The chloride effect is related to anion binding in determining the rate of iron release from the human transferrin N-lobe

Qing-Yu HE*[1], Anne B. MASON*, Vinh NGUYEN†, Ross T. A. MacGILLIVRAY† and Robert C. WOODWORTH*

*Department of Biochemistry, College of Medicine, University of Vermont, Burlington, VT 05405, U.S.A., and †Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

The major function of human transferrin is to deliver iron from the bloodstream to actively dividing cells. Upon iron release, the protein changes its conformation from 'closed' to 'open'. Extensive studies *in vitro* indicate that iron release from transferrin is very complex and involves many factors, including pH, the chelator used, an anion effect, temperature, receptor binding and intra-lobe interactions. Our earlier work [He, Mason and Woodworth (1997) Biochem. J. **328**, 439–445] using the isolated transferrin N-lobe (recombinant N-lobe of human transferrin comprising residues 1–337; hTF/2N) has shown that anions and pH modulate iron release from hTF/2N in an interdependent manner: chloride retards iron release at neutral pH, but accelerates the reaction at acidic pH. The present study supports this idea and further details the nature of the dual effect of chloride: the anion effect on iron release is closely related to the strength of anion binding to the apoprotein. The negative effect seems to originate from competition between chloride and the chelator for an anion-binding site(s) near the metal centre. With decreasing pH, the strength of anion binding to hTF/2N increases linearly, decreasing the contribution of competition with the chelator. In the meantime, the 'open' or 'loose' conformation of hTF/2N, induced by the protonation of critical residues such as the Lys-206/Lys-296 pair at low pH, enables chloride to enter the cleft and bind to exposed side chains, thereby promoting cleft opening and synergistically allowing removal of iron by the chelator, leading to a positive anion effect. Disabling one or more of the primary anion-binding residues, namely Arg-124, Lys-206 and Lys-296, substantially decreases the anion-binding ability of the resulting mutant proteins. In these cases, the competition for the remaining binding residue(s) is increased, leading to a negative chloride effect or, at most, a very small positive effect, even at low pH.

Key words: anion effect, kinetics, metal protein, pH effect.

INTRODUCTION

Human serum transferrin (hTF) belongs to a group of iron-binding proteins that includes ovotransferrin and lactoferrin (for recent reviews, see [1–3]). Holo-transferrin is composed of two halves, designated the N-lobe and C-lobe, linked by a short peptide; each lobe contains an iron-binding site seated in a deep cleft. Iron is held octahedrally by co-ordination to four amino acid residues, i.e. Asp-63, Tyr-95, Tyr-188 and His-249, in the hTF/2N polypeptide (recombinant N-lobe of human transferrin comprising residues 1–337), and to two oxygen atoms from the synergistic anion, carbonate [4–6]. When iron is released, the two domains of each lobe, termed the NI- and NII-domains and the CI- and CII-domains, rotate around a hinge to change the protein conformation from 'closed' to 'open' [5,6]. While the iron ligands play a primary role in iron binding, other residues (including Gly-65, Glu-83, Tyr-85, Arg-124, Lys-206, Ser-248 and Lys-296 in the transferrin N-lobe) make up a second shell network that also contributes to the stabilization of the iron-binding site [5,7]. For example, Arg-124 is hydrogen-bonded to the synergistic anion. Also, Lys-206 and Lys-296, which come from opposite domains, are hydrogen-bonded to each other in the closed form; protonation of this dilysine pair appears to be a driving force to trigger cleft opening of hTF/2N at low pH [7,8].

Since the primary function of hTF *in vivo* is to transport iron from the bloodstream to cells by a process of pH-dependent iron binding and release, great effort has been focused on defining the exact mechanism of iron release from transferrin. Numerous experiments *in vitro* have shown that iron release is very complex and involves many factors that may or may not be independent of each other. Those factors that modulate iron release from hTF include receptor binding, lobe–lobe interactions, temperature, pH, chelator and anion concentration or ionic strength [4,9]. In particular, low pH appears able to induce the conformational change that is critical to iron release [4].

Discrepant results regarding iron release from full-length transferrin have been reported. Unfortunately, comparisons are difficult owing to the use of different conditions and measurement techniques by different research groups. The exact effect of anions on iron release is a major source of controversy. Earlier studies showed that chloride retards iron release from the transferrin N-lobe, but enhances release from the C-lobe in the presence of EDTA at pH 7.4 [10,11]. A different study showed that chloride exerted a similar accelerating effect on iron release from each transferrin-binding site at pH 7.4 when citrate was used as the chelator [12]. The same result was achieved with 1,2-dimethyl-3-hydroxy-4-pyridinone (L1) and acetohydroxamic acid [13]. When pyrophosphate (PP_i) was used as the chelator, a positive chloride effect on iron release from Fe_C-hTF (C-terminal monoferric human transferrin) at both pH 5.6 and pH 7.4 was noted [9,14–16], but no significant chloride effect on iron release from Fe_N-hTF (N-terminal monoferric human transferrin) was observed at pH 7.4 [17]. In the meantime, Marques et al. [18]

Abbreviations used: hTF, human serum transferrin; Fe_N-hTF and Fe_C-hTF, N- and C-terminal monoferric human transferrin respectively; hTF/2N, recombinant N-lobe of human transferrin comprising residues 1–337; NTA, nitrilotriacetate; Tiron, 4,5-dihydroxy-1,3-benzenedisulphonate.

[1] To whom correspondence should be addressed (e-mail: qhe@zoo.uvm.edu).

reported that, at pH 7.4, chloride had a very slight effect on iron release from Fe_N-hTF at 5 mM PP_i, but a significant positive effect when the concentration of PP_i was increased to 100 mM.

