A New Method for Obtaining Human Transferrin C-Lobe in the Native Conformation: Preparation and Properties†

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ABSTRACT: Eukaryotic transferrins comprise a class of bilobal iron-binding proteins in which each lobe carries a single binding site. Although expression of full-length transferrins and their N-terminal lobes, in wild-type and mutated forms, has been successfully accomplished by several laboratories, expression of C-lobes has been much less satisfactory. A possible explanation of the difficulty is that proper folding of the C-lobe, with its 11 disulfide bonds, depends on prior synthesis and proper folding of the N-lobe. We have therefore developed a new strategy, introducing a specific factor Xa cleavage site in the interlobe-connecting strand to permit separation of the lobes after expression of the full-length protein. The resulting protein was expressed in satisfactory yield, >20 mg/L, and could be easily and completely cleaved to yield two distinguishable fragments representing N- and C-lobes, respectively. Retaining the glycosylation sites, found only in the C-lobe, made it possible to separate the fragments from each other by ConA affinity chromatography. The isolated C-lobe so obtained displayed spectroscopic and kinetic features of the C-lobe in native transferrin and was competent as an iron donor for K562 cells to which it bound in saturable fashion inhibitable by native diferric transferrin. Since the N-lobe by itself will neither bind nor donate iron to cells, the primary receptor-recognition site of transferrin resides in its C-lobe.

Eukaryotic transferrins comprise a class of bilobal iron-binding proteins, each lobe bearing a single site capable of reversibly binding iron and accounting for the physiological roles of the proteins in iron transport and iron withholding as a defense against infection (*1*). Present day transferrins probably arose from duplication and fusion of a gene encoding a single-sited ancestral protein (*2*), thereby accounting for the sequence identities and similarities in the two lobes. Despite such similarities, the lobes exhibit distinguishable chemical and physiological properties, so that studies of isolated lobes and the intact proteins from which they derive have proven valuable in understanding the molecular basis of iron binding and release in transferrin function (*3*−*7*).

Transferrin normally provides iron for cellular needs, and for most cells the delivery of transferrin-borne iron depends on association of the protein with transferrin receptors, TfR1 and TfR2 (*12*), on plasma membranes. The transferrin binding site of transferrin receptors is not known, although a conserved Arg-Gly-Asp sequence in TfR1 has been implicated by site-directed mutagenesis studies (*13*). Even less is clear about the receptor-recognition site of the transferrin molecule. General agreement prevails that the N-lobe by itself is not capable of delivering iron to cells by a receptor-dependent route, but the functional capability of the C-lobe in this respect is still controversial.

Expression of wild-type and mutated holotransferrins and their N-lobes has been accomplished in several laboratories, but expression of C-lobes in the verified native conformation has been more problematic (*8*−*10*). Recombinant human C-lobe has been produced in *Escherichia coli* in the only report verifying native conformation, but renaturation of the product was necessary to achieve a yield of 5% of the total C-lobe protein (*11*). A possible explanation of the difficulty is that proper folding of the C-lobe, with its 11 disulfide bonds and 231 combinations of ways they might be formed, is normally guided by prior synthesis and folding of the N-lobe which then acts as a kind of chaperone for construction of the C-lobe. Incorrectly folded C-lobe may exhibit intracellular instability, resulting in low yields of expressed protein that fails to bind metal ions properly. We have therefore turned to a new strategy, inserting a factor Xa cleavage site in the β-strand connecting the two lobes of human transferrin. The resulting protein could be quantitatively cleaved to yield both lobes, with the C-lobe in the verified native conformation. We report now on the preparative procedures and the chemical and functional properties of the C-lobe so obtained.

MATERIALS AND METHODS

Materials. Human diferric transferrin was purchased in lyophilized form from Boehringer-Mannheim and kept at −20 °C until used. Established methods were used to prepare apotransferrin and monoferric transferrin loaded in the C-lobe (*4*). Radioisotopes were obtained from Amersham Pharmacia Biotech, and other reagents were from Fisher Scientific,

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Aldrich, and Sigma. Culture media were from Gibco, Ultroser G was from Crescent Chemical, and fetal bovine serum was from Atlanta Biologicals. Factor Xa was provided by Novagen. Poros 50 HQ anion-exchange resin was obtained from PerSeptive Biosystems, and other chromatography media were from Amersham-Pharmacia.

Spectroscopy. EPR spectra were recorded at X-band using a Bruker 200D spectrometer with ESP 300 upgrade and Gunn diode as the microwave source. Temperatures were maintained at 100 K by a Bruker ER 4111 variable temperature apparatus. Microwave power was 10 mW for all spectra, and microwave frequencies were in the range 9.514−9.518 GHz. An Aviv-Cary 14DS spectrophotometer was used for optical scanning. Kinetics of iron release from proteins were measured in a Photon Technology QM-200−4SE spectrofluorometer by methods previously described (*15*).

Electrophoresis Studies. SDS−PAGE[1] studies were performed under reducing conditions with a Pharmacia Phast-System using 12.5% homogeneous polyacrylamide gels. Molecular weight standards were from Pharmacia.

Kinetics of iron release from transferrins to pyrophosphate were measured using a Photon Technology spectrofluorometer following previously reported methods (*4*). Excitation slits were set at 1 nm and emission slits at 2 nm.

