A Kinetically Active Site in the C-Lobe of Human Transferrin†

Olga Zak,‡ Beatrice Tam,§ Ross T. A. MacGillivray,§ and Philip Aisen*,‡

Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461, and Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada

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ABSTRACT: Release of iron from transferrin, the iron-transporting protein of the circulation, is a concerted process involving remote amino acid residues as well as those at the two specific iron-binding sites of the protein. Previous studies of fluoresceinated transferrin have suggested Lys 569 as a kinetically active site in the C-terminal lobe of the protein. We have therefore turned to site-directed mutagenesis to investigate the role of Lys 569 in the release process at pH 5.6, the pH of the endosome where iron is transferred from transferrin to the iron-dependent cell. Mutation of positively charged Lys 569 to an uncharged Gln results in a protein in which release of iron from the mutated lobe to pyrophosphate is slowed by a factor of 15−20 and in which release kinetics switch from a complex saturation-linear to a simple saturation function. Acceleration of release by chloride is also substantially less than in native transferrin. When Lys 569 is replaced by a positively charged Arg, in contrast, observed release rates and chloride dependence are close to those of the native protein. The mechanism of release from the C-lobe site therefore appears to be sensitive to positive charge at position 569. Binding of chloride or other simple anion accelerates and is essential for release from the C-lobe; a muted response of K569Q to chloride concentration suggests that Lys 569 may function as a kinetically active anion-binding residue in the C-lobe. Despite the kinetic effects of the K569 mutation on iron release, rates of iron uptake by K562 cells from the C-lobes of native, K569Q, and K569R proteins are almost identical. In contrast to the C-lobe, iron release from the N-lobe is insensitive to charge at residue 233, the site in that lobe homologous to residue 569, with chloride retarding rather than accelerating release. K233, therefore, is not a kinetically active anion-binding site in the N-lobe. Release mechanisms differ substantially in the two lobes of transferrin despite the identity of ligands and their nearly identical arrangements in the lobes.

The single polypeptide chain of human serum transferrin is made up of 679 amino acid residues arranged in two homologous lobes, each bearing a single iron-binding site. Internal similarity in the intron−exon organization of the transferrin gene (Park et al., 1985) and 45% identity in amino acids of the lobes (MacGillivray et al., 1983) provide compelling evidence that the modern molecule arose from duplication and fusion of a precursor gene specifying a single-sited transferrin precursor. Such an event must have occurred more than 500 million years ago, since the cockroach expresses a two-sited transferrin (Gasdaska et al., 1996), but the evolutionary advantage conferred by the duplication is still unclear.

Serum transferrin is the chief, and in normal circumstances the only, source of iron for the metabolic needs of most cells. Each transferrin molecule undergoes 100−200 cycles of iron binding, transport, and release to cells during its lifetime in the circulation (Katz, 1961), so that understanding the molecular mechanisms governing iron release from transferrin is essential for understanding the physiological functions of the protein. Despite the identical iron-binding ligands in each lobe and the near-identity of their 3-dimensional arrangements (Haridas et al., 1995; Bailey et al., 1988; Zuccola, 1992), the iron-binding properties and the mechanisms of iron release are distinctly different in the two lobes. Among the factors that distinguish iron release from each lobe is the response to simple anions when pyrophosphate is the iron-sequestering ligand that is necessary for release to occur under physiological circumstances. In the C-terminal lobe of native transferrin, chloride (or another simple anion) is essential for and accelerates release (Egan et al., 1992), but in the isolated N-lobe as well as the N-lobe of native transferrin, chloride retards release (Zak et al., 1995). Remarkably, release from both lobes of human transferrin and lactoferrin to the synthetic catechol-based iron chelator 3,4-LICAMS[1] displays an absolute dependence on ionic strength (Kretchmar & Raymond, 1988), presumably indicating an anion requirement in both lobes when LICAMS serves as the required iron acceptor.

The kinetically active anion binding site or sites of either lobe are not known. In one study of human transferrin, iron release to EDTA was found to be substantially slower from

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* Address correspondence to this author at Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

‡ Albert Einstein College of Medicine.

§ University of British Columbia.

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[1] Abbreviations: 3,4-LICAMS, *N*,*N*′,*N*″-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HEPES, 4-(2−hydroxyethyl)-1-piperazineethanesulfonic acid; hTf/2N, recombinant human transferrin N-lobe (residues 1−337); KISAB, kinetically significant anion binding; MES, 4-morpholinoethanesulfonic acid; NTA, nitrilotriacetate; PCR, polymerase chain reaction; PPi, pyrophosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tf-Fe_C, monoferric transferrin loaded in the C-lobe.

