The Metal-binding Properties of Ovotransferrin
AN INVESTIGATION OF COBALT(II) DERIVATIVES

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Cobalt(II) ovotransferrin bicarbonate and oxalate ternary complexes were prepared and investigated in the pH range 7–10.5. Cobalt(II) provides an excellent and unique spectroscopic probe to monitor subtle structural differences in solution between the two sites of ovotransferrin and to investigate the structural dependence on pH. CD spectroscopy on one side and 1H NMR spectroscopy of isotropically shifted signals on the other are extremely sensitive techniques and are particularly suited for high spin cobalt(II)-containing compounds. In the case of the oxalate derivative the metal-binding ability of the protein is different at the two binding sites and is pH dependent; the CD spectra reveal two different sites, one of which is clearly pH dependent with a pK of 9.5. On the contrary the bicarbonate analogue does not show any spectral difference between the two sites; both of them change with pH, the pK being again 9.5. 1H NMR spectra of the oxalate derivatives at pH 7–8 reveal the presence of conformers, the distribution of which depends on the H2O/D2O ratio. Such conformers are not revealed in the bicarbonate system; at pH around 10 the NMR spectra of both systems show inequivalence between the two sites and/or the presence of different conformers for each site. Such differences are discussed in terms of the possible implications in mechanism and function. The overall spectral data are consistent with the donor groups being two histidines, two tyrosines, the synergistic anion, and possibly a solvent molecule.

Ovotransferrin (conalbumin, Otf) is an iron-binding protein of the class of the transferrins. The chemistry of this class of proteins has been recently reviewed (1–3). Otf is a single chain protein with a two-domain structure (M, 79,500); each domain contains an iron-binding site respectively designated as C-terminal (A site) and N-terminal (B site). The metal-binding capacity of the protein depends on the presence of a synergistic anion. Many derivatives of Otf and transferrin (Tf) with tripotassic and bipositive metal ions have been prepared (4–16) in order to study the structural properties of the binding sites and to relate them with their function. The spectroscopic data obtained on transferrins suggest that the metal ions are six coordinated (3) with a pseudo-octahedral arrangement of the ligands which presumably are two tyrosinates (4, 7, 9, 15–18), two histidines (7, 19–21), the synergistic anion (8, 22–27), and a water or hydroxido moiety (15, 28–31). The primary structure has been recently determined for both Otf and Tf (32, 33), and the identification of the protein residues involved in iron binding is in progress (2). Particular attention has been devoted to the characterization of the structural differences between the two sites at physiological pH and at high pH (1–3, 5, 6). Differences in the metal uptake at low pH are also looked for (34, 35). Finally the presence of different conformers in equilibrium for each site has been proposed from EPR spectroscopic studies of iron transferrin (36–38), and further evidences are found from EPR on oxovanadium(IV) and copper(II) derivatives of both Tf and Otf (6, 31, 39, 40). The presence of conformers may be meaningful as far as the mechanism of metal release is concerned, as suggested by Bates from kinetic measurements (41).

To further investigate the structural and conformational features of the binding sites we have prepared both bicarbonate and oxalate derivatives of Co(II)-Ot and studied them through CD and 1H NMR spectroscopies. The systems contain the chromogenic high spin cobalt(II) ion which can be properly studied through electronic spectroscopy; the 1H NMR of such paramagnetic derivatives allows us to record the signals of protons attached to the metal ligands. This is a powerful technique to detect structural variations in solution (42). The comparison between bicarbonate and oxalate may be meaningful in terms of the induced structural changes when passing from the natural synergistic anion to oxalate. Indeed, these variations appear to have relevance with respect to the metal release mechanism since the oxalate derivative of iron transferrin has been found to donate the metal ion to reticulocytes much slower than the native protein (43).