Radiolabeling. Proteins were labeled with ^{125}I by the Bio-Rad Enzymobead method or by the iodine monochloride procedure (*16*). After chromatography on Bio-Rad PD10 columns more than 91%, and usually more than 94%, of the radioactivity in labeled proteins was precipitable in 20% trichloracetic acid/4% phosphotungstic acid. Labeling with ^{59}Fe was carried out with [^{59}Fe]nitrilotriacetate, as previously described (*4*). Both isotopes were measured in a TM Analytic Model 1193 γ counter.

Sequencing. DNA sequencing was performed in the sequencing facility of the Department of Molecular Genetics and protein sequencing in the Laboratory for Macromolecular Analysis and Proteomics using an Applied Biosystems Procise 494 sequencer.

Mutagenesis, Expression, and Purification of Recombinant Transferrins. Expression of the C-lobe, residues 332−679 of the transferrin sequence, was attempted in *Drosophila* S2 insect cells using the expression vector pMT/BiP (Invitrogen) with its own signal sequence and in BHK cells using the pSecTag2/Hygro vector (Invitrogen), also with its own signal sequence. Because of disappointing results with these approaches, which resulted in products with expected mobilities on SDS−PAGE but incapable of EPR-verified specific binding of Fe^{3+} or Cu^{2+}, we turned to a new strategy, inserting a cleavage site for factor Xa, a highly specific protease, in the interlobe β-strand.

Mutagenesis in the pNUT vector bearing the full-length transferrin coding sequence (*4*) was carried out using the Stratagene QuikChange kit. The mutagenic primers were

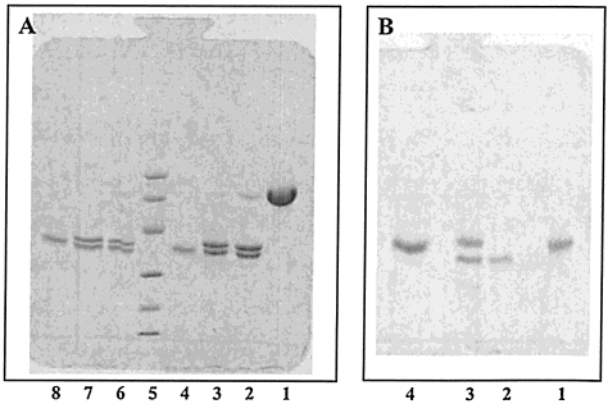

FIGURE 1: SDS−PAGE electrophoresis of rTf-Xa and its cleavage products. Panel A: Nonglycosylated rTf-Xa and its products. Lanes: 1, untreated rTf-Xa; 2 and 6, rTf-Xa after digestion with factor Xa at 37 °C for 20 h; 3 and 7, rTf-Xa after digestion with factor Xa for 44 h; 4 and 8, expressed rTfN-lobe; 5, MW markers (phosphorylase B, 97000; albumin, 66000; ovalbumin, 45000; carbonic anhydrase, 30000; trypsin inhibitor, 20100; α-lactalbumin, 14400). Panel B: Glycosylated rTf-Xa and its products. Lanes: 1 and 4, cleaved rTfC-lobe after ConA chromatography; 2, rTfN-lobe; 3, rTf-Xa after 44 h digestion with factor Xa.

GAAGGCACATGCATAGAAGGCAGAGCCCCAACAG and its complement, which effected the following change verified by DNA sequencing:

...Thr-Cys-Pro-Glu-Ala-Pro-Thr-Asp-Glu...
(wild type)

...Thr-Cys-Ile-Glu-Gly-Arg- Ala-Pro-Thr-Asp-Glu...
(mutant)

Factor Xa cleaves between the Arg and Ala of the mutant, producing a C-lobe starting at Ala 334.

Expression of the mutated transferrin, designated rTf-Xa, was accomplished in BHK 21 cells in DMEM-F12 medium containing 0.5−1% Ultroser G, again as previously described. Purification of the recombinant factor Xa protein was carried out by anion-exchange chromatography with 20 mM Tris, pH 7.0, and a gradient of 0−200 mM NaCl using an Amersham-Pharmacia Explorer 10 system and Poros 50 HQ (PerSeptive Biosystems), a strong anion exchanger. Expressed rTf-Xa was easily separated from the bovine transferrin[2] present in Ultroser G; the former eluted between 10% and 20% NaCl and the latter between 30% and 38% NaCl. The final yield of purified rTf-Xa was >20 mg/L.

After about 60 h digestion at 25 °C with factor Xa, following the provider's instructions, the mutant protein was virtually completely cleaved to yield two fractions distinguishable by SDS−PAGE (Figure 1). To ensure removal of undigested mutant transferrin and any residual bovine transferrin, the digestion mixture was subjected to size-exclusion chromatography using a HiLoad 16/60 Superdex 75 column. The recovered slow-moving fraction was then taken for ConA chromatography, following the manufacturer's directions for elution of bound protein with 300 mM

[1] Abbreviations: Con A, concanavalin A; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; rTf-Xa, recombinant human transferrin bearing a factor Xa cleavage site in the interlobe-connecting strand; rTfC-lobe, cleaved C-lobe from rTf-Xa; rN-lobe, recombinant N-lobe, residues 1−337; FBS, fetal bovine serum; BSA, bovine serum albumin; Tf-Fe_C, monoferric transferrin selectively loaded in the C-lobe; Fe_2Tf, diferric transferrin; ELISA, enzyme-linked immunosorbent assay; K_a, association constant for specific binding of protein to K562 cells.

