Iron Release from Transferrin, Its C-Lobe, and Their Complexes with Transferrin Receptor: Presence of N-Lobe Accelerates Release from C-Lobe at Endosomal pH†

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ABSTRACT: Human transferrin, like other members of the transferrin class of iron-binding proteins, is a bilobal structure, the product of duplication and fusion of an ancestral gene during the course of biochemical evolution. Although the two lobes exhibit 45% sequence identity and identical ligand structures of their iron-binding sites (one in each lobe), they differ in their iron-binding properties and their responsiveness to complex formation with the transferrin receptor. A variety of interlobe interactions modulating these iron-binding functions has been described. We have now studied the kinetics of iron release to pyrophosphate from the isolated recombinant C-lobe and from that lobe in the intact protein, each free and bound to receptor. The striking finding is that the rates of iron release at the pH of the endosome to which transferrin is internalized by the iron-dependent cell are similar in the free proteins but 18 times faster from full-length monoferric transferrin selectively loaded with iron in the C-lobe than from isolated C-lobe when each is complexed to the receptor. The possibility that the faster release in the receptor complex of the full-length protein at endosomal pH contributes to the evolutionary advantage of the bilobal structure is considered.

Human serum transferrin, a bilobal protein capable of bearing a single Fe(III) atom in each of its similar but not identical lobes, functions in delivering iron to meet the needs of iron-dependent cells. Because each molecule of transferrin experiences 100−200 cycles of reversible iron uptake and delivery during its lifetime in the circulation (*1*), the kinetics and mechanism of iron release are of fundamental physiological interest. Many studies over the years have therefore been directed at elucidating the rates and mechanisms of iron release from full-length transferrin (*2*−*5*) and each of its constituent lobes (*6*−*11*). In the aggregate, these studies have clearly demonstrated differences between the iron-binding properties of the lobes and the effects of mutating iron ligands and remote residues on these properties. With few exceptions (*12*−*15*), these and similar studies have dealt with free transferrin or free recombinant lobes. In the iron-dependent cell, however, free transferrin is not found: during its iron-donating journey through the cell, transferrin remains complexed to its receptor. This classical receptor is now sometimes designated transferrin receptor 1 since the discovery of a second receptor (*16*) with an as yet incompletely understood function in iron metabolism; our present studies are confined to transferrin receptor 1. Binding to the transferrin receptor significantly alters the kinetics of iron release from the two lobes of transferrin, with distinctive differences in effects on each lobe (*12*, *13*), so that the physiologically relevant problems are to discern the effects of receptor on iron release from receptor-transferrin complexes, the amino acids of receptor and transferrin participating in such effects, and how the lobes of transferrin interact with each other in such complexes. In this paper, we focus on the last of these concerns.

Our approach was to prepare complexes of transferrin receptor with full-length monoferric transferrin selectively loaded in the C-lobe ($Tf\text{-}Fe_C$)[1] or with recombinant C-lobe half-transferrin (*17*) for the measurement of rates of iron release at extracellular pH 7.4 and endosomal pH 5.6 (*18*). These rates were compared with corresponding rates from free $Tf\text{-}Fe_C$ and free C-lobe. In doing so, we were able to ascertain the effect of the N-lobe on the release of iron from the C-lobe of full-length transferrin, free and complexed to transferrin receptor. Similar studies with the receptor and N-lobe were not feasible since the isolated N-lobe does not bind to receptor (*19*, *20*).

MATERIALS AND METHODS

Proteins and Reagents. Lyophilized human serum transferrin was purchased from Boehringer-Mannheim (Indianapolis) and shown to be homogeneous by SDS−PAGE, indistinguishable in its spectroscopic properties from native transferrin isolated in this laboratory from Cohn fraction IV-7, and effective in donating iron to hepatic and erythroid/myeloid cells (*11*, *21*). $Tf\text{-}Fe_C$ was prepared by previously reported methods (*11*) and verified by urea-gel electrophoresis. The soluble exocytic portion of the human transferrin receptor was provided by Dr. Peter Snow of the California

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[1] Abbreviations: $Tf\text{-}Fe_C$, monoferric transferrin selectively loaded in the C-lobe; Tf, full-length transferrin; TfR, recombinant human transferrin receptor 1 (soluble exocytic portion); PPi, pyrophosphate.