MATERIALS AND METHODS

Chicken egg apo-ovotransferrin was purchased from Sigma and purified through repeated dialysis against 0.1 M citrate-acetate buffer at pH 4.5, then against 0.1 M perchlorate, and finally against freshly distilled water following a procedure largely used (44). All reagents were analytical grade. The protein concentration was measured through UV absorption spectroscopy by monitoring the band at 280 nm (ε = 92,300 M\(^{-1}\) cm\(^{-1}\)). Cobalt derivatives were prepared by addition of aliquots of cobalt(II) sulfate solutions to apoprotein solutions. All sample handling was performed in exclusion of air, and solutions were degassed in order to avoid CO\(_2\) contamination. Deuterated samples were obtained by adding deuterium oxide to the lyophilized apoprotein. To allow for complete deuteration of possible slow exchanging protons some samples were incubated in deuterium oxide at 37 °C up to over a week.

The UV difference spectra were performed on a Cary 17D spectrophotometer by monitoring the development of the typical tyrosinate bands at 290 and 250 nm upon titration with cobalt(II) sulfate. The protein concentration in the above experiments was about 1.5 × 10\(^{-5}\) M in 1 × 10\(^{-2}\) M Tris-HCl buffer, with synergistic anion concentration about 1 × 10\(^{-3}\) M.

The CD spectra in the visible region were performed on a Jasco J500C spectropolarimeter; the transition energies were determined...
through spectral simulation (45). CD samples were 5 × 10⁻⁴ M in protein and 5 × 10⁻³ M in Tris-HCl buffer; synergetic anion concentrations were either 3 × 10⁻³ M or 0.1 M.

The NMR spectra were performed on a CXP Bruker spectrometer equipped with a Varian DA 60 electromagnet operating at 1.4 tesla. The spectra were recorded in quadrature detection using the modified DEFT sequence (46). The T₁ values were measured through saturation recovery experiments. The samples were about 1.5 × 10⁻³ M in protein concentration.

**RESULTS**

**CD Spectra of Cobalt(II)-Otf-Bicarbonate**—The absorption spectra in the d-d region of cobalt(II)-Otf derivatives are very low in intensity (εₜₘₓ of the order of 20 M⁻¹ cm⁻¹/cobalt ion) consistent with a pseudo-octahedral geometry of the chromophore (Fig. 1A) (42). Such spectra are of little help in monitoring differences between the two sites or minor changes in the coordination polyhedron as a function of pH. The CD spectra in the visible region, on the contrary, show many intense transitions which make this technique quite suitable for the above purposes. They can also be used for determining the stoichiometry of the metal-protein derivatives at different pH values. The spectra of the cobalt-Otf-bicarbonate complex in the 400-700-nm range at different cobalt to protein ratios at pH 8.3 are reported in Fig. 1B. The spectra show an increase in intensity with increasing cobalt to protein ratio until a 1:1 stoichiometry is reached. Further additions of cobalt(II) do not alter the final spectrum. The spectrum of the 1:1 derivative is characterized by two negative bands at 450 and 490 nm and two positive bands at 560 and 590 nm.

It was earlier shown that the first cobalt(II) preferentially occupies the C-terminal site (7); therefore, it can be assumed that such a spectrum is due to cobalt(II) in the C-terminal site. At higher cobalt to protein ratios the spectrum increases in intensity without any detectable change in the shape, indicating that cobalt(II) in the N-terminal site has essentially the same CD spectrum. This is in agreement with the already reported equivalence of the ¹H NMR spectra of the mono- and dicobalt-substituted Otf-bicarbonate derivatives at the same pH (7).

By lowering the pH the intensities of the CD transitions decrease, but again no changes are detectable in the general shape of the spectrum. This probably indicates that cobalt(II) is gradually detaching from the protein; the pKₐ for such a process in the presence of 0.1 M bicarbonate was calculated to be around 7.3. The above result was confirmed through UV difference spectroscopy experiments performed in the pH region 8-6.8, at intervals of 0.2 pH unit. Again, a pKₐ value of about 7.3 was found for the release of cobalt(II), and no evidence for stepwise depopulation of the sites was detected.

