Complexes of Human Lactoferrin with Vanadium in Oxidation States +3, +4 and +5*

Clyde A. Smith, Eric W. Ainscough and Andrew M. Brodie
Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand

Complexes of human lactoferrin with vanadium in the +3, +4 and +5 oxidation states have been characterised, as biological models for vanadium–protein interactions, by UV difference spectroscopy. The results indicate that the metal binds to lactoferrin in the specific iron(III) binding sites with a metal : protein stoichiometry of 2:1. Electron spin resonance spectroscopy shows oxygen or hydrogen peroxide rapidly oxidise vanadium(III)–lactoferrin to the vanadium(IV) complex which is then itself oxidised to vanadium(V)–lactoferrin. The metal-binding sites of the three vanadium–lactoferrin complexes have been modelled assuming a common protein–ligand donor set. The results show that the metal ion can be accommodated by the protein in the oxidation states +3, +4 and +5 as the V^{3+}, VO^{2+} and VO_2^+ ions respectively, the increase in V–O(oxo) co-ordination being compensated for by decreasing the V–O(carbonate) interaction.

Vanadium has an important role in many biological processes. For example it is present in the active site of bromoperoxidases found in algae, seaweeds and lichens and in some nitrogenases. It is found in ascidian (sea squirt) blood cells and has been observed as an insulin mimetic.[1] Furthermore it has been proposed that delivery of vanadium in higher organisms is performed by transferrin.[2] In an attempt to gain insight into the biological roles of vanadium, many recent studies have focussed on the co-ordination chemistry of this metal, in the oxidation states +3, +4 and +5, with biologically relevant ligands.[3] In contrast to the approach using small molecule ligands, we have chosen to use the well characterised and defined protein molecule, lactoferrin, as a biological model for studying protein–vanadium interactions.

Lactoferrin, an iron-binding glycoprotein found in human milk, is structurally related to members of the transferrin family of proteins: namely the serum transferrins and ovotransferrins from various species[4,5] and the membrane-associated melanotransferrin.[6] These proteins, with the exception of the latter[7] and an insect transferrin isolated from the tobacco hornworm,[8] have the ability to bind two Fe^{3+} ions concomitantly with two carbonate ions. In addition, they are also capable of binding a host of other metal ions, including most first-row transition metals, several second- and third-row transition metals, the Group 13 metals and a number of lanthanides and actinides.[5]

Lactoferrin consists of a single polypeptide chain of 691 amino acid residues arranged into two halves (the N-terminal and C-terminal lobes) joined by an α-helix. Each lobe can be further divided into two dissimilar domains, with the metal-binding sites located in the interdomain cleft.[9] In diiron(III) lactoferrin, each iron is co-ordinated to two tyrosine residues, one histidine and an aspartic acid, in addition to a bidentate carbonate. The carbonate anion is hydrogen bonded to the N-terminus of a helix and the sidechains of two amino acid residues and serves as a bridge between the metal ion and a positively charged region of the protein.[9]

The recent structural determination of dicopper(II) lactoferrin[10] shows that the Cu^{2+} binds in the same specific metal-binding site that is occupied by Fe^{3+} in the native diiron(III) lactoferrin complex. It is, therefore, reasonable to assume that other metal ions which are known to bind to lactoferrin[5] may be accommodated in this specific metal-binding site and could interact with the protein in a similar fashion to iron(III) and copper(II) This will, however, be dependent upon the flexibility (or lack thereof) in the specific binding sites.

Complexes of serum transferrin with vanadium in the +3,[11,12] +4[12–17] and +5[16,18,19] oxidation states have been reported previously by several groups and in all cases the metal to protein stoichiometry of vanadium binding was reported as 2:1. A limited amount of work has been done on vanadium binding to other members of the transferrin family, with studies of the vanadium(IV) complexes of ovotransferrin,[20,21] bovine lactoferrin[22] and human lactoferrin[17,23] only being reported.

In our continuing study of the interaction of metal ions with lactoferrin,[24–26] we have undertaken a comparative study of the binding of vanadium in its +3, +4 and +5 oxidation states to human lactoferrin. In this paper we report these results and compare them with those obtained with serum transferrin. Postulated modes of vanadium(IV) and vanadium(V) co-ordination to lactoferrin have been modelled. Overall the results give further insight into the redox stability and co-ordination behaviour of vanadium in its different oxidation states in biological processes.

Experimental

Apolactoferrin was prepared following previously reported methods,[27] which involved an initial ammonium sulfate precipitation and chromatography on a Sephadex CM-50 column. The purified apolactoferrin was stored in 0.025 mol dm^{-3} Tris–HCl, 0.1 mol dm^{-3} NaCl, 0.01 mol dm^{-3} $NaHCO_3$, pH 7.8 at 4 °C [Tris = tris(hydroxymethyl)methylamine]. The concentration and iron saturation of the lactoferrin was estimated from the absorbances at 280 and 466 nm using absorption coefficients for a 1% (10 mg cm^{-3}) solution of 10.9 and 0.51 dm^3 mol^{-1} cm^{-1} respectively.[24] Iron saturation was typically below 10%. The purity of the isolated protein was determined by gel electrophoresis in denaturing conditions.[28]

Instrumentation and Titration Procedures.—Electron spin resonance spectra were recorded at −160 °C on a Varian E104A spectrometer fitted with a Varian E-257 variable-temperature accessory. Conditions: frequency ≈9 GHz, microwave power 6 mW, scan time 4 min, modulation

* *Non-SI unit employed:* G = 10^{-4} T.

amplitude 20 G, time constant 0.25 s. Spectral g values were calibrated with a 1,1-diphenyl-2-picrylhydrazyl (dpph) internal standard. Protein concentrations were typically 7 mg cm^{-3}.

