Measurement of Nonsynergistic Anion Binding to Transferrin by EPR Difference Spectroscopy

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Abstract: The effects of salts on the two iron centers of human serum transferrin were investigated by EPR difference spectroscopy. The change in the EPR spectrum with increasing salt concentration is shown to arise from the binding of anions to a novel class of sites that are distinct from those occupied by the synergistic anion carbonate. The interaction of these nonsynergistic anions with the protein is very unusual in that the anions bind pairwise with strong positive cooperativity. A mathematical model describing anion binding to this multisited protein is presented. From this model and from measurements on diferric and the two monoferric transferrins, it is inferred that four anions bind to the protein, two in each domain. The values of the apparent overall association constant for anion binding in each domain are presented and follow the sequence thiocyanate > perchlorate > pyrophosphate > adenosine triphosphate > chloride ≫ tetrafluoroborate, orthophosphate, adenosine monophosphate, fluoride, sulfate, and bicarbonate. The above sequence parallels the lyotropic series for the strength of anion interactions with proteins. The results of this study suggest that the binding of physiologically relevant nonsynergistic anions such as chloride, ATP, and pyrophosphate to transferrin may be important in influencing the iron binding properties of this important protein. The relationships between anion binding, iron binding, and the kinetics of iron release are discussed.

The iron transport protein human serum transferrin binds two Fe^{3+} ions tightly but reversibly in separate domains of the molecule.[1-5] It is well established that a synergistic anion, carbonate or bicarbonate in physiological media, must bind in order for the protein to sequester the iron.[6-8] Neither the metal ion nor the anion binds appreciably without the other. In the absence of bicarbonate, certain organic anions with a carboxylate group and a second electron-withdrawing group can also facilitate iron binding.[8] There is good evidence that the synergistic anion binds directly to the metal and is probably linked to the protein as well.[8-14] This anion requirement for metal binding is unique to transferrin. Several workers have suggested that the anion may play a central role in the release of iron from the protein to iron-requiring tissues.[15-17] Experiments lend support to this view.

Our knowledge of the importance of anion binding to the iron-binding properties of transferrin is, however, incomplete. Recent studies have shown that various salts of nonsynergistic inorganic anions have a rather pronounced effect on the kinetics of iron release in vitro.[18,19] Moreover, ATP and pyrophosphate are efficient catalysts of iron release although neither functions as a synergistic anion.[20-22] Williams and Moreton have discovered that the distribution of iron between the N- and C-terminal metal binding sites in equilibrated serum is significantly changed by dialysis.[23] Chloride has recently been implicated as the low molecular weight component largely responsible for this change.[18]

In the present paper, we report anion binding experiments using EPR difference spectroscopy of the high-spin Fe^{3+} centers in transferrin. These studies demonstrate that a novel class of anion binding sites exist on the protein in addition to those that bind the synergistic anion. Occupation of these sites by physiologically relevant anions, e.g., Cl^-, ATP, and pyrophosphate, perturbs the Fe(III) EPR spectrum in a similar way for all of the anions. Moreover, the interaction of these anions with transferrin is unusual in that they bind pairwise in each domain with strong positive cooperativity, indicating that the protein has structurally unusual binding sites. The stoichiometry and association constants for binding of these anions to transferrin are reported.

Experimental Section

Commercial preparations of iron-free human serum transferrin of stated 99% purity were purchased from Behring Diagnostics Corp. and used without further purification. Protein concentrations were determined spectrometrically on a Spectronic 200 or a Cary 219 spectrophotometer with $E_{1cm}^{1\%} = 11.4$ at 280 nm[24] and a molecular weight of 78 000.[25]

The protein was brought to near 100% saturation by the addition of 2 equiv of Fe^{II} as 0.1 M $Fe^{II}(NH_4)_2(SO_4)_2$ to ~1 mM apotransferrin in 0.1 M Hepes (*N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid)/NaOH, 0.01 M $NaHCO_3$, pH 7.5. The salmon pink color characteristic of diferric transferrin slowly developed. The solution was in contact with the air for a minimum of 24 h or until no further increase in absorption at 465 nm was observed.

Buffers and salt solutions were rendered metal free by ion-exchange chromatography on Chelex-100. Glassware was cleansed of metals by soaking in 1:1 concentrated H_2SO_4–HNO_3 followed by rinsing in doubly distilled deionized water. All solutions were prepared in doubly distilled water and buffered at pH 7.5 in 0.1 M Hepes buffer (Sigma).