Upon raising the pH of the cobalt(II)₁-derivative above 9, the general shape of the CD spectrum changes markedly, as shown in Fig. 2. From the analysis of the variation of the negative band at 450 nm with pH (Fig. 2, inset), a pKₐ value of 9.5 ± 0.1 could be estimated. The spectral variation is essentially complete at pH 10.5. If cobalt(II) is added to an apo-Otf solution at pH 10.5 in the presence of 0.1 M bicarbonate, the shape of the spectrum remains substantially the same at 1:1 and 2:1 ratios. This indicates either that the two sites are still spectroscopically indistinguishable or that cobalt(II) binding is no longer sequential, or both.

Finally, an experiment was attempted in order to establish whether strong coordinating anions could displace the presumed water moiety from the coordination sphere of the metal; addition of either azide or cyanide to the cobalt-Otf-bicarbonate adduct at pH 8.3 resulted in progressive removal of the metal without apparent detection of intermediate adducts.

**CD Spectra of Cobalt(II)-Otf-Oxalate**—When cobalt(II) sulfate is added at pH 7 to a 5 × 10⁻⁴ M solution of apo-Otf containing 3 × 10⁻³ M sodium oxalate, the CD spectrum of Fig. 3 develops. Such spectrum is characterized by at least four overlapping transitions, two negative at 450 and 490 nm, and two positive at 520 and 550 nm. The molar ellipticity of these bands increases until a 1:1 cobalt to protein ratio is reached; then any further addition of cobalt(II) has no effect on the above spectrum. If the same experiment is performed at pH 8, the spectra shown in Fig. 4A are obtained. It appears that two cobalt(II) ions are now bound per protein molecule. Furthermore, the CD spectral features appear to be noticeably different between the two sites. This is clearly indicated by the negative band at 450 nm whose ellipticity increases linearly with cobalt concentration up to a 1:1 cobalt to protein ratio, whereas, when a second equivalent of cobalt(II) is added, a slight decrease in the absolute value is observed (Fig. 4A, inset). The difference between the final spectrum and that of the 1:1 derivative shows that the CD spectrum of the second site is characterized by only two positive bands at 480 and 520 nm, possibly indicating a more symmetric coordination for the second cobalt ion as compared to the first one. The CD spectra relative to the first and to the second site are reported in Fig. 4, B and C, respectively.

By stepwise increasing the oxalate concentration in Co₂Otf-oxalate solutions up to a final value of 0.1 M, the intensities of the CD transitions of the second site decrease; the
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Fig. 2. Visible CD spectra of the Co(II)$_2$Otf-bicarbonate complex at pH 8.2 (---) and pH 10.8 (-----). The inset shows the variation of the ellipticity value at 450 nm as a function of pH. Same conditions as Fig. 1. The pH was raised by addition of sodium hydroxide.

Fig. 3. CD spectrum of the Co(II)-Otf-oxalate complex after addition of 1 eq of cobalt(II) at pH 7. The inset shows the variation of $\theta_{450}$ as a function of cobalt(II) to protein ratio. Otf concentration is $5 \times 10^{-4}$ M, sodium oxalate $3 \times 10^{-3}$ M, Tris-HCL 50 mM.

The final shape and intensity of the spectrum resemble that of the first site. This indicates that oxalate is competing with the second protein-binding site for cobalt complexation. The observations that only one cobalt(II) ion binds at pH 7, that at pH 8 the binding of cobalt(II) is sequential, and that excess oxalate removes cobalt(II) from the second site may be consistent with the first site being the C-terminal as in the case of the bicarbonate derivative.