Ultraviolet difference spectrophotometric titrations were recorded on a Hewlett-Packard HP8452A diode array spectrophotometer. For a typical titration the apolactoferrin sample was loaded into two sealed argon-flushed 3 cm^3 quartz cuvettes. One was used as a reference, while aliquots of the metal ions in the order of 2–10 μl were added to the other with the mixtures being stirred thoroughly after each addition. An equivalent volume of distilled deionised water was added to a second apolactoferrin solution and the difference spectrum in the UV region (230–350 nm) was recorded. The peak near 240 nm was monitored for at least 10 min until the absorbance values remained constant.

Vanadium Solutions.—Stock solutions (≈0.01 mol dm^{-3}) of the vanadium ions were prepared from anhydrous VCl_3, $VOSO_4 \cdot H_2O$ and NH_4VO_3. Vanadium(III) solutions were prepared and handled under argon as the $V^{3+}(aq)$ ion is very unstable towards oxidation. A sample of VCl_3 was transferred into a pre-weighed, argon-flushed 20 cm^3 Schlenk tube[29] inside an argon-filled glove bag. The sealed tube was weighed and the VCl_3 dissolved in a known volume of degassed distilled deionised water injected through a rubber septum. The final concentration was determined by titration of aliquots of the V^{III} solution with $KMnO_4$ previously standardised with oxalate.[30] The concentrations of vanadium(IV) solutions were determined either by direct titration with $KMnO_4$ or by measuring the visible absorption at 750 nm and using the molar absorption coefficient of 18.0 dm^3 mol^{-1} cm^{-1} to calculate the concentration.[31] The two methods agreed to within 0.5%. The vanadium(V) solution concentrations were determined by reduction of vanadium(V) to vanadium(IV) by ammonium iron(II) sulfate, followed by oxidation by $KMnO_4$.[30] The excess Fe^{II} was destroyed by persulfate prior to the addition of $KMnO_4$. Persulfate ions react rapidly with Fe^{2+} ions but only slowly with vanadium(IV), so that if the $KMnO_4$ titration is completed quickly, the loss of vanadium(IV) is negligible.

Preparation of the Vanadium–Lactoferrin Complexes.—*Vanadium(III)–lactoferrin.* Apolactoferrin solutions were thoroughly degassed by repeated evacuation and argon flushing in a Schlenk tube to remove all traces of oxygen. Sufficient solid sodium bicarbonate was added to the apolactoferrin solution to make it 0.01 mol dm^{-3} in carbonate before its removal from the Schlenk tube, as the evacuation/flushing steps removed virtually all of the carbonate which was initially present. The pH of the apolactoferrin solution was also monitored at this stage and adjusted to 7.8 if necessary. The pH electrode was immersed into the Schlenk tube in a steady positive flow of argon. The protein solution was then transferred by syringe to either an argon-flushed 3.5 cm^3 quartz cuvette for spectrophotometric titrations, or into an argon-flushed system consisting of a 15 cm^3 Thunberg tube joined to a quartz ESR tube for the recording of ESR spectra. Sufficient aliquots (1–2 μl) of the vanadium(III) solution were then added using a syringe.

Vanadium(IV)–lactoferrin. Although stable in air at acidic pH, vanadium(IV) is rapidly converted to the vanadium(V) ion at physiological pH.[15,16] In order to avoid this, the vanadium(IV) solution was added to the apolactoferrin solution which had been treated as described above for the vanadium(III) case, in that all traces of oxygen were rigorously excluded by the application of repeated evacuation and flushing with argon. The vanadium(IV) solution was added to the apolactoferrin in either a quartz cuvette or the Thunberg ESR tube, depending upon the type of experiment being undertaken.

Vanadium(V)–lactoferrin. Vanadium(V)–lactoferrin was prepared by adding the vanadium(V) solution directly to an apolactoferrin solution at pH 7.8. The resulting solutions were colourless but the formation of a specific ternary complex was monitored by UV difference spectroscopy.

Modelling Studies.—Modelling of the binding of vanadium to lactoferrin in the +3, +4 and +5 oxidation states was carried out using the program FRODO[32] on an Evans and Sutherland PS300 interactive graphics terminal. Atomic coordinate sets used were those of diiron(III) lactoferrin refined at 2.2 Å resolution (R factor = 0.187, E. N. Baker, personal communication) and dicopper(II) lactoferrin[10] refined at 2.1 Å resolution (R factor = 0.198).