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Table 1: Recombinant Proteins

protein	mutagenic primer[a]	molecular weight expected	molecular weight found	[Tris] at peak in chromatogram (mM)
K569Q	5′-GGTACCAGGCAACCTGTGGAG-3′	75 146	75 155	136
Q569K	5′-GGTACCAGGAAACCTGTGGAG-3′ [b]	75 146	75 155	120
K569R	5′-GGTACCAGGAGACCTGTGGAG-3′ [b]	75 174	75 182	117
N-lobe	---	37 151	37 152	121
K233Q	5′-CCTAGACAACACCCGGCAGCCGGTAGATGAATACAAGG-3′ [b,c]	37 151	37 153	161

[a] Mutated nucleotides are underlined. [b] Complementary sequence also required. [c] In N-lobe sequence (hTf/2N).

fluoresceinated than from unmodified protein (Egan, 1993). Fluorescence energy transfer calculations implicated modification of a lysine lying 21 −25 Å from the iron site as responsible for the retardation of release, with Lys 569 as one of the candidates for the kinetically active site in the C-lobe. In the present study we have turned to site-directed mutagenesis of Lys 569 and its homologue in the N-lobe, Lys 233, for exploring the functions of these residues in modulating iron release from transferrin.

MATERIALS AND METHODS

Reagents and Enzymes. All reagents were reagent-grade or the highest quality commercially obtainable. Pyrophosphoric acid was purchased from Aldrich Chemical Co. (Milwaukee, WI); because of its deliquescence, reproducibility of results was best when a 1 M stock solution was divided into aliquots which were frozen and thawed for use as needed. Restriction endonucleases and T4 ligase were purchased from Pharmacia Biotech Inc. (Piscataway, NJ) , [^{35}S]dATP from NEN−DuPont (Boston, MA), and Taq polymerase from Perkin-Elmer Cetus (Foster, CA).

Transferrins. Lyophilized human iron-saturated transferrin was supplied by Boehringer Mannheim Corp. (Indianapolis, IN) and freed of iron and extraneous chelators by established methods (Aisen et al., 1978; Price & Gibson, 1972). Selective reloading of transferrin at the C-site was achieved with minor modifications of published procedures (Baldwin & de Sousa, 1981). Briefly, loading of the C-lobe was accomplished by presenting a stoichiometric quantity of iron as its 1:2 complex with NTA to the protein at pH 6.5, at which pH binding to the N-lobe is very much weaker than binding to the C-lobe. Use of NTA also helps direct iron to the C-lobe (Aisen et al., 1978). C-lobe monoferric transferrins labeled with ^{59}Fe were prepared with ^{59}Fe(NTA)$_2$, using the same stock solution for all proteins to minimize errors. Preparations were freed of extraneous chelators by dialysis against 0.1 M sodium perchlorate (Price & Gibson, 1972).

Site-Directed Mutagenesis. The K569Q mutation was introduced into the sequence coding for full-length nonglycosylated human transferrin in the pNUT expression vector (Mason et al., 1993) using a PCR-based mutagenesis procedure (Nelson & Long, 1989). A *Pst*I−*Hin*dIII fragment of nonglycosylated human transferrin cDNA, resulting from mutagenesis of the glycosylation residues Asn 413 and Asn 611 to Asp, was cloned into the plasmid Bluescript SK; this fragment corresponds to nucleotides 1510 −1868 of transferrin cDNA (Yang et al., 1984). Mutagenesis was performed by PCR with Bluescript-specific flanking primers, following procedures described previously (Mason et al., 1993); the mutagenic primer is shown in Table 1. The nucleotide sequence was determined to confirm the mutation and the absence of other mutations introduced during the PCR steps. The mutated *Pst*I−*Hin*dIII fragment was then reintroduced into the nonglycosylated human transferrin cDNA cloned into pNUT for expression in BHK-21 cells.

Other mutations were carried out using the Stratagene QuikChange mutagenesis kit. The mutagenesis protocol employed with this kit directly mutates double-stranded plasmid DNA using Pfu DNA polymerase, which replicates both plasmid strands with high fidelity without displacing mutagenic oligonucleotide primers. Manufacturer's directions were followed without modification in the mutagenesis procedure. K569R and the recombinant protein lacking glycosylation sites but otherwise with the wild-type sequence (Q569K) were prepared from pNUT bearing the K569Q construct. K233 was generated from pNUT bearing the sequence encoding the leader and first 337 residues of the human N-lobe, hTf/2N (Funk et al., 1990). After mutation and expansion, the Qiagen Miniprep kit was used to isolate the resulting plasmid DNA for sequencing and transfection. Sequencing was carried out in the sequencing facility of the Albert Einstein College of Medicine, using Perkin-Elmer Models ABI 373A or 377 automated sequencers and AmpliTaq DNA polymerase FS.

Expression, Purification, and Characterization of Recombinant Proteins. BHK-21 cells were transfected by the calcium phosphate coprecipitation (Searle et al., 1985; Chen & Okayama, 1987) or Lipofectamine (Gibco, Grand Island, NY) methods, with equivalent results using either procedure. Transfected cells were selected with 500 μM methotrexate and then cultured in DMEM−F12 medium (Gibco) with 0.5−1.0% UltroSer G for expression of recombinants (Funk et al., 1990). Media were harvested at 48−72 h intervals, pooled, and concentrated with an Amicon ultrafiltration cell using YM30 membranes for holotransferrin recombinants and PM10 membranes for N-lobe recombinants. Fe(NTA)$_2$, pH 5.0, was added to harvested culture media to promote stability of recombinant transferrins by saturating them with iron. The expressed proteins were purified by anion-exchange chromatography on a 15/300 column of POROS 50 HQ (PerSeptive Biosystems, Framingham, MA) using a linear gradient of 25−200 mM tris·HCl, pH 7.0. Pink fractions were pooled, concentrated, and rechromatographed on a 4.5/100 POROS QE/M column, with a linear gradient of 20−200 mM tris·HCl, pH 7.0. This pH results in improved separation of nonglycosylated recombinants from native transferrin found in UltroSer G presumably because the relative charge difference between sialylated and nonsialylated molecules is increased as their isoelectric points are approached. The concentration of tris at the chromatographic peak of each protein is shown in Table 1. For isolation and

