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Metal Site Conformational States of Vanadyl(IV) Human Serotransferrin Complexes†

N. Dennis Chasteen,* Lawrence K. White, and Robert F. Campbell‡

ABSTRACT: This study was undertaken to investigate the conformational states of the two metal sites in the human serum transferrin molecule. The 9.2 GHz electron paramagnetic resonance spectra of frozen solutions of divanadyl(IV) transferrin consist of a superposition of two sets of resonances, A and B, due to the magnetically nonequivalent binding environments of the VO^{2+} ion. Examination of the intensities of the A and B resonances as a function of pH from 6.0 to 10.7 reveals that they arise from two conformational states of the metal sites in which the geometrical arrangement and/or identity of one or more ligands in the first coordination sphere are different. From pH 7.5 to 9.0, the metal sites exist in A and B conformations but above pH 9.0 the A conformation undergoes a transition to the B conformation. This transformation is coupled to the ionization of an apparently noncoordinating protein functional group with a $pK = 10.0 \pm 0.1$. Below pH 7.0, binding in the B conformation is rapidly lost, driven in part by the protonation of a functional group, possibly the anion, with a $pK = 6.6 \pm 0.1$. In 90% D_2O, this pK is elevated to 7.8 ± 0.1. At pH 6.0 in H_2O, essentially one VO^{2+} ion remains bound to the protein with the metal site in the A conformation. Experiments with mixed VO^{2+}–Fe^{3+} transferrin complexes indicate that the same may be true of Fe^{3+}. At pH 10.7, a new set of VO^{2+} resonances, labeled C, are observed; they possibly arise from a third conformation of the metal site. One bicarbonate or carbonate is required per VO^{2+} ion bound to the protein. 2.7 H^+ are released per VO^{2+} bound in either the A or B conformations. The above results are discussed in terms of the "equivalence" and "nonequivalence" of the metal sites.

The iron transport protein transferrin has two Fe(III) binding sites which exhibit similar spectroscopic and thermodynamic properties (Gafni and Steinberg, 1974; Luk, 1971; Binford and Foster, 1974; Aasa and Aisen, 1968; Aisen et al., 1969; Aasa, 1972; Price and Gibson, 1972a). Transferrin has a molecular weight of around 80 000 and consists of a single polypeptide chain (Mann et al., 1970) with two identical carbohydrate side chains (Spik et al., 1975). Human serotransferrin has been recently reviewed (Chasteen, 1977).

A suitable anion must be present in a 1:1 stoichiometric ratio with the iron in order for the metal to bind (Aisen et al., 1967;

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‡ Present address: Department of Chemistry, Cornell University, Ithaca, N.Y.

Price and Gibson, 1972b; Aisen et al., 1969; Bates and Schlabach, 1975; Aisen and Leibman, 1973). In physiological media, the anion is carbonate or possibly bicarbonate (Harris et al., 1974). The structural and functional roles of the anion are not known with any certainty, although recent spectroscopic experiments employing a variety of anions suggest that the anion may be directly coordinated to the metal (Aisen and Leibman, 1973; Schlabach and Bates, 1975; Harris et al., 1974; Harris and Aisen, 1975a; Aisen et al., 1973).

DEAE-cellulose chromatography and hydrodynamic data indicate that diferric, monoferric, and apotransferrin exist in different conformational states (Lane, 1971, 1973; Rosseneu-Motreff et al., 1971; Bezkorovainy, 1966). The chaotropic agent perchlorate perturbs the electron paramagnetic resonance (EPR) spectrum of iron transferrin or conalbumin by inducing conformational changes at the metal site (Price and Gibson, 1972a). Apparently, either site can exist in one of two conformations, B or S, which are responsible for the broad and sharp features, respectively, in the EPR spectrum. Addition of perchlorate to transferrin solutions shifts the equilibrium toward the B conformation. Perchlorate has the opposite effect on conalbumin, shifting the equilibrium toward the S conformation. Similar properties are observed with the C- and N-terminal iron binding fragments isolated from *conalbumin* (Williams, 1974; Butterworth et al., 1975).

