Studies of Anion Binding by Transferrin Using Carbon-13 Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The ^{13}C NMR spectra of apotransferrin and the Co^{3+} and Fe^{3+} complexes of transferrin with bound ^{13}C-enriched (bi)carbonate have been studied at 68 MHz. Information has been obtained about the mechanism of metal binding, the spatial relationship of the metal and the anion binding sites, the ionization state of the anion, the protein ligation of the anion, and differences in the properties of the two anion binding sites. The spectrum of the $Co^{3+}{}_2$ complex contains a doublet resonance due to nonexchanging anion and three resonances due to exchanging anions. The nonexchanging anion is bound at the B site, and on the basis of its chemical shift value and its pH behavior we concluded that it is carbonate. The exchanging anions are bound at the A site, and they are assigned to bound bicarbonate and a protein–carbamino adduct. In the spectra of the $Fe^{3+}{}_2$ complex, no resonances corresponding to specifically bound anion are observed since these ^{13}C resonances are broadened beyond detection by interaction with the paramagnetic Fe^{3+}. In a previous study at 25 MHz, it was similarly observed that the ^{13}C resonances were broadened beyond detection, but due to limitations of the signal to noise ratio it was only determined that the metal–anion distance is less than 9 Å [Harris, D. C., Gray, G. A., & Aisen, P. (1974) *J. Biol. Chem. 249*, 5261–5264]. In the present study, it is calculated that the Fe^{3+}–C distance must be less than 4.9 Å, which implies that the anion is directly bound to the Fe^{3+} in the physiologically important Fe^{3+}–transferrin–carbonate complexes. Two resonances are observed in the spectra of apotransferrin which appear to be due to specific anion binding. This indicates that anion binding to transferrin occurs prior to metal binding and that anion binding may be a preliminary step for metal binding. On the basis of the spectra and their pH behavior, it is concluded that the anion binding ligand at the B site is probably a guanidino group of arginine, while that at the A site may be either a guanidino group of arginine or an ϵ-amino group of lysine. The difference in the properties of the two anion sites and the state of the anion bound at the two sites could explain the functional difference in their iron-donating properties.

Transferrin, the iron-transport protein of human serum, has two metal binding sites, each of which binds a ferric ion only when a stereochemically suitable anion is concomitantly bound (Warner & Weber, 1953; Aisen et al., 1967; Price & Gibson, 1972; Bates & Schlabach, 1975). The metal binding sites are capable of binding a number of di- and trivalent metal ions, including Co^{3+} and Cu^{2+} (Aasa & Aisen, 1968; Aisen et al., 1969; Zweier & Aisen, 1977). The role of the anion in Fe^{3+} binding and release has been questioned, and there is evidence that the mechanism for the delivery of iron to the reticulocyte involves an attack by the reticulocyte on the bound anion (Aisen & Leibman, 1973). A number of studies have been performed to determine the spatial relationship of the bound metal and anion (Harris et al., 1974; Harris & Aisen, 1975b; Najarian et al., 1978). Recently, it has been demonstrated that the anion in the Cu^{2+}–transferrin–oxalate complex is directly bound to the metal (Zweier et al., 1979). However, it has not been determined if the anion is bound to Fe^{3+} in the physiologically important Fe^{3+}–transferrin–carbonate complex. The state of the bound anion is not known; it may be carbonate, bicarbonate, or some other form (Warner & Weber, 1953; Aisen & Brown, 1977). The identity of the ligands involved in anion binding is also not known.

Recently, techniques have been developed for preparing and characterizing single-site transferrin complexes (Aisen et al., 1978). Using these methods, it has been determined that the two sites have a number of different properties. The A site binds Fe^{3+} and Cu^{2+} at a lower pH than the B site (Princiotto & Zapolski, 1975; Zweier, 1978). Ferric iron added as a nitrilotriacetate chelate binds preferably to the A site, whereas Fe^{3+} added as a citrate or oxalate chelate binds preferably to the B site (Aisen et al., 1978).

The ^{13}C NMR technique was used several years ago by Harris et al. (1974) in a specific attempt to determine if the anion is directly bound to the Fe^{3+} ion. Due to severe limitations of the signal to noise ratio attainable with the instrumentation available at that time, they were unable to answer this question, although they showed that the Fe^{3+}–anion distance was less than 9 Å. In the Initial study, it was also found that while the anion bound at one site of the $Co^{3+}{}_2$–transferrin complex exchanges rapidly with free bicarbonate the anion bound at the other site does not exchange; only the ^{13}C resonance of one site, the site whose anion did not exchange, was observed.