purification of K233Q, a size-exclusion chromatographic step with Sephacryl 100 HR was inserted between the two anion-exchange chromatographies. Final preparations were characterized by SDS−PAGE, EPR spectroscopy, and electrospray mass spectrometry.

Other Methods. SDS−PAGE was carried out with the Pharmacia PhastSystem. EPR spectra of recombinant proteins selectively loaded in the mutated lobe were obtained with a Bruker 200D spectrometer upgraded to ESP300 specifications. Electrospray mass spectrometry was performed on an API-III triple-quadrupole mass spectrometer (PE-SCIEX, Ontario) in the Laboratory for Macromolecular Analysis of the Albert Einstein College of Medicine, using the SCIEX ion spray interface with nitrogen as the injection gas.

Binding of transferrins to K562 cells was measured using proteins labeled with ^{125}I by the Enzymobead (Bio-Rad) procedure, as previously described (Zak et al., 1994).

Uptake of iron by K562 cells from native and mutant transferrins was studied using ^{59}Fe-labeled proteins (Zak et al., 1994). Incubations were carried out in a shaking water bath at 37 °C. In each study the incubation medium was RPMI 1640 containing 1% BSA to suppress nonspecific interactions of transferrins with cells. Transferrin concentrations were 2.5 μM, more than sufficient to saturate transferrin receptors, and cell suspensions contained 7.5×10^6 cells/mL. For radioactive counting, cells were separated from media by centrifugation through a 9:1 mixture of dibutyl phthalate and mineral oil. Because the human transferrin receptor does not recognize N-lobe half-transferrin (Zak et al., 1994), binding and uptake studies could only be carried out with bilobal transferrins.

Kinetics of Iron Release. Iron release from native and recombinant proteins was studied with a spectrofluorometric method that requires only microgram quantities of protein while providing a continuous display of reaction progress (Egan et al., 1993). Most measurements were carried out at 25 °C in duplicate or triplicate; where shown, error bars reflect the latter. In some instances, error bars are too small to be seen. Observed rate constants in the range of 0.01−0.0005 s^{-1}, where problems arising from mixing artifacts and instrumental drift are generally negligible, were reproducible to ±8%. As in earlier studies from this laboratory, pyrophosphate was chosen as the required iron-sequestering agent because of its high affinity for iron and its role as the active group of ATP, a principal intracellular iron-binding species. Chloride concentrations were chosen to provide for progress curves with optimum release rates for analysis. Progress curves of iron release were analyzed by nonlinear least-squares curve fitting using the Levenberg−Marquardt algorithm of the SigmaPlot graphics program (Jandel).

RESULTS

Characteristics of Recombinant Proteins. All recombinant proteins used in this work gave single bands by SDS−PAGE (PhastSystem, Pharmacia Bio-Tech; data not shown) and single peaks by mass spectrometry with molecular weights within about 0.01% of expected values (Table 1). Full-length nonglycosylated transferrins showed no evidence of glycosylated native transferrin present in UltroSer G. Except for minor variations in apparent line widths, EPR spectra of Fe(III) specifically bound in mutated or wild-type recombinant lobes (Figure 1) were virtually indistinguishable from corresponding spectra of Fe(III) in native transferrin (Zak & Aisen, 1985), indicating that mutations did not disturb the ligand structure of binding sites. Elution peaks of recombinants by POROS QE/M anion-exchange chromatography are shown in Table 1.

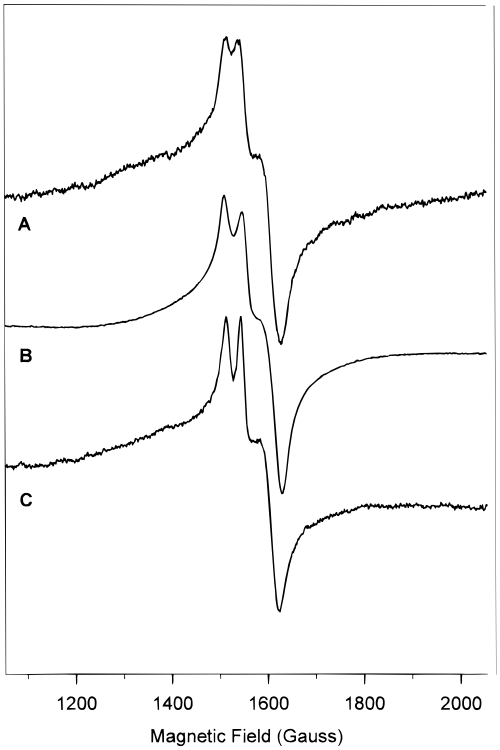

FIGURE 1: EPR spectra of recombinant transferrins. (A) Monoferric K569Q loaded in C-lobe; (B) monoferric K569R loaded in C-lobe; (C) K233Q. Concentrations of protein vary, so spectra are digitally scaled to provide for approximately equal peak-to-peak amplitudes. Microwave frequency, 9.298 GHz; microwave power, 20 mW; modulation amplitude, 10 G; time constant, 655 ms; sweep time, 671 s; temperature, 77 K.