Conformational states are most likely important for the interaction of the transferrin molecule with the receptor site of the reticulocyte membrane. The observed heterogeneity in the iron-donating capability of the two sites (Fletcher and Huehns, 1967; Harris and Aisen, 1975b) may be related to conformational differences between them.

Recently, it was reported that the EPR[1] spectrum of divanadyl transferrin with or without perchlorate present can be used to distinguish between different metal binding environments in the transferrin molecule, denoted as A and B (Cannon and Chasteen, 1975). Most of the previous work was performed in the presence of perchlorate. Subsequent investigations in our laboratory in perchlorate-free media under numerous conditions have revealed shifts in the relative intensities of the A and B resonances of fully saturated vanadyl transferrin. We report here a summary of these measurements showing that the EPR spectrum distinguishes between A and B conformations of the metal sites and under what conditions interconversion between these conformations takes place. Several properties of the vanadyl and iron transferrins are shown to be similar.

Experimental Section

Commercial iron-free human serum transferrin (99% pure) was obtained from Behring Diagnostics. Procedures for handling the protein and introducing the vanadyl ion were identical to those detailed elsewhere (Cannon and Chasteen, 1975). EPR spectra were measured on a Varian E-9 spectrometer operating at X-band and 100 kHz modulation. The g values were measured relative to Varian strong pitch (g = 2.0028) or diphenylpicrylhydrazyl radical, DPPH (g = 2.0036). The magnetic field was calibrated with a proton/lithium NMR gauss-meter.

"Bicarbonate-free" transferrin solutions were prepared by a number of published methods (Aisen et al., 1967; Bates and Schlabach, 1975; Fraenkel-Conrat, 1950). Generally, the method and apparatus of Aisen et al. (1967) were used. The protein solution was adjusted to pH 4.5 with 0.1 M HCl, while rapidly stirring with a spin bar. One-half milliliter of 2×10^{-4} M acidified protein solution containing 20 μl of 0.1% bromthymol blue in water was placed in a double Thunberg apparatus and alternately evacuated and flushed with prepurified nitrogen (Aisen et al., 1967). The Thunberg apparatus was then tipped allowing the solution to run to the top bulb to dissolve the $VOSO_4$, sufficient to 95% saturate the protein. Finally, the pH was adjusted between 7.0 and 9.0 by diffusing ammonia gas into the solution from one-half of the apparatus until the bromthymol blue indicator changed from yellow to light blue. By tipping the apparatus, the solution was loaded into a side-arm quartz tube for EPR measurements. At the end of the experiment, the solution was opened to the atmosphere and the pH was measured with a Ag/AgCl-glass combination electrode. For frozen solution spectra, the double Thunberg apparatus was identical to that of Aisen et al. (1967). For room-temperature solution measurements, the Thunberg tube (under positive nitrogen pressure) had a side arm fitted with a serum stopper through which the protein was withdrawn with a syringe and transferred to a serum stoppered N_2-fshed flat cell. The addition of increments of bicarbonate to the flat cell was accomplished by a method similar to that previously outlined for other titrations (Fitzgerald and Chasteen, 1974). A serum-stoppered nitrogen-flushed EPR tube was used for bicarbonate titrations involving EPR spectroscopy of frozen solutions.

Proton-release studies were carried out using the pH titration vessel described previously (Fitzgerald and Chasteen, 1974). In a typical experiment, 0.3 ml of 0.6 mM apotransferrin containing no added bicarbonate was adjusted to pH 6.0, 8.0, or 10.0 with 0.02 M NaOH or HCl while stirring. Then 1 equiv of 0.0200 M $VOSO_4$ (Alfa Inorganics) was introduced, resulting in a drop in pH. NaOH (0.0200 M) was added and the equivalents of base per VO^{2+} required to restore the pH in its original value was noted.

