4-chlorophenolate, 1121-75-1; lithium 4-chloro-3,5-dimethylphenolate, 114299-84-2; lithium 4-fluorophenolate, 114299-85-3; lithium 4-methoxyphenolate, 1122-94-7; lithium 3,5-dimethyl-4-methoxyphenolate, 114299-86-4; lithium 4-(trifluoromethyl)phenolate, 114299-87-5; lithium 2-methylphenolate, 83859-26-1; lithium 2-ethylphenolate, 114299-88-6; lithium 2-propylphenolate, 114299-89-7; lithium 2-isopropylphenolate, 114299-90-0; lithium 2-*tert*-butylphenolate, 114299-91-1; lithium 2-(methoxymethyl)phenolate, 114299-92-2; lithium 2-(trifluoromethyl)-phenolate, 114299-93-3; lithium 3,5-dimethoxyphenolate, 114299-94-4; lithium 3,5-diethylphenolate, 114299-95-5; phenol, 108-95-2; 3,5-dimethylphenol, 108-68-9; 4-bromophenol, 106-41-2; 4-chlorophenol, 106-48-9; 4-chloro-3,5-dimethylphenol, 88-04-0; 4-fluorophenol, 371-41-5; 4-methoxyphenol, 150-76-5; 3,5-dimethyl-4-methoxyphenol, 4962-29-2; 4-(trifluoromethyl)phenol, 402-45-9; 2-methylphenol, 95-48-7; 2-ethylphenol, 90-00-6; 2-propylphenol, 644-35-9; 2-isopropylphenol, 88-69-7; 2-*tert*-butylphenol, 88-18-6; 2-(methoxymethyl)phenol, 5635-98-3; 2-(trifluoromethyl)phenol, 444-30-4; 3,5-dimethoxyphenol, 500-99-2; 3,5-diethylphenol, 1197-34-8.

Low-Spin Cyanide Adduct of Transferrin

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Abstract: The interaction between cyanide and the high-spin iron(III) centers of diferric human serum transferrin was investigated to gain insight into the nature of mixed-ligand complexes that are formed between iron, the protein, and exogenous chelators. The binding of cyanide forms a low-spin adduct at only the C-terminal iron(III) binding site of transferrin. The characteristic $g' = 4.3$ EPR spectrum of the high-spin Fe(III) at this site is converted to a low-spin spectrum exhibiting $g_x = 2.34$, $g_y = 2.15$, and $g_z = 1.92$. Analysis of the g factors according to a crystal field perturbation model indicates that the unpaired electron is in a d_{xy} orbital and that the metal site has nearly complete "rhombic" symmetry. The cyanide adduct is formed according to the reaction Fe_c–Tf–$HCO_3^z + 3CN^- \rightarrow Fe_c$–Tf–$(CN)_3^{z-2} + HCO_3^-$ where the synergistic anion HCO_3^- normally required for iron(III) binding to the protein is displaced from the first-coordination sphere by cyanide. This result demonstrates that the presence of bicarbonate is not essential for the formation of stable mixed-ligand complexes with the protein. The stoichiometry of the reaction implies that three CN^- anions are bound to the metal and, by inference, three coordination sites are accessible to exogenous ligands in the iron(III)–protein complex. The possibility that some of the CN^- simply binds elsewhere on the protein is not precluded by the data, however. Ultraviolet difference and visible spectral measurements on the C-terminal monoferric transferrin suggest that the phenolate groups of two tyrosine residues are coordinated to the iron. A possible structure for the metal site is proposed.

The transferrins are a class of iron-binding and -transport proteins that play an essential role in the metabolism of iron in vertebrates. All transferrins contain two metal-binding sites, bind iron strongly in the 3+ oxidation state, and display EPR signals at $g' = 4.3$, which are characteristic of high-spin Fe(III) in a ligand environment of low symmetry.[1,2] While serum transferrin functions as the iron-transport protein of the circulation, ovotransferrin from egg white and lactoferrin from milk and other secretions have bacteriostatic functions as well as other possible roles.[3-5]

The mechanism by which serum transferrin exchanges iron with its environment is not well understood, but it is clear that anions are important in this process. The binding of (bi)carbonate to the iron is required for the metal to bind at the specific sites of the protein.[6] In addition, anions such as chloride and perchlorate are known to affect the kinetics of iron exchange between chelators and the protein and to influence the relative thermodynamic stability of iron binding in the two sites.[7-11] Recent studies of iron exchange between transferrin and anionic chelators have detected the formation of unstable mixed-ligand intermediate complexes of the type chel–Fe–transferrin in which both the chelator and protein are presumably coordinated to the iron.[12,13] Little is known about the metal-site coordination in these intermediates and whether the (bi)carbonate remains bound.

Knowledge of labile coordination sites in the iron–transferrin complex is particularly important in light of the recently published X-ray structure of lactoferrin, which indicates that the protein contributes four amino acid ligands to the metal: two tyrosines, one histidine, and one aspartate.[14] It is likely that the remaining two positions are filled by (bi)carbonate and possibly water (or hydroxide) to give a six-coordinate complex. Therefore, it appears that at least two ligand sites on the iron may be accessible to small anions and chelators, which participate in removal of the metal from the protein.