Iron Release from K569Q. The kinetics of iron release from the C-lobe of recombinant K569Q were investigated at pH 5.6, near the pH of the acidified endosome where iron is released from transferrin within the cell (Klausner et al., 1983). Three features distinguish release kinetics of K569Q from release kinetics of the C-lobe of native transferrin. First, and most striking, is the slowness of release from the K569Q mutant (Figure 2). Since iron release from the C-lobe is almost absolutely dependent on binding of an anion to a *k*inetically *s*ignificant *a*nion *b*inding or KISAB site (Egan et al., 1992), a profile of observed release rates as a function of chloride concentration was determined. Throughout the range of chloride concentrations from 0 to 1 M, release from the mutant is $^1/_{15}$−$^1/_{20}$ the rate from the native C-lobe. Second, the response to chloride is dampened in the mutant, which exhibits more than a 4-fold increase in release rates as chloride concentration is increased from 0 to 1 M chloride, while the corresponding increase is 9-fold in the native protein. For both proteins, however, observed release rates are nearly linear functions of chloride concentration and approach zero as chloride concentration approaches zero (an ionic strength of zero cannot be achieved in a buffered protein solution containing an anionic iron acceptor). Thus, the nearly absolute dependence of release on ionic strength (Kretchmar & Raymond, 1988) or anion concentration (Egan

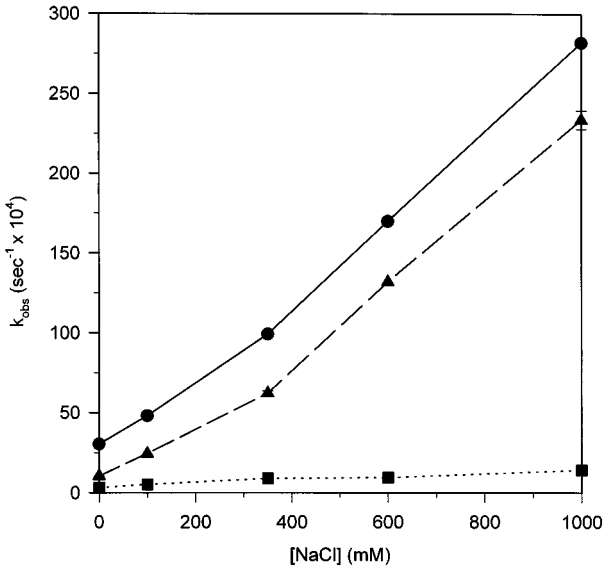

FIGURE 2: Effects of chloride concentration on observed rates of iron release from native and recombinant transferrins. (●), Tf-Fe_C; (▲), K569R-Fe_C; (■), K569Q-Fe_C. [PP_i], 10 mM; [MES], 100 mM; pH 5.6.

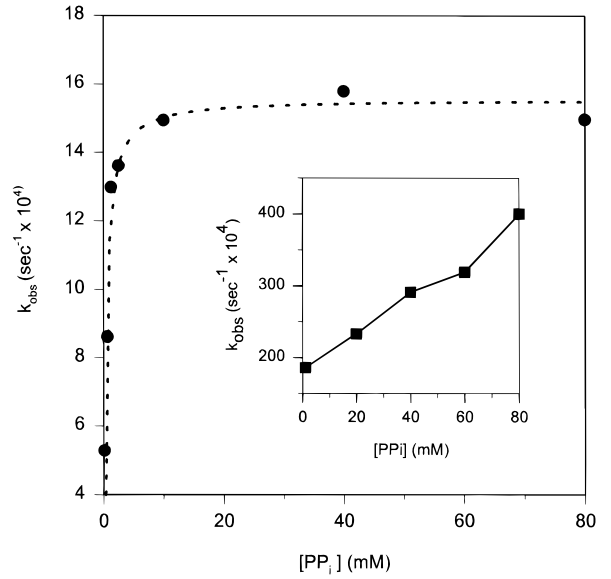

FIGURE 3: Iron release from K569Q as a function of pyrophosphate concentration. Buffer: 100 mM MES and 1 M NaCl, pH 5.6. The dotted line is a least-squares fit of a saturation function to the data points. Inset, release from Tf-Fe_C under the same conditions.

et al., 1992) is preserved in the K569Q mutant. Finally, observed release rates from K569Q saturate with increasing pyrophosphate concentration (Figure 3), reaching a plateau by 10 mM pyrophosphate. In contrast, release rates from the C-lobe of native protein under identical conditions continue to increase in a nearly linear manner at least until a pyrophosphate concentration of 80 mM is reached. Saturation in the kinetic profile is inconsistent with the dual-pathway model of iron release, in which release is dependent upon occupancy of an anion-binding (KISAB) site by a simple anion such as chloride or by an anionic iron-accepting chelator such as pyrophosphate as well as on the concentration of chelator. Rather, saturation of release rates accords with the conformational change model of Bates and coworkers (Cowart et al., 1982; Nguyen et al., 1993; Bali et al., 1991a) in which the rate-limiting step in release at higher concentrations of iron-accepting ligand is the transition from