C-Terminal monoferric transferrin (~1 mM) was prepared by adding 1 equiv of iron (as ferrous ammonium sulfate) to apotransferrin at pH 6. The solution was allowed to stand for 1–2 days until no further increase in the A_{465} was observed. The purity of C-terminal monoferric transferrin was verified by urea/polyacrylamide gel electrophoresis and shown to be greater than 90% pure.[26] N-Terminal monoferric transferrin was prepared by the method of Williams.[27] Apotransferrin was dissolved in 0.1 M Hepes/NaOH, 0.01 M $NaHCO_3$, pH 7.5, such that

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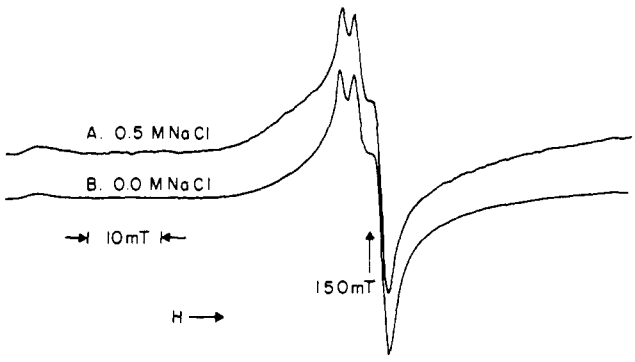

Figure 1. Effect of chloride ion on the X-band EPR spectrum of diferric transferrin. (A) 0.5 M NaCl; (B) no NaCl. Conditions: 1 mM protein, 0.1 M Hepes, 0.01 M $NaHCO_3$, pH 7.5, 98 K. Instrument settings: power = 20 mW, modulation amplitude = 10 G, sweep rate = 2000 G/16 min, time const = 0.3 s. Peak-to-peak amplitude of spectrum A scaled to that of spectrum B.

the concentration was 20 mg/mL. Enough iron, as ferrous ammonium sulfate, was introduced to saturate the protein. The solution was allowed to stand for 24 h. The protein was then diluted to 4 mg/mL with a 0.1 M Hepes, 0.5 M NaCl, 1.0 mM $Na_4P_2O_7$, pH 7.5 solution. Enough desferrioxamine (CIBA Pharmaceutical Co.) was added to complex 50% of the iron. Under these conditions iron is preferentially removed from the C-terminal site.[27] When the reaction reached completion (~6 h after addition of the desferrioxamine), the protein was dialyzed and then concentrated in an Amicon Model 3 ultrafiltration apparatus fitted with a PM 10 membrane. Urea/polyacrylamide gel electrophoresis showed the presence of only N-terminal monoferric transferrin and a small amount of apotransferrin.

EPR spectra were measured at X-band (9.5 MHz) frequency on Varian E-4 and E-9 spectrometers interfaced with a MINC-11 laboratory computer (Digital Equipment Corp.). Samples were placed in calibrated quartz tubes (approximately 4 mm o.d., 3 mm i.d.) for spectra recorded at 98 K. Titrations of the protein with the various anions were carried out by delivering a calculated volume of 1 M titrant in 0.1 M Hepes/NaOH buffer, pH 7.5, into 0.4 mL of buffered 1 mM protein. Approximately 300 μL of solution was withdrawn and frozen in a quartz tube by immersion in liquid N_2. Temperature of the sample was maintained at 98 K by employing a Varian V-4257 variable-temperature cavity insert on the spectrometer. Temperature was determined by immersing a copper-constantan thermocouple in the sample. Spectra were recorded, digitized, and stored on floppy disk after each addition of titrant.

The EPR first derivative peak-to-peak amplitude of the Fe(III) signal at ~150 mT decreases with the addition of salts, e.g., NaCl or $NaClO_4$, but the integrated intensities of the sample samples remain nearly constant. At 0.5 M Cl^-, the amplitude corrected for dilution is reduced by approximately 40% while the intensity obtained from double integration of the spectrum between field values of 50 and 250 mT is reduced by only 10%. Price and Gibson have observed a reduction in amplitude with perchlorate as well.[28] The small reduction in integrated intensity with increasing salt concentration could be due to a slight change in dielectric properties of the sample or to some EPR intensity outside the range of the limits of integration. The reproducibility of the double integral between samples did not warrant extending the limits further.

Power saturation studies of diferric transferrin samples with 0.5 M chloride and with no chloride reveal that for microwave power settings between 0.01 and 30 mW no saturation is occurring. Furthermore, the perturbations on the iron EPR spectrum due to salts are evident for all microwave settings investigated. The EPR spectrum is independent of the method of freezing the sample, slow immersion in liquid nitrogen or quick freezing in *n*-pentane slush.

All Fe(III) spectra as a function of salt were computer scaled either to the same peak-to-peak amplitude of the $g' = 4.3$ signal or to the same integrated intensity prior to subtraction. Because the analysis of the data in terms of anion binding to the protein involves taking the ratio of the amplitudes of the difference spectra, the stoichiometries and binding constants obtained are not very sensitive to the method of scaling and are the same for both methods within experimental error (Table I).

Results

Figure 1 shows the effect of chloride on the iron EPR spectrum of diferric human serum transferrin. Both the $g' \simeq 9.7$ and $g' = 4.3$ signals are changed. The feature showing the greatest change, a broad shoulder on the low-field side of the characteristic doublet at $g' = 4.3$, increases in amplitude as the concentration of chloride is increased. This increase as well as that in the $g' = 9.7$ line is enhanced in the computer-subtracted difference spectra of Figure 2. In addition to chloride, this effect was also observed for the sodium salts of perchlorate, thiocyanate,[29] adenosine triphosphate (ATP), and pyrophosphate. The sodium salts of the anions tetrafluoroborate, orthophosphate, bicarbonate, sulfate, fluoride, and adenosine monophosphate (AMP), tested

