Iron release from recombinant N-lobe and single point Asp⁶³ mutants of human transferrin by EDTA

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Transferrins bind ferric ion and deliver the iron to cells. The mechanism of the iron release has been studied kinetically, *in vitro*, with the aid of single point mutants in which the iron-binding ligand, Asp⁶³ (aspartic acid-63, D63), has been changed to Ser, Asn, Glu and Ala. Iron release from the unmutated N-lobe of human serum transferrin (hTF/2N) by EDTA is influenced by a variety of factors. The rate-determining conformational-change mechanism may be a major pathway for iron release from hTF/2N's having a 'closed' conformation, which leads to a saturation kinetic mode with respect to ligand concentration. The effect of chloride depends on the protein conformation, showing a negative action in the case of tight binding and a positive action when the protein has an 'open' or 'loose' conformation. The negative effect of chloride could originate from

the binding competition between chloride and the chelate to the active site for iron release, and the positive effect could derive from the synergistic participation of chloride in iron removal. The 'open' conformation may be induced by decreasing pH: the transitional point appears to be at about pH 6.3 for the wild-type hTF/2N; the 'loose' conformation may be facilitated by mutations at D63, which result in the loss of a key linking component in interdomain interactions of the protein. In the latter case, structural factors dominate over other potential negative effects because the weak interdomain contacts derived from the mutation of D63 cause the binding site to open easily, even at pH 7.4. Therefore chloride exhibits an accelerating action on iron release by EDTA from all the D63 mutants.

INTRODUCTION

Transferrins are single-chain iron-binding and transporting proteins found in the blood plasma and lymph of many species. Two iron-binding sites are distributed in N- and C-lobes, each comprised of two domains (I and II) [1]. Iron is held in each binding site by ligation to two tyrosine residues, one histidine residue, one aspartic acid residue and a bidentate carbonate anion. As documented by the crystal structures of apo- and iron-containing human lactoferrin (hLF), a substantial conformational change accompanies iron binding; the N II domain rotates 54° relative to the N I domain in a rigid body motion [2]. In cells, iron is released readily from human serum transferrin (hTF) via receptor-mediated endocytosis in an acidified endosome (pH ≈ 5.5) [3,4]. Ionic strength, pH and binding to the transferrin receptor are all functional variables in this process. Extensive studies on iron release from full-length transferrin *in vitro* by a variety of chelating ligands have been conducted to assist in our understanding of the nature of iron delivery to cells by transferrins [5–8]. Recently, Zak et al. reported kinetic studies for iron release from the recombinant N-lobe of human transferrin (hTF/2N) and several single point mutants [9]. They showed that mutations of 'kinetically active residues' may accelerate or retard release of iron from the protein to pyrophosphate. Recombinant DNA technology provides an excellent means for investigating the role of individual residues in the release of iron.

The aspartic acid residue at position 63 (Asp⁶³, D63) is one of the metal-binding ligands in the binding site of the N-lobe of

human transferrin (hTF/2N). In hLF, besides binding iron, this aspartic acid residue [Asp⁶⁰ (D60) in this case] also provides important stabilizing interactions in the 'closed' structure through a carboxylate oxygen atom which forms hydrogen bonds linking two helices in domains I and II [2]. Thus the aspartic acid ligand appears to play a particularly important role in both iron binding and domain closure [10,11]. Recent studies from our laboratory showed that mutations of the D63 residue of hTF/2N substantially alter the metal ion- and anion-binding properties of the protein [12]. The substitution of serine, asparagine, glutamic acid or alanine for the aspartic acid at position 63 results in the loss of this metal-binding ligand and weakens the interdomain contacts. This appears to be similar to the situation in the closely related mutant, D60S (Asp⁶⁰ → Ser), from the N-lobe of hLF. In this hLF mutant, the substituted serine does not co-ordinate to iron directly; instead, a water molecule fills the iron co-ordination site and takes part in interdomain hydrogen bonding [13]. The structural alterations caused by replacement of D63 in hTF/2N result in a preference for bigger anions, namely nitrilotriacetate (NTA) and 4,5-dihydroxy-1,3-benzenedisulphonic acid (Tiron), which act as 'synergistic' anions, so that kinetic studies of iron release from the mutants by Tiron were compromised. However, iron release from the D63 mutants could be achieved and monitored by using EDTA as the chelating agent. A comprehensive kinetic study for the wild-type recombinant N-lobe and each of the D63 mutants with EDTA is presented here and shows both a significant effect of chloride and the individual mutation on the iron-release

Abbreviations used: hTF, human serum transferrin; hLF, human lactoferrin; hTF/2N, recombinant N-lobe of human transferrin comprising residues 1–337; mutants of hTF/2N are designated by the wild-type amino acid residue, the position number and the amino acid to which the residue was mutated; D60, D63, aspartic acid-60 and -63 of hLF and hTF respectively; BHK, baby-hamster kidney cells; NTA, nitrilotriacetate; Tiron, 4,5-dihydroxy-1,3-benzenedisulphonic acid, disodium salt; D60S, D63S, Asp⁶⁰ → Ser mutant of hLF and Asp⁶³ → Ser mutant of hTF; KISAB, kinetically significant anion-binding.

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process. A detailed pH profile presented for the wild-type hTF/2N shows that the chloride effect can be either positive or negative, depending on the pH.

EXPERIMENTAL

Materials

Chemicals were reagent grade. Stock solutions of Hepes, Mes and other buffers were prepared by dissolving the anhydrous salts in Milli-Q (Millipore)-purified water and adjusting the pH to desired values with 1 M NaOH or HCl. EDTA stock solutions were prepared by dissolving the EDTA (acid form; Mann Research Laboratories, New York, NY, U.S.A.) in the appropriate buffers and adjusting the pH to the desired values with 1 M NaOH. For the pH profile of iron release, a series of buffer solutions of constant ionic strength were prepared by diluting the concentrated stock solutions to the desired value (the conductivity was 0.88 ± 0.01 mS/cm). The final concentrations of the buffers were approx. 37 (pH 5.93), 28 (pH 6.14), 23 (pH 6.25), 20 (pH 6.40), 18 (pH 6.55) and 17 mM (pH 6.69) respectively. The ionic strength was measured directly with a CDM80 conductivity meter (Radiometer, Copenhagen, Denmark). The pH values are the averages of those measured immediately before and after iron-release reactions (variations $\leq \pm 0.02$).

Methods

Expression, isolation and purification of proteins

The construction of the D63 mutants and wild-type hTF/2N has been described previously [12]. The N-lobe of hTF and the D63-series single-point mutants were expressed into the medium of baby-hamster kidney (BHK) cells containing the relevant cDNA in the pNUT vector and were purified as reported previously [14,15]. The preparation of apo- and Fe-loaded protein samples was accomplished by following the procedure described previously [12]. Sodium bicarbonate solution (50 mM) was used in preparing the Fe-loaded mutant samples.

Kinetics of iron release

Iron-release reactions were performed in a quartz cuvette (3 ml, maintained at 25 ± 0.1 °C) and monitored by UV-visible spectroscopy with a Cary-219 spectrophotometer (Varian) equipped with a thermostatically controlled cell. The computer program Olis-219s-Assay (OLIS, Bogart, GA, U.S.A.) was used to run the time-based spectral measurements. Iron-loaded transferrin was filtered (0.2 μ m Acrodisc) and added into the sample cuvette containing the desired buffer. The final concentration of the protein was 40–45 μ M. All solutions were measured against a reference containing the appropriate amount of buffer, KCl and chelator. The pair of matched cuvettes containing the sample and the reference was equilibrated at 25 °C for 10–15 min before starting the measurement.

The time of mixing in the EDTA was recorded as zero time. For the iron release from wild-type hTF/2N, the absorbance decrease at 294 or 470 nm was monitored and recorded every 2–30 s, depending on the reaction rate, until at least three half-lives were completed. Iron release with chloride alone was monitored for 2 h ($\approx 4\%$ and 13% complete at pH 6.14 and 5.93 respectively). For iron release from the mutant proteins, the decrease in absorbance at 294 nm or around the absorbance maximum ($\lambda_{\max}$) in the visible region (426, 450, 422 and 414 for the D63S, D63E, D63N and D63A mutants respectively) was monitored and recorded every 2 s until the reaction was near completion (5–20 min). Rate constants were obtained by fitting

the absorbance-versus-time data to either a single- or a double-exponential function with the user-defined non-linear equations in the Axum program (MathSoft 1996 version). For most of the calculations the initial and final absorbance values (A_0 and A_∞) were treated as adjustable parameters as long as their values stayed within experimentally reasonable boundaries [7,12,16]. Measurements were made at least in duplicate.

RESULTS

Iron release from wild-type hTF/2N

Iron release from native full-length transferrin by EDTA at pH 7.4 is very slow, taking more than 20 h to reach completion even at 37 °C [5]. This reaction for wild-type hTF/2N at pH 7.4 and 25 °C is even slower, too slow indeed for reliable measurements because of the potential protein decomposition and instrumental drift during such a long determination. Alternatively, it was found that the use of lower pH values (pH 5.9–6.7) gave satisfactory kinetic curves for iron release to EDTA at 25 °C over an appropriate range of ligand and chloride concentrations.

Difference spectral measurements showed that, when EDTA was added to the solution of the protein, two negative peaks at around 295 nm and at the $\lambda_{\max}$ in the visible region (about 470 nm) were observed (Figure 1A). These spectral changes clearly showed that EDTA was removing iron from the proteins, resulting in the disappearance of the Fe–O(Tyr) interaction.

Figure 1 (A) Difference spectra for iron removal from wild-type hTF/2N by EDTA (0.8 mM; 50 mM Mes, pH 6.5, 25 °C; recorded with 2.5 min intervals) and (B) the dependence of absorbance versus time for A_{470} (□) and A_{295} (○) and fitted to a pseudo-first-order kinetic mode.

Table 1 Variation of k_{obs} for iron release from wild-type hTF/2N with EDTA ([KCl] = 0) and KCl ([EDTA] = 0.8 mM) concentrations

Mes was present at 50 mM. The pH was 6.5 and the temperature was 25.0 ± 0.1 °C. Values are mean $\pm$ S.D.

EDTA-dependence ([KCl] = 0)		KCl-dependence ([EDTA] = 0.8 mM)	
[EDTA] (mM)	k_{obs} (min ⁻¹)	[KCl] (M)	k_{obs} (min ⁻¹)
0.4	0.057 ± 0.003	0.05	0.080 ± 0.004
0.8	0.087 ± 0.002	0.10	0.066 ± 0.004