lutions will be presented in a subsequent paper.

Conclusion

This study confirms that perchlorate ions form inner-sphere complexes with lanthanide ions in weakly to moderately coordinating solvents. Evidence for such complexes is still rare,[26,28] but their existence is being increasingly invoked, even in aqueous solutions. For instance, the decrease of the hydration number of the Lu(III) ion upon addition of an excess of perchlorate has been tentatively attributed to coordination of the perchlorate ion.[12] Similarly, the decrease in the lifetime of the $^5D_0$ excited state of the Eu(III) ion in aqueous perchlorate solutions upon addition of sodium perchlorate or upon an increase in concentration was explained by a change in the hydration number consecutive with the penetration of a perchlorate ion in the Ln(III) coordination sphere.[31,32] From the quantitative data that we report for anhydrous acetonitrile solutions, it appears that the Ln(III)/ClO_4^- interaction remains moderate and that steric and electrostatic effects are of the same order of magnitude, which explains the larger concentration of associated perchlorate observed for the lighter neodymium ion. Finally, one notes that within the series of investigated ions the largest differences occur between Nd(III) and Eu(III), with most of the data for the intermediate and heavier ions being more or less equal within experimental error.

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Registry No. $Nd(ClO_4)_3$, 13498-06-1; $Eu(ClO_4)_3$, 13537-22-9; $Tb(ClO_4)_3$, 14014-09-6; $Er(ClO_4)_3$, 14017-55-1; MeCN, 75-05-8.

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Characterization of the Binding, Kinetics, and Redox Stability of Vanadium(IV) and Vanadium(V) Protein Complexes in Serum

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The redox kinetics and complexation of vanadium(IV) and vanadium(V) with albumin and transferrin in serum and buffer solution were studied. ^{35}Cl and ^{14}N NMR line width measurements of chloride, perchlorate, and nitrate binding to transferrin, ultraviolet difference spectroscopy, equilibrium dialysis, and ultrafiltration experiments confirm that a specific 2:1 complex is formed between vanadium(V) and apotransferrin. In contrast, vanadium(V) binds only weakly to albumin. EPR data reveal the presence of reducing agents in fresh serum that quantitatively convert vanadate(V) to vanadyl(IV), resulting in formation of specific VO^{2+}-albumin and $(VO^{2+})_2$-transferrin complexes. Half-lives for air oxidation of vanadium(IV) albumin and transferrin complexes and for the reduction of vanadium(V) by endogenous reducing agents in serum fall in the range 5-30 min at pH 7.5. Thus, the interconversion between oxidation states is rapid relative to the normal residence time of vanadium in the circulation. The data suggest that the metal is probably present in both oxidation states in the bloodstream and that albumin as well as transferrin may be involved in the transport of vanadium(IV) whereas vanadium(V) is possibly carried by transferrin alone.

Introduction

Interest in the physiology and biochemistry of vanadium has grown enormously in recent years.[2] Orthovanadate(V), an orthophosphate analogue, is a potent inhibitor of many phosphate metabolizing enzymes and has been used extensively to probe enzyme mechanisms.[2a-d] EPR studies with erythrocytes,[3] fat cells,[4] and yeast[5] have shown that vanadate(V) is rapidly acquired by cells and reduced intracellularly to the vanadyl(IV) ion, VO^{2+}, complexed with glutathione or proteins. However, dimeric vanadate(V) and decavanadate(V) as well as vanadyl(IV) have also been shown to accumulate within cells when yeast cultures are exposed to toxic levels of vanadate(V).[5] In the vanadium-rich blood cells of the tunicates *Ascidia ceratodes* and *A. nigra*[6] the V^{3+} ion, possibly complexed to sulfate or tunichrome, is the principal species present. Small amounts of vanadyl(IV) ion are often found as well. Thus, under physiological conditions the +3, +4, and +5 oxidation states of the metal are accessible both kinetically and thermodynamically. The facility with which vanadium changes oxidation state may be a key to its biological function.

The physiological effects of vanadium on mammals are currently being studied in many laboratories.[2e,f] Early feeding studies suggest that trace amounts of vanadium are essential for normal growth and development of rats and chicks.[7] More recent work suggests that dietary vanadium may have cancer-preventative properties.[8] Although the iron-transport protein transferrin appears to transport dietary vanadium also,[9,10] the species of vanadium present in the circulation have not been completely identified. Vanadium administered intravenously to rats or dogs separates with the transferrin component of plasma during chromatography and electrophoresis.[9a] Similar results are obtained when the metal as vanadate(V) or vanadyl(IV) is administered to rats by gastric intubation.[10] Since recoveries are often in-

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complete, it is possible that other serum proteins or small chelating agents also play some role in binding and transport of vanadium. There is evidence that such species are present.[9] The metal may exist in more than one oxidation state in the physiological milieu of plasma as well. For example, transferrin complexes of vanadium in the +3, +4, and +5 oxidation states have all been prepared in vitro[11] and conceivably could be present in the circulation also. However, there is limited data available on the redox stability and rates of interconversion between oxidation states of vanadium in serum or other physiological fluids.

In this paper we examine the oxidation–reduction and metal binding properties of vanadium with serum proteins in buffer and in freshly drawn serum. The spectroscopic, binding, and kinetic studies described herein provide some insight into the solution chemistry of vanadium in serum.