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Cyanide is a useful probe of the accessibility to exogenous ligands of the coordination sites in metal–protein complexes. While low-spin complexes between ferriheme and cyanide are well-known (e.g. ferrihemoglobin cyanide[15]), there appears to be only one previous report of a cyanide adduct formed with an iron(III)-containing protein of the $g' = 4.3$ type, namely the protocatechuate 3,4-dioxygenase.[16] In the present study, we demonstrate that a stable low-spin adduct is also formed with transferrin. In this case the cyanide replaces the natural anion (bi)carbonate, the first example where another inorganic anion can form a stable iron(III)–transferrin–anion ternary complex. This unusual low-spin complex has been characterized thermodynamically and spectroscopically. The results further clarify the role of chelators and anions in the mechanism of iron exchange with transferrin.

Methods

Human serum apotransferrin of 98% stated purity was obtained from Calbiochem-Behring and used without further purification. The concentration was determined spectrophotometrically by the absorbance of a 1% solution, 11.4,[17] and the molecular weight of 79 600.[18]

Diferric transferrin was prepared by slow addition, with stirring, of 1.8 equiv of 0.02 M ferrous ammonium sulfate in 0.01 M HCl to apotransferrin in 25 mM sodium bicarbonate, 20–100 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4. After 24 h the percent saturation was determined with ϵ_{465} 5000 M^{-1} cm^{-1} per two iron(III) bound.[19] The diferric solution was then ultrafiltered with CO_2-free distilled deionized water with an Amicon ultrafiltration cell fitted with a PM 10 membrane until the concentration of Hepes was less than 0.1% of the original. The pH was adjusted to 10.4 with 1 M NaOH with stirring. This stock protein solution was stored at −20 °C and thawed prior to each experiment and the pH finally adjusted under argon or dinitrogen.

Samples of transferrin with a single ferric ion at the C-terminal or N-terminal binding site were prepared as previously described.[11] Their purity was confirmed by urea polyacrylamide gel electrophoresis,[8,20] and they were stored at −100 °C and thawed immediately prior to use.

For the preparation of the cyanide adduct of transferrin, 2 M sodium cyanide was dissolved in water and slowly brought down to pH 10.4 by 1 M HCl. Diferric transferrin at pH 10.4 was then mixed with the appropriate amount of sodium cyanide and water for dilution. Developed samples were stored frozen at −100 °C. When not specified, the concentration of carbonate in solution was that from atmospheric CO_2.

The bicarbonate equilibrium studies were carried out in an ultrafiltration cell. The diferric protein in bicarbonate/carbonate solution, pH 10.6, was added to the cell followed by the pH 10.6 sodium cyanide solution. The bicarbonate/carbonate concentration was then decreased by ultrafiltration using a 0.2 or 0.4 M cyanide solution prepared from boiled water and NaCN (parts A and B of Figure 4, respectively). The ultrafiltration and all solution transfers were done under purified argon gas. Dilution of the original bicarbonate/carbonate solution was calculated from the amounts of ultrafiltrate removed and sodium cyanide solution added. For each point in the experiment, an aliquot was removed from the cell for EPR measurement at 77 K and then returned to the cell, and the ultrafiltration process was continued.

The cyanide equilibrium studies were carried out in individual calibrated EPR tubes. Transferrin, water, and sodium cyanide solutions were added to the EPR tubes and mixed to give a solution 0.5 mM in protein, pH 10.4, with variable NaCN concentration and added Na_2SO_4 to give a total ionic strength of 0.5 M (see Results). All samples were developed for 90 min at room temperature and then frozen in dry ice/acetone, and the EPR spectrum was recorded at 77 K.

The iron titration of apotransferrin was done by preparing samples of iron transferrin with ferrous ammonium sulfate as described above to obtain iron/protein ratios ranging from 0.4 to 2.40:1. After the samples were allowed to develop for 48 h at room temperature, they were mixed with equal volumes of 1 M NaCN to give a final concentration of 0.5 M NaCN, pH 10.4, and ~0.5 mM protein, and the solution was developed for 90 min.