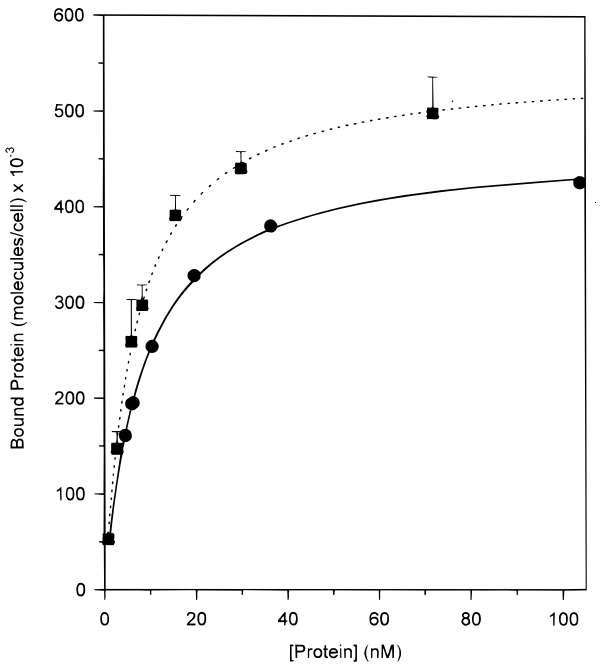

FIGURE 4: Binding of K569Q and native transferrins to K562 cells at 4 °C. (■), diferric K569Q; (●), diferric transferrin. Continuous curves are least-squares fits of binding isotherms; values of fitted parameters for K569Q are $n = 548\,000$ and $K_a = 1.44 \times 10^8\ M^{-1}$; for native transferrin, $n = 465\,000$ and $K_a = 1.65 \times 10^8\ M^{-1}$. Error bars are from measurements in triplicate.

"closed" to "open" states of the releasing lobe.

Full-length transferrin lacking glycosylation sites but otherwise bearing the wild-type sequence was indistinguishable from native protein in iron release from the C-site (data not shown). In contrast to observations of slightly reduced hepatic iron uptake from enzymatically deglycosylated transferrin (Hu et al., 1991), the carbohydrate moieties play no direct role in iron release from transferrin to iron chelators.

Binding of K569Q to Cell Surface Receptors of K562 Cells. Because iron release from transferrin is modulated by binding of the protein to its receptor (Bali et al., 1991b), it was of interest to determine whether mutating K569 perturbs binding of transferrin to receptor. Measurements of ^{125}I-labeled transferrins to cell surface receptors of erythroleukemic K562 cells, which express transferrin receptors in substantial numbers, were therefore carried out. As shown in Figure 4, the strength of binding of K569Q to K562 cells at 4 °C , and the number of binding sites accessible to the mutant protein, are substantially the same as for the recombinant protein lacking glycosylation sites but otherwise with the native sequence. We consider the small difference in behaviors of the two proteins to be within experimental error and biological variation of the cells.

Iron Uptake from Native and Mutant Transferrins by K562 Cells. To determine whether the retarding effects of the K569Q mutation on iron release *in vitro* are paralleled by effects on iron uptake by cells, we examined iron uptake by K562 cells from monoferric transferrins loaded in the C-lobes. Uptake was virtually linear with time over the course of a 3 h incubation for wild-type, K569Q, and K569R proteins, with relatively little difference in average rates of uptake among the proteins: 61 000 atoms $cell^{-1}$ min^{-1} for wild type, 66 000 atoms $cell^{-1}$ min^{-1} for K569Q and 47 000 atoms $cell^{-1}$ min^{-1} for K569R (Figure 5).

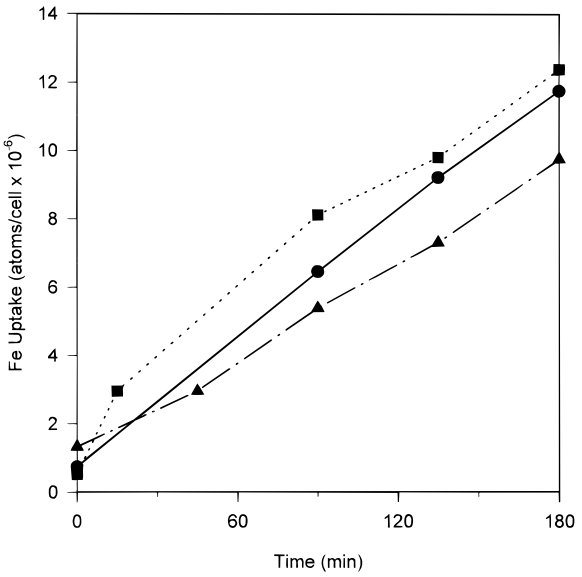

FIGURE 5: Uptake of iron by K562 cells from C-lobes of native (●), K569Q (■), and K569R (▲) transferrins. Incubations carried out at 37 °C in RPMI 1640 buffer containing 10 mM HEPES, 1% BSA, 2.5 μM ^{59}Fe-labeled transferrin, and suspensions of 7.5 × 10^6 cells/mL. Experimental points are means of measurements in duplicate.