Results and Discussion

Ultraviolet Difference Spectra.—The UV difference spectra obtained from spectrophotometric titrations of human apolactoferrin with vanadium solutions in oxidation states +3, +4 and +5 are shown in Fig. 1. Molar absorption coefficients of the three vanadium–lactoferrin complexes, along with other metal–lactoferrin and some metal–transferrin complexes are given in Table 1. In the case of the two lower oxidation states,

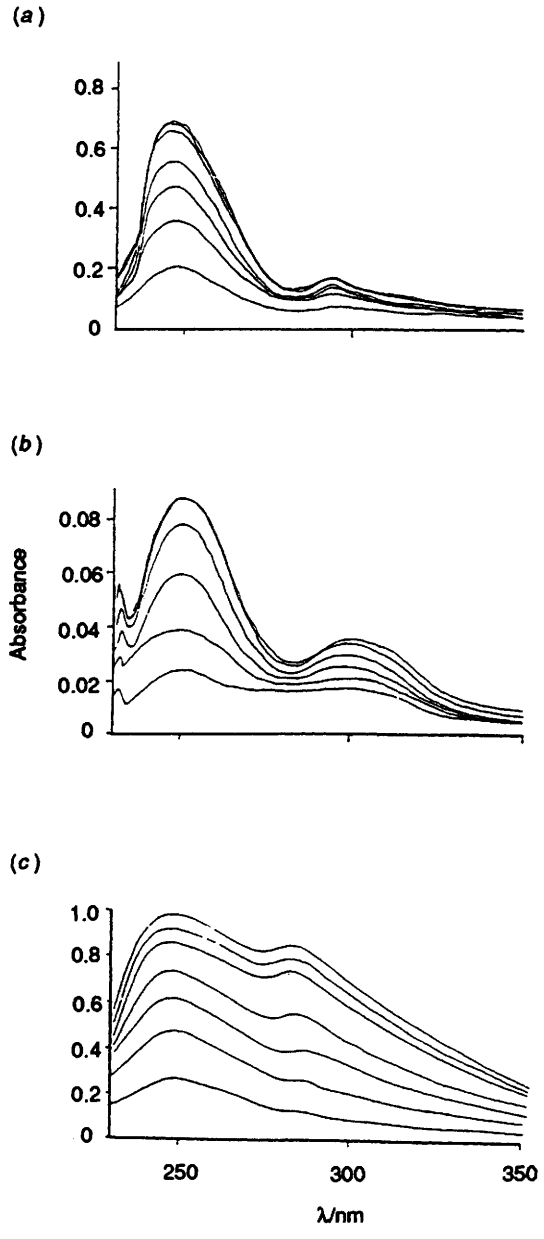

Fig. 1 The UV difference spectra for the addition of vanadium solutions to apolactoferrin in 0.025 mol dm^{-3} Tris–HCl, 0.01 mol dm^{-3} $NaHCO_3$, 0.01 mol dm^{-3} NaCl, pH 7.8. (*a*) Vanadium(III) addition, (*b*) vanadium(IV) addition, (*c*) vanadium(V) addition. For clarity, not all spectra are depicted

Table 1 Molar absorption coefficients at 245 nm obtained from difference spectra of metal–ion lactoferrin complexes[a]

Metal	$\Delta\varepsilon_1$/dm^3 mol^{-1} cm^{-1}	$\Delta\varepsilon_2$/dm^3 mol^{-1} cm^{-1}
V^{III}	19 580	28 700[b]
V^{IV}	11 350	17 300[c]
V^{V}	6 700	11 300[d]
Fe^{III}	12 600	21 500
Cu^{II}	9 850	17 500
Nd^{III}	29 120	45 950
Sm^{III}	31 720	51 250

[a] In 0.025 mol dm^{-3} Tris–HCl, 0.01 mol dm^{-3} NaCl, 0.01 mol dm^{-3} $NaHCO_3$, pH 7.8; $\Delta\varepsilon$ values based on a molecular weight of 80 000 for human lactoferrin. [b] For vanadium(III)–transferrin $\Delta\varepsilon_1$ = 17 100, $\Delta\varepsilon_2$ = 32 000 dm^3 mol^{-1} cm^{-1} (ref. 12). [c] For vanadium(IV)–transferrin $\Delta\varepsilon_2$ = 30 000 dm^3 mol^{-1} cm^{-1} (ref. 12). [d] For vanadium(V)–transferrin $\Delta\varepsilon_1$ = 9400, $\Delta\varepsilon_2$ = 18 500 dm^3 mol^{-1} cm^{-1} (ref. 18).

+3 and +4, two distinct maxima are observed, one at 245 nm and a second less-intense peak at 295 nm, consistent with the perturbation of the phenolate $\pi \longrightarrow \pi^*$ transition following deprotonation of the metal-binding tyrosine residues.[33] In the case of vanadium(IV), comparison of the absorption coefficients observed for vanadium(IV)–transferrin with values obtained for a hexadentate tyrosinate model compound [ethylenediamine-*N*,*N*′-di(*o*-hydroxyphenylacetate)] led to the conclusion that two tyrosine ligands were bound to each vanadium(IV) ion.[33] The difference spectra obtained with vanadium(V) appear somewhat different due to the high absorption of vanadate in the UV region[18] although a peak near 250 nm can be identified, along with a less intense peak at 295 nm. This is again consistent with the deprotonation of tyrosine residues.

The results from the vanadium(III) and vanadium(IV) titrations were plotted as the change in the molar absorption coefficient ($\Delta\varepsilon$) against the metal to protein ratio (r), as shown in Fig. 2. In addition in the vanadium(V) case, a correction for the absorbance of free vanadate was made using the method described by Harris and Carrano[18] and plotted as the average number of metal ions bound per lactoferrin molecule ($\bar{\nu}$) against the metal to protein ratio, r [Fig. 2(*c*)]. In all cases, the molar stoichiometry of vanadium to protein binding is 2:1.