Table I. Binding Data for Anions with Human Transferrin[a]

anion	protein	n[b]	log K[c]	K[d]	r[e]
SCN^-[f]	FeTfFe[g]	2.39 (0.34)	3.56 (0.09)	3600	0.995
ClO_4^-	FeTfFe	2.34 (0.31)	2.90 (0.06)	800	0.997
$HP_2O_7^{3-}$	FeTfFe	1.85 (0.37)	2.86 (0.07)	730	0.970
ATP^{3-}	FeTfFe	1.99 (0.36)	2.62 (0.12)	420	0.989
Cl^-[h]	FeTfFe	2.15 (0.15)	1.97 (0.05)	93	0.997
Cl^-	FeTf[i]	2.02 (0.41)	2.75 (0.24)	560	0.946
Cl^-	TfFe[j]	2.13 (0.27)	2.11 (0.08)	130	0.991
HPO_4^{2-}	FeTfFe			⩽2	
AMP^{2-}	FeTfFe			⩽2	
SO_4^{2-}	FeTfFe			⩽2	
F^-	FeTfFe			⩽2	
BF_4^-	FeTfFe			⩽2	

[a] Conditions: 1 mM protein in 0.1 M Hepes/NaOH buffer, pH 7.5. Spectra measured at 98 K and scaled to the same peak-to-peak amplitude of the $g' = 4.3$ signal before substraction. The difference spectrum parameter Δ_i measured from the broad spectral feature at 140 mT (Figure 2). [b] Hill coefficient with the 95% confidence interval from the linear regression (Figure 4) given in parentheses. [c] Log of the overall apparent association constant K for the binding of n anions. 95% confidence interval in parentheses. Because the measurement is performed on a frozen solution, the temperature corresponding to the value of K is unknown. [d] The experimental reproducibility of these values is about 10%. [e] Correlation coefficient of the linear regression (Figure 4). [f] Analysis of the $g' = 9.7$ line at 70 mT with Δ_i measured at this field and spectra scaled to the same peak-to-peak amplitude at $g' = 4.3$ gives the following results: $n = 2.31$ (0.35), log $K = 3.47$ (0.09), and $r = 0.994$. [g] Diferric transferrin. [h] Analysis of the $g' = 4.3$ signal with the spectra scaled to the same integrated intensity before subtraction gives the following results: $n = 2.32$ (0.33), log $K = 1.95$ (0.11), and $r = 0.990$, where Δ_i is measured from the broad feature at 140 mT, and $n = 2.22$ (0.23), log $K = 1.88$ (0.07), and $r = 0.993$, where Δ_i is measured from the sharp feature at 158.5 mT. [i] N-Terminal monoferric. [j] C-Terminal monoferric.

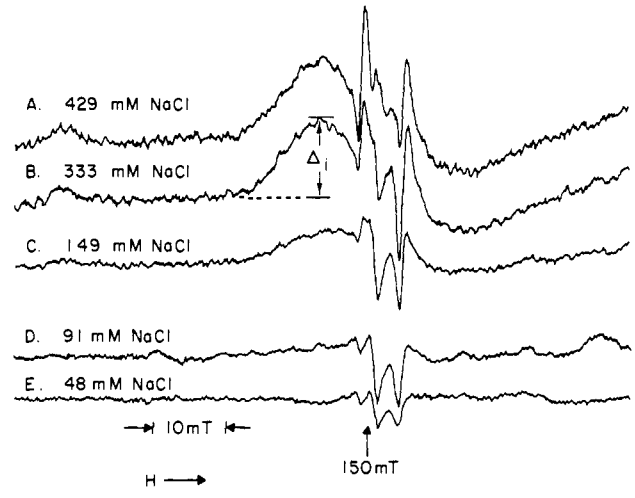

Figure 2. Computer-subtracted EPR difference spectrum as a function of chloride concentration. Spectrum B subtracted from spectrum A of Figure 1. The difference spectrum parameter Δ_i is indicated. Conditions as in Figure 1.

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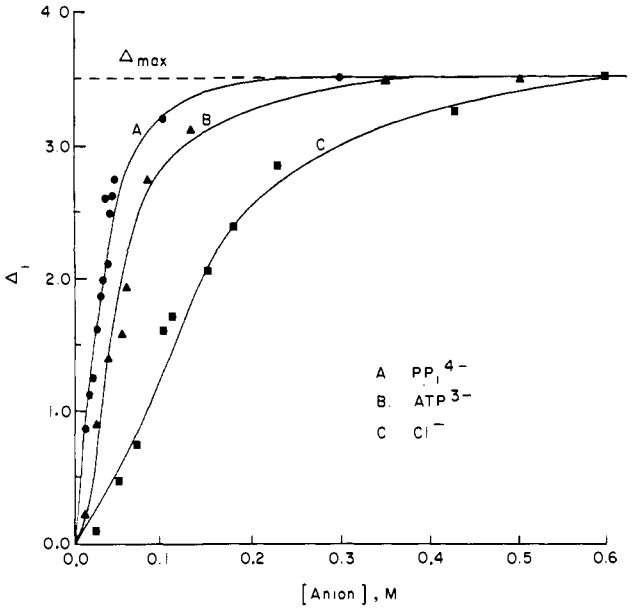


Figure 3. Difference spectrum parameter Δ_i is arbitrary units as a function of anion concentration for different anions. The maximum value Δ_{max} at saturating levels of anion is indicated. Conditions as in Figure 1.

at a concentration of 0.5 M, showed little effect.