Experimental Section

Lyophilized human serum apotransferrin of stated 98% purity was purchased from Calbiochem Corp., and crystallized–lyophilized human serum albumin from Sigma Chemical Corp. The proteins were exhaustively dialyzed against 0.1 M $NaClO_4$ (pH 6–7) and then against deionized water. Protein solutions were then adjusted to 0.1 M Hepes buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 25 mM $NaHCO_3$, pH 7.45) by the addition of appropriate stock solutions. To remove adventitious metal ions, all stock solutions were treated with Chelex-100 chelating resin prior to use. Transferrin and albumin concentrations were determined spectrophotometrically from the molar absorptivities of 9.23×10^4 and 4.2×10^4 cm^{-1} M^{-1} at 280 nm for the two proteins, respectively.[12,13]

A few lots of apotransferrin from Calbiochem were found to contain a reducing agent contaminant that reduced vanadate(V) to vanadyl(IV). During kinetic runs, its presence retarded the rate of VO^{2+} oxidation by as much as a factor of 3 and introduced nonlinearity in the first-order plots. Dialysis against 0.1 M $NaClO_4$, a common method of removing low molecular weight species that bind to transferrin, was ineffective in removing the reductant. The protein was therefore pretreated in air with 50 mM vanadate(V) as an oxidant for 4 h and then subjected to exhaustive dialysis. Protein prepared in this way displayed kinetic behavior identical with that of transferrin containing no reducing agent contaminant.

Samples of apotransferrin containing the contaminating reducing agent were subjected to gel filtration chromatography on Sephadex G200 and to sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis with and without the presence of 2-mercaptoethanol. The results indicated that 8–14% of the protein was present as a disulfide-bridged dimeric species.[14b] The isolated dimer exhibited an Fe^{3+} EPR signal indistinguishable from that of the native protein.

In a typical kinetic run of vanadium(IV) oxidation, 300 μL of 1 mM apotransferrin in the above Hepes/bicarbonate buffer or of serum was made anaerobic under an atmosphere of moist prepurified nitrogen or argon gas. Sufficient 0.1 M $VOSO_4$ was added to 90% saturate the transferrin binding sites with vanadium. After the solution was stirred for 15 min, the reaction was initiated by adding 2.7 mL of oxygen-equilibrated serum or buffer (pH 7.45), giving a total volume of 3.0 mL, and the nitrogen atmosphere immediately changed to the appropriate oxygen atmosphere, P_{O_2} = 0.2 atm or 1.0 atm. The pH was monitored with an in situ electrode and maintained at pH 7.45 by the addition of microliter quantities of 0.1 M HCl as needed. At various time intervals, 300-μL aliquots of the reaction mixture were withdrawn, loaded into calibrated sample tubes, and quickly frozen in dry ice–acetone for later EPR measurement.

To oxidize endogenous reducing agents in serum and thus eliminate their effect on the kinetics of vanadium(IV) oxidation, serum was first made 5 μM in vanadate(V) followed by incubation for 4 h under a 5% CO_2/95% O_2 atmosphere. That all of the endogenous reducing agents were consumed was verified by the failure to generate a VO^{2+} spectrum upon addition of vanadate(V) anaerobically to the serum at a concentration of 0.1 mM. The oxidation of vanadyl(IV) in the pretreated serum (containing no vanadate(V)) was carried out by making the serum anaerobic followed by addition of vanadyl(IV) to a concentration of 0.5 mM and initiating the oxidation reaction as described above.

Vanadate(V) solutions were prepared from reagent grade ammonium metavanadate(V) (Aldrich Chemical Co.). The sparingly soluble salt was oven-dried at 110 °C for $^1/_2$ h and then slowly dissolved in water by stirring. The resultant 25 mM solution was then diluted to 0.70 mM in buffer and allowed to equilibrate for 24 h prior to use in the spectrophotometric titrations of apotransferrin and albumin. Ultraviolet difference spectra were measured on a Cary 219 spectrophotometer. Samples were equilibrated for 15 min prior to measuring the spectrum.

Ultrafiltration experiments were performed using an Amicon 3-mL cell fitted with a PM10 membrane having a nominal molecular weight exclusion limit of 10000. The solution contained 0.1 mM vanadate(V) and 0.2 mM apotransferrin or albumin in 0.15 M NaCl/25 mM $NaHCO_3$/50 mM Hepes at pH 7.5. Equilibrium dialysis was carried out for 5 days using a Plexiglass cell with chambers separated by dialysis tubing (A. H. Thomas No. 3787-D42) having a molecular weight exclusion limit of 12000. The two chambers initially contained 40 μM vanadate(V) and either 0.4 mM albumin or 0.2 mM apotransferrin, respectively, in the above buffer. Samples were taken every 24 h for vanadate(V) analysis.

Vanadate(V) concentrations were determined by the addition of 10 μL of 1 M HCl to 50 μL of unknown solution or standard solution containing 0.1 M Hepes, 0.15 M NaCl, and 25 mM $NaHCO_3$ at pH 7.5 with or without 0.2 mM protein, followed by the addition of 10 μL of freshly prepared 0.1 M ascorbic acid to reduce the vanadate(V) to EPR-active vanadyl(IV). The amplitude of the fourth EPR line from low field of the aquo VO^{2+} ion was measured at room temperature in a 1 mm i.d./2 mm o.d. quartz capillary fitted inside a 2 mm i.d./4 mm o.d. quartz tube permanently positioned in the EPR cavity. Linear standard curves (correlation coefficient >0.999) in the concentration range 10–200 μM vanadium were obtained. The relative precision of the vanadium determination was ±6% (95% confidence level) at a metal concentration of 0.1 mM. The detection limit was approximately 5 μM vanadium, corresponding to a signal/noise ratio of 2:1. The E-4 instrument parameters were as follows: field set 330 mT, scan range 100 mT, scan time 30 min, gain 10^4, 100-KHz modulation amplitude 1.0 mT, time constant 3 s, power 100 mW, frequency 9.47 GHz. Only the EPR line of interest was scanned. A weak background cavity signal was subtracted from the signals of the standards and unknowns.