[2] An earlier report indicated the presence of human transferrin in Ultroser G, but its manufacturer recently advised the U.S. Department of Agriculture that it contained only bovine proteins. We have verified this by western blotting with polyclonal antisera to bovine and human transferrins, each of which cross-reacts poorly to the heterologous antiserum.

methyl α-D-glucopyranoside, to separate the glycosylated C-lobe from the nonglycosylated N-lobe (Figure 1).

Cell Studies. K562 cells were grown in RPMI 1640 medium containing 10% FBS and harvested when cell densities were near 10^7/mL. To free them of adventitious transferrin, cells were incubated twice for 15 min at 37 °C in RPMI 1640 without serum and washed each time. Binding experiments were carried out in RPMI 1640 with 10 mM HEPES, pH 7.4, containing 2% BSA and diluted with equal volumes of labeled transferrin in PBS to final cell densities near 1.5×10^6/mL. After 1 h incubation at 4 °C, cells were isolated in triplicate by centrifugation through a 9:1 mixture of dibutyl phthalate−mineral oil, and the pellets were taken for counting. Variations in counts were within 5% of the means. Data were analyzed by curve fitting to a saturable binding isotherm using the Levenberg−Marquardt algorithm, as described earlier (*14*). Nonspecific binding of the iron-bearing rTfC-lobe was determined with a 100-fold molar excess of unlabeled native diferric transferrin at three different concentrations of labeled protein, with results fitted to a linear regression function. Calculated nonspecific binding, using the regression parameters, was then subtracted from each data point. In evaluating binding of native diferric transferrin, a linear term for nonspecific binding was added to the saturable binding isotherm before curve fitting (*17*), and specific binding was calculated by subtracting nonspecific binding at each data point.

Iron uptake by K562 cells in RPMI 1640/10 mM HEPES/1% BSA, pH 7.4, was measured at 37 °C, as previously described (*4*).

RESULTS

Expression of C-Lobes. Although expression of recombinant products of C-lobe vectors for *Drosophila* and BHK 21 cells, with the expected mobilities in SDS−PAGE and immunoreactive by western blotting or ELISA, was obtained (data not shown), in neither case did these products show the characteristic optical and EPR spectra of Fe^{3+}−transferrin complexes. Further efforts to obtain isolated C-lobes by these means were therefore abandoned.

Characterization of the C-Lobe from rTf-Xa. The N-terminal sequence of the cleaved C-lobe was determined in seven Edman cycles as Ala-Pro-Thr-Asp-Glu- -Lys (where the blank represents cysteine that is not recognized by the sequencer), as expected. There was no evidence of a second sequence, which would have been detected at a molar concentration less than 1/200th of that of the found sequence.

Eukaryotic transferrins exhibit a ligand to metal (tyrosinate to Fe^{3+}) charge-transfer band with a maximum near 465 nm and an absorptivity (per Fe^{3+}) in the range 2000−2500 M^{-1} cm^{-1}. As shown in Figure 2, the cleaved C-lobe displays a visible absorption spectrum with the expected properties: A_{max} at 461 nm with absorptivity of 2200 M^{-1} cm^{-1}.

A diagnostic feature of human transferrin and its constituent lobes is provided by their EPR spectra. The signal at $g' = 4.3$, typical of high-spin Fe^{3+} with a rhombic fine-structure tensor and small axial perturbation, shows an unusual splitting of about 2.7 mT in the N-lobe proteolytically derived from native transferrin (*18*) and 3.9 mT in the proteolytically cleaved C-lobe (*19*) or 3.8 mT in Tf-Fe_C measured in the spectrometer in current use in our laboratory. A splitting of 3.7 mT is present in the recombinant C-lobe (Figure 3), in good agreement with splittings previously observed in the C-lobe fragment and selectively loaded C-lobe of native transferrin, given the relatively broad lines in question.

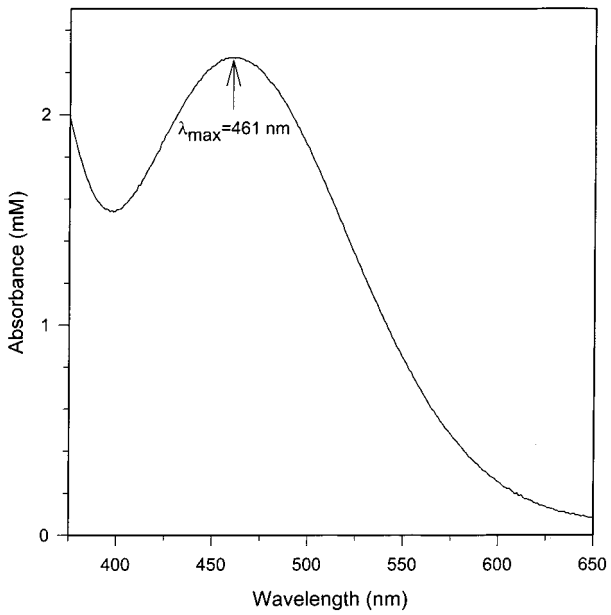

FIGURE 2: Visible absorbance spectrum of the cleaved iron-bearing rTfC-lobe. The concentration of the iron-loaded C-lobe was determined from its absorbance at 280 nm. The molar absorptivity used, 59300 M^{-1} cm^{-1}, was calculated from the amino acid composition of the C-lobe using the protein parameter tool of the ExPASy web site and corrected for the 31% increase due to binding of iron. The latter figure was estimated from the molar absorptivities of apotransferrin (86700 M^{-1} cm^{-1}) and diferric transferrin (114000 M^{-1} cm^{-1}) as determined from dry weights in our laboratory (unpublished results).