To simplify studies of iron binding and release, the recombinant N-lobe of transferrin (hTF/2N) has been generated; the hTF/2N protein has proved to be an excellent model for the N-lobe in holo-transferrin [19,20]. Work with the isolated recombinant hTF/2N has enabled us to focus specifically on the effects of pH and anions on iron release without the complications of receptor binding and lobe–lobe interactions. Extensive experimentation in our laboratory using both conventional and stopped-flow kinetic measurements under carefully controlled conditions has shown that pH and anions exert their effects on iron release from hTF/2N in an inter-dependent manner: chloride has a negative effect at pH 7.4, but a positive effect at pH 5.6 [7,21,22]. It is clear that the chloride effect may be physiologically significant, given the fact that the physiological concentration of chloride is approx. 0.14 M, which is high enough to influence the kinetics of iron release from transferrin.

The experimental results presented here support our previous findings and further define the nature of the pH–anion inter-dependent mechanism of iron release from hTF/2N. The structural basis for these behaviours is also considered by examining iron release from three hTF/2N mutants: R124A (Arg-124 mutated to Ala), H249E and K206A/K296A. These three mutant proteins demonstrate completely different iron-binding abilities; all feature loss of one or two residues involved in anion binding (see Discussion). The three mutants are excellent representatives of those hTF/2N proteins with weak anion binding due to a structural change caused by mutation. A comparative study at different pH values indicates that, under identical conditions, the chloride effect on iron release from hTF/2N depends both on the strength of anion binding to the protein and on the availability of anion-binding residues in the protein.

EXPERIMENTAL

Materials

Chemicals were reagent grade. Stock solutions of Hepes and Mes were prepared by dissolving the anhydrous salts in Milli-Q (Millipore) purified water, and adjusting the pH to the desired value with 1 M NaOH. 4,5-Dihydroxy-1,3-benzenedisulphonate (Tiron) was from Fisher Scientific Co., EDTA was from Mann Research Laboratories, and nitrilotriacetate (NTA) was from Sigma. Centricon 10 microconcentrators were from Amicon. Tiron and EDTA stock solutions were prepared by dissolving these chelators in the appropriate buffers and adjusting the pH to the desired value with 1 M NaOH; no chloride is present in these stock solutions.

Molecular biology

Mutation of Arg-124 and Lys-206/Lys-296 to alternative amino acids was carried out by using a PCR-based mutagenesis procedure [23]. PCR products of the mutagenesis reactions were ligated into Bluescript SK containing the hTF/2N cDNA. The nucleotide sequences of the inserts were determined to confirm the introduction of the specific mutations and the absence of any other mutations. The mutated hTF/2N cDNAs were excised from the Bluescript vector, blunt-ended and ligated into the *Sma*I site of the pNUT expression vector. Restriction endonuclease mapping was used to confirm the correct orientation [7,20,21,24].

Expression, purification and preparation of proteins

The N-lobe of hTF and the single and double mutants of hTF/2N were expressed by using the pNUT–BHK cell system. Isolation and purification of the recombinant proteins followed the general strategy used previously [7,19]. Fe(III)-loaded protein was prepared by adding a slight excess of $Fe(NTA)_2$ to a pH 7.4 buffer containing apo-protein or Fe-unsaturated protein and bicarbonate. The Fe(III)–hTF/2N samples were then exchanged into 10 mM Hepes (pH 7.4) using Centricon 10 microconcentrators, and were stored as concentrated stock solutions ($\sim$ 2.5 mM). To prepare the pure Fe–R124A–CO_3 and Fe–R124A–NTA complexes, exhaustive exchange with 50 mM Hepes (pH 7.4) containing 100 mM bicarbonate or 1 mM NTA respectively was performed before a final exchange of each protein into 10 mM Hepes. The preparation of most apo-protein samples followed the procedure described in detail previously [7,21]. The apo-form of the K206A/K296A mutant, which binds iron with high affinity, was prepared by adding at least a 20-fold excess of EDTA into the buffer solution (pH 4.9) containing the protein and allowing it to stand at 4 °C for 2 days.

Electronic spectra

UV–visible spectra were recorded on a Cary 219 spectrophotometer under the control of the computer program Olis-219s (Online Instrument Systems, Inc., Bogart, GA, U.S.A.). Hepes buffer (50 mM, pH 7.4) served as the reference for full-range spectra from 250 to 650 nm. Difference spectra were generated by storing the spectrum of the apo-protein as the baseline and subtracting it from the sample spectra.

Kinetics of metal removal

The detailed settings for the kinetics of metal release from transferrin and the processing of the data have been described previously [7,22,25]. Tiron was used as the chelator for iron removal at pH 7.4 (50 mM Hepes), and EDTA was used as the chelator at pH 6.1 and 5.6 (50 mM Mes). Slow iron-release kinetics were determined by a conventional method involving monitoring of the absorbance increase at 480 nm [for Fe(III)–Tiron] or the absorbance decrease at 293 or 470 nm (for iron removal by EDTA). The protein concentration used in this case was approx. 40 μM. Iron release from wild-type Fe–hTF/2N by chloride alone at pH 5.6 followed the conditions used to monitor the slow reaction. For fast iron-release reactions, the kinetics were determined by employing an Olis-RSM 1000 stopped-flow spectrophotometer, which is able to record the spectral change in real time. The same wavelengths as cited above were used as the central wavelength in each assay, and the protein concentration was approx. 6.5 μM [7]. The pH was measured after each assay to ensure that it remained constant. The protein samples used for the kinetic measurements were the natural iron-containing complexes with carbonate, unless stated otherwise. All kinetic assays were carried out at 25 °C.

Anion-binding titration

The anion titrations of apo-protein and the calculation of the binding constants were carried out as described previously [26]. Hepes (50 mM) was used for titrations at pH 7.4, 7.2 and 6.8, and Mes (50 mM) was used for titrations at pH 6.2. In each case the protein concentration was approx. 13 μM.

