction of antigenous vanadyl ion that may arise in *A. ceratodes* blood cells could never represent a large fraction of the total intracellular vanadium.

Thus fits to two of the three vanadium K-edge XAS spectra obtained on blood cells treated with DTT indicated that about 0.5% of the total vanadium could be represented by the $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ model. In contrast, fits to all the control sample XAS spectra rejected this component, with these tests exhibiting final fractions of $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})] \sim 10^{-6}$ or less. Similar results ensued when the XAS spectrum of $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ was tested in fits to the XAS of blood cell samples incubated with both DTT and VO^{2+} . These positive results attending DTT alone are tentative considering the very small magnitude of the fitted $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ fractions. However, absent the EDTA component the fits to the XAS of the DTT-treated cells were uniformly, if marginally, poorer. The uniform exclusion of the $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ model from all of the fits to the control samples, plus the small improvements in goodness-of-fit to two of the DTT-incubated samples allow a tentative conclusion that this fraction may be real, and not just the result of an additional degree of freedom in the fit.

A more striking result came from fits to the K-edge XAS spectra of whole blood cells incubated with both DTT and $\text{VO}(\text{aq})^{2+}$, which uniquely and consistently required a significant fraction of the XAS spectrum of $[\text{VO}(\text{edta})]^{2-}$. The Series 1 blood cells also uniquely showed a significant increase in $[\text{VO}(\text{H}_2\text{O})_5]^{2+}$, represented as the $[\text{VOCl}_2]$ model (Table 2). None of these fits showed any noteworthy increase in the percent of the $\text{K}_2[\text{VO}(\text{catecholate})_2]$ model.

A second and more subtle result of DTT plus VO^{2+} incubation was that the calculated proportion of $[\text{V}(\text{H}_2\text{O})_5\text{SO}_4]^+$ uniformly diminished across all three experiments (Table 2, Figure 6, Table S1). On the other hand, the proportion of the hexaaqua ion $[\text{V}(\text{H}_2\text{O})_6]^{3+}$ remained nearly constant, while the $\text{K}_3[\text{V}(\text{catecholate})_3]$ model, already very low in the control and DTT-incubated blood cells, was found uniformly absent from the blood cells incubated with DTT and $\text{VO}(\text{aq})^{2+}$.

Finally, a linear association emerged between the intracellular proportions of $\text{VO}(\text{aq})^{2+}$ and bis-chelated vanadyl ion. The same association was previously observed in data from fits to the vanadium K-edge XAS spectra of a prior collection of *A. ceratodes* whole blood cells [22]. The combined data sets from the two independent collections are shown plotted in Figure 7. The proportions of free and complexed vanadyl ion found in fits to the current set of samples fall near the same straight line as found previously, which is the line shown in Figure 7. The slope of the line resulting from a linear fit to the composite data set has a slope ($m=0.22$) very similar to that found originally.

In addition, a second linear relation emerged between $\text{VO}(\text{aq})^{2+}$ and the proportion of the $[\text{VO}(\text{edta})]^{2-}$ model in fits to the cells incubated with DTT and VO^{2+} , and this result is also shown in Figure 7. The line through these points has a slope nearly identical to the line fitting the composite $[\text{VO}(\text{bis-chelate})]$ data points (see Figure 7 Legend).

UV/visible Studies

Kanamori, et al. were first to report that $[\text{VO}(\text{edta})]^{2-}$, but not the very similar $[\text{VO}(\text{trdta})]^{2-}$, could be reduced to the V(III) complex with cysteine methyl ester under the mild conditions of aqueous pH 6.5 solution [35]. The difference in reactivity was rationalized in terms of the 7-coordinate V(III) product available to $[\text{VO}(\text{edta})]^{2-}$ but not to $[\text{VO}(\text{trdta})]^{2-}$. This point is discussed further below. Reported here are experiments carried out to test the generality of the reductive chemistry. Confirming the previously reported results, $[\text{VO}(\text{edta})]^{2-}$ was found to be reduced by cysteine methyl ester, producing 7-coordinate $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$ (Supplemental Figure 6) while the homologous $[\text{VO}(\text{trdta})]^{2-}$ complex was unaffected.

In testing the generality of this reductive chemistry, another ligand system and another reductant were explored. Thus, vanadyl ion was treated with two equivalents of cysteine methyl ester either in the presence of equi-molar nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA), or in the presence of equi-molar NTA but without added IDA. Although aqueous $[\text{V}(\text{nta})(\text{aq})]$ was originally deduced to be 6-coordinate $[\text{V}(\text{nta})(\text{H}_2\text{O})_2]$ [45], the crystalline complex is 7-coordinate $[\text{V}(\text{nta})(\text{H}_2\text{O})_3]$ [46]. In solution, the UV/visible absorption spectrum of $[\text{V}(\text{nta})(\text{aq})]$ exhibits the near-IR bands (Supplemental Figure 7) that are diagnostic of 7-coordinate V(III) [47,48], consistent with the crystal structure. With vanadyl ion, NTA forms the $[\text{VO}(\text{nta})(\text{H}_2\text{O})]^-$ complex ($\log\beta=12.3$) [49]. UV/visible absorption spectra of this complex, on treatment with cysteine methyl ester in pH 6.5 solution, gave evidence of a slow production of 7-coordinate $[\text{V}(\text{nta})(\text{H}_2\text{O})_3]$ (Supplemental Figure 8).

