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A Spectroscopic Study of Bovine Lactoferrin†

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ABSTRACT: Bovine lactoferrin was examined spectroscopically in order to determine the constituents of the iron-binding site and the relationship of iron binding to conformation. In aqueous solutions of metal-free lactoferrin, one or two more tryptophan residues were accessible to the perturbants sucrose, ethylene glycol, glycerol, and dimethyl sulfoxide than in similar solutions of iron(III)-lactoferrin. At pH 11, six more tyrosine residues were ionized in lactoferrin than in iron(III)-lactoferrin. Difference absorption spectra indicated a shift of tyrosine and tryptophan residues from a nonpolar to a more polar environment in the presence of guanidine · HCl and aqueous acid. The shift was seen with both forms of the protein, but higher concentrations of denaturants were required when iron was present. The circular dichroism (CD) spectrum of iron(III)-lactoferrin was compared with that of the metal-free protein. Small changes in the ellipticities of the ultraviolet bands near 295, 291, 275, and 254 nm were seen and additional bands appeared at 455 and 330–310 nm when iron was bound. Below 240 nm, the CD spectra of the two forms were identical. The results suggest that tryptophan residues may not be bound directly to iron, but tyrosine residues probably are, and iron binding stabilizes the spectroscopically observed native conformation of lactoferrin.

Lactoferrin is a nonheme, iron-binding protein originally isolated from milk (Groves, 1960), but since identified in a variety of mammalian secretions (Masson *et al.*, 1966). It is similar to the serum protein, transferrin, and the egg-white protein, ovotransferrin, which is also called conalbumin. Each of these proteins consists of a single polypeptide chain which contains two specific metal binding sites.

The iron-binding characteristics of human transferrin and chicken ovotransferrin have been extensively studied. Feeney and Komatsu (1966) reviewed the early studies; more recent reports include those of Aisen *et al.* (1967), Lehrer (1969), Tan and Woodworth (1969), and Nagy and Lehrer (1972). Teuwissen *et al.* (1972) studied the role of tyrosine residues in iron binding by human lactoferrin. Aisen and Leibman (1972) compared the absorption and electron paramagnetic resonance spectra of human and bovine lactoferrin with those of human transferrin.

In each of these proteins, iron binding appears to occur at chemically equivalent, noninteracting sites (Aisen and Leibman, 1972), and includes the binding of one small anion, normally bicarbonate, with each atom of iron. This iron–bicarbonate–protein complex develops a salmon color with an absorption maximum between 470 and 460 nm.

The presence of two or three residues of tyrosine per iron-binding site in each of the transferrin-like proteins has been indicated by electrometric and spectrophotometric titrations (Tan and Woodworth, 1969; Teuwissen *et al.*, 1972; Wishnia *et al.*, 1961). Chemical modification and spectral studies including electron paramagnetic resonance, fluorescence, and circular dichroism (CD) have implicated histidine and tryptophan as well as tyrosine in the binding sites (Aasa *et al.*, 1963; Lehrer, 1969; Phillips and Azari, 1972; Tan, 1971).

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TABLE I: Summary of Solvent Perturbation of Lactoferrin.

Perturbant (%)	Apparent Number of Exposed Tryptophan Residues			
	Lactoferrin			
	8 M Urea Mercaptoacetic Acid Treated	8 M Urea	Aqueous	Iron(III)–Lactoferrin Aqueous
Sucrose (20)	14.5 ± 0.8	11.5 ± 1.0	7.1 ± 0.8	5.4 ± 0.6
Ethylene glycol (20)	11.2 ± 1.1	9.3 ± 0.6	5.6 ± 0.3	5.8 ± 0.5
Glycerol (20)	14.3 ± 1.6	10.1 ± 1.2	6.2 ± 0.4	4.5 ± 0.3
Dimethyl sulfoxide (20)	11.7 ± 0.3	9.6 ± 0.5	3.8 ± 0.1	3.0 ± 0.6

[a] Protein concentrations were 1.5–3.3 × 10^{-5} M.

Lactoferrin differs from transferrin and ovotransferrin in the conditions needed for the removal of iron from the protein. Metal-free transferrin and ovotransferrin can be obtained by dialysis against a competitive chelator such as citrate or ethylenediaminetetraacetic acid at pH 4. Removal of iron from lactoferrin can be accomplished only at pH 2, suggesting a difference either in the composition of the binding site, or in the overall conformation of the molecule. Therefore, we have undertaken this study of bovine lactoferrin to obtain further information about the binding of iron to this protein, the composition of the binding sites, and any conformational changes which might accompany iron binding.

