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Nonequivalence of the Metal Binding Sites in Vanadyl-Labeled Human Serum Transferrin†

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ABSTRACT: Vanadyl ion, VO(IV), has been used as an electron paramagnetic resonance (EPR) spin label to study the metal-binding properties of human serum transferrin in the presence of bicarbonate. Iron-saturated transferrin does not bind the vanadyl ion. Room temperature titrations of apotransferrin with VO(IV) as monitored by EPR indicate the extent of binding to be pH dependent, with a full 2.0 VO(IV) ions per transferrin molecule bound at pH 7.5 and 9, but only about 1.2 VO(IV) ions bound at pH 6. The EPR spectra of frozen solutions with or without 0.1 *M* $NaClO_4$ at 77 K show that there are two spectroscopically nonequivalent binding sites (A and B) with a slight difference in binding constants. One site (A site) exhibits essentially constant binding capacity in the pH range 6-9, but the other (B site) becomes less available as the pH is reduced below 7. Results with mixed Fe(III)-VO(IV) transferrin complexes suggest that iron shows a slight tendency to bind at the B site over the A site at pH 7.5 and 9.0. Only the B site in both vanadyl and iron transferrins is perturbed by the presence of perchlorate.

Human serum transferrin is an Fe(III) transport protein of about 80,000 molecular weight. It gives up ferric ions to bone marrow and placental tissues in preference to other cells such as liver cells (Morgan, 1972, and references cited therein). The protein is capable of binding two irons per molecule but an anion such as bicarbonate (in biological systems) or oxalate must be present for binding to occur (Aisen et al., 1967; Price and Gibson, 1972a). It has been shown that HCO_3^- is bound stoichiometrically to transferrin in a 1:1 ratio with Fe(III) (Aisen et al., 1969).

Fletcher and Huehns (1967) and Harris and Aisen (1975) have shown that the two sites differ in their ability to donate iron to reticulocytes. Aisen et al. (1973) also report that two different rate constants are needed to describe the exchange of bound CO_3^{2-} during dialysis. Additional evidence for nonequivalence of the sites is the report by Luk (1971) that transferrin binds a full two rare earth ions per protein molecule for the smaller ions Tb^{3+}, Eu^{3+}, Er^{3+}, and Ho^{3+}, but smaller amounts for the ions with larger ionic radii, such as Nd^{3+} and Pr^{3+}.

Circular polarization of luminescence has failed to show any differences in the ligand fields of the two sites of the protein when Tb^{3+} is bound (Gafni and Steinberg, 1974). Solution calorimetry has yielded values of ΔH for addition of the first and second moles of iron to transferrin which are the same within experimental error (Binford and Foster, 1974). EPR experiments and magnetic susceptibility measurements with Fe(III), Co(III), Cu(II), and Mn(III) bound to transferrin in frozen solutions have not demonstrated the presence of more than one type of binding (Aasa and Aisen, 1968; Aisen et al., 1969). However, a careful reexamination of the Fe(III) EPR spectrum suggests that differences do exist between the two metal sites (Aasa, 1972). In the case of Cr(III), EPR spectra indicate the presence of two metal environments. The lines attributed to these species lose spectral identity in a sequential manner when Fe(III) displaces the Cr(III) (Aisen et al., 1969).

Price and Gibson (1972b) have observed that addition of $NaClO_4$ to Fe(III) transferrin produces changes in the EPR spectrum of frozen solutions of the protein suggestive of changes in one site per protein molecule. The relatively sharp peak characteristic of the perchlorate-free species decreases in intensity as $NaClO_4$ is added, and a broader band with a similar *g* value grows in.

Fourier transform ^{13}C nuclear magnetic resonance (NMR) of Fe-transferrin in the presence of $K_2{}^{13}CO_3$ suggests that the anion actually bound may be CO_3^{2-} rather than HCO_3^- (Harris et al., 1974). Other anions reported to bind in place of bicarbonate include oxalate, nitrilotriacetate, and ethylenediaminetetraacetate (Aisen et al., 1967).