The amount of "free acid", presumably due to sulfuric acid in commercial vanadyl sulfate, was found by titration of the $VOSO_4$ solution with standard base while monitoring the loss of the EPR spectrum due to the formation of $VO(OH)_2$(s) (Francavilla and Chasteen, 1975). The amount of base used in excess of two per VO^{2+} was attributed to free acid. A value of 0.3 H^+/VO^{2+} was obtained, in agreement with the literature (Tapscott and Belford, 1967; Tapscott, 1968). This value was subtracted from the number of equivalents of NaOH required per VO^{2+} to restore the protein to its original pH; this yielded the number of protons released by the protein per VO^{2+} bound.

pH measurements were done with a Radiometer pHM meter. pD values were obtained from the equation pD = meter reading +0.4, where the meter was standardized against H_2O buffers. The protein was dissolved in 99.9% D_2O. Various stock solutions in H_2O were subsequently added giving a final solution 90% in D_2O.

Results

EPR Spectra. Figure 1 shows the frozen solution spectrum of divanadyl transferrin at 77 K in 0.01 M sodium bicarbonate, 90% D_2O, pD = 8.4. The spectrum is a superposition of two sets of resonances, labeled A and B, due to the magnetic nonequivalent binding environments of the metal. The resolution of the spectrum in Figure 1 is improved by the use of D_2O and a smaller magnetic-field modulation amplitude over that reported previously (Cannon and Chasteen, 1975).

[1] Abbreviations used: NTA, nitrilotriacetate; EPR, electron paramagnetic resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

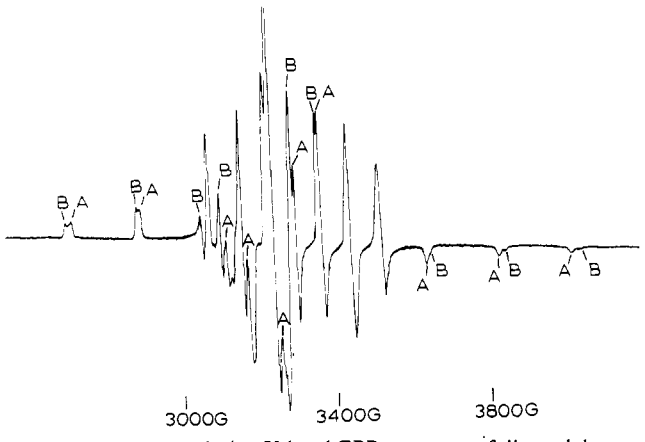

FIGURE 1: Frozen solution X-band EPR spectrum of divanadyl transferrin, 0.6 mM protein in 90% D_2O, pD = 8.4, 77 K. Instrument settings: power = 5 mW, modulation amplitude = 5 G, sweep rate = 2000 G/16 min, and time constant = 0.30 s. Resonances for the A and B metal ion environments are noted.

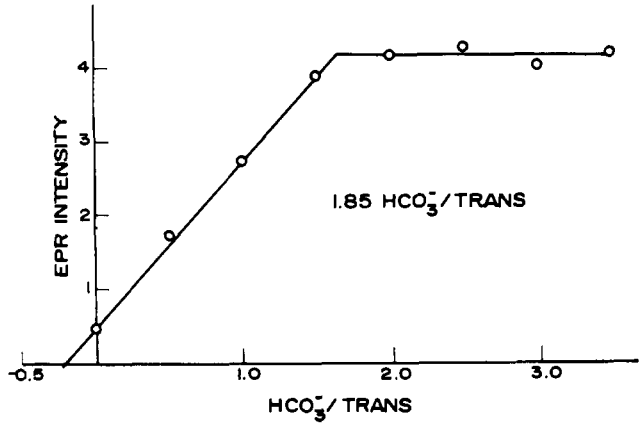

FIGURE 2: Dependence of the peak-to-peak first derivative intensity of the strong central line in the room-temperature EPR spectrum on added bicarbonate. The nonzero intercept corresponds to 0.30 mol of residual bicarbonate/mol of transferrin. Conditions: 0.21 mM protein, 25 °C, pH 8.2 (after).