Materials and Methods

Ultra Pure urea and Gdn · HCl[1] from Schwarz/Mann Biochemicals[2] were used without further purification. Mercaptoacetic acid was freshly distilled immediately before use. The iron-binding protein was purified as described by Parry and Brown (1974) from fresh milk, colostrum, and the secretion of the involuted mammary gland. Each of the three preparations appeared homogeneous on polyacrylamide gel electrophoresis at pH 3 and were indistinguishable under our experimental conditions. Iron(III)-lactoferrin[3] (1.95 ± 0.25 mol of Fe/86,000 g of protein) was prepared by adding a slight excess of 1:1 $FeCl_3$–nitrilotriacetic acid to the protein solution and removing the excess by passage through Sephadex G-25. Iron was determined colorimetrically as the 1,10-phenanthroline complex. Protein of acceptable purity showed absorbance ratios of A_{280}/A_{465} between 27 and 28 and A_{410}/A_{465} between 0.80 and 0.85. Iron was removed from the protein by the method of Johanson (1960), leaving not more than 0.1 mol of iron/mol of lactoferrin.

Concentrations of protein solutions were estimated spectrophotometrically using the absorption at 280 nm. The molar absorptivities ϵ_{280} 1.35 × 10^5 M^{-1} cm^{-1} for iron(III)-lactoferrin and ϵ_{280} 1.09 × 10^5 M^{-1} cm^{-1} for the metal-free protein were determined on a dry weight basis using a molecular weight of 86,000 (Groves, 1960; Parry and Brown, 1974). The spectrophotometric method of Edelhoch (1967) was used to determine the numbers of tryosine and tryptophan residues.

Absorption spectra were obtained with a Cary Model 14 recording spectrophotometer, and single wavelength absorbances with a Zeiss PMQ-2. Beer's law was shown to hold over the range of concentrations used. All solutions, except where specifically noted, contained 0.1 M KCl in addition to the specified buffers or perturbants. Neutral, aqueous solutions were either unbuffered at pH 6.5–7.5, or buffered at pH 7.0 with Hepes. No differences were noted due to the presence or absence of buffer.

Solvent perturbation difference spectra were obtained using a minimum of two different concentrations of iron(III)–lactoferrin in aqueous solution, lactoferrin in aqueous solution and in 8 M urea, and 8 M urea solution of lactoferrin which had been disulfide cleaved with mercaptoacetic acid by the method of Herskovits and Laskowski (1962). Concentrations were selected so that A_{280} was between 1.0 and 2.2. The perturbants used were sucrose, ethylene glycol, glycerol, and dimethyl sulfoxide in aqueous and 8 M urea solutions with a final perturbant concentration of 20%. The data were obtained and analyzed using the method of Herskovits and Sorensen (1968a,b).

The circular dichroism spectra were recorded using a Jasco Model ORD/UV/CD-5 or a Cary 60 recording spectropolarimeter with a Model 6002 circular dichroism attachment. Ellipticities in the 600–300-nm region are expressed as the mean molar ellipticity $[\theta]$ (deg cm^2)/dmol of iron bound, and below 300 nm in terms of protein concentration as the mean molar ellipticity $[\theta]_{MRW}$, using 113 as the mean residue weight.

Results

For each 86,000 g of lactoferrin, 19.8 ± 0.1 tyrosine and 12.8 ± 0.1 tryptophan residues were found by the spectrophotometric method of Edelhoch (1967).

Solvent Perturbation. Table I summarizes the results of solvent perturbation of lactoferrin in terms of apparent tryptophan exposure. When the protein was dissolved in 8 M urea and reduced with mercaptoacetic acid, there was 100% exposure of tryptophan residues (12.6 ± 0.5) to the perturbants. In 8 M urea solution where disulfide bonds were still intact, the exposure was about 80%; 45% of the total tryptophan exposure was obtained in aqueous solutions of lactoferrin, and 40% in aqueous solutions of iron(III)-lactoferrin. Figure 1 presents the difference spectrum for an

[1] Abbreviations used are: Gdn · HCl, guanidine hydrochloride; Hepes, buffer consists of 5 × 10^{-3} M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 2 × 10^{-3} M NaOH at pH 7.0.

