A Determination of the Reduction Potentials for Diferric and C- and N-Lobe Monoferric Transferrins at Endosomal pH (5.8)

Daniel C. Kraiter,[†] Olga Zak,[‡] Philip Aisen,[‡] and Alvin L. Crumbliss*,[†]

Department of Chemistry, Duke University, Durham, North Carolina 27708-0346, and Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received May 30, 1997

Human transferrin is a bilobal protein, with two Fe(III) binding sites displaying different spectroscopic, kinetic, and thermodynamic properties despite identical ligand donor groups. We present a spectroelectrochemical determination of the formal Fe(III/II) redox potentials for all possible permutations of this Fe-binding protein: Fe-saturated diferric (Fe_2Tf), monoferric C lobe (Fe_CTf), and monoferric N-lobe (Fe_NTf), at endosomal pH (5.8), 20 °C, and 0.5 M KCl. The spectroelectrochemical technique utilized an anaerobic optically transparent thin-layer electrochemical (OTTLE) cell and methyl viologen mediator. Correction was made for the Fe^{II}_nTf ($n = 1$, 2) dissociation equilibrium using an estimated value for the Fe(II)−transferrin binding constant at pH 7.4 in order to calculate upper limits of the redox potentials at pH 5.8 for Fe_2Tf, Fe_CTf, and Fe_NTf of −526, −501, and −520 mV (vs NHE), respectively. Reduction of Fe_2Tf is by two-electron transfer. This study represents the first direct determination of the Fe(III/II) transferrin redox potentials for all Fe-bound permutations of the protein at physiological conditions relevant to the endosomal release of Fe. Our results suggest that the two redox sites are similar and that the redox potentials are too negative for Fe(III) reduction by a physiological reducing agent to be involved in endosomal release of Fe from transferrin in the absence of a reaction sequence sequestering Fe(II) or effecting a conformational change in transferrin that substantially raises the reduction potential of its Fe(III). No such sequence is known.

Introduction

The iron-binding protein transferrin has evolved for transporting iron in the circulation of all vertebrates and a number of invertebrates,[1] conveying the metal from cells where it is released to cells where it is stored or incorporated into essential proteins such as hemoglobin and the cytochromes. The human transferrin molecule is a single-chain glycopeptide of 679 amino acids, disposed in two similar lobes with about 60% sequence identity between them.[2] Each lobe consists of two rigid domains hinged to form a cleft bearing an iron-binding site. Four of the six coordinating atoms of bound iron are provided by the protein: two tyrosyl phenolic oxygens, one carboxylate oxygen of an aspartate, and one histidyl nitrogen. The remaining iron coordination sites are satisfied by two oxygen atoms of a "synergistic" carbonate anion, linked to the protein by a network of hydrogen and electrostatic bonds. In the absence of carbonate, or a substitute for it, iron is not bound by transferrin, so that the requirement for a synergistic anion may be taken as a defining feature of the transferrin class of iron-binding proteins. For recent reviews of transferrin structure, see Baker and Lindley[3] and Baker.[4]

Iron resists hydrolysis by tight, but reversible, binding to transferrin, with apparent stability constants greater than 10^{20} M^{-1}.[5] During its lifetime in the circulation, the human transferrin molecule carries out more than 100 cycles of iron binding, transport, and release.[6] Understanding the physiological activity of the protein therefore depends on understanding the mechanism of iron release. Iron is taken up from transferrin by the iron-requiring cell in a complex series of events initiated by a binding of the transferrin molecule to its specific receptor on cell membranes. The receptor−transferrin assembly is internalized in a membrane-bound endosome in which proton pumping lowers the pH to 5.4−6.0.[2,7,8] Iron is freed from transferrin within the endosome and the resulting apotransferrin returned to the cell surface, where it is released to the circulation for another cycle of iron transport. No more than 2−3 min is required for the entire process to be completed.[6]

Although the low pH of the endosome facilitates iron release from transferrin, it is not sufficient to bring about such release: a self-buffered solution of transferrin at pH 5.0 requires hours to release half of its load of iron.[9] Other factors, such as chelation and labilization of the iron−transferrin bound by the transferrin receptor, must also participate in the release mech-

* Address correspondence to this author. E-mail: alc@chem.duke.edu. Fax: 919-660-1605. Tel: 919-660-1540.
[†] Duke University.
[‡] Albert Einstein College of Medicine.

