Gay, S., & Miller, E. J. (1979) *Arch. Biochem. Biophys. 198*, 370–378.
Glanville, R. W., Rauter, A., & Fietzek, P. P. (1979) *Eur. J. Biochem. 95*, 383–389.
Hayashi, T., Curran-Patel, S., & Prockop, D. J. (1979) *Biochemistry 18*, 4182–4187.
Heathcote, J. G., Sear, C. H. J., & Grant, M. E. (1978) *Biochem. J. 176*, 283–294.
Hong, B.-S., Davison, P. F., & Cannon, D. J. (1979) *Biochemistry 18*, 4278–4282.
Kefalides, N. A. (1971) *Biochem. Biophys. Res. Commun. 45*, 226–234.
Kefalides, N. A., Alper, R., & Clark, C. C. (1979) *Int. Rev. Cytol. 61*, 167–228.
Killen, P. D., & Striker, G. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3518–3522.
Kresina, T. F., & Miller, E. J. (1979) *Biochemistry 18*, 3089–3097.
Minor, R. R., Clark, C. C., Strause, E. L., Koszalka, T. R., Brent, R. L., & Kefalides, N. A. (1976) *J. Biol. Chem. 251*, 1789–1794.
Piez, K. A. (1968) *Anal. Biochem. 26*, 305–312.
Rhodes, R. K., & Miller, E. J. (1978) *Biochemistry 17*, 3442–3448.
Sage, H., & Bornstein, P. (1979) *Biochemistry 18*, 3815–3822.
Sage, H., Woodbury, R. G., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 9893–9900.
Sato, T., & Spiro, R. G. (1976) *J. Biol. Chem. 251*, 4062–4070.
Schenkein, I., Levy, M., Franklin, E. C., & Frangione, B. (1977) *Arch. Biochem. Biophys. 182*, 64–70.
Schwartz, D., & Veis, A. (1978) *FEBS Lett. 85*, 326–332.
Sykes, B. C., & Bailey, A. J. (1971) *Biochem. Biophys. Res. Commun. 43*, 340–346.
Timpl, R., Martin, G. R., Bruckner, P., Wick, G., & Wiedemann, H. (1978) *Eur. J. Biochem. 84*, 43–52.
Timpl, R., Bruckner, P., & Fietzek, P. (1979a) *Eur. J. Biochem. 95*, 255–263.
Timpl, R., Martin, G. R., & Bruckner, P. (1979b) *Front. Matrix Biol. 7*, 130–141.
Trelstad, R. L. (1979) *Front. Matrix Biol. 7*, 9–18.
Tryggvason, K., & Kivirikko, K. I. (1978) *Nephron 21*, 230–235.

Studies on Human Lactoferrin by Electron Paramagnetic Resonance, Fluorescence, and Resonance Raman Spectroscopy†

Eric W. Ainscough,* Andrew M. Brodie,* Jeffrey E. Plowman, Stephen J. Bloor, Joann Sanders Loehr, and Thomas M. Loehr*

ABSTRACT: Investigations of metal-substituted human lactoferrins by fluorescence, resonance Raman, and electron paramagnetic resonance (EPR) spectroscopy confirm the close similarity between lactoferrin and serum transferrin. As in the case of Fe(III)- and Cu(II)-transferrin, a significant quenching of apolactoferrin's intrinsic fluorescence is caused by the interaction of Fe(III), Cu(II), Cr(III), Mn(III), and Co(III) with specific metal binding sites. Laser excitation of these same metal-lactoferrins produces resonance Raman spectral features at ca. 1605, 1505, 1275, and 1175 cm^{-1}. These bands are characteristic of tyrosinate coordination to the metal ions as has been observed previously for serum transferrins and permit the principal absorption band (λ_{max} between 400 and 465 nm) in each of the metal-lactoferrins to be assigned to charge transfer between the metal ion and tyrosinate ligands. Furthermore, as in serum transferrin the two metal binding sites in lactoferrin can be distinguished by EPR spectroscopy, particularly with the Cr(III)-substituted protein. Only one of the two sites in lactoferrin allows displacement of Cr(III) by Fe(III). Lactoferrin is known to differ from serum transferrin in its enhanced affinity for iron. This is supported by kinetic studies which show that the rate of uptake of Fe(III) from Fe(III)–citrate is 10 times faster for apolactoferrin than for apotransferrin. Furthermore, the more pronounced conformational change which occurs upon metal binding to lactoferrin is corroborated by the production of additional EPR-detectable Cu(II) binding sites in Mn(III)–lactoferrin. The lower pH required for iron removal from lactoferrin causes some permanent change in the protein as judged by altered rates of Fe(III) uptake and altered EPR spectra in the presence of Cu(II). Thus, the common method of producing apolactoferrin by extensive dialysis against citric acid (pH 2) appears to have an adverse effect on the protein.

Human lactoferrin is one of the class of nonheme, iron-binding proteins generally designated as the transferrins. However, because the importance of lactoferrin in human physiology has only recently become apparent, it has not been as extensively studied as the other members of the class, serum transferrin and ovotransferrin. Lactoferrin occurs in high concentration in human milk and has been found in a variety of other bodily secretions and in intracellular components (Aisen, 1973; Bezkorovainy, 1977; Feeney & Komatsu, 1966). Because of its avidity for Fe(III), it has been postulated to have a bacteriostatic function in depriving microorganisms of essential iron required for their growth (Bullen et al., 1974). A nutritional role for human lactoferrin as an iron (McMillan et al., 1976) as well as a zinc carrier (Ainscough et al., 1980) has also been suggested.

All the transferrins have a molecular weight of ~80 000 and are capable of binding specifically two Fe(III) ions in

† From the Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand (E.W.A., A.M.B., J.E.P., and S.J.B.), the Department of Chemistry, Portland State University, Portland, Oregon 97207 (J.S.L.), and the Department of Chemistry and Biochemical Sciences, Oregon Graduate Center, Beaverton, Oregon 97006 (T.M.L.). *Received February 1, 1980.* This work was supported by the New Zealand University Grants Committee and the U.S. Public Health Service, National Institutes of Health (Grant GM 18865).

0006-2960/80/0419-4072$01.00/0

conjunction with two bicarbonate (or carbonate) ions (Aisen, 1973; Bezkorovainy, 1977; Feeney & Komatsu, 1966). Chemical modification, titration, and spectroscopic studies on human lactoferrin have shown that the amino acid side chains probably involved in binding the Fe(III) are two or three tyrosyl groups (Teuwissen et al., 1972, 1973) and at least one histidyl residue (Aisen & Leibman, 1972; Krysteva et al., 1975). Other metal ions, e.g., Cr(III), Mn(III), Co(III), and Cu(II), bind to lactoferrin (Ainscough et al., 1979; Masson & Heremans, 1968) and the other transferrins (Aisen et al., 1969; Tomimatsu et al., 1976) in an apparently similar specific fashion. However, the proteins do differ in immunological properties, amino acid composition, and electrophoretic mobility (Morgan, 1974). Another chemical difference is in the conditions needed to remove the iron from the proteins. Metal-free transferrin and ovotransferrin can be produced at pH ~4 by dialysis against a suitable chelating agent whereas for lactoferrin a pH of ~2 is required to release the metal ion completely (Groves, 1960; Johansson, 1960). Equilibrium dialysis studies show that human lactoferrin binds iron up to several hundred times more tightly than either serum transferrin or ovotransferrin (Aisen & Leibman, 1972).