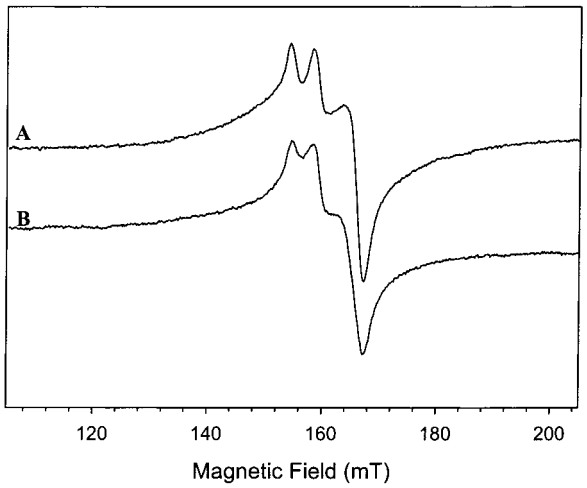

FIGURE 3: EPR spectra of the iron-bearing rTfC-lobe and native Tf-Fe_C. Curves: A, native Tf-Fe_C; B, rTfC-lobe. EPR parameters: microwave frequency, 9.517 GHz; microwave power, 10 mW; modulation amplitude, 1 mT; temperature, 100 K.

Further verification of the native conformation in the recombinant C-lobe is provided by comparison of the kinetics of iron release to pyrophosphate from Tf-Fe_C and the iron-bearing rTfC-lobe (Figure 4). Iron release rates from both proteins, and their response to chloride concentrations, are almost identical.

We conclude, therefore, that the iron-binding properties of the C-lobe cleaved from rTf-Xa are substantially identical

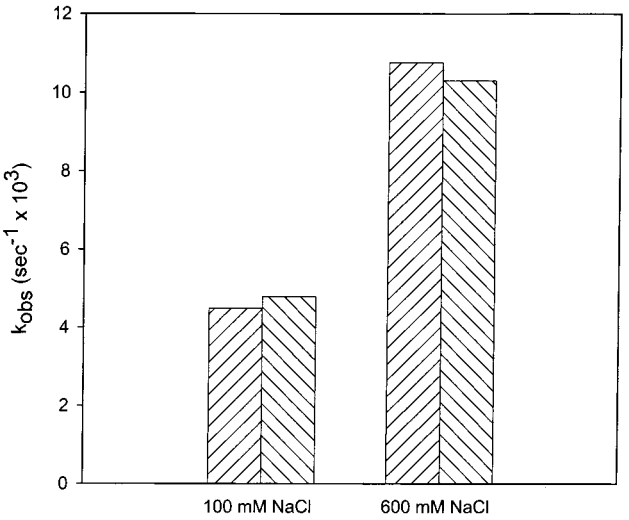

FIGURE 4: Comparison of iron release kinetics from the Tf-Fe_C and rTfC-lobe. Buffer: 100 mM MES and 10 mM pyrophosphate, pH 5.6. Data were collected in duplicate, with variations less than 3% of the mean. The rate constant, k_{obs}, is obtained by curve fitting to a single-exponential rate function. Plots: Tf-Fe_C, ///; rTfC-lobe, \\\.

to those of the C-lobe in native transferrin and that the cleaved C-lobe retains its native conformation.

Binding of the Cleaved C-Lobe to K562 Cells. The ability of the cleaved iron-loaded C-lobe to recognize and be recognized by transferrin receptors was tested by measuring binding to K562 cells at 4 °C as a function of concentration (Figure 5A). After correction for nonspecific binding, measured in the presence of a 100-fold molar excess of unlabeled native diferric transferrin, the binding isotherm shows saturation behavior, as expected of binding to specific cell-surface transferrin receptors. Parameters derived from fitting are as follows: number of specific binding sites, 1.5×10^6; K_a, 1.3×10^6 M^{-1}. For comparison, parameters for the binding of native diferric transferrin to the same harvesting of K562 cells are as follows: specific binding sites, 4.6×10^5; K_a, 9.7×10^7 M^{-1}. From the binding constants the free energy of C-lobe binding, calculated from the relation $-\Delta G = RT \ln K_a$, is -32.4 kJ, while that of diferric native transferrin is -42.4 kJ. Thus, the free energy of formation of the C-lobe−receptor complex is 76% of that of the transferrin−receptor complex, indicating that the C-lobe is a major contributor to the binding of the intact protein.

Nonspecific, nonsaturable binding of C-lobe, as revealed by the linear portion of the binding isotherm, is about twice as large on a molar basis as that observed with native diferric transferrin measured with the same harvesting of K562 cells (Figure 5) but still linear with respect to protein concentration. The larger nonspecific binding of the C-lobe presumably reflects the smaller size and different surface characteristics of the isolated C-lobe compared to the intact transferrin molecule. Its smaller size may also help to account for the larger number of specific binding sites accessible to the C-lobe as for the intact protein; in other studies, ratios of binding sites from curve fits for the C-lobe and diferric transferrin ranged from 1.6 to 3.0.