The Anion Requirement. The room-temperature solution spectrum of divanadyl transferrin (Cannon and Chasteen, 1975) is characteristic of the VO^{2+} ion bound to a slowly tumbling macromolecule. The intensity of this spectrum in H_2O is greatly reduced (10 to 15% of original) when steps are taken to remove CO_2 prior to addition of the VO^{2+} ion. Addition of $NaHCO_3$ to the substantially CO_2-free VO^{2+}-transferrin mixture causes an immediate increase in the intensity of the room temperature solution EPR spectrum. The increase in signal intensity cannot be due to the variation in pH, since before the addition of HCO_3^- the pH was greater than 7.0 where VO^{2+} binding is complete. Figure 2 shows the peak-to-peak first derivative signal intensity of the strongest line in the spectrum as a function of added HCO_3^-. The signal grows linearly and then levels off. The nonzero intercept is due to the presence of residual CO_2 at the start of the titration. Use of the plot (Figure 2) as a standard additions curve yields 1.85 HCO_3^- per transferrin; that is, 0.92 HCO_3^- is required per VO^{2+} bound. Repeat experiments gave values of 0.94 and 0.96 HCO_3^- per VO^{2+} for solutions in the pH range 7.5 to 9.0. A titration performed at pH 6.6 (measured at the end of the experiment) yielded only 1.32 HCO_3^- per transferrin molecule, consistent with the previous observation that less than two

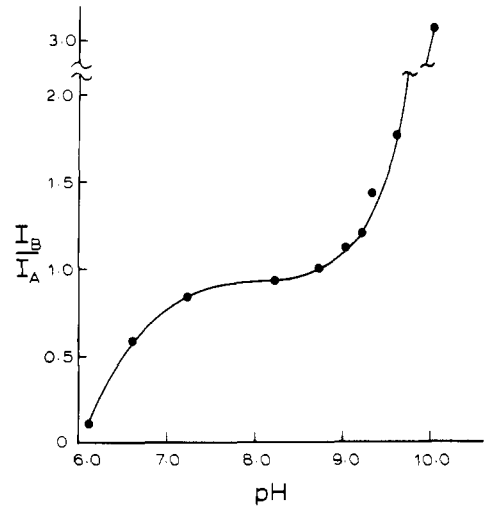

FIGURE 3: Plot of the ratio of the first derivative EPR intensities, I_B/I_A,[3] of the $M_I = -7/2$ low-field parallel lines of the A and B conformations as a function of pH. Each point represents a different sample preparation. Divanadyl transferrin concentration was typically 0.5 mM in 0.01 M $NaHCO_3$.

VO^{2+} ions bind to transferrin below pH 7 in 0.01 M $NaHCO_3$ (Cannon and Chasteen, 1975).

Titration of "bicarbonate-free" transferrin solutions containing 2.0 equiv of VO^{2+} at pH ~7.5 while monitoring the frozen solution spectrum reveals that the A and B resonances grow in at about the same rate. This indicates that the equilibrium constant for the reaction is essentially the same for either metal environment.

$$VO^{2+} + HCO_3^- + \text{Apo-TRN} \rightarrow VO^{2+}\text{-TRN-}CO_3^{2-} + 3\ H^+$$

In the presence of perchlorate, a *small* but definite preference of VO^{2+} for the B environment was noted (Cannon and Chasteen, 1975).

Conformational States. The nonequivalent metal ion environments can be best understood in terms of a difference in the conformational states of the two metal sites. Here, we use the broad term conformation to include geometrical changes and/or changes in the ligands bound to the metal.

Figure 3 shows the ratio of the intensities, I_B/I_A, as a function of pH for vanadyl-saturated transferrin.[2] In the pH range 7.5 to approximately 9.0, $I_B/I_A \sim 1$, corresponding to binding of one VO^{2+} ion in each environment. In the presence of perchlorate, the EPR spectrum is invariant in this pH range (Cannon and Chasteen, 1975). As the pH is increased above 9.0, the B resonances gain intensity at the expense of the A resonances. Titrations, such as previously described (Cannon and Chasteen, 1975), of the apoprotein with VO^{2+} while monitoring the room-temperature spectrum or the frozen solution spectrum reveal that nominally two VO^{2+} ions are bound at pH 10. The frozen solution spectrum at pH 10 is composed of almost all B resonances, as shown in Figurg 4B. The amount of A contributing to the spectrum can be estimated from the asymmetry in the lines. Deconvolution of the low-field parallel line yields $I_B/I_A = 3.1$ at pH 10.0.