In the pH range 8.5-10.5 the stoichiometry of the binding of cobalt(II) to the protein is again 2:1, and it is no longer affected by oxalate concentration. The shape of the spectrum changes significantly with pH as shown in Fig. 5. The most remarkable change is observed on the negative band at 450 nm; a best fitting analysis of such spectral variation provided a $pK_a$ value of 9.5 ± 0.1 (Fig. 5, inset). This spectral behavior can be interpreted by assuming that the shape of the CD spectrum of the C-terminal site is pH dependent and transforms into a high pH spectrum similar to the N-terminal site spectrum, while the spectrum of the N-terminal site remains almost unaltered. Indeed, the final spectrum at pH 10.4 (Fig. 5) is reminiscent of the N-terminal site spectrum (Fig. 4C). The experimental spectra, simulated through gaussian line-shape analysis (45), were reproduced through linear combination of the spectrum of the N-terminal site and two (high and low pH) spectra of the C-terminal site.

At pH 10.5 there is no longer a definite stoichiometry in the binding of the metal ion and dramatic changes in the shape of the spectra are observed, which may indicate availability of further binding sites or the occurrence of some denaturation or oxidative processes.

$^1$H NMR Spectra—The $^1$H NMR spectra of monocobalt and dicobalt Otf-oxalate derivatives were performed at different pH values. The $^1$H NMR spectrum of the monocobalt derivative at pH 7 is reported in Fig. 6B. Consistent with the CD results, this is the spectrum of a single site, possibly the C-terminal site. Several signals, many of them well resolved, are detected both downfield and upfield (see Table I for chemical shift values). On the basis of our previous assign-
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ment of the cobalt-Otf bicarbonate derivative (Fig. 6A), essentially based on the measure of nuclear T1 values (7), the imidazole proton signals of the two coordinated histidines (two NH, two ortho-like, and two meta-like) are expected in the downfield region, while the tyrosine ortho signals are expected in the upfield region; the tyrosine meta signals, because of the difficulty of predicting the extent of dipolar and contact contributions, could be found either upfield or downfield, and in any case near the diamagnetic region. In the downfield region a larger number of signals than in the case of the bicarbonate analogue is observed. Signals b through h can be assigned to either NH or meta-like histidine protons, whereas signal a is typical of an ortho-like histidine proton; another ortho-like proton could be under the b-d group, as found in the bicarbonate analogue, or very far upfield shifted and broadened beyond detection.

Discrimination between NH and meta-like histidine protons is generally performed through deuteron exchange of the former and recording of the spectrum in D2O (42). In this case, however, the spectrum in D2O looks different in the downfield region with respect to the H2O spectrum, especially for the increase of signal e and decrease in intensity of signals c, d, g, h (Fig. 6C). Such behavior has been observed on all the samples investigated, independently on the time and temperature of incubation of the apoprotein in D2O (see “Materials and Methods”). Since it is not reasonable to have as many as four NH protons in the monocobalt derivative and there is no reason for a signal to increase when the spectrum is recorded in D2O, it is proposed that an equilibrium between two different conformers is present, the distribution of which is affected by the isotope composition of the solvent. Substitution of protons with deuterons could destabilize some hydrogen bonding of the chromophore and favor one configurational state with respect to another. The changes of the spectra are smooth with the change of the H2O/D2O ratio; the CD spectra are, however, insensitive to the change of the solvent, thus ruling out the possibility that the changes in the NMR spectra are due to migration of the metal from one site to the other. The spectrum in D2O would show predominance of a conformer displaying the following signals in the down-

Fig. 4. A, visible CD titration of Otf with cobalt(II) at pH 8 in 1 x 10^{-3} M oxalate. The inset shows the molar ellipticity values at 520 nm (○) and 450 nm (■) as a function of cobalt(II) to protein ratio. B, CD spectrum of the C-terminal site. C, CD spectrum of the N-terminal site as obtained by subtracting spectrum B from the final spectrum in A. Same conditions as Fig. 3.

Fig. 5. Visible CD spectra of the Co2-Otf-oxalate complex at pH 8.2 (-----) and pH 10.4 (-----). In the inset the variation of θ450 as a function of pH is shown. Otf concentration is 5 x 10^{-4} M, sodium oxalate 3 x 10^{-3} M, Tris-HCl 50 mM. The pH was raised by addition of sodium hydroxide under nitrogen atmosphere.