In the present work, we have repeated the experiments performed previously, but we have used a modern high-field NMR spectrometer capable of attaining a signal to noise ratio as much as a factor of 100 above that obtained in the initial study. This enabled us to determine if the anion is in fact directly bound to the Fe^{3+} ion. In addition, resonances of anions bound at both transferrin binding sites are observed, assigned, and studied in the present work. The techniques of preparing and characterizing single-site transferrin complexes recently introduced have been used to determine differences in the two anion binding sites and the state of the anion bound at each site.

Experimental Procedures

Materials. Purified human transferrin, either obtained from Behring Diagnostics or prepared from Cohn fraction IV-7 (Aisen et al., 1966), was freed of metal ions and chelating

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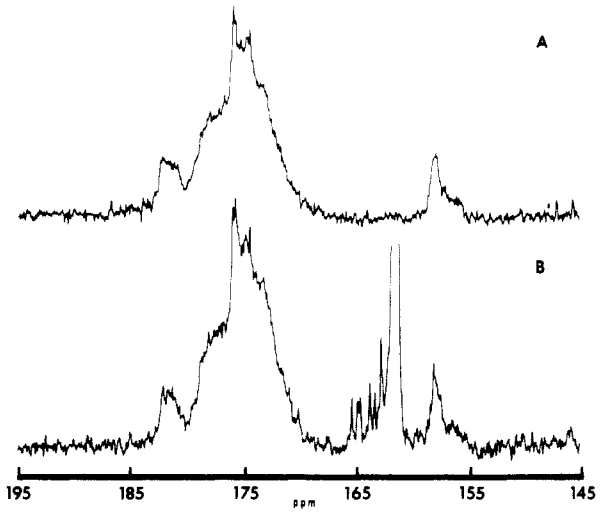

FIGURE 1: (A) Carbonyl region of a 67.9-MHz ^{13}C NMR spectrum of a preparation of apotransferrin, pH 7.4. (B) Same as in (A), with excess [^{13}C]carbonate added.

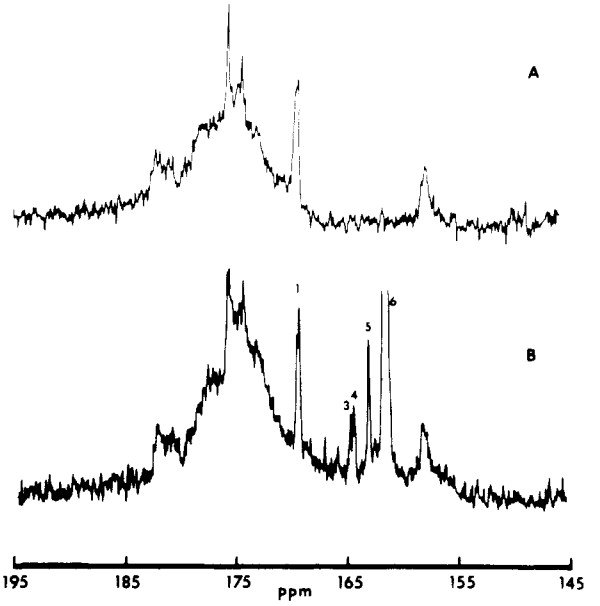

FIGURE 2: (A) Carbonyl region of a 67.9-MHz ^{13}C NMR spectrum of a preparation of Co^{3+}_2–transferrin prepared in the presence of excess $^{13}CO_3^{2-}$ which was then dialyzed out, final pH 7.4. A sharp resonance is observed at 169.4 ppm (1) with a line width of 28 Hz, and this resonance corresponds to nonexchanging ^{13}C-enriched anion bound at the B site. (B) Preparation in (A) with excess $^{13}CO_3^{2-}$ added and pH adjusted to 7.4. Three new resonances are observed in addition to the large resonance of free bicarbonate. At 163.0 ppm (5), a resonance is seen with an amplitude approximately equal to that of the resonance of the nonexchanging bound anion while at 164.4 (4) and 164.7 (3) ppm two smaller overlapping resonances are seen. These resonances correpond to anion bound at the A site.

agents by accepted methods (Bates & Schlabach, 1973). The protein was brought to a final concentration of (1.5–3.0) × 10^{-3} M, taking the millimolar absorption coefficient at 280 nm as 83.8, by a vacuum ultrafiltration with a Schleicher & Schuell collodion bag suction apparatus. The buffer used in all experiments was 0.05 M *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) and 0.1 M KCl.