[2] The peak height of the $M_I = -7/2$ low-field parallel line is a good measure of the amount of A and B present, since the line shape is nearly that of an absorption curve with a width at half-height, which is the same for both the A and B resonances. M_I labeling assumes a negative nuclear hyperfine interaction.

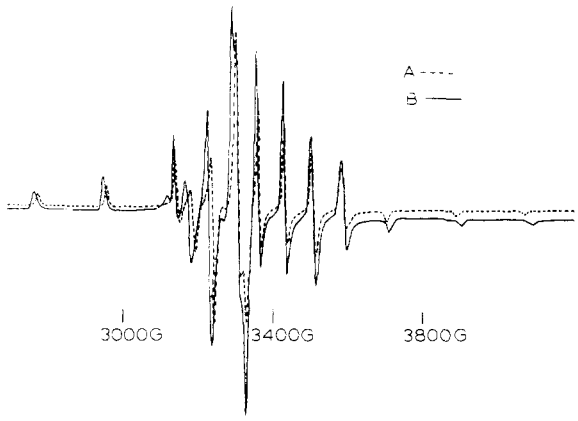

FIGURE 4: Frozen solution X-band EPR spectra of the A (- - -) and B (—) conformations of vanadyl transferrin. (A) 0.5 mM protein, 0.5 mM $VOSO_4$, pH 7.4, adjusted with NH_3(g) after removing HCO_3^- to less than 1 equiv remaining (see text). (B) 0.5 mM divanadyl transferrin, 0.01 M $NaHCO_3$, pH 10.0, 77 K.

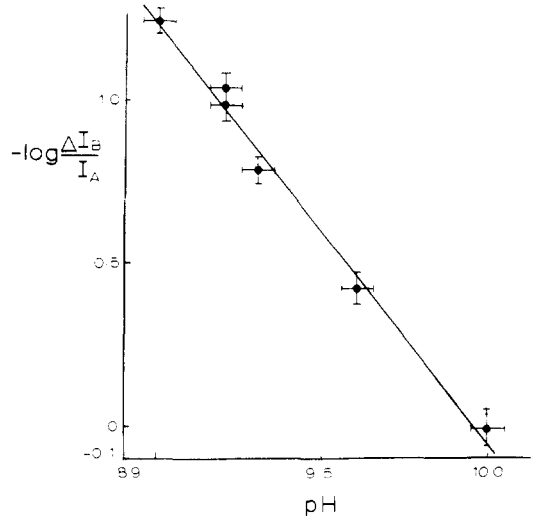

FIGURE 5: Henderson–Hasselbalch plot for the conversion of the A to the B conformation, $-\log(\Delta I_B/I_A)$ vs. pH. Linear regression line with a slope of -1.27 is shown. For a single ionizing functional group, the slope theoretically should be -1.00. pK = 10.0 ± 0.1. pH was adjusted with ammonia gas. Each point represents a different sample preparation.

The ratio $\Delta I_B/I_A$ can be used to determine the pK of the functional group responsible for the conformational change of A to B above pH 9.0. Here, ΔI_B is the increase in the intensity of the B resonances over that when A and B resonances are equally intense and is equal to $(I_B - I_A)/2$. A plot of $-\log(\Delta I_B/I_A)$ vs. pH is shown in Figure 5. The pH was adjusted with gaseous NH_3. Each point represents a different sample preparation. The data obey the Henderson–Hasselbalch equation quite well, pH = pK + log $(\Delta I_B/I_A)$ for a single ionizing group with an apparent pK = 10.0 ± 0.1.