X-Band EPR spectra were measured on a Varian E-4 spectrometer interfaced to a MINC-23 laboratory computer. Spectra consisting of 2048 data points were measured at 77 K with a liquid-nitrogen Dewar insert in a TE_{102} rectangular cavity. ^{35}Cl and ^{14}N NMR line width measurements at 26 °C were made on a JEOL FX-90Q spectrometer at 8.72 and 6.42 MHz, respectively. Line widths were measured from the full width at half-height of the plotted spectrum.

Results

Kinetic Studies. Oxidation of Vanadyl(IV) Transferrin in Buffer. Kinetic studies were carried out to qualitatively assess the redox stability of vanadium–protein complexes and to determine the rates of interconversion between oxidation states. Initially, we studied the rate of oxidation of $(VO^{2+})_2$–transferrin in 0.1 M Hepes/25 mM bicarbonate buffer at 37 °C under an oxygen tension of 0.2 atm. The characteristic EPR spectrum of divanadyl(IV) transferrin (Figure 1) rapidly disappears as the metal is oxidized by molecular oxygen. The C and N spectral components from VO^{2+} binding in the two specific sites of the protein decay at the same rate, indicating no site dependence of vanadium(IV) oxidation. The loss in intensity of the central portion of the EPR spectrum during a typical kinetic run is shown in Figure 2. The first-order plot (Figure 2, inset) is linear over 3 half-lives, giving $t_{1/2}$ = 8.1 ± 1.3 min (average of four runs) under the conditions in Figure 1. The half-life of oxidation of the 1:1 vanadyl(IV) albumin complex is 6.5 ± 1.4 min under the same conditions. Thus, vanadium(IV) oxidation occurs relatively rapidly with similar rates for both proteins.

The effects of the concentrations of apotransferrin and bicarbonate and the partial pressure of oxygen on the rate were briefly investigated to determine the sensitivity of vanadium(IV) oxidation to these parameters. The presence of excess apotransferrin (0.2 mM apoprotein, 0.1 mM VO^{2+}) decreased the rate of oxidation by 40%, resulting in an increase in $t_{1/2}$ from 8.1 ± 1.3 to 11.3 ± 1.1 min at an oxygen partial pressure of 0.2 atm. Since excess apoprotein favors VO^{2+}–transferrin complex for-

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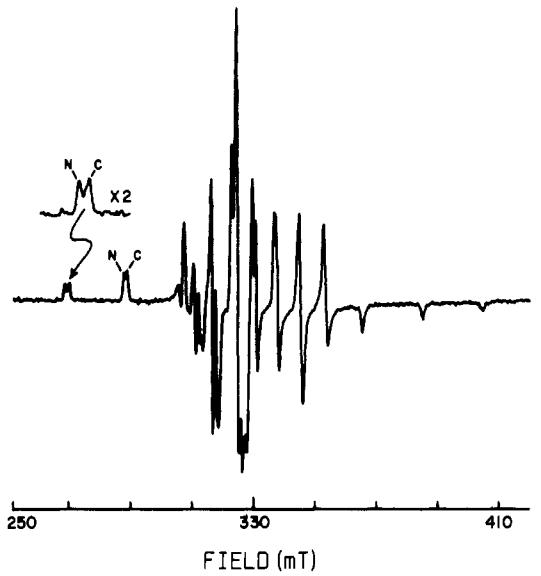

Figure 1. Frozen-solution EPR spectrum of vanadyl(IV) transferrin. The C and N labels denote resolved signals from VO^{2+} binding in the N- and C-terminal metal-binding domains of the protein. Conditions: 0.2 mM vanadyl(IV) sulfate, 0.3 mM apotransferrin in 0.1 M Hepes, 25 mM $NaHCO_3$, pH 7.5, 77 K. Instrument settings: field set 330 mT; scan range 200 mT; time constant 0.3 s; scan time 16 min; modulation amplitude 0.5 mT; gain 2000; microwave power 5 mW; microwave frequency 9.112 GHz.

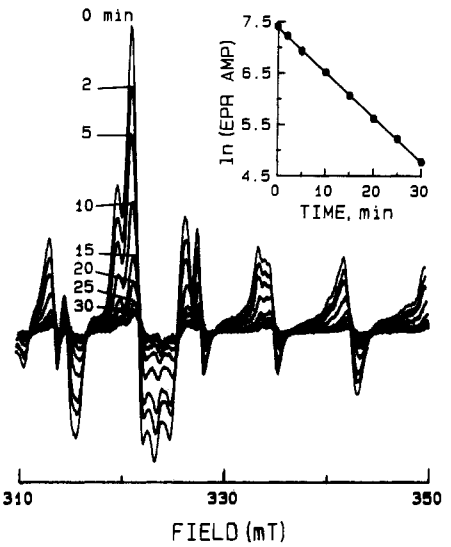

Figure 2. Oxidative decay of the vanadyl(IV) transferrin EPR spectrum. The central portion of spectrum is shown. Inset: semilogarithmic first-order plot. Conditions: 0.2 mM vanadyl(IV) sulfate, 0.1 mM apotransferrin, 0.1 M Hepes, 25 mM $NaHCO_3$, pH 7.5, 37 °C. Instrument settings: as in Figure 1 except scan range 40 mT, time constant 0.3 s, scan time 4 min, and gain 1500.

mation, we conclude that oxidation of the vanadium probably occurs via the "free metal ion". The polymeric hydroxide species of unchelated vanadium(IV) are known to be highly susceptible to air oxidation whereas the +4 oxidation state is stabilized by complexation.[2a,b]

Bicarbonate binding to apotransferrin is required for VO^{2+} binding,[14a] and therefore the absence of excess bicarbonate was expected to increase the rate of vanadium(IV) oxidation. However, with only 0.3 mM bicarbonate from ambient CO_2[15] available for VO^{2+} binding, the rate of oxidation instead decreased, $t_{1/2}$ = 12.4 ± 1.3 min (3 runs). An enhanced rate of exchange of VO^{2+} between protein-bound and free forms may account for the higher

