Communications to the Editor

Specific Binding of Cerium by Human Lactoferrin Stimulates the Oxidation of Ce^{3+} to Ce^{4+}

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Received April 28, 1994

Proteins of the transferrin family, serum transferrin, lactoferrin (Lf), and ovotransferrin, have the characteristic ability to bind, tightly but reversibly, two Fe^{3+} ions together with two associated CO_3^{2-} ions.[1] Many other metal ions can be bound in place of Fe^{3+}, not only other transition-metal ions such as Cr^{3+}, Mn^{3+}, Co^{3+}, and Cu^{2+} but also species such as Al^{3+} and Ga^{3+} and larger cations such as lanthanides and actinides. This binding ability is of potential importance for the control of trace element levels, for medical purposes, and for the metabolic fate of heavy metals ingested from the environment.

For serum transferrin, the larger lanthanides appear to bind in only one of the two specific sites, either because of size restrictions or because of weakness of binding.[2] Here we show that for human lactoferrin, lanthanides as large as Ce^{3+} do bind in *both* sites but that in the case of cerium this stimulates a remarkably facile oxidation of the bound Ce^{3+} to Ce^{4+} in the presence of molecular oxygen. This is attributed to the steric demands of the metal sites. The binding of this larger metal ion is also found to accentuate differences between the two sites with respect to metal ion release.

Human apo-lactoferrin was prepared as described previously,[3] but without any use of EDTA in the isolation procedures, in order to avoid the possibility that residual EDTA might inhibit metal binding. The concentration and iron saturation of the lactoferrin were estimated from the absorbances at 280 and 465 nm using extinction coefficients for a 1% (10 mg/mL) solution of 10.9 and 0.51, respectively.[4] Iron saturation was typically around 8%.

Solutions of Ce^{3+}, prepared from $Ce(NO_3)_3 \cdot 5H_2O$(BDH), were adjusted to pH 3 to prevent the absorption of CO_2, and their concentrations were determined by titration with EDTA. UV difference titrations,[5] covering the range 240–400 nm, were carried out by adding aliquots of Ce^{3+} solution to a 1.5 mg/mL solution of apo-lactoferrin in 0.1 M Tris-HCl buffer, pH 7.5 containing 0.1 M NaCl. This showed clearly that two Ce^{3+} ions were bound per molecule of lactoferrin (Figure 1); a similar 2:1 ratio was obtained from measurements of fluorescence quenching as the metal ion was added (data not shown). The resulting colorless Ce_2Lf complex was stable at 4 °C over a period of 2 weeks, providing that all solutions had previously been degassed with O_2-free argon. The difference spectrum of this complex was

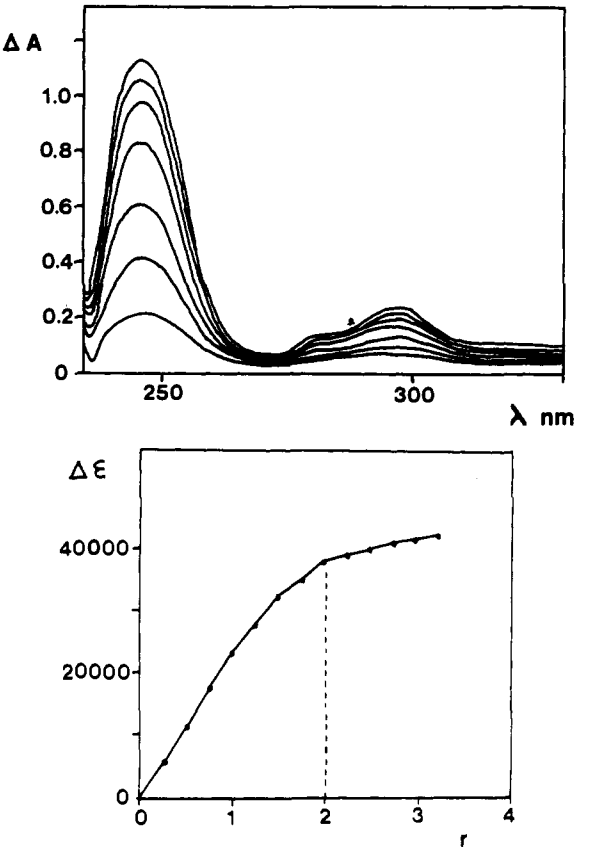

Figure 1. Results of metal titration of human apo-lactoferrin with Ce^{3+}. In the top, UV difference spectra for the addition of aliquots of 2.3 mM Ce^{3+} to 27.5 μM apo-lactoferrin are shown. In the bottom, the corresponding titration curve shows that binding is complete at a ratio (r) of 2 mol of Ce^{3+} per mole of lactoferrin.