The vanadyl(IV) ion, VO^{2+}, has been used to probe metal binding sites in a number of proteins (Chasteen et al., 1973; Fitzgerald and Chasteen, 1974a,b; DeKoch et al., 1974; Francavilla and Chasteen, manuscript in preparation). The success of this probe is due in part to the fact that its EPR spectrum consists of relatively sharp lines which make it possible to distinguish small differences in the spectroscopic *g* and *A* parameters which reflect differences in the metal ion environment. In addition, vanadyl(IV) species exhibit reasonably well-resolved room temperature solution spectra thus avoiding some of the inherent difficulties of

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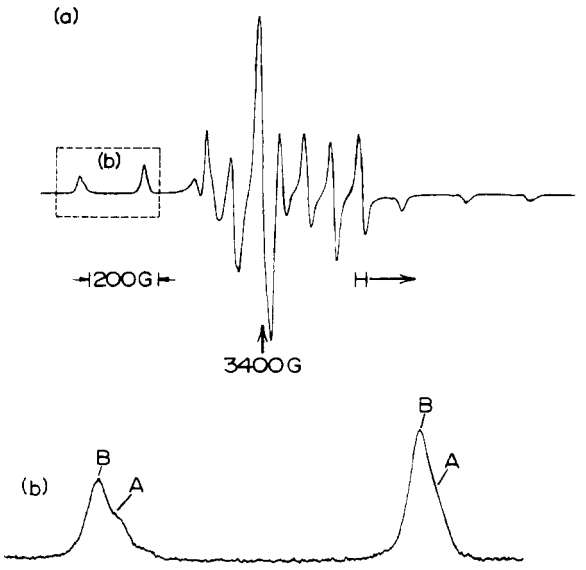

FIGURE 1: (a) Perchlorate-free, room temperature spectrum of 2×10^{-4} M divanadyl transferrin in 0.01 M $NaHCO_3$ at pH 8.56. The inset region of (a) is shown in (b) with the field expanded 5×. A and B refer to the two sites in transferrin. Instrument settings: power = 100 mW, modulation amplitude = 10 G, sweep rate = 2000 G/0.5 hr, and time constant = 1.0 sec.

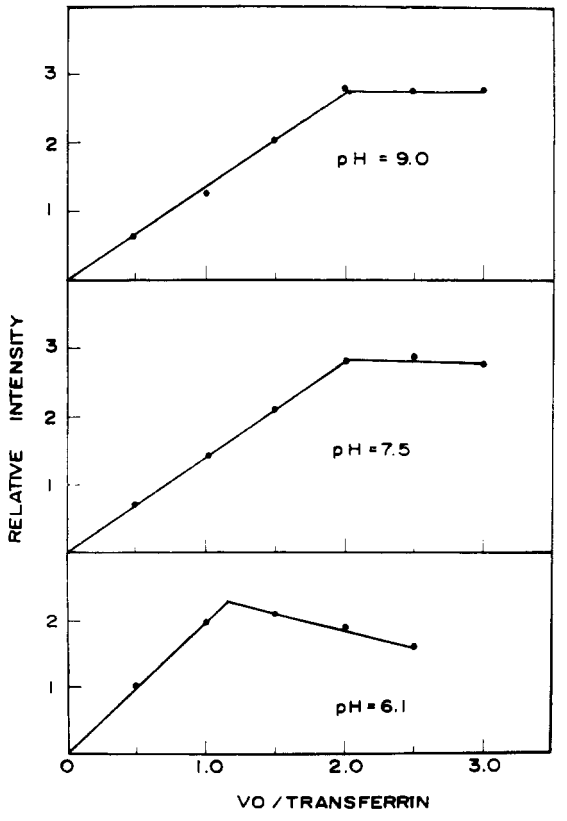

FIGURE 2: EPR titration of 2×10^{-4} M apotransferrin in 0.1 M $NaClO_4$ and 0.01 M $NaHCO_3$ with VO^{2+} ion. Intensity is based on signal-to-gain ratio for the central peak of the room temperature spectrum.

frozen solution EPR spectroscopy of biochemical samples. Room temperature EPR spectra of vanadyl(IV) solutions provide a means of distinguishing between binding to small ligands and tight binding to the protein which is characterized by a very anisotropic spectrum arising from the slow molecular tumbling of the macromolecule.