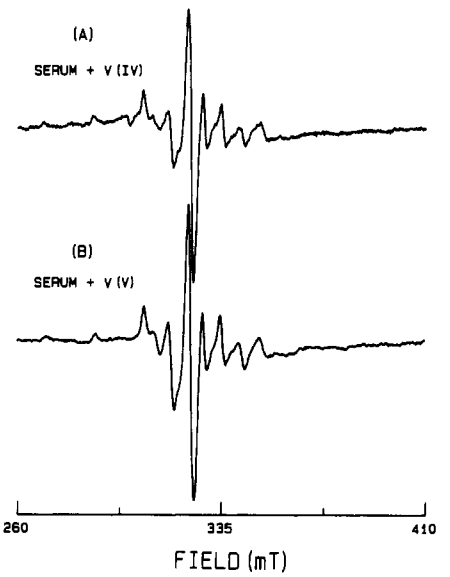

Figure 3. Frozen-solution EPR spectra of vanadium in serum. The metal is added as vanadyl(IV) sulfate (upper spectrum) or ammonium metavanadate(V) (lower spectrum). In both instances, the spectrum is principally due to a VO^{2+}–albumin complex. Conditions: 0.1 mM vanadium, pH 7.5, 77 K. Instrument parameters: field set 330 mT; scan range 200 mT; time constant 3 s; scan time 30 min; modulation amplitude 0.5 mT; power 5 mW; frequency 9.113 GHz.

oxidation rate in 25 mM bicarbonate. Excess bicarbonate is known to significantly facilitate the exchange of free and bound carbonate in Fe(III)–transferrin–CO_3.[16a] Since metal and bicarbonate binding to the protein are intricately related, metal ion exchange might be enhanced also.[16b]

An increase in oxygen partial pressure from 0.2 to 1.0 atm decreased the half-life of vanadium(IV) oxidation by a factor of 2.8 ± 0.6 (four measurements). This value is much less than the fivefold change expected for a first-order dependence on P_{O_2}. A factor of 2.23 is expected for a square-root dependence.

Redox Studies of Vanadium in Serum. Initial studies of vanadyl(IV) oxidation in serum showed markedly curved semilogarithmic first-order plots yielding apparent half-lives of 30 min or more. These effects were traced to the presence of reducing agents in serum that retarded the net rate of vanadium(IV) oxidation. When vanadate(V) was added anaerobically to fresh serum followed by an incubation period of $^1/_2$ h, the EPR spectrum of vanadium(IV) shown in Figure 3B was obtained. The spectrum is principally due to the formation of the 1:1 vanadyl(IV) albumin complex, which has been previously characterized in this laboratory.[17] A spectrum of the same amplitude was obtained upon addition of vanadyl(IV) directly to serum (c.f. Figure 3A,B), indicating quantitative reduction of vanadate(V) to vanadyl(IV) had occurred. Comparison of the double integral of spectrum B in Figure 3 with that of a 0.5 mM $VOSO_4$ EPR intensity standard showed that all of the vanadium was accounted for by the EPR spectrum. Little, if any, EPR-silent vanadium(III) must be present. Reduction of vanadate(V) to vanadyl(IV) was only observed with fresh serum.

The rates of oxidation of vanadium(IV) and reduction of vanadium(V) in pooled fresh serum were investigated. In the reduction reaction, vanadate(V) was added to serum anaerobically and the growth in the EPR spectrum of the vanadyl(IV) albumin complex measured. In the oxidation reaction, vanadyl(IV) was added to anaerobic serum that had been previously treated to remove reducing agents (see Experimental Section). The serum was then brought to 20% oxygen tension by the addition of oxygen-saturated (P_{O_2} = 1 atm) serum and the sample exposed to

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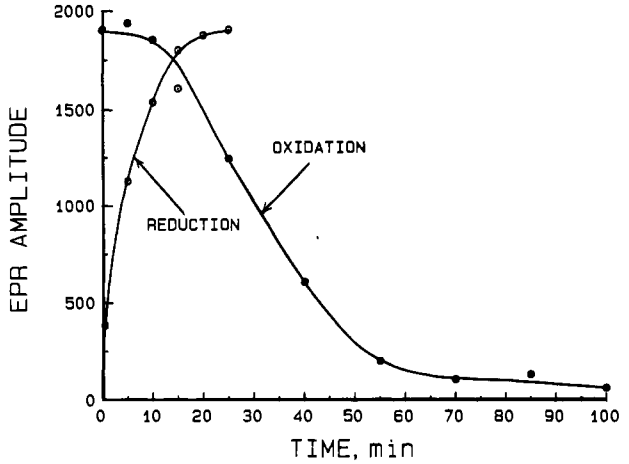

Figure 4. Oxidation of vanadium(IV) and reduction of vanadium(V) in serum as a function of time. Oxidation conditions: 50 μM vanadyl(IV) sulfate added anaerobically to serum at pH 7.5 followed by exposure to the atmosphere at 37 °C. The serum was previously treated with vanadate(V) to remove reductants prior to introduction of vanadyl(IV) (see Experimental Section). Reduction conditions: 50 μM ammonium metavanadate added to fresh serum anaerobically at pH 7.5 and 37 °C under dinitrogen.