In the presence of equimolar NTA and IDA, the $\sim 10^3$ difference in binding constants [49] assures virtually 100% of VO^{2+} will be in the $[\text{VO}(\text{nta})(\text{H}_2\text{O})]^-$ complex. The equimolar IDA gave no UV/visible spectroscopic evidence of additional binding to this vanadyl complex. However, the ligands NTA and IDA can together potentially facilitate reduction of VO^{2+} by producing an N_2O_5 7-coordinate $[\text{V}(\text{nta})(\text{ida})]^{2-}$ product, making a structural analogy with $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$.

Figure 8 shows the time course of reduction of vanadyl ion by cysteine methyl ester in the additional presence of equi-molar NTA and IDA. The inset to Figure 8 shows the electronic absorption spectrum of a freshly made pH 6.5 aqueous solution of V^{3+} with equimolar NTA and IDA. In Figure 8, the appearance of the $[\text{V}(\text{nta})(\text{ida})]^{2-}$ complex that should be the VO^{2+} reduction product with cysteine methyl ester is indicated by the growing band at 460 nm, which is prominently present in Figure 8, inset. Supplemental Figure 7 shows that the absorption spectrum of $[\text{V}(\text{nta})(\text{ida})]^{2-}$ is similar to, but not identical with, that of $[\text{V}(\text{nta})_2]^{3-}$. The difference in the first and second binding constants of NTA with V^{3+} [45] ($\log\beta_1=13.4$, $\log\beta_2=8.7$) ensures that equimolar NTA and IDA produces virtually no $[\text{V}(\text{nta})_2]^{3-}$. That is, all the equi-molar NTA is commanded by the first binding constant with V(III). Thus, the NTA/IDA reduction chemistry reported here is not an artifact of reductive disproportionation, and reaction 1,

does not occur under the experimental conditions. With IDA present, cysteine methyl ester reduced $[\text{VO}(\text{nta})(\text{H}_2\text{O})]^-$ about $20\times$ faster than when IDA was absent.

The specificity of cysteine thiol as reducing agent was tested by substituting ascorbate into the reaction with $[\text{VO}(\text{edta})]^{2-}$. In contrast with the lack of reductive reactivity with NADPH [50], at pH 6.5 ascorbate successfully but slowly reduced $[\text{VO}(\text{edta})]^{2-}$ to $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$. With $[\text{VO}(\text{trdta})]^{2-}$, no evidence of reduction by ascorbate was seen even after 30.8 hr sealed

under dinitrogen (Supplemental Figure 9). In passing, we note that ascorbate is a far weaker reducing agent ($E_0' = 58$ mV) [51] than cysteine or NADH ($E_0' = -245$ mV or -316 mV, respectively) [52,53], and the rate of reduction of $[\text{VO}(\text{edta})]^{2-}$ with ascorbate was observed to be far slower than with cysteine methyl ester (compare Supplemental Figure 8 with Supplemental Figure 6). The appearance of the $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$ product despite the weaker driving force of ascorbate relative to NADPH reinforces the inference [50] that vanadyl reduction likely occurs by an inner sphere mechanism.

Discussion

It is now known that whole blood cells of *A. ceratodes* can directly take up added vanadyl ion in addition to added vanadate [32–34], *in vitro*. The incubation experiments reported here were composed to test whether *A. ceratodes* blood cells, like those from *P. nigra*, would produce a new chelated form of V(III) when exposed to DTT. Aquated vanadyl ion represents about 30% of the ~ 0.1 M average [V] in blood cells from *P. nigra*. Any significant reductive diversion of this vanadyl ion into V(III) is readily detected by vanadium K-edge XAS [19]. In *A. ceratodes*, however, aqua vanadyl ion is very scarce [22]. Therefore, only very low levels of any new endogenous form of V(III) could be produced from this source, if VO^{2+} reduction followed exposure to DTT.

Fits to the vanadium K-edge spectra of two of the three DTT-incubated blood cell samples showed an improvement in the goodness-of-fit 'F' when the XAS of $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ was added to the suite of model spectra (Table 2, Supplementary Table 1). The level of this component was on the order of $\sim 0.5\%$, consistent with the low level of the endogenous VO^{2+} . A fit component at the 0.5% level is only marginally significant except for, e.g., vanadyl ion or vanadate, which display an especially intense pre-edge feature. The only reason to suppose significance here is that the $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ model was retained only in fits to two of the blood cell samples that had been treated with DTT alone. In the third sample, an analogously small positive fraction for $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ was found but only with the same magnitude as the statistical fit error, making the fraction indistinguishable from zero. In contrast an added $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ component was definitively zeroed out ($\sim 10^{-4} - 10^{-7} \%$) by the algorithm in fits to both the non-treated controls, and the blood cell samples treated with both DTT and VO^{2+} . These results are thus a marginal though perhaps real corroboration of the analogous but much more pronounced effect previously reported for *P. nigra*. Thus it appears that in two ascidian species representing two genera, whole blood cells treated with DTT produce a new V(III) species bound in a 7-coordinate biological site that is structurally similar to the ligand set provided by EDTA plus a water ligand. This V(III) species almost certainly results from the reduction of intracellular VO^{2+} [19].