The fact that some salts are relatively ineffective in bringing about a change in the EPR spectrum precludes the possibility that sodium is significantly involved or that the effect is due to variation in ionic strength of the solutions. Moreover, absorbance measurements of the ion–transferrin complex at 465 nm indicate that, except for pyrophosphate, none of the anions are removing iron from the protein, eliminating the possibility that small Fe(III)–anion complexes are contributing to the EPR difference spectrum. Also, solutions of 0.5 M ATP, chloride, perchlorate, or thiocyanate containing 1 mM Fe^{3+} at pH 7.5 in the absence of protein do not exhibit $g' = 4.3$ signals. Solutions of pyrophosphate and iron under the same conditions do exhibit a featureless signal at $g' = 4.3$ that may contribute up to 5% to the observed EPR difference spectrum. The possibility that the difference spectrum arises from nonspecifically bound iron was also ruled out by eluting diferric transferrin on Sephadex G-75, a procedure known to remove such iron.[30] The EPR spectrum of the protein before and after this treatment responded to perchlorate in the same way. In protein preparations in which iron(III) nitrilotriacetate 1:2 was used to saturate the protein with iron, no anion effect was observed.

The value of Δ_i in the difference spectrum as a function of salt concentration for three different anions is shown in Figure 3. The curves display saturation behavior, indicative of a binding process. To a first approximation, the binding of these anions to an iron-containing domain of the protein can be represented by the following simplified equilibrium,

$$\text{domain} + n\text{A} \rightleftharpoons \text{domain}' \qquad (1)$$

where domain′ represents an anion complex in which the electronic environment of the metal center is altered, giving rise to a new EPR spectrum. The apparent overall association constant, K, is given by

$$K = \frac{[\text{domain}']}{[\text{domain}][\text{A}]^n} = \frac{\theta}{(1-\theta)[\text{A}]^n} \qquad (2)$$

where θ ($= \Delta_i/\Delta_{max}$) is the degree of saturation of the anion binding sites. Δ_i and Δ_{max} are determined from the graphs in Figure 3. Equation 2 is equivalent to the Hill equation and predicts that a plot of $-\log [\theta/(1-\theta)]$ vs. $-\log$ [A] will yield a straight line with a slope of n and an intercept of $-\log K$. Straight-line plots are obtained for all of the anions that perturb the EPR spectrum (Figure 4). The values of the Hill coefficient n are generally 2.0 within the 95% confidence interval for all the effective anions (Table I). The overall apparent association constants are also summarized in Table I. Similar results are obtained if the analysis is performed on the $g' = 9.7$ signal instead (cf. SCN^- in Table I).

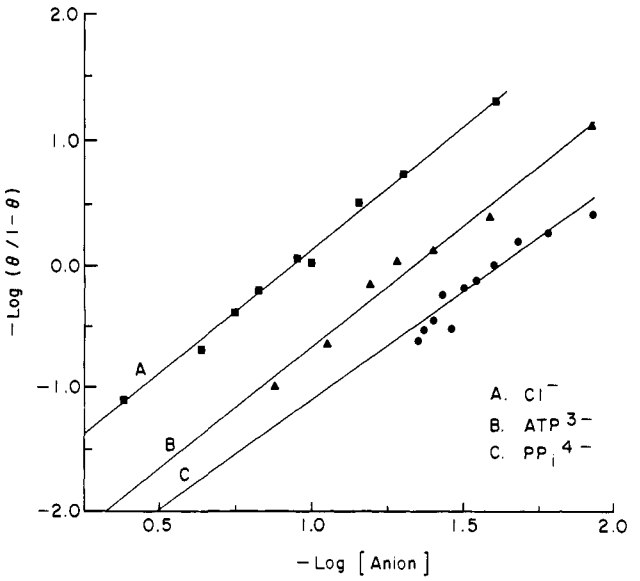
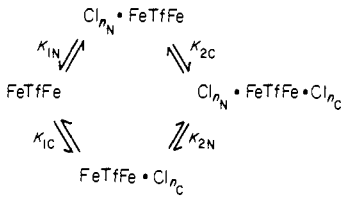


Figure 4. Hill plots of the data in Figure 3 for several anions. The slope is the Hill coefficient n, and the intercept of the ordinate is $-\log K$. Values from the linear regression are presented in Table I.