Since in some cases it may be not known whether histidines coordinate the metal ions through N(1) or N(3), the standard numbering scheme cannot be used to indicate ring position adjacent to the coordinating nitrogen. Such positions will be labeled ortho-like, while the H(4) proton of a histidine coordinated through N(1) will be labeled meta-like.
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Fig. 6. A, 60-MHz $^1$H NMR spectrum of the Co$_2$-Otf bicarbonate complex at pH 8.3 (Ref. 7); B, 60-MHz $^1$H NMR spectrum of the complex Co-Otf-oxalate at pH 7; C, B upon deuteration. Otf concentration is $1.5 \times 10^{-3}$ M, sodium oxalate $5 \times 10^{-3}$ M, Tris-HCl 50 mM.

TABLE I

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chemical shift values of the $^1$H NMR signals of the Co-Otf-oxalate complex at pH 7 (see Fig. 6B)

The signals are labeled according to Fig. 6B. Chemical shifts are given in ppm from Me$_4$Si.

field region: a (ortho-like), b (meta-like), e (meta-like). Some effects of D$_2$O in the EPR spectra of oxovanadium(IV) Otf bicarbonate had already been noted (6).

At variance with the bicarbonate analogue which shows four ortho-tyrosinate protons in the region -30/-100 ppm (Fig. 6A) (7), in the oxalate derivative only two ortho-tyrosinate signals of unit intensity (1, m) are observed at -36 and -63 ppm. Such large upfield values were attributed to overwhelming dipolar contributions which would be operative in a pseudo-tetragonal chromophore with the tyrosines in trans position to each other (7). The lack of the other two ortho-tyrosinate signals in the spectrum of the oxalate derivative could either mean that they are less upfield shifted and lie within the large envelope of the diamagnetic protein signals or that they are broadened beyond detection. From the point of view of the contact contribution the meta-tyrosinate signals would be expected to be downfield shifted; however, the dipolar contribution which is strong for at least one of the tyrosines may justify their being slightly upfield shifted. Therefore, signals 1-k may at least in part be attributed to meta-tyrosinate protons.

Upon changing pH from 7 to 9 there are slight changes in the relative intensity of the NMR signals which, however, did not allow us to perform an analysis of the conformers distribution.

At pH 8, using a low concentration of oxalate, the protein can bind two cobalt(II) ions, which are distinguishable through CD spectroscopy (see preceding section). UV difference spectroscopy in the tyrosinate absorption region shows that the spectrum is similar to that of the bicarbonate derivative (22,000 against 20,000 M$^{-1}$ cm$^{-1}$ for the latter) and similar to that found for other bipositive metal ion Otf derivatives (4); these data suggest the presence of two tyrosine residues coordinated at the metal ion in each binding site. The NMR spectra of the monocobalt and dicobalt derivatives, respectively, are reported in Fig. 7, A and B. The spectrum of the dicobalt adduct shows only very slight differences with respect to the monocobalt. In an attempt to have an overall look at the N-terminal site spectrum the free induction decay of the monocobalt derivative was subtracted from the free induction decay of the bis-cobalt derivative obtained under the same experimental conditions. The transformed spectrum is reported in Fig. 7C. Despite its low quality and its overall similarity to the parent spectra, some peculiar features like the broad signal at -70 ppm and the sharp signal at 30 ppm seem characteristic of the N-terminal site.