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Table 1: Iron Release at pH 7.4 (50 mM HEPES, 100 mM PPi) from Tf-Fe_C, C-Lobe, and Their Complexes with Transferrin Receptor

	k_{obs} ($s^{-1} \times 10^3$) ± SD	
protein	100 mM NaCl	600 mM NaCl
Tf-Fe_C	1.6 ± 0.05	3.2 ± 0.04
C-lobe	1.6 ± 0.1	4.1 ± 0.1
Tf-Fe_C/TfR	0.36 ± 0.08	2.9 ± 0.4
C-lobe/TfR	1.8 ± 0.04	5.3 ± 0.1

Table 2: Iron Release at pH 5.6 (100 mM MES, 0.125 mM PPi) from Tf-Fe_C, C-Lobe, and Their Complexes with Transferrin Receptor

	k_{obs} ($s^{-1} \times 10^3$) ± SD	
protein	100 mM NaCl	600 mM NaCl
Tf-Fe_C	1.9 ± 0.1	11.2 ± 0.7
C-lobe	1.8 ± 0.15	9.5 ± 0.3
Tf-Fe_C/TfR	109 ± 23[a]	>300[b]
C-lobe/TfR	6.1 ± 0.2	13.1 ± 1.0

[a] $n = 5$. [b] Too fast for reliable measurement.

Institute of Technology; its preparation and properties are described elsewhere (*22*). Removal of the 6-His tag was accomplished with Factor Xa and confirmed by Western blotting with an anti-His tag antibody (Sigma-Aldrich, St. Louis, MO). The C-lobe of human transferrin was prepared and purified as previously described, taking advantage of the poly-His tag introduced in the N-lobe (*17*). Standard laboratory reagents were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich.

Kinetics of Iron Release. Rates of iron release from Tf-Fe_C and the recombinant C-lobe, each free or complexed to the transferrin receptor, were measured by the spectrofluorometric method previously described (*15*), using a Photon Technology International (Lawrenceville, NJ) QuantaMaster spectrofluorometer with a high-sensitivity photomultiplier tube. (It should be noted that the present results cannot be directly compared to those of the earlier study, which was carried out in detergent-solubilized full-length placental TfR.) To minimize photobleaching in receptor complexes, a narrow entry slit, 0.25 mm (corresponding to 1 nm resolution), was used, with an exit slit of 1.0 mm (4 nm) with a 75 W short-arc xenon lamp source. Sample cuvettes were maintained at 25 °C using a recirculator from Neslab (Portsmouth, NH), and reactions were initiated by pipetting 4−10 μL of protein samples into 1.8 mL of magnetically stirred release buffer in the sample cuvette thermally equilibrated for at least 15 min. For studies at extracellular pH 7.4, release buffer was 0.1 M HEPES, 0.1 M PPi, and NaCl at indicated concentrations. For studies at endosomal pH 5.6, release buffer was 0.1 M MES, 0.125 mM PPi, and indicated concentrations of NaCl. Final concentrations of proteins were near 10 nM. Fitting of progress curves, collected at 0.5−1.0 s intervals, to exponential functions (*15*) was carried out using the Levenberg−Marquardt algorithm provided in SigmaPlot 2001 (SPSS Science, Chicago, IL). In all cases, progress curves were well-fit by single-exponential functions, as expected from proteins occupied by single iron atoms with little likelihood of intersite exchange (in the case of Tf-Fe_C). Studies were carried out in triplicate (except as indicated), and results are presented as means ± SD (Tables 1 and 2).

Glycosylated and nonglycosylated proteins were studied interchangeably since control experiments under identical conditions indicated that release rates varied for the two types of proteins by less than ±5%, within experimental error. Previous studies by Mason et al. indicated that glycosylation also has no effect on receptor binding (*23*). In all cases, pyrophosphate was used as the iron-accepting agent necessary to induce release, with concentrations chosen to achieve release rates optimized for spectrofluorometric measurement since spontaneous release of iron is exceedingly slow even at endosomal pH (*24*, *25*).