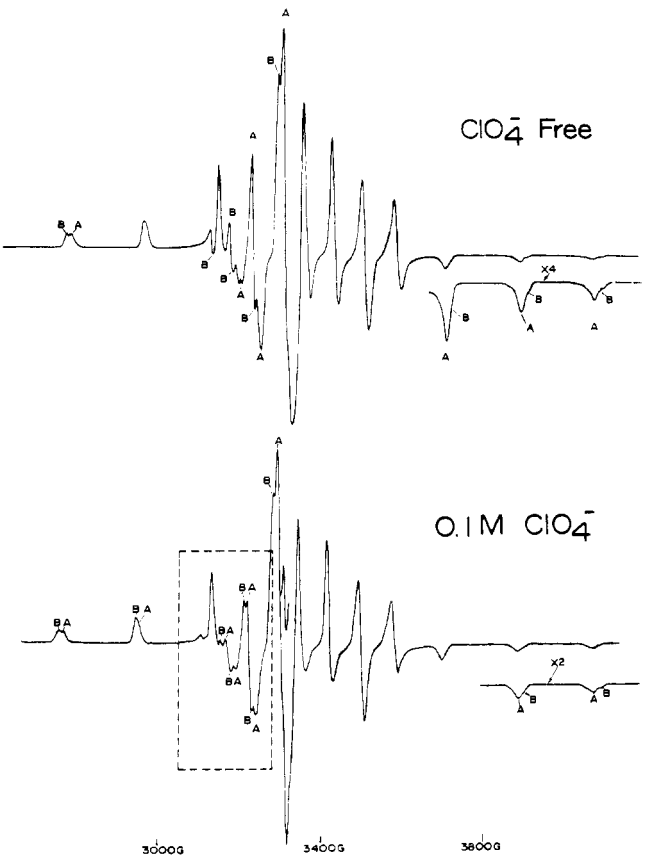

FIGURE 3: Divanadyl transferrin, 2×10^{-4} M, at 77 K: (top spectrum) perchlorate-free, 0.01 M $NaHCO_3$ at pH 8.56; (bottom spectrum) 0.1 M $NaClO_4$-0.01 M $NaHCO_3$ at pH 9.0. A and B resonances are clearly seen in both spectra with only the B resonances appreciably shifted by perchlorate. The inset region in the bottom spectrum and a similar region of other spectra are shown in Figure 4. Instrument settings: power = 1.0 mW, modulation amplitude = 10 G, sweep rate = 2000 G/0.5 hr, and time constant = 0.3 sec.

The VO^{2+} ion was chosen as a probe of metal binding in transferrin in order to obtain information about the binding of metal ions as a function of pH and perchlorate concentration. It was intended that this information might suggest a chemical basis for the apparent biological nonequivalence of the metal binding sites.

Experimental Section

Commercial preparations of iron-free human serum transferrin (Sigma Chemical Co., St. Louis, Mo.) were found to be contaminated with a low molecular weight chelating agent as evidenced by the appearance of an isotropic, rotationally averaged EPR spectrum (in addition to the anisotropic spectra discussed below) when VO^{2+} was added to a solution of the undialyzed commercial protein. After dialysis against 0.1 M $NaClO_4$ (Price and Gibson, 1972a) this species was no longer observed. The protein contained nominally 0.05 g-atom of Fe/mol of protein as determined by atomic absorption spectroscopy. The purity of the dialyzed protein was further verified by gel electrophoresis. Protein concentrations were determined using a Beckman Model DU spectrophotometer. Measurements were made at 280 nm using 81,000 as the molecular weight and 11.4 as the 1% (by weight) absorbance in 1-cm cells (Aisen et al., 1967).

