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The Chromium, Manganese, Cobalt and Copper Complexes of Human Lactoferrin

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The human milk protein, lactoferrin forms complexes of the type M_2Lf (where M = Cr(III), Mn(III) or Co(III) and Lf = lactoferrin). The properties of these complexes and their electronic spectra are reported along with those of the known iron and copper complexes, Fe_2Lf and Cu_2Lf. For the latter complex, a d–d band, not previously reported, is seen at 677 nm. The spectral data confirm the suggestion that the metal binding sites in the protein are two or three tyrosyl residues and at least one nitrogen (probably from a hystidyl residue).

Introduction

Lactoferrin is an iron binding protein which occurs in high concentration in human milk and has also been identified in other bodily secretions and intracellular components [1]. Its physiological role has yet to be defined with certainty, but, because of its ability to bind iron(III) extremely tightly, it has been postulated to have a bacteriostatic function in depriving microorganisms of essential iron required for their growth [2]. A nutritional role as an iron carrier has also been suggested [3]. Like the related proteins, human serum transferrin and ovotransferrin (from egg whites), lactoferrin has a molecular weight of around 80,000, and specifically and strongly binds two high spin iron(III) ions concomitantly with two bicarbonate (or carbonate) ions [1]. Proposed binding sites are two or three tyrosyl groups and at least one hystidyl residue [1, 4, 5]. However as human lactoferrin has been less studied than transferrin or ovotransferrin, and does show distinct immunological and physiological properties [1], as well as having a greater affinity for iron(III) [4], it seemed worthwhile to probe its metal binding sites further. Thus we have prepared the human lactoferrin complexes of chromium, manganese, and cobalt (Cr_2Lf, Mn_2Lf and Co_2Lf) and report their absorption spectra along with that of the previously prepared [6] copper–lactoferrin complex (Cu_2Lf).

Experimental

All glassware was treated with Triton X100 and A.R. concentrated nitric acid and then thoroughly rinsed with distilled, deionized water before use, to minimise metal ion contamination. Human apolactoferrin was isolated directly from fresh human colostrum using a modification of a method previously described [7]. Percentage iron saturation, estimated using the absorbances at 280 nm and 465 nm [4], was generally around 6%.

The manganese and cobalt complexes (Mn_2Lf and Co_2Lf) were prepared by a modification of the procedure described for transferrin metal complexes [8]. A five-fold excess of the appropriate metal(II) ion citrate complex was added to a 1% solution of apolactoferrin in 0.05*M* Tris buffer, pH 7.6. After the addition of excess $NaHCO_3$ the solutions were allowed to stand at 4 °C for one week. Increments of 5 μl 1.0% H_2O_2 were added to the protein solution during this period until the intensities of the visible absorption bands maximised. Uncombined metal ions were removed by Sephadex G-25 gel filtration followed by chromatography on Chelex 100. The solutions were concentrated to about 1% by ultrafiltration using a Chemlab Ultrafiltration Cell fitted with Amicon Diaflo Membranes. The average number of metal ions per molecule of lactoferrin was 2.0 ± 0.2. In the case of the chromium–lactoferrin complex the initial pH was below 7.0 to avoid precipitation of $Cr(OH)_3$. The metal was added as $CrCl_3$ and after the pH was increased to 7.6 by the addition of NaOH, excess $NaHCO_3$ was added. Excess chromium ions were not readily removed by chromatography on Sephadex and Chelex thus the chromium–lactoferrin complex (Cr_2Lf) was best prepared by the addition of two mol of chromium per mol of protein. Electronic absorption spectra were recorded on a Shimadzu MPS 5000 spectrophotometer and metal ion concentrations were measured on a Varian Techtron AA5 atomic absorption spectrophotometer. Protein concentrations were determined from the absorbances at 280 nm.

TABLE I. Electronic Spectral Data for Human Lactoferrin Complexes.

Complex[a]	Colour	λ_{max} (nm)	$E^{1\%}_{1cm}$ [b]	ϵ(l mol^{-1} cm^{-1})[d]
ApoLf[c]	colourless	283	10.9	–
Fe_2Lf[c]	salmon pink	283	14.3	–
		465	0.51	4140
Cu_2Lf	lemon	283	12.5	–
		438	0.60	4800
		677	0.08	620
Co_2Lf	yellow	283	13.6	–
		405	1.28	10340
Mn_2Lf	brown	283	14.7	–
		340(sh)	1.23	9950
		435	1.19	9620
		520(sh)	0.60	4850
		640(sh)	0.28	2310
Cr_2Lf	grey green	283	12.2	–
		442	0.064	520
		612	0.035	280

[a]Lf = Human lactoferrin. [b]$E^{1\%}_{1cm}$ is absorbance of 1% protein solution in a 1 cm cell. [c]Data from ref. 4. [d]Assuming MW of 81000.