Since transferrin has two EPR-active Fe^{3+} centers, the precise meanings of the values of n and K obtained from the plots in Figure 4 are not clear. An obvious question is whether one or both metal binding domains are influenced by the binding of anions. It is not possible to tell from the above treatment whether the values of $n \sim 2$ (Table I) reflect the binding of two anions in each of the two domains ($n_N = 2$, $n_C = 2$), two in only one domain (e.g., $n_N = 2$, $n_C = 0$), one in each domain ($n_N = 1$, $n_C = 1$), or some other combination. A more elaborate model of anion binding to diferric transferrin was developed to examine these possibilities. The overall binding scheme is given by



(3)

where

$$K_{1N} = \frac{[\text{Cl}_{n_N}\text{·FeTfFe}]}{[\text{FeTfFe}][\text{Cl}]^{n_N}} \qquad (4)$$

[Cl_{n_N}·FeTfFe] is the analytical concentration of the diferric species with n_N mol of chloride bound in the N-terminal domain. The other concentrations are defined similarly. The K_i values are conditional overall stability constants for the binding of n_N and n_C chloride ions in the N- and C-terminal domains, respectively. This scheme also allows for positive ($K_{2N}/K_{1N} = K_{2C}/K_{1C} > 1$) or negative ($K_{2N}/K_{1N} = K_{2C}/K_{1C} < 1$) cooperativity between domains in chloride binding. A similar scheme for the binding of iron to transferrin has been presented in detail elsewhere.[2,26]

When chloride is added to diferric transferrin, a distribution of anion-containing species will be formed, each of which will contribute to the observed difference spectrum parameter Δ_i. The calculated value, Δ_{calcd}, is given by

$$\Delta_{calcd} = \Delta_N X_N + \Delta_C X_C + \Delta_{N,C} X_{N,C} \qquad (5)$$

(30) Leinjnse, S. S.; van der Kraan, A. M. *J. Inorg. Biochem.* **1981**, *15*, 329.

Table II. Results of Model Calculations for Chloride Binding to Human Serum Transferrin[a]

case	n_N	n_C	cooperativity	K_{1N}	K_{1C}	K_{2N}	K_{2C}	n_{calcd}[b]	$(\log K)_{calcd}$[c]	r[d]	S_y[e]
I	1	1	no	7.71	7.71			1.02	0.967	0.9960	0.455
II	1	1	yes	−10.44	8.44	10.87	−8.79	2.08	1.91	0.9982	0.0654
III	2	2	no	64.95	68.39			2.00	1.85	0.9992	0.095
IV	2	2	yes	52.61	52.63	73.06	73.08	2.13	1.95	0.9995	0.069
V	2	0		67.23				2.00	1.84	0.9999	0.088
exptl								2.15 ± 0.15[f]	1.97 ± 0.05[f]	0.9969	0.066[g]

[a] Calculations based on eq 5-8 with $\Delta_N = \Delta_C = \Delta_{(N,C)}/2 = 1.75$. K_i values are subject to the constraint $K_{2N}/K_{1N} = K_{2C}/K_{1C}$ imposed by the cyclic equilibrium. [b] Calculated from the linear regression of $\log [\theta/(1-\theta)]_{calcd}$ vs. $\log [Cl]_{exptl}$. [c] Calculated from the intercept of the regression line in footnote b. [d] Correlation coefficient of the regression in footnote b. r is a measure of the linearity of the line predicted by the model and does not reflect the fit of the model to the data. [e] The residual is defined as $S_y = [(Y_{calcd} - Y_{obsd})^2/n' - p]^{1/2}$ where $Y_{calcd} = \log [\theta/(1-\theta)]_{calcd}$, $Y_{obsd} = \log [\theta/(1-\theta)]_{obsd}$, n' (= 9) is the number of experimental points, and p (= 2 or 3) is the number of independent parameters in the equation. S_y is a measure of the goodness of the fit of the model to the data. [f] Experimental values, Table I. [g] The residual of the experimental data is defined by $S_y = [(Y_{obsd} - Y)^2/(n'-2)]^{1/2}$, where Y is the calculated value of $-\log [\theta/(1-\theta)]$ from the linear regression line at each value of $-\log [Cl]_{exptl}$.

where X_N, X_C, and $X_{N,C}$ are the mole fractions of the transferrin species Cl_{n_N}·FeTfFe, FeTfFe·Cl_{n_C}, and Cl_{n_N}·FeTf·Cl_{n_C}, respectively. The values of Δ_N, Δ_C, and $\Delta_{N,C}$ are the respective "extinctions" in the EPR difference spectrum of each of these species.

$\Delta_{N,C}$ is taken as the value of Δ_{max} obtained when diferric transferrin is titrated with chloride (Figure 3). The values of Δ_N and Δ_C were chosen as $\Delta_N = \Delta_C = \Delta_{N,C}/2$, corresponding to chloride inducing the same maximum spectral change in both domains, and $\Delta_N = \Delta_{N,C}$ with $\Delta_C = 0$, corresponding to a change in one domain only. Here we have assumed that the spectra of the two domains are additive.

Cooperativity between domains enters eq 5 through the values off X_N, X_C, and $X_{N,C}$. The expressions for the mole fractions derived by a procedure analogous to that outlined for iron binding to transferrin[26] are given by the following:

$$X_N = \frac{1}{1 + 1/(K_{1N}[Cl]^{n_N}) + \frac{K_{1C}[Cl]^{n_C}}{K_{1N}[Cl]^{n_N}} + K_{2C}[Cl]^{n_C}} \quad (6)$$

$$X_C = \frac{1}{1 + 1/(K_{1C}[Cl]^{n_C}) + \frac{K_{1N}[Cl]^{n_N}}{K_{1C}[Cl]^{n_C}} + K_{2N}[Cl]^{n_N}} \quad (7)$$

$$X_{N,C} = \frac{1}{1 + \frac{K_{2C}[Cl]^{n_C} + K_{2N}[Cl]^{n_N}}{K_{2C}K_{2N}[Cl]^{n_C}[Cl]^{n_N}} + 1/(K_{2C}K_{1N}[Cl]^{n_C}[Cl]^{n_N})} \quad (8)$$

where the constants are subject to the constraint $K_{2N}/K_{1N} = K_{2C}/K_{1C}$ imposed by the cyclic equilibrium (3).