Fe(III) was added to the apoprotein as the 1:2 complex

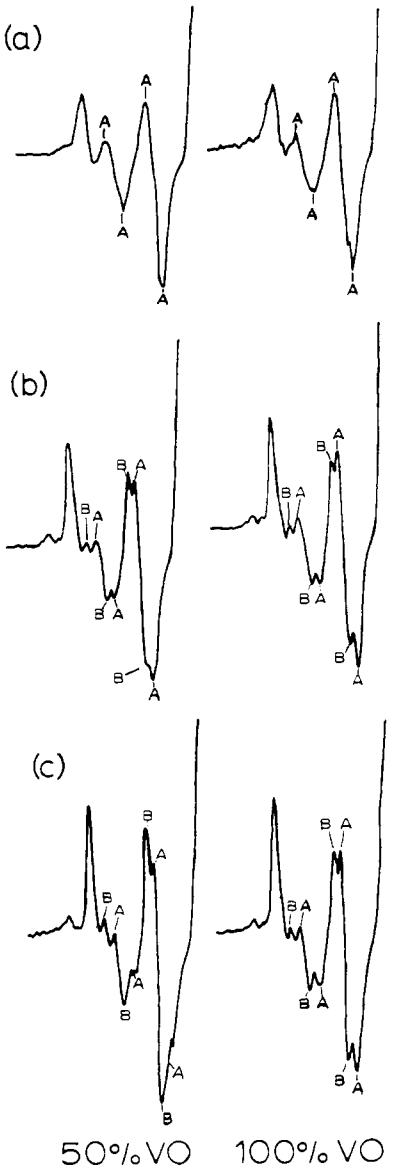

FIGURE 4: Effect of pH and the degree of vanadyl saturation on features of the 77 K EPR spectra in 0.1 *M* $NaClO_4$ and 0.01 *M* $NaHCO_3$. The region shown corresponds to the inset portion of Figure 3: (a) pH 6.1; (b) pH 7.5; (c) pH 9.0; instrument settings are as in Figure 3.

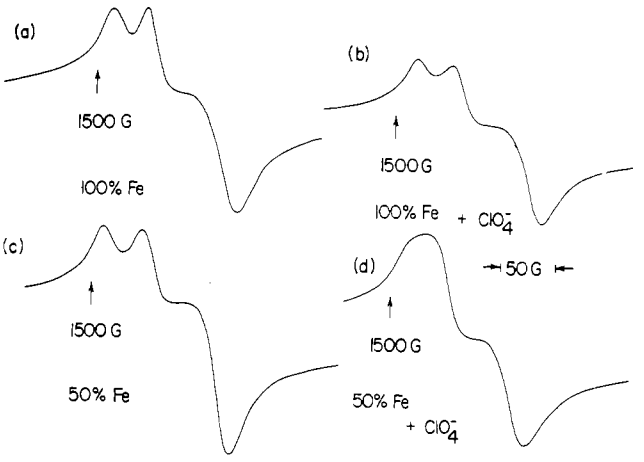

FIGURE 5: Effect of ClO_4^- and degree of iron saturation on 77 K Fe(III) EPR spectrum of 2×10^{-4} *M* transferrin in 0.01 *M* $NaHCO_3$: (a) 100% saturation, no $NaClO_4$, pH 8.5, gain = 1.0×10^3; (b) 100% saturation, 0.1 *M* $NaClO_4$, pH 8.5, gain = 1.0×10^3; (c) 50% saturation, no $NaClO_4$, pH 7.8, gain = 2.5×10^3; (d) 50% saturation, 0.1 *M* $NaClO_4$, pH 7.8, gain = 2.0×10^3. In this range, the pH has little effect on the spectrum. Instrument settings: power = 40 mW, modulation amplitude = 10 G, sweep rate = 400 G/16 min, and time constant = 0.3 sec.

with nitrilotriacetic acid (NTA).[1] Hydrolysis of the complex was avoided by following the precautions suggested by Bates and Wernicke (1971). After combining the protein and the complex in the presence of excess HCO_3^-, the NTA was removed by dialysis against 0.1 *M* $NaClO_4$ (pH 7.0). The resultant iron content of the protein was determined on a Varian Techtron AA-3 atomic absorption spectrometer.