[2] Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

[3] The designation iron(III)-lactoferrin refers to the iron-saturated complex of lactoferrin containing 2 mol of iron(III)/mol of protein. Lactoferrin refers to the metal-free form of the protein.

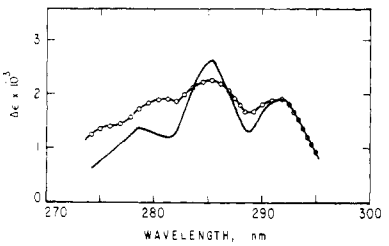

FIGURE 1: The effect of 20% ethylene glycol on the absorption spectrum of lactoferrin in 0.1 M KCl (pH 7): (- O - O -) experimental curve; and (—) the computer generated "best fit" representing perturbation of 6 tryptophan and 18 tyrosine residues.

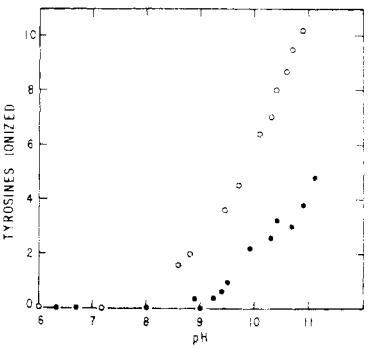

FIGURE 2: Ionization of tyrosine residues in (O) lactoferrin and (●) iron(III)-lactoferrin. The number of residues is calculated using $\Delta\epsilon_{295nm} = 2.33 \times 10^3$ cm^{-1} per ionized tyrosine.

aqueous solution of lactoferrin perturbed by 20% ethylene glycol. Fitting the experimental curve with the tryptophyl and tyrosyl model compound data of Herskovits and Sorensen (1968a,b) indicated approximately 18 tyrosine and 6 tryptophan residues exposed to the solvent. The calculated curve (solid line, Figure 1) was generated according to

$$\Delta\epsilon_{\lambda(\text{protein})} = a\Delta\epsilon_{\lambda(\text{Trp})} + b\Delta\epsilon_{\lambda(\text{Tyr})}$$

with $a = 6$, $b = 18$. The general shape of the curve and the degree of correspondence with a calculated curve was similar when dimethyl sulfoxide, sucrose, or glycerol was used as the perturbant. When the protein was dissolved in 8 M urea or urea-mercaptoacetic acid solution and exposed to each of the above perturbants, the curves were similar in shape to those obtained in aqueous solution, but the degree of exposure was greater. The apparent number of exposed tryptophan residues was consistently higher when the long range perturbants sucrose (mean diameter, 9.4 Å) and glycerol (5.2 Å) were used than when the shorter range ethylene glycol (4.4 Å) and dimethyl sulfoxide (4.0 Å) were used. The differences between aqueous solutions of lactoferrin and iron(III)-lactoferrin are most easily seen with the longer range perturbants.

The portion of the difference spectrum above 290 nm is due almost entirely to perturbed tryptophan residues. In this region the experimental and calculated curves coincide very well. At lower wavelengths, where both tyrosine and tryptophan make significant contributions to the spectrum, the agreement between calculated and experimental curves is not good. The high tyrosine to tryptophan ratio makes it difficult to clearly separate the effects of the two types of residues, therefore, we have not tried to quantitate the numbers of perturbed tyrosine residues in these experiments.

Tyrosine Ionization. The difference spectrum of metal-free lactoferrin, pH 12.4 *vs.* pH 7.0, corresponded to 20.6 ± 1.5 ionizable tyrosine residues. The ultraviolet difference spectrum of lactoferrin at alkaline *vs.* neutral pH has maxima at 245 and 295 nm and a negative absorption in the 275-270-nm region, resembling the difference spectrum of acetyltyrosine (Donovan, 1964). A neutral reference solution was used with both lactoferrin and iron(III)-lactoferrin so that the curves obtained would be comparable, and to avoid spectral complications due to loss of iron binding at low pH. Spectra at pH 12.4 using the neutral reference solution gave an absorption maximum at 295 nm as for acetyltyrosine, and accounted for ionization of all 20 tyrosines in metal-free lactoferrin. When the pH of the solution is 11 or lower, A_{245}/A_{295} is about 4.7 as would be expected for tyrosine ionization; when the pH is higher, the ratio becomes larger indicating other contributions to the spectrum. These could be perturbation of some of the disulfide bonds, or possibly a slight turbidity which would exhibit a greater effect at 245 nm than at 295 nm.