The results show that vanadium is able to bind specifically to lactoferrin in three oxidation states, +3, +4 and +5. The difference spectra obtained from the vanadium(III) and vanadium(IV) titrations resemble those obtained for lanthanide ions,[34] in that the bands at 245 and 295 nm are sharp and clearly defined. However, in terms of intensity they are more similar to other transition metals (see Table 1), as $\Delta\varepsilon_2$ values (the change in molar absorption coefficient at a metal:protein ratio of 2:1) for the lanthanide ions range between 35 000 and 58 000 dm^3 mol^{-1} cm^{-1}, while the equivalent values for the transition metals are near 20 000 dm^3 mol^{-1} cm^{-1}. Based on the intensities of these bands at full saturation and the associated $\Delta\varepsilon_1$ (see Table 1), it is reasonable to suggest that in both oxidation states +3 and +4, two vanadium atoms bind specifically in the two metal-binding sites normally occupied by iron(III) and that they each interact with two tyrosine residues.

Although the difference spectra of the vanadium(V)–lactoferrin complex were more difficult to interpret, the observations that there is a significant increase in absorbance at about 250 nm, consistent with tyrosine deprotonation, and that the stoichiometry of metal:protein binding is 2:1, indicate that the binding of vanadate also takes place in the specific metal-binding sites. The absence of a charge-transfer absorption in the visible region, assignable to a phenolate to vanadium(V) transition, eliminates the presence of a VO^{3+} species co-ordinated in the specific site.[18] From the plot of $\bar{\nu}$ against r [Fig. 2(*c*)], an estimate of the association constant for the second equivalent of vanadium(V) can be calculated. As the gradient of the plot between r = 0 and 1 is equal to unity, the assumption that the binding of the first equivalent of vanadium(V) is quantitative[18] is essentially valid (the concentration of apolactoferrin is zero after the addition of 1 equivalent of metal ion). Therefore, the concentrations of the three species (lf = lactoferrin) present at equilibrium were calculated using equations (1)–(3)[18] and the binding constant calculated from equation (4).

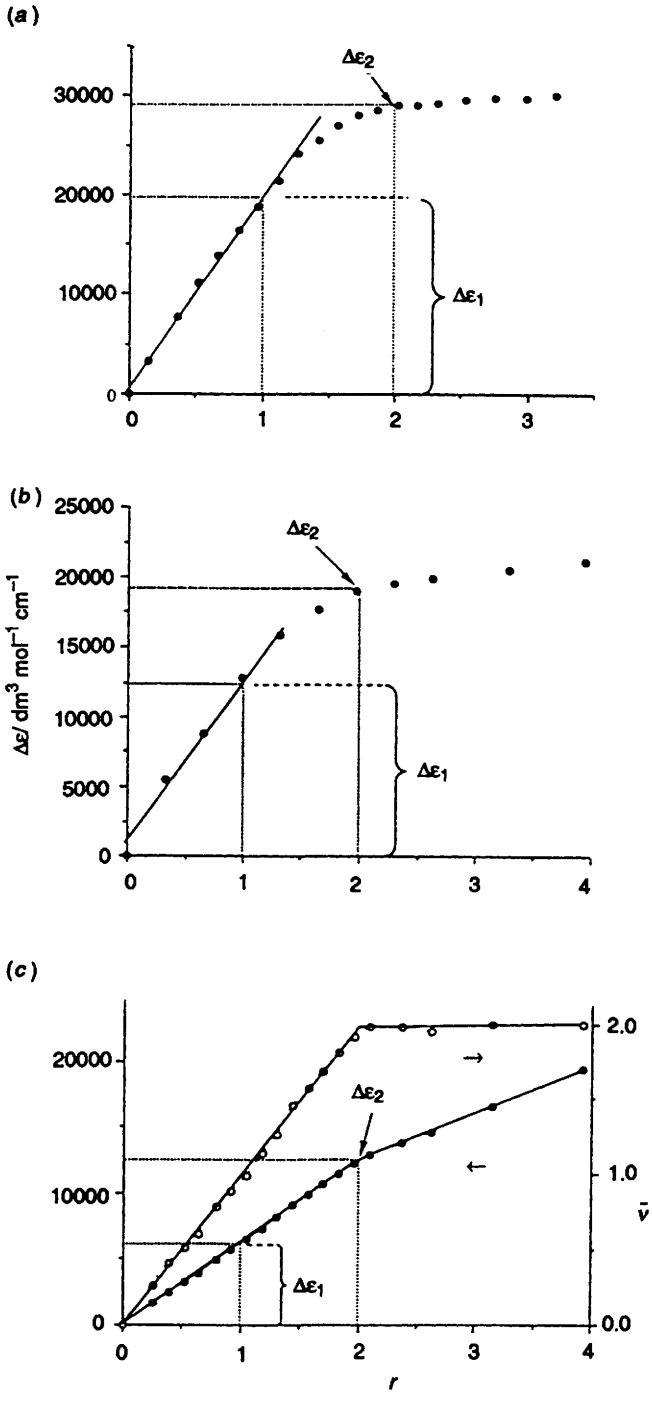

Fig. 2 Titration curves for the addition of vanadium solutions to apolactoferrin. (*a*) Vanadium(III) addition, (*b*) vanadium(IV) addition; (*a*) $\Delta\varepsilon$ = change in the molar absorption coefficient at 245 nm, r = metal:protein ratio. (*c*) Vanadium(V) addition; $\Delta\varepsilon$ *vs.* r (●) and $\bar{\nu}$ *vs.* r (○); $\Delta\varepsilon$ = change in molar absorption coefficient at 250 nm, r = metal:protein ratio, $\bar{\nu}$ = average number of vanadium ions bound per lactoferrin molecule

$$[V_2\text{-lf}] = (\bar{\nu} - 1)\,[\text{lf}]_{\text{total}} \quad (1)$$

$$[V^{V}]_{\text{free}} = (r - \bar{\nu})\,[\text{lf}]_{\text{total}} \quad (2)$$

$$[V\text{-lf}] = [\text{lf}]_{\text{total}} - [V_2\text{-lf}] \quad (3)$$

$$K_2 = [V_2\text{-lf}]/[V\text{-lf}]\,[V^{V}]_{\text{free}} \quad (4)$$

The value of log K_2 at pH 7.8 is 6.22. This compares favourably with the results obtained in two independent studies on the binding of vanadium(v) to human serum transferrin[18] and rat serum transferrin,[35] where the log K_2 values were reported as 6.5 and 6.36 respectively.