In order to obtain a better understanding of the structural and functional differences between lactoferrin and serum transferrin, we have undertaken a series of spectroscopic investigations of metal binding by lactoferrin. The present work summarizes the results with fluorescence, resonance Raman, and electron paramagnetic resonance (EPR)[1] spectroscopy.

Materials and Methods

All glassware was treated with Triton X-100 and AR concentrated HNO_3 and then thoroughly rinsed with distilled and deionized water before use to minimize metal ion contamination. Human apolactoferrin was isolated from fresh human colostrum by using a modification of a method reported previously (Querinjean et al., 1971). Final purification was achieved by CM-Sephadex C-50 chromatography in 0.01 M (pH 7.6) phosphate buffer by eluting with a 0–0.5 M NaCl salt gradient. $NaDodSO_4$–acid gel electrophoresis indicated a purity of 99%. The percentage of iron saturation, estimated by using the absorbances at 280 and 465 nm (Aisen & Leibman, 1972), was generally near 6%. Apolactoferrin, as isolated, was used since metal ion uptake and EPR experiments (see Results) indicated that the recommended dialysis (Masson & Heremans, 1968) against 0.01 M citric acid (pH 2) adversely affected the protein, and dialysis against 0.1 M $NaClO_4$ to remove extraneous citrate, as suggested for transferrin by Price & Gibson (1972), led to protein precipitation. The metal ion–lactoferrin complexes were prepared as previously described (Ainscough et al., 1979).

Metal Ion Uptake Experiments. The kinetics of the reaction of metal chelates with apolactoferrin were followed by the absorbance changes at 465 nm for Fe(III) or 438 nm for Cu(II), by using a Shimadzu MPS 5000 spectrophotometer. Solutions were thermostated and maintained at 25 °C with a circulating water bath. The metal chelates were prepared by dissolving $Fe(NO_3)_3 \cdot 9H_2O$ or $CuCl_2 \cdot 2H_2O$ in solutions containing equimolar amounts of citric acid and EDTA or the trisodium salt of NTA. Solutions were all freshly prepared before use. Studies were conducted both in phosphate buffer, 0.01 M (pH 7.6), that was 0.33 M in NaCl, and in Tris-HCl buffer, 0.05 M (pH 7.4), that was 0.5 M in NaCl. Runs were made in duplicate, and, in all cases, 2 molar equiv of the appropriate metal ion were added per mol of protein.

Fluorescence Spectra. Measurements were made on a Turner Model 430 spectrofluorometer. Two molar equivalents of the appropriate metal ion were added to the protein solution as follows: Fe(III) and Pb(II) as their NTA complexes, Mn(II) and Co(II) as their citrate complexes, and Cu(II), Ni(II), Zn(II), and Hg(II) as their chloride salts. Solutions were prepared in duplicate and allowed to stand overnight at 4 °C before measurements were made except in the case of the fluorescence titrations where measurements were made 45 min after incremental metal ion addition.

Resonance Raman Spectra. Samples for resonance Raman spectroscopy were transferred to standard melting point capillaries and held in a variable-temperature nitrogen-flow assembly maintained at −40 °C. It was found that higher quality Raman spectra were obtained for the 1900–900-cm^{-1} region at this temperature, although the low-frequency region, 900–100 cm^{-1}, was largely obscured by strong scattering from the frozen buffer solution. Additionally, the low temperature minimizes thermal denaturation of the protein samples. Light from a Coherent Radiation Laboratory CR-04 argon ion laser was passed through narrow-band interference filters to eliminate plasma emission at each of the wavelengths chosen for study. Samples were irradiated through the flow assembly after passage of the laser light through a cylindrical lens to obtain a slit-shaped focus. Scattered Raman light was collected in a ~180° geometry and analyzed by a computer-controlled laser Raman spectrophotometer which has been previously described (Loehr et al., 1979). The detector employed in these studies was an ITT FW-130 (S-20) photomultiplier.

EPR Spectra. These were recorded at ~100 K by using a Varian E-104A spectrometer equipped with a Varian E-257 variable temperature accessory. Spectral g values were calibrated with a DPPH standard. Samples containing Cu(II) were prepared from solutions of $^{65}CuCl_2$. Isotopically pure ^{65}Cu was obtained as its oxide from Oak Ridge National Laboratory and dissolved in 0.1 M HCl before use. Bicarbonate-free ^{65}Cu(II)–lactoferrin was prepared with a Thunberg apparatus by using a modification of the method described for transferrin (Aisen et al., 1967). Apolactoferrin in 0.01 M phosphate buffer was titrated to pH 4 with 1 M H_3PO_4, and then $^{65}CuCl_2$ was added to the protein in a 2:1 molar ratio. After evacuation and flushing with N_2 for 1.5 h, solid Na_2HPO_4 was added to give a final pH in the range 7–8.

Results

Metal Ion Binding Studies. The rate of Fe(III) uptake by apolactoferrin is dependent on the nature of the chelating agent bound to the iron (Figure 1a). Apolactoferrin (isolated from human colostrum and used directly) reacts rapidly with Fe(III)–NTA and achieves saturation of the two specific metal binding sites within seconds. For Fe(III)–citrate the time required to half-saturate the sites, $t_{1/2}$, is 4 min and for Fe(III)–EDTA the $t_{1/2}$ value is ~3 days. The relative rates of reaction are similar to those observed for serum transferrin (Bates et al., 1967), but the absolute rates for lactoferrin are greater than for transferrin with the same Fe(III) complexes. For example, the $t_{1/2}$ values for transferrin are 8 h when the iron is added as the Fe(III)–citrate complex and ~4 days when added as Fe(III)–EDTA.

The rate of Cu(II) uptake by apolactoferrin is similar to Fe(III) with a $t_{1/2} < 10$ s when the metal is added as cop-

[1] Abbreviations used: DPPH, diphenylpicrylhydrazyl; EDTA, (ethylenedinitrilo)tetraacetic acid; EPR, electron paramagnetic resonance; Lf, lactoferrin; NTA, nitrilotriacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

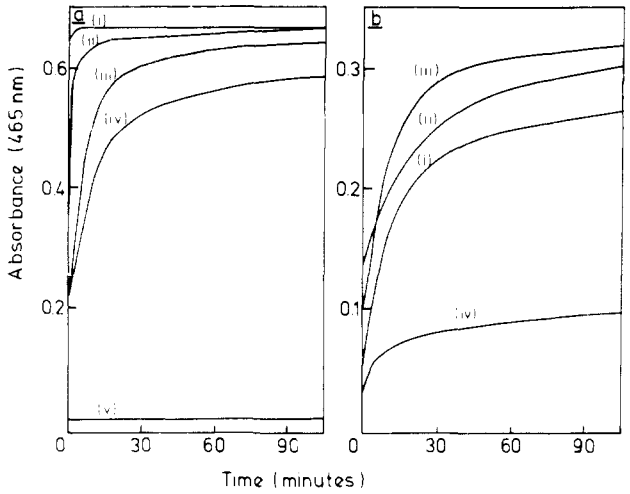

FIGURE 1: (a) Uptake of Fe(III) from various chelates by apolactoferrin: (i) Fe–NTA (citrate-treated protein); (ii) Fe–NTA; (iii) Fe–citrate (citrate-treated protein); (iv) Fe–citrate; (v) Fe–EDTA. The protein concentration is 1.6×10^{-4} M in 0.01 M phosphate buffer, pH 7.6; [NaCl] = 0.33 M. (b) Effect of other metal ions on Fe(III) uptake from Fe–citrate by apolactoferrin: (i) no other metal ions present; (ii) Zn(II); (iii) Hg(II); (iv) Cr(III). The protein concentration is 0.89×10^{-4} M in 0.05 M Tris-HCl buffer, pH 7.4; [NaCl] = 0.5 M. Metal ion/protein molar ratio = 2:1.