Equations 5-8 were used to compute Δ_{calcd} at the experimental concentrations of chloride. From this, $\log (\theta/(1-\theta))_{calcd}$, where $\theta = \Delta_{calcd}/\Delta_{N,C}$, was evaluated and compared with the experimentally obtained quantity. Initially, a set of equilibrium constants and integer values of n_N and n_C were assumed. A nonlinear regression employing a Simplex optimization procedure was performed in which the values of the K_i were varied to provide a least-squares fit to the $\log (\theta/(1-\theta))$ data for the given choice of n_N and n_C. It was believed that through this procedure we would be able to simulate the experimental curve of Figure 3 for the correct values of n_N and n_C. The results of this analysis for different values of n_N and n_C, with and without cooperativity between domains in chloride binding, are presented in Table II.

All models in Table II provide straight-line plots of $-\log (\theta/(1-\theta))_{calcd}$ vs. $-\log [Cl]_{exptl}$, but several of them can be discarded as providing unsatisfactory simulations of the experimental curve. The slope n_{calcd} of the line in case I is only 1 (compared to an experimentally obtained value of 2), and the negative binding constants obtained in case II indicate that (for this case) we are attempting to fit the experimental curve with an inappropriate model. Of the remaining models, the last three listed in Table II provide satisfactory fits to the experimental data (cf. model

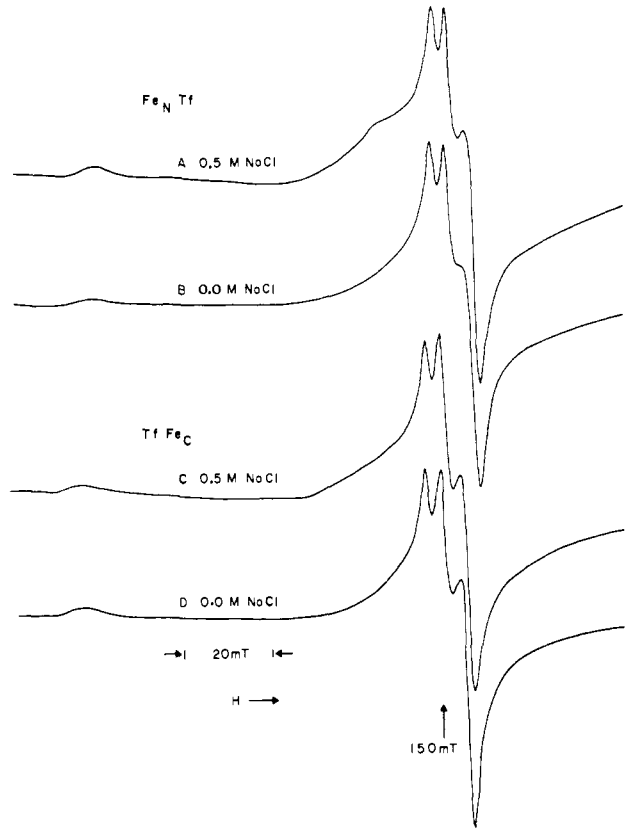


Figure 5. Effect of chloride on the X-band EPR spectra of the monoferric transferrins. (A) N-Terminal monoferric with 0.5 M NaCl; (B) N-terminal monoferric without NaCl; (C) C-terminal monoferric with 0.5 M NaCl; (D) C-terminal monoferric without NaCl. Conditions and instrument settings as in Figure 1. All spectra scaled to the same peak-to-peak amplitude.

S_y values with the experimental value). The calculated values of n and $\log K$ for all three models are also close to the experimental values. A comparison of cases III and IV reveals that only a slight improvement in fit is obtained when cooperativity between domains is introduced (case IV). We conclude that if chloride binds in both domains of transferrin it does so essentially independently. Therefore, only cases III and V need be considered further.

To determine which of the two satisfactory cases (III or V) of Table II applies to diferric transferrin, we prepared N- and C-terminal monoferric transferrins and titrated each with chloride. The EPR spectra off the monoferric species with and without chloride shown in Figure 5 indicate that the effect on the spectrum occurs in both halves of the protein but is greater in the N-terminal domain than in the C-terminal domain; i.e., $\Delta_C \sim \Delta_N/2$ when referenced to the same peak-to-peak EPR amplitude for both