Much more robust is the finding that *A. ceratodes* blood cells treated with both DTT and $[\text{VO}(\text{aq})]^{2+}$ uniquely revealed the presence of a new vanadyl ion complex that was best modeled with the XAS spectrum of $[\text{VO}(\text{edta})]^{2-}$. The new vanadyl component averaged $\sim 6\%$, which is very significant and well within the resolution of the experiment. In two of the three samples all the new vanadyl ion appeared in the form modeled by this complex (see Table S1). Thus, an inference can be made that the incubation conditions induced the production of a bound form of vanadyl ion within an EDTA-like environment. The uniformly EDTA-like nature of the induced complexes in *A. ceratodes* marks an identity with the response of *P. nigra* blood cells to added DTT alone.

The specificity of the fits for the EDTA complex can be evaluated by consideration of the range of other complexation environments tested. Apart from solution-phase $[\text{VO}(\text{edta})]^{2-}$, other tested vanadyl ligand environments included the pentaqua complex, $[\text{VO}(\text{oxalate})_2]^{2-}$, $\text{K}_2[\text{VO}(\text{catecholate})_2]$, $[\text{VO}(\text{acac})_2]$, $\text{Na}_2[(\text{VO})_2(3\text{-methylglutarate})_2]$, and $\text{K}[\text{VO}(\text{hedtra})]$

(HEDTRA=ethylenediamine-N(2-hydroxyethyl)-N,N',N'-triacetic acid). The ligand environment about vanadyl ion in $[\text{VO}(\text{hedtra})]^-$ is nearly identical to that provided by the EDTA ligand [54] and the fit was nearly as good. The results showing discrimination among the many ligand environments, along with the very good fits modeling the new intracellular vanadyl environment with $[\text{VO}(\text{edta})]^{2-}$, lend confidence that the N_2O_3 environment of EDTA contains essential elements of the induced biological ligation site.

Following uptake of vanadyl ion, the total intracellular $[\text{V}]$ must have increased. If the only change were vanadium uptake the normalized percents of the original vanadium species would have declined in the blood cell samples containing added vanadyl ion. Looking at Table 2, it is evident that rather than declining the normalized percent of intracellular $[\text{V}(\text{H}_2\text{O})_6]^{3+}$ remained nearly constant. This result implies that the absolute concentration of $[\text{V}(\text{H}_2\text{O})_6]^{3+}$ increased in the blood cells exposed to both DTT and VO^{2+} . In contrast, the normalized percents of $[\text{V}(\text{SO}_4)(\text{H}_2\text{O})_5]$, i.e., the principle sulfate complex ion of V(III), decreased even more than would be expected by the absolute increase in intracellular vanadium due to endocytosed vanadyl ion. If the aqua and sulfated forms of V(III) reside in the same vacuole, as is virtually certain [9,12,15,38,42,55], then this reciprocal response is consistent with an increase in acidity following uptake of vanadyl ion. Such an increase in acidity could be accomplished either by influx of $[\text{H}^+]$ to the vacuole, or efflux of $[\text{SO}_4^{2-}]$ from the vacuole. Each process would have to be coupled to further ion transport, e.g., substitution of sulfate by chloride, to maintain electroneutrality. In either case the endogenous pH would decrease, driving up the equilibrium intravacuolar concentration of $[\text{V}(\text{H}_2\text{O})_6]^{3+}$.

There is an alternative possible explanation, namely that exposure of blood cells to DTT and VO^{2+} caused an oxidative shift of V(III) from $[\text{V}(\text{SO}_4)(\text{H}_2\text{O})_5]^+$ into VO^{2+} . This possibility is excluded by equilibrium theory, however, which would not allow removal of the sulfate complex ion without re-equilibration of some of the remaining endogenous $[\text{V}(\text{H}_2\text{O})_6]^{3+}$ into $[\text{V}(\text{SO}_4)(\text{H}_2\text{O})_5]^+$. The lowered fraction of the V(III) hexaaqua ion this possibility requires is not observed in the results.

Thus, the combination of metabolic theory [56–59] and chemical thermodynamics support that the discerned changes in endogenous V(III) equilibrium ratios following vanadyl uptake and complexation reflect a systematic biological response, namely an endogenous decrease in pH following uptake of vanadyl ion (and possibly DTT). Significantly, a decreased pH would facilitate the proton-dependent reduction of vanadyl ion.

In contrast to the above, the response of the normalized percent of the hydrolyzed V(III) complexes $[\text{V}(\text{H}_2\text{O})_5(\text{OH})]^{2+}$ is within error of the change expected from the increase in total blood cell vanadium. Vacuolar regions containing hydrolyzed V(III) complexes are clearly less acidic than the average. These hydrolyzed V(III) complexes may represent a sub-population of signet ring cells with more alkaline vacuoles that do not participate in vanadyl reduction. The fact that hydrolyzed V(III) complexes exist at all in blood cells against a strong oxidation gradient (e.g., at pH 4 E1/2 of $[\text{V}(\text{aq})]^{3+} \sim -0.14 \text{ V}$) will eventually demand a metabolic explanation.