The titration of lactoferrin (Figure 2) represents a continuing ionization of tyrosines similar in appearance to the curves for human lactoferrin (Teuwissen *et al.*, 1972). The apparent number of ionized tyrosine residues was calculated on the basis of $\Delta\epsilon_{295} = 2.33 \times 10^3$ M^{-1} cm^{-1} (Beaven and Holiday, 1952). In metal-free lactoferrin, two tyrosines are ionized at pH 8.6 and six more by pH 10.5. In iron(III)-lactoferrin, there is no apparent tyrosine ionization at pH 8.6; at pH 10.5 the difference spectrum will account for the ionization of three tyrosines. At pH 11, the apparent numbers of ionized tyrosines are 10.4 in lactoferrin and 4.4 in iron(III)-lactoferrin. At higher pH's the absorptivity of iron(III)-lactoferrin solutions increased with time and determinations were complicated by the release of iron and the formation of Fe_2O_3.

Effects of Guanidine, Urea, and Acid on the Absorption Spectra. In the presence of Gdn · HCl, urea, or acid, the absorption maximum of lactoferrin was shifted from 280 to 277-278 nm accompanied by a general decrease in the absorptivity of the 330-250-nm region. Difference spectra showed minima at 292 and 286 nm characteristic of the transfer of tryptophan and tyrosine residues from a hydrophobic portion of the molecule into the aqueous phase. The difference spectrum of the iron(III)-lactoferrin also had a broad band in the 320-300-nm region. Acid difference spectra for the two forms are shown in Figure 3. At pH 4.5, there was no acid difference spectrum for lactoferrin; at pH 3, the difference spectrum of lactoferrin had the same general characteristics as the pH 2 spectrum and was about two-thirds as intense. The acid difference spectrum of iron(III)-lactoferrin developed slowly as the debinding pH was approached. The denaturation effects of urea and Gdn · HCl on the spectrum of lactoferrin were immediate, however, with iron(III)-lactoferrin these effects were seen to increase over a several hour period which may correspond to a slow release of iron. The absorptivity changes for iron(III)-lactoferrin were larger than those of the metal-free protein because of the greater absorptivity of the normal form of the iron-protein complex. The maximum spectral change was achieved with a 2 M Gdn · HCl solution in the case of lactoferrin, while a 6 M solution was required for iron(III)-lactoferrin to reach the same observed state. The native lactoferrin spectrum was recovered when the urea or Gdn · HCl was removed, or the pH adjusted back to neutrality. Recovery of the iron(III)-lactoferrin spectrum was slower and never complete, probably because of the formation of Fe_2O_3 under these conditions.

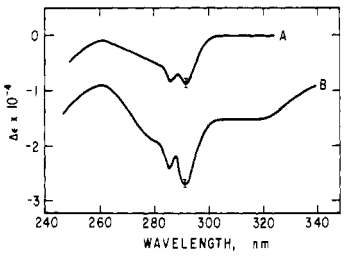

FIGURE 3: The effect of acid on the absorption spectra of lactoferrin (A) and iron(III)-lactoferrin (B) in 0.1 M KCl. In each case the sample solution is at pH 2 and the reference at pH 6.5-7.5. Protein concentrations are 10^{-5}-10^{-6}. $\Delta\epsilon$ is per mol of protein. The error bar represents the maximum variation at a specific wavelength in this case for four experiments.

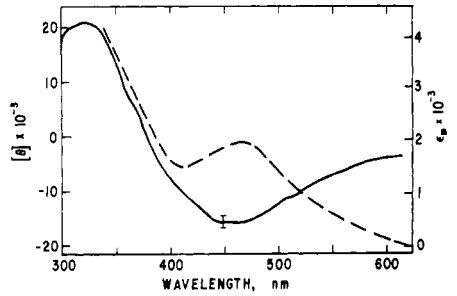

FIGURE 4: Visible CD(—) and absorption (- - -) spectra of iron(III)-lactoferrin in aqueous solution. The CD spectrum is the average of six experiments; two each in water, 0.1 M KCl, and Hepes all at pH 6.5-7.5, the units of $[\theta]$ and ϵ are deg cm^2 dmol^{-1} Fe and cm^{-1} M^{-1} Fe, respectively. Corresponding values in terms of protein concentration would be twice as great.