In all, binding of three different preparations of the C-lobe, each to a different harvesting of K562 cells, was measured,

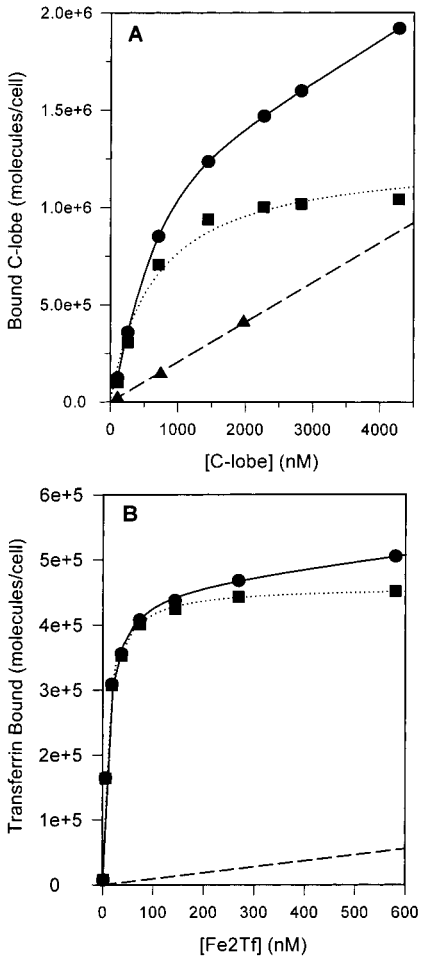

FIGURE 5: Binding of the iron-bearing rTfC-lobe and Fe_2Tf to K562 cells at 4 °C. Symbols: total binding, ●; calculated specific binding, ■; fitted binding isotherm, ⋯; nonspecific binding, ▲. In panel B nonspecific binding was calculated by inclusion of a linear term in the binding isotherm (*17*).

Table 1: Binding of Iron-Bearing Transferrin C-Lobes to K562 Cells

protein	K_a (M^{-1})
proteolytic C-lobe fragment from native transferrin[a]	4.2×10^6
C-lobe fragment + N-lobe[a]	4.7×10^7
rTfC-lobe (preparation 1, factor Xa cleavage)	1.7×10^6
rTfC-lobe (preparation 1) + rN-lobe	1.7×10^7
rTfC-lobe (preparation 2, Figure 5)	1.3×10^6
rTfC-lobe (preparation 3)	1.6×10^6

[a] Reference *14*.

with resulting data summarized in Table 1. The binding constants fall within a narrow range, $1.3-1.7 \times 10^6$ M^{-1} and within a factor of 3 of that reported earlier for a proteolytically cleaved C-lobe derived from native transferrin bearing a disulfide-linked fragment of the N-lobe (*14*). This consistency is further evidence in support of specific binding of the recombinant C-lobe to transferrin receptors.

No evidence of saturable binding to K562 cells could be obtained with the iron-free rTfC-lobe. Only nonspecific binding, linear in concentration and not inhibitable by native diferric transferrin, was observed (Figure 6).

Uptake of Iron by K562 Cells from the rTfC-Lobe and Native Transferrin. At equimolar concentrations of the proteins, 250 nM, uptake of iron is about four times faster

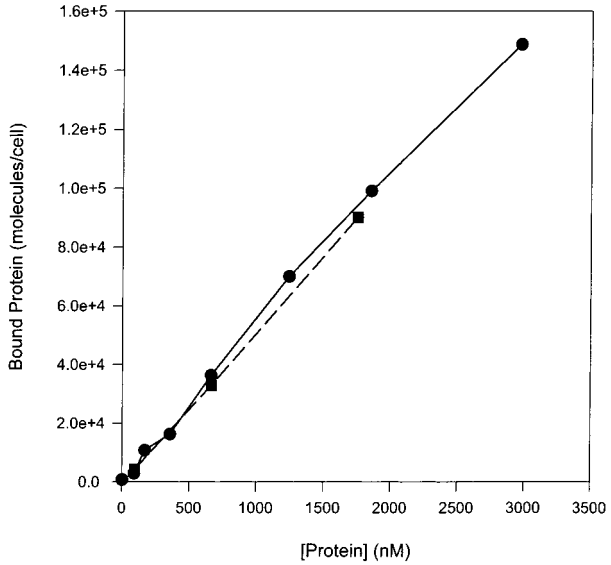

FIGURE 6: Binding of the iron-free rTfC-lobe to K562 cells at 4 °C. Symbols: iron-free rTfC-lobe, ●; iron-free rTfC-lobe + 100-fold molar excess native diferric transferrin, ■.

from the native protein (Figure 7). Increasing the concentration of the rTfC-lobe to five times that of the native protein results in approximately equal rates of iron uptake from the two proteins. We conclude, therefore, that the C-lobe by itself is competent to deliver iron to K562 cells.

DISCUSSION

Binding of transferrin to its receptor is the first step in the receptor-dependent uptake of iron by cells from transferrin. A conserved tripeptide motif, Arg-Gly-Asp, has been incriminated as a transferrin-binding sequence in transferrin receptor secreted by COS-7 cells (*13*), but relatively little progress beyond speculation has been made in identifying the receptor-recognition site(s) of the human transferrin molecule. Even whether one or both lobes of the transferrin molecule are required for binding to receptor has been controversial. Earlier work from this laboratory with a C-lobe derived by limited proteolysis of native human transferrin indicated that the C-lobe alone is sufficient for saturable binding and delivery of iron to cells, with binding enhanced by the presence of the intact N-lobe (*14*). Another publication reported both lobes of transferrin to be required for specific receptor binding and iron donation to HeLa cells (*10*), which is contrary to our previous findings. The suggestion was made that an N-lobe fragment, joined by a disulfide bond from Cys137 to Cys331 of the proteolytically derived C-lobe which began at Leu326, contributed to the binding and iron-donating properties we observed in that lobe. We have therefore undertaken further examination of transferrin−receptor interactions, a fundamental aspect of iron metabolism, by developing a new method for preparing the C-lobe free of the N-lobe remnants and in the verified native conformation.