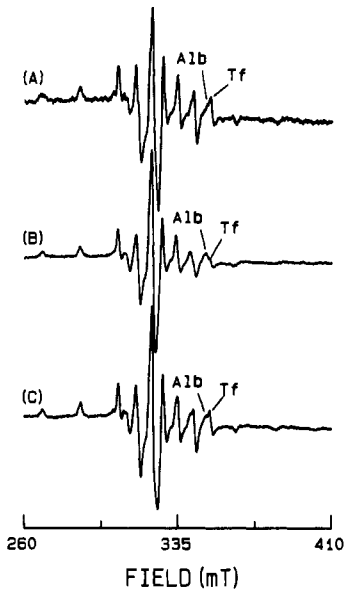

Figure 5. Frozen-solution EPR spectra of vanadyl(IV) in serum: (A) 25 μM vanadium; (B) 50 μM vanadium; (C) 50 μM vanadium plus 50 μM added apotransferrin. Partially resolved peaks due to transferrin and albumin are denoted. Spectral analyses (see text) indicate that in all instances binding to albumin is favored. Conditions: N_2 atmosphere, pH 7.45, 77 K. Instrument settings: field set 330 mT; scan range 200 mT; scan time 30 min; time constant 3 s; modulation amplitude 0.5 mT; gain 10000 (A) or 6200 (B, C); power 5 mW; frequency 9.112 GHz; modulation amplitude 0.5 mT. Spectra scaled to the same amplitude.

the air. The decay of the vanadyl(IV) EPR spectrum was recorded. The results for both reactions are shown in Figure 4. Approximate half-lives of 5 min (reduction) vs. 15 min (oxidation) were obtained. After an initial 10-min lag the oxidation reaction showed first-order kinetics over 3 half-lives whereas the reduction reaction did not. The kinetics of these reactions were dependent on the serum sample. The rate of reduction decreased with the age of the serum.

Protein Binding Studies. Equilibrium Distribution of Vanadium(IV) between Albumin and Transferrin in Serum. EPR spectroscopy was used to estimate the partitioning of VO^{2+} between transferrin and albumin in serum under equilibrium conditions. VO^{2+} was added to pooled serum at pH 7.45 under dinitrogen and allowed to incubate for 15 min, and the EPR spectrum was recorded (Figure 5). The transferrin and albumin signals are

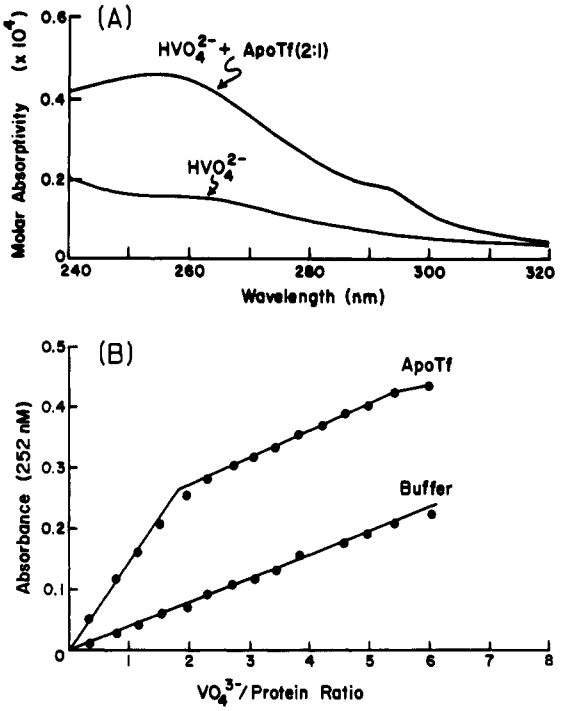

Figure 6. (A) Ultraviolet difference spectra. Conditions: 28 μM vanadate(V) plus 14 μM apotransferrin (upper curve) or 28 μM vanadate(V) alone (lower curve) in 0.1 M Hepes, 20 mM $NaHCO_3$, pH 7.5, 20 °C in sample cell and buffer alone or with apotransferrin in the reference compartment. (B) Spectrophotometric titration of apotransferrin with vanadate(V): apotransferrin in buffer (upper curve) or buffer alone (lower curve).

most resolved in the upfield perpendicular region of the spectra (Figure 5). Although vanadyl(IV) transferrin is present in smaller amounts, its EPR lines are more pronounced because of their relatively narrow widths. To obtain the relative amounts of the two protein complexes present, the EPR spectra in Figure 5 were compared with spectra obtained by summing appropriately weighted spectra of the pure protein complexes. At a VO^{2+} concentration of 25 μM V^{2+} the metal is distributed between albumin and transferrin in a 2.3:1 ratio (Figure 5A). This ratio increases to 6.4:1 at 50 μM VO^{2+} (Figure 5B). Addition of apotransferrin (50 μM apotransferrin/50 μM VO^{2+}) reduces the ratio from 6.4:1 to 2.1:1 (Figure 5C).

In serum, transferrin is typically 30% saturated with iron,[12] the average concentrations of albumin and transferrin in serum are 400 and 35 μM, respectively.[18] From this information and the above ratios of VO^{2+}–protein complexes in serum, we estimate that the ratio of VO^{2+} association constants for these proteins falls in the range $K_{tf}/K_{alb} \sim 2-6$, transferrin having a slightly larger affinity than albumin for VO^{2+}.

To obtain an independent measure of K_{tf}/K_{alb}, a competitive binding experiment was performed with a solution equimolar in sites of the two proteins (100 μM albumin, 50 μM apotransferrin, and 80 μM VO^{2+} in 0.1 M Hepes/25 mM $NaHCO_3$ at pH 7.45). The EPR spectrum indicated a distribution of vanadium(IV) between transferrin and albumin of ~3:1. The same result was obtained whether the VO^{2+}–albumin complex was first formed followed by addition of the apotransferrin or vice versa, indicating that equilibrium had been achieved. From these results, we calculate a value of $K_{tf}/K_{alb} \sim 6$ in accord with the findings for serum above. Although transferrin has a somewhat higher association constant for VO^{2+}, the higher concentration of albumin in serum results in vanadium(IV) being bound principally to the latter protein.