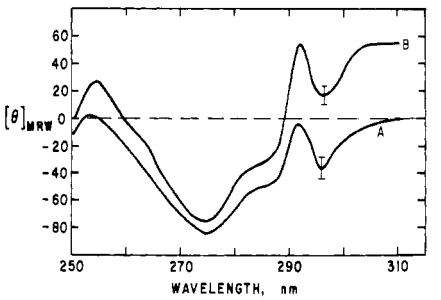

FIGURE 5: Near-ultraviolet CD spectra of lactoferrin (A) and iron-(III)-lactoferrin (B) in neutral, aqueous solution. $[\theta]_{MRW}$ in units of deg cm^2 dmol^{-1} mean residue concentration would correspond to $[\theta] \cdot MRW \cdot M^{-1}$ in terms of protein concentration where M is the gram molecular weight, and MRW the mean residue weight, 113 calculated from the amino acid analysis of Gordon *et al.* (1963). Each curve is an average of eight to ten experiments with varying protein concentrations and buffer conditions.

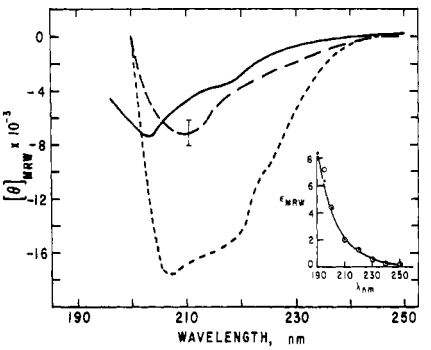

FIGURE 6: Far-ultraviolet CD spectra of lactoferrin. The pH 2 spectrum (—) is for lactoferrin in an aqueous 0.1 M KCl-HCl mixture at pH 2.0, an average of six experiments. The trifluoroethanol spectrum (- - - -) is for lactoferrin in a solution which is 90% trifluoroethanol, an average of two experiments. Native (— —) is either lactoferrin or iron-(III)-lactoferrin in neutral, aqueous solution, an average of 10 experiments each. Inset shows the corresponding absorption spectrum: (○) lactoferrin, and (●) iron(III)-lactoferrin.

Circular Dichroism Spectra. The visible absorption spectrum of iron(III)-lactoferrin has been reported (Aisen and Leibman, 1972), and is presented in Figure 4 for comparison with the visible circular dichroism (CD) spectrum. The major feature of the CD spectrum between 600 and 350 nm (Figure 4) is a broad negative band centered at 455 nm ($[\theta]_{455} = -16 \pm 3 \times 10^3$) which corresponds to the absorption maximum near 460 nm. The near-ultraviolet portion of the CD spectrum is characterized by a positive band or possibly an unresolved doublet in the 330-310-nm region. ($[\theta]_{320} = 21 \pm 3 \times 10^3$, corresponding to the similarly located band in the acid difference spectrum (Figure 3).)

The CD spectrum of lactoferrin in the 310-250-nm region (Figure 5) consists of a trough at 295-296 nm, a peak at 291-292 nm, a poorly resolved negative band centered at 274-276 nm, and a positive band at 254 nm. Iron binding had little effect on the shape of the curve, but ellipticities for iron(III)-lactoferrin were more positive than those for lactoferrin, particularly above 290 nm where the ellipticities for iron(III)-lactoferrin were themselves positive. At 295 nm $[\theta]_{MRW}$ is 18 ± 3 for iron(III)-lactoferrin and −38 ± 5 for lactoferrin.