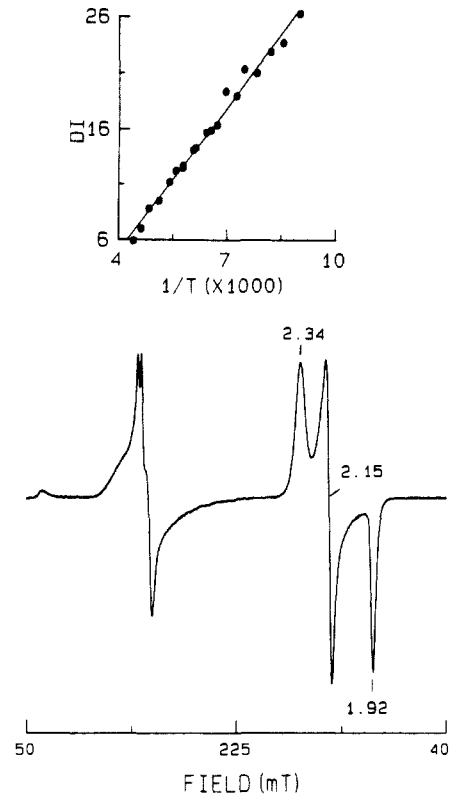

Figure 1. EPR spectrum of iron(III) transferrin at 77 K in the presence of 0.82 M NaCN (lower) and temperature dependence of the double integral (DI) of the low-spin signal (upper). A total of 47% of the iron is low-spin as determined by the double integral evaluated between 242 and 370 mT. Conditions: 0.58 mM protein, 90% Fe saturated, pH 10.6. EPR spectrometer settings: field set 250 mT, scan width 400 mT, time constant 1 s, modulation amplitude 1.25 mT, scan time 16 min, power 20 mW.

The UV difference spectrum of the cyanide adduct of the C-terminal monoferric transferrin vs the native C-terminal monoferric protein was obtained by introducing the C-terminal monoferric transferrin in 0.5 M NaCN, pH ~10.4, into the sample cell and an identical sample of C-terminal transferrin without cyanide into the reference cell. After approximately 1 h, equal volumes of 1 M Hepes, pH 7, were added to both cells to reduce the pH to 7.1 as verified at the end of the experiment. The UV difference spectrum was then recorded after approximately 1 h.

The 1:1 iron(III) complex with EHPG (ethylenebis[(*o*-hydroxyphenyl)glycine]) was prepared by adding a stoichiometric amount of 18 mM $FeCl_3$, pH 1.2, in HCl solution (Fisher Scientific, 1000 ppm Fe atomic absorption standard) to a 2 mM EHPG solution, pH 11, adjusted with NaOH. An equal volume of 2 M NaCN, pH ~12, was then added and the EPR spectrum recorded.

EPR measurements were made as previously described.[10,11,13] Double integrals were calculated from the first-derivative spectra, assuming a linear base-line correction with limits of 51.6–242 mT for the high-spin and 242–370 mT for the low-spin regions of the EPR spectrum. The precision in the double integrals was nominally ±10% and ±5% for the two regions, respectively, the high-spin $g = 4$ region being more sensitive to variations in base line. Double integrals were related to the concentrations of high- and low-spin Fe(III) with Fe_2-transferrin as an $S = {}^5/_2$ standard for the $g = 4$ region and a 1 mM copper chloride solution (1:2 (v/v) glycerol/water, pH 2.0) as an $S = {}^1/_2$ standard for the $g = 2$ region. Integrals were corrected for the dependency of the transition probability on the g factor.[21]

Results

Chemistry of Adduct Formation. The addition of NaCN to a solution of differric transferrin produces the EPR spectrum shown in Figure 1. The signal at $g' = 4.3$ is characteristic of high-spin iron(III) transferrin and corresponds to approximately 50% of

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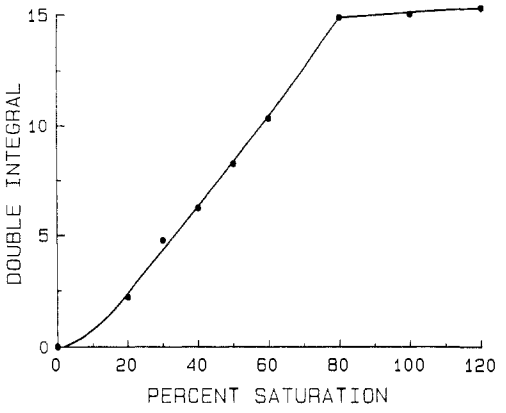

Figure 2. EPR spectrometric Fe(III) titration. Iron(II) (as ferrous ammonium sulfate, 0.02 M, pH 2) was added to apotransferrin (1 mM) in a solution of 25 mM $NaHCO_3$, 100 mM Hepes, pH 7, and allowed to oxidize. After 48 h, 1 M NaCN was added to the samples to give a final cyanide concentration of 0.5 M, pH 10.4. After 1 h, EPR spectra were taken, and the double integral of the low-spin signal was evaluated between 242 and 307 mT. EPR instrument parameters are the same as in Figure 1.

the iron present in the sample as determined from the EPR double integral (Figure 1 legend). The remaining iron gives rise to a three-component signal near $g' = 2$, typical of a low-spin Fe(III) complex with rhombic symmetry.

To verify that the low-spin signal arose from an iron–protein complex and not from a low molecular weight cyanide complex, a sample containing differric transferrin in the presence of cyanide was ultrafiltered with an Amicon PM 10 membrane having a nominal molecular weight exclusion limit of 10000. EPR spectra were taken of the retentate (and of the ultrafiltrate) before and after the ultrafiltration. Both the $g' = 2$ and the $g' = 4.3$ portions were observed in the retentate but not in the ultrafiltrate, indicating that they are due to protein-bound Fe(III).[22]

To obtain evidence for the formation of a specific cyano complex with iron(III) transferrin, an EPR spectrometric titration was done. The sharp break in the value of the double integral of the low-spin signal at 80% saturation (Figure 2), the maximum level typically obtained with ferrous ammonium sulfate, implies the formation of a specific complex. For each sample in the titration, approximately half of the iron was found to contribute to the low-spin signal and half to the high-spin signal. This result is consistent with the fact that the iron(III) in transferrin prepared from ferrous ammonium sulfate at pH 7 distributes approximately equally between the two iron-binding sites in partially saturated samples.[8] Since it was not possible to convert more than 50% of the iron to the low-spin form at cyanide concentrations as great as 1.5 M, it appeared likely that the adduct is formed at only one site. Monoferric transferrins were prepared to investigate this possibility.