(1) Aisen, P. in *Iron Metabolism in Health and Disease*; Brock, J. H., Halliday, J. W., Pippard, M. J., Powell, L. W., Eds.; W. B. Saunders: London, 1994; p 1.
(2) Klausner, R. D.; Ashwell, G.; van Renswoude, J.; Harford, J. B.; Bridges, K. R. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2263.
(3) Baker, E. N.; Lindley, P. F. *J. Inorg. Biochem.* **1992**, *47*, 147.
(4) Baker, E. N. *Adv. Inorg. Chem.* **1994**, *41*, 389.
(5) Aisen, P.; Leibman, A.; Zweier, J. *J. Biol Chem.* **1978**, *253*, 1930.
(6) Theil, E. C.; Aisen, P. in *Iron Transport in Microbes, Plants, and Animals*; Winkelmann, G., van der Helm, D., Neilands, J. B., Eds.; VCH: Weinheim, 1987; p 491.
(7) van Renswoude, J.; Bridges, K. R.; Harford, J. B.; Klausner, R. D. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6186.
(8) Dautry-Varsat, A.; Ciechanover, A.; Lodish, H. F. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2258.
(9) Zak, O.; Aisen, P. Unpublished observations.

S0020-1669(97)00644-7 CCC: $15.00
Published on Web 02/14/1998

anism. The weaker binding of Fe(II) to transferrin (log K = 2−3)[10] as well as its higher lability relative to Fe(III),[11] is also consistent with a reductive elimination of the iron. Therefore, a study of the reduction of the iron bound to transferrin at low pH is of considerable importance in gaining an understanding of the physiological activity of this iron transport protein.

Earlier studies focused on a determination of the redox potential of iron in transferrin at pH 7.4.[10,12,13] D. C. Harris, et al.[12] reported a reduction potential of −400 mV (vs NHE) for Fe_2Tf at pH 7.4, using dithionite as the reducing agent and methyl viologen as a mediator. However, no correction was made for the dissociation of Fe(II) in the reduced product, $Fe^{II}{}_2$-Tf; such a correction would make the redox potential more negative. Raymond et al.[13] reported the reduction potential of $Fe^{III}{}_2Tf$ at high electrolyte concentration (2 M KCl) and pH 7.4 using bulk electrolysis and spectrophotometric detection. Extrapolation of a concentration/potential plot results in a calculated redox potential which, when corrected for $Fe^{II}Tf$ dissociation, is −520 mV. Neither of these studies[12,13] distinguished between diferric transferrin (Fe_2Tf) and C-terminal (Fe_CTf) and N-terminal (Fe_NTf) monoferric transferrin. An indirect approach was taken by W. R. Harris,[10] who combined Fe(II) binding constants for $Fe^{II}{}_CTf$ and $Fe^{II}{}_NTf$, calculated from a linear free-energy relationship between Fe(II) and Ni(II), with the corresponding $Fe^{III}Tf$ binding constants to estimate formal reduction potentials of −340, −280, and −338 mV for C-terminal, N-terminal, and diferric transferrin, respectively, at pH 7.4. W. R. Harris considered the dissociation of Fe(II) from transferrin in his calculations as well.[10]

The goal of this investigation was to directly determine the formal redox potential of Fe in Fe_CTf, Fe_NTf, and Fe_2Tf at endosomal pH, 5.8. Our approach was to utilize an anaerobic optically transparent thin-layer electrochemical (OTTLE) cell in conjunction with the spectroelectrochemical technique.[14] Relatively short measurement times (0.5−4 h), the absence of O_2, and the use of a methyl viologen (MV^{2+}) mediator facilitated precise measurement of the redox potentials without complicating side reactions. The overall system, including correction for Fe(II) dissociation, may be represented as follows.

$$MV^{2+} + e^- \rightleftharpoons MV^{\bullet+} \quad \text{(at the electrode)} \tag{1}$$

$$MV^{\bullet+} + Fe^{III}Tf \rightleftharpoons MV^{2+} + Fe^{II}Tf \quad \text{(in solution)} \tag{2}$$

violet (λ = 602 nm); orange (λ = 465 nm); colorless; colorless

$$Fe^{II}Tf \overset{1/K_b}{\rightleftharpoons} Fe_{aq}{}^{2+} + Tf \tag{3}$$

Experimental Section

Materials. Diferric transferrin, Boehringer Mannheim Corp., and C-terminal and N-terminal monoferric transferrins prepared by the method of Baldwin and de Sousa[15] were freed of extraneous chelators by dialysis against 0.1 M $NaClO_4$ followed by dialysis against 0.05 M HEPES buffer at pH 6.5.[16] For spectroelectrochemical experiments, diferric and C-terminal monoferric transferrin samples were further dialyzed overnight against a 0.05 M MES, 0.2−0.4 mM methyl viologen (MV^{2+}), 0.5 M KCl solution at pH 5.8, 4−6 °C. The final protein concentration with respect to Fe was 0.11−0.22 mM. The N-terminal monoferric transferrin samples were dialyzed similarly, but for 7 h with a complete replacement of solution after 3 h, and stored at 4−6 °C ([Fe] = 0.68 mM). The diferric and C-terminal monoferric transferrins were shown to be stable for several weeks when kept cold. The N-terminal monoferric transferrin was used within 24 h of dialysis. The purity of the preparations was verified by urea gel electrophoresis.[5]

Methyl viologen dichloride hydrate (98%, Aldrich), 2-[*N*-morpholine]ethanesulfonic acid monohydrate, MES (>99.5%, Sigma), and certified ACS KCl (Fisher Scientific) were free of any electrochemical impurities and used as received. A 1 N KOH solution (Fisher Scientific) was used to adjust the solution pH to 5.8. Doubly distilled water was used at all times. Glassware was soaked in a detergent or a 30% H_2O_2 in 0.1 M HNO_3 solution, thoroughly washed with water, rinsed with deionized water, and finally rinsed with doubly distilled water prior to use.