Vanadium(V) Binding to Transferrin and Albumin. Ultraviolet Difference Spectroscopy. While the binding of VO^{2+} to transferrin

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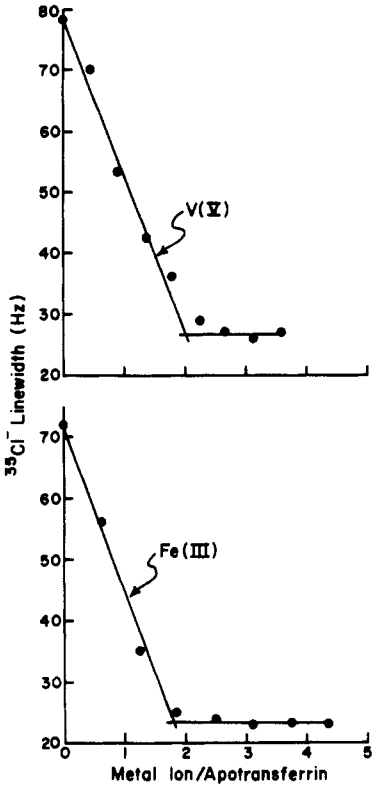

Figure 7. ^{35}Cl NMR line width of chloride ion as a function of metal:apoprotein ratio: vanadate(V) (upper curve) and iron(III) (lower curve). Conditions: 0.3 M NaCl, 50 μM apotransferrin, 0.1 M Hepes, 20 mM $NaHCO_3$, pH 7.5, 28 °C.

and albumin has been extensively studied,[11d,14,17] the binding of vanadium(V) has not. Ultraviolet difference spectroscopy is frequently used to study metal ion binding to transferrin (e.g. ref 19) and was employed here to study vanadium(V) binding. The ultraviolet difference spectrum obtained with vanadate(V) and apotransferrin in a 2:1 ratio measured against apotransferrin as a reference is shown in Figure 6A. A maximum absorbance at 252 nm and a shoulder at 290 nm are observed. The spectrum of vanadate(V) in buffer alone is also shown for comparison (Figure 6A). An interaction between vanadium(V) and the protein is evident. The bands suggest tyrosine coordination with the protein.[19] The results are in general agreement with those recently published by Harris and Carrano.[11a]

The spectrophotometric titration curve shown in Figure 6B indicates that a 2:1 complex is formed, a stoichiometry commonly found for metal ion complexes of transferrin.[12,19,20] From the linear portion of the curve, we calculate $\epsilon_{290nm} = 5900 \pm 150$ M^{-1} cm^{-1} and $\epsilon_{252nm} = 9750 \pm 220$ M^{-1} cm^{-1} for the first metal bound (average of two titrations), in agreement with the reported value of 9400 M^{-1} cm^{-1}.[11a] Beyond a 2:1 metal/protein ratio the slope of the titration curve is 22% higher than that with the buffer alone (Figure 6B), suggesting some additional vanadium(V) binding. A spectrophotometric titration of diferric transferrin with vanadate(V) showed no break at a 2:1 stoichiometry and produced a line similar to that obtained with buffer alone. Thus, iron(III) binding blocks vanadium(V) binding at the high affinity sites. In contrast to the results with transferrin, a spectrophotometric titration of albumin showed no evidence for the formation of a strong complex with vanadate(V).

^{35}Cl and ^{14}N NMR Quadrupolar Relaxation Measurements. The interaction of vanadium(V) with apotransferrin was studied further by NMR line width measurements employing the probe anions $^{35}Cl^-$, $^{35}ClO_4^-$, and $^{14}NO_3^-$. NMR is useful here since the binding

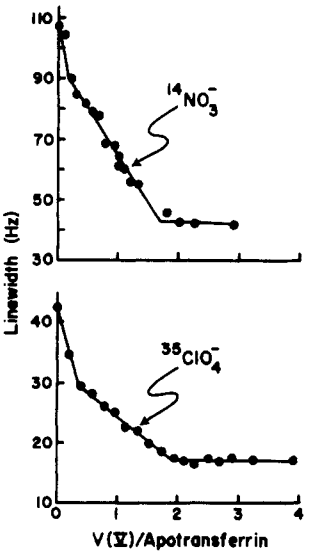

Figure 8. ^{14}N and ^{35}Cl NMR line widths of nitrate and perchlorate ions as a function of vanadate(V):apoprotein ratio. Conditions: 0.2 M $NaNO_3$ or $NaClO_4$, 0.2 mM apotransferrin, 0.1 M Hepes, 20 mM $NaHCO_3$, pH 7.8.

of these anions is strongly influenced by the binding of metals to the protein. The fast-exchange limit is almost always achieved for binding of inorganic anions to proteins and has been demonstrated for transferrin.[21] In this instance the observed line width is the weighted average of line widths of the free and bound forms of the anion.

Figure 7A illustrates the effect of vanadate(V) on the ^{35}Cl NMR line width of solutions of apotransferrin containing NaCl. The addition of vanadium causes a pronounced decrease in the line width, reaching a minimum value at a metal:protein molar ratio of approximately 2:1, a result confirming the stoichiometry obtained from the spectrophotometric titration (Figure 6B). A similar change in line width is observed when the apoprotein is titrated with iron (Figure 7B), indicating that anion binding is similarly reduced by the binding of either metal.

^{35}Cl and ^{14}N NMR experiments with the anions perchlorate and nitrate yielded similar results (Figure 8). The pronounced reduction in line width in the early part of both titrations in Figure 8 may reflect nearly sequential binding of vanadium to the two sites of the protein. The interactions of anions with the two domains of iron(III) transferrin are not identical and differ for different anions.[22] The association constants for the binding of the first and second vanadium(V) to transferrin also are not the same.[11a] The contributions of each metal binding domain of the protein to the net rates of ^{35}Cl and ^{14}N relaxation are probably different as well.