As the pH is lowered below 7.0, the B resonances rapidly lose intensity, while the A resonances remain relatively constant. At pH ~5.9, only one VO^{2+} is bound; it displays an A spectrum. A plot of $-\log(I_B/(I_A - I_B))$ vs. pH shows that the protonation of a single functional group with an apparent pK = 6.6 ± 0.1 is at least partly responsible for the loss of VO^{2+} binding in the B conformation (Figure 6). Here, it is assumed that $I_A - I_B$ is proportional to the concentration of the functional group in the acid form; I_B is proportional to the concentration in the basic form. When D_2O is employed instead

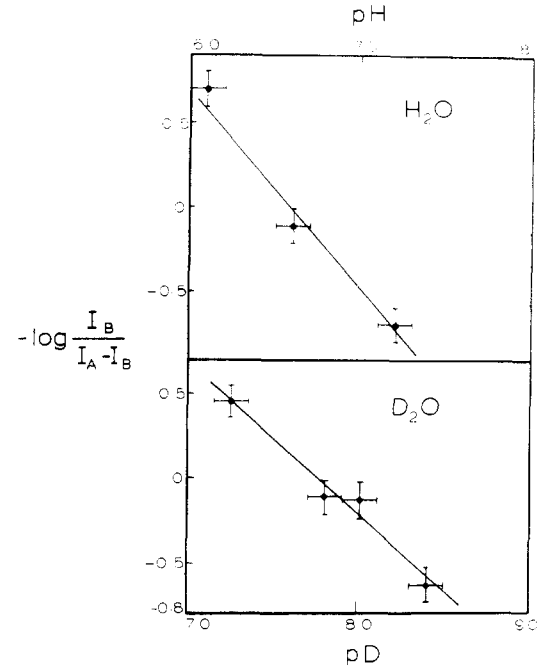

FIGURE 6: Henderson–Hasselbalch plot for the loss of binding at the B conformation. $-\log(I_A/(I_B - I_A))$ vs. pH (upper curve), vs. pD (lower curve). Slope of the linear regression line is -1.17 (upper curve), -0.911 (lower curve). pK = 6.6 (upper curve), 7.8 (lower curve). Each point represents a different sample preparation.

of H_2O, the apparent pK is elevated to pK = 7.8 on the pD scale (Figure 6).

There are 2.7 ± 0.1 H^+ released per VO^{2+} bound to the protein, regardless of the pH (6.0, 8.0, or 10.0). Under these different pH conditions, the EPR spectrum consists of only A resonances, A and B resonances, and nearly complete B resonances, respectively. Thus, both the A and B conformations release the same number of protons. Metal-ion hydrolysis as well as protein functional groups could be the source of protons. 2.6 H^+ are released per Fe^{3+} bound (Bates and Schlabach, 1973; Aisen et al., 1966, Aasa et al., 1963).

Like VO^{2+}, only one Fe^{3+} ion binds to transferrin at pH ~5.8 (Princiotto and Zapolski, 1975; Lestas, 1976). When Fe^{3+} was added as a fresh $Fe(NO_3)_3$ solution to a pH 4.0 protein solution and the pH was raised to 6.0 with microliter increments of 0.1 M NaOH and 0.2 M $NaHCO_3$, the formation of an orange color was noted within seconds.[3] The sample was refrigerated overnight and any unbound Fe^{3+} was removed by dialysis against 0.1 M succinate pH 6.0 buffer. The succinate was removed by dialysis against pH 9.0, 0.02 M $NaHCO_3$. The Fe/transferrin solution was then buffered at pH 7.5 with 0.1 M Hepes,[4] followed by flushing with prepur-

[3] This procedure is reported by Bates and Schlabach (1973). Also, in accord with this reference, we observe that NTA binds strongly to the protein when Fe^{3+} is added as a 1:2 Fe^{3+}–NTA complex. This leads to a vanadyl–NTA complex when the vanadyl label is added. Our attempts to remove the NTA by using Sephadex G-25 columns eluted with pH 6.0 succinate buffer or pH 7.5 Tris buffer were unsuccessful. We also attempted to remove NTA from the monoferric transferrin complex (prepared at pH 6.0) by dialysis at pH 6.0 with 0.1 M $NaClO_4$, followed by dialysis against 0.02 M $NaHCO_3$ (pH 9.0) to remove the $NaClO_4$. This procedure gave broad EPR spectra in which the A and B features could not be resolved when the 1 equiv of vanadyl label was added at pH 8.0.