Methods. An optically transparent thin-layer electrochemical (OTTLE) cell was constructed with separate compartments for the working, auxiliary, and reference electrodes and thermostated at 20 °C.[14,17] The reference compartment was filled with deaerated dialysis solution (supporting electrolyte, buffer, and methyl viologen mediator at pH 5.8) and separated from the working electrode compartment by an agar plug. A Ag/AgCl in 3 M NaCl reference electrode was used for all measurements, and a gold wire, 0.05-cm diameter, was used as an auxiliary electrode. The working electrode was gold gauze (1 × 0.4 in.^{2} plain weave mesh, 82 × 82 wires/in.^{2}, 65% open surface, Goodfellow Inc.) sandwiched between the transparent cell wall and a quartz plate to give a 0.035(5)-cm light path. The working electrode was conditioned before each potential step by soaking in a 0.1 M HNO_3 solution (>15 min) followed by sonication in ethanol (15 min). The electrode was then held at −76 mV (15 min) and scanned in 1 M H_2SO_4 between 1.80 and −0.01 V until a reproducible cyclic voltammogram was obtained.[18] The electrode was then rinsed with doubly distilled water and allowed to dry. The electrode potential was controlled by an external P.A.R. Model 363 potentiostat. All potentials are reported relative to the NHE.

Before each experiment, the empty cell and transferrin solution were repeatedly subjected to several careful vacuum−ultrapure Ar purges, frothing being avoided as much as possible. A permanent flow of ultrapure Ar was maintained over the cell during the spectroelectrochemical experiment in order to prevent any O_2 contamination which would react with Fe(II) transferrin and the reduced form of the mediator.

Spectroelectrochemical experiments were conducted using solutions consisting of transferrin ([Fe] = 0.11−0.68 mM), MES buffer (0.05 M), KCl supporting electrolyte (0.5 M), methyl viologen mediator (MV^{2+}; 0.2−0.4 mM), and CO_2 ([HCO_3^-] < 0.007 mM). The decrease in the concentration of Fe(III) transferrin by reduction at various fixed electrode potentials was monitored at 465 nm ($\epsilon_{Fe(N)Tf}$ = 2130 M^{-1} cm^{-1}, $\epsilon_{Fe(C)Tf}$ = 2670 M^{-1} cm^{-1}, and ϵ_{Fe_2Tf} = 4800 M^{-1} cm^{-1})[12] using a Cary 2300 UV-vis spectrophotometer. Initially the potential was set at −206 mV, where the Fe transferrin is fully oxidized ($Fe^{III}Tf$-measured absorption denoted A_{to}). Then the potential was stepped to more negative values between −386 and −566 mV (measured absorption values denoted A_e) in 20−40-mV increments, where the Fe centers were partially reduced. The final step was to −656 mV, where the Fe transferrin was fully (>99.5%) reduced ($Fe^{II}Tf$ and $Fe_{aq}{}^{2+}$ measured absorption denoted A_{tr}). All absorption changes (at 465 nm) were corrected for the absorption of the mediator at that potential, which usually accounted for less than 10% of the total absorption. Equilibrium at each potential was reached in 0.5−4 h. When the cell was kept at the same potential for a long time after reaching equilibrium, a decrease in the absorbance of $MV^{\bullet+}$ was observed, probably due to the formation of dimers by reactions between fully oxidized and reduced mediator species.[19,20] In such experiments, the spectroelectrochemical process became irreversible due to mediator side reactions. After each potential/

(10) Harris, W. R. *J. Inorg. Biochem.* **1986**, *27*, 41.
(11) Wilkins, R. G. *Kinetics and Mechanism of Reactions of Transition Metal Complexes*, 2nd ed.; VCH: Weinheim, 1991.
(12) Harris, D. C.; Rinehart, A. L.; Hereld, D.; Schwartz, R. W.; Burke, F. P.; Salvador, A. R. *Biochim. Biophys. Acta* **1985**, *838*, 295.
(13) Kretchmar, S. A.; Reyes, Z. E.; Raymond, K. N. *Biochim. Biophys. Acta* **1988**, *956*, 85.
(14) Faulkner, K. M.; Bonaventura, C. J.; Crumbliss, A. L. *Inorg. Chim. Acta* **1994**, *226*, 187.
(15) Baldwin, D. A.; de Sousa, D. M. R. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1101.
(16) Bates, G. W.; Schlabach, M. R. *J. Biol. Chem.* **1973**, *248*, 32289.
(17) Faulkner, K. M.; Bonaventura, C. J.; Crumbliss, A. L. *J. Biol. Chem.* **1995**, *270*, 13604.
(18) Hoare, J. P. *J. Electrochem. Soc.* **1984**, *131*, 1808.
(19) Bird, C. L.; Khun, A. T. *Chem. Soc. Rev.* **1981**, *10*, 49.