Buffers and salt solutions were rendered metal free by phase extraction with 0.001% dithiophenol carbazone in carbon tetrachloride. Glassware was cleansed of metals by soaking in 1:1 concentrated H_2SO_4–HNO_3 followed by rinsing in doubly distilled deionized water and polyethylene or polypropylene containers were used where possible. All solutions were purged with prepurified nitrogen gas prior to addition of vanadyl ion to minimize oxidation. Transfers were made between serum-stoppered vessels using nitrogen purged syringes. Direct solution contact with the stoppers was avoided.

EPR spectra were measured on a Varian E-9 spectrometer using Varian strong pitch, $g = 2.0028$, as a reference material. EPR titrations were carried out by injecting increments of titrant into the aqueous solution flat cell containing the sample being titrated, and then mixing by withdrawing the sample into a syringe and returning under nitrogen.

Results

When VO^{2+} is added to an oxygen-free solution of perchlorate free apotransferrin containing 0.01 *M* $NaHCO_3$, no color change is observed, but an anisotropic room temperature EPR spectrum is produced (Figure 1a) which is indicative of tight binding of the vanadyl ion to the slowly tumbling protein. The line shapes (Figure 1b) indicate that more than one vanadyl environment (labeled A and B) is contributing to the spectrum. The room temperature spectrum obtained when 0.1 *M* $NaClO_4$ is added is similar, although there is less evidence of two environments of the metal.

The extent of vanadyl binding to transferrin in the presence of perchlorate and bicarbonate is pH dependent. Figure 2 shows the results of room temperature EPR titrations at pH 6.1, 7.5, and 9.0. Here, the peak-to-peak intensity of the first-derivative curve is plotted as a function of VO^{2+} added to apotransferrin. At the higher pH values, a full 2.0 VO^{2+} ions are bound per transferrin molecule, but at pH 6.1 only about 1.2 sites are available. Similar results are found in the absence of perchlorate.

Spectra in Figure 3 obtained at liquid nitrogen temperature clearly show evidence of two environments for the metal ion in the presence or absence of perchlorate. Hereafter, we refer to these environments as the A and B sites of transferrin. Overlaying of the two spectra in Figure 3 reveals that only the positions of the B resonance are appreciably affected by perchlorate.

Examination of a portion of the "perpendicular region" (see Figure 3) of frozen solutions at pH 6.1, 7.5, and 9.0

[1] Abbreviation used is: NTA, nitrilotriacetic acid.

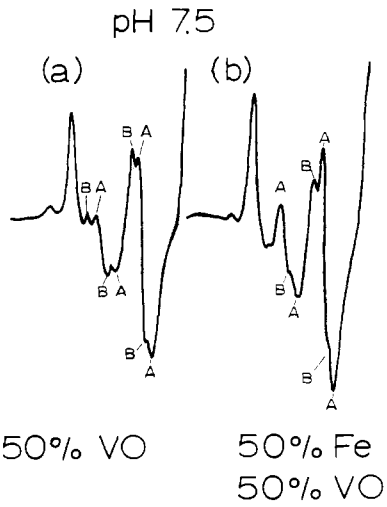

FIGURE 6: Spectra of transferrin, pH 7.5, 0.01 *M* $NaHCO_3$, 0.1 *M* $NaClO_4$: (a) 50% saturated with VO^{2+}; (b) 50% saturated with Fe^{3+} followed by addition of 2 equiv of VO^{2+} to yield a protein 50% saturated in VO^{2+}. Instrument settings are as in Figure 3.

with perchlorate present (Figure 4) indicates that essentially only the A site is occupied at pH 6.1. Binding at the B site occurs only at pH 7.5 and 9.0. This is consistent with the results presented in Figure 2. Moreover, comparison of the relative intensities of the A and B resonances for 50 and 100% saturated vanadyl transferrin shows that the vanadyl ion shows a slight binding preference for the B site at pH 7.5 and 9.0. This is most evident at pH 9.0 (Figure 4c).