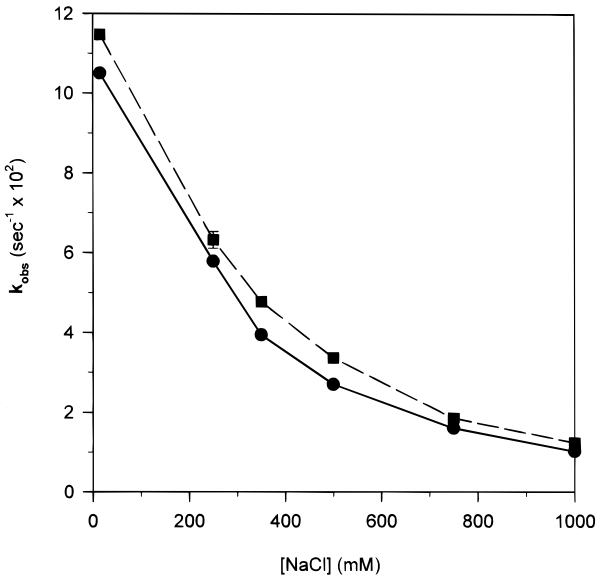

FIGURE 6: Effects of chloride on observed rates of iron release from wild-type and mutant N-lobe recombinants. (●), Wild type; (■), K233Q. [PP_i], 0.1 mM; [MES], 100 mM; pH 5.6.

Iron Release from K233Q. The homologue of K569 in the N-lobe, K233, has also been implicated as a kinetically active residue (Egan, 1993) and was therefore mutated to a glutamine in recombinant N-lobe half-transferrin. Unlike the mutation in the C-lobe, the N-lobe mutation has no significant effect on iron release rates throughout the range of chloride concentrations from 0 to 1 M (Figure 6). In wild-type and mutated recombinants alike, chloride exerts a retarding action on release from the N-lobe. This retarding action can be modeled by assuming competition between chloride and pyrophosphate for binding to a site where ligand exchange between sequestering anion and transferrin can take place (Zak et al., 1995), analogous to competitive inhibition in the Michaelis−Menten model of enzyme kinetics.

DISCUSSION

Factors Regulating Iron Release from Transferrin. At least five factors are known to modulate the kinetics of iron release from human transferrin: (a) the site from which release takes place; (b) ionic composition of the supporting buffer; (b) pH; (d) the sequestering ligand for released iron; and (e) binding of transferrin to its receptor. The molecular basis underlying actions of all of these factors is poorly understood. In the present study we have focused on the first two of these factors, using site-directed mutagenesis to explore and localize their effects on iron release to pyrophosphate from transferrin. Because release of iron from transferrin to the iron-needy cell occurs in an endocytic vesicle at a pH near 5.6, we have initiated our studies at this pH. Efforts to examine iron release kinetics at pH 7.4 by the sensitive spectrofluorometric method were frustrated by the slowness of release, so that analysis of progress curves was confounded by problems of instrumental drift.

Location of Mutated Residues. Each lobe of the human transferrin molecule comprises two dissimilar domains surrounding a deep hydrophilic cleft bearing an iron-binding site (Figure 7). The first domain in each lobe, designated

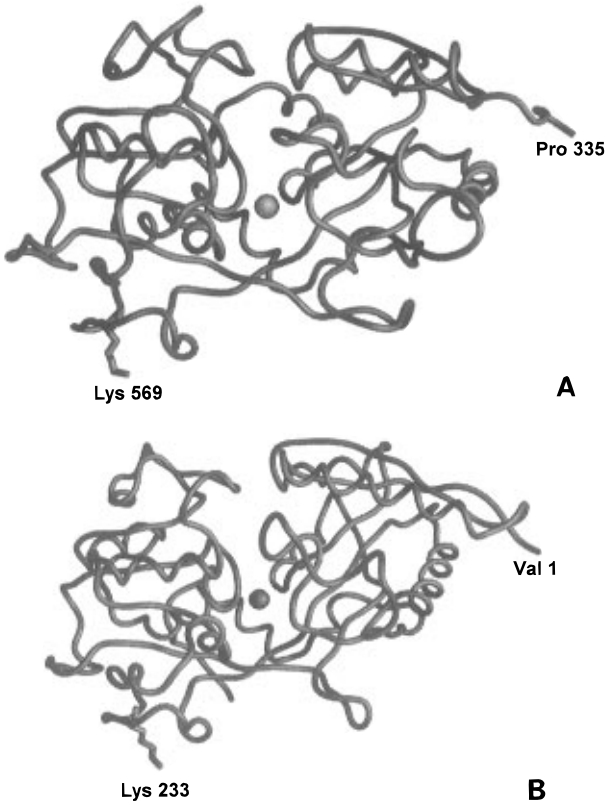

FIGURE 7: Location of mutated residues K569 and K233 in human transferrin C-lobe and N-lobe, respectively. Iron atoms are shown as spherical balls. (A) C-lobe (residues 335−679), from the structure of native human transferrin. (B) recombinant N-lobe (residues 1−337). Images were generated with the GRASP program (Nicholls et al., 1991).