Figure 3 shows the spectra of the C-terminal monoferric and N-terminal monoferric transferrins in the presence of NaCN. The formation of the low-spin adduct in the C-terminal site alone is clearly demonstrated. It is noteworthy that the EPR spectrum of the N-terminal monoferric transferrin shows a broad feature at $g' \sim 7$ ($\sim$90 mT), indicating that this center is perturbed by the presence of cyanide also. This feature disappears in the presence of added bicarbonate, suggesting that cyanide and bicarbonate may compete for binding at the N-terminal site but that the metal is not converted to low-spin in this instance. The $g' = 7$ signal was generally not observed unless care was taken to protect the pH 10.4 solutions against atmospheric CO_2. This signal was not present for samples where double integrals were evaluated.

(22) A low-spin iron(III) EPR spectrum similar to that observed with diferric transferrin can be generated by simply mixing ferric chloride with 1 M sodium cyanide at pH 11 in the absence of protein. A control ultrafiltration experiment with a solution containing only iron(III) and cyanide showed that the low-spin ferric cyanide complex is not retained by the membrane.

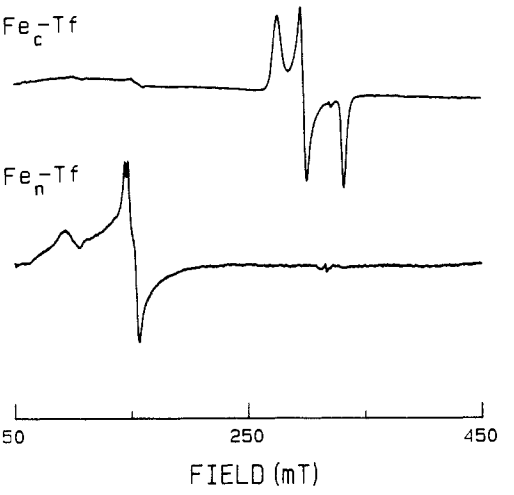

Figure 3. C-Terminal and N-terminal monoferric transferrins in the presence of 0.5 M NaCN, pH 10.5, after 1 h at room temperature. Instrument parameters are the same as in Figure 1.

In order to quantify the stoichiometry of binding, the equilibrium for adduct formation was studied in some detail, viz.

$$Fe_c\text{-Tf-}HCO_3^z + nCN^- \rightleftarrows Fe_c\text{-Tf-}(CN)_n^{z-n+x} + xHCO_3^-$$

Here Fe_c-Tf-HCO_3^z is the C-terminal iron transferrin complex; $x = 0$ indicates no bicarbonate displacement, $x = 1$ indicates one bicarbonate is displaced, and n is the number of cyanides involved in the reaction.

The conditional equilibrium constant K for adduct formation is given by $K = [Fe_c\text{-Tf-}(CN)_n^{z-n+x}]([HCO_3^-] + [CO_3^-])^x / [Fe_c\text{-Tf-}HCO_3^z][CN^-]^n$. When this equation is plotted in logarithmic form, i.e. ln ($[Fe_c\text{-Tf-}HCO_3]/[Fe_c\text{-Tf-}(CN)_n]$) vs ln ($[CO_3^{2-}] + [HCO_3^-]$), for two values of constant cyanide concentration (0.2 and 0.4 M), straight lines are obtained with slopes $x \sim 1$ (parts A and B of Figure 4). These results indicate that the synergistic anion (bi)carbonate is displaced from the protein during adduct formation. From the intercept in Figure 5B, the conditional equilibrium constant was calculated to be 0.55 ± 0.12 M^{-2} (95% confidence interval) with $x = 1$ and $n = 3$ (vide infra).

To determine the value of n, the concentration of cyanide was varied in the presence of ambient bicarbonate/carbonate at pH 10.4. The appropriate logarithmic plot is shown in Figure 4C from which a value of $n = 3.01 \pm 0.20$ is obtained from the slope, indicating the involvement of three cyanide ions in the reaction. Double integrations of the high- and low-spin signals during the course of the titration indicated that the EPR spectra accounted for all of the iron within a ±3% root-mean-square deviation for all samples and a 7% maximum deviation.

The majority of the experiments were performed at pH 10.4 to prevent outgassing the HCN. Under these conditions the iron is slowly lost from the C-terminal site of the protein. (Diferric transferrin in the absence of cyanide is stable toward loss of iron up to pH 12.2.) When the excess cyanide was removed from the protein by ultrafiltration and the pH reduced to pH $\sim$7.5, the cyanide was observed to dissociate very slowly from the protein with a half-life of 59.2 ± 0.4 h at 37 °C. Attempts to prepare stable adducts with fluoride, imidazole, and azide under various conditions were unsuccessful.