Stability towards Oxidation.—The relative stabilities of the three oxidation states of lactoferrin-bound vanadium towards oxidation were investigated using ESR spectra. Vanadium(IV) ($3d^1$) is the only oxidation state of vanadium which is ESR active. Of the other states, vanadium(III) ($3d^2$) transitions are outside the range of an X-band spectrometer and vanadium(V) ($3d^0$) has no unpaired electrons. The vanadium(IV)–lactoferrin complex displays a characteristic vanadyl-transferrin type ESR spectrum[13,22,23,36] as shown in Fig. 3. It should be noted that at physiological pH, any unbound vanadium(IV) would be rapidly hydrolysed to species such as $VOOH^+$ and $(VOOH)_2^{2+}$ which are ESR silent,[15] indicating that the observed signal is specific for vanadium bound to lactoferrin and transferrin as VO^{2+}.

Vanadium(IV) ions, when bound to human lactoferrin, are unstable towards aerobic oxidation. When samples of the 2:1 vanadium(IV)–lactoferrin complex (prepared under argon) were opened to the air and oxygen bubbled through, the complete loss of ESR activity was observed within 1 h (Fig. 3). It has been reported previously that vanadium(IV) ions bound to human serum transferrin are unstable towards aerobic oxidation[16] and the half-life for the oxidation has been estimated to be between 5 and 13 min. A vanadium(IV)–lactoferrin sample, treated under argon with a small volume of 5% H_2O_2, showed a much more rapid decrease in the VO^{2+} ESR signal (about 5 min for complete loss).

The ESR spectra of vanadium(III)–lactoferrin solutions in the absence and presence of oxygen are shown in Fig. 4. In the absence of oxygen, the spectrum is essentially featureless. The discernible signal near 3000 G [Fig. 4(*a*)] can be attributed to a small (2–3%) amount of VO^{2+} contamination in the vanadium trichloride used to prepare the standard vanadium(III) solution, as estimated by comparison with standard vanadium(IV) solutions. Once opened to the air (with oxygen bubbled through) the vanadium(III) is rapidly oxidised to vanadium(IV) and a strong VO^{2+}-type signal is observed after 5 min incubation [Fig. 4(*b*)]. This signal decays over time and a flat line results once again as the vanadium(IV) is further oxidised to vanadium(V).

The preparation of an air-stable 2:1 vanadium(III)–transferrin complex has been reported previously with the unexpected stability of the V^{III} state being attributed to the function of the transferrins to stabilise metal ions in the +3 oxidation state.[11] Although other workers[18] questioned these observations, the redox stability of transferrin-bound vanadium(III) was later reiterated.[12]

The present results show that the vanadium(III) complex of human lactoferrin is *not* stable towards oxidation and it is not clear why there is an apparent discrepancy between the transferrin and lactoferrin results. The oxidation of both bound Mn^{II} and Co^{II} to Mn^{III} and Co^{III} shows that both transferrin[37] and lactoferrin[24] have metal-binding sites accessible to oxygen. The rapid oxidation of lactoferrin-bound vanadium(III) to vanadium(IV) is as would be expected given the redox instability of vanadium(III). It is only stable towards oxidation at low pH.[38] It is very unlikely that the pH in the vicinity of the metal binding site would be significantly lower than that of the external medium. The results of titration experiments[39] indicate

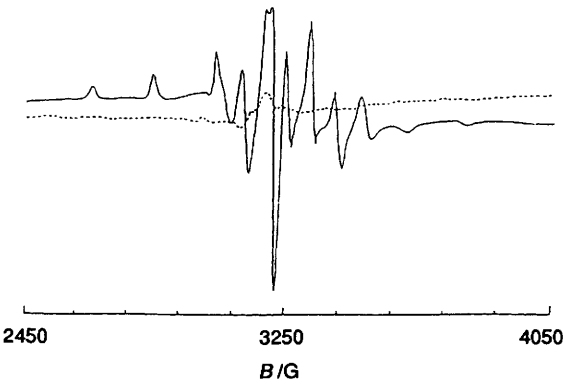

Fig. 3 The ESR spectra of the vanadium(IV)–lactoferrin complex. Under argon (——) and after exposure to air (– – – –). See Experimental section for conditions