The Co^{3+} complexes of transferrin were prepared by adding the appropriate amount of $CoCl_2$ standard to a solution of transferrin containing 0.001% H_2O_2 (by weight) and an excess of $^{13}CO_3^{2-}$ or $^{12}CO_3^{2-}$. This mixture was left in a stoppered tube overnight at 4 °C and then dialyzed twice against 500-mL portions of buffer. The diferric complexes were prepared by adding the iron as Fe^{3+}–nitrilotriacetate (1:2), pH 4.5, in the presence of a large excess of $^{12}CO_3^{2-}$ or $^{13}CO_3^{2-}$.

Monoferric A-site transferrin was prepared by using a method described previously (Zweier, 1978) similar to that of Princiotto & Zapolski (1975). These preparations were verified to be pure A-site monoferric transferrin by using both urea gel electrophoresis (Aisen et al., 1978) and Cu^{2+} probe EPR (Zweier, 1978).

The $CoCl_2$ standard was obtained from Varian, and anhydrous $K_2{}^{13}CO_3$ (90% enriched) was purchased from either Thompson-Packard or Stohler Isotope Chemicals. Doubly distilled water was used in all experiments, and reagents were of the highest quality commercially obtainable.

Methods. Proton-decoupled ^{13}C NMR spectra were obtained at 67.89 MHz on the NIH 270-MHz spectrometer, which is a modified system based on a Bruker 6.3 T superconducting magnet and a Nicolet 1180 data system. A minimum of 30 000 scans were obtained per spectrum, which resulted in 16-h accumulations when 16K data points and a 2-s pulse delay were employed. In some spectra, a 16K zero filling was made to allow a 32K transform for maximum digital resolution. A spectral window of ±7500 Hz and quadrature detection was employed throughout. In most of the spectra, a 3-Hz filter function was applied. The sample tubes were maintained at a temperature of 15 °C during the spectral acquisition.

Chemical shifts were measured with respect to a dioxane standard but are reported relative to tetramethylsilane (Me_4Si), the widely accepted ^{13}C chemical shift reference (Stothers, 1972; Shindo et al., 1977). The chemical shift of dioxane with respect to Me_4Si is 67.4 ppm.

Results

Apotransferrin. The spectra of preparations of apotransferrin possess broad natural-abundance ^{13}C resonances which are characteristic of a protein (Egan et al., 1977). All the observed resonances are quite broad with line widths greater than 100 Hz (Figure 1A). When a large excess of $^{13}CO_3^{2-}$ is added to the apotransferrin at pH 7.4, a large resonance is observed due to $H^{13}CO_3^-$ at 161.4 ppm, and several small resonances are also observed. Two of these resonances, those at 162.4 and 164.9 ppm, have intensities greater than the others (Figure 1B). The intensities of each of these two resonances correspond to approximately 0.2 ^{13}C/transferrin while each of the other resonances corresponds to less than 0.1 ^{13}C/transferrin. These two resonances are not seen in similar preparations of diferric transferrin while all of the other resonances are (Figure 4B). When Fe^{3+} is added to these apotransferrin preparations, these two resonances disappear while the others remain unchanged. Therefore, these two resonances may be due to a specifically bound anion at transferrin's two anion binding sites.

Dicobalt Transferrin. Preparations of Co^{3+}–transferrin which have been dialyzed to remove excess $^{13}CO_3^{2-}$ give spectra with a clear sharp peak at 169.4 ppm with a line width of 28 Hz (Figure 2A). This peak is not seen in the spectrum of complexes of apotransferrin, or in the spectrum of complexes of Co^{3+}–transferrin formed in the presence of $^{12}CO_3^{2-}$. By comparing the intensity of this peak to that of a standard of HCO_3^- of known concentration, it was determined that this peak corresponds to 1 ^{13}C/transferrin molecule. Therefore, this sharp resonance corresponds to a nonexchanging bound $^{13}CO_3^{2-}$ or $H^{13}CO_3^-$ at one of the transferrin's anion binding sites. The chemical shift of this peak remains constant at 169.4 ppm over the pH range 7.0–11.0, and the intensity of this peak also remains constant, with no observed decrease in the in-