In the far-ultraviolet (240-190 nm) both the CD and absorption spectra of iron(III)-lactoferrin and lactoferrin in neutral aqueous solution were indistinguishable (Figure 6). The method of Chen *et al.* (1972) gave values of about 15% helix and 50% unordered structure for either form of the protein. Addition of helix-forming solvents such as acidic methanol or trifluoroethanol to lactoferrin solutions increased the helical content to about 25 or 40%, respectively, showing that the low helicity of the native protein is not due entirely to the rigidity imparted by disulfide bonds. In 6 M Gdn · HCl, 8 M urea, or aqueous acid solution (pH 2), the far-ultraviolet CD spectrum was that of a completely unordered protein. These solvents also affected the aromatic region of the CD spectrum by decreasing the magnitude and resolution of the bands. The effect of Gdn · HCl on the 300-290-nm portion of the CD spectra is illustrated by the variation of $[\theta]_{MRW}$ at 295 nm with Gdn · HCl concentration. In the absence of Gdn · HCl $[\theta]_{MRW}$ for lactoferrin was −36, in 2 M Gdn · HCl it was −11. No further change was seen with higher Gdn · HCl concentrations. The CD spectrum of native iron(III)-lactoferrin with $[\theta]_{MRW}$ at 295 nm of 18 was obtained when the Gdn · HCl concentration was 2 M or less. In 4 M Gdn · HCl $[\theta]_{MRW}$ was decreased to 12 and in 6 M Gdn · HCl to −11. Accompanying the change in ellipticity was a decrease in resolution, the final result being the same whether iron was present or not. The effect of increasing Gdn · HCl concentration on the direct ultraviolet absorption spectrum of either lactoferrin or iron(III)-lactoferrin was to shift the absorption maximum from 280 to 276 nm and to cause a slight decrease in intensity. When Gdn · HCl was removed by dialysis, the native protein spectra were recovered.

Discussion

Tyrosine and tryptophan are major contributors to the absorption spectrum of lactoferrin, and one might expect that some of the spectral differences between lactoferrin

and iron(III)-lactoferrin could be interpreted in terms of the binding of these chromophores to iron. The tyrosine ionization study would seem to lend itself to this kind of interpretation. It is tempting to say that the difference of about four ionized tyrosines between the two forms below the normal ionization point of tyrosine means that these more easily ionized tyrosines are involved in the binding. Michaud (1967) showed that four residues of ovotransferrin were distinctly not normal in that two were ionized at pH 8.5 and two more below pH 10, leading him to conclude that these tyrosines are involved in metal binding. Our data for lactoferrin would tend to support this hypothesis with respect to those tyrosines which are ionizable at pH 8.6, but the ionization of two tyrosines at pH 10 in the iron(III)-lactoferrin would indicate that while these are not normal, they are also not involved in metal binding. Also, the difference of six ionized tyrosines at pH 11, which is just below the point at which the protein unfolds and the iron is released, could be used as evidence of three tyrosines per binding site. Another possibility is that the iron stabilizes a conformation of the molecule in which certain tyrosine residues are not readily accessible to the titrant. Teuwissen *et al.* (1972) achieved similar results with human lactoferrin and concluded that a definite answer was not possible from these data. The most probable explanation for our tyrosine titration data is the combined effects of iron binding to some residues, and the stabilization by iron of a conformation in which other tyrosine residues are less subject to environmental change when the pH is increased.

The perturbation of tryptophan residues makes a stronger argument for the theory of increased conformational stability due to the binding of iron. Comparison of aqueous solutions shows that one (with short range perturbants) or two (with long range perturbants) more tryptophan residues are exposed in lactoferrin than in iron(III)-lactoferrin. Assuming the two iron binding sites have the same amino acid composition, a difference of one perturbable tryptophan per molecule would not indicate direct involvement in metal binding by that particular residue. Perturbation of one additional residue per molecule by the longer range perturbants sucrose and glycerol can be taken as further evidence of conformational stability enhanced by the presence of bound iron.

Both absorption and CD visible spectra of the iron complexes of lactoferrin, ovotransferrin, and transferrin are quite similar. All three have absorption bands with maxima at 460–470 nm and the corresponding negative CD bands at 465–455 nm (Tan, 1971; Nagy and Lehrer, 1972). The strong positive band or doublet near 320 nm in the CD spectrum of iron(III)-lactoferrin corresponds to that reported for iron(III)-ovotransferrin by Tan (1971) who was able to resolve maxima at 325 and 305 nm. Nagy and Lehrer (1972) found a less intense band at 360 nm for iron(III)-transferrin. The 320-nm band is not well defined in the direct absorption spectrum of iron(III)-lactoferrin where it is overlapped to some extent by both the 465- and the 280-nm bands. In absorption spectra of neutral, aqueous solutions of iron(III)-lactoferrin against a 6 M guanidine or aqueous pH 2 solution of the same protein, the band appears as a broad shoulder. Tan (1971) has suggested that the 320-nm CD band in iron(III)-ovotransferrin may be due to a change in the dihedral angle of one or more of the disulfide bonds when iron is bound to the protein. Beychok (1966) observed CD spectra of perturbed tryptophan residues at wavelengths as high as 320 nm. While perturbation of either tryptophan residues or of disulfide bonds is a possible explanation for this band, our preliminary results using other trivalent metals with ionic radii similar to that of iron(III) tend to indicate that the optically active 320-nm band is unique to the iron(III) complex (Parry and Brown, 1974). Since it does not appear with other metals, we speculate that this 320-nm band may simply be characteristic of an iron–oxygen bond in an octahedral configuration.