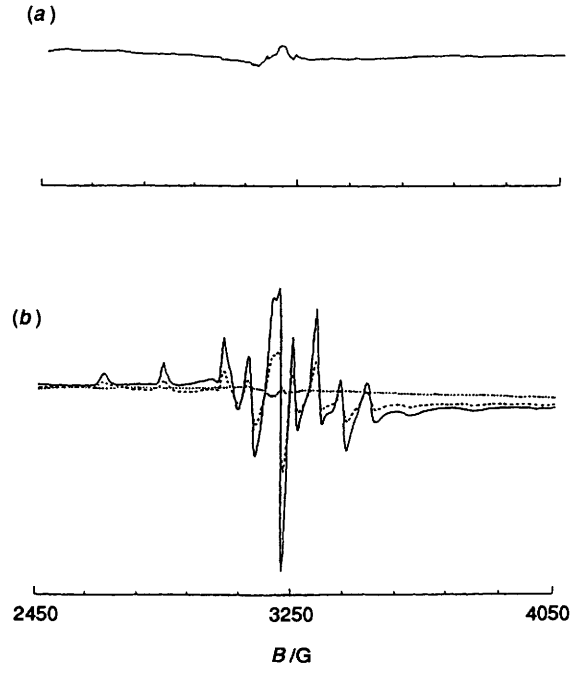

Fig. 4 (*a*) The ESR spectrum of the vanadium(III)–lactoferrin complex. (*b*) The ESR spectra upon exposure to air: after 5 min (——), after 30 min (– – – –) and after 6 h (· · · ·). See Experimental section for conditions

that the protons released from the tyrosines (and possibly the bicarbonate) upon metal binding are released to the external medium and do not remain in the vicinity of the bound metal ions. Even if this were not the case, hydrogen ions lost from the ligands would probably be taken up by nearby basic residues of which there are several in each binding cleft and thus would not result in a significant lowering of the local pH.

The statement by Bertini *et al.*[11] that the transferrins stabilise metal ions in the +3 oxidation state is misleading and all the results from metal-binding studies to date [with the possible exception of vanadium(III)–transferrin] seem to point to the transferrins being stabilisers of metals in their highest readily accessible oxidation states. This has been pointed out previously[18] based on the observation that vanadium(IV)–transferrin is oxidised to vanadate readily by atmospheric oxygen and the fact that the divalent ions of Fe, Mn and Co are all oxidised to their trivalent counterparts in the presence of the transferrins. The present results with vanadium(IV)–lactoferrin and the oxidation of vanadium(III)–lactoferrin are also consistent with this, as is a recent result with cerium(III)–lactoferrin where the +3 ion is readily oxidised to the +4 state.[40]

Nature of Vanadium Bound to Lactoferrin. Modelling Studies.—Evidence presented in this and previous reports points to vanadium in oxidation states +3, +4 and +5 binding to the transferrins in the two specific binding sites.[11–23] Computer simulations, carried out in this study using the atomic coordinates of the iron(III)– and copper(II)–lactoferrin complexes, show that indeed vanadium, in these oxidation states, can be modelled into the specific binding sites if a common protein–ligand donor set is assumed. Based primarily on the results of UV difference spectrophotometric titrations of lactoferrin with vanadium(III) and on the known co-ordination preferences of vanadium(III),[38] it seems reasonable to assume that the vanadium will be bound as the bare V^{3+} ion in the two specific binding sites of the protein. Although small movements of the protein ligands and the carbonate ion cannot be discounted, it is envisaged that the co-ordination environment around the vanadium(III) ions will be similar to the rhombic geometry observed with Fe^{3+}.[9] This is shown in Fig. 5(*a*).

The ESR studies on the transferrins and other biological molecules,[15] show that the form of the bound vanadium(IV) ion is VO^{2+} (vanadyl). Of the vanadium(IV) species present in aqueous solution, only the VO^{2+} and the $VO(OH)_3^-$ ions are ESR active, the former being stable below pH 4 in the absence of chelating agents, and the latter above pH 11.[15] When bound to the transferrins at physiological pH under an inert atmosphere, the characteristic VO^{2+} spectrum is observed, with any excess vanadyl being ESR silent.

It has been established that when two vanadyl ions bind to serum transferrin, 2 equivalents of a suitable synergistic anion also bind.[14,41] However, it has been reported that vanadium(IV) is able to bind in the specific sites of ovotransferrin in the absence of an anion[20,21] and conformational changes and increased flexibility in ovotransferrin have been suggested as possible reasons for this difference.[5] In the present model for VO^{2+} binding to human lactoferrin [Fig. 5(*b*)], a monodentate carbonate has been assumed [as is observed in the N-lobe site of copper(II) lactoferrin]. This results in a six-co-ordinate geometry at the vanadium atom with very little disruption to the surrounding structure, and with the metal atom occupying the same position as copper. The hydrogen-bonding interactions involving the anion are identical to those seen in the five-co-ordinate copper case.[10] In addition, the vanadyl oxygen, which occupies the sixth position (vacant in the case of copper), could possibly interact *via* hydrogen bonds with the Arg-121 sidechain, as indicated in Fig. 5.

Five-co-ordinate vanadyl complexes can be square pyramidal or trigonal bipyramidal. In the former case a sixth ligand may be attached *trans* to V=O[38,42] and in this respect, the VO^{2+} model shown here is consistent both with such small molecule vanadyl complexes and with the observed geometry in the N-lobe of copper(II) lactoferrin. The vanadyl oxygen is *trans* to the phenolate O of Tyr-92, which, in copper(II) lactoferrin, is co-ordinated to the copper with a long (≈2.7 Å) bond.[10] When copper is substituted for iron, the position of the metal-binding ligands and the extent of closure of the domains are essentially identical.[10] It can therefore be predicted that if vanadyl ions bind to lactoferrin as depicted in Fig. 5, there would be little disruption to the overall structure of the protein.