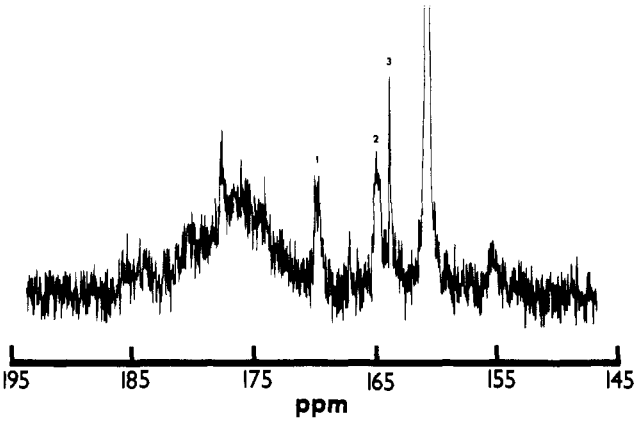

FIGURE 3: Carbonyl region of a 67.9-MHz ^{13}C NMR spectrum with higher point resolution and no applied filter function of a preparation of $Co^{3+}{}_2$–transferrin in the presence of excess $^{13}CO_3^{2-}$, pH 8.8. The nonexchanging anion resonance at 169.4 ppm (1) is demonstrated to be a doublet. At 164.7 ppm (3), a resonance is seen with an amplitude almost twice that of the doublet, and this resonance corresponds to exchangeably bound anion at the A site. A nonspecific protein carbamino resonance (2) is also observed at 165.5 ppm.

Table I: Chemical Shifts (ppm) for the Peaks (1-6) of Co^{3+}-Transferrin

	protein-bound anion					free anion $HCO_3^- \rightleftharpoons CO_3^{2-}$
	nonexchanging	exchanging				
pH	1	2	3	4	5	6
7.4	169.4		164.7	164.4	163.0	161.4
7.6	169.4		164.7	163.7	162.6	161.4
7.7	169.4		164.7	163.7	162.5	161.4
8.3	169.4		164.7			161.4
8.8	169.4	165.5	164.7			162.0
9.2	169.4	165.5	164.7			162.2
9.7	169.4	165.5				
10.2	169.4	165.5				166.7
11.3	169.4	165.5				169.4

tensity of this peak up to pH 11.0. Spectra taken with greater point resolution show that this peak is actually a doublet consisting of two peaks with line widths of 12 Hz separated by 16 Hz (Figure 3). The relative intensities of the two components of the doublet vary with pH. A 1:1 ratio is observed at pH 8.8, while below pH 8.8 the higher field component predominates and above pH 8.8 the lower field component predominates.

If the excess $^{13}CO_3^{2-}$ is not dialyzed out, or if excess $^{13}CO_3^{2-}$ is added after dialysis to preparations at pH 7.4, a peak due to $HCO_3^- \rightleftharpoons CO_3^{2-}$ and an additional resonance is observed at 163.0 ppm with a line width of 14 Hz and an amplitude about equal to that of the nonexchanging anion resonance. Two smaller resonances are also seen at 164.4 and 164.7 ppm (Figure 2B). These resonances are not seen in similar preparations of apotransferrin (Figure 1B) or diferric transferrin (Figure 4B), and the intensities of these three peaks sum to 1 ^{13}C/transferrin. Therefore, these resonances appear to correspond to specifically bound anion. In addition, these resonances disappear when Fe^{3+} is added to these preparations; the Fe^{3+} displaces the bound Co^{3+} as indicated by the change in the optical absorption spectra. As the pH is raised over the range 7.4–9.0, the chemical shift of these peaks changes, and the intensity of each of these peaks changes while the summed intensity remains constant (Table I). At pH 8.8, only a single resonance is seen at 164.7 ppm with a line width of 10 Hz, whose amplitude is twice that of the doublet resonance of the nonexchanging anion (Figure 3). This resonance is not observed in similar preparations of apotransferrin or diferric transferrin at pH 8.8, and it also disappears when Fe^{3+} displaces the Co^{3+} bound at transferrin's two sites. The intensity of this resonance corresponds to 1 ^{13}C/transferrin; therefore, this resonance may correspond to exchanging bound anion at one of transferrin's specific sites. Above pH 8.8, an additional resonance appears at 165.5 ppm, corresponding to more than 1 ^{13}C/transferrin (Figure 3). This resonance is also observed in preparations of apotransferrin and diferric transferrin above pH 8.8 so it is probably due to nonspecific [^{13}C]carbamino derivatives of transferrin (see Discussion).