The CD spectra of lactoferrin and iron(III)-lactoferrin in the 310–250-nm region closely resemble those of ovotransferrin and its iron complex as reported by Tan (1971) and Gaffield *et al.* (1966). It was found by Tan (1971) and Tomimatsu and Vickery (1972) that copper binding to ovotransferrin had even less effect on the aromatic CD spectrum than did iron binding. The 310–250-nm portion of the CD spectrum of transferrin has more fine structure than do those of lactoferrin and ovotransferrin. The shape of this fine structure is not affected by the binding of copper or zinc to transferrin although the actual ellipticities all become more negative (Tomimatsu and Vickery, 1972; Nagy and Lehrer, 1972).

A comparison of the ultraviolet CD spectra of lactoferrin and iron(III)-lactoferrin shows that the two forms are quite similar. The shapes of the two curves are the same and except in the 300–290-nm region, the ellipticities are not very different. Analysis of the 300–250-nm portion of the CD spectrum in terms of specific residues is complicated by the lack of resolution, however, it is due mainly to the combined effects of tryptophan, tyrosine, and disulfide bonds. Tryptophan is the most likely source of fine structure in the 300–290-nm region, but caution must be used in attributing the positive ellipticities in this region to any direct iron–tryptophan interaction as they may well be due to overlap of the 320-nm band discussed earlier. In the 290–275-nm portion and around 254 nm where the spectral details are mainly attributable to tyrosine residues and disulfides, respectively, the differences are much smaller. The general impression is that the environments of the chromophores in lactoferrin and iron(III)-lactoferrin are not very different; the slight increase in resolution in the presence of iron may mean that a greater conformational stability allows less opportunity for free rotation of the individual residues.

In the far-ultraviolet region (240–190 nm) where the CD spectrum is dependent directly on the protein conformation, the two spectra are identical as are the absorption spectra at these wavelengths. The helical content, which we estimate by the method of Chen *et al.* (1972) to be about 15%, compares well with the range of 15–21% for transferrin and ovotransferrin when calculated by this method (Tomimatsu and Vickery, 1972).

Evidence that lactoferrin can be made to undergo a reversible conformational change can be seen in the effects of acid, urea, and Gdn · HCl on the absorption and CD spectra. The difference peaks at 291 and 286 nm indicate that tryptophan and tyrosine residues are being shifted from less polar to more polar environments. The decrease in magnitude and resolution of the CD spectrum between 300 and 250 nm appears to be due to a greater degree of free rotation of the chromophoric residues. These spectral effects are symptomatic of an unfolding of the molecule, and are reversed by the removal of the perturbing agent. Iron stabilizes the normal conformation as seen by the fact that higher concentrations of acid or Gdn · HCl are needed to initiate the unfolding process in iron(III)-lactoferrin than in lactoferrin. The maximum unfolding is obtained only under con-

ditions where iron is no longer bound by the protein. The far-ultraviolet CD spectra show that exposure of either lactoferrin or iron(III)–lactoferrin to 6 M Gdn · HCl or acid (pH 2) results in a completely unordered protein. Glazer and McKenzie (1963) observed a similar reversible conformation change for ovotransferrin under denaturing conditions. Further evidence of the unfolding of the protein is provided by the drop in the sedimentation coefficient from 5.3 for lactoferrin in neutral, aqueous solution to 3.4 at pH 2 (Parry and Brown, 1974), and the corresponding drop was reported for ovotransferrin by Phelps and Cann (1956).

The results of this study indicate that iron(III)–lactoferrin may not contain any tryptophan–iron bonds; it probably does contain two or three tyrosine–iron bonds per atom of iron. The spectroscopically observed native conformation of lactoferrin is stabilized by the binding of iron as indicated by the higher concentrations of denaturants needed to induce the reversible unfolding of the iron(III)–lactoferrin molecule.

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