It has been observed that vanadium(V) is able to bind to serum transferrin in the absence of a suitable synergistic anion[18] and it is the nature of the bound vanadium(V) which confers this unique property. The dioxovanadium(V) ion, VO_2^+ (for which the *cis*-oxo geometry has been confirmed in small molecule complexes[38]) or the protonated form, $VO(OH)^{2+}$, could occupy essentially the same position as the Fe^{3+}, interact with the same four protein ligands but not require a carbonate anion as the two *cis* oxygen atoms attached to the vanadium would serve to bridge between the positively charged anion pocket and the metal centre [Fig. 5(*c*)]. Computer modelling studies suggest that it is possible a water molecule could fit between the vanadium oxyanion and the residues comprising the anion pocket, in roughly the same position as the third carbonate oxygen. In this way, the general structure of the metal- and anion-binding sites would not be greatly altered when vanadium(V) binds.

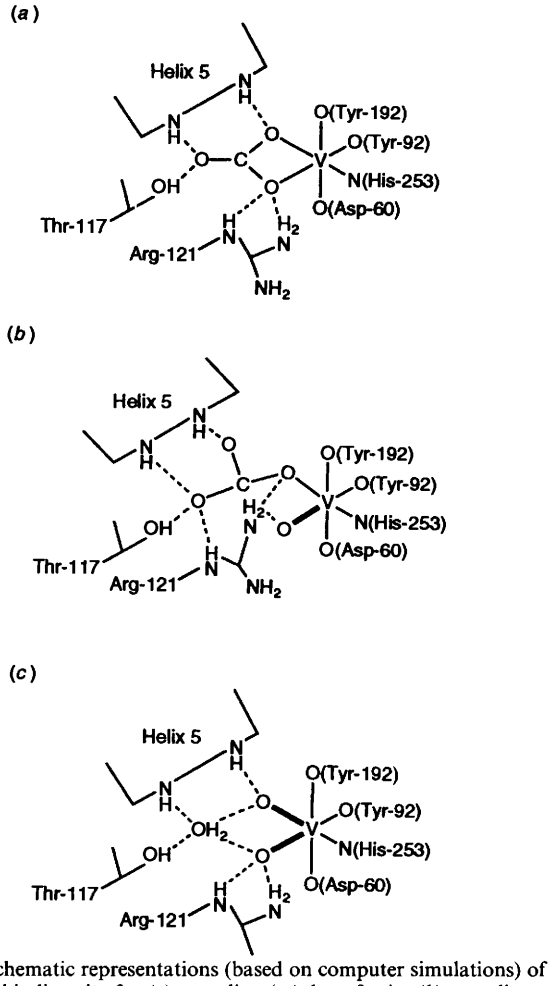

Fig. 5 Schematic representations (based on computer simulations) of the metal-binding site for (*a*) vanadium(III)–lactoferrin, (*b*) vanadium(IV)–lactoferrin and (*c*) vanadium(V)–lactoferrin

The binding of vanadium in the three different oxidation states modelled in this study is broadly consistent with the concept of 'rack-induced' bonding,[43] whereby hydrogen bonding and other ionic or hydrophobic interactions with neighbouring groups hold the protein ligands in fairly fixed positions. Incoming metal ions are required to fit into a pre-specified site and interact with the ligands in a co-ordination geometry defined by the protein–ligand donor set. However, it is possible as in the case of vanadium binding proposed here, for the increased vanadium–oxo co-ordination on going from V^{3+} to VO^{2+} to VO_2^+ to be compensated for by decreasing the synergistic CO_3^{2-} anion co-ordination mode from bidentate to monodentate and then finally expelling the ion altogether. Although there are uncertainties concerning the details of vanadium–lactoferrin binding which will only be resolved by further X-ray crystallographic studies on a range of metal-substituted transferrins, it can be concluded that in biological processes under oxidising conditions a co-ordination sphere containing mainly oxygen donors, such as phenoxides and carboxylates, will favour the +5 oxidation state for vanadium.

Acknowledgements

We greatly appreciate the support, encouragement and interest shown by Professor E. N. Baker and Mrs. H. M. Baker during the course of this work.