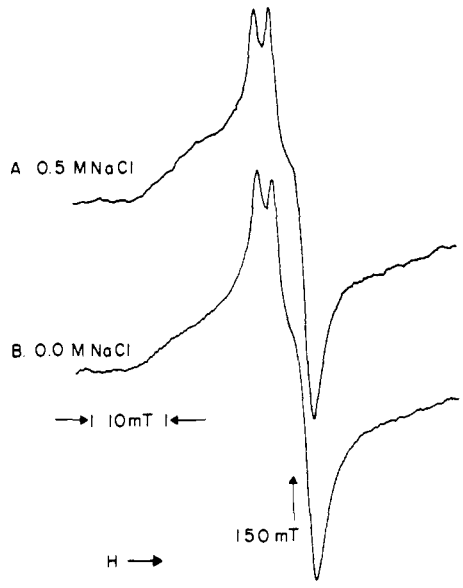


Figure 6. Effect of chloride on the X-band EPR spectrum of diferric ovotransferrin. (A) 0.5 M NaCl; (B) no NaCl. Conditions and instrument settings as in Figure 1. Spectra scaled to the same peak-to-peak amplitude.

monoferrics. The chloride binding data for the monoferric transferrins are also summarized in Table I. The results indicate that two chloride ions bind to both the N-terminal and C-terminal monoferric species. By inference, four anions bind to diferric transferrin. The value of K for the C-terminal monoferric is about the same as observed for the diferric protein while the value of K for the N-terminal monoferric is 6-fold higher.

The effects of perchlorate on the iron EPR spectrum of diferric transferrin observed in this study are similar to those reported by Price and Gibson.[28] Their early work demonstrated that perchlorate produced opposite effects on the EPR spectra of human serum transferrin and ovotransferrin. We have also observed this difference in behavior for the two proteins with perchlorate; however, both proteins respond to chloride in the same manner (cf. Figures 1 and 6). A logarithmic plot of the data from titration of diferric ovotransferrin with chloride likewise yields a straight line with $n = 2.00 \pm 0.356$, $\log K = 1.29 \pm 0.079$, and a correlation coefficient $r = 0.986$, indicating that chloride also binds to ovotransferrin but more weakly than to serum transferrin.

Discussion

On the basis of the observations reported here, human serum transferrin has binding sites for nonsynergistic anions that affect the metal centers of the protein. Titration of the monoferric transferrins indicate that there are two sites in each domain of the protein, in accord with the predicted results for case III of our computer modeling study. Previous investigations also lend support to this conclusion. For example, perchlorate has been shown to affect both the N- and C-terminal metal binding fragments of ovotransferrin, and chloride is known to affect the kinetics of iron release from both domains in diferric transferrin.[18,19,31] Moreover, the EPR spectrum of both sites of divanadyl human transferrin is changed by perchlorate.[32]

The existence of these anion binding sites raises the question as to their location on the protein. In diferric transferrin it is unlikely that the anions bind to a significant extent at the sites occupied by the synergistic anion since none of them facilitate iron binding in the absence of bicarbonate. The observation that all of the anions alter the EPR spectrum in a similar manner seems to preclude the possibility that they are directly ligated to the metal. With various metal-coordinated synergistic anions, different spectra are produced in both Fe(III) and VO(II) transferrins.[9,33] Perchlorate is a relatively tightly held anion (see Table I) but is known to be a poor ligand for metal ions while fluoride, which has little or no effect on the EPR spectrum (vide supra), is a good ligand for Fe^{3+}. It is more likely that these anions bind to positively charged amino acids or possibly to amide dipoles of the protein.

The origin of the changes observed in the EPR spectra is not clear. One possible explanation is that anion binding results in an allosteric effect on the metal centers as a result of a conformational change in the protein. There is ample evidence in the literature that transferrin exists in more than one conformation.[15-17] EPR spectral changes in Gd(III), Cu(II), VO(II) transferrins have also been observed attending the addition of ClO_4^- and Cl^-, indicating that the anion sites may be preserved in all of these metal derivatives of the protein.[34,35]

The effects of anions on the conformational states of proteins often follow the lyotropic series $F^- < SO_4^{2-} < HPO_4^{2-} < CH_3CO_2^- < Cl^- < Br^- < NO_3^- < I^- < CCl_3CO_2^- < ClO_4^- < SCN^-$.[36] This series is approximately the same sequence for the affinity of anions for positively charged sites on proteins. Inspection of Table I reveals that the strength of interaction for the anions reported here also follows this series.

It may also be significant that ATP and pyrophosphate, which bind to the protein (Table I), are very efficient catalysts for mediating the removal of iron from transferrin to desferrioxamine in vitro.[20,21] The kinetics of this process show saturation behavior with respect to the anion, a result consistent with, but not proof of, anion binding to the protein. Furthermore, the diphosphate structure appears to be important. Neither AMP nor orthophosphate function as an efficient catalyst of iron removal; nor do they bind appreciably to the anion sites observed here.

It is interesting that solutions of the diferric protein that have been prepared from iron(III) nitrilotriacetate do not show the effect of anions on the EPR spectrum. Price and Gibson have observed that the presence of NTA in solutions of transferrin negates the response of the EPR spectrum to perchlorate.[28] NTA is also known to bind tenaciously to transferrin and can only be removed by dialysis or gel permeation chromatography in the presence of perchlorate.[37]

The calculations indicate that cooperativity between domains in anion binding is negligible; however, the Hill plots for diferric and monoferric transferrins (Figure 4 and Table I) show that within each domain two anions bind with strong positive cooperativity. ^{35}Cl NMR studies recently completed in our laboratory confirm this result.[38] The strong positive cooperativity in anion binding is unexpected since normally binding of a second ligand is more difficult than that the first ($K_2 << K_1$). This implies that, in the protein, the interaction of the first anion causes structural changes that facilitate binding of the second anion. Such a change would be consistent with an allosteric mechanism discussed above. We are unaware of any other protein that also displays strong positive cooperativity in binding of nonstereospecific anions. In this regard, transferrin appears to be unique.