Before discussing the deductions that can flow from the appearance of EDTA-like complexes in ascidian blood cells incubated with DTT or DTT plus VO^{2+} , the two correlations depicted in Figure 7 are noted. Correlation is not causation [60]. However within the bounds of metabolic theory, closely related substances that consistently change concentrations in concert are potentially evidence of a unified underlying driver. In this case, it appears that there is a common vanadium regulatory mechanism that now twice has revealed itself governing the endogenous distribution of the vanadyl ion between bound and free states.

If the correlations in Figure 7 are indeed a result of biochemical feedbacks, it is reasonable to suppose that a regulatory pathway exists consisting of proteins sensitive to the concentrations and oxidation states of all three forms of biological vanadium, namely vanadic, vanadyl, and vanadate. It further seems reasonable that there should exist an effector protein subject to a vanadium-regulated conformational state that responsively binds to some specific DNA sequence. This DNA sequence in turn should code for a vanadium reductase. This metabolic system, if found, would testify to an ancient heritage for genomic metal regulation because it would have evolved into being well before the fossiliferous vanadium-accumulating ascidians that have been found in Cambrian sediments [1–3]. Similar metal-based eukaryotic regulation of metalloproteins exists for the copper oxidase ceruloplasmin, and for metallothionein [61–63].

The complexes $[\text{VO}(\text{edta})]^{2-}$ and $[\text{VO}(\text{trdta})]^{2-}$ provide nearly identical ligand environments to vanadyl ion. Each vanadyl is nearly surrounded by a highly distorted N_2O_4 octahedron with a very short axial vanadium-oxygen bond and a distant axial amine, as typified by the $\text{K}[\text{VO}(\text{hedtra})]$ structure [64]. However, the V(III) complexes are very different. $\text{Na}[\text{V}(\text{trdta})]$ is 6-coordinate pseudo-octahedral about V(III) [65]. With EDTA, however, V(III) yields the 7-coordinate $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ complex featuring an extra equatorial water ligand. $\log \beta = 26.5$ for formation of 7-coordinate $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$ relative to $\log \beta = 18.5$ for $[\text{V}(\text{trdta})]^-$ [66], indicating the far greater stability of the former complex. The difference in stability has been traced to a relaxation of ligand-metal electronic repulsion in the equatorial off-axis ligation provided by 7-coordination [66]. Analysis of the K-edge XAS spectra of $\text{Na}[\text{V}(\text{edta})(\text{H}_2\text{O})]$ and $\text{Na}[\text{V}(\text{trdta})]$ showed the median 3d-orbital energy of V(III) is lower by about 0.5 eV in the EDTA complex [27]. This energy difference almost exactly reconciles the 10^8 difference in aqueous V(III) binding constants favoring EDTA over TRDTA [66]. The added stability of the EDTA complex, in turn, translates to a 470 mV disparity in reductive EMF from a common N_2O_3 VO^{2+} precursor. The lower energy of the d-orbitals of 7-coordinate V(III) in these two otherwise homologous ligand systems [27] thus rationalizes the different reductive reactivities of the respective vanadyl complexes with cysteine methyl ester. That is, the availability of a 7-coordinate V(III) product greatly facilitates the reduction of VO^{2+} by enhancing the reductive $-\Delta G$ by about 0.5 eV.

The Biological Reduction Mechanism of Vanadate/Vanadyl in Ascidians: A Complete Hypothesis. Previous sulfur K-edge XAS studies of blood cells from *A. ceratodes* revealed large amounts of endogenous sulfur, much of it at the thiol-disulfide valence levels [12,15, 38]. XAS experiments, including the new results reported here, have indicated that biological vanadium sites similar to $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$ or $[\text{VO}(\text{edta})]^{2-}$ are induced in ascidian blood cells when DTT or DTT plus VO^{2+} are present, respectively. The vanadyl reduction chemistry reported earlier and confirmed and extended here demonstrated that if a 7-coordinate V(III) site is available, VO^{2+} can be reduced by some biological reductants under the mild cytosol-like conditions of aqueous pH 6.5 solution. These findings provide all the necessary elements of a biological vanadyl reduction pathway that is metabolically analogous to the inorganic chemistry described above.

Therefore a detailed hypothesis is made here that proposes the existence of a vanadium reductase in the blood cells of Phlebobranch ascidians. This new and detailed hypothesis predicts the mode of vanadium binding, the mechanism of vanadium reduction, and that the reductase active site will have a ligand array similar to that of EDTA. The proposed site readily accommodate the several bonding requirements unique to the vanadate, vanadyl, and vanadic ions and facilitates reductive conversion of bound VO^{2+} to 7-coordinate V(III). The transitions among these modes of bonding can be accomplished with minimal amino acid side-chain motions and thus minimal Franck-Condon barriers. Further, a thiol binding site is proposed to be situated near the locale of vanadium reduction. As noted above, the hypothesis of an

endogenous vanadium reductase is independent of the mechanisms of biological vanadium uptake or transport.