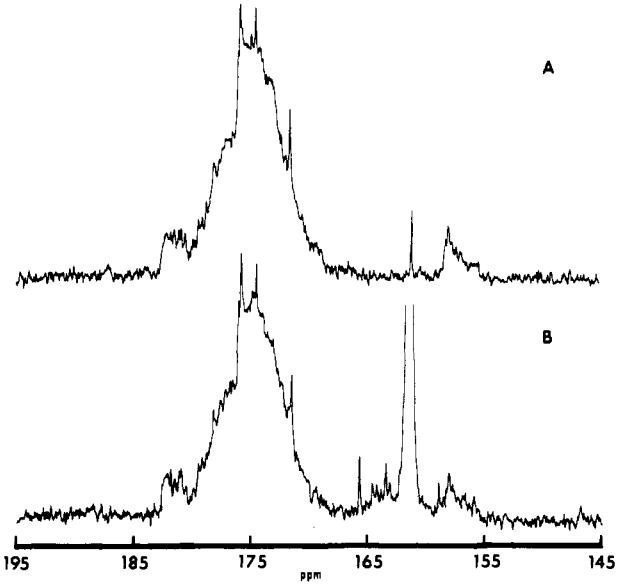

FIGURE 4: (A) Carbonyl region of a 67.9-MHz ^{13}C NMR spectrum of a preparation of $Fe^{3+}{}_2$–transferrin prepared in the presence of an excess of natural-abundance carbonate, pH 7.4. (B) As in (A) but prepared in the presence of an excess of ^{13}C-enriched carbonate.

Diferric Transferrin. Complexes of diferric transferrin formed in the presence of excess $^{13}CO_3^{2-}$ give rise to a spectrum which appears to be identical with similar complexes prepared in the presence of an excess of $^{12}CO_3^{2-}$. Only the broad resonances of the natural-abundance ^{13}C present in the protein's amino acids are observed, and no resonance peak due to bound anion was apparent (Figure 4). The signal to noise ratios obtained in these spectra were as high as 40:1, assuming the signal to be of the amplitude of the nonexchanging peak of the Co^{3+}–transferrin spectrum. This indicates a line width of greater than 1120 Hz for the resonance of the bound anion. Since the $Fe^{3+}{}_2$–transferrin complex has two nonexchanging molecules of bound anion, rather than one as in the Co^{3+} complex (Harris et al., 1974), one might expect the signal amplitude to be as much as twice that of the Co^{3+} complex. Therefore, the line width may actually be greater than 2240 Hz. The difference spectrum [$Fe^{3+}{}_2$–transferrin–(^{13}C anion)$_2$] – [$Fe^{3+}{}_2$–transferrin–(^{12}C anion)$_2$] was plotted in an effort to observe a broad ^{13}C anion signal in the absence of the broad background protein resonances, and only a flat base line was observed.

When a large excess of $^{13}CO_3^{2-}$ is added to preparations of diferric transferrin at pH 7.4, a large $H^{13}CO_3^- \rightleftharpoons {}^{13}CO_3^{2-}$ peak appears, and several small-amplitude peaks are observed, whose intensities sum to less than 0.2 ^{13}C/transferrin (Figure 4B). The three exchangeable peaks seen in the spectrum of the Co^{3+} complexes are not observed, and this confirms that these peaks correspond to specifically bound ^{13}C anion. The small exchangeable peaks observed in the diferric complex probably correspond to nonspecific ionic binding of HCO_3^-

to postively charged protein groups.

Monoferric Monocobalt Transferrin. Single-site Co^{3+} complexes were prepared in the presence of excess $^{13}CO_3^{2-}$ by using transferrin with the A site occupied by Fe^{3+}. In spectra of these complexes, the nonexchanging anion resonance is observed with an intensity corresponding to 1 ^{13}C/transferrin while the three exchanging resonances are not observed even when excess $^{13}CO_3^{2-}$ is added. Thus, it appears that the B site is the site at which the anion does not exchange in the Co^{3+} complexes while the A site is the site at which exchange does occur.

The line width of the nonexchanging anion resonance was carefully measured from a spectrum plotted with no filter function applied, and a value of 28 Hz was determined, which is identical with the value observed in preparations of dicobalt transferrin. The error in line width measurements is less than 1 Hz, so the paramagnetic broadening of the ^{13}C resonance of the anion bound at the B site from Fe^{3+} bound at the A site is less than 1 Hz.

Monocobalt Transferrin. Preparations of Co^{3+}–transferrin were prepared in the same manner as the $Co^{3+}{}_2$ complexes, adding 1 Co^{3+}/transferrin instead of 2 Co^{3+}/transferrin. The spectrum of these complexes showed the nonexchanging anion resonance with an amplitude of 0.8 ^{13}C/transferrin while the exchanging resonances were not seen. This indicates that Co^{3+} preferentially binds at the B site when the complex is formed by oxidation of $CoCl_2$.

