LOW pH BINDING OF VANADYL TO BOVINE LACTOFERRIN

Electron spin resonance studies

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1. Introduction

The iron binding protein in milk, lactoferrin, has 2 Fe(III) binding sites which have similar if not identical spectroscopic and thermodynamic properties [1–3]. These binding properties are also similar to those of the serotransferrins and ovotransferrin [4–11]. However, the diferic lactoferrin complex is stable over a pH range of ~10–2 whereas the serotransferrins only binds 2 Fe(III) ions/protein at pH > 6 and loses iron at pH 4 [12]. In the course of studying the replacement of iron by vanadyl in the lactoferrin complex we have found that the divanadyl lactoferrin is stable at pH 4.2 and this article describes the VO^{2+} binding of bovine lactoferrin in the presence of the oxalate anion at pH 4.2 as studied by electron paramagnetic resonance (ESR). The vanadyl is assumed to bind at the same site as Fe(III) does in lactoferrin. This was shown to be the case for serotransferrin in [9,10].

Lactoferrin also requires an anion for metal binding, a property shared by the transferrins [4–11]. The bicarbonate or carbonate ion serves as the anion under normal pH conditions in the native protein. However, at low pH other anions, such as oxalate and succinate, must be used instead of bicarbonate. This study uses the oxalate anion.

2. Experimental

Bovine lactoferrin was isolated from the fluid expressed from the mammary gland of non-lactating cows by the method in [13]. The lactoferrin saturated with iron had A_{280}/A_{465} and A_{410}/A_{465} of 27–30 and 0.8, respectively. Also, the lactoferrin traveled as a single component of ~80 000 mol. wt in SDS–acrylamide slab-gel electrophoresis (with both 10% and 15% acrylamide). The SDS running buffer was at pH 8.3 and consisted of 0.05 M Tris, 0.4 M glycine and 0.1% SDS.

Apolactoferrin was prepared by dialysis of the diferric lactoferrin against 0.1 M citric acid at pH 2 and continued dialysis against several changes of metal-free water followed by dialysis against the 0.01 M acetate buffer at pH 4.2.

Buffers made with doubly distilled and deionized water were extracted with 10^{-5} M dithizone in carbon tetrachloride to eliminate unwanted metal ions and subsequently heated in a boiling water bath to rid the solutions of the organic material. Plastic ware was used whenever practical and glassware was soaked in 1:1 concentrated nitric and sulfuric acid prior to rinsing well with metal free water.

The vanadyl solutions were prepared and stored under an oxygen-free nitrogen atmosphere. All transfers and mixtures of solutions were carried out under a pure nitrogen atmosphere.

The ESR spectra of lactoferrin solutions drawn into 1 mm i.d. quartz capillaries were observed at 10°C or at liquid nitrogen temperature, 77 K, with a Varian E-4 spectrometer.

The pH was measured after each series of experiments and in each case remained at pH 4.2.

3. Results

The typical ESR spectrum of divanadyl lactoferrin

in solution and frozen at 77 K is a normal glass-type bound VO^{2+} spectrum as illustrated in fig.1. The solution spectrum at 10°C in fig.1A shows evidence of the unbound VO^{2+} lines which increase in intensity as the VO^{2+} concentration increases above the stoichiometric amount.

When apolactoferrin is titrated with a solution of vanadyl oxalate at pH 4.2 in an acetate buffer the intensity of the ESR spectrum of the bound vanadyl

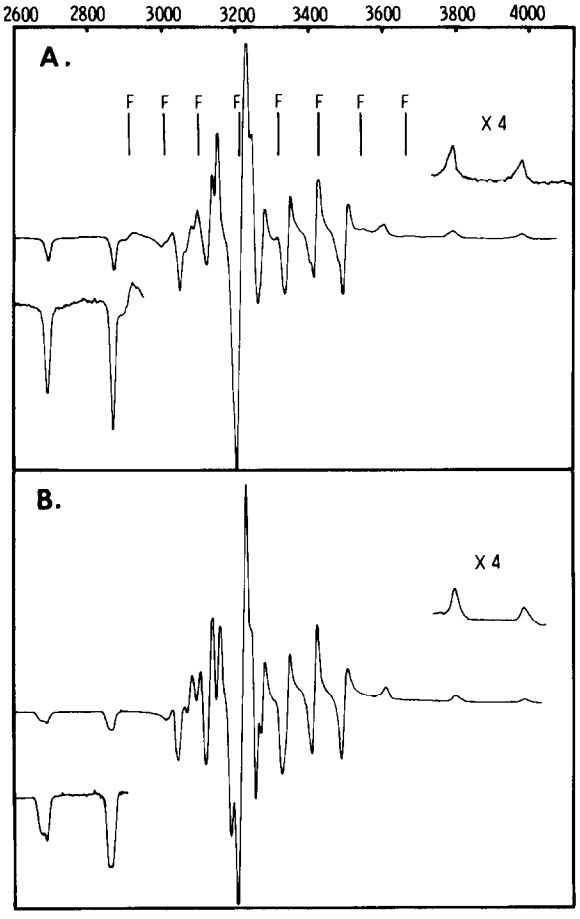

Fig.1. The ESR spectrum of 2×10^{-3} M divanadyl lactoferrin in 0.025 M (pH 4.2) acetate buffer in solution at 10°C (A) and frozen at the temperature of liquid nitrogen (B). A small amount of the free vanadyl is observable in the solution spectrum (A) and is indicated by vertical lines with F above. The remaining lines are due to the bound VO^{2+}. The ESR was observed at X band with 40 mW incident power and a modulation amplitude of 5 G. The magnetic field markings are in gauss.