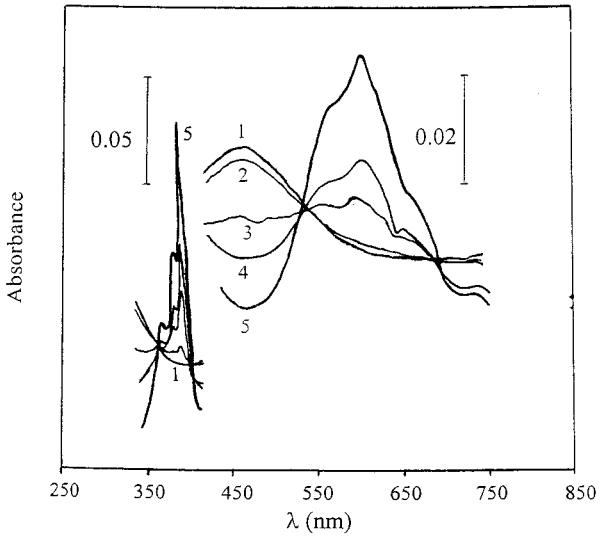

Figure 1. A typical collection of spectra for the reduction of $Fe^{III}Tf$ in the presence of methyl viologen at various applied potentials. The solution contains 0.20 mM Fe_CTf and 0.4 mM MV^{2+} in 0.5 M KCl, 0.05 M MES at pH = 5.8, and 20 °C: $Fe^{III}Tf$ peak at 465 nm; MV^{2+} peaks at 395 and 602 nm. The equilibrium spectra were obtained at −206 (1), −386 (2), −426 (3), −486 (4), and −506 mV (5).

absorbance measurement the cell was opened and the solution changed in order to replenish the mediator, and the electrode was chemically and electrochemically cleaned in order to remove any protein which may have adsorbed on the electrode surface. At negative potentials below −526 mV, an amalgamated Au gauze working electrode was used to prevent H_2 evolution. The amalgamation procedure consisted of electrochemically depositing Hg (E_{dep} = 544 mV) from a 0.05 M $Hg(NO_3)_2$ solution in 2 M HNO_3 for 10−15 min. The electrode surface was renewed before each potential step by soaking the Au(Hg) gauze in anhydrous ethanol for 30 min, followed by a short Hg electrochemical deposition step.

The stability of solutions in the OTTLE cell was established by monitoring a solution containing 0.234 mM Fe_CTf (or Fe_2Tf) and 0.2 mM MV^{2+} in 0.5 M KCl and 0.05 M MES at pH 5.8 over a 2-h period (the average time span for an experiment) and noting an absorbance drift of <2.5% from 340 to 750 nm. Reversibility was tested for diferric transferrin (0.13 mM) in the presence of 0.4 mM MV^{2+}, 0.5 M KCl, 0.05 M MES, and pH 5.8 to establish that large negative potentials did not reduce the protein disulfide bridges (an irreversible process).[12] The system in its fully oxidized state at −206 mV was stepped to −656 mV and allowed to reach equilibrium in the fully reduced form. The solution was then reoxidized by exposure to air and the spectrum indicated that >85% of the initial protein concentration was regenerated.

Spectroelectrochemical measurements in our OTTLE cell at the conditions of the transferrin experiments resulted in a formal redox potential of −474(3) mV (number of electrons transferred n = 1.06) for methyl viologen and +416 mV (n = 0.94) for ferricyanide ($Fe^{III/II}(CN)_6^{3-/4-}$). These are consistent with literature values,[13,21] once allowance is made for the ohmic drop and liquid junction potential in the thin-layer cell. The $Fe^{III/II}(CN)_6^{3-/4-}$ redox potential reported here may be used as a reference point for comparison of our transferrin results with those obtained in other laboratories.