Discussion

Spatial Relationship of the Metal and Anion. Comparison of the spectra of the Fe^{3+}–transferrin complexes to those of the Co^{3+}–transferrin complexes indicates that there is a large paramagnetic broadening of the ^{13}C anion resonance due to the Fe^{3+}, in the Fe^{3+}–transferrin complexes. No ^{13}C anion resonance was observed in either the $Fe^{3+}{}_2$–transferrin–^{13}C anion complexes or the $^{13}C - {}^{12}C$ difference spectra. This indicated that the Fe^{3+} broadens the ^{13}C anion resonance by more than 1120 Hz. By use of the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957; Mildvan & Cohn, 1970), an upper limit can be determined for the Fe^{3+}–anion distance. This calculation was performed as described previously (Harris et al., 1974) with the contact term set to zero, since the magnitude of this term is significant only if the anion is directly bound to the metal. It was determined that the Fe^{3+}–^{13}C distance must be less than 4.9 Å. The carbonyl bond length in carbonate or bicarbonate is 1.3–1.4 Å (Edsall, 1969), and typical metal–ligand bond lengths are in the range 2–3 Å (Freeman, 1967). Thus, the maximum distance of the carbonyl oxygen from the Fe^{3+} (3.6 Å) is so close to the bond length which would be expected if the Fe^{3+} was bound to carbonate or bicarbonate that one may infer that the Fe^{3+} is bound directly to the anion.

State of the Bound Anion. It was first discovered in 1953 that a synergistic anion is necessary for the binding of iron to the transferrins (Warner & Weber, 1953). The physiological anion bound to transferrin was identified as some form of CO_2 in equilibrium with water, since in CO_2-free preparations transferrin does not specifically bind Fe^{3+} or other metal ions. The anion is thought to be bound as either carbonate or bicarbonate (Aisen & Brown, 1977). Over the last 30 years, although many studies of the anion dependence of transferrin have appeared, the form of the bound anion is still not known. The ^{13}C NMR spectra of ^{13}C-enriched carbonate can provide direct information regarding the state of the bound anion which cannot be obtained by the indirect measurements used in previous studies (Warner & Weber, 1953; Aisen et al., 1973a,b; Gaber et al., 1974; Cannon & Chasteen, 1975; Schlabach & Bates, 1975; Zweier & Aisen, 1977; Zweier, 1978), since carbonate, bicarbonate, and the other forms of CO_2 in equilibrium with water have very different chemical shift values.

Since the ^{13}C anion resonance is broadened beyond detection, no information can be inferred about the ferric transferrin complexes. However, the ^{13}C anion resonances are clearly seen in the diamagnetic Co^{3+}–transferrin complexes. The ^{13}C anion bound at the B site of transferrin has a sharp resonance observed at 169.4 ppm. The chemical shift of this resonance is close to that of free carbonate, which is 169.6 ppm, but far from that of bicarbonate, which is 161.4 ppm (Morrow et al., 1974). The effect of positive ligands which may bind to the anion is to shift the resonance downfield by up to 4 ppm (Pople et al., 1959; Levy, 1976; Wüthrich, 1976), while the effect of Co^{3+} bound to the anion is to shift the resonance upfield (Stothers, 1972). If this resonance was due to bound bicarbonate, the shift downfield due to the ionic binding to positive protein ligands would have to be 8.2 ppm + the magnitude of the upfield shift due to Co^{3+}, which is more than twice the largest expected chemical shift value of 4 ppm. Thus, the chemical shift values of this resonance can be assigned to bound carbonate but not bicarbonate. In addition, the chemical shift of this resonance is constant over the pH range 7.0–11.2, and its intensity remains constant as 1 ^{13}C/transferrin up to pH 10.5. If this resonance was due to bicarbonate, one might expect it to shift or decrease in amplitude upon raising the pH.

High-resolution spectra of this nonexchanging resonance show that it actually consists of two overlapping peaks of line width 12 Hz. This doublet must be due to two peaks with slightly different chemical shift values rather than a hyperfine splitting since 25-MHz NMR spectra show only one peak with a line width of 14 Hz (Harris et al., 1974). In addition, the relative amplitude of the two peaks varies as a function of pH. Therefore, there must be two conformations at the B site which result in slightly different electron shielding at the ^{13}C nucleus of the bound carbonate. This is consistent with electron paramagnetic resonance studies of the Cu^{2+} complexes of transferrin, which have also determined that the B site exhibits two conformations for the binding of Cu^{2+} (Zweier, 1978).