A cartoon depicting the proposed mode of vanadyl complexation and reduction within a vanadium reductase is illustrated in Figure 9. The hypothetical vanadium reductase site is composed of four carboxyl and two nitrogenous amino acid residues. The nitrogenous amino acids are left unspecified, but analogy to other protein metal binding sites makes imidazole nitrogen the most reasonable choice. Together, these residues are arranged to provide a binding site for vanadyl ion that is entirely analogous to the complexation array provided by the EDTA ligand. One of the carboxyl residues remains uncoordinated, as in $[\text{VO}(\text{edta})]^{2-}$. This carboxyl is proposed to remain in the acid form at this stage of the reductase cycle, providing an important H-bond to the oxo-group of bound vanadyl ion. This H-bond should facilitate subsequent VO^{2+} reduction by increasing the redox potential of the bound metal ion relative to the free aqua ion [67–69]. The biological excess EMF driving the reduction of enzyme-bound VO^{2+} to 7-coordinate V(III) should thus be more than the 470 mV increase produced by 7-coordinate EDTA ligation relative to the 6-coordinate $[\text{V}(\text{trdta})]^{2-}$ complex.

Following nearby binding of a thiol, VO^{2+} is reduced to V(III), while the oxo-group of the vanadyl ion smoothly converts to a water ligand. Production of this water is facilitated by the H-bonded carboxylic proton plus a proton from the thiol. That is, the proposed mechanism requires no long-range proton transfer. The new carboxylate residue translates $\sim 2 \text{ \AA}$ to become the seventh ligand to V(III).

The site-complexed vanadium ion can readily adjust to these events because a nascent V(III) can rotate much faster ($\sim 10^{-11}$ sec [70]) than the usual rate of electron transfer even from a proximate reductant ($\sim 5 \times 10^{-5}$ sec [71]). This rapid rotation minimizes electronic repulsions that might otherwise arise between the electrons in the equatorial 3d-orbitals and the ligand lone pairs in the emergent 7-coordinate site during the reduction event [66].

As depicted, the proposed site is naturally arranged to complex vanadyl ion with high affinity, to activate VO^{2+} towards reduction, and to facilitate reduction by providing a smooth transition over a low energy barrier to the 7-coordinate V(III) product. This site should be capable of taking full advantage of the increased EMF provided by a 7-coordinate V(III) EDTA-like reduction product [27]. This hypothesized site offers a natural solution to all the structural requirements of biological vanadyl ion reduction, including displacement of the oxo group, accommodation of the very different geometric requirements of VO^{2+} and V^{3+} , and stabilization of the final V(III) product. All of this is accompanied by minimal adjustments of the amino acid side-chains, obviating any significant Franck-Condon barrier to reduction.

Figure 10 shows the same site can just as readily support the ligation and reduction of vanadate. The resting site of the vanadium reductase is depicted with the four carboxylic acid side-chains fully protonated. Complexation of H_2VO_4^- vanadate, the predominant monomeric form of milliMolar V(V) at neutral pH, is accompanied by loss of two water molecules. The two new carboxylate residues, along with the two nitrogenous side-chains, become ligands to bound vanadate. This vanadate-binding array has structural analogies in the inorganic complexation chemistry of VO_2^+ [72–74]. The two remaining carboxylic acid residues are then available to contribute one hydrogen bond to each of the two remaining oxo-groups of complexed vanadate. As with complexed vanadyl ion discussed above, these H-bonds can facilitate reduction by increasing the V(V) redox potential, as well as by stabilizing and positioning VO_2^+ within the active site. The reducing agent can again be thiol, bound to the same hypothetical enzymatic site as before. During reduction to vanadyl ion, loss to water of one of the two oxo groups is again readily facilitated by way of protons from thiol and one of the H-bonding carboxylic acid side-chains. Again, no long distance proton transfer is required. The resultant carboxylate then

becomes a ligand to the new in-site VO^{2+} , by way of a small translational movement. The reduction product is VO^{2+} bound in the reductase active site as depicted in Figure 9, ready to undergo final reduction to V(III).

Figure 11 illustrates these two reactions combined into the full vanadium reductase hypothesis. The various steps are presented as a complete reduction cycle, catalytic in terms of the enzyme, with all the intermediate steps represented. Once again, an enzyme site providing an EDTA-like ligand array plausibly supports the full reduction of vanadate through to 7-coordinate V(III). Hydrogen bonds to metal-oxo groups activate both vanadate and vanadyl for reduction, and go on to facilitate conversion of these oxo-groups into water. Following loss of the side-chain protons and the metal oxo-groups, the previously H-bonded carboxylate residues are positioned to smoothly convert into ligands to VO^{2+} or V(III). The consumption of four protons over the enzymatic cycle predicts that reduction of vanadate and vanadyl ions will likely depend upon the action of a biological proton pump. This latter prediction is already partially verified by the apparent increase in endogenous $[\text{V}(\text{H}_2\text{O})_6]^{3+}$ following incubation of blood cells with DTT and VO^{2+} , as described above.