Results

Spectral changes associated with the reaction of $Fe^{III}Tf$ with MV^{2+} (eqs 1−3) at various potentials are illustrated in Figure 1. Initially, at −206 mV, only the absorbance of $Fe^{III}{}_nTf$ is observed (MV^{2+} has a negligible absorbance over the wavelength range scanned). When stepping the potential to more

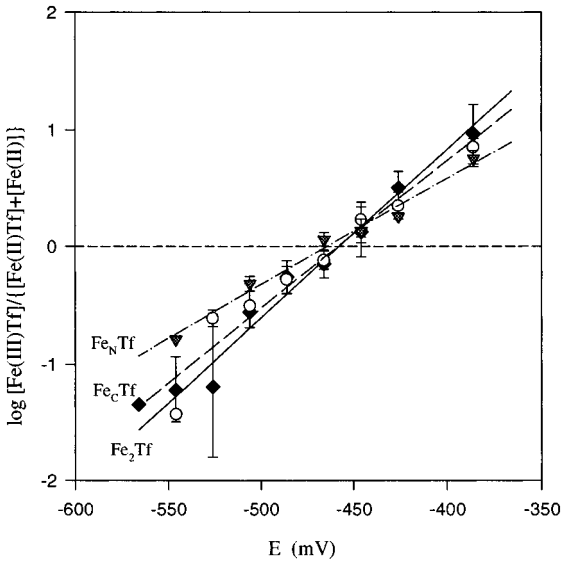

Figure 2. Nernst plots for the reduction of iron transferrin species according to eqs 5 and 6. Each data point represents the average of 2−3 independent measurements of different transferrin solutions. Conditions: 0.2−0.4 mM MV^{2+} in 0.5 M KCl, 0.05 M MES at pH = 5.8, and 20 °C. Diamonds: Fe_2Tf (0.11−0.19 mM in Fe), circles: Fe_C-Tf (0.18−0.22 mM in Fe). Triangles: Fe_NTf (0.68 mM in Fe). Error bars represent standard deviations for the average of 2−3 independent experiments.

negative values, the characteristic peaks for $MV^{\bullet+}$ (396 and 602 nm) increase and the $Fe^{III}{}_nTf$ peak (465 nm) decreases. An equilibrium position is reached between the oxidized and reduced forms of the protein and the mediator at each externally applied potential, with the absorption spectrum remaining constant. Due to the relatively low affinity of apotransferrin for Fe(II) and the relatively high lability of Fe(II),[22] the overall process that takes place upon reduction in the OTTLE cell is illustrated in eq 4 and characterized by a potential denoted as

$$Fe^{III}{}_nTf + ne^- \rightleftharpoons nFe_{aq}^{2+} + \text{apo-Tf} \qquad (4)$$

$$(n = 1 \text{ or } 2)$$

E_{rxn}. The relationship between the concentration ratio of the oxidized and reduced transferrins and their absorbance in solution is shown in eq 5, where C_{ox} is the concentration of the

$$C_{ox}/C_{red} = [Fe^{III}Tf]/\{[Fe_{aq}^{2+}] + [Fe^{II}Tf]\} = (A_e - A_{tr})/(A_{to} - A_e) \qquad (5)$$

oxidized species present, $Fe^{III}Tf$, C_{red} is the concentration of both reduced species present, $Fe^{II}Tf$ and Fe^{2+}(aq), and A_{to}, A_{tr}, and A_e are the absorbances obtained at potentials where the transferrin is totally oxidized, totally reduced, and at intermediate potentials, respectively, as described in the Experimental Section. Equation 6 expresses the Nernst relationship in terms

$$\log\{(A_e - A_{tr})/(A_{to} - A_e)\} = (n_{app}/58.1)E - (n_{app}/58.1)E_{rxn} \qquad (6)$$

of the dependence of the ratio of the oxidized to reduced species on the imposed solution potential, E, written as a function of the characteristic absorbance values at 465 nm. n_{app} in eq 6 is the apparent number of electrons transferred in the process, and E_{rxn} is the apparent redox potential for eq 4. Figure 2 is a plot

(20) Zak, O.; Leibman, A.; Aisen, P. *Biochim. Biophys. Acta* **1983**, *742*, 490.
(21) Kolthoff, I. M.; Tomsicek, W. J. *J. Phys. Chem.* **1935**, *39*, 945.
(22) Gaber, B. P.; Aisen, P. *Biochim. Biophys. Acta* **1970**, *221*, 228.

Table 1. Redox Parameters for Mono- and Diferric Transferrins at pH 5.8[a]

transferrin species	conc mM	E_{rxn}[b] mV (vs NHE)	$n_{app}b$	$E°'$,[c] mV (vs NHE)	n[c]
Fe_2Tf	0.11−0.19	−458(9)	0.84(19)	−526[d] (−621)[e]	2.1
Fe_CTf	0.18−0.22	−460(8)	0.68(8)	−501[f] (−576)[g]	1.2
Fe_NTf	0.68	−466(5)	0.51(2)	−520[h] (−649)[g]	0.7