RESULTS

Preparation of iron-containing and apo-proteins

The final step of purification for the recombinant N-lobe of transferrin involves passage over a gel-filtration column in 0.1 M NH_4HCO_3. The eluted proteins are the normal iron-containing complexes with carbonate as the synergistic anion. Electronic absorption spectra were obtained for the eluted protein samples to allow comparison with that for wild-type hTF/2N. Table 1 lists the spectral characteristics for wild-type hTF/2N and for the mutants R124A and K206A/K296A. The spectral features of each of these mutants are similar to those of the wild-type protein, indicating that the iron-binding core is essentially unchanged in the mutant proteins.

Consistent with the report of Li et al. [20], the R124A mutant has two forms, the carbonate complex and the NTA complex. These two forms are interconvertible, giving rise to an intermediate species, a feature also found for other mutants such as D63S and H249A [21,25]. Iron binding to the R124A mutant is quite labile (see below), allowing easy preparation of the apo-protein. In contrast, iron binding to the K206A/K296A mutant is extremely tight, similar to other mutants in which either K206 or K296 is changed [7,20,27]. Preparation of apo-K206A/K296A required a higher chelator concentration and a longer incubation period.

Anion-binding titration

The apo-proteins were titrated with sulphate, as reported previously for wild-type apo-hTF/2N at pH 7.4 [7,26]. To observe the effect of the hydrogen ion concentration on anion binding, the titrations with sulphate for wild-type apo-hTF/2N were performed at three additional pH values. The resulting binding constants (K) and maximum absorptivities ($\Delta\epsilon_{max}$) are summarized in Table 2 and plotted in Figure 1. When the pH is decreased, the anion-binding constant increases linearly, while the maximum absorptivity decreases in a linear fashion. These data are similar to previously reported results [26].

Anion-binding experiments were also performed with apo-R124A and apo-K206A/K296A. Compared with that to wild-type hTF/2N, sulphate binding to apo-R124A at pH 7.4 has a

Table 1 Summary of spectral characteristics for iron-loaded wild-type (WT) hTF/2N and mutants

Spectra were taken usually in 50 mM Hepes buffer, pH 7.4. Proteins were in carbonate complex form, unless stated otherwise.

Protein	λ_{max} (nm)	λ_{min} (nm)	A_{max}/A_{min}	A_{280}/A_{max}
hTF/2N (WT)	472	410	1.37	23.5
R124A	460	403	1.28	25.4
R124A(NTA)	471	406	1.32	23.3
H249E*	457	400	1.30	25.3
K206A/K296A	470	409	1.33	24.1

* From He et al. [21].

Table 2 Sulphate-binding titration of apo-R124A and wild-type (WT) apo-hTF/2N at different pH values

Protein	pH	K (M^{-1})	$\Delta\epsilon_{max}$ ($M^{-1} \cdot cm^{-1}$)
hTF/2N (WT)	7.42	5840	5185
	7.15	14100	4230
	6.82	25400	3175
	6.16	39100	960
R124A	7.42	425	4870

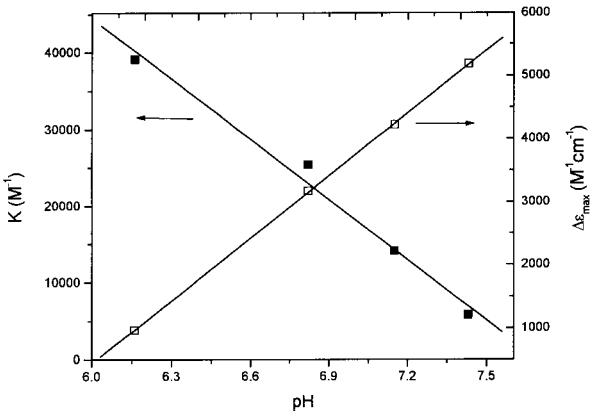

Figure 1 Sulphate-binding titration of wild-type apo-hTF/2N at different pH values

See the Experimental section for detailed conditions and data processing. ■, Binding constant (K); □, maximum absorptivity ($\Delta\epsilon_{max}$).

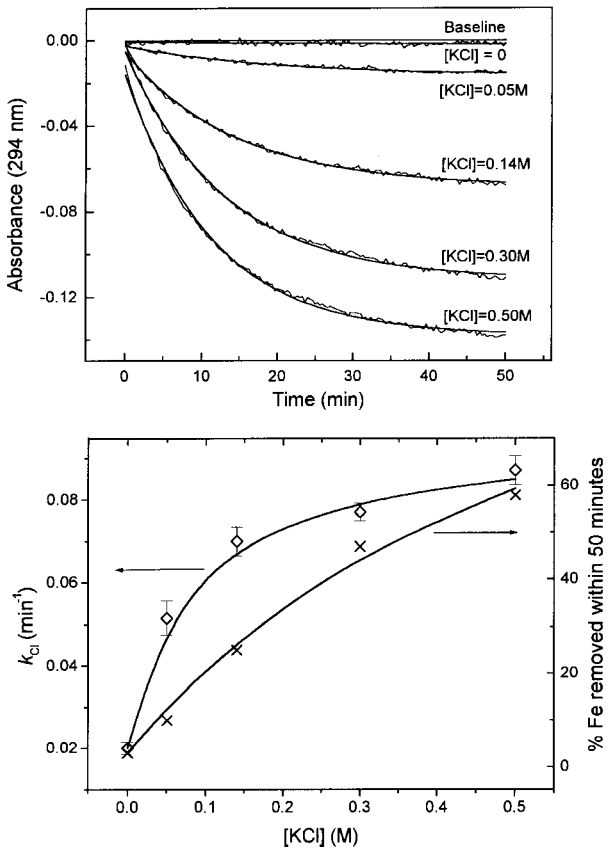

Figure 2 Iron release from wild-type hTF/2N by chloride alone at pH 5.6 (50 mM Mes) and 25 °C

Lower panel: ◇, Rate constant (k_{Cl}); ×, % Fe removed.