Receptor Complexes. The concentration of receptor was determined by the Bradford method, and concentrations of Tf-Fe_C and the C-lobe were calculated from absorbances at 465 nm, taking the molar extinction of each as 2780 (*26*). Complexes of the receptor and transferrin or the C-lobe were prepared by incubating a molar excess of full-length monoferric transferrin loaded in the C-lobe (Tf-Fe_C) or recombinant C-lobe with ~10^{-4} M receptor subunits for 1 h at 37°, then overnight at 4°. Size-exclusion chromatography on a Hi-Load 16/60 Superdex 200 column (Amersham Biosciences, Piscataway, NJ) or a BioSil 250-5 column (BioRad, Hercules, CA) separated complexes from uncomplexed components (Figure 1 A,B), thereby verifying the complex formation. For both columns, the buffer was 50 mM HEPES, 100 mM NaCl, pH 7.4, with flow rates of 0.8 mL/min for the larger Hi-Load column and 0.3 mL/min for the BioSil column. Chromatograms were obtained using a Pharmacia Äkta Explorer 10 system, and preparations were stored in the eluting buffer at 4 °C. Size-exclusion chromatography also demonstrated that a complex of the receptor and C-lobe survived in a single peak after freezing and thawing for EPR spectroscopy (Figure 1C). Persistence of the intact complex is consistent with the finding that 76% of the free energy of binding of Tf to TfR comes from the C-lobe (*20*). A comparison of release kinetics of complexes with a His-tagged receptor and receptor freed of the His tag showed no detectable influence of the tag on release rates.

Spectroscopy. EPR spectroscopy was carried out using a Bruker 200D X-band spectrometer with an ESP 300 upgrade and ER 4111 variable temperature apparatus. Sample buffer was 50 mM HEPES, 100 mM NaCl, pH 7.4. Instrumental parameters were as follows: microwave frequency, 9.514 GHz; microwave power, 10 mW; modulation amplitude, 1 mT; and temperature, 100 K. Optical spectra were recorded on a Shimadzu UV 2401 PC spectrophotometer in the same buffer.

RESULTS AND DISCUSSION

Experimental Design. Iron release rates were measured at extracellular pH 7.4, at which transferrin first encounters its receptor, and at endosomal pH 5.6, at which iron is released from the protein for export to the cytoplasm. Because release may take hours in the absence of an iron sequestering agent even at low pH (*25*), it was necessary to include an iron sequestering reagent to achieve measurable rates. We have chosen pyrophosphate as the required iron binder, to model the pyrophosphate moiety of ATP that has been suggested as an intracellular chelator (*27*). The concentration of ATP (or of other candidate chelators such as citrate and oxalate) within the transferrin-bearing endosome is not known, so