Spectroscopic Measurements. Changes occur in the visible and UV spectra upon addition of cyanide to the C-terminal monoferric transferrin (parts A and B of Figure 5). In the visible spectrum, λ_{max} shifts from 460 nm for the native protein to 420 nm for the cyanide adduct while the corresponding extinction coefficient remains essentially the same (Figure 5A). For the native protein, this band has been attributed to a ligand-to-metal $p\pi \rightarrow d\pi^*$ charge-transfer transition originating on the phenolate oxygen of the coordinated tyrosine.[23] Similar bands observed for several

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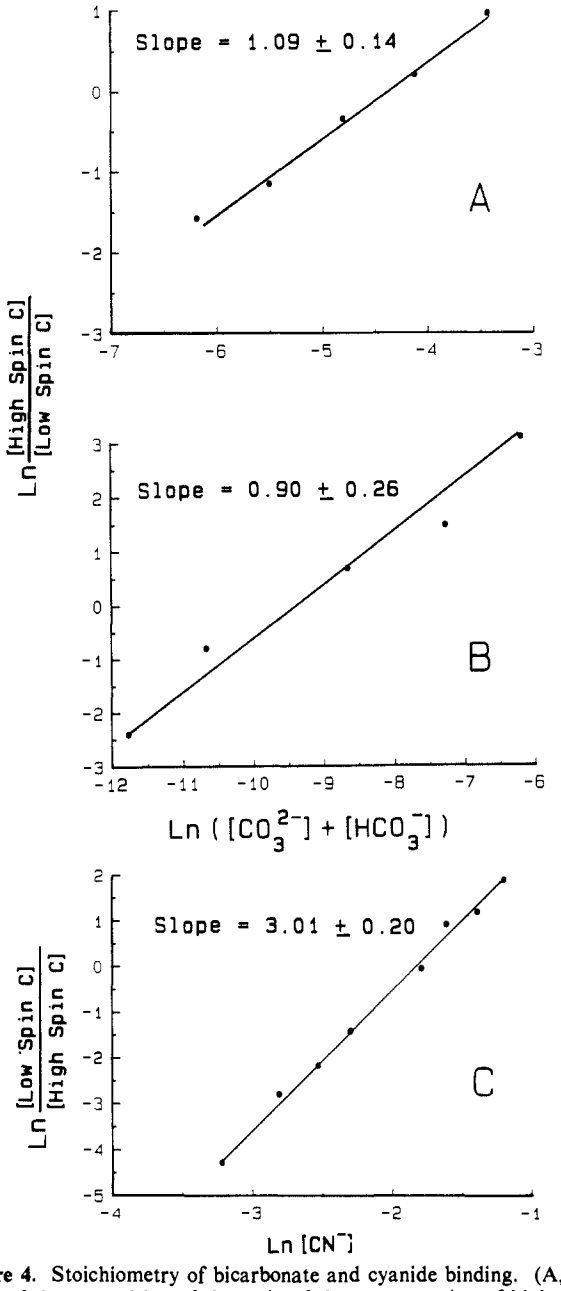

Figure 4. Stoichiometry of bicarbonate and cyanide binding. (A, B) Plots of the natural log of the ratio of the concentration of high-spin iron(III) in the C site to low-spin iron(III) in the C site as a function of the concentration of bicarbonate. Conditions: 0.2 mM protein, 90% Fe saturated, pH 10.6, 0.2 M NaCN (A), and 0.4 M NaCN (B). (C) Plot of the natural log of the ratio of the concentration of low-spin iron(III) in the C site to high-spin iron(III) in the C site as a function of the concentration of cyanide. Conditions: 0.50 mM protein, 90% saturated, pH 10.4, no added $NaHCO_3$. Errors in slopes are given at the 95% confidence level. The concentration of high-spin iron in the C site was calculated from the value of the double integral of the $g' = 4.3$ signal minus half the maximum value when two Fe^{3+} are bound to the protein. The concentration of low-spin iron was calculated from the double integral of the $g' = 2$ signals. The ratio of high- and low-spin forms in the C-terminal site was also calculated independently from $(I_{max} - I)/I$, where I_{max} is the maximum value of the low-spin double integral under conditions of complete adduct formation and I is an intermediate value. In this instance slopes of $x = 1.00$ and $n = 3.12$ were obtained from the plots corresponding to B and C.

metal derivatives of transferrin have also been assigned to charge-transfer transitions.[24]

The UV difference spectrum for native C-terminal monoferric transferrin, employing apotransferrin as the reference, has bands at 295 and 245 nm, which can be assigned to $\pi \rightarrow \pi^*$ transitions