Anion-binding experiments were also performed with apo-R124A and apo-K206A/K296A. Compared with that to wild-type hTF/2N, sulphate binding to apo-R124A at pH 7.4 has a

Table 3 Rate constants for iron removal from the mutants of hTF/2N at various KCl concentrations and pH values, at 25 °C

WT, wild type.

	k_{obs} (min^{-1})					
Protein KCl (mM)...	0	25	50	140	300	500
Iron release by Tiron (12 mM) at pH 7.4						
hTF/2N (WT)	$(2.25 \pm 0.09) \times 10^{-2}$		$(2.05 \pm 0.04) \times 10^{-2}$	$(1.65 \pm 0.03) \times 10^{-2}$	$(1.31 \pm 0.04) \times 10^{-2}$	$(1.26 \pm 0.08) \times 10^{-2}$
H249E	$(7.36 \pm 0.14) \times 10^{-2}$		$(6.80 \pm 0.33) \times 10^{-2}$	$(5.21 \pm 0.12) \times 10^{-2}$	$(4.75 \pm 0.05) \times 10^{-2}$	$(4.49 \pm 0.11) \times 10^{-2}$
R124A	2.21 ± 0.10	1.84 ± 0.09	1.68 ± 0.05	1.25 ± 0.06	1.01 ± 0.04	0.936 ± 0.071
K206A/K296A	4.36×10^{-4}		1.36×10^{-4}			
Iron release by EDTA (4 mM) at pH 5.6						
hTF/2N (WT)	4.99 ± 0.08		22.6 ± 0.8	39.5 ± 0.2	47.2 ± 0.5	46.4 ± 0.5
H249E	11.5 ± 0.3	13.0 ± 0.2	11.9 ± 0.3	9.12 ± 0.15	6.87 ± 0.19	5.87 ± 0.26
R124A	$(2.55 \pm 0.15) \times 10^{2}$	$(2.61 \pm 0.10) \times 10^{2}$	$(2.70 \pm 0.11) \times 10^{2}$	$(2.89 \pm 0.10) \times 10^{2}$	$(3.14 \pm 0.12) \times 10^{2}$	$(3.42 \pm 0.13) \times 10^{2}$
K206A/K296A	$(2.48 \pm 0.15) \times 10^{-2}$		$(1.88 \pm 0.05) \times 10^{-2}$	$(1.12 \pm 0.05) \times 10^{-2}$	$(7.08 \pm 0.80) \times 10^{-3}$	$(7.42 \pm 0.70) \times 10^{-3}$

much smaller K value ($\sim$ 13-fold less) and a slightly smaller $\Delta\epsilon_{max}$ value (Table 2). The titration of apo-K206A/K296A with sulphate under identical conditions did not yield the characteristic absorbance bands for normal anion binding; only very weak and featureless spectral curves were observed. A similar spectrum was found on titration of the apo-H249E mutant with sulphate [25].

Iron-release kinetics

Iron release from wild-type Fe-hTF/2N by chloride alone at pH 5.6 is summarized and plotted in Figure 2. In 50 mM Mes solution, pH 5.6, Fe–hTF/2N was essentially stable, with a decrease of only about 3 % iron. Addition of chloride to the solution led to removal of iron from the protein. The rate constants follow a saturation profile with respect to the chloride concentration (Figure 2). The reaction was at least 90 % complete within 50 min, with removal of approx. 10, 25, 47 and 58 % of the iron for KCl concentrations of 50, 140, 300 and 500 mM respectively.

For iron release by Tiron and EDTA, Table 3 lists the rate constants for wild-type hTF/2N and the three mutants (R124A, H249E and K206A/K296A) at various chloride concentrations and at both pH 5.6 and pH 7.4. Figure 3 shows iron release from wild-type hTF/2N at three different pH values. It is clear that chloride retards iron release at neutral pH (Figure 3A), but accelerates it at acidic pH (Figure 3C), with saturation modes. At pH 6.1, the chloride effect has two phases (Figure 3B); with increasing chloride concentration, the rate constant increases, reaching a maximum at a KCl concentration of approx. 0.1 M, which is followed by a decrease.

The iron-release rates for the different mutants are extremely variable. Compared with that for wild-type hTF/2N, in the absence of chloride, the iron-release rates are slightly higher ($\sim$ 3 times) for the H249E mutant [25], much higher (50–100 times) for the R124A mutant and significantly lower (50–200 times) for the K206A/K296A mutant (Table 3). Figure 4 shows a comparison of the chloride effect on the rates of iron release from wild-type hTF/2N and the H249E and R124A mutants at pH 7.4. In the case of both the H249E and R124A mutants, chloride inhibits iron release in a saturation mode, similar to the wild-type protein but with a greater sensitivity. Chloride appears to have a negative effect on iron release from the K206A/K296A mutant (Table 3). A detailed chloride-dependence study was not performed for this mutant at pH 7.4, due to the low rate of iron release which is exacerbated by additional chloride.

An interesting effect of chloride on iron release was found when a comparison was made between wild-type hTF/2N and the three mutants at pH 5.6 (Figure 5). When the chloride concentration was increased from 0 to 0.5 M, the iron-release rate for the wild-type protein increased by 10-fold (Figure 5A); iron release from the R124A mutant also showed a positive but very small anion effect (less than 0.5-fold increase) (Figure 5C). In contrast, chloride exerted an apparent retarding effect on iron release from the K206A/K296A mutant (Figure 5D). Interestingly, both the positive and negative chloride effects were observed on the iron-release rates from the H249E mutant (Figure 5B). The rate constant showed a slight increase at a low chloride concentration, and then decreased significantly when more chloride was added.