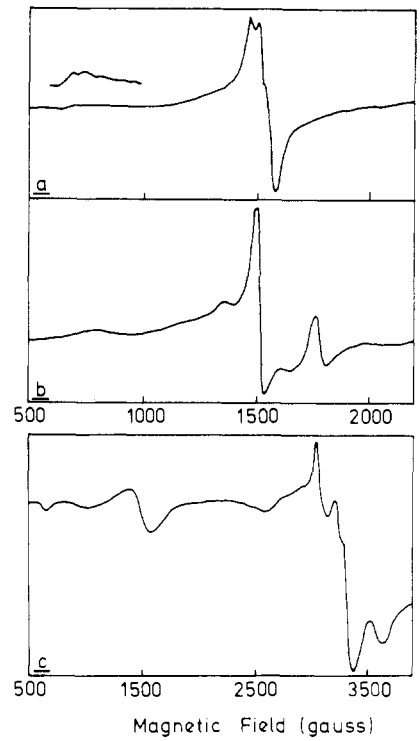

FIGURE 2: Frozen solution X-band EPR spectra for Fe(III) species. (a) Fe(III)–lactoferrin (specifically bound) in 0.01 M phosphate buffer, pH 7.6. The protein concentration is 4×10^{-4} M. (b) Fe(III)–lactoferrin titrated to pH 2.0 with 1 M citric acid. (c) Fe(III)–lactoferrin titrated to pH 2.0 with 1 M phosphoric acid.

per(II) chloride or as Cu(II)–citrate, but no binding is observed with Cu(II)–EDTA. The uptake of Fe(III)–citrate is considerably diminished with Cr(III)–lactoferrin (Figure 1b). While the first phase (~5 min) is fast, the second phase is slow, and only 45% of the chromium is displaced by the end of 7 days. This result strongly contrasts the behavior of the more weakly bound Zn(II) and Hg(II) ions which actually enhance Fe(III) uptake (Figure 1b). Such enhancement may be due to increased dissociation of the Fe(III)–citrate complex or to an alteration of protein conformation which increases iron affinity.

When iron uptake at pH 7.6 is measured by using apolactoferrin prepared by dialyzing the Fe(III)–protein against 0.1 M citric acid (pH 2), a more rapid incorporation of Fe(III) is observed than when untreated lactoferrin is used. The pH 2 treatment causes complete removal of iron, as evidenced by the change in the EPR spectrum from that of the Fe(III)–lactoferrin complex (Figure 2a) to that of a 1:3 Fe(III)–citrate complex (Figure 2b). Similarly, the removal of Fe(III) by treatment with phosphoric acid at pH 2 gives an EPR signal characteristic of iron(III) phosphate and near octahedral FeO_6 coordination (Figure 2c). Thus, although the pH 2 treatment is useful in removing the remaining Fe(III), it apparently causes some permanent structural change in the protein which alters its rate of metal binding. Because of the uncertain effects of citric acid, directly isolated and untreated protein was used as a source of apolactoferrin in all subsequent experiments.

A further indication of structural modification during citric acid treatment of lactoferrin is seen in the complexation of Cu(II). In this case an additional weak signal appears in the EPR spectrum near 3300 G which is not observed in Cu(II)–lactoferrin prepared from untreated lactoferrin (Table I). Some time ago, Aasa et al. (1963) observed an identical high-field bump for Cu(II)–transferrin and ascribed it to nonspecifically bound Cu^{2+} ions. More recently, Mazurier et al. (1977) have suggested that the appearance of this resonance at 3300 G and other weak signals in Cu(II)–transferrin are evidence for one of the specific-site Cu(II) ions being coordinated to three or four nitrogen donor residues. Furthermore,

Table I: EPR Spectral Parameters for ^{65}Cu(II) Complexes

species	pH	$g_\parallel$	$A_\parallel$ (G)	$g_\perp$
Cu(II)-lactoferrin (specific)	7.6	2.315	151	2.057
Cu(II) + Fe(III)-lactoferrin[a]	7.6	2.276	150	2.072
Cu(II) + Mn(III)-lactoferrin	7.6	2.230	202	2.05
Cu(II)-lactoferrin (bicarbonate free)	7.4	2.372	145	2.076
Cu(II)-lactoferrin (low pH)	3.9	2.372	145	2.076
Cu(II)-tetraammine[b]	>9	2.241	185	2.06

[a] Data given are for the nonspecifically bound Cu(II); the observed spectrum is a composite of both the specifically and nonspecifically bound Cu(II)'s. [b] From Aisen et al. (1967).

because these "extra" signals are modified and weakened on carbethoxylation of histidine residues, it was postulated that this Cu(II) binding site is more accessible to the diethyl pyrocarbonate reagent. However, the apolactoferrin used by Mazurier et al. had been dialyzed at low pH to achieve iron removal and then lyophilized. This treatment presumably modified its structure enough to expose histidyl residues capable of reacting with diethyl pyrocarbonate or Cu(II), with a resultant heterogeneity in the EPR spectrum.

Fluorescence Spectra. For apolactoferrin in phosphate buffer (0.01 M; pH 7.6), the wavelength of excitation was found to be 292 nm and the maximum emission was at 348 nm, whereas in Tris-HCl (0.05 M; pH 7.6) the corresponding wavelengths are 290 and 334 nm. The addition of Fe(III) or Cu(II) ions causes a quenching of the fluorescence, which is maximal upon addition of two metal ions per protein molecule. As in the case of serum transferrin and ovotransferrin (Lehrer, 1969), it is probable that tryptophan residues are not directly bound to the metal ions but that their fluorescence is quenched by a long-range energy transfer to an absorption band produced by the metal–protein interaction. Moreover, the fluorescence titration curve for Fe(III) binding is not linear

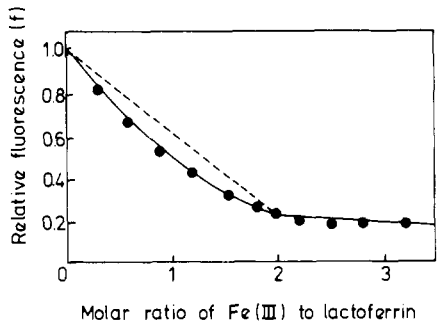

FIGURE 3: Fluorescence titration for Fe(III) uptake (as Fe–NTA) by apolactoferrin. Excitation λ = 292 nm; emission, λ = 348 nm. The protein concentration is 1.25×10^{-5} M in 0.01 M phosphate buffer, pH 7.6.