Expression of the Human Transferrin C-Lobe. Expression of transferrin and lactoferrin N-lobes in good yield and in the verified native conformation has been reported by several laboratories and has made these N-lobes excellent subjects for mutagenesis studies (*5*, *6*, *20*−*23*). In contrast, expression of transferrin C-lobes has been less satisfactory, with much

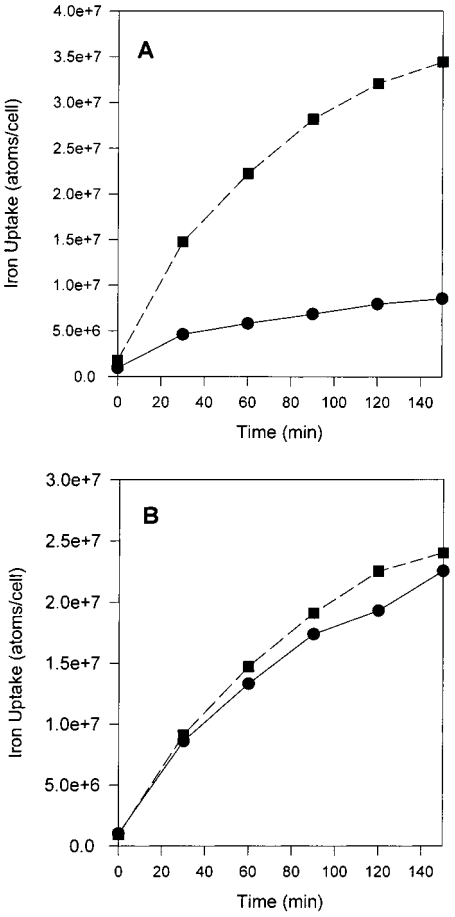

FIGURE 7: Uptake of iron from the rTfC-lobe and Fe_2Tf at 37 °C. Panel A: [rTfC-lobe], 2.5×10^{-7} M; [Fe_2Tf], 2.5×10^{-7} M. Panel B: [rTfC-lobe], 12.5×10^{-7} M; [Fe_2Tf], 2.5×10^{-7} M. Symbols: Fe_2Tf, ■; rTfC-lobe, ●.

poorer yields and limited evidence of structural integrity (*8*, *10*). A previous study of recombinant human transferrin C-lobe, expressed by *E. coli* as inclusion bodies requiring solubilization and renaturation in urea and reducing solutions, reported spectroscopic and iron-binding verification of the native conformation but small yield (*11*). As indicated, we have obtained expression of immunoreactive human C-lobes in insect cells and in mammalian cells, but also in poor yield of a product unable to bind iron or copper in a specific manner. The human transferrin C-lobe has 22 cysteine residues, all of which participate in disulfide bond formation so that aberrant disulfide bonding with consequent misfolding and degradation of the nascent protein in expressing cells may occur. Because recombinant expression of full-length transferrin has been quite successful (*24*), suggesting a protective or "chaperoning" function of the N-lobe in guiding folding of the C-lobe during biosynthesis of full-length transferrin, we turned to the strategy of inserting a specific factor Xa site in the interlobe-connecting strand of human transferrin. The resulting protein was easily cleaved by the protease, yielding the C-lobe (residues 334−679 of native transferrin) with intact iron-binding properties providing evidence that the native conformation is preserved.

An additional advantage of the present method for preparing the C-lobe is that it makes possible comparative studies of C-lobe properties, chemical or functional, in the isolated lobe and in the full-length protein where it is joined to the

N-lobe. Interlobal interactions are known to affect the properties of the individual lobes (*3*, *19*, *25*), and such interactions can now be readily explored by comparative studies of the mutated C-lobe in the full-length protein and in the isolated form.

The investigation by Mason and co-workers (*10*) employed HeLa cells for studying receptor-dependent iron uptake from transferrin and did not accept our conclusion that the "primary receptor-recognition site of human transferrin is in the C-lobe". That study employed recombinant immunoreactive C-lobe beginning at residue Thr 336 preceded by the first four residues of the N-lobe, included to ensure cleavage of the wild-type signal sequence. The molecular weight and N-terminal sequence of the recombinant protein were as expected, but no report of the protein's iron-binding properties was offered. Limited uptake of 200 nM recombinant C-lobe (designated hTf/2C) by cells at 37 °C was observed, but this uptake was not effectively suppressible by excess diferric transferrin. A full binding isotherm was not reported to substantiate the conclusion that both lobes are needed for full binding. The N-lobe (hTf/2N) alone neither bound nor donated iron to HeLa cells in significant fashion, in accord with earlier results from other laboratories (*14*, *26*).

In the present study we have chosen K562 cells for examining the receptor-binding and iron-donating abilities of the rTfC-lobe. The K562 cell, of human hematopoietic origin, can be induced to express hemoglobin and has often been taken as a model of the transferrin-dependent iron-requiring cell. It offers the advantage of a high receptor number, in the range of 350000−1000000, and relatively little nonspecific binding of transferrin. We find that the recombinant iron-loaded C-lobe, entirely free of N-lobe fragments and extraneous amino acid residues, binds to K562 cells in saturable fashion inhibitable by native diferric transferrin. The protein is competent to deliver iron to the cells and, when offered in adequate concentration, matches the capabilities of native transferrin as the iron donor. The iron-free C-lobe binds to K562 cells only in a nonspecific fashion that cannot be inhibited by a 100-fold molar excess of native diferric transferrin. We conclude that the C-lobe is necessary and sufficient for binding transferrin and delivering its iron to cells.