DISCUSSION

Anion and iron release

It has long been known that anions bind to transferrin and that there are at least two functional types of anion binding. The first is the synergistic anion binding that must be completed for high-affinity iron binding to occur, and the second is the so-called kinetically significant anion binding (KISAB) [14]. As pioneered by Harris [28], anion binding can be observed by titrating an anion into apo-transferrin and analysing the resulting negative electronic absorbance bands in difference UV spectra. A recent study from our laboratory revealed that the spectral change caused by anion binding originates from the direct or indirect interaction with the liganding tyrosine residues Tyr-95 and Tyr-188, and this explains why no spectral absorbance is observed when anion is added to Fe–transferrin in which these tyrosine ligands are already bound to iron [7]. Our experiments also indicated that the Lys-206/Lys-296 pair in hTF/2N [7] is a major KISAB site. Disturbing the binding sites directly by mutating the lysine residues or indirectly through the second-shell network causes changes in anion binding. The dramatically decreased anion-binding ability of the R124A, H249E and K206A/K296A mutants in the present study supports this concept (see below).

It has been reported that anions are required for iron release from transferrin [16,29]. Iron release from transferrin by 'non-

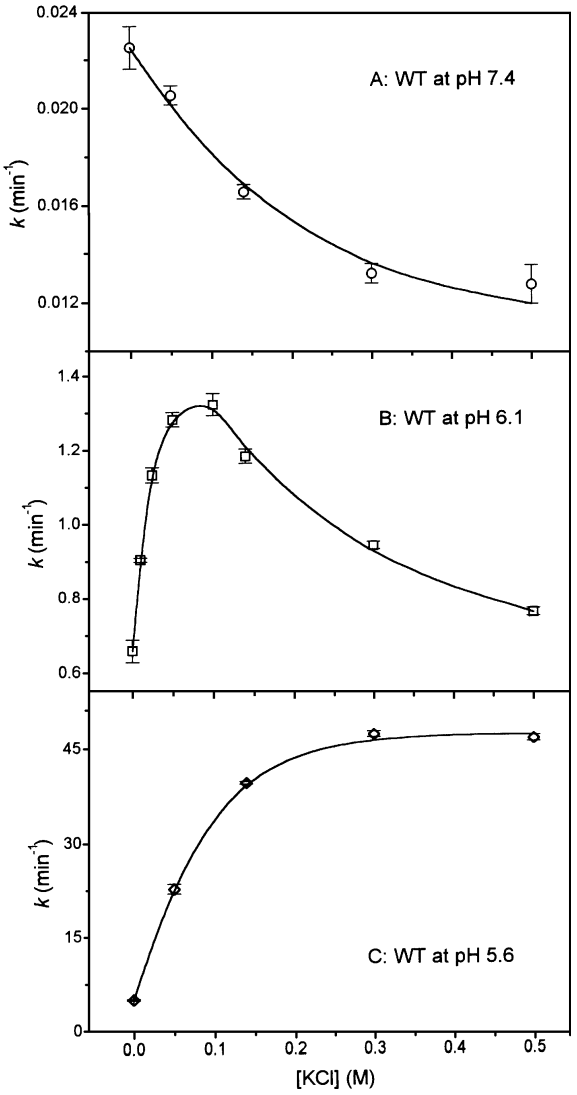

Figure 3 Chloride effect on iron release from wild-type (WT) hTF/2N at three different pH values

Tiron (12 mM) was used for the release assay at pH 7.4 (50 mM Hepes), and EDTA (4 mM) was used at pH 6.1 and pH 5.6 (50 mM Mes).

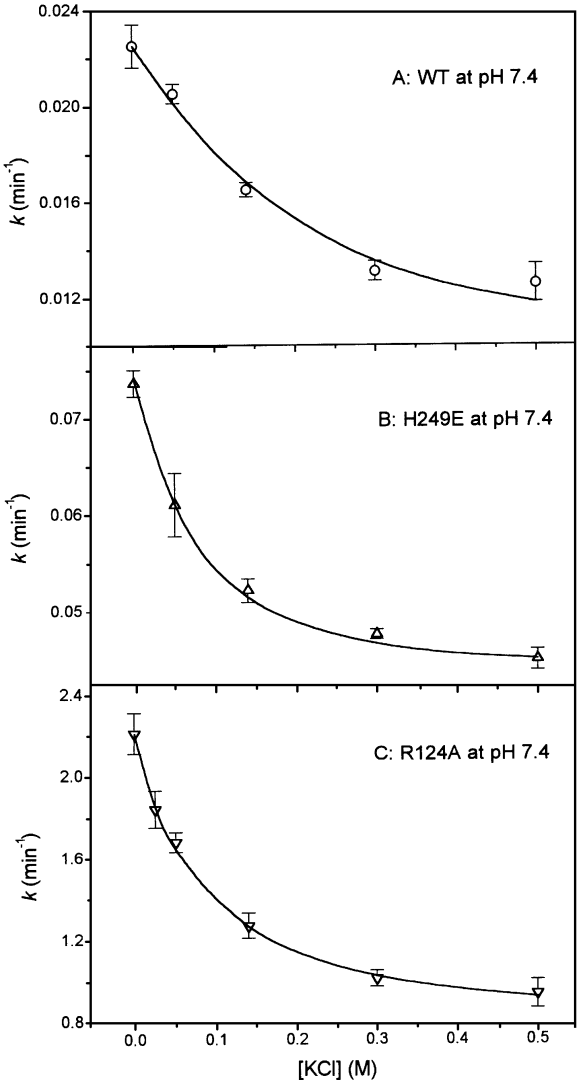

Figure 4 Comparison of the chloride effect on iron release from wild-type (WT) hTF/2N and the mutants H249E and R124A by Tiron (12 mM) at pH 7.4 (50 mM Hepes)

chelating' anions is demonstrated by the kinetic results presented in this paper and in other work using an equilibrium approach [25,30]. At pH 5.6, slow but significant removal of iron by chloride is observed (Figure 2). Foley and Bates [30] showed that a series of anions could promote iron release from transferrin, with an order of effectiveness of $SO_4^{2-} > NO_3^- > Cl^- > ClO_4^-$. We believe that an anion helps promote and maintain the conformational change from closed to open by binding to the side chains along the cleft, in addition to acting directly as a weak chelator to remove iron. An anion can also decrease the rate of iron release from transferrin under certain conditions. For example, chloride inhibits iron release from hTF/2N by both PP_i and Tiron at pH 7.4 [21,31]. Zak et al. [31] proposed, and we agree, that this negative chloride effect may be due to competition between chloride and these chelators for the anion-binding site(s) near the metal centre.