Since carbonate is negatively charged, it is probably electrostatically bound to the protein by a positively charged basic amino acid group. The pH dependence of the binding of carbonate at the B site can be used to obtain information about which ligands make up this anion binding site. Up to pH 10.5, less than 10% of the specifically bound carbonate is released as the intensity of the nonexchanging [^{13}C]carbonate resonance still corresponds to 1 ^{13}C/transferrin molecule. If the anion binding ligand is assumed to deprotonate in accordance with the Henderson–Hasselbalch equation, the pK would have to be greater than 11.5. The guanidino group of arginine and the ϵ-amino group of lysine are ligands which could be involved in ionic binding of the carbonate anion. The guanidino group of arginine has a pK of 12.5, with a range in proteins from 11.5 to 13.0, while the ϵ-amino group of lysine has a pK of 10.5, with a range in proteins from 9.6 to 10.7 (Steinhart & Reynolds, 1969). Therefore, the ligand involved in the binding of carbonate at the B site is probably the guanidino group of arginine. This is consistent with chemical modification experiments, which have demonstrated that modification of arginine results in a decrease in iron binding, and with amino acid sequence data, which have identified two homologous regions, each containing an arginine, postulated to be the loci

of the metal binding sites (MacGillivrary & Brew, 1975; Rogers et al., 1977, 1978).

The exchanging anion peaks which correspond to anion bound at the A site have chemical shift values in the range 164.7–162.5 ppm. Over the pH range 7.4–8.3, two sets of exchangeable peaks are observed just downfield of the free $HCO_3^- \rightleftarrows CO_3^{2-}$ resonance (Table I, second and third columns from right). These peaks shift as the pH is raised, and their amplitude varies with the pH value. These two peaks are demonstrated to correspond to anion specifically bound at the A site of transferrin since they are not observed in similar preparations of A-site monoferric transferrin. The chemical shift values of these peaks, which are close to those of free bicarbonate, and their pH behavior suggest that they probably correspond to bound bicarbonate rather than carbonate. Over the pH range 7.4–9.7, another exchanging anion peak is observed at 164.7 ppm. This resonance is also demonstrated to correspond to anion bound at the A site since it is not seen in preparations of A site monoferric transferrin. As the pH is raised, the intensity of this resonance increases up to 1 ^{13}C/transferrin while the intensities of the other two resonances decrease correspondingly. The chemical shift value of this resonance is close to that expected for protein–carbamino adducts (Morrow et al., 1974, 1976). Thus, both the pH dependence and the chemical shift values are consistent with this peak being a protein–carbamino derivative.

Above pH 8.8, another peak is observed with a chemical shift value of 165.5 ppm, and the amplitude of this peak also increases as the pH is raised. This peak corresponds to nonspecifically bound ^{13}C anion since its intensity is greater than 2 ^{13}C/transferrin, and it is seen in preparations of apotransferrin and diferric transferrin. The chemical shift value and its pH dependence suggest that this peak also corresponds to protein–[^{13}C]carbamino derivatives. Therefore, it appears that the exchanging anion bound at the A site may be bound primarily either as bicarbonate or as a protein–carbamino derivative, depending on the pH of the sample preparation.

The formation of a protein–carbamino derivative at the A site indicates that the ligand involved in anion binding must have an amino group. Both lysine and arginine satisfy this requirement while no other basic amino acid side-chain groups do. Above pH 9.6, the exchangeable peaks disappear, and the anion at the A site is apparently released. If the loss of the anion is due to deprotonation of the anion binding ligand, the pH behavior would be more suggestive of a lysine ligand. However, the release of the anion occurs too abruptly to correspond with the simple deprotonation of a single amino acid group. Therefore, either arginine or lysine could be the ligand responsible for anion binding at the A site.

The multiplicity in the forms of the bound anion revealed by these ^{13}C NMR observations may account for the confusion and conflicting data in the literature about the state of the specifically bound anion.

Mechanism of Metal Binding. The spectra of preparations of apotransferrin in the presence of ^{13}C-enriched anion exhibit resonances due to the binding of bicarbonate to the protein. Two resonances are observed which appear to be due to specifically bound anion since these resonances are not seen in similar complexes of diferric transferrin and they disappear upon the addition of Fe^{3+} to the protein. The presence of these resonances suggests that specific anion binding does occur in the absence of metal ions. Numerous studies have determined that Fe^{3+} or other metal ions cannot specifically bind to the two sites of transferrin in the absence of a concomitantly bound anion (Price & Gibson, 1972; Zweier & Aisen, 1977); therefore, specific anion binding may be a preliminary step in the mechanism of metal binding.