Table II: Relative Fluorescence of Metal Ion–Lactoferrin Complexes

metal ion	f^a	metal ion	f^a
Fe(III)[b]	0.22	Co(II)	0.77
Cu(II)[b]	0.28	Ni(II)	0.81
Mn(III)	0.34	Zn(II)	0.82
Co(III)	0.47	Cd(II)	0.77
Cr(III)	0.63	Hg(II)	0.81
Mn(II)	0.78[c]	Pb(II)	0.74

[a] f = fluorescence relative to apolactoferrin; metal ion/protein ratio = 2:1. Protein concentration, 1.0×10^{-6} M in 0.05 M Tris-HCl, pH 7.6. [b] In 0.01 M phosphate buffer, pH 7.6. [c] After 5 days, f = 0.57; after 7 days, f = 0.38.

(Figure 3) and, as for serum transferrin (Lehrer, 1969), may be approximated by using a random binding model in which $k_1 = 0.80k_2$ (where k_1 and k_2 represent the fraction of the original fluorescence quenched by the binding to one and to two sites, respectively, on the same molecule). For serum transferrin, an almost identical result has been obtained, i.e., $k_1 = 0.75k_2$ (Lehrer, 1969). The titration data for Cu(II) binding to lactoferrin are also curved, in contrast to the result for transferrin where a linear fluorescence dependence was found (Lehrer, 1969), but the total quenching produced by the addition of 2 molar equiv of copper to each protein is the same (final relative fluorescence ~0.3).

Fluorescence quenching is also exhibited by the other M(III)–lactoferrin complexes (Table II) and is most significant for Mn(III) and Co(III), in line with a specific-site interaction by these ions. In contrast, the more weakly bound divalent ions Mn(II), Co(II), Ni(II), Zn(II), Cd(II), Hg(II), and Pb(II) do not give intensely colored solutions and exhibit a lesser quenching. Increasing the added metal ion to protein ratio above 2:1 for these divalent ions does not alter the fluorescence, but the addition of Fe(III)–NTA causes an immediate increase in quenching (relative fluorescence ~0.30) as the Fe(III) enters the specific sites. An interesting difference in behavior is seen for the Mn(II) and Co(II) solutions which both can form trivalent ion complexes with lactoferrin. Whereas the Mn(II)–lactoferrin solution shows an increased quenching with time as the metal is oxidized to Mn(III) (addition of H_2O_2 induces a similar effect), the Co(II) does not readily undergo air oxidation in the presence of the protein and the fluorescence remains constant with time.

Resonance Raman Spectra. The visible absorption spectral data for the more strongly complexed metal lactoferrins, Fe(III)–, Cr(III)–, Mn(III)–, Co(III)–, and Cu(II)–lactoferrins, are given in Table III, along with the corresponding data for ovotransferrins and serum transferrins. It is apparent that the spectral characteristics for a given metal ion are quite similar for all three proteins. The absorptions at 465 nm in Fe(III)-substituted and 438 nm in Cu(II)-substituted proteins have been assigned to a charge-transfer transition between tyrosinate ligand pπ orbitals and metal ion dπ* or dσ* orbitals on the basis of the strong enhancement of tyrosinate ligand modes in the resonance Raman spectra (Gaber et al., 1974; Tomimatsu et al., 1976).

Table III: Visible Absorption Spectral Parameters of Metal Ion Substituted Lactoferrin, Ovotransferrin, and Transferrin

	λ_{max} (nm) [ϵ (M^{-1} cm^{-1}/M^{n+})]			
protein	Fe(III)	Cu(II)	Mn(III)	Co(III)
lactoferrin[a]	465 (2070)	438 (2400)	435 (4810)	405 (5170)
ovotransferrin[b]	465 (2000)	435 (2500)	430 (4000)	415 (4500)
transferrin[b]	465 (1875)	430 (1900)	430 (4400)	400 (3900)

[a] Values for native lactoferrin are from Aisen & Leibman (1972); other values are from Ainscough et al. (1979). [b] Tomimatsu et al. (1976).

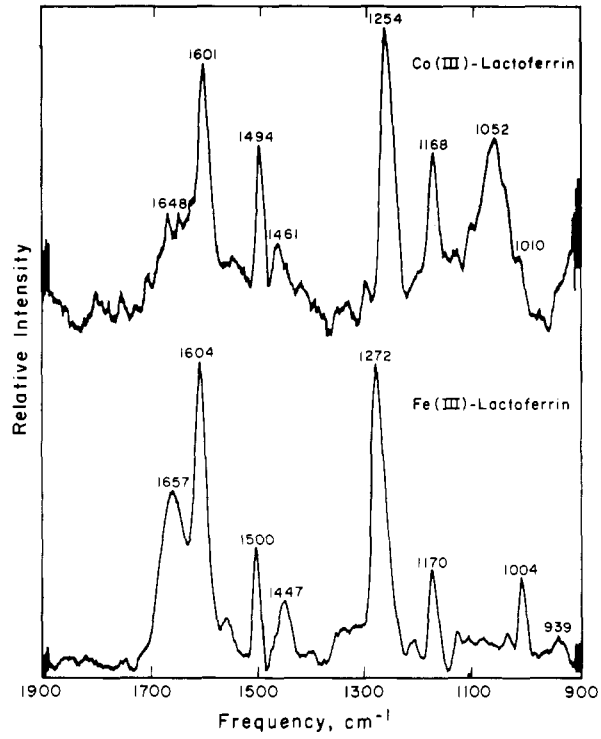

FIGURE 4: Resonance Raman spectra of Co(III)–lactoferrin and Fe(III)–lactoferrin obtained with 457.9 nm (25 mW at the sample) and 514.5 nm (~100 mW at the sample), respectively. Instrumental conditions: resolution, 8 cm^{-1}; scan rate, 5.0 cm^{-1} s^{-1}; digitizing increment, 1.0 cm^{-1}; number of scans, for Co(III)–Lf, 50, and for Fe(III)–Lf, 10.

Resonance Raman spectra of these lactoferrins have been obtained in frozen aqueous buffer solutions, and representative spectra for the Fe(III)– and Co(III)–lactoferrins are shown in Figure 4. It is immediately apparent that these spectra are dominated by strong resonance-enhanced vibrational modes of metal ion coordinated tyrosinates, similar to those observed for serum transferrin, ovotransferrin, and uteroferrin (Carey & Young, 1974; Gaber et al., 1974, 1979; Tomimatsu et al., 1976). Raman data for the Fe(III)–, Cu(II)–, Mn(III)–, Co(III)–, and Cr(III)–lactoferrins are given in Table IV. The four peaks at approximately 1600, 1500, 1260–1290, and 1170 cm^{-1} are vibrational modes of metal-coordinated tyrosinates. Tomimatsu et al. (1976), using data from a normal coordinate analysis on bis(2,4,6-trichlorophenolato)diimidazolecopper(II), have assigned the two highest frequencies principally to CC stretching, the 1260-cm^{-1} band to CO stretching, and the lowest frequency to CO bending of the phenolate ligand.