Despite its failure to recognize transferrin receptors by itself, the N-lobe plays a role in the binding of transferrin to its receptor. The presence of the N-lobe enhances the binding strength of the C-lobe by a factor of about 10 (Table 1) and augments the uptake of iron from the C-lobe (*10*, *14*). What cannot yet be ascertained is whether the N-lobe binds directly to the receptor in the presence of the bound C-lobe or affects the binding of the C-lobe by allosteric interaction with that lobe rather than by directly binding to the receptor. Since direct binding of the N-lobe to receptor has not been demonstrable, we favor the latter mechanism. Extrapolation of the temperature-dependent binding constants for the association of recombinant N- and C-lobes from the calorimetric study by Mason et al. (*10*) to a temperature of 4 °C in a van't Hoff plot (ln K_a vs $1/T$) yields an association constant of 2.7×10^5 M^{-1}. The addition of 2×10^{-6} M rN-lobe to 3.34×10^{-8} M iron-bearing rTfC-lobe increases the binding strength of the C-lobe to K562 cells by a factor of 10 (Table 1); the predicted concentration of associated N- and C-lobes in solution would then be about 12 nM, which could substantially account for the observed increase in binding strength.

Receptor saturation by diferric transferrin is >95% in both panels of Figure 7, while that of the C-lobe increases from 27% (panel A) to 67% (panel B). Nothing is known of the relative cycling times of diferric transferrin and the C-lobe within the K562 cell, nor of the relative efficiencies of iron uptake from the two proteins, to help to account for the nearly equal rates of iron uptake from the two proteins in panel B. Nevertheless, receptor occupancy at the higher concentration of the C-lobe is two-thirds that of diferric transferrin, so that the similarity in rates is not very surprising.

The exocytic region of a recently discovered second transferrin receptor, TfR2 (*12*), exhibits 45% identity and 66% similarity with the corresponding region of the transferrin receptor. TfR2 is transcribed in K562 cells, but its level of expression has not been determined. It is not now possible, therefore, to evaluate its role in the present studies of transferrin binding and iron delivery to K562 cells.

Investigations with isolated transferrin lobes cannot fully explain the receptor-binding properties of full-length transferrin. When either lobe of rabbit transferrin is occupied by iron, the protein binds to rabbit reticulocytes with about one-fourth the affinity of the diferric protein (*27*) in a study in which apotransferrin was also found to bind in a saturable manner with an association constant only 24-fold less than that of diferric transferrin. The latter finding is at variance, however, with results from other laboratories indicating virtual absence of apotransferrin binding to solubilized or cell surface receptors at pH 7.4 (*28*, *29*). Undetected pH variations may help to account for the discordance, since apotransferrin binds well to the receptor at pH 5.0−5.6 (*30*, *31*). A naturally occurring human transferrin variant bearing the C-lobe mutation Gly 394 → Arg was found to have iron bound to both lobes. Nevertheless, the C-lobe was in the open conformation, as revealed by low-angle solution X-ray scattering, rather than the closed conformation characteristic of iron-bearing lobes and was found competent to bind to K562 cells (*32*). In that study the relative roles of iron binding and lobe conformations could not be distinguished. Since interlobe interactions affect the properties of transferrin lobes (*3*, *19*, *33*), isolated lobes may function differently in receptor binding than do the corresponding lobes in full-length transferrin. The suggestion that the energy barriers in conformational transitions of transferrin lobes are small (*34*) may also help to explain the ability of the variant to adopt a receptor-binding conformation of the iron-occupied C-lobe.

By exploiting the inability of chicken transferrin receptor to recognize human transferrin, the transferrin-binding site of the human transferrin receptor has been localized to the C-terminal region in studies of human and chicken receptor chimeras (*35*). Consistent with this finding is the demonstration that a conserved Arg-Gly-Asp sequence (residues 646−648 of 758) in the C-terminal region of the human receptor has a critical role in capturing transferrin; substitution of the Arg or Gly by Ala abolished detectable transferrin binding, while replacement of the Asp by Ala reduced binding to 16% of controls (*13*). The crystal structure of the exocytic domain of the receptor (*36*) shows that the tripeptide is solvent accessible, with the Arg side chain protruding out from the helix in which it is located. A reasonable speculation,

therefore, is that a substantial part of the binding energy in the receptor−transferrin interaction comes from relatively small areas of protein−protein contact, consistent with our present and earlier claim that the primary receptor-recognition site of transferrin is in the C-lobe.