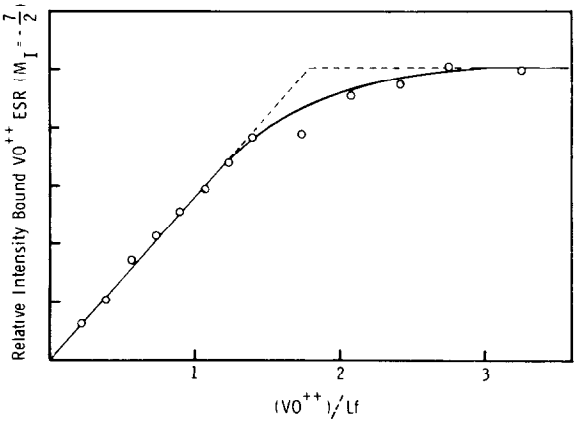

Fig.2. The relative intensity of the bound vanadyl ESR signal is plotted versus the number of moles of vanadyl oxalate per mole lactoferrin (Lf) added to a 5×10^{-4} M apolactoferrin solution in a 0.01 M (pH 4.2) acetate buffer. The intersection indicates 1.8 molecules of vanadyl oxalate are bound per lactoferrin molecule.

shows a distinct end point corresponding to 1.8:1 mol VO^{2+} to protein. The peak height of the $M_I = -7/2$ line of the bound vanadyl ESR spectrum is plotted in fig.2 versus the number of moles per protein of vanadyl oxalate added to 5×10^{-4} M apolactoferrin in 0.01 M (pH 4.2) buffer. The peak height of this line adjusted for spectrometer gain closely approximates the relative concentration of the bound vanadyl over the experimental concentration ranges. At this pH the unbound VO^{2+} ESR is also observable. As more vanadyl is added to a solution containing >2 VO^{2+}/protein several distinct VO^{2+} solution lines are apparent and can yield the concentration of the unbound vanadyl in solution [14]. The points in fig.3 are a Scatchard plot of the number, $\bar{\nu}$, of bound VO^{2+}/protein divided by the free VO^{2+} concentration versus $\bar{\nu}$. The solid line is the theoretical line calculated assuming 2 groups of binding sites: one with 1.9 identical sites having a binding constant (K_b) of 5×10^6 M^{-1}; the other having 3.8 identical sites with K_b 5×10^3 M^{-1}. The similarity of the calculated curve and experimental points indicates that there are 2 sets of binding sites for vanadyl: a weak binding group of ~4 equivalent sites; 2 sites which bind vanadyl strongly.

The strong binding VO^{2+} sites are the same as the iron binding sites and the vanadyl binding is

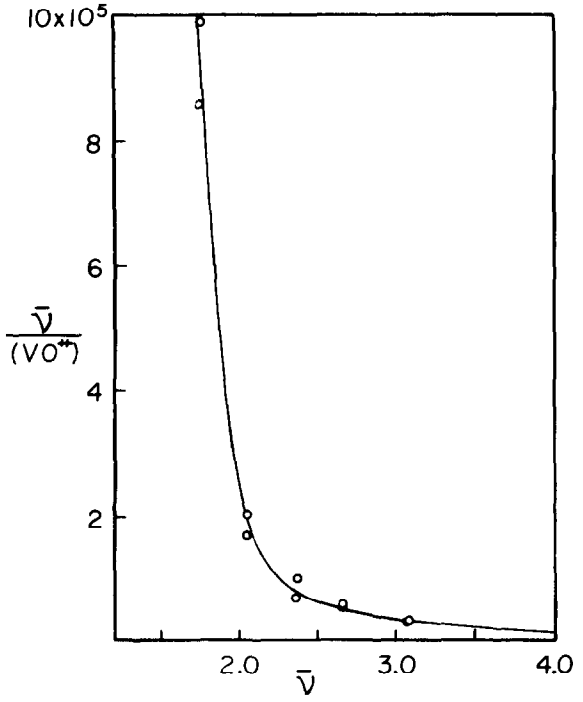

Fig.3. A Scatchard plot of the average number ($\bar{\nu}$) of vanadyl cations bound per molecule of lactoferrin divided by the free vanadyl concentration (VO^{2+}) versus $\bar{\nu}$. The solid line is calculated assuming 2 groups of sites – one group consisting of 1.9 equivalent sites with K_b 5 × 10^6 M^{-1} and the other group of 3.8 equivalent sites with K_b 5 × 10^3 M^{-1}. The circles are the experimental points obtained when vanadyl oxalate was added to a 5 × 10^{-4} M apolactoferrin solution in 0.01 M (pH 4.2) acetate buffer. The M_I = −5/2 line of the free vanadyl ESR was monitored to provide the free vanadyl concentration.