When iron-saturated transferrin (two irons per protein molecule) is prepared, and VO^{2+} is added to the solution, no vanadyl(IV) EPR spectrum (or a very weak one) is observed.[2] The 100% saturated iron EPR frozen solution spectra (Figure 5) exhibit a dependence on perchlorate concentration like that reported by Price and Gibson (1972b). When perchlorate-free half-saturated transferrin is prepared, the iron EPR spectrum is essentially like the 100% (two iron) spectrum, but with approximately one-half the intensity (cf. Figures 5a and c). When 0.1 *M* $NaClO_4$ is added, however, the change in the iron spectrum is much more pronounced (cf. Figures 5b and d).[3]

VO^{2+} binds to 50% Fe-saturated transferrin with the vanadyl EPR spectrum having about one-half of the intensity obtained from divanadyl transferrin of the same protein concentration. This reduction is observed both with and without perchlorate being present. The ratios of intensities of the vanadyl(IV) EPR spectral features of the two sites are not the same for the 50% iron-saturated transferrin as for the iron-free protein. These features are compared in Figure 6. Note that for 50% VO^{2+}--50% Fe^{3+}-transferrin the B resonances are much less intense than the A resonances.

Discussion

Evidence of Vanadyl Binding at the Iron Sites. The very anisotropic room temperature spectrum is indicative of tight binding of the VO^{2+} ion to the protein moiety. This would be expected for multidentate coordination, i.e., VO^{2+} binding at the site normally occupied by Fe^{3+}. Apotransferrin exhibits a maximum binding capacity of two VO^{2+} ions per protein molecule which is consistent with the fact that there are two iron binding sites in transferrin.

The effect of iron on transferrin's ability to bind the vanadyl ion is quite pronounced. When transferrin is fully iron saturated, less than 5% of the VO^{2+} in solution binds. When the protein is 50% iron saturated, VO^{2+} binding is approximately 50%. Thus, the bindings of Fe^{3+} and VO^{2+} are mutually exclusive.

We have shown that VO^{2+}, like iron, requires bicarbonate or other suitable anions in order to bind to the protein (J. C. Cannon and N. D. Chasteen, manuscript in preparation). The ultraviolet spectrum of vanadyl transferrin with apotransferrin as a blank likewise displays the characteristic 296- and 246-nm bands attributed to tyrosine binding in iron transferrin (Teuwissen et al., 1972, and references therein).

The Nonequivalence of the Vanadyl Binding Sites of Transferrin. The doubling of the room temperature, perchlorate-free solution EPR spectrum of vanadyl transferrin indicates that two spectroscopically nonequivalent species are present (Figure 1). The fact that this doubling is observed in a perchlorate-free room temperature solution (Figure 1) rules out the possibility that it might be an artifact of either freezing or chaotropism, changes in hydrogen bonding properties of water due to perchlorate (Price and Gibson, 1972b). EPR spectroscopic titrations have shown that the maximum vanadyl binding capacity of transferrin is pH dependent (Figure 2).

Again, the observation cannot be an artifact, as this behavior occurs with both room temperature and frozen solutions in the presence or absence of perchlorate. However, the nonequivalence is most easily studied in frozen solutions containing 0.1 *M* $NaClO_4$. This is because the line widths are narrower in frozen solution and because perchlorate happens to shift the resonances of the B site in such a way as to facilitate intensity comparisons of certain doubled features in the spectrum.

The frozen solution spectra, particularly in the region displayed in Figure 4, show that when two VO^{2+} ions are available per protein molecule, the occupancy of the A site is essentially constant, while binding at the B site takes place only at higher pH. However, at pH 7.5 or 9.0 where both sites are capable of binding, vanadyl seems to prefer the pH-dependent B site. This is indicated by the greater intensity of the features attributed to this site relative to the A site when less than 2 equiv of VO^{2+} are present (Figure 4).

The Nonequivalence of the Iron Binding Sites of Transferrin. If iron exhibited no preference for either site, 50% saturation with Fe^{3+} followed by addition of a slight excess of VO^{2+} ion should have the same effect on the vanadyl EPR spectrum as reducing the transferrin concentration by one-half with the A and B resonances equally intense. How-

[2] At pH 7-8, the uncomplexed VO^{2+} ion primarily exists as a suspension of $VO(OH)_2$, which is difficult to detect by EPR.