Table IV: Raman Spectral Data for Native and Metal-Substituted Lactoferrins[a]

Raman spectral data (cm^{-1})					
$Fe(III)_2Lf$	$Cu(II)_2Lf$	$Mn(III)_2Lf$	$Co(III)_2Lf$	$Cr(III)_2Lf$	assignments[b]
~1657	~1660	~1660	~1650	~1656	ν_2 (H_2O)
1604	1603	1601	1601	1604	Tyr ligand
1500	1501	1499	1494	1505?	Tyr ligand
1447	1450	1462	1462	1461	δ (CH_2)
1272	1275	1253	1254	1267?	Tyr ligand
1170	1169	1169	1168		Tyr ligand
1004	1002		~1010		ν (Phe)
939	936		933	943	ν (C–C)

[a] Spectra were obtained by using 457.9–514.5-nm laser excitation. [b] Based on Gaber et al. (1974) for tyrosine ligand assignments and Frushour & Koenig (1975) for the nonresonant contributions to the spectrum.

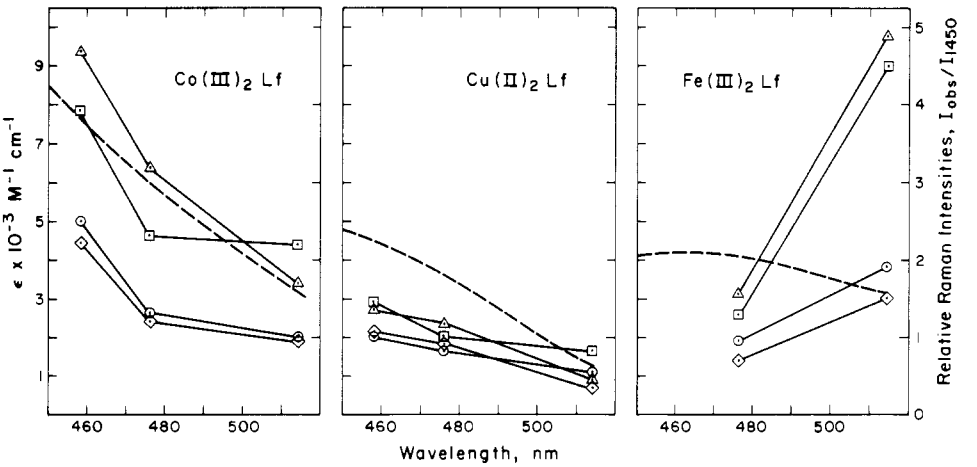

FIGURE 5: Resonance Raman excitation profiles of Co(III)–, Cu(II)–, and Fe(III)–lactoferrins. The dashed lines are the optical absorption spectra for each sample (left ordinate axis). The wavelength dependence of the Raman intensities, relative to the CH_2-deformation mode at ~1450 cm^{-1}, is indicated for the tyrosinate ligand frequencies at ca. 1600 (□), 1500 (○), ~1260 (△), and 1170 cm^{-1} (◇) (right ordinate axis).

Nonresonant features which arise from protein motions are at ~1450 (CH_2 deformation), ~1005 (phenylalanine ring breathing mode), and ~940 cm^{-1} (backbone CC stretching); the broad feature at ~1660 cm^{-1} arises from solvent (H_2O deformation) (Frushour & Koenig, 1975). Spectral lines in the region below 900 cm^{-1} were largely obscured by contributions from the frozen solvent.

The resonance Raman spectra of the Fe(III)– and Cu(II)–lactoferrins are nearly identical, as are the spectra of the Co(III)– and Mn(III)–lactoferrins. However, the two sets differ in that the ~1253-cm^{-1} band is ~20 cm^{-1} lower in frequency in the case of Co(III) and Mn(III) than with Fe(III) and Cu(II). The other three tyrosinate modes, ~1602, 1500, and 1169 cm^{-1}, are nearly coincident in the four proteins except that the 1500-cm^{-1} peak is shifted to 1494 cm^{-1} in Co(III)–lactoferrin. These results for the metal-substituted lactoferrins are nearly identical with those reported for the corresponding ovotransferrins and serum transferrins by Tomimatsu et al. (1976). It is difficult to determine the position of Cr(III)–lactoferrin in the above classification because its low absorptivity led to weak resonance enhancement. Only the 1604-cm^{-1} line was clearly evident among the four tyrosinate modes, and the spectrum was dominated by the nonresonant peaks.

Relative Raman intensities were calculated by using the CH_2 deformation at ~1450 cm^{-1} as an internal intensity standard since the large contribution from the buffer at ~1050 cm^{-1} apparent in some of the spectra (Figure 4) precluded the use of the phenylalanine peak at ~1004 cm^{-1}. An examination of the intensities of the four resonance-enhanced vibrational modes as a function of excitation wavelength for Co(III)–, Cu(II)–, and Fe(III)–lactoferrins reveals interesting differences (Figure 5). In the Co(III)– and Cu(II)–lactoferrins, the excitation profiles of the individual vibrations parallel the optical absorption spectrum in the region between 514.5 and 457.9 nm, as would be expected for resonance enhancement by single electronic states whose respective optical transitions are given in Table III. However, for the Fe(III)–lactoferrin, the tyrosinate frequencies appear to decrease in intensity as the λ_{max} is approached. Such behavior is probably due to an excitation profile fine structure similar to that observed for Fe(III)–transferrin (Gaber et al., 1974). Since fewer excitation wavelengths were used in the present study, we were not able to resolve the fine structure in the excitation profiles.

Table V: EPR Spectral Parameters for Cr(III) Complexes[a]

	spectral parameters	
site	Cr(III)–Lf	Cr(III)–NTA
type 1	5.43	
type 2	5.62, 5.15, 2.42	
nonspecific	2.05	1.98

[a] Assignments for g values by analogy to Cr(III)–transferrin; from Aisen et al. (1969).

EPR Spectra. The EPR spectra of Fe(III)– and Cu(II)–lactoferrin (Figures 2a and 6a) are similar to those reported previously for Fe(III)– and Cu(II)–transferrins (Aisen & Leibman, 1972; Aisen et al., 1973; Pinkowitz & Aisen, 1972). The Fe(III)–lactoferrin spectrum is characteristic of the metal ion being high spin in a rhombic environment. Computer simulation of the Cu(II)–lactoferrin spectrum points to one nitrogen bound per metal ion. The best fit between the observed and calculated spectrum is given by the following pa-