References

1 *Vanadium in Biological Systems*, ed. N. D. Chasteen, Kluwer Academic Publishers, Dordrecht, 1990.
2 L. C. Cantley, M. Resh and G. Guidotti, *Nature (London)*, 1978, **272**, 552.
3 J. Chakravarty, S. Dutta and A. Chakravorty, *J. Chem. Soc., Chem. Commun.*, 1993, 1091; T. A. Kabanos, A. D. Keramidas, A. B. Papaioannou and A. Terzis, *J. Chem. Soc., Chem. Commun.*, 1993, 643; A. Neves, A. S. Ceccato, C. Erasmus-Buhr, S. Gehring, W. Hasse, H. Paulus, O. R. Nascimento and A. A. Batista, *J. Chem. Soc., Chem. Commun.*, 1993, 1782; A. Neves, A. S. Ceccato, I. Vencato, Y. P. Mascarenhas and C. Erasmus-Buhr, *J. Chem. Soc., Chem. Commun.*, 1992, 652; L. J. Calviou, J. M. Arber, D. Collinson, C. D. Garner and W. Clegg, *J. Chem. Soc., Chem. Commun.*, 1992, 654.
4 J. H. Brock, in *Metalloproteins*, ed. P. Harrison, Macmillan, London, 1985, Part 2, p. 183.
5 D. C. Harris and P. Aisen, in *Iron Carriers and Iron Proteins*, ed. T. Loehr, VCH, New York, 1989, p. 241.
6 T. M. Rose, G. D. Plowman, D. B. Teplow, W. J. Dreyer, K. E. Hellström and J. P. Brown, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 1261.
7 E. N. Baker, H. M. Baker, C. A. Smith, M. R. Stebbins, M. Kahn, K. E. Hellström and I. Hellström, *FEBS Lett.*, 1992, **298**, 215.
8 N. S. Bartfeld and J. H. Law, *J. Biol. Chem.*, 1990, **265**, 21684.
9 B. F. Anderson, H. M. Baker, G. E. Norris, D. W. Rice and E. N. Baker, *J. Mol. Biol.*, 1989, **209**, 711.
10 C. A. Smith, B. F. Anderson, H. M. Baker and E. N. Baker, *Biochemistry*, 1992, **31**, 4527.
11 I. Bertini, G. Canti and C. Luchinat, *Inorg. Chim. Acta*, 1982, **67**, L21.
12 I. Bertini, C. Luchinat and L. Messori, *J. Inorg. Biochem.*, 1985, **25**, 57.
13 J. C. Cannon and N. D. Chasteen, *Biochemistry*, 1975, **14**, 4573.
14 N. D. Chasteen, L. K. White and R. F. Campbell, *Biochemistry*, 1977, **16**, 363.
15 N. D. Chasteen, in *Biological Magnetic Resonance*, eds. L. Berliner and J. Reuben, Plenum Press, New York, 1981, vol. 3, p. 53.
16 N. D. Chasteen, J. K. Grady and C. E. Holloway, *Inorg. Chem.*, 1986, **25**, 2754.
17 S. S. Eaton, J. Dubach, K. M. More, G. R. Eaton, G. Thurman and D. R. Ambruso, *J. Biol. Chem.*, 1989, **264**, 4776.
18 W. R. Harris and C. J. Carrano, *J. Inorg. Biochem.*, 1984, **22**, 201.
19 A. Butler, M. J. Danzitz and H. Eckert, *J. Am. Chem. Soc.*, 1987, **109**, 1864.
20 J. D. Casey and N. D. Chasteen, *J. Inorg. Biochem.*, 1980, **13**, 111.
21 J. D. Casey and N. D. Chasteen, *J. Inorg. Biochem.*, 1980, **13**, 127.
22 A. Carmichael and J. S. Vincent, *FEBS Lett.*, 1979, **105**, 349.
23 J. Mazurier, J.-M. Lhoste, J. Montreuil and G. Spik, *Biochim. Biophys. Acta*, 1983, **745**, 44.
24 E. W. Ainscough, A. M. Brodie and J. E. Plowman, *Inorg. Chim. Acta*, 1979, **33**, 149; E. W. Ainscough, A. M. Brodie, J. E. Plowman, S. J. Bloor, J. S. Loehr and T. M. Lohr, *Biochemistry*, 1980, **19**, 4072.
25 M. S. Shongwe, C. A. Smith, E. W. Ainscough, H. M. Baker, A. M. Brodie and E. N. Baker, *Biochemistry*, 1992, **31**, 4451.
26 E. W. Ainscough, A. M. Brodie, S. J. McLachlan and V. S. Ritchie, *J. Inorg. Biochem.*, 1983, **18**, 103.
27 G. E. Norris, H. M. Baker and E. N. Baker, *J. Mol. Biol.*, 1989, **209**, 329.
28 U. K. Laemmli, *Nature (London)*, 1970, **227**, 680.
29 D. F. Shriver, *Manipulations of Air-sensitive Compounds*, McGraw-Hill, New York, 1969.
30 A. I. Vogel, *Quantitative Inorganic Analysis including Instrumental Analysis*, 3rd edn., Longmans, London, 1961.
31 J. F. Fitzgerald and N. D. Chasteen, *Anal. Biochem.*, 1974, **60**, 170.
32 T. A. Jones, *J. Appl. Crystallogr.*, 1978, **11**, 268.
33 V. L. Pecoraro, W. R. Harris, C. J. Carrano and K. N. Raymond, *Biochemistry*, 1981, **20**, 7033.
34 C. A. Smith, Ph.D. Thesis, Massey University, Palmerston North, 1992.
35 N. D. Chasteen, E. M. Lord, H. J. Thompson and J. K. Grady, *Biochim. Biophys. Acta*, 1986, **884**, 84.
36 S. S. Eaton and G. R. Eaton in ref. 1, p. 201.
37 P. Aisen, R. Aasa and A. C. Redfield, *J. Biol. Chem.*, 1969, **244**, 4628.
38 J. Vilas-Boas and C. Pessoa, in *Comprehensive Coordination Chemistry*, eds. G. Wilkinson, R. Gillard and J. McCleverty, Pergamon Press, Oxford, 1987, vol. 3, p. 453.
39 M. H. Gelb and D. C. Harris, *Arch. Biochem. Biophys.*, 1980, **200**, 93.
40 C. A. Smith, E. W. Ainscough, H. M. Baker, A. M. Brodie and E. N. Baker, *J. Am. Chem. Soc.*, 1994, **116**, 7889.
41 R. F. Campbell and N. D. Chasteen, *J. Biol. Chem.*, 1977, **252**, 5996.
42 N. D. Chasteen, *Struct. Bonding (Berlin)*, 1983, **53**, 105.
43 H. B. Gray and B. G. Malmström, *Comments Inorg. Chem.*, 1983, **2**, 203.

Received 29*th June* 1994; *Paper* 4/03951C