[3] It should be noted that the spectrum shown in Figure 5d for 50% Fe-saturated transferrin has not been observed by other workers. To verify our results, the experiments were repeated with transferrin obtained from Behring Diagnostics which is guaranteed 98% pure. The protein was half-saturated by adding the required amount of the Fe(III)-NTA complex (1:2) to the protein in 0.1 *M* $NaClO_4$ and 0.01 *M* $NaHCO_3$, pH 8.4, followed by dialysis against the same solution to remove excess NTA. The protein solution had the characteristic salmon pink color of Fe(III) transferrin and exhibited the spectrum shown in Figure 5d. Dialysis of the protein against 0.01 *M* $NaHCO_3$, pH 8.5, to remove the perchlorate produced the spectrum in 5c. Readdition of $NaClO_4$ to the same protein solution changed the spectrum back to that of Figure 5d. This confirms our observation with the Sigma protein. Moreover, the vanadyl spectra of the Behring transferrin were very similar to those obtained with the Sigma protein (Figure 3).

ever, the spectra of the preparations which are 50% saturated with Fe^{3+} followed by 50% saturation with VO^{2+} do not conform to this expectation (Figure 6b). The vanadyl peak heights are not equal. The A-site resonances are much more intense than those of the B site. Since there is sufficient VO^{2+} present to fill all of the sites not occupied by iron, one must conclude that iron has not chosen its sites in a random manner. Since the A site is more frequently occupied by VO^{2+} when Fe^{3+} is present, one must conclude that iron prefers to bind at the B site, the pH-dependent site that would otherwise be preferentially occupied by the vanadyl ion at pH 7.5 and 9.0. This behavior is a strong indication that the two metal binding sites in iron(III) transferrin are nonequivalent.

It has been previously reported (Price and Gibson, 1972b) and verified by us that perchlorate affects the EPR spectrum of iron(III) transferrin, with the behavior being attributed to a chaotropically induced change in protein configuration. This is consistent with the observation that in the case of vanadyl(IV) transferrin the two metal binding sites feel the effects of perchlorate to different extents, with the B resonances primarily shifted.

In the case of iron, it can be seen that the spectrum at 50% saturation (Figures 5c and 5d) is most broadened by perchlorate. We have already shown above that at 50% saturation, the iron preferentially binds at the B site. Thus, it appears that only the B site in both iron and vanadyl transferrin is appreciably affected by perchlorate.

In our notation, the B site is the same as the *B*-site (*B* referring to the site exhibiting the broad iron signal in the presence of perchlorate) of Price and Gibson (1972b) and the A site is synonomous with their *S*-site (sharp iron signal). In their model, apparently either of the two sites in transferrin can be affected by perchlorate to become the *B*-site through a conformational change in half of the protein while the other site remains as the *S*-site.

Conclusion

In conclusion, it has been shown that two metal binding sites of transferrin are nonequivalent in several ways. The availability of one site (B) for metal binding is dependent on pH, while the other (A) exhibits no such dependence in the range of pH 6 to 9. At pH values where both sites are available, one is favored over the other (B over A) by both VO^{2+} and Fe^{3+} in the presence of bicarbonate. In addition, the sites differ at least slightly in the nature and/or arrangement of the ligands, as evidenced by slight, but definite differences in their spectra. The sites also differ in the sort of interaction or configurational change which occurs when perchlorate is present with the B site of both iron and vanadyl transferrins primarily affected. Although this evidence is not sufficient for construction of an explicit model describing the interaction of metal ions with transferrin, it seems likely that these observations relate to the chemical basis for the previously reported (Fletcher and Huehns, 1967; Harris and Aisen, 1975) biological nonequivalence of the metal binding sites of human serum transferrin. For example, if loss of iron to the reticulocytes involves a proton transfer to the protein (possibly to the anion), one might speculate that the pH dependent B site would more readily give up its iron.

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