N1 or C1 depending on the lobe in which it is found, is half-completed by the first 90 or so amino acid residues of the peptide chain in that lobe. The chain then crosses to the second domain, N2 or C2, which is fully formed by the next ~160 residues, before returning to complete the first domain. The domains are thus joined by two connecting antiparallel β-strands, each of which contributes a ligand to the iron-binding site (Tyr 95/426; His 249/585), with each domain

also contributing a ligand (Asp 63/392; Tyr 188/517). The coordination requirements of Fe(III) are then completed by the synergistic carbonate anion, secured to the protein by a network of hydrogen and electrostatic bonds.

Binding of iron is associated with a conformational change from an "open" disposition of the two domains surrounding the iron-binding cleft to a configuration with the domains closed around the cleft. Pivoting between open and closed conformations can be viewed as a rigid rotation of the domains with respect to each other. The major part of the rotation occurs as a "see-saw" action about a major axis through antiparallel β-strands linking the two domains (Gerstein et al., 1993), with resulting change in their interfacial contacts. In the larger interface on one side of the rotation axis, residues are solvent-accessible in the open form but become buried in the closed form, while the reverse situation obtains in the smaller interface. The connecting β-strands and their surroundings are therefore logical regions to search for kinetically active residues, and such a search is supported by the nonradiative energy transfer studies of fluoresceinated transferrin implicating Lys 233/569 as kinetically active residues (Egan, 1993). Lys 233/569 each lie at the start of a coil or loop near the N-terminus of one of the strands connecting the two domains of their respective lobes (Figure 7), near the iron ligands of the strands. Thus, location and charge of these lysine residues, although not in the interfacial cleft where mutation of other nonligand residues has been shown to modulate iron release kinetics (Zak et al., 1995), would be consistent with a kinetic role for them.

Mutation of Lys 569. The more than 15-fold retardation in rate of iron release from the C-lobe of K569Q, compared to release from the C-lobe of native transferrin, supports and extends the observation of Egan (1993) that fluoresceination of Lys 569 inhibits iron release from its lobe. A nearly linear dependence of iron release upon ionic strength reported earlier by Kretchmar and Raymond (1988) suggests an ion-binding requirement for release. This inference is strengthened by studies and a model derived from them implicating an anion-binding (KISAB) site, most clearly demonstrated in the C-lobe, that must be occupied for release to occur (Egan et al., 1992). Could Lys 569 be the kinetically active anion-binding site?

At pH 5.6 all lysine residues would be expected to be fully protonated and positively charged, so that Lys 569 is a reasonable candidate for the kinetically significant anion-binding (KISAB) site. In support of this candidacy, replacement of Lys 569 to an uncharged glutamine results in a protein from which release is sluggish compared to native transferrin. Further support would be provided by loss of anion responsiveness in K569Q, but such responsiveness is still evident in the mutant, albeit substantially muted. Although kinetic analysis favors only one KISAB site per lobe (Egan et al., 1992), the possibilities that more than one exists or that more than one residue participates in the function of a single site are not rigorously excluded. We suggest, therefore, that Lys 569 is a kinetically active anion-binding site but possibly not the only such site in the C-lobe. Binding of transferrin to its receptor at pH 5.6 accelerates release of iron from the C-lobe while preserving the release-accelerating effect of chloride, so it is likely that the two effects involve different mechanisms or different sites (Egan et al., 1993). We also note that lost or diminished anion responsiveness would lead to simple saturation kinetics, as predicted by Bates and co-workers (Cowart et al., 1982) and as observed in Figure 3.

The precise mechanism by which a kinetically active lysine at position 569 in the C-lobe sequence might exert its kinetic effects eludes facile explanation. One possibility is that Lys 569 forms a stabilizing salt bridge with a carboxylate oxygen of Glu 573. Oppositely charged groups of these residues in Zuccola's 3.3 Å structure lie about 4 Å apart, rather long for a salt bridge but perhaps not excludable given the relatively low resolution of the X-ray structure and the flexibility of the interacting side chains. Anion binding to the positively charged ϵ-amino nitrogen of Lys 569 would then disrupt the salt bridge to facilitate release. The release-retarding effect of the K569Q mutation, particularly at pH 5.6, might then be due to stabilizing hydrogen-bond formation between Gln 569 and Glu 573 that would not be disrupted by anion binding. This hypothesis is supported by the present results with K569R, in which positive charge is preserved and anion responsiveness close to that of native transferrin C-lobe persists. Other stabilizing interactions of a glutamine at position 569 are also possible but not immediately evident from the crystal structure of the human transferrin C-lobe. Many subtle interactions are known to accelerate or retard iron release from transferrins, so the kinetic effects of the K569Q mutation still defy simple analysis and explanation.