In Figures 9–11, the reducing agent is shown as a generic thiol. However, elements of glutathione metabolism have been found in ascidians [75,76], and a glutathione reductant is not inconsistent with the copious reduced sulfur detected in whole blood cells using sulfur K-edge XAS [12,15,38]. The successful chemical production of 7-coordinate V(III) from complexed VO^{2+} by ascorbate or cysteine methyl ester, but not by NADPH [50], implies that a direct metal-reductant interaction is necessary. For that reason, a vanadium reductase is likely to have a thiol-binding region near the vanadium reduction site.

Finally, the hypothesis of vanadate/vanadyl reduction is extended to include some consideration of the cellular locale of the biochemical machinery. The percents of $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$ and $[\text{VO}(\text{edta})]^{2-}$ needed to fit the vanadium K-edge XAS spectra of blood cells from *P. nigra* or *A. ceratodes* after treatment with DTT- or DTT plus VO^{2+} respectively, are much too great to represent membrane-bound proteins. This is true even for the very small percents of $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$ possibly found in the DTT-treated blood cells from *A. ceratodes* (Table 2). For example, it can be estimated from the dense protein populations on membrane lipid rafts that, at maximal protein density, a cell membrane might support about one protein molecule per 66 nm^2 [77]. A typical signet-ring cell of $\sim 10 \mu$ diameter represents $3.14 \times 10^{-10} \text{ m}^2$ of surface, relative to $5.2 \times 10^{-16} \text{ m}^3$ of volume. The upper limit estimate of 4.8×10^6 protein molecules supportable by that surface equates to a cell-volumetric concentration of $15 \mu\text{M}$. Thus, for a fully saturated membranous vanadium reductase and a typical average whole blood cell concentration of 0.1 M vanadium, an analysis of K-edge XAS intensity would yield an *upper-limit* normalized mole fraction of 0.00015 relative to total intracellular vanadium. This is well below the current XAS quantitation limit and $27\times$ smaller than even the small $[\text{V}(\text{edta})(\text{H}_2\text{O})]^-$ fraction reported in Table 2. Therefore, for both *P. nigra* and *A. ceratodes*, the XAS fitting results are consistent only with a soluble cytoplasmic enzyme with an estimated average cytosolic concentration of $\sim 6 \text{ mM}$ (assuming one vanadium binding site per enzyme molecule). This cytoplasmic vanadium reductase should be most richly present in signet ring cells, where the large bulk of cellular V(III) resides.

Figure 12 shows a phase-contrast photomicrograph of a signet ring cell. Most of the interior volume is filled with the acidic vanadium-containing vacuolar solution. Previous fits to both the vanadium K-edge and sulfur K-edge spectra of whole blood cells have been suggestive that internal acidity can reach as low as pH 0 in these spaces, and vanadyl EPR experiments have revealed an average 1.8 pH. The presence of a reductase working as outlined above seems unlikely within the highly acidic vacuole, where protons can effectively compete with VO_2^+ for the active site residues, and where thiol is minimally effective as a reducing agent (e.g., at

pH 1, the $E_{1/2}$ of glutathione ~120 mV vs NHE [53]). A more likely intracellular region where the reductase may operate is in the neutral cytosol in the intermediate space between the cell membrane and the vacuolar membrane. This is reasonable in terms of the average ~6 mM cytosolic reductase concentration inferred above. In a 10 μ diameter signet ring cell with an 8 μ diameter vacuole, remaining neutral cytosol still comprises ~49% of the volume of the cell. This translates the inferred average reductase concentration to ~12 mM, which is reasonable for a small (~20 kD) protein [78,79]. If indeed V(III) is produced in the intermembrane space, then it may be hypothesized that there is a vanadate/vanadyl transporter in the outer cell membrane [20,32], and a V(III) endo-transporter in the vacuolar membrane. The intermembrane space should also be rich with thiol, such as, e.g., glutathione.

Taken together, the results and hypotheses presented here provide an outline of the metabolic regulation of intracellular vanadium by ascidians. The hypothesis is fully consistent with chemical and biochemical theory and with all known aspects of vanadium and sulfur biochemistry in ascidians. There are presently no known vanadium reductases, nor any known redox biochemistry of vanadium in any metalloenzyme. Apart from the redox-inert V(V) haloperoxidases and the vanadium nitrogenases, no native vanadium-protein assembly is known. However, the vanadium biochemistry of ascidians, now under investigation for nearly a century, has ensured the existence of an extensive vanado-metabolic machinery and its equally extensive genomic regulation.