The chloride effect depends on the strength of anion binding to hTF/2N

Our earlier work [22] showed that the chloride effect on iron release from wild-type hTF/2N is pH dependent: chloride retards the reaction at higher pH, but enhances iron release at lower pH, with an isotropic point at pH 6.26. The data in the present paper support the idea of this dual role of chloride, and provide further insight into the nature of the pH-dependent mechanism: the chloride effect is directly dependent on the strength of anion binding to the protein. Titration of apo-proteins with sulphate shows that the anion-binding strength of hTF/2N increases with decreasing pH (Figure 1). The increased capability of the protein for anion binding appears to dominate the competition between chloride and the chelator. The parallel relationship between sulphate and chloride in anion binding to hTF/2N has been described [7]. The positive effect of chloride may become dominant when the protein conformation becomes 'open' or 'loose', induced by the protonation of the dilysine pair and other critical residues at low pH, so that chloride can easily enter the cleft,

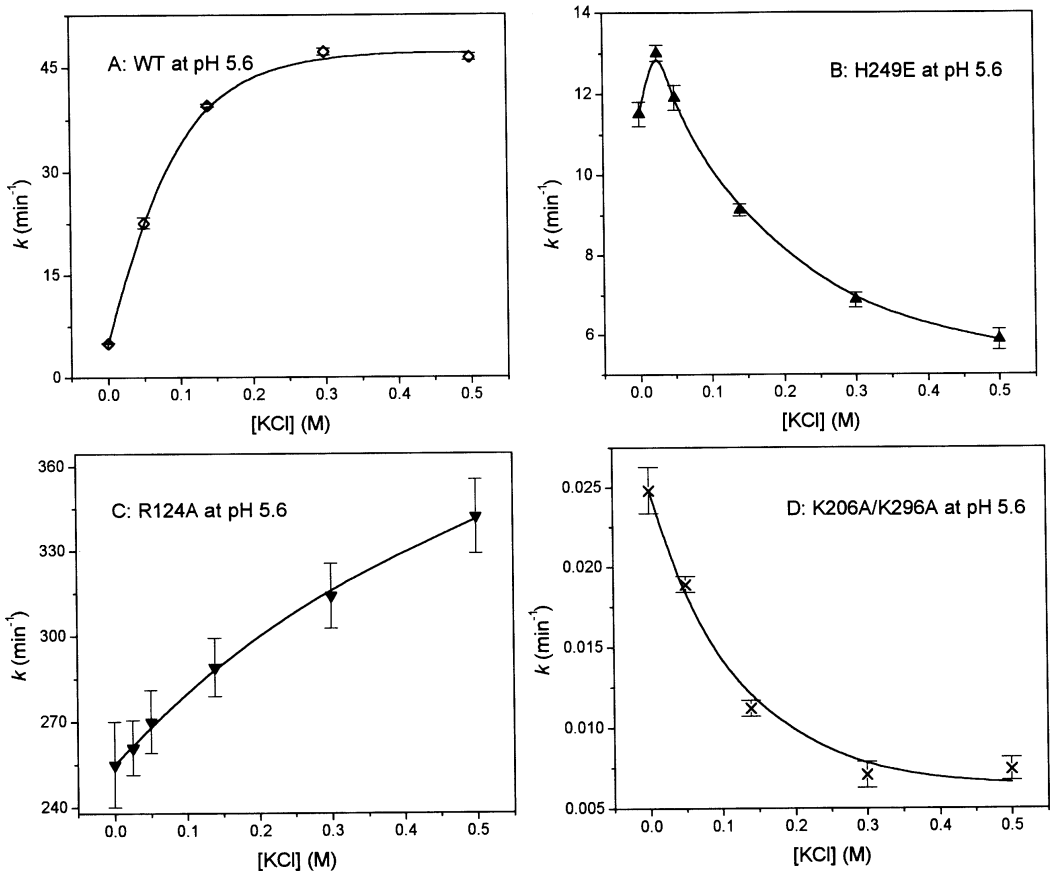

Figure 5 Comparison of the chloride effect on iron release from wild-type (WT) hTF/2N and the mutants H249E, R124A and K206A/K296A by EDTA (4 mM) at pH 5.6 (50 mM Mes)

bind to the exposed residues to keep the cleft open and also help to remove iron. The moderate decrease in the iron-release rate at pH 7.4 (Figure 3A) reflects the negative effect of chloride. The significant increase in the release rate at pH 5.6 (Figure 3C) appears to correspond to the positive effect of chloride when the protein has a strong anion-binding capability and an 'open' conformation. The two-phase curve shown in Figure 3(B) demonstrates the dynamic balance of the dual effect of chloride when iron is released from hTF/2N at pH 6.1, near to the isotropic point. At a low concentration ([KCl] < 0.1 M), chloride accelerates iron release because the protein has ample capability to bind anion, but at [KCl] > 0.1 M, the increasing concentration of chloride dominates over the chelator as it competes for binding to the protein and thus retards iron release.