Intersite Distance. By use of the spectra of preparations of dicobalt transferrin and preparations of monoferric monocobalt transferrin, an assessment of the distance between transferrin's two sites can be made. The line width of the nonexchanging anion ^{13}C resonance was identical within experimental error in these two preparations. This means that the paramagnetic broadening caused by Fe^{3+} bound at the A site on the ^{13}C anion resonance at the B site is less than 1 Hz. Utilizing the Solomon–Bloembergen equation, it can be calculated that the Fe^{3+} (A site) to ^{13}C anion (B site) distance is greater than 16 Å. This probably implies that there is no direct steric influence of one site on the other, although the binding of metal at one site may indirectly affect the other site by inducing changes in the protein conformation. This latter type of effect may explain how Fe^{3+} bound at the A site alters the binding of Cu^{2+} at the B site, which has been demonstrated in a previous study (Zweier, 1978).

Properties of the Metal and Anion Binding Sites. It has been determined that one site donates iron to the reticulocyte more effectively than the other (Fletcher & Huehns, 1967; Fletcher, 1969; Awai et al., 1972, 1975a,b; Lane, 1973; Harris & Aisen, 1975a). Because of this property, Fletcher & Huehns (1968) hypothesized that transferrin has a role in regulating the absorption and distribution of iron in the body. This hypothesis generated considerable interest in the heterogeneity of the two sites. Numerous studies were performed which have elucidated differences in the properties of the metal binding sites. The A site has been shown to bind Fe^{3+} more strongly than the B site (Aisen et al., 1978). The A site binds both Fe^{3+} and Cu^{2+} at a lower pH than the B site (Princiotto & Zapolski, 1975; Zweier, 1978). Iron binds preferentially to the A site when added as a nitrilotriacetate chelate, but when added as a citrate or oxalate chelate it binds to the B site (Aisen et al., 1978). When iron is added in the ferrous form and then oxidized, it binds preferentially to the B site (Aisen et al., 1978). The B site exhibits two different conformations for the binding of Cu^{2+} while the A site has only one (Zweier, 1978). The two sites have distinguishable Fe^{3+}, Cr^{3+}, Cu^{2+}, and VO^{2+} EPR spectra (Chasteen, 1977; Harris, 1977; Aisen et al., 1978; Zweier, 1978). In this study, it was determined that cobalt binding is similar to that of iron in that it preferentially binds to the B site when added as the divalent form, Co^{2+}, and then is oxidized to the trivalent form, Co^{3+}.

The two anion binding sites also have several different properties. It has been found that the B site will accept oxalate as the synergistic anion for Cu^{2+} binding while the A site will not (Zweier & Aisen, 1977). It also has been determined that two rate constants are necessary to describe the exchange of bound $^{14}CO_3^{2-}$ in the diferric complex while only one rate constant is necessary to describe the exchange in monoferric transferrin (Aisen et al., 1973). In this study, we have shown that the anion is bound directly to Fe^{3+} at both sites of the diferric transferrin complex. For the cobalt transferrin complexes, it was demonstrated that the anion bound at the B site does not exchange and is bound as carbonate, while that bound at the A site exchanges and is bound either as bicarbonate or as a carbamino derivative. The B site anion binding ligand appears to be a guanidino group of arginine. The A site anion binding ligand is either a guanidino group of arginine or an ϵ-amino group of lysine. The difference in the exchange of the anion bound at the two sites must reflect a difference in the anion binding sites. This difference could be explained if different ligands bind the anion at each site or if there is

a spatial–geometric difference in the position of the anion binding sites with respect to the metal.

The Fe^{3+}–transferrin–oxalate complexes are not very effective in donating iron to the reticulocyte, although they bind to the reticulocyte receptors in the same way as the ternary carbonate complex, and it has been concluded that the reticulocyte requires the presence of (bi)carbonate at the anion binding site if it is to remove specifically bound iron. It was postulated that the mechanism of iron delivery may entail an attack by the reticulocyte on the bound (bi)carbonate anion (Aisen & Leibman, 1973). If this hypothesis is correct, the difference in the properties of the two anion binding sites or in the state of the bound anion could explain the functional difference of the two sites.

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