The field of vanadium metalloenzyme redox biochemistry should provide rich rewards for the inorganic biochemist intrepid enough to pursue it. Nevertheless, this field of fascinating biochemistry and molecular biology has been badly neglected, principally, it seems, because no one has known where, or for what, to look. These impediments are now being removed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Table of Abbreviations and Definitions

XAS	x-ray absorption spectroscopy
DTT	dithiothreitol
nta	nitrilotriacetic acid
ida	iminodiacetic acid
edta	ethylene-1,2-diamine-N,N',N'-tetraacetic acid
trdta	trimethylene-1,3-diamine-N,N,N',N'-tetraacetic acid
MES	2-[N-morpholino]ethanesulfonic acid
PBS	phosphate-buffered saline
antegenous	prior origination <i>in situ</i>

Acknowledgments

This work was supported by grant NIH RR-01209 (to KOH). XAS data were measured at SSRL, which is supported by the Department of Energy, Office of Basic Energy Sciences Division. The SSRL Structural Molecular Biology Program is supported by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program and by the Department of Energy, Office of Biological and Environmental Research (BER). The project described was also supported by Grant Number RR001209 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH) and its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH.

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Figure 1.

a. Vanadium K-edge XAS Absorption, and b. First derivative of the XAS spectra of the untreated control samples for the series of three *Ascidia ceratodes* whole blood cell experiments after: (—), 40 minutes incubation on ice; (---), 35 minutes incubation on ice, and; (...), 30 minutes on ice. Insets: Expansion of the pre-edge energy region of the spectra.

Figure 2. Vanadium K-edge XAS spectra of *A. ceratodes* whole blood cells after incubation for 40 minutes on ice following treatment with: (—), no reagents (control sample); (---), 25 mM dithiothreitol (DTT), and; (...), 25 mM each of DTT and $[\text{VO}(\text{aq})]^{2+}$. Inset: Expansion of the pre-edge energy region.

Figure 3.

First derivatives of the vanadium K-edge XAS spectra of the *A. ceratodes* whole blood cells sample of Figure 2: (—), no reagents (control sample); (---), 25 mM dithiothreitol (DTT), and; (...), 25 mM each of DTT and $[\text{VO}(\text{aq})]^{2+}$. Inset a: Expansion of the pre-edge energy region. Inset b: Comparison of the first derivatives of the pre-edge energy region of the vanadium K-edge XAS spectra of: (—), *A. ceratodes* whole blood cells after treatment with 25 mM each of DTT and $[\text{VO}(\text{aq})]^{2+}$ (intensity is left ordinate), and; (---) 0.1 M VOSO_4 in pH 1.0 H_2SO_4 frozen solution (intensity is right ordinate).

Figure 4.

Vanadium K-edge XAS spectra of *A. ceratodes* whole blood cells, (—), after incubation for 40 minutes on ice with 25 mM each of DTT and $[\text{VO}(\text{aq})]^{2+}$; (---), the fit to the spectrum, and; (...), the components of the fit. Inset: Expansion of the pre-edge energy region of the XAS spectrum and the fit to the spectrum. Bottom: The data minus fit residuals. The symbols represent: ($\circ$), 50 mM V(III) with 521 mM SO_4^{2-} in pH 0.0 solution; ($\bullet$), 50 mM V(III) with 75 mM SO_4^{2-} in pH 3.0 solution; (Δ), 0.10 M VOCl_2 in 1 M HCl solution; ($\square$), $\text{K}_2[\text{VO}(\text{catecholate})_2]$ finely ground in boron nitride, and; ($\blacksquare$), 0.10 M $[\text{VO}(\text{edta})]^{2-}$ in pH 6.6 solution with equimolar free edta ligand.

Figure 5.

First derivative of: (—), the vanadium K-edge XAS spectrum of the *A. ceratodes* whole blood sample of Figure 4, and; (---), of the fit to the spectrum. Inset: Expansion of the pre-edge energy region of the spectrum and of the fit.

Figure 6.

Stick diagram of the average vanadium speciation within the experimental series of whole blood cell samples: a. Major components (left ordinate); b. lesser components (right ordinate). The colors represent: Black, untreated control; White, treated with 25 mM DTT; Hatched, treated with 25 mM each of DTT and vanadyl ion. The error bars represent $\pm 12.25\%$. The $[VO(edta)]^{2-}$ component appears only in fits to cells treated with both DTT and vanadyl ion.

Figure 7.

Linear least squares line fit to data relating the $[\text{VO}(\text{aq})]^{2+}$ component points to: (▲), the $[\text{VO}(\text{edta})]^{2-}$ component points. The fitted line is $y = 0.0492 + 0.225x$, $r = 0.98$, and; (●), the $[\text{VO}(\text{bis-chelate})]$ component. The square points, (■), are data representing the $[\text{VO}(\text{aq})]_{2+} : [\text{VO}(\text{bis-chelate})]$ component ratio obtained from the fits to the vanadium K-edge XAS spectra of the prior collection of whole blood cell samples [22]. In this case, the line is identical to that which was fit to the prior data set, namely $y = -0.0726 + 0.278x$, $r = 0.95$. This line is seen to pass through the middle of the combined data set. The fitted line to the combined data set has the slope $m=0.22$, ($r=0.81$).

Figure 8.

Time-course of the reduction of 10.1 mM vanadyl ion in the presence of 7.0 mM NTA plus 7.1 mM IDA and 20.2 mM cysteine methyl ester, all in pH 6.5 MES buffer. The dashed-line spectrum shows the result of adding a further 3 mM each of NTA and IDA, bring into stoichiometric equivalence the previous deficient complexation environment of vanadyl ion. Inset: the absorption spectrum of $[V(nta)(ida)]^{2-}$ in pH 6.5 MES buffer. See Supplemental Figure 7 for the spectrum of $[V(nta)(H_2O)_2]$ alone in the same pH 6.5 buffer.