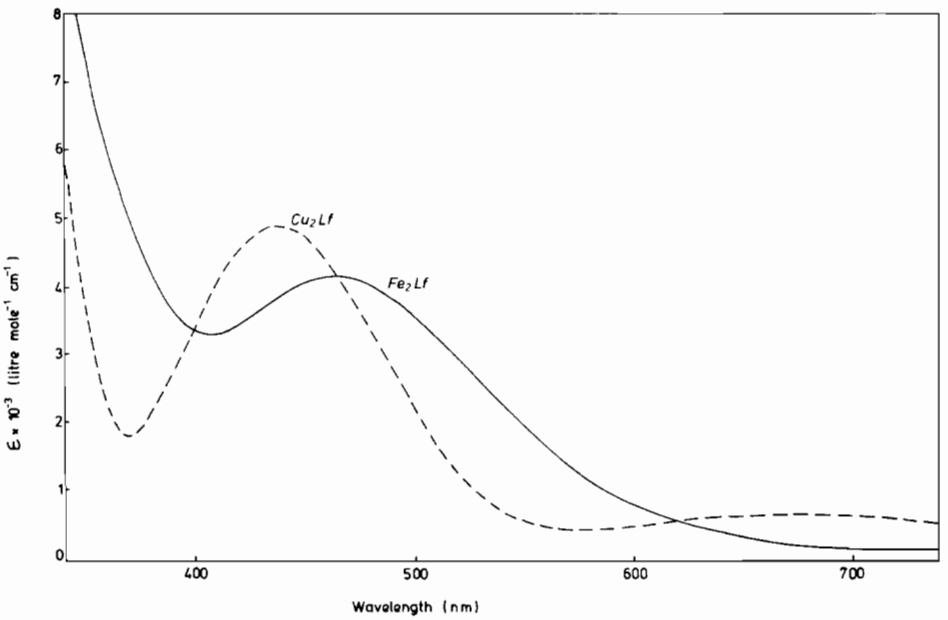

Figure 1. Visible absorption spectra of Fe_2Lf (——) and Cu_2Lf (- - - -), phosphate buffer, pH 7.6, 0.01 *M*.

Results and Discussion

Previously human lactoferrin has been shown to form the complexes Fe_2Lf and Cu_2Lf [1, 4, 6] and we now find that chromium, manganese and cobalt form similar M_2Lf (M = Cr(III), Mn(III), and Co(III)) complexes. However unlike iron(III) and copper(II) these latter ions do not bind to lactoferrin in phosphate buffer (0.01 *M*, pH 7.6) necessitating the use of the more weakly coordinating Tris buffer (0.05 *M*, pH 7.6) system.

All the complexes show an increased absorbance in the ultraviolet near 295 nm, when compared with the apoprotein, pointing to deprotonated tryosyl residues coordinated to metal ions [4, 8]. The visible spectra (Figures 1–3, Table I) resemble those reported for the human serum transferrin and ovotransferrin complexes [8–10], thus reinforcing the postulate that the metal binding sites in all these proteins are very similar and involve the same ligating donor atoms. Thus by analogy with the transferrin complexes [8], manganese and cobalt ions

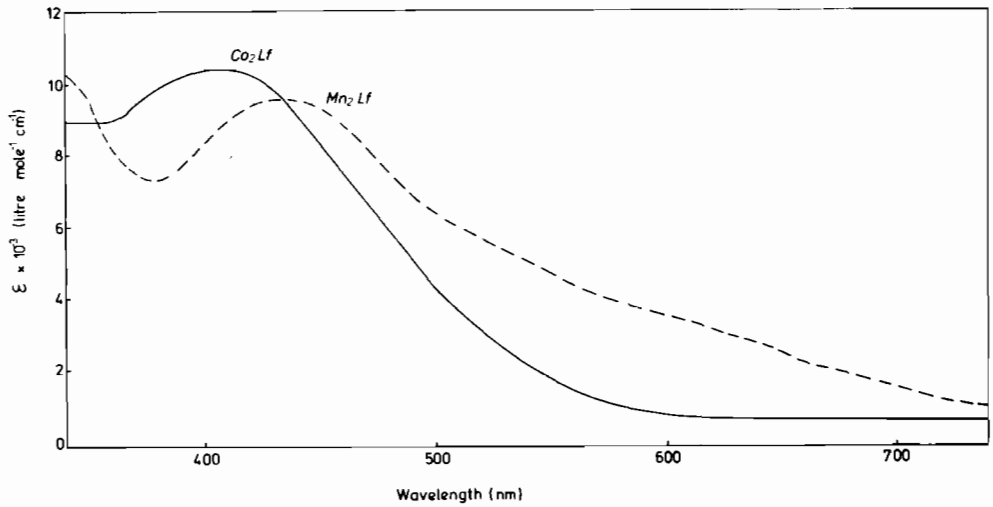

Figure 2. Visible absorption spectra of Co_2Lf (———) and Mn_2Lf (-----), Tris buffer, pH 7.6, 0.05 *M*.

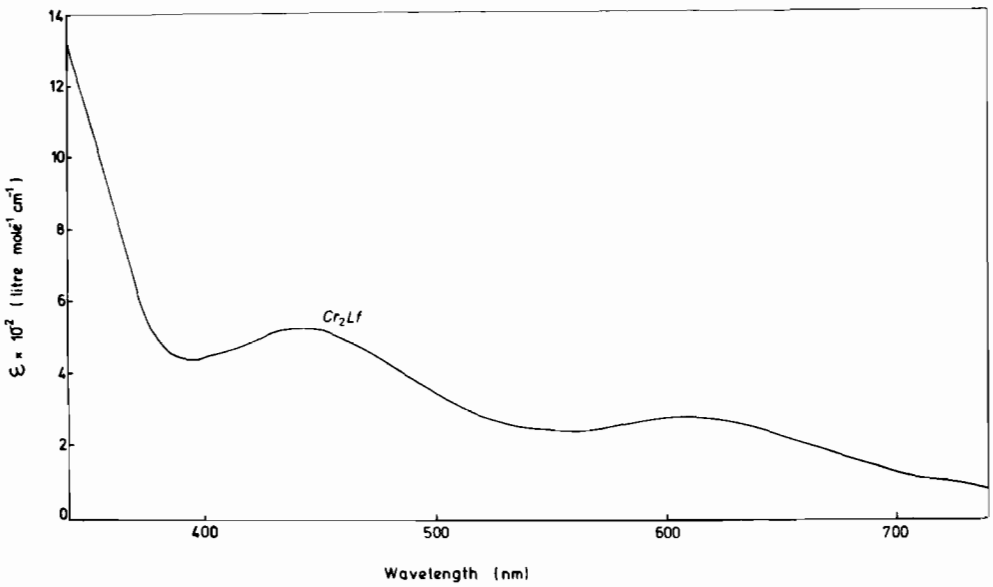

Figure 3. Visible absorption spectrum of Cr_2Lf, Tris buffer, pH 7.6, 0.05 *M*.

are most likely in the trivalent state *viz.* high spin Mn(III) and low spin Co(III). Moreover, to form the intensely coloured complexes, Mn_2Lf and Co_2Lf, the addition of hydrogen peroxide to protein solutions containing the appropriate divalent ions, is essential. The formation of the trivalent ions is in line with the expected behaviour of complexes containing phenolate type ligands [11]. For example Martell *et al.* [12] found meaningful titration curves could not be obtained for the interaction of Mn(II) and Co(II) with ethylenediamine di(*o*-hydroxyphenylacetate) because of irreversible oxidation of these ions in the presence of the ligand. As with lactoferrin itself, the oxidation is even more apparent with the Fe(II) ion. Fe(II) is rapidly oxidized to Fe(III) when added to apolactoferrin to form the Fe_2Lf complex [6]. Both Mn_2Lf and Co_2Lf show intense bands in the 400 nm region (Figure 2) which are tentatively assigned, as for Fe_2Lf, to a phenolate → metal charge transfer type transition, however data for suitable small molecule manganese and cobalt complexes containing simple phenolate type ligands are not available for comparison. In the case of Mn_2Lf, at least two shoulders are discernible (Figure 2) on the longer wavelength side of the 435 nm band which, although of high intensity, are presumably a result of d–d absorptions "stealing intensity" from the nearby charge transfer band. Certainly for a range of high spin Mn(III) complexes (which like Cu(II) complexes are expected to be

subject to Jahn–Teller distortions [13]) up to three d–d bands are generally observed in the 500–1000 nm range [14].

In agreement with previous reports [6, 15] the copper(II) complex, Cu_2Lf, exhibits a band at 438 nm (Figure 1) which for the analogous transferrin complex has been assigned to a phenolate → copper, or alternatively, a copper → phenolate, charge transfer transition [9, 10]. The wavelength of this absorption, when compared with the data for small molecule complexes (*e.g.* bis(2,4,6-trichlorophenolato)di-imidazole copper(II)) containing phenolate ligands [9, 16], is indicative of tyrosyl residues equatorially bound to copper(II) in a tetragonal environment. However not previously reported for Cu_2Lf, is a weaker band at 677 nm (Figure 1), assignable to a d–d transition [17]. Assuming the rule of average environment [18] the position of the absorption points to the coordination of one, or at the most two nitrogen donor ligands bound to a copper(II) ion in a distorted tetragonal or rhombic site. Aisen *et al.* [4], from e.s.r. studies on Fe_2Lf, have demonstrated a rhombic coordination sphere, and studies on the copper, although results from a recent e.s.r. study led presence of at least one nitrogen ligand bound to the copper, although results from recent e.s.r. study led to the suggestion that one of the copper ions, interacts with three or four nitrogen atoms [15].

Finally we turn to the Cr_2Lf complex, which does not exhibit a charge transfer band in the visible region, thus allowing the observation (Figure 3) of the two spin-allowed d–d transitions $^4A_{2g} \rightarrow {}^4T_{1g}$ and $^4A_{2g} \rightarrow {}^4T_{2g}$ (at 442 nm and 612 nm respectively) expected for an octahedral chromium(III) complex [19]. Since the binding of chromium to apolactoferrin is more rapid in the presence of excess bicarbonate and the chromium, once bound, is not readily displaced by iron, to form the salmon pink Fe_2Lf complex, it appears that the chromium is involved in the specific metal binding site. Moreover the electronic spectrum is very similar to the analogous chromium transferrin complex, for which evidence has also been presented [8] (*e.g.* two mol of CO_2 are liberated when the two chromium(III) ions are released) to support specific site binding. The electronic spectrum of Cr_2Lf is similar to those of chromium(III) complexes known to have a CrO_6 coordination (Table II) and does not seem consistent with the involvement of both nitrogen and oxygen ligands only as suggested by studies on other lactoferrin complexes. However the absorption maxima do in fact resemble those of chromium(III) complexes containing chloride ions coordinated, as well as nitrogen and oxygen donors (Table II), which may indicate that because of the kinetic inertness of the Cr(III) ion, at least one chloride ion has been retained in the coordination sphere rather than a water molecule (since the metal ion is added as commercial chromium(III) chloride which contains $[Cr(H_2O)_4Cl_2]^+$ ions [20]). A water molecule has been suggested as a ligand in the iron(III) transferrin complex [21]. Thus, taking into account results presented here and elsewhere by others, it appears that that metal binding sites in human lactoferrin contain two or three tyrosyl phenolates and at least one nitrogen (from a hystidyl residue). In the Cu_2Lf complex, two tyrosyls and one nitrogen ligand at least, are likely to be occupying the equatorial positions of a distorted tetragonal coordination sphere. As the donor sites appear to be identical to those of human serum transferrin, the differences between the behaviour of the two proteins could well be due to the reduced accessibility of the metal binding sites in lactoferrin to solvent and other molecules [22].

TABLE II. Electronic Spectral Data for Cr(III) Complexes.

Complex[a]	λ_{max} (nm)		Donor Set	Ref
Cr_2Lf	442	612	?	[b]
Cr_2Tf	440	615	?	8
$[Cr(H_3edta)Cl_2(H_2O)_2]$	450	620	CrO_3NCl_2	23
$[Cr(H_2O)_5Cl]^{2+}$	428	608	CrO_5Cl	24
$[Cr(cat)_3]^{3-}$	475	592	CrO_6	25
$[Cr(H_2O)_6]^{3+}$	407	573	CrO_6	26
$[Cr(H_2O)_5(NH_3)]^{3+}$	397	545	CrO_5N	26
$[Cr(H_2O)_4(en)]^{3+}$	385	512	CrO_4N_2	27
$[Cr(edta)(H_2O)]^-$	395	545	CrO_4N_2	27

[a]Abbreviations: Lf = human lactoferrin, Tf = human serum transferrin, edta = ethylenediaminetetracetato, cat = catecholato, en = ethylenediamine. [b]This work.

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