[4] We thank Professor Daniel Harris for the preprint of his manuscript where he suggests the use of Hepes buffer. The 0.1 M Hepes buffer serves to sharpen the vanadyl EPR spectrum, allowing better resolution of the A and B features.

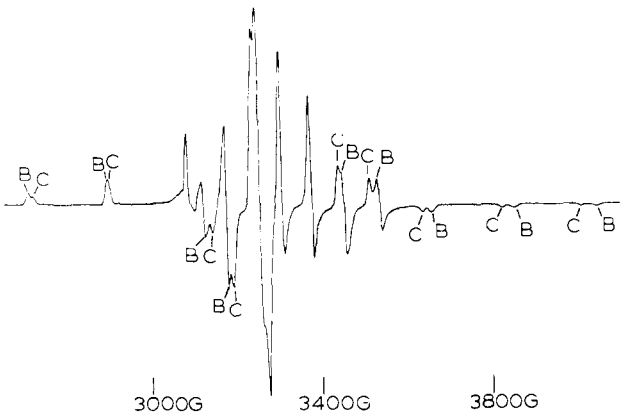

FIGURE 7: Frozen solution X-band EPR spectrum of divanadyl transferrin at pH 10.7; 0.52 mM protein, 0.01 M $NaHCO_3$, 77 K. C resonances are observed in addition to the B resonances. Instrument conditions: power = 5 mW, sweep rate = 2000 G/30 min, modulation amplitude = 5 G, and time constant = 0.3 s.

ified nitrogen gas. Addition of 1 equiv of VO^{2+} resulted in a clean, sharp B-type vanadyl spectrum in frozen solution, similar to that shown in Figure 4B.[4] *Under these conditions,* in the absence of iron, an A, B type vanadyl spectrum is obtained. These results suggest that at pH ~6, Fe^{3+} binds in the environment that would otherwise be occupied by VO^{2+} at this pH and which exhibits the vanadium A resonances.

When the pH is increased from 10.0 to 10.7 with gaseous ammonia, the vanadyl spectrum dramatically changes from that in Figure 4B to the one in Figure 7 in which a new set of resonances, C, are observed. A similar but less well-resolved anisotropic spectrum is obtained with room-temperature solutions, which indicates that the VO^{2+} responsible for the C resonances is bound to the protein. The spectrum in Figure 7 can only be obtained when the VO^{2+} ion is introduced at pH 10 or below, where it binds at the specific sites, followed by elevation of the pH. Hydrolysis of the VO^{2+} ion at pH 10.7 appears to interfere when the metal is introduced to the apoprotein at this pH and no spectrum is obtained. This new spectrum has not been examined in detail; however, it probably is due to a third conformation of the metal site. The fact that the VO^{2+} ion must be first bound to the specific sites before elevation of the pH to produce the C resonances suggests that nonspecific binding is probably not responsible for the new spectrum.

Discussion

One fundamental question in transferrin chemistry is the extent to which the two metal binding sites differ structurally and functionally. The two sites in diferric human transferrin differ in their ability to donate iron to rabbit reticulocytes (Fletcher and Huehns, 1967; Harris and Aisen, 1975a) and exchange carbonate at different rates (Aisen et al., 1973). On the other hand, human transferrin donates iron to human reticulocytes equally from both sites (Harris and Aisen, 1975c). The two sites appear to be equivalent or nonequivalent depending on the metal derivative and the spectroscopic or thermodynamic method employed (Luk, 1971; Gafni and Steinberg, 1974; Binford and Foster, 1974; Aasa and Aisen, 1968; Aisen et al., 1969; Aasa, 1972; Cannon and Chasteen, 1975). These apparent discrepancies could well arise from changes in conformational states of the two metal sites in which they are equivalent or nonequivalent, depending on the circumstances.