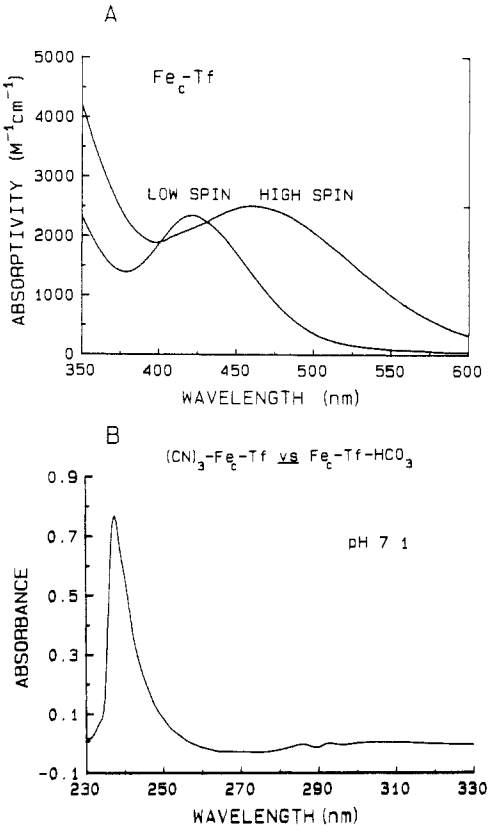

Figure 5. Visible and UV difference spectra: (A) visible spectrum of C-terminal monoferric transferrin in the absence and presence of 0.5 M NaCN, pH 10.4; (B) UV difference spectrum of the low-spin cyanide adduct of C-terminal monoferric transferrin vs the native C-terminal monoferric transferrin in the reference cell. Protein concentration for both cells 0.0123 mM, 500 mM Hepes, pH 7.1.

of the ionized phenolate groups of two tyrosines bound at the metal site.[25] Figure 5B shows the UV difference spectrum of the cyanide adduct of C-terminal transferrin vs native C-terminal transferrin. The absence of an absorbance in the region of 295 nm suggests that the same number of tyrosines (two) are bound to the metal in both the adduct and native protein. The band observed at 238 nm in the difference spectrum (Figure 5B) may be due to a cyanide-to-iron charge-transfer transition as has been suggested for ferricyanide itself.[26]

***g*-Factor Analysis.** Analysis of the g factors of the adduct provides information about the coordination environment and electronic states of the metal. In order to simplify the analysis, it is assumed that the t_{2g} and e_g orbitals are well separated in energy. This assumption is supported by the Curie behavior of the intensity of the adduct signal vs temperature (Figure 1), indicating that $S > {}^1/_2$ excited states involving the e_g orbitals are not thermally accessible.

The perturbation Hamiltonian used for the analysis is[27]

$$\hat{H}' = -\lambda\vec{\hat{l}}\cdot\vec{s} + (\mu/9)[3\hat{l}_z^2 - l(l+1)] + (R/12)(\hat{l}_+^2 + \hat{l}_-^2)$$

where λ is the spin–orbit coupling constant and μ and R are the tetragonal and rhombic crystal field splitting constants. Here, the "hole" formalism is used where the t_{2g}^5 configuration is considered a hole in the t_{2g}^6 closed shell.

The eigenfunctions of $\hat{H}'$ in terms of the familiar d orbital functions have the form[27]

$$|\psi_i^+\rangle = D|d_{yz}, \alpha\rangle + E|d_{xy}, \beta\rangle + F|d_{xz}, \alpha\rangle$$

$$|\psi_i^-\rangle = D|d_{yz}, \beta\rangle + E|d_{xy}, \alpha\rangle + F|d_{xz}, \beta\rangle$$

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Table I[a]

complex[b]	g_x (±0.01)	g_y (±0.01)	g_z (±0.01)	μ/λ (±0.12)	R/λ (±0.19)	$\lvert R/\mu\rvert$ (±0.04)	ref[c]
			Category I				
$FeAcen(Im)_2$	2.14 (2.36)	−1.94 (2.14)	−2.36 (1.94)	9.46 (−8.88)	5.55 (6.71)	0.59 (0.76)	28
$FeAcen(CN)_2$	2.13 (2.32)	−1.96 (2.13)	−2.32 (1.96)	10.91 (−10.22)	6.36 (7.73)	0.58 (0.76)	28
$FeAcen(Apy)_2$	2.10 (2.31)	−1.91 (2.10)	−2.31 (1.91)	9.74 (−9.14)	5.68 (6.91)	0.58 (0.76)	28
			Category II				
FeEHPG[d]	2.33	2.15	1.92	−8.11	4.88	0.60	this work
dioxygenase	2.43	2.21	1.89	−6.25	3.51	0.56	16
transferrin	2.34	2.15	1.92	−8.07	5.05	0.63	this work

[a] The sign and ordering of the g factors according to x, y, and z were chosen in all cases such that the calculated value of R/λ is positive and $|R/\mu|$ is less than or equal to $^2/_3$ according to the convention proposed by Bohan.[27] Alternate assignments that do not conform to this convention are given in parentheses. The stated uncertainty in the crystal field parameters is based on the uncertainty in the measurement of the g factors. [b] Acen, *N,N'*-bis(salicylidene)ethylenediamine; Im, imidazole; Apy, 4-aminopyridine; EHPG, ethylenebis[(*o*-hydroxyphenyl)glycine]. [c] References for the g factors. [d] The EPR signal arises from a short-lived species in solution (see Methods).