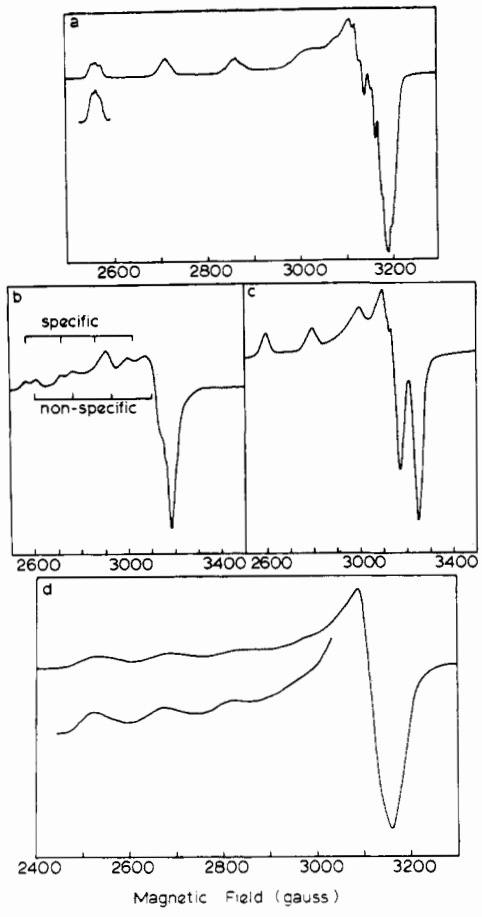

FIGURE 6: Frozen solution X-band EPR spectra for $^{65}Cu(II)$ species: (a) Cu(II)–lactoferrin (specifically bound) in 0.01 M phosphate buffer, pH 7.6; (b) Cu(II) addition to the Fe(III)–lactoferrin in 0.01 M phosphate buffer, pH 7.6, Cu(II)/protein molar ratio = 2:1; (c) Cu(II) addition to Mn(III)–lactoferrin in 0.05 M Tris-HCl buffer, pH 7.6, Cu(II)/protein molar ratio = 2:1; (d) Cu(II)–lactoferrin in 0.05 M Tris-HCl buffer titrated to pH 3.9 with 1 M HCl. Protein concentrations are all 4×10^{-4} M.

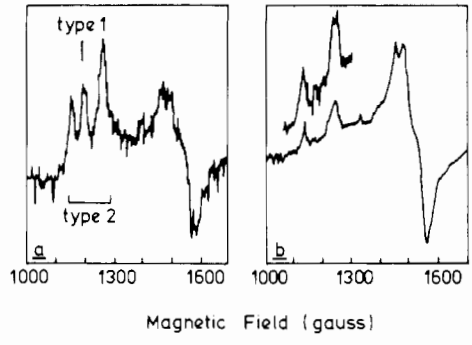

FIGURE 7: Frozen solution X-band EPR spectra for Cr(III)–lactoferrin: (a) specifically bound Cr(III)–lactoferrin; (b) Fe(III) addition to Cr(III)–lactoferrin, Fe–NTA/protein molar ratio = 2:1. Protein concentration is 4×10^{-4} M in 0.05 M Tris-HCl buffer, pH 7.6.

rameters: g_z, 2.353, A_z, 151 G, and A_z^N, 10 G; g_x, 2.088, A_x, 20 G, and A_x^N, 12 G; g_y, 2.006, A_y, 10 G, and A_y^N, 12 G (W. C. Tennant, personal communication). However, it is evident that the low-field hyperfine line, in which the nitrogen superhyperfine structure is best displayed, is slightly asymmetric. This asymmetry is identical with that observed for Cu(II)–transferrin (Zweier & Aisen, 1977), where evidence has been presented to support the suggestion that it is a result of the two metal binding sites being spectroscopically inequivalent.

More convincing evidence for spectroscopically distinguishable sites comes from the spectrum of the Cr(III)–lactoferrin complex prepared by the addition of Cr(III)–NTA to the apoprotein. The spectrum (Figure 7a) is essentially the same as that of Cr(III)–transferrin, which can be approximated by using different Hamiltonians with rhombic symmetry (Aisen et al., 1969). The resulting assignments of the type 1 and type 2 resonances for Cr(III)–lactoferrin are shown in Figure 7 and Table V. Support for these assignments comes from reconstitution experiments with chromium(III) chloride in the absence of excess bicarbonate. The type 1 signal is selectively observed when the reconstitution is performed at pH 4.8, while the type 2 signal is selectively observed when the reconstitution is performed at pH 7.6. Site selectivities were also observed with Cr(III)–transferrin, but the pH effects on the two sites were reversed with the Cr(III) ion binding to the type 2 site at lower pH (Harris, 1977).

A further difference between the two sites in Cr(III)–lactoferrin is that addition of Fe(III) in a 1:1 molar ratio to the protein will displace Cr(III) bound to the type 1 site and cause a rapid disappearance of that signal. The type 2 signal remains intact, and resonances characteristic of Fe(III)–lactoferrin appear (Figure 7b). This is in agreement with the results of the iron uptake studies (Figure 1b) which showed that only one chromium is readily displaced by Fe(III) in a 2:1 molar ratio to the protein. The addition of Fe(III) even in excess of the 2:1 molar ratio has no effect on the intensity of the type 2 signal and, hence, the Cr(III) bound to the type 2 site.

The distinctive EPR spectrum of Cu(II)–lactoferrin (Figure 6a) can be used as an indication of the nature of Cu(II) binding to lactoferrins. The addition of Cu(II) in a 2:1 molar ratio to Cr(III)–lactoferrin results in the appearance of Cu(II) resonances and the disappearance of the type 1 Cr(III)–lactoferrin signal. The addition of Cu(II) to Fe(III)–lactoferrin results in the appearance of both specifically bound and nonspecifically bound Cu(II) resonances (Figure 6b and Table I), indicating that only part of the Fe(III) is readily displaced by Cu(II). In contrast, no resonances characteristic of specifically bound Cu(II) appear when Cu(II) is added to Mn(III)–lactoferrin, and there is no change in the Mn(III)–lactoferrin charge-transfer band at 435 nm. Thus, none of the Mn(III) is displaced by Cu(II), and the new Cu(II) resonances which do appear in the EPR spectrum (Figure 6c and Table I) must be due to binding of Cu(II) at a new site in the protein. The addition of more than 2 molar equiv of Cu(II) causes no further increase in the unusual Cu(II) EPR resonances, suggesting that the number of new Cu(II) sites is limited. The EPR spectral properties of these Cu(II) sites in Mn(III)–lactoferrin are consistent with the presence of at least three nitrogen donor groups per site (Peisach & Blumberg, 1974; Sakaguchi & Addison, 1979).

EPR spectra also show that Cu(II)–lactoferrin differs from Cu(II)–transferrin in its response to pH. Treatment of Cu(II)–lactoferrin with aqueous ammonia (pH >10) results in the reversible formation of a species with an EPR spectrum identical with that of Cu(II)–tetraammine (Table I). Similarly, Cu(II)–transferrin (Zweier & Aisen, 1977) and Cu(II) added to Fe(III)–lactoferrin under pH >10 conditions exhibit the same Cu(II)–tetraammine EPR spectrum. In the pH range 4–10, Cu(II)–lactoferrin exhibits the EPR spectrum characteristic of specifically bound Cu(II), whereas Cu(II)–transferrin shows different EPR spectra below pH 6, between pH 7 and pH 8.4, and at pH 9.4 (Aasa & Aisen, 1968; Zweier & Aisen, 1977). Furthermore, in contrast to the behavior of Cu(II)–lactoferrin, at pH 9.4 transferrin containing only one copper is colorless and shows an EPR spectrum which is consistent with copper coordination by three

to four nitrogens, but in the presence of excess copper, a biuret-type EPR spectrum is observed (Zweier & Aisen, 1977). Thus, transferrin appears to have a greater variety of Cu(II) binding sites than does lactoferrin.