Ultrafiltration and Equilibrium Dialysis Studies. Vanadium(V) binding to transferrin and albumin was also studied by ultrafiltration and by equilibrium dialysis experiments under conditions outlined in the Experimental Section. In the ultrafiltration experiment, the retentate and ultrafiltrate were analyzed for vanadate(V) after the volume was reduced by 50% in the filtration cell. A solution containing only vanadate(V) in buffer served as a control to check for vanadium retention by the membrane. None was found. Complete retention of the metal by the transferrin solution was observed, the vanadium in the ultrafiltrate being below the detection limit for the metal. From this result we estimate

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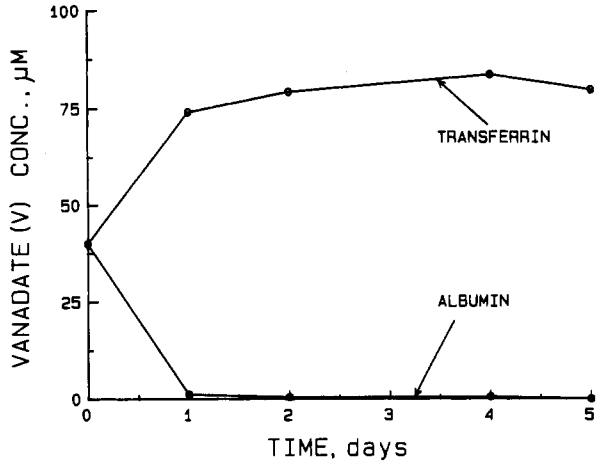

Figure 9. Competitive binding between albumin and apotransferrin for vanadium(V). The equilibrium dialysis was performed with 0.2 mM transferrin and 0.4 mM albumin in separate chambers containing 40 μM vanadate(V) in each. At equilibrium the vanadium partitions to the transferrin chamber. The first data point corresponds to the initial vanadium(V) concentration in each chamber; subsequent points are measured values as outlined in the Experimental Section. Conditions: 0.15 M NaCl, 25 mM $NaHCO_3$, 50 mM Hepes, pH 7.5, 20 °C.

that the formation constant for the vanadium(V) transferrin complex must be greater than 6000 M^{-1}, in accord with the reported value of 3.2×10^6 M^{-1} derived from spectrophotometric data.[11a]

In contrast to transferrin, little vanadium(V) was retained by the albumin solution, i.e. 0.096 ± 0.006 mM vanadium in the retentate vs. 0.078 ± 0.006 mM in the ultrafiltrate. These values correspond to a formation constant of nominally 600 M^{-1}, assuming a 1:1 complex.

Figure 9 summarizes the results of a competitive binding experiment between apotransferrin and albumin for vanadium(V) using equilibrium dialysis. Equilibrium was rapidly established with virtually complete partitioning of the vanadium(V) to the transferrin compartment, a finding in agreement with the ultrafiltration results. Thus, albumin is ineffective in competing with apotransferrin for vanadium(V).

Discussion

The data presented in Figures 6–9 confirm the formation of a specific 2:1 complex of vanadium(V) with transferrin. The identity of the vanadium(V) species bound to transferrin is not completely clear, however. At pH 7.5 and a vanadium concentration of 15 μM employed in the UV experiments, the dominant forms of free vanadate(V) in solution are the phosphate analogue anions $H_2VO_4^-$ and HVO_4^{2-} with polynegative oligomeric forms accounting for less than 20% of the total.[2a] However, upon complexation by the protein, oxo ligand elimination reactions may occur that lead to protein-coordinated cationic species such as the vanadyl(V) ion, VO^{3+}, or the pervanadyl(V) ion, VO_2^+. For example, VO_2^+ is formed when orthovanadate(V), VO_4^{3-}, is complexed by EDTA.[23] On the basis of the use of the ligand ethylenebis((*o*-hydroxyphenyl)glycine) as a model for the binding sites of transferrin, Harris and Carrano propose that vanadium(V) binds to the protein in the form of the VO_2^+ ion and is coordinated to the phenolate groups of two tyrosine residues.[11a] Such as assignment is certainly reasonable in view of current knowledge of the chemistry of vanadium and transferrin.

The anion NMR data (Figures 7, 8) provide some insight into the conformational properties of transferrin and its metal ion complexes. A number of studies have shown that the kinetic and thermodynamic properties of transferrin in solution change markedly in the presence of salts, effects attributed in part to the binding of anions to the protein.[22] The similar reduction in NMR line width upon binding of either vanadium(V) or iron(III) to apotransferrin (Figure 7) suggest that both metals affect the conformation of the protein in the same way. Iron(III) transferrin is known to have smaller hydrodynamic radii than the apoprotein;[24] protein folding could result in a decrease in available sites for anion binding, causing a decrease in NMR line width as observed. Recent models for the metal and bicarbonate binding sites of the transferrin show the presence of cationic groups of lysine and arginine near the probable iron-binding ligands.[25] Anions such as chloride and perchlorate may be denied access to these groups in the more compact structure of the metallotransferrin complex.