The chloride effect depends on the availability of anion-binding site(s)

This idea is supported by comparison of the iron-release behaviour of wild-type hTF/2N and the three mutants that are missing one or two anion-binding residues, i.e. R124A, H249E and K206A/K296A. The importance of the dilysine pair for anion binding has been demonstrated [7], and is further confirmed by the absence of specific sulphate binding by the K206A/K296A mutant. The 13-fold smaller anion-binding constant for the R124A mutant (Table 2) suggests that Arg-124 is also a significant contributor to non-synergistic as well as synergistic anion binding. Also, the H249E mutant does not show specific anion binding. A structural basis for this has been identified: the glutamic acid substituted at position 249 forms an ion pair with Lys-296, precluding anion binding [32].

A significant negative chloride effect on iron release from all three mutants is observed at pH 7.4 (Table 3), indicating that, when any one of the critical anion-binding residues is lacking, the competition between chloride and the chelator for the available binding sites becomes stronger. In particular, the weak iron binder, R124A, probably has a 'loose' conformation due to the lack of the scaffolding support from Arg-124 to the synergistic anion, carbonate. Chloride usually enhances iron release from weak iron-binding mutants that are believed to have a loose or open conformation [22,25,33,34]. The negative rather than positive chloride effect on iron release from the R124A mutant at pH 7.4 obviously must be attributed directly to the loss of this major anion-binding residue in the mutant.

The effect of losing an anion-binding residue applies also at pH 5.6 (Figure 5). Corresponding to an increase in the strength of anion binding to hTF/2N as the pH decreases, chloride substantially accelerates iron release from the wild-type protein (Figure 5A). In the case of the R124A mutant, even the weak anion binding at pH 7.4 would be strengthened at pH 5.6, thereby converting the negative chloride effect at pH 7.4 into a small positive effect (Figure 5C). Since the dilysine pair appear to be the main anion-binding residues or site [7], eliminating both lysines means that the chloride effect should remain negative at pH 5.6, as seen in Figure 5(D). The situation for the H249E mutant appears to be a combination of these two cases (Figure

5B). Although the major part of the dilysine pair has been lost due to the pairing of Lys-296 to Glu-249, anion can still bind to Arg-124 and Lys-206 in the H249E mutant. This remaining anion-binding ability would be strengthened at pH 5.6, leading to a small positive chloride effect at low concentrations of chloride. However, the loss of the primary anion-binding residue Lys-296 may result in strong competition between chloride and the chelator for the remaining anion-binding site(s), leading to a significant negative chloride effect on iron release from the H249E mutant.

Conclusion

For the isolated transferrin N-lobe, the effect of anions on iron release from the protein is closely related to the anion-binding ability of the protein. The fact that chloride retards iron release from wild-type hTF/2N at neutral pH, but enhances the reaction at acidic pH, corresponds to the observation that the anion-binding strength of the protein increases linearly with decreasing pH. When one or two important anion-binding residues are disabled, the anion-binding ability of the resulting mutants is substantially reduced. In this case, chloride has significant negative effects or slight positive effects even at the low pH of 5.6. Clearly, the chloride effect depends on the strength of anion binding to the protein and the availability of anion-binding site(s) in the protein.

This work was supported by USPHS grant R01 DK 21739 (to R. C. W.) from the National Institute of Diabetes and Kidney Diseases.

REFERENCES

1 Evans, R. W., Crawley, J. B., Joannou, C. L. and Sharma, N. D. (1999) Iron proteins. In Iron and Infection: Molecular, Physiological and Clinical Aspects (Bullen, J. J. and Griffiths, E., eds), pp. 27–86, John Wiley and Sons, Chichester

2 Aisen, P. (1998) Transferrin, the transferrin receptor, and the uptake of iron by cells. In Iron Transport and Storage in Microorganisms, Plants and Animals (Sigel, A. and Sigel, H., eds), vol. 35, pp. 585–631, Marcel Dekker, New York

3 Wessling-Resnick, M. (1999) Biochemistry of iron uptake. Crit. Rev. Biochem. Mol. Biol. **34**, 285–314

4 Baker, E. N. (1994) Structure and reactivity of transferrins. Adv. Inorg. Chem. **41**, 389–463

5 MacGillivray, R. T. A., Moore, S. A., Chen, J., Anderson, B. F., Baker, H., Luo, Y. G., Bewley, M., Smith, C. A., Murphy, M. E., Wang, Y. et al. (1998) Two high-resolution crystal structures of the recombinant N-lobe of human transferrin reveal a structural change implicated in iron release. Biochemistry **37**, 7919–7928

6 Jeffrey, P. D., Bewley, M. C., MacGillivray, R. T. A., Mason, A. B., Woodworth, R. C. and Baker, E. N. (1998) Ligand-induced conformational change in transferrins: crystal structure of the open form of the N-terminal half-molecule of human transferrin. Biochemistry **37**, 13978–13986

7 He, Q.-Y., Mason, A. B., Tam, B. M., MacGillivray, R. T. A. and Woodworth, R. C. (1999) Dual role of Lys206-Lys296 interaction in human transferrin N-lobe: Iron-release trigger and anion-binding site. Biochemistry **38**, 9704–9711

8 Dewan, J. C., Mikami, B., Hirose, M. and Sacchettini, J. C. (1993) Structural evidence for a pH-sensitive dilysine trigger in the hen ovotransferrin N-lobe: Implications for transferrin iron release. Biochemistry **32**, 11963–11968

9 Zak, O., Tam, B., MacGillivray, R. T. A. and Aisen, P. (1997) A kinetically active site in the C-lobe of human transferrin. Biochemistry **36**, 11036–11043

10 Baldwin, D. A. and DeSousa, D. M. R. (1981) The effect of salts on the kinetics of iron release from N-terminal and C-terminal monoferric transferrins. Biochem. Biophys. Res. Commun. **99**, 1101–1107

11 Williams, J., Chasteen, N. D. and Moreton, K. (1982) The effect of salt concentration on the iron-binding properties of human transferrin. Biochem. J. **201**, 527–532