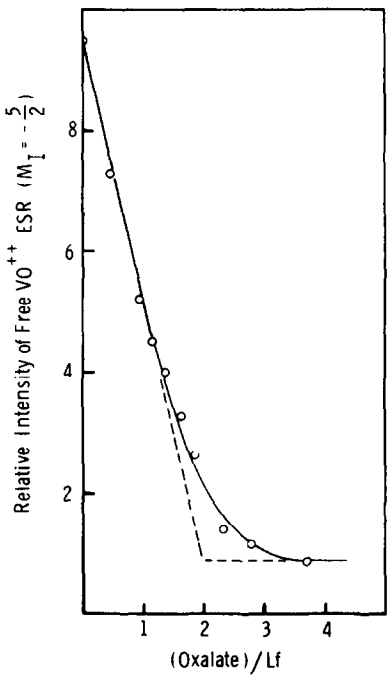

Fig.4. The relative intensity of the free vanadyl (VO^{2+}) ESR signal (M_I = −5/2 line) is plotted versus the number of moles of oxalate per mole of lactoferrin (Lf) as oxalate is added to a 5 × 10^{-4} M apolactoferrin solution in 0.01 M (pH 4.2) acetate buffer containing 3 × 10^{-2} M vanadyl sulfate.

approximately as strong as the iron binding. When a solution of divanadyl lactoferrin is incubated overnight with a large excess of Fe(III) the bound VO^{2+} ESR intensity decreases, the free vanadyl ESR spectrum and the iron lactoferrin visible spectrum appears. Also when diferric lactoferrin with oxalate as the anion is treated with excess vanadyl oxalate the bound vanadyl lactoferrin ESR spectrum appears, the free vanadyl spectrum decreases, and the 465 nm Fe(III) lactoferrin absorption decreases.

Lactoferrin is also similar to transferrin and conalbumin in that strong metal binding only occurs in the presence of carbonate, oxalate or other suitable anions. A titration which demonstrates the oxalate requirement is illustrated in fig.4. The intensity of the ESR of free VO^{2+} in a solution containing 5 × 10^{-4} M apolactoferrin is plotted as oxalate is added. The free VO^{2+} ESR levels off at a minimum close to 2 equiv. oxalate/protein and the bound VO^{2+} signal becomes maximal at this point as well.

The 2 strong binding sites in transferrin are not equivalent in binding either iron or vanadyl. ESR signals have been observed [9–11] for each site in the divanadyl transferrin complex around pH 7.5. The isotopic substitution studies [9] using ^{55}Fe and ^{59}Fe have shown that the 2 sites are also distinct for iron binding. The EPR spectrum of divanadyl lactoferrin at pH 4.2 with oxalate anion in solution at 10°C (fig.1A) appears to result from identical binding sites as it is impossible to distinguish 2 different spectra at this pH and temperature. However, if the temperature of this sample is reduced to 77 K the spectrum in fig.1B indicates 2 slightly different sites or conformations with nearly identical

populations. The thermal motion near room temperature effectively averages these 2 distinct but similar conformations yielding the spectrum with only 1 observable type of binding site.

4. Conclusion

Lactoferrin, although similar to the serotransferrins in binding metals at physiological pH ranges, is distinctly different in its ability to bind vanadyl at low pH. There are 2 strong binding sites which appear identical at pH 4.2 with $K_b \sim 5 \times 10^6\ M^{-1}$. One mole of oxalate is required for each mole of VO^{2+} bound. In addition there are also ~4 relatively weak non-specific binding sites with $K_b \sim 5 \times 10^3\ M^{-1}$. The ESR of VO^{2+} bound at these sites are not observable in solution probably due to incomplete immobilization in binding but are still too immobile to contribute to the free VO^{2+} spectra. The ESR of the divanadium lactoferrin in the pH 4.2 acetate buffer does not indicate non-equivalence of the 2 strong binding sites near room temperature. There is no observable splitting of the vanadyl ESR lines at 10°C. However, the 77 K spectrum does exhibit either 2 conformations of the 2 identical sites or 2 different sites which have similar binding properties at room temperature. The replacement of iron with other metals such as Cu^{2+}, and VO^{2+} have provided most of the evidence for non-equivalent lactoferrin binding sites. The lactoferrin ^{57}Fe Mössbauer spectra [15] were interpreted to be due to 2 equivalent sites. The ESR studies [16] on lactoferrin in which Cu^{2+} replaces iron demonstrates the 2 distinct Cu(II) binding sites. One site has only 1 nitrogen ligand whereas the other has >1. Upon carbethoxylation one site becomes totally inactive leaving only 1 bound copper/lactoferrin.

The vanadyl binding properties of lactoferrin at pH 4.2 differ sufficiently from those of serrotransferrin to suggest mechanistic differences in the details of binding. Further study of the vanadyl and other metal binding is necessary before proposing a model for complex formation.

Acknowledgements

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