By increasing the pH above 9 the NMR spectrum changes dramatically; at pH 10.1 the general shape of the spectrum is that shown in Fig. 8A. Drastic variations can be noted both in the upfield and in the downfield region; these abrupt changes in the NMR spectrum reflect the above estimated pK$_\alpha$ value of 9.5 and indicate that some large distortion of the chromophore occurs. Three very broad downfield signals are observed (a', b', c') which can be assigned to ortho-histidine protons; the meta and NH histidine protons are less resolved than in the low pH spectrum and lie in the 70-20-ppm region. Upfield at least eight signals are observed; two of these are very broad and comprised in the range between -130 and -160 ppm while three signals with relative intensity 1:2:1 are relatively broad and are in the region between -40 and -100 ppm; finally at least three other signals, much narrower, are observed near the diamagnetic region. The two very broad and far upfield shifted signals are very likely to be assigned to ortho-tyrosinate protons. In order to assign the three signals in the region -40/-100 ppm, their $T_2$ values were measured. The values were around 3-4 ms; therefore, also these signals, in our opinion, are to be assigned to ortho-tyrosinate protons (7).

The $^1$H NMR spectrum of the bicarbonate adduct at pH 10.4 (Fig. 8B) also differs from the already reported one at pH 8.3 (7) (Fig. 6A), indicating that large distortions are again introduced in the chromophore by raising the pH. On the
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other hand, this spectrum closely resembles that of the analogous high pH oxalate derivative, particularly in the downfield region. Again, it exhibits a very broad and far shifted signal at about 220 ppm, two broad resonances in the region between 150 and 100 ppm, a group of three signals at 70–50 ppm, and some narrower signals near the diamagnetic region. Conversely the pattern of the resonances in the upfield region is quite different; however, we can recognize a kind of parallelism assuming that the resonances $k$, $l$, and $m$ (Fig. 7A) correspond to $k'$, $l'$, and $m'$ (Fig. 7B), respectively and that the far shifted $n$ resonance corresponds to the broad signals $n'_1$ and $n'_2$ (see Table II for chemical shift values). From the above observations it can be inferred that the high pH behavior of the bicarbonate derivative is substantially parallel to that shown by the oxalate derivative.

**DISCUSSION**

The peculiar characteristics of the cobalt(II) ion both from the point of view of the electronic transitions and of the induced isotropic shifts on the neighboring atoms prompted us to use this ion for investigating the structural properties of the binding sites of Otf. As the binding of the metal occurs

![Fig. 7. 60-MHz $^1$H NMR spectra of the monocobalt (A) and dicobalt (B) Otf-oxalate derivatives at pH 8. In C the difference spectrum obtained through computer subtraction of A from B is reported. Conditions as in Fig. 6.](image)

![Fig. 8. A, 60-MHz $^1$H NMR spectrum of Co$_2$ Otf-oxalate at pH 10.1. Same conditions as Fig. 6. The sample was prepared under nitrogen atmosphere. B, $^1$H NMR spectrum of Co$_2$-Otf-bicarbonate at pH 10.3. Otf 1.5 x $10^{-4}$ M, sodium bicarbonate 20 mM.](image)

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in the presence of synergistic anions, we have investigated both cobalt-Otf-bicarbonate and cobalt-Otf-oxalate in order to try to understand the role of the synergistic anion and to monitor the flexibility of the coordination sites which can accommodate differently charged metals and/or different synergistic anions.

The most striking difference between the two investigated derivatives is that in the case of the bicarbonate adduct the CD spectra of the two sites appear to be spectroscopically equivalent, whereas in the case of the oxalate adduct they are different at least in the pH region 8–9. Possibly the oxalate anion, imposing sterical constraints different from those of the natural synergistic anion, enhances the subtle structural differences between the two sites. Oxalate is believed to bind in a monodentate fashion, bridging both the metal ion and some positive charged protein residue according to the interlocking sites model proposed by Bates (47). A common feature to both systems, however, is that at least in the pH range 7–8.5, one site appear to bind cobalt more tightly than the other site. Indeed, in the case of oxalate, the second site is able to
bind the metal only at pH 8. These data are consistent with those obtained on all the other metal derivatives in the sense that different synergistic anions provide different pH dependence of metal binding and different structural properties (48, 49).