Assignments of the g values for the low-spin adduct, based on convention III of Bohan,[27] are $g_x = 2.34$, $g_y = 2.15$, and $g_z = 1.92$. Determination of the coefficients and diagonalization of the matrix representing $\hat{H}'$ result in the following relative energies in units of λ and the eigenstates for the three lowest Kramers' doublets of transferrin where $i = -1^{1/2}$.

$$E_1/\lambda = 5.41,\ D = -0.994i,\ E = -0.042i,\ F = 0.095$$

$$E_2/\lambda = 0.17,\ D = -0.089i,\ E = -0.098i,\ F = -0.991$$

$$E_3/\lambda = -5.37,\ D = -0.052i,\ E = 0.994i,\ F = -0.094$$

This result indicates that the hole, and therefore the unpaired electron, resides in the ψ_3 orbital, which is largely d_{xy} in character, and that the doubly occupied $\psi_1 \sim d_{yz}$ orbital is the most stable of the t_{2g} set.

The non-heme iron microbial enzyme, protocatechuate 3,4-dioxygenase, forms a low-spin cyanide adduct of rhombic symmetry characterized by $g_x = 2.43$, $g_y = 2.21$, and $g_z = 1.89$.[16] Analysis of these data leads to the following Kramers' doublet energies and eigenfunctions:

$$E_1/\lambda = 3.91,\ D = -0.989i,\ E = -0.053i,\ F = 0.131$$

$$E_2/\lambda = 0.33,\ D = -0.124i,\ E = -0.123i,\ F = -0.984$$

$$E_3/\lambda = -4.25,\ D = -0.069i,\ E = 0.991i,\ F = -0.115$$

where the unpaired electron again resides in a molecular orbital largely d_{xy} in character. Table I is a compilation of g factors and crystal field parameters for various low-spin iron(III) chelates and the two protein complexes.

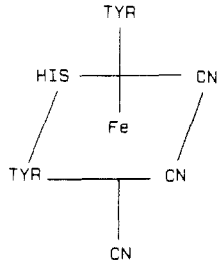
Discussion

Metal-Site Structure. The equilibrium and spectral data provide some insight into the structure of the metal site and the mechanism of iron removal from the protein. The stoichiometric equation for adduct formation is

$$Fe_c\text{–}Tf\text{–}HCO_3^z + 3CN^- \rightleftarrows Fe_c\text{–}Tf\text{–}(CN)_3^{z-2} + HCO_3^-$$

suggesting the coordination of three cyanides to the iron in the product. The possibility that one or two of the cyanides bind solely to cationic sites on the protein is not precluded by the data however.[1,7-11]

Consistent with the X-ray structure of lactoferrin, we propose the following six-coordinate complex for the adduct. The *fac* arrangement shown for the cyanide ligands is expected to be particularly stable, providing each cyanide with a separate d orbital for back-bonding.



The proposed structure is consistent with the UV and visible spectral data (Figure 5), both of which suggest that the iron remains coordinated to two tyrosines in the adduct. The structure is also in accord with the displacement of (bi)carbonate and the expected displacement of hydroxide (or water) upon cyanide binding. Of the remaining protein ligands, histidine and aspartate, we favor the coordination of histidine since nitrogen donor ligands are preferred by low-spin iron(III) complexes. In the case of the related cyanide adduct of protocatechuate 3,4-dioxygenase, available data suggest that the coordination environment of the iron(III) consists of two tyrosines, two histidines, and two cyanides,[16,29] a complex differing from the proposed structure for transferrin by the presence of a histidine rather than a third cyanide.

Previous studies of transient mixed-ligand protein complexes have provided no evidence concerning (bi)carbonate binding to the metal in such complexes.[12,13] Our data indicate that, at least in the case of cyanide, the synergistic anion (bi)carbonate is displaced from the first coordination sphere of the metal by the exogenous ligand. The structural requirement that synergistic anions must contain a carboxyl group and a second adjacent function group[6] does not hold here. Moreover, the data, while not conclusive on this point, suggest that three coordination sites on the iron may be available for complexation by exogenous chelators during iron-removal reactions. In contrast to studies with other complexing agents that form transient mixed-ligand complexes, the cyanide adduct of transferrin can be considered an example of a particularly stable mixed-ligand complex in which loss of iron from the C-terminal site to the competing CN^- in solution occurs only slowly.

It is interesting to note that only the C site of transferrin forms the cyanide adduct. Bates and co-workers[12] in their studies of the kinetics of iron removal from transferrin have provided evidence for a requisite change in the protein to an "open" conformation in which the metal is exposed to attacking chelators prior to the formation of a mixed-ligand intermediate complex. We suggest that a similar conformational change occurs here in which the otherwise buried iron(III) in the C-terminal site becomes exposed to cyanide. Consistent with this view is the fact that the C-terminal site in the native protein is known to be destabilized both kinetically and thermodynamically through a conformational change induced by the presence of high concentrations of salts[7-9] comparable to the concentration of NaCN employed in our experiments.