The observation of reducing agents for vanadium(V) in serum (Figure 3) is of particular interest since extracellular vanadium has been generally thought to exist predominantly in the +5 oxidation state. Agents present in serum that may serve as reductants for vanadium(V) include ascorbate,[26] catecholamines,[27] and cysteine,[28] among others.[2a-d] The in vitro data (Figures 3, 5) suggest that a significant amount of the reduced vanadium is probably complexed to albumin as VO^{2+}. Although fractionation of plasma by chromatography or electrophoresis in our laboratory and elsewhere has invariably shown vanadium-48 to be almost exclusively associated with the transferrin component,[9,10] occasionally low levels of the metal have been observed in the albumin fraction also.[9a] With the exception this latter work,[9a] the effect of the vanadium(IV) oxidation on the results of the separation has not been taken into account. The data in Figures 2 and 4 indicate that both vanadyl(IV) albumin and vanadyl(IV) transferrin would rapidly oxidize during the time required to fractionate plasma unless precautions are taken against exposure of the sample to air. Since vanadium(V) binds strongly to transferrin but only weakly to albumin (Figure 9), vanadium separation with the transferrin fraction is the expected result. Thus, vanadyl(IV) albumin, if it were present initially in plasma, would escape detection. Therefore, the possibility that albumin plays a role in vanadium transport cannot be excluded at this point. Direct EPR measurement of the distribution of VO^{2+} in serum of animals on vanadium-supplemented diets is precluded by its low concentration, ~1 μM in the circulation.[2a]

Vanadokinetic studies indicate half-lives of approximately 14 h, 24 h, and 8.5 days for the clearance of different vanadium pools from the circulation of experimental animals following intravenous injection of solutions of vanadium salts.[9] A rapidly clearing pool, $t_{1/2} < 0.5$ h, has been also observed and attributed to low molecular weight vanadium(V) species that are readily taken up by cells. Our in vitro kinetic measurements with serum (Figure 4) indicate that the interconversion between vanadium oxidation states is short relative to the residence time of most of the metal in the circulation. Endogenous reducing agents and dissolved oxygen ensure that both the +4 and +5 oxidation states are present in serum. However, the actual distribution of vanadium between oxidation states and between transferrin and albumin will depend on kinetic factors such as the concentration and identity of reductants in plasma, the fluctuation in oxygen tension between 40 and 100 torr for venous and arterial blood, respectively, and the flux of the metal in and out of cells. In the case of yeast cells, intracellular VO^{2+} from vanadate(V) reduction is subsequently exported to the external medium.[5] Such a process may be a feature of cells in general, contributing significant amounts of VO^{2+} to the plasma pool of vanadium. In this connection, vanadyl(IV), as well as vanadate(V), has recently been found to inhibit the Na,K-ATPase enzyme.[29,30] Several aspects of the clearance of vanadium from

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the blood have been nicely discussed by Harris et al.[9a]

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Complexation of Copper(II) with a Macrocyclic Peptide Containing Histidyl Residues: Novel Observation of NMR Spectra of Paramagnetic Copper(II) Compounds

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Interaction of Cu(II) and *cyclo*-(Gly-His-Gly-His-Gly-His-Gly), a synthetic macrocyclic peptide, was investigated by electronic absorption, EPR, and ^1H and ^{13}C NMR spectroscopy. These experiments have been conducted in aqueous solution as a function of pH and for different ligand:Cu(II) ratios. The results indicate the presence of two species. The species found under neutral conditions is described as one involving the three N(1) nitrogens of imidazole residues, while under basic conditions, the complexation occurs via four GlyNH deprotonated peptide nitrogens. Both systems are found to be in slow exchange over the wide pH range, with dissociation constants of 1.25×10^3 s^{-1} and $\ll 100$ s^{-1}, respectively. An unexpected behavior was observed at basic pH by ^1H and ^{13}C NMR techniques. The data are interpreted as Cu(II) being firmly trapped inside the cyclic cavity. The results revealed for the first time some important properties that may have significance in studying the paramagnetic behavior of biologically relevant Cu(II) compounds by NMR spectroscopy.

Interest in synthetic multidentate macrocyclic compounds is continually increasing because of their unique properties. Among these compounds, cyclic peptides, which owe their functional properties to their macrocyclic structure, have attracted much attention in recent years.[1] Cyclic peptides belong to a special class of peptides well-known for their biological functions as hormones, antibiotics, toxins, and regulators of ion transport.[2-4] The reduced flexibility of the peptide backbone and the absence of both N- and C-terminal functional groups make the cyclic peptides attractive models to study the structural and functional aspects of proteins and enzymes.[5-8] In such a model, by varying the number and the nature of the amino acid units, one can obtain cyclic peptides with different cavity sizes, different coordination environments, and different amino acid side chains that are important for the functional sites of proteins and enzymes. Among the amino acid residues, the histidyl side chain is probably the most frequently encountered metal-binding site in biological systems. The histidyl residue plays an important role in the active sites of many enzymes[9-13] and the metal-transport sites of several transport proteins.[14-20] Thus, information obtained from metal complexes with synthetic model compounds containing histidyl residues are very useful.

Our investigations, thus far, have been devoted to the interactions of diagmagnetic metals with the side-chain residues of model cyclic peptides.[5,6] In the present investigation, we chose to synthesize *cyclo*-(Gly-L-His-Gly-L-His-Gly-L-His-Gly) (herinafter denoted as G4H3) and study its complexation properties with the paramagnetic metal Cu(II) in aqueous solution over a wide pH range using electronic absorption, EPR, and ^1H and ^{13}C NMR spectroscopy. From analyses of the spectral data, it is concluded that Cu(II) binds to G4H3 in two modes: one involving three imidazole residues at neutral pH and the other involving four deprotonated peptide amide nitrogens at basic pH. An unusual NMR observation of a paramagnetic Cu(II) compound is reported.

Table I. Spectroscopic Data for G4H3–Copper(II) Complexes[a]

pH	λ_{max}, nm (ϵ)	$A_\parallel$, G	$g_\parallel$	$g_\perp$
5.3	620 (9)	176	2.254	2.058
6.3	590 (19)	175	2.252	2.059
7.0	560 (27)	174	2.250	2.060
8.5	610 sh 540 (33)	174 188	2.252 2.204	2.059 2.052
10.0	540 (35)	190	2.202	2.052
11.2	540 (43)	190	2.200	2.050

[a] sh = shoulder.

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