REFERENCES

1. Aisen, P. (1998) in *Metal Ions in Biological Systems* (Sigel, A., and Sigel, H., Eds.) Vol. 35, pp 585−631, Marcel Dekker, New York.
2. Park, I., Schaeffer, E., Sidoli, A., Baralle, F. E., Cohen, G. N., and Zakin, M. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3149−3153.
3. Day, C. L., Stowell, K. M., Baker, E. N., and Tweedie, J. W. (1992) *J. Biol. Chem. 267*, 13857−13862.
4. Zak, O., Tam, B., MacGillivray, R. T. A., and Aisen, P. (1997) *Biochemistry 36*, 11036−11043.
5. Nurizzo, D., Baker, H. M., He, Q. Y., MacGillivray, R. T. A., Mason, A. B., Woodworth, R. C., and Baker, E. N. (2001) *Biochemistry 40*, 1616−1623.
6. He, Q. Y., Mason, A. B., Nguyen, V., MacGillivray, R. T. A., and Woodworth, R. C. (2000) *Biochem. J. 350*, 909−915.
7. Muralidhara, B. K., and Hirose, M. (2000) *J. Biol. Chem. 275*, 12463−12469.
8. Sheth, B., Stowell, K. M., Day, C. L., Baker, E. N., and Tweedie, J. W. (1994) *Adv. Exp. Med. Biol. 357*, 259−263.
9. Steinlein, L. M., and Ikeda, R. A. (1993) *Enzyme Microb. Technol. 15*, 193−199.
10. Mason, A. B., Tam, B. M., Woodworth, R. C., Oliver, R. W. A., Green, B. N., Lin, L. N., Brandts, J. F., Savage, K. J., Lineback, J. A., and MacGillivray, R. T. A. (1997) *Biochem. J. 326*, 77−85.
11. Hoefkens, P., De Smit, M. H., De Jeu-Jaspars, N. M. H., Huijskes-Heins, M. I. E., de Jong, G., and van Eijk, H. G. (1996) *Int. J. Biochem. Cell Biol. 28*, 975−982.
12. Kawabata, H., Yang, S., Hirama, T., Vuong, P. T., Kawano, S., Gombart, A. F., and Koeffler, H. P. (1999) *J. Biol. Chem. 274*, 20826−20832.
13. Dubljevic, V., Sali, A., and Goding, J. W. (1999) *Biochem. J. 341*, 11−14.
14. Zak, O., Trinder, D., and Aisen, P. (1994) *J. Biol. Chem. 269*, 7110−7114.
15. Egan, T. J., Zak, O., and Aisen, P. (1993) *Biochemistry 32*, 8162−8167.
16. McFarlane, A. S. (2001) *J. Clin. Invest. 42*, 346−361.
17. Osterloh, K., and Aisen, P. (1989) *Biochim. Biophys. Acta 1011*, 40−45.
18. Zak, O., Leibman, A., and Aisen, P. (1983) *Biochim. Biophys. Acta 742*, 490−495.
19. Zak, O., and Aisen, P. (1985) *Biochim. Biophys. Acta 829*, 348−353.
20. Mason, A. B., Funk, W. D., MacGillivray, R. T., and Woodworth, R. C. (1991) *Protein Expression Purif. 2*, 214−220.
21. Faber, H. R., Baker, C. J., Day, C. L., Tweedie, J. W., and Baker, E. N. (1996) *Biochemistry 35*, 14473−14479.
22. He, Q. Y., Mason, A. B., Woodworth, R. C., Tam, B. M., MacGillivray, R. T. A., Grady, J. K., and Chasteen, N. D. (1998) *J. Biol. Chem. 273*, 17018−17024.
23. Steinlein, L. M., Ligman, C. M., Kessler, S., and Ikeda, R. A. (1998) *Biochemistry 37*, 13696−13703.
24. Mason, A. B., Miller, M. K., Funk, W. D., Banfield, D. K., Savage, K. J., Oliver, R. W. A., Green, B. N., MacGillivray, R. T. A., and Woodworth, R. C. (1993) *Biochemistry 32*, 5472−5479.
25. Aisen, P., Leibman, A., and Zweier, J. (1978) *J. Biol. Chem. 253*, 1930−1937.
26. Lineback-Zins, J., and Brew, K. (1980) *J. Biol. Chem. 255*, 708−713.
27. Young, S. P., Bomford, A., and Williams, R. (1984) *Biochem. J. 219*, 505−510.
28. Lebrón, J. A., Bennett, M. J., Vaughn, D. E., Chirino, A. J., Snow, P. M., Mintier, G. A., Feder, J. N., and Bjorkman, P. J. (1998) *Cell 93*, 111−123.
29. Tsunoo, H., and Sussman, H. H. (1983) *Arch. Biochem. Biophys. 225*, 42−54.
30. Ecarot-Charrier, B., Grey, V. L., WilczynsKa, A., and Schulman, H. M. (1980) *Can. J. Biochem. 58*, 418−426.
31. Klausner, R. D., Ashwell, J. V., VanRenswoude, J. B., Harford, J., and Bridges, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2263−2266.
32. Evans, R. W., Crawley, J. B., Garratt, R. C., Grossmann, J. G., Neu, M., Aitken, A., Patel, K. J., Meilak, A., Wong, C., Singh, J., Bomford, A., and Hasnain, S. S. (1994) *Biochemistry 33*, 12512−12520.
33. Beatty, E. J., Cox, M. C., Frenkiel, T. A., Tam, B. M., Mason, A. B., MacGillivray, R. T. A., Sadler, P. J., and Woodworth, R. C. (1996) *Biochemistry 35*, 7635−7642.
34. Rawas, A., Muirhead, H., and Williams, J. (1997) *Acta Crystallogr. D 53*, 464−468.
35. Buchegger, F., Trowbridge, I. S., Liu, L. F., White, S., and Collawn, J. F. (1996) *Eur. J. Biochem. 235*, 9−17.
36. Lawrence, C. M., Ray, S., Babyonyshev, M., Galluser, R., Borhani, D. W., and Harrison, S. C. (1999) *Science 286*, 779−782.

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