Our data is best interpreted in terms of a conformational difference between the two sites, 1 and 2, of the vanadyl transferrin molecule. Results with mixed VO^{2+}-Fe^{3+} transferrins reported here and elsewhere (Cannon and Chasteen, 1975) suggest that similar conformations exist for iron transferrin as well. There are at least two explanations of the data for the VO^{2+} system. First, with the vanadyl system between pH 7.5 and 9.0, sites 1 and 2 exist in the A and B conformations, respectively, i.e. A-B (the first letter denotes site 1 and the second site 2). Above pH 9.0, site 1 begins to undergo a conformational change from A to B. When this is complete, the molecule exists in the B-B configuration. This change is coupled to the ionization of a single functional group with a pK = 10.0 ± 0.1, which is "associated" with site 1. Tyrosine or arginine is a likely candidate. In the second explanation, both sites exist in A and B conformations in the pH range 7.5 to 9.0; the molecular configurations are A-B and B-A (which are equally stable), but not B-B or A-A. In order for both sites within the same molecule to exist in the B conformation, B-B, the pK = 10 functional group must be ionized. The absence of a B-B configuration below pH 9 precludes significant amounts of the A-A configuration, since the A and B signals are observed to be about equally intense in the pH range 7.5 to 9.0. The present data do not enable us to distinguish between the above two explanations.

Upon ionization, the pK = 10 functional group does not appear to coordinate to the metal, although we cannot be certain on this point. This tentative conclusion is based on the observation that an additional proton is not released when the VO^{2+} ion binds in the B conformation.

B-conformation binding is lost below pH ~7.0. The loss is complete at pH ~6.0 and is governed by a functional group, possibly the anion, with an apparent pK of 6.6 in H_2O. This pK is elevated to pK = 7.8 in 90% D_2O. Isotope effects on the pK's of functional groups are well known (Li et al., 1961). Invariably, pK's are raised by D_2O with increases in the range of 0.33 to 0.80 for a variety of functional groups, including those of amino acids and proteins (Li et al., 1961, Meadows, 1972). The larger than expected isotope shift in the pK for transferrin relative to known substances suggests that more is involved in the release of the metal than simply the protonation of a functional group. Other structural changes likely occur which help to trigger the release of the metal. It is known that different hydrogen-bonding characteristics of D_2O and H_2O affect the stability of proteins and their conformations (Hvidt and Nielsen, 1966). Conformational differences between diferric, monoferric, and apotransferrin do exist (Bezkorovainy, 1966, Rosseneu-Motreff et al., 1971; Lane, 1971, 1973) and the relative stabilities of these conformations undoubtedly are affected by D_2O.

One can account for the spectral differences between the A and B conformations in at least three ways. (1) The geometrical arrangement of the ligands about the metal is different in the two environments. (2) In the B conformation, an additional protein ligand, which is already ionized, coordinates to the metal with a concomitant displacement of a water molecule or expansion of the coordination sphere. (3) One protonated functional group displaces another in going from A to B such that the total number of protons released upon metal binding is the same for either conformation.

One remarkable feature of the transferrin molecule is the equal or near equal affinities of the A and B conformations for metals at physiological pH (despite the obvious differences in the metal environments as manifested in the vanadyl EPR spectrum). On the other hand, a reduction in pH causes a selective release of iron and vanadyl from the B conformation.

These observations are consistent with one proposed model for the regulation of iron metabolism (Fletcher and Huehns, 1968) in which uptake of iron is homogeneous with respect to the metal sites while release is heterogeneous.

Of particular interest is the role of the anion in metal binding. It has been established that bicarbonate (or carbonate) binding to the protein is 1:1 with Fe^{3+}, Cu^{2+}, Cr^{3+}, Mn^{3+}, Co^{3+} (Aisen et al., 1969; Bates and Schlabach, 1975), and VO^{2+} (vide supra). Given the four to six protein ligands at the metal site, it is surprising that such a diversity of metal ions all require an anion in order for the metal to bind at the specific sites of the protein. It is plausible that the anion, through binding to the protein, neutralizes a positively charged group, such as an arginine, or induces a conformational change which brings the ligands into the correct geometrical configuration for metal ion coordination or both (Gaber et al., 1974; Schlabach and Bates, 1975; Bates and Schlabach, 1973).

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