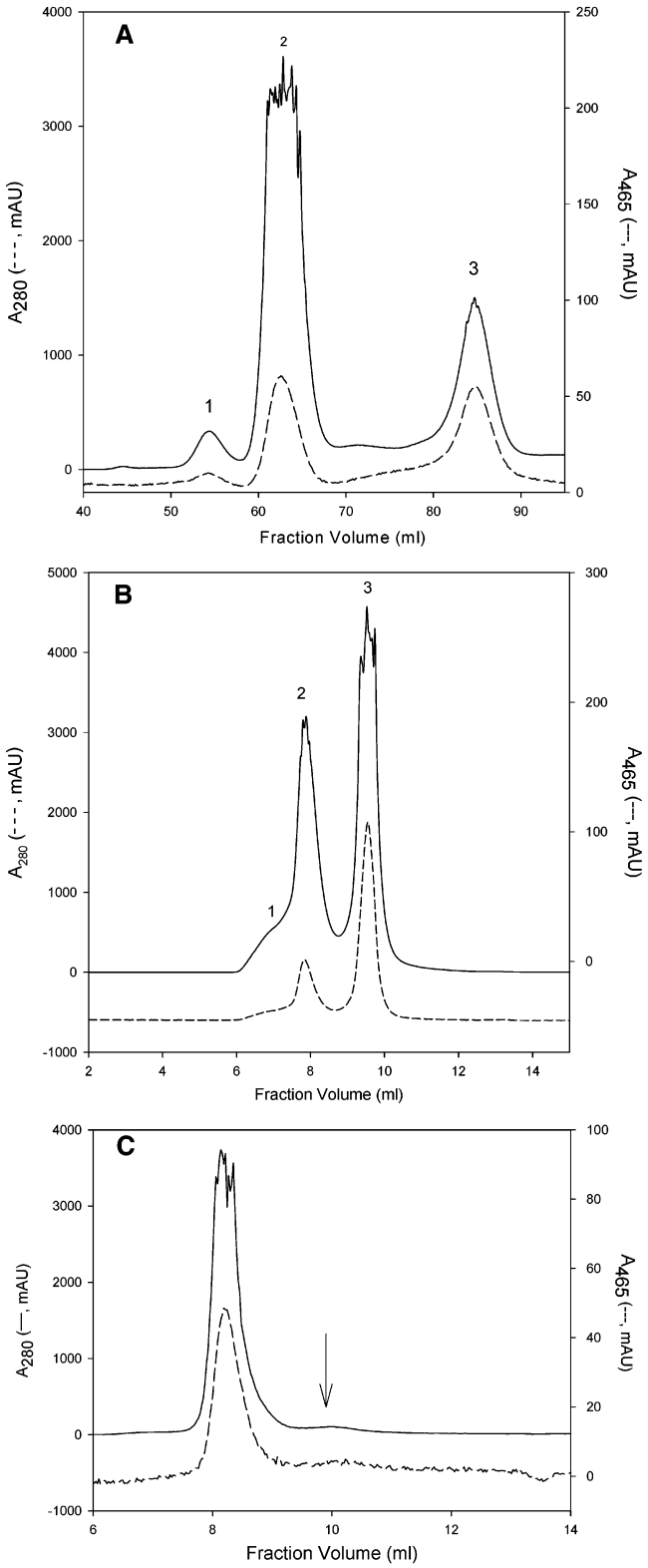

FIGURE 1: (A) Size-exclusion chromatogram for isolation of the complex of transferrin C-lobe and TfR using a Superdex 200 16/60 (Amersham Biosciences, Piscataway, NJ) column. (B) Chromatogram of full-length Tf and TfR using a BioSil 250-5 (BioRad, Hercules, CA) column. (C) Chromatogram of C-lobe/TfR after EPR spectroscopy showing the only peak present. For all chromatograms, A_{280}, —; A_{465}, - - -. For chromatograms A and B: peak 1, protein aggregates; peak 2, complex of C-lobe or Tf and TfR; and peak 3, uncomplexed transferrin or C-lobe. The arrow in chromatogram C indicates the expected elution volume of uncomplexed proteins. Baseline correction has been applied to all chromatograms, and scales have been adjusted for clarity.

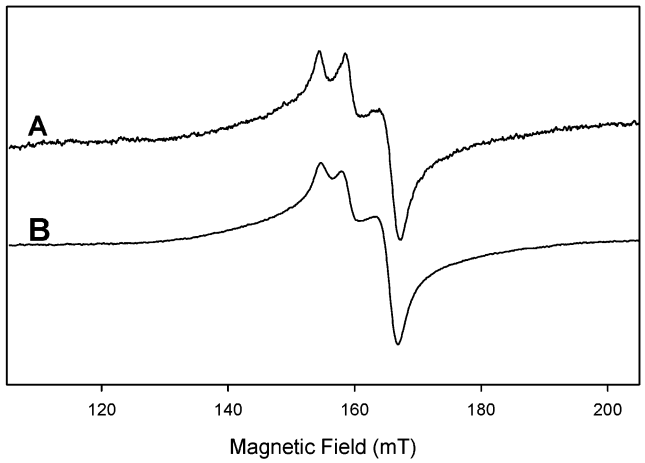

FIGURE 2: X-band EPR spectra of (A) C-lobe/TfR complex and (B) C-lobe, each at pH 7.4. The splitting of the $g' = 4.3$ line is 4.0 mT in spectrum A and 3.5 mT in spectrum B. EPR parameters: microwave frequency, 9.514 GHz; microwave power, 10 mW; and temperature, 100 K.