[a] Experimental conditions: 0.2−0.4 mM MV^{2+}; 0.5 M KCl; 0.05 M MES; pH = 5.8; 20 °C. Results are averages of three independent determinations with standard deviations in parentheses for $Fe^{III}{}_2Tf$ and $Fe^{III}{}_CTf$, and two independent determinations for $Fe^{III}{}_NTf$ with average deviations in parentheses. [b] Apparent reduction potential and apparent number of electrons transferred for reaction 4 calculated from the slope and *x*-intercept of a plot of eq 6. Numbers in parentheses represent the uncertainties in the last significant digit obtained from the standard deviation of the mean of the slope and intercept of three independent data sets and corresponding plots of eq 6. [c] Parameters in columns 3 and 4 corrected for $Fe^{II}{}_nTf$ dissociation to give formal $Fe^{III/II}Tf$ redox potential for reaction 7. [d] Upper limit assuming $K_b = 10^{5.7}$ (eq 10b). [e] Lower limit assuming $K_b = 10^2$ (eq 10b). [f] Upper limit assuming $K_b = 10^{2.7}$ (eq 10a). [g] Lower limit assuming $K_b = 10^1$ (eq 10a). [h] Upper limit assuming $K_b = 10^{3.0}$ (eq 10a).

of eq 6 for Fe_2Tf, Fe_CTf, and Fe_NTf, where the slope and *x*-axis intercept represent n_{app} and E_{rxn}, respectively. These parameters are listed in Table 1.

To obtain the formal potential for $Fe^{III/II}{}_nTf$ as shown in eq 7, each experimental point on the Nernst plots in Figure 2 was

$$Fe^{III}{}_nTf + ne^- \rightleftharpoons Fe^{II}{}_nTf \quad (7)$$

corrected using $Fe^{II}Tf$ and $Fe^{II}{}_2Tf$ stability constants.[10] For each potential step, the equilibrium absorbance (A_e) at 465 nm can be written as

$$A_e = A_{[Fe^{III}Tf]e} + A_{[Fe^{II}Tf]e} + A_{[Fe_{aq}{}^{2+}]e} = \epsilon_{Fe^{III}Tf} l[Fe^{III}{}_nTf]_e + \epsilon' l\{[Fe_{aq}{}^{2+}]_e + [Fe^{II}{}_nTf]_e\} \quad (8)$$

where l is the light path length (0.035(5) cm), and the total concentration of Fe may be written as

$$[Fe]_{tot} = [Fe^{III}{}_nTf]_o = n[Fe^{III}{}_nTf]_e + n[Fe^{II}{}_nTf]_e + [Fe_{aq}{}^{2+}]_e = n[Fe^{II}{}_nTf]_r + [Fe_{aq}{}^{2+}]_r \quad (9)$$

where $[Fe^{III}{}_nTf]_o$ is the total concentration of Fe when it is all oxidized and $\{n[Fe^{II}{}_nTf]_r + [Fe_{aq}{}^{2+}]_r\}$ is the total concentration of Fe when it is all reduced. The extinction coefficient ϵ' in eq 8 was determined to be 0 from the total absorbance at −656 mV, where transferrin is fully reduced. This is consistent with the fact that ligand-to-metal charge transfer, which is responsible for the absorption peak at 465 nm, is not possible for Fe(II)-transferrin complexes.[22] The concentration of aqueous Fe(II), $[Fe_{aq}{}^{2+}]$, was estimated from the stability constant expressions (eq 10) and the literature values for $K_b{}^{Fe^{II}Tf}$ ($10^{3.0}$ for $Fe^{II}{}_NTf$,

$$K_b{}^{Fe^{II}Tf} = [Fe^{II}Tf]/\{[Fe_{aq}{}^{2+}][Tf]\} \quad (10a)$$

$$K_b{}^{Fe^{II}2Tf} = [Fe^{II}{}_2Tf]/\{[Fe_{aq}{}^{2+}]^2[Tf]\} \quad (10b)$$

$10^{2.7}$ for $Fe^{II}{}_CTf$) and for $K_b{}^{Fe^{II}2Tf}$ ($10^{5.7}$ calculated from $10^{3.0} \times 10^{2.7} = 10^{5.7}$).[10] Estimated values of $[Fe_{aq}{}^{2+}]$ calculated in this way were assumed to be lower limits, since the literature K_b values[10] were obtained from a linear free energy correlation at pH 7.4 where transferrin binding to Fe(II) is expected to be greater than at pH 5.8. Since transferrin binding to Fe(II) at pH 5.8 is very weak, the free apotransferrin concentration was taken as equal to $[Fe_{aq}{}^{2+}]$ for monoferric transferrin and equal to $^1/_2[Fe_{aq}{}^{2+}]$ for diferric transferrin. The concentrations of $Fe^{III}{}_nTf$ and $Fe^{II}{}_nTf$ obtained from eqs 8−10 were used in the Nernst equation (11). Consequently, the corrected Nernst plot

$$\log\{[Fe^{III}{}_nTf]/[Fe^{II}{}_nTf]\} = (n/58.1)E - (n/58.1)E°' \quad (11)$$

of $\log\{[Fe^{III}{}_nTf]/[Fe^{II}{}_nTf]\}$ versus E exhibits an *x*-intercept representing an upper limit for $E°'$ (the $Fe^{III/II}{}_nTf$ redox potential corresponding to the half-reaction shown in eq 7), and a slope of n/58.1. Corrected plots of the Nernst equation (11) are given in Figure 3 for all three transferrin species, and the upper limit reduction potentials $E°'$ for eq 7 and the n values are listed in Table 1.