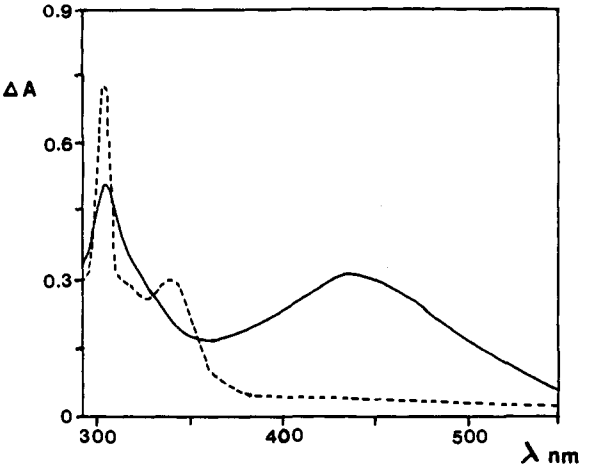

Figure 2. UV–visible difference spectra for $Ce(III)_2$ lactoferrin (dashed line) and $Ce(IV)_2$ lactoferrrin (full line), showing the change to the 442-nm charge-transfer absorption characteristic of the Ce(IV) complex.

characterized by a peak at ~340 nm, assigned as the expected phenolate → metal LMCT band (Figure 2).

On exposure of the colorless Ce(III) complex to air, however, the solution became red-brown in color within 2 days, with the appearance of an intense visible absorption band at λ_{max} = 442 nm (ϵ = 4640 M^{-1} cm^{-1}) (Figure 2). This change occurred instantly if 5 μL of 5% H_2O_2 was added, confirming that Ce(III)

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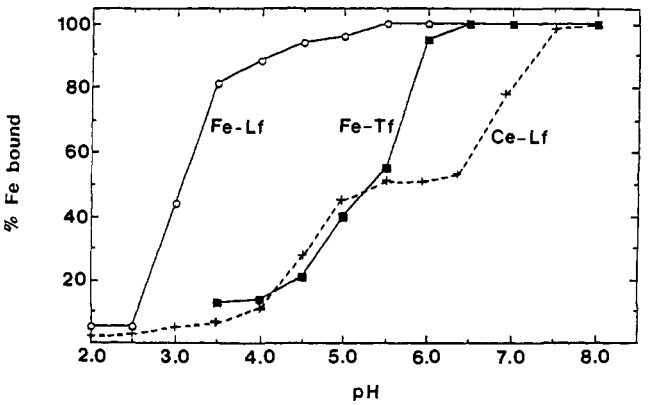

Figure 3. pH-mediated release of cerium from $Ce(IV)_2$ lactoferrin (broken line), showing the distinctly biphasic release of the metal ion from the two sites of lactoferrin compared with that for Fe(III) release (Fe-Lf). The Fe(III) release profile for transferrin (Fe-Tf) is also shown for comparison. The percent saturation was determined from the decrease in absorbance at 442 (for Ce(IV)) and 465 nm (for Fe(III)).

had been oxidized to Ce(IV)[6] and that the 442-nm band represented the phenolate → metal LMCT transition for this metal ion. The energy and extinction coefficient for this transition are similar to those for the LMCT bands for Fe(III) (λ_{max} = 465 nm, ϵ = 4160 M^{-1} cm^{-1}), Cu(II) (λ_{max} = 434 nm, ϵ = 4880 M^{-1} cm^{-1}), and Mn(III) (λ_{max} = 435 nm, ϵ = 9520 M^{-1} cm^{-1})[7]. The similarity in these values also suggests a similar metal site. The change in λ_{max} from 340 to 442 nm going from Ce(III) to Ce(IV) closely matches that for manganese, from ~310 nm for Mn(II) to 435 nm for Mn(III).