we have used concentrations that provide reasonable rates for measurement by the spectrofluorometric method. Substantial differences in release rates from free and receptor-complexed transferrins were verified at both pH values studied, in accord with our earlier studies with detergent-solubilized receptor (*12*, *15*). Considerable differences in the release rates from the recombinant C-lobe and the C-lobe in full-length Tf-Fe_C, and their complexes with the transferrin receptor, were noted for the first time. The release-accelerating effect of high salt concentration on release was found in all cases studied, as expected from previous studies (*15*).

Integrity of Transferrin/Receptor Complexes. The optical spectrum of the C-lobe/TfR complex showed the broad tyrosinate-to-Fe(III) charge-transfer band centered at 460 nm, almost indistinguishable from that of the free C-lobe (the slight hypsochromic shift in the absorption maximum may be due to spillover from the 280 nm band of the receptor). Similarly, the EPR spectra of the C-lobe/receptor complex at pH 7.4 was virtually indistinguishable from that of the free C-lobe, showing the characteristic ~3.8 mT splitting of the $g' = 4.3$ line in all cases (Figure 2). These traditional measures therefore substantiate the integrity of the complexes.

Iron Release at pH 7.4. Rates of iron release to pyrophosphate from free Tf-Fe_C and the C-lobe were identical at 100 mM NaCl, but a slightly greater effect of increasing NaCl to 600 mM was found in the C-lobe (Table 1). The accelerating effect of high salt concentration was also observed in the complexes of the proteins with receptor. Binding to the receptor effected a nearly 4-fold decrease in the release rate from Tf-Fe_C, corroborating earlier studies with detergent-solubilized transferrin/receptor complexes (*12*, *15*). In contrast, binding to the receptor had a minimal accelerating effect on iron release from the C-lobe, although the accelerating effect of high salt was preserved in this and the other proteins studied. Thus, the N-lobe exerts a release-retarding action on the C-lobe of full-length transferrin bound to receptor, again demonstrating interlobe interactions previously observed in several contexts (*28*−*32*). The molecular mechanisms underlying such interactions are not clear. NMR studies of recombinant transferrin selectively labeled with

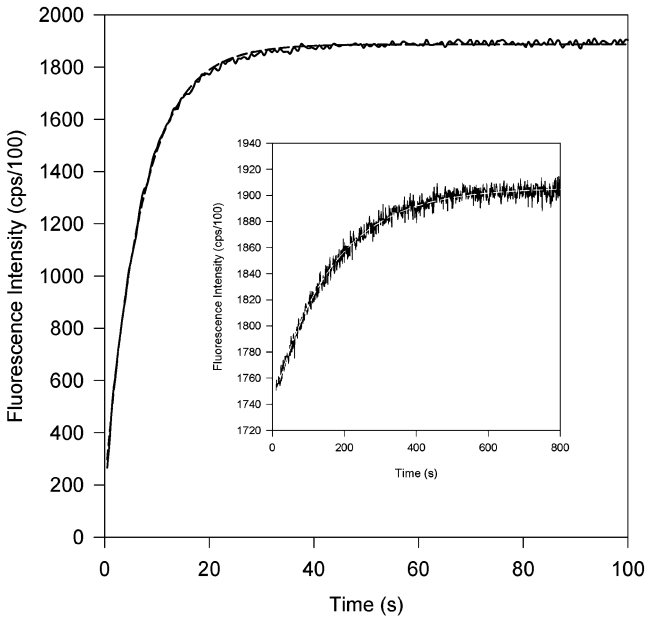

FIGURE 3: Spectrofluorometric progress curves of iron release from the Tf-Fe_C/TfR complex and (inset) the C-lobe/TfR complex at pH 5.6 and 100 mM NaCl. Solid lines are single-exponential fits to the experimental curves; note difference in time axes. Rate constant (k_{obs}) for Tf-Fe_C/TfR: 140×10^{-3} s^{-1} and for C-lobe/TfR: 6.2×10^{-3} s^{-1}.