12 Marques, H. M., Watson, D. L. and Egan, T. J. (1991) Kinetics of iron removal from human serum monoferric transferrins by citrate. Inorg. Chem. **30**, 3758–3762

13 Li, Y. J. and Harris, W. R. (1998) Iron removal from monoferric human serum transferrins by 1,2-dimethyl-3-hydroxypyridin-4-one, 1-hydroxypyridin-2-one and acetohydroxamic acid. Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. **1387**, 89–102

14 Egan, T. J., Ross, D. C., Purves, L. R. and Adams, P. A. (1992) Mechanism of iron release from human serum C-terminal monoferric transferrin to pyrophosphate: kinetic discrimination between alternative mechanisms. Inorg. Chem. **31**, 1994–1998

15 Marques, H. M., Walton, T. and Egan, T. J. (1995) Release of iron from C-terminal monoferric transferrin to phosphate and pyrophosphate at pH 5.5 proceeds through two pathways. J. Inorg. Biochem. **57**, 11–21

16 Egan, T. J., Zak, O. and Aisen, P. (1993) The anion requirement for iron release from transferrin is preserved in the receptor-transferrin complex. Biochemistry **32**, 8162–8167

17 Harris, W. R. and Bali, P. K. (1988) Effects of anions on the removal of iron from transferrin by phosphonic acids and pyrophosphate. Inorg. Chem. **27**, 2687–2691

18 Marques, H. M., Egan, T. J. and Pattrick, G. (1990) The non-reductive removal of iron from human serum N-terminal monoferric transferrin by pyrophosphate. S. Afr. J. Sci. **86**, 21–24

19 Woodworth, R. C., Mason, A. B., Funk, W. D. and MacGillivray, R. T. A. (1991) Expression and initial characterization of five site-directed mutants of the N-terminal half-molecule of human transferrin. Biochemistry **30**, 10824–10829

20 Li, Y. J., Harris, W. R., Maxwell, A., MacGillivray, R. T. A. and Brown, T. (1998) Kinetic studies on the removal of iron and aluminum from recombinant and site-directed mutant N-lobe half transferrins. Biochemistry **37**, 14157–14166

21 He, Q.-Y., Mason, A. B., Woodworth, R. C., Tam, B. M., Wadsworth, T. and MacGillivray, R. T. A. (1997) Effects of mutations of aspartic acid 63 on the metal-binding properties of the recombinant N-lobe of human serum transferrin. Biochemistry **36**, 5522–5528

22 He, Q.-Y., Mason, A. B. and Woodworth, R. C. (1997) Iron release from recombinant N-lobe and single point Asp 63 mutants of human transferrin by EDTA. Biochem. J. **328**, 439–445

23 Nelson, R. M. and Long, G. L. (1989) A general method of site-specific mutagenesis using a modification of the Thermus aquaticus polymerase chain reaction. Anal. Biochem. **180**, 147–151

24 Funk, W. D., MacGillivray, R. T. A., Mason, A. B., Brown, S. A. and Woodworth, R. C. (1990) Expression of the amino-terminal half-molecule of human serum transferrin in cultured cells and characterization of the recombinant protein. Biochemistry **29**, 1654–1660

25 He, Q.-Y., Mason, A. B., Pakdaman, R., Chasteen, N. D., Dixon, B. L., Tam, B. M., Nguyen, V., MacGillivray, R. T. A. and Woodworth, R. C. (2000) Mutations at the histidine 249 ligand profoundly alter the spectral and iron-binding properties of human serum transferrin N-lobe. Biochemistry **39**, 1205–1210

26 Cheng, Y. G., Mason, A. B. and Woodworth, R. C. (1995) pH dependence of specific divalent anion binding to the N-lobe of recombinant human transferrin. Biochemistry **34**, 14879–14884

27 Steinlein, L. M., Ligman, C. M., Kessler, S. and Ikeda, R. A. (1998) Iron release is reduced by mutations of lysine 206 and 296 in recombinant N-terminal half-transferrin. Biochemistry **37**, 13696–13703

28 Harris, W. R. (1985) Thermodynamics of anion binding to human serum transferrin. Biochemistry **24**, 7412–7418

29 Nguyen, S. A. K., Craig, A. and Raymond, K. N. (1993) Transferrin: The role of conformational changes in iron removal by chelators. J. Am. Chem. Soc. **115**, 6758–6764

30 Foley, A. A. and Bates, G. W. (1988) The influence of inorganic anions on the formation and the stability of Fe(3+)-transferrin-anion complexes. Biochim. Biophys. Acta **965**, 154–162

31 Zak, O., Aisen, P., Crawley, J. B., Joannou, C. L., Patel, K. J., Rafiq, M. and Evans, R. W. (1995) Iron release from recombinant N-lobe and mutants of human transferrin. Biochemistry **34**, 14428–14434

32 MacGillivray, R. T. A., Bewley, M. C., Smith, C. A., He, Q.-Y., Mason, A. B., Woodworth, R. C. and Baker, E. N. (2000) Mutation of the iron ligand His 249 to Glu in the N-lobe of human transferrin abolishes the dilysine 'trigger' but does not significantly affect iron release. Biochemistry **39**, 1211–1216

33 He, Q.-Y., Mason, A. B., Woodworth, R. C., Tam, B. M., MacGillivray, R. T. A., Grady, J. K. and Chasteen, N. D. (1998) Mutations at nonliganding residues Tyr-85 and Glu-83 in the N-lobe of human serum transferrin - Functional second shell effects. J. Biol. Chem. **273**, 17018–17024

34 He, Q.-Y., Mason, A. B., Woodworth, R. C., Tam, B. M., MacGillivray, R. T. A., Grady, J. K. and Chasteen, N. D. (1997) Inequivalence of the two tyrosine ligands in the N-lobe of human serum transferrin. Biochemistry **36**, 14853–14860

Received 16 March 2000/26 June 2000; accepted 13 July 2000