Chasteen and Williams have recently shown that two functional groups undergo proton dissociation with strong positive cooperativity in the monoferric transferrins to bring about a change in the binding affinities of the two iron sites.[26] The phenomena of cooperativity of anion binding and of proton dissociation may be related in some way.

The results presented here do not demonstrate that the observed anion sites are directly involved in the kinetics of iron removal from transferrin. However, the parallels between the kinetics of

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iron removal[18-21] and the strength of anion binding to transferrin raise this question. In addition, the unusual property of positive cooperativity exhibited in both anion binding and proton dissociation by transferrin poses new questions about this important protein. Further studies are clearly indicated to fully understand the role that anions play in the structure of transferrin, in the thermodynamics of iron binding, and in the mechanism of iron removal.

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Registry No. ATP, 56-65-5; AMP, 61-19-8; SCN^-, 302-04-5; ClO_4^-, 14797-73-0; $HP_2O_7^{3-}$, 42499-21-8; Cl^-, 16887-00-6; BF_4^-, 14874-70-5; HPO_4^{2-}, 14066-19-4; F^-, 16984-48-8; SO_4^{2-}, 14808-79-8; HCO_3^-, 71-52-3; Fe, 7439-89-6.

Communications to the Editor

Effects of Wavelength on the Photochemistry of Provitamin D_3[1]

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Wavelength effects in solution photochemistry can arise from excited-state or ground-state properties.[2] This variant has been studied in the photochemistry of polyenes, and it generally has been found that wavelength-dependent photochemistry arises from a variety of ground-state effects such as a secondary reaction of the primary photoproduct,[3] independent excitation of conformational[4-10] and structural isomers,[11] and excitation of ground-state complexes.[12,13] However, it has been suggested that wavelength dependencies may also result from the involvement of higher excited states or from the involvement of activation barriers in a single excited state. These latter processes permit new vibrational modes for relaxation into new minima on the excited state hypersurfaces.[8,14-16]

Scheme I

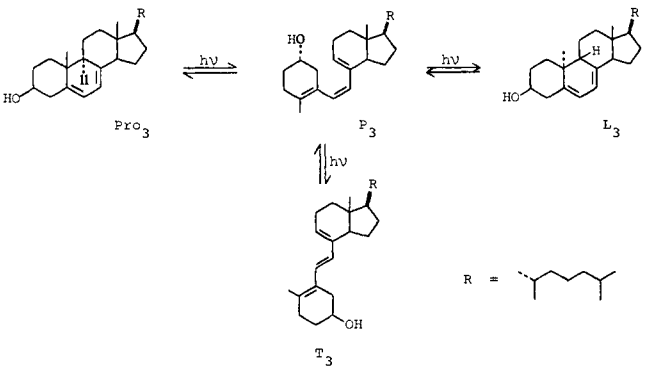

Table I. Relative Quasi-photostationary State Concentrations

λ, nm	Pro_3	P_3	L_3	T_3
295.0	2.0	71	4.0	23
297.5	3.0	70	6.0	21
300.0	5.0	68	8.0	19
302.5	8.0	65	13.0	14
305.0	15.0	50	25.0	10

A detailed study using light of different wavelengths (225–400 nm) for the formation of "potential vitamin D_2" found that 295-nm light was the most effective.[17] More recently, wavelength effects upon the four components in the previtamin D_3 (P_3) photostationary state (Scheme I) have been reported for two wavelengths (254.0 and 302.5 nm)[18] and it was concluded that "it is not yet possible to quantitatively assess the separate contributions of the ground-state and the excited-state properties to the wavelength effects observed". We report results that clearly indicate that excited-state properties are involved in the wavelength effects found in the transformation of P_3 to Pro_3 and L_3.

As an extension of an earlier investigation into the effect of incident irradiation wavelength on the formation of previtamin D_3 (P_3) from 7-dehydrocholesterol (Pro_3),[19] a 1.15×10^{-4} M solution of Pro_3 in anhydrous ether at 0 °C was irradiated with a Chromatix CMX-4 coaxial flash-lamp pumped dye laser.[20] In

(1) This investigation was supported by PHS Grant 00709, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases. The laser study was partially supported by the National Science Foundation Grant CHE 78-6484.

(2) For an excellent review of wavelength effects on organic photoreactions in solution, see: Turro, N. J.; Ramamurthy, V.; Cherry, W.; Farneth, W. *Chem. Rev.* **1978**, *78*, 125.

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(20) The laser power at each wavelength was as follows: 295 nm, 3.5 mW; 297.5 nm, 4.0 mW; 300 nm, 4.0 mW; 302.5 nm, 3 mW; 305 nm, 5 mW.

0002-7863/82/1504-5780$01.25/0