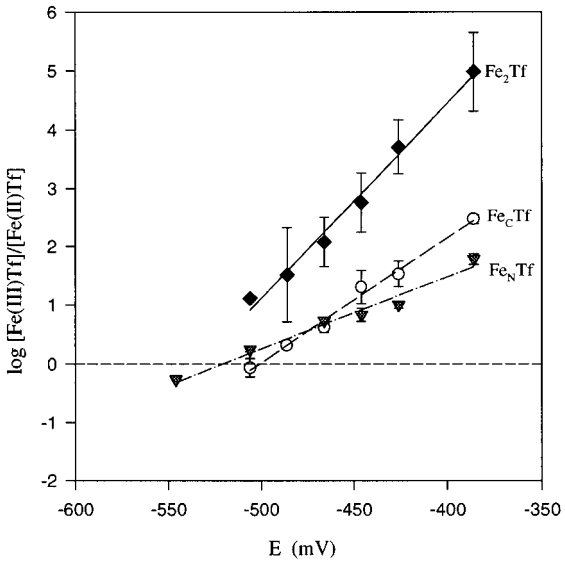

Figure 3. Corrected Nernst plots for the reduction of Fe(III) transferrin species. The data from Figure 2 for Fe_2Tf, Fe_NTf, and Fe_CTf obtained at the six most positive potentials were corrected for the dissociation of $Fe^{II}{}_nTf$ using eqs 8−11. Data obtained below −530 mV for Fe_2Tf and Fe_CTf in Figure 2 were not used for the corrected Nernst plots due to the low absorbance changes measured at low $\{Fe^{III}]/\{[Fe^{III}Tf] + [Fe^{II}]\}$ ratios, which results in less reliable measurements. Diamonds, Fe_2Tf (0.11−0.19 mM in Fe), circles: Fe_CTf (0.18−0.22 mM in Fe). Triangles: Fe_NTf (0.68 mM in Fe). Error bars represent standard deviations for the average of 2−3 independent experiments.

Discussion

Direct spectroelectrochemical investigation of mono- and diferric transferrins at pH 5.8 results in a determination of E_{rxn} for the overall reaction 4, where E_{rxn} is the potential required to reduce 50% of the ferric transferrin present. Correction of E_{rxn} for the dissociation of Fe(II) from reduced transferrin (eq 3) shifts the Fe(III/II) redox potential to a more negative value. This corrected value corresponds to the formal half-potential ($E°'$) for the $Fe^{III/II}Tf$ couple at pH 5.8 (eq 7). The magnitude of the shift to more negative potentials depends on the estimated value for the affinity of Fe(II) for transferrin at pH 5.8 (K_b in eqs 10a and 10b). The values in column 5 of Table 1 are an upper limit based on K_b values estimated at pH 7.4.[10] K_b values at pH 5.8 will undoubtedly be lower, and the $E°'$ values listed in parentheses in column 5 of Table 1 represent a calculated lower limit for arbitrarily assumed K_b values of 10^1 for $Fe^{II}{}_CTf$ and $Fe^{II}{}_NTf$ and 10^2 for $Fe^{II}{}_2Tf$. For Fe_2Tf, $n \approx 2$ (Table 1) for the process described in eq 7, as expected for two Fe sites with

similar redox potentials acting as a unit.[23] For Fe_CTf, $n \approx 1$ as expected for a single redox-active site. For Fe_NTf, n appears to be slightly less than 1 (Table 1). The corrected n value may be slightly low for Fe_NTf because this is a more labile binding site than the C-terminal lobe and some Fe dissociation or scrambling between N and C sites may have occurred during the course of our measurements.[24−26] This lability difference between sites may have been amplified by the low-pH conditions of our study.

Comparison of our results with those reported by Raymond et al[13] ($E^{\circ\prime} = -520$ mV for Fe_2Tf at pH 7.4) suggests that there is little dependence of the $Fe^{III/II}Tf$ redox potential on pH. An n value of 1.1 can be calculated from the results of Raymond et al. for Fe_2Tf.[13] This suggestion of a single-electron transfer for Fe_2Tf may be due to a conformational change at the high salt concentration used (2.0 M KCl), where the protein adopts a more open conformation, resulting in the two Fe centers operating independently. Our uncorrected results are comparable to those of D. C. Harris et al. for Fe_2Tf at pH 7.3 (−400 mV).[12] No correction was made by these authors for Fe(II) dissociation. Our results are significantly more negative than those indirectly calculated from a LFER and corrected for Fe(II) dissociation by W. R. Harris at pH 7.4 ($E^{\circ\prime} = -340$, −280, and −338 mV for Fe_CTf, Fe_NTf, and Fe_2Tf, respectively).[10] We are reluctant to ascribe this difference to a pH effect, since these values were not directly measured and are based on a LFER extrapolation of Fe(II) and Ni(II) binding to transferrin.