The EPR spectrum of bicarbonate-free Cu(II)–lactoferrin at pH 7.4 (Table I) is similar to that of Cu(II)–lactoferrin at low pH (Figure 6d). The broad features distinguish it from the spectrum for Cu(II) binding in the presence of bicarbonate (Figure 6a). The EPR parameters ($g_{\parallel}$ = 2.372, $A_{\parallel}$ = 145 G, and $g_{\perp}$ = 2.076) point to oxygen donor ligands binding to the Cu(II) ion (Peisach & Blumberg, 1974; Sakaguchi & Addison, 1979), and the lack of the visible Cu(II)–phenolate charge-transfer band would seem to exclude tyrosines as ligands. Thus, the most likely candidates for Cu(II) binding in the absence of bicarbonate are protein carboxylate residues, and the normal coordination of Cu(II) to tyrosinate does not occur without bicarbonate. A previously reported spectrum (Aisen & Leibman, 1972) for bicarbonate-free Cu(II)–lactoferrin had somewhat different parameters ($g_{\parallel} \simeq 2.25$, $A_{\parallel} \simeq 200$ G, and $g_{\perp} \simeq 2.06$), which could be due to the fact that the apoprotein was treated with citric acid in that study.

Discussion

Human lactoferrin, while clearly similar in many respects to the related serum protein, transferrin, also exhibits some intriguing differences. The Fe(III) uptake studies indicate that lactoferrin's greater affinity for iron is a kinetic as well as a thermodynamic effect. The greater stability of the iron(III)–lactoferrin complex has also been demonstrated (Teuwissen et al., 1974) by denaturation experiments, since the concentration of urea which caused 50% denaturation at pH 7.4 is 8 M for Fe(III)–lactoferrin but only 5.5 M for Fe(III)–transferrin.

The complete amino acid sequences for lactoferrin and transferrin are not available, but results for the N-terminal end show ~40% homology between the two proteins (Jollès et al., 1976). The results from the fluorescence measurements, which explore regions around the binding sites, show that this homology must extend to certain aspects of the proteins' three-dimensional structures. From the similarities of the fluorescence titration curves for the interaction of Fe(III)–NTA with the apoproteins, it is apparent that the spatial relationship of the critical quenched tryptophan residues to the iron binding sites in both lactoferrin and transferrin must be almost identical.

Resonance Raman Spectra. The resonance Raman spectral properties are also nearly identical with those of the other metal–transferrins. The appearance of resonance-enhanced tyrosinate vibrational modes in the Raman spectra of all of the metal-substituted lactoferrins and transferrins upon excitation close to the visible absorption maxima means that these absorption bands are primarily due to charge-transfer transitions between tyrosinate and metal. The fact that the lactoferrin tyrosinate vibrations (particularly at the peak at ~1270 cm^{-1}) are similarly affected by changing the metal ion indicates that the tyrosinate coordination of metals is the same in lactoferrin as in ovotransferrin and serum transferrin.

Two non-heme-iron dioxygenases, protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenase, have recently been found to exhibit a similar set of four resonance-enhanced tyrosinate ring modes at ca. 1605, 1505, 1275, and 1175 cm^{-1} (Tatsuno et al., 1978; Keyes et al., 1978; Que & Heistand, 1979). This has led us to propose the existence of an entire class of iron–tyrosinate proteins in which tyrosinate coordination dominates the visible absorption and resonance Raman spectra (Keyes et al., 1978). Comparison of spectral properties with those of model iron compounds for both the transferrins and dioxygenases suggests the presence of at least two tyrosinate groups as iron ligands (Gaber et al., 1974; Felton et al., 1978). The remaining ligand sites for octahedral iron coordination have been assigned to histidines and bicarbonate in the case of transferrins (Aisen, 1973; Feeney & Komatsu, 1966) but are unidentified in the dioxygenases except for an available site for substrate binding (Felton et al., 1978; Que & Heistand, 1979). The lack of contribution of histidine, bicarbonate, or other iron ligands to the ~465-nm absorption band has prevented these ligands from being identified in the resonance Raman spectra. Enzyme–substrate complexes of the iron dioxygenases have an additional band at ~650 nm which has substrate → Fe(III) charge-transfer character and results in the appearance of resonance-enhanced substrate vibrational modes (Felton et al., 1978; Que & Heistand, 1979).

Of the four characteristic tyrosinate vibrational frequencies, the one at ~1270 cm^{-1} appears to be the most sensitive to metal substitution and active-site structure. In the iron–tyrosinate proteins, it varies from 1293 cm^{-1} in catechol 1,2-dioxygenase (Que & Heistand, 1979) and uteroferrin (Gaber et al., 1979) to 1285–1270 cm^{-1} in the transferrins and 1265 cm^{-1} in protocatechuate 3,4-dioxygenase (Tatsuno et al., 1978; Keyes et al., 1978), while substitution of Co(III) or Mn(III) for Fe(III) or Cu(II) in lactoferrin and in the transferrins results in an ~20-cm^{-1} decrease. It is reasonable that this frequency be more responsive to changes in the metal ion environment since it contains a substantial contribution from CO stretching motion whereas the other tyrosinate vibrational modes involve primarily phenyl ring deformations (Tomimatsu et al., 1976). However, the observed decrease in the CO stretching frequency cannot be explained merely on the basis of increased metal–oxygen bond strengths. In the case of the metal-substituted transferrins, there is no correlation between the CO stretching frequency and the electronegativity of the metal (Tomimatsu et al., 1976), and, in the case of the iron–transferrins, there is no correlation between the CO stretching frequencies and the stability constants for iron binding. Human lactoferrin has been shown to bind iron 60 times more strongly than ovotransferrin (Aisen & Leibman, 1968; Aisen & Leibman, 1972), yet its CO vibration at 1272 cm^{-1} is actually higher in energy than the corresponding 1270-cm^{-1} peak in ovotransferrin (Tomimatsu et al., 1976). Thus, the variability in the frequency of the ~1280-cm^{-1} vibration is most likely due to small changes in the geometry of the iron–tyrosinate moiety. A similar conclusion was drawn for the observed spectral changes in the iron–tyrosinate chromophore of protocatechuate 3,4-dioxygenase following its reaction with substrate and oxygen (Keyes et al., 1979).

EPR Spectra. As is the case for electronic and resonance Raman spectra, the specific metal binding sites of lactoferrin and transferrin exhibit parallel EPR behavior with Fe(III), Cu(II), and Cr(III) substituents. The EPR signals from Cr(III)–lactoferrin are consistent with the presence of a type 1 and a type 2 metal site, as in Cr(III)–transferrin (Aisen et al., 1969). However, the effect of pH on the preferential binding of Cr(III) to the type 1 or type 2 site is opposite in the two proteins.