The CD spectrum of the tighter binding site (C-terminal) of the oxalate derivative is very similar, although less intense, to that of the cobalt bicarbonate derivative, especially for the presence of two negative bands at 450 and 490 nm. The positive bands are a little different among the two derivatives, oxalate showing maxima at 520 and 550 nm and bicarbonate at 560 and 590 nm. The presence of four well separated CD maxima suggests that the coordination polyhedron of the cobalt(II) ion has a very low symmetry. The NMR spectrum of the oxalate derivative at pH 7 differs, however, from the analogous spectrum of the bicarbonate derivative not only because it is indicative of the presence of different conformers but also with respect to the number and position of the high field signals which monitor the tyrosine ortho-protons. Indeed the oxalate derivative shows only two ortho-tyrosine signals instead of the four which are detected in the bicarbonate derivative, and, in addition, these two signals are much less high field shifted than those of the bicarbonate derivative. UV difference measurements confirm that, also in the oxalate adduct, two tyrosines are bound; therefore, a reasonable conclusion is that, although there are very strict analogies between the CD spectra of the oxalate C-terminal site derivative and the bicarbonate adduct, probably the tyrosine binding is somehow different in the two chromophores. The existence of different conformers for this site as well as the sizable conformational changes which occur for simple deuteration of the sample support the idea of a “stressed” coordination polyhedron in which some ligand changes its position in dependence, for example, of the weakening of some hydrogen bond.

The existence of different conformers for one or both sites has already been proved in the oxovanadium(IV) and copper(II) derivatives of ovotransferrin (5, 6). In particular, Chas-teen in his work (6) on the EPR spectra of the oxovanadium(IV) derivative described three conformational states for the metal in both the sites and calculated their relative population as a function of pH. At pH values below 8.5 only two of them are predominant, whereas at pH 9 the three conformational states are comparably populated (6). It is possible that one or more of these conformers are important in metal uptake and release.

Although the N-terminal site of the oxalate derivative has a CD spectrum different from that of the C-terminal site, its NMR spectrum, obtained through computer subtraction, seems to indicate that the difference is not substantial in terms of ligand arrangement around the metal.

When the pH is increased both the bicarbonate and the oxalate derivative show drastic spectroscopic changes which can be related to the ionization of a group with a $pK_a$ of about 9.5. It appears that deprotonation of the synergistic bicarbonate anion is not responsible for this interconversion, since the oxalate derivative shows this very same equilibrium. Nor is the bound water molecule responsible since a similar $pK_a$ is observed for the copper bicarbonate derivative (5), and in this case, if a water molecule is present, in our opinion it is semicoordinated in an axial position; its $pK_a$ would be expected to be higher than in the case of the cobalt(II) complex. Reasonable groups with a $pK_a$ of 9.5 are a tyrosine and a lysine. A third tyrosine has indeed been proposed to be relatively close to the metal-binding site (2). It would be tempting to propose that the latter group coordinates the metal ion with a $pK_a$ of 9.5, as already suggested by Zweier for the copper ovotransferrin derivative (5), thus accounting for the many NMR signals observed upfield. However, the observation of three broad signals attributable to ortho-like histidine protons in the far downfield region is more indicative of inequivalence of the metal sites rather than of coordination of a third tyrosine. Such an inequivalence might also be due to the presence of conformers for each site.

The presence of a synergistic anion different from the natural one, i.e. oxalate, enhances the structural differences between the two sites providing a large variety of conformers. In the most general sense the structural dependence of the sites on pH and on synergistic anions has provided much information on the reactivity of the system. Particularly, the flexibility of the binding sites as it results from the existence of conformers and from conformational changes detected here can be meaningful in the understanding of the mechanism of uptake and release of metal ions. Indeed, it has been proposed that the rate-limiting step in the iron release is a conformational transition of the protein (41, 50).

On the other hand, the equivalence of the two binding sites in the bicarbonate derivative at physiological pH, as it results from NMR and CD data, is consistent with the essentially similar functional role performed by both sites in the “in vivo” systems, in agreement with the latest physiological studies (51–54).

REFERENCES

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