This study represents the first direct determination of the Fe(III/II) transferrin redox potentials for all of the Fe-bound permutations of the protein (Fe_2Tf, Fe_CTf, Fe_NTf) at physiological conditions relevant to the endosomal release of iron. Our results at endosomal pH, 5.8, illustrate that, in reducing $Fe^{III}{}_2Tf$, the two Fe centers act as a unit ($n = 2$),[23] although this does not necessarily mean that release of the Fe from Fe_2Tf is cooperative. The small difference we see in the upper limit reduction potentials for Fe in the N and C lobes may be ascribed to a small difference in Fe(II) affinity between the two sites.

Our results directly address the issue of whether reduction of $Fe^{III}{}_2Tf$ or $Fe^{III}Tf$ is a likely mechanism for Fe release from transferrin within the endosome. Pyridine nucleotides (NADH and NADPH), the electron source for reductases, have a reduction potential at pH 5.8 of −284 mV. Reductive Fe release is therefore unlikely due to the negative potentials reported here as an upper limit at pH 5.8 for Fe_2Tf, Fe_CTf, and Fe_NTf, unless some process is operative which shifts the redox potential positive, such as a conformational change or a cascade of reactions involving Fe(II) chelation after reduction. For example, $Fe^{III}{}_nTf$ reduction followed by dissociation (eq 4), coupled with Fe(II) chelation (eq 12) to provide an overall

$$Fe_{aq}{}^{2+} + \text{chel} \rightleftharpoons Fe^{II}\text{chel} \qquad (12)$$

$$Fe^{III}{}_nTf + ne^- + \text{chel} \rightleftharpoons nFe^{II}\text{chel} + \text{apo-Tf} \qquad (13)$$

reduction−ligand exchange process, shown in eq 13, would be thermodynamically favorable at pH 5.8 with a NAD(P)H/NAD(P) reductant if the effective equilibrium constant for eq 12 were greater than 10^3 and the Fe(II) chelator were present in sufficient concentration to capture most of the released Fe. No such Fe(II)-sequestering agent, or cascade of reactions, has yet been identified. Although reduction of Fe must occur before the metal can be deposited in the ferritin core or incorporated into heme by ferrochelatase, our results suggest that this reduction occurs subsequent to release of Fe from transferrin.

Acknowledgment. We gratefully acknowledge financial support from the NIH (DK 15056 (P.A.); HL58248-01 (A.L.C.)) and the Duke University Arts & Sciences Research Council (A.L.C). We thank F. Bedioui (ENSCP) for helpful discussions.

IC970644G

(23) As can be seen by comparing columns 4 and 6 in Table 1, correction of the spectroelectrochemical data for the dissociation of $Fe^{II}Tf$ influences the value obtained for the n parameter. However, our conclusions that electrochemical reduction of $Fe^{III}{}_2Tf$ involves a two-electron-transfer process and that electrochemical reductions of $Fe(III)_CTf$ and $Fe(III)_NTf$ are one-electron processes are not dependent on the assumed value for K_b (eq 10). For example, the calculated n value for $Fe^{III}{}_2Tf$ varied between 2.1 and 2.2 on changing the K_b value from 1 to 10^7. The same consistency in calculated corrected values for n (≈1) for the monoferric transferrin species was also observed. Furthermore, our conclusion that reduction of Fe_2Tf occurs by a two-electron-transfer process is not dependent upon an assumed model. Equation 6 treats the observed absorbance changes in a way that is not model-dependent. A plot of eq 6 is shown in Figure 2, which clearly shows a greater slope for the Fe_2Tf system than for either Fe_C-Tf or Fe_NTf. This is supportive of a difference between Fe_2Tf and FeTf. When correction is made for $Fe^{II}{}_nTf$ dissociation using an $Fe^{III}{}_2$-Tf model as shown in Figure 3, we obtain $n = 2.1$ and $E^{\circ\prime} = -526$ mV, as listed in Table 1. When the correction is made for $Fe^{II}{}_nTf$ dissociation using a per site model for diferric transferrin, ignoring the distinction between the N- and C-terminal sites (i.e., $[Fe^{III}] = 2[Fe^{III}{}_2Tf]$), we obtain $n = 1.6$ and $E^{\circ\prime} = -504$ mV. This is not consistent with a one-electron transfer and suggests that $Fe^{III}{}_2Tf$ is best treated as a 2-electron system.

(24) Kristensen, E. W.; Igo, D. H.; Elder, R. C.; Heineman, W. R. *J. Electroanal. Chem.* **1991**, *309*, 61.
(25) Princioto, J. V.; Zapolski, E. J. *Nature* **1975**, *255*, 87.
(26) Lestas, A. M. *Br. J. Haematol.* **1976**, *32*, 341.