The pH dependence of metal ion release from the Ce_2Lf complex was monitored by following the decline of the 442-nm charge-transfer band as the pH was incrementally lowered from 8.0 to 2.0 by titration with small (1 μL) aliquots of 0.1 M HCl. Release was distinctly biphasic, with one Ce(IV) ion being lost at pH 7 and the second at pH 5; this is a marked contrast to iron release, for which, under the same conditions, both Fe(III) ions are lost essentially together over the pH range 4.0–3.0 (Figure 3). It clearly illustrates the way in which the binding of nonnative metal ions accentuates the differences between the two sites.[8]

Proteins of the transferrin family are known for their preferential binding of metal ions in their higher oxidation states, e.g., Fe(III), Mn(III), and Co(III) are strongly favored over the corresponding M(II) ions.[9] In these cases, the preference probably derives from the electronic environment provided by the metal site, with three anionic ligands (one Asp, two Tyr) to match the metal 3+ charge (the charge on the CO_3^{2-} ion is matched by the positive charge of its protein binding site).[10]

The very facile oxidation of bound Ce(III) to Ce(IV) was unexpected, given the normal preference for M(III) ions and the high redox potential (~1.7 V) of the $Ce^{3+}(aq)/Ce^{4+}(aq)$ couple.[11] Although appropriate ligands can reduce this significantly, in the case of Ce_2Lf it seems likely that steric factors also play a large part. Fe(III) binding to transferrins is marked by a large-scale conformational change, from an open form to a closed form, with the metal site only being fully formed in the closed configuration.[12] Although binding of larger cations such as Ce(III) (radius 1.01 Å[13]) clearly occurs, for lactoferrin, the usual methods of detection, such as UV–visible difference spectroscopy, only monitor binding to the two Tyr ligands; these are both associated with the same protein domain,[12] and binding to them could occur in either open or closed forms.[14] Low-angle X-ray scattering measurements[15] suggest that larger cations may not be able to stabilize the closed configuration. Thus, In(III) stabilizes the closed form of transferrin but Hf(IV) does not, suggesting a critical maximum radius of around 0.8–0.9 Å. Within this range, however, the ability to stabilize the closed form will also depend on the bonding and stereochemical preferences of a given metal ion. Although crystallographic data are lacking, these ideas offer an explanation for the oxidation of Ce(III) seen here. We suggest that although Ce(III) binds to lactoferrin at both of the sites, it may be too large to stabilize the closed form and remains bound only to the two Tyr residues and CO_3^{2-} anion on one domain of the open form. Ce(IV), however, with its smaller radius (0.87 Å for an octahedral geometry[13]), may be just small enough to fit into the closed configuration and complete its coordination to the remaining Asp and His ligands. This stabilization of bound Ce(IV), relative to Ce(III), is then what facilitates the oxidation.

The implications for the binding of other large metal ions are important, since if Ce(IV) is indeed able to bind to lactoferrin with a closed structure similar to that for Fe(III), then binding to cell receptors could also be similar, even given the greater pH lability of bound Ce(IV). Other metal ions of similar charge and size, e.g., Pu(IV) (radius 0.86 Å), may show the same behavior, although we stress that for ions such as these, whose size and coordination characteristics may be marginal for specific binding, small differences between different transferrins and between the two sites may become critical.

Acknowledgment. We gratefully acknowledge support from the U.S. National Institutes of Health (Grant No. HD-20859), the Wellcome Trust, and the Health Research Council of New Zealand, and from Massey University in the award of a Graduate Assistantship (to C.A.S.). E.N.B. also receives support as an International Research Scholar of the Howard Hughes Medical Institute. We thank Dr. P. F. Lindley for discussions that stimulated these experiments.

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(9) Binding constants approximately 10^3 for Fe(II) (Harris, W. R. *J. Inorg. Biochem.* **1986**, *27*, 41–52) and 10^{20} for Fe(III) (Aisen, P.; Leibman, A.; Zweier, J. *J. Biol. Chem.* **1978**, *253*, 1930–1937).

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(12) Crystallographic analyses show that in the open form, seen for apo-lactoferrin, the CO_3^{2-} ion and the two Tyr ligands are associated with domain 2, while the His and Asp ligands are some 8–9 Å away on domain 1 (Anderson, B. F.; Baker, H. M.; Norris, G. E.; Rumball, S. V.; Baker, E. N. *Nature (London)* **1990**, *344*, 784–787). In the closed form, seen for diferric and dicupric lactoferrins (refs 8 and 10), the two Tyr, His, Asp, and bidentate CO_3^{2-} all coordinate the metal ion.

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