Figure 9.

The proposed enzyme active site for complexation and reduction of inorganic vanadyl ion to 7-coordinate V(III) in the blood cells of ascidians. The site has been constructed to produce the EDTA-like ligation sphere deduced from model-based fits to the vanadium K-edge XAS spectra of ascidian blood cells treated with DTT alone or with DTT plus VO^{2+} . The semi-circles represent an unspecified protein matrix. The brackets represent the nitrogens as general nitrogenous amino acid residues, most reasonably imidazole. The oxo-group is trans to a nitrogenous ligand, as in $[\text{VO}(\text{edta})]^{2-}$ and is H-bonded to a nearby carboxylic acid residue. This last helps activate the vanadyl ion toward reduction. In the 7-coordinate V(III) product, the H-bonded carboxylate is now a ligand, and the new water ligand derives from the prior oxo-group. The water hydrogens come from the prior H-bonded carboxylic acid and the thiol reductant. During electron transfer, the V(III) can rotate within the ligand sphere to the low-energy position, attained when the two half-filled 3d orbitals point between the equatorial ligands. See text for discussion.

Figure 10.

The hypothetical reductase site of Figure 9 extended to the homologous reduction of complexed vanadate. Note that there are now two reduction-activating H-bonds offered in a natural way by the proposed enzyme active site. Either carboxylic acid is well-positioned to convert easily into a ligand to the vanadyl reduction product. An inorganic structural model for this site is found in reference [72]. Following reduction, the oxo-group that was converted to water is displaced by the nearby carboxylate group. The hydrogens for the new water again come from the displacing carboxylic acid and the thiol reductant.

Figure 11.

The full hypothesis for the operation of the ascidian vanadium reductase. Vanadate complexation, the subsequent reduction to vanadyl and vanadic ions, and the final dissociative hydrolysis to $[V(H_2O)_6]^{3+}$ are shown. Four protons are consumed during reduction of $H_2VO_4^-$ to $[V(H_2O)_6]^{3+}$. Two moles of thiol are consumed, and the sulphhydryl protons end up on water.

Figure 12.

Phase-contrast photomicrograph of a signet ring cell from *A. ceratodes* whole blood. The large blue off-center circle is the vacuole containing sulfuric acid and vanadium. The full arrows point to: a, the intermembrane space where the soluble vanadium reductase is expected to operate; b., the outer and inner membranes where the vanadate/vanadyl, and the V(III) transporters, respectively, are expected to reside, and; c., to the cell nucleus.

Table 1

Trypan-Blue Assay of Dead *A. ceratodes* Blood Cells per 100 Counted Under Incubation Conditions

Minutes →	0	10	20	30	40	120
Sample ↓						
Control	0	1	0	0	0	0
Treatment 1	1	0	0	1	0	0
Treatment 2	1	1	2	1	0	0

Treatment 1: 25 mM DTT in cold PBS buffer. Treatment 2: 25 mM DTT with 25 mM VO²⁺ in cold PBS buffer.

Table 2

Average Blood Cell Vanadium

	Component	No Treatment	DTT only	DTT+VO ²⁺
[V ^{III} (aq)]	[V(H ₂ O) ₆] ³⁺	0.56±0.03	0.56±0.05	0.60±0.08
	[V(SO ₄)(H ₂ O) ₅] ⁺	0.20±0.01	0.19±0.01	0.11±0.01
	[V(SO ₄) ₂ (H ₂ O) ₄] ⁻	0.01±0.001	0.01±0.001	0.01±0.001
[V ^{III} (hydr)]	[V(OH)(H ₂ O) ₅] ²⁺	0.10±0.01	0.11±0.01	0.09±0.01
	[V(OH) ₂ (H ₂ O) ₄] ⁺	0.02±0.001	0.02±0.003	0.02±0.003
	[V(OH)(SO ₄)]	0.01±0.001	0.01±0.002	0.01±0.001
	[(V ₂ O)(H ₂ O) ₁₀] ⁴⁺	0.02±0.001	0.02±0.003	0.02±0.002
[V ^{III} (complex)]	[V(cat) ₃] ³⁻	0.02±0.002	0.03±0.004	0.00
	[V(edta)(H ₂ O)] ⁻	0.00	(0.004±0.002)	0.00
[V ^{IV}]	[VO(aq)] ²⁺	0.03±0.002	0.03±0.002	0.05±0.003
	[VO(cat) ₂] ²⁻	0.01±0.001	0.01±0.001	0.01±0.002
	[VO(edta)] ²⁻	0.00	0.00	0.06±0.003

a. Components may not add up to 100% due to round-off error. b. Fraction errors are the averages of the errors given in Table S1. c. The fractions of V(III) complex ions have been calculated from the known compositions of the model solutions using well-established equilibrium constants [40] and the Medusa application [39] (see Materials and Methods section).