Spectral Properties. Que and co-workers[29] in studies of model compounds for iron tyrosinate proteins have observed correlations between the reduction potential of the Fe(III), the crystal field strength of the other ligands, and energy of the charge-transfer band. The blue shift observed here for the charge-transfer band of the C-terminal site of transferrin from 460 to 420 nm upon cyanide binding is consistent with decreased Lewis acidity of the metal ion and the strong ligand field provided by cyanide. In contrast, the cyanide adduct of protocatechuate 3,4-dioxygenase

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exhibits a red shift from 460 to about 620 nm.[16] The difference between the two proteins may reside in the different number of cyanide anions bound to the metal. The additional cyanide in the transferrin complex suggested by our data would impart one more negative charge to the metal center, hence reducing the Lewis acidity of the iron in transferrin relative to the dioxygenase.

Analysis of the g factors of model compounds and proteins indicates that the complexes fall into two categories (Table I). The Schiff base complexes in the first category are similar to the type O low-spin heme complexes categorized by Blumberg and Peisach[30] where, according to Bohan,[27] g_x is positive and g_y and g_z are negative with the unpaired electron located in a d_{xz} orbital ($\mu > 0$). In the Schiff base complexes the adduct ligands are coordinated trans to one another and perpendicular to the plane of the chelate ring of the base, a situation analogous to heme. The second category consists of the commonly employed model compound for transferrin FeEHPG,[23,25,31] dioxygenase, and transferrin, all having positive g factors and the unpaired electron in a d_{xy} orbital ($\mu < 0$). In contrast to the first category, the cyanide ligands of the dioxygenase and transferrin complexes are thought to be cis.

The values of the axial crystal field parameter μ of the compounds in Table I are rather large, falling in the range 2500–4400 cm^{-1} where λ is taken as $\sim$400 cm^{-1}.[32] A significant lifting of the t_{2g} d-orbital degeneracy is required for the observation of electron paramagnetic resonance.[33] The complexes are all highly rhombic in their crystal fields as reflected in the values of R/μ. Transferrin has the lowest "symmetry" with $R/\mu = 0.63$, a value approaching the theoretical limit of 0.67 for rhombic symmetry.[27]

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Infrared Spectra and Structures of Lithium–Benzene and Lithium–Dibenzene Complexes in Solid Argon

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Abstract: Cocondensation of lithium and sodium atoms with benzene diluted in argon produced new infrared absorptions attributable to mono- and dibenzene complexes in the case of lithium but nothing with sodium. Isotopic studies (^{6}Li, ^{7}Li, C_6H_6, C_6D_6) of the observed fundamentals suggest that the Li position in the monobenzene complex is axial and that the ligand is distorted from 6-fold symmetry. The symmetric ring-breathing mode, forbidden for C_6H_6, gives a strong 924-cm^{-1} absorption with a 2-cm^{-1} lithium isotopic shift for LiC_6H_6. The dibenzene species exhibits a strong ring-breathing mode at 901 cm^{-1}, and motions of the two spectroscopically equivalent benzene ligands are coupled. Interaction of ring-breathing and Li–ring stretching modes indicates a significant electronic interaction between lithium and benzene in these complexes.

Lithium forms insertion compounds of formula LiC_6 with graphite as well as complexes with large benzenoid polycyclic molecules.[1,2] These compounds, owing to their physical properties as conducting materials, have been the subject of experimental and theoretical investigations; several of their physical properties (C–C distance increase and ^{7}Li magnetic coupling) have been discussed in terms of charge transfer of the alkali valence electron, the extent of which is still a matter of current controversy. Recently two theoretical studies[3,4] have been devoted to interaction between Li and C_6H_6, hoping to attain better understanding of a "less complex" system than large aromatic molecules. In the course of our investigation of alkali metal atom reactivity with unsaturated hydrocarbons in solid argon, it was desired to determine if aromatic molecules form strong complexes with Li, as do alkenes[5] and alkynes.[6] Here is reported an infrared study of the interaction between Li atoms and benzene.

Experimental Section

The cryogenic refrigeration system, vacuum vessel, alkali-atom source, and experimental techniques have been described elsewhere.[7] Isotopically enriched samples of lithium metal, 99.99% ^{7}Li and 95.6% ^{6}Li (ORNL), and natural sodium metal (Baker and Adamson, 99.9%) were evaporated directly from a heated Knudsen cell. High-purity benzene (Baker, spectroscopic grade) and benzene-d_6 (MSD, 98% D) were frozen, thawed, and outgassed at liquid nitrogen temperature before use. Argon gas (99.995%, Air Products) was used without purification. Gaseous benzene/argon mixtures were deposited at about 2 mmol/h simultaneously with the alkali-metal beam on a CsI (mid-IR) or on a polished copper block (Raman). The lithium concentration was modified by varying the temperature of the Knudsen effusion cell, resulting in a 10-fold change in metal vapor pressure. The highest Li/Ar concentration used here is estimated at 1/600.

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