Iron Release from K569R. To explore further the kinetic role of a positively charged residue at position 569 of the transferrin amino acid sequence the K569R mutant was expressed for iron release studies. Absolute rates of release as a function of chloride concentration at fixed pyrophosphate concentration were only about 20% slower in K569R loaded in the C-lobe than in native monoferric Tfn-Fe_C (Figure 2). Although the side chain of arginine is longer than that of lysine, and the p*K* of arginine is generally 2−3 pH units higher than that of lysine, at pH 5.6 native transferrin and K569R each bear a full positive charge at position 569. The loss of this charge in K569Q, therefore, probably contributes to the retarded iron release kinetics in that mutant by a mechanism yet to be discovered. The nearly intact responsiveness to chloride in K569R points to an anion-binding function for a positively charged residue in position 569, although other functions of a positive charge are not excluded.

Iron Uptake by K562 Cells from Wild-Type and Mutant Transferrins. Despite the striking differences in iron release to pyrophosphate from the C-lobes of native and K569Q transferrins, K562 cells take up iron at similar rates from the C-lobes of native, K569Q, and K569R transferrins. The kinetic effects of the K569Q mutation in a simple system are therefore masked by cellular mechanisms. Although little is known of the ionic composition within the endosome where iron is freed from transferrin within the cell, it is reasonable to assume that the ionic milieu is not very different from that of the cytoplasm or the extracellular fluid. The restricting action on iron release of low ionic strength or low chloride concentration is therefore not likely to be relevant to iron release within the endosome. One important difference between iron release to chelators and iron release to the cell is that the latter takes place from transferrin bound to the transferrin receptor. Binding of transferrin to its receptor has been shown to facilitate iron release from the

C-lobe at endosomal pH (Bali & Aisen, 1991; Egan et al., 1993), and it is possible that in the cell such binding overcomes the release-retarding effect of the K569Q mutation. The detailed mechanism of iron release within the cell is largely unknown, however, and other factors may also be operative. Actions of iron-accepting ligand or ligands are also important in regulating iron release. It is hardly surprising, therefore, that release rates to pyrophosphate do not correlate simply with rates of iron uptake by cells.

Release from K233Q. The mutation in the recombinant N-lobe homologous to K569Q, K233Q, did not affect iron release. The ϵ-amino group of Lys 233 lies 23 Å from the Fe(III), in a coil near the N-terminal end of one of the two domain-connecting β-strands of the N-lobe, similar to the disposition of Lys 569 in the C-lobe (Figure 7). A critical difference between the lobes, however, is the presence of a candidate for electrostatic bonding with the positively charged ϵ-amino nitrogen of K233. A negatively charged carboxylate oxygen of Glu 237 lies 2.9 Å from that nitrogen. Electrostatic interactions between OE2 of Glu 237 and NZ1 of Lys 233 in the wild-type N-lobe might then be insensitive to facilitating effects of anion concentration on release, so that the previously described inhibition of iron release by chloride competing with an anionic sequestering ligand is exhibited (Zak et al., 1995). Such competitive inhibition by chloride is observed in K233Q (Figure 6), which would lack the putative anion-binding site presented by Lys 233 in the wild-type N-lobe. Replacement of Lys 233 by a glutamine has no appreciable effect on observed rates of iron release to pyrophosphate, perhaps because the repulsive interactions of Lys 206 and Lys 296, lying on opposite domains of the N-lobe, dominate the release mechanism in that lobe. Such repulsive interactions may be less important in the C-lobe, where the homologue of Lys 206 is Lys 534 and that of Lys 296 is Arg 632. The positive charge of Arg 632 is divided among its three side-chain nitrogen atoms, with a distance of 4.2 Å separating the closest-lying nitrogen atoms of Lys 534 and Arg 632, with repulsive interactions correspondingly diminished.

The present results again highlight differences between the iron-binding properties of transferrin N- and C-lobes. Iron and synergistic anion ligands of the two lobes are identical, and their dispositions are nearly so. Nevertheless, the lobes differ in their response to anions when pyrophosphate serves as the iron-accepting ligand required for iron release from the specific site of each lobe. Chloride accelerates release from the C-lobe of full-length transferrin to chelators but retards release from the N-lobe, whether in recombinant half-transferrin or in the full-length structure (Zak et al., 1995). Our present studies are consistent with the accelerating effect of chloride on release from the C-lobe to a variety of iron-accepting species (Kretchmar & Raymond, 1988; Marques et al., 1991, 1995) and support the role of a KISAB site (or sites) that must be occupied for release to occur from that lobe (Kretchmar & Raymond, 1988; Egan et al., 1992). Lys 569 acts as such a site, and effects originating at loci remote from the iron-binding sites are therefore of critical importance in governing iron release. The anionic effect on iron release from the N-lobe is less clear, however. Although release rates from N-lobe to LICAMS show a nearly linear increase with ionic strength, similar to release from the C-lobe (Kretchmar & Raymond, 1988), release to pyrophosphate is competitively inhibited rather than accelerated by chloride (Zak et al., 1995), while mutagenesis of K233, the N-lobe homolog of K569, does not perturb release. The identities of kinetically active anion-binding loci in the N-lobe remain obscure. Spectroscopic, kinetic, and thermodynamic differences between the two lobes of transferrin have long been appreciated. Site-directed mutagenesis provides a powerful tool for examining these differences and for exploring release-modulating actions of residues remote from the specific iron-binding sites.

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