^{13}C at the methyl groups of methionine residues showed that subsequent loading of the N-lobe with gallium perturbed the ^{13}C resonance of the gallium-loaded C-lobe residue Met382, situated in the interlobe contact region (*31*). Such interlobe contacts, and possibly long-range allosteric effects as well, are presumably responsible for interlobe interactions.

Iron Release at pH 5.6. Because of the accelerating effect of the lower pH, the concentration of pyrophosphate had to be reduced from 100 to 0.125 mM to achieve release rates amenable to study by the spectrofluorometric method. As at pH 7.4, release rates from free Tf-Fe_C and the C-lobe were similar, with both proteins responding to a high salt concentration with substantial rate increases (Table 2). Marked differences in iron release between free and receptor-bound proteins were found, however. Binding to the receptor increases the release rate from the C-lobe by more than three times. An even more striking effect was seen in the release from Tf-Fe_C bound to receptor, in which the release is 18 times faster than from the free protein at 100 mM NaCl (Figure 3), and at least 23 times faster at 600 mM NaCl. Thus, the presence of the N-lobe, even though devoid of iron, accelerates iron release from the C-lobe at endosomal pH.

Iron release from the N-lobe is known to be much more facile than from the C-lobe at endosomal pH and therefore requires a much lower concentration of pyrophosphate to monitor release (*11*) so that the correspondingly facile cellular acquisition of the N-lobe iron would also be expected. Iron borne in the C-lobe, however, is relatively resistant to lowering the pH (*33*). A biological advantage is therefore offered by the accelerating effect of the N-lobe on the release of iron from the C-lobe of bilobal transferrin: efficiency of release from both lobes is assured. This effect complements the release-promoting action of the receptor at endosomal pH, thereby contributing to the more facile release of iron from the C-lobe of transferrin bound to the receptor at endosomal pH (*13*). It may also help explain the predominance of iron in the N-lobe of circulating transferrin (*34*, *35*).

Biological Advantage of a Bilobal Transferrin. Although sequence homologies between the lobes of known eukaryotic transferrins and the organization of the human transferrin gene offer compelling evidence that the bilobal protein arose during the course of evolution by duplication and fusion of an ancestral gene specifying a single-lobed protein, no eukaryotic single-lobed transferrin is known. A reasonable supposition, therefore, is that the emergence and persistence of a bilobal structure offers substantial advantages to organisms dependent upon transferrin for circulatory transport and the cellular delivery of iron.

The nature of this advantage is speculative. The suggestion that the twinned protein resists loss through glomerular filtration in the kidney (*36*) is appealing but has been questioned because the bilobal structure may have evolved and prevailed before the filtration kidney appeared (*37*). In earlier studies, we have reported that the stability constant for iron binding in the C-lobe of full-length transferrin is about 4 times greater than that of the isolated C-lobe derived by selective proteolysis of native transferrin at pH 7.4 and about 25 times greater as the pH is lowered to 6.7 (*38*). If this stabilizing effect of interlobe interactions is unopposed, then iron release from the C-lobe at endosomal pH might be too slow for cellular needs, but binding to receptor overcomes the effect. Accordingly, we hypothesize that the efficiency of iron release from the C-lobe of native transferrin is impaired by stabilizing interactions of the lobes with each other that retard the release of iron but is enhanced at the pH within the endosome by release-promoting interactions with the transferrin receptor. Thus, the bilobal structure is favored during the course of biochemical evolution because of an interlobe interaction that promotes iron release from the receptor complexed Tf. Molecular mechanisms involved in such interactions must await structural, imaging, and mutagenesis studies beyond those now available. In any case, the present observations provide a striking example of interlobe interactions in a receptor-complexed transferrin.

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