The finding of an additional set of metal binding sites from the EPR spectra of Cu(II)-treated Mn(III)–lactoferrin offers another example of probable differences between transferrin and lactoferrin. Lactoferrin is believed to undergo considerable structural alteration upon Fe(III) binding (Querinjean et al., 1971), with the structural change being more pronounced than in Fe(III) binding to transferrin (Teuwissen et al., 1974). If

Mn(III) binding induces a somewhat different conformational change than Fe(III) in lactoferrin, then this could explain the exposure of a new site on the protein for binding Cu(II) ions. Although Mn(III) and Fe(III) probably bind to the same sites in lactoferrin, they might differ enough in coordination geometry to account for the production of different protein conformations. Whereas high-spin Fe(III) complexes tend to adopt regular octahedral structures, high-spin Mn(III) is subject to a considerable Jahn–Teller distortion which results in increased axial bond lengths. This is borne out by X-ray crystallographic structures of relevant Mn(III) complexes in which the axial Mn–N and Mn–O distances are about 0.2 Å longer than the equatorial distances (Figgis et al., 1978; Stutts et al., 1979).

The current spectroscopic studies on metal-substituted lactoferrins indicate that the specific metal binding sites in lactoferrin must be very similar to those in serum transferrin. The factors responsible for the greater stability and rate of Fe(III) binding in lactoferrin must be due to subtle differences in protein conformation.

Acknowledgments

We thank Dr. W. C. Tennant (Chemistry Division, D.S.I.R., Petone) for recording some of the EPR spectra and for many useful discussions, Dr. K. N. Mickleson (Middlemore Hospital, Aukland), the Palmerston North Hospital Neonatal Unit for a supply of human colostrum, and Dr. W. E. Keyes for assistance with the resonance Raman experiments.

References

Aasa, R., & Aisen, P. (1968) *J. Biol. Chem. 243*, 2399.

Aasa, R., Malmström, B. G., Saltman, P., & Vänngård, T. (1963) *Biochim. Biophys. Acta 75*, 203.

Ainscough, E. W., Brodie, A. M., & Plowman, J. E. (1979) *Inorg. Chim. Acta 33*, 149.

Ainscough, E. W., Brodie, A. M., & Plowman, J. E. (1980) *Am. J. Clin. Nutr.* (in press).

Aisen, P. (1973) *Inorg. Biochem. 1*, Chapter 9.

Aisen, P., & Leibman, A. (1968) *Biochem. Biophys. Res. Commun. 30*, 407.

Aisen, P., & Leibman, A. (1972) *Biochim. Biophys. Acta 257*, 314.

Aisen, P., Aasa, R., Malmström, B. G., & Vänngård, T. (1967) *J. Biol. Chem. 242*, 2484.

Aisen, P., Aasa, R., & Redfield, A. G. (1969) *J. Biol. Chem. 244*, 4628.

Aisen, P., Pinkowitz, R. A., & Leibman, A. (1973) *Ann. N.Y. Acad. Sci. 222*, 337.

Bates, G. W., Billups, C., & Saltman, P. (1967) *J. Biol. Chem. 242*, 2810, 2816.

Bezkorovainy, A. (1977) *J. Dairy Sci. 60*, 1023.

Bullen, J. J., Rogers, M. J., & Griffiths, E. (1974) in *Microbial Iron Metabolism* (Neilands, J. B., Ed.) Chapter 20, Academic Press, New York.

Carey, P., & Young, N. M. (1974) *Can. J. Biochem. 52*, 273.

Feeney, R. E., & Komatsu, S. S. (1966) *Struct. Bonding (Berlin) 1*, 149.

Felton, R. H., Cheung, L. D., Phillips, R. S., & May, S. W. (1978) *Biochem. Biophys. Res. Commun. 85*, 844.

Figgis, B. N., Raston, C. L., Sharma, R. P., & White, A. H. (1978) *Aust. J. Chem. 31*, 2545.

Frushour, B. G., & Koenig, J. L. (1975) *Adv. Infrared Raman Spectrosc. 1*, 35.

Gaber, B. P., Miskowski, V. M., & Spiro, T. G. (1974) *J. Am. Chem. Soc. 96*, 6868.

Gaber, B. P., Sheridan, J. P., Bazer, F. W., & Roberts, R. M. (1979) *J. Biol. Chem. 254*, 8340.

Groves, M. C. (1960) *J. Am. Chem. Soc. 82*, 3345.

Harris, D. C. (1977) *Biochemistry 16*, 560.

Johansson, B. G. (1960) *Acta Chem. Scand. 14*, 510.

Jollès, J., Mazurier, J., Boutigue, H. H., Spik, G., Montrieul, J., & Jollès, P. (1976) *FEBS Lett. 69*, 27.

Keyes, W. E., Loehr, T. M., & Taylor, M. L. (1978) *Biochem. Biophys. Res. Commun. 83*, 941.

Keyes, W. E., Loehr, T. M., Taylor, M. L., & Loehr, J. S. (1979) *Biochem. Biophys. Res. Commun. 89*, 420.

Krysteva, M. A., Mazurier, J., Spik, G., & Montrieul, J. (1975) *FEBS Lett. 56*, 337.

Lehrer, S. S. (1969) *J. Biol. Chem. 244*, 3613.

Loehr, T. M., Keyes, W. E., & Pincus, P. A. (1979) *Anal. Biochem. 96*, 456.

Masson, P. L., & Heremans, J. F. (1968) *Eur. J. Biochem. 6*, 579.

Mazurier, J., Lhoste, J. M., Spik, G., & Montrieul, J. (1977) *FEBS Lett. 81*, 371.

McMillan, J. A., Landaw, S. A., & Oski, F. A. (1976) *Pediatrics 58*, 686.

Morgan, E. H. (1974) in *Iron in Biochemistry and Medicine* (Jacobs, A., & Worwood, M., Eds.) Chapter 2, p 29, Academic Press, New York.

Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691.

Pinkowitz, R. A., & Aisen, P. (1972) *J. Biol. Chem. 247*, 7830.

Price, E. M., & Gibson, J. F. (1972) *Biochem. Biophys. Res. Commun. 46*, 646.

Que, L., Jr., & Heistand, R. H., II (1979) *J. Am. Chem. Soc. 101*, 2219.

Querinjean, P., Masson, P. L., & Heremans, J. F. (1971) *Eur. J. Biochem. 20*, 420.

Sakaguchi, U., & Addison, A. W. (1979) *J. Chem. Soc., Dalton Trans.* 600.

Stutts, B. R., Marianelli, R. S., & Day, V. W. (1979) *Inorg. Chem. 18*, 1853.

Tatsuno, Y., Saeki, Y., Iwaki, M., Yagi, T., Nozaki, M., Kitagawa, T., & Otsuka, S. (1978) *J. Am. Chem. Soc. 100*, 4614.

Teuwissen, B., Masson, P. L., Osinski, P. A., & Heremans, J. F. (1972) *Eur. J. Biochem. 31*, 239.

Teuwissen, B., Masson, P. L., Osinski, P. A., & Heremans, J. F. (1973) *Eur. J. Biochem. 35*, 366.

Teuwissen, B., Schanck, K., Masson, P. L., Osinski, P. A., & Heremans, J. F. (1974) *Eur. J. Biochem. 42*, 411.

Tomimatsu, Y., Kint, S., & Scherer, J. R. (1976) *Biochemistry 15*, 4918.

Zweier, J. L., & Aisen, P. (1977) *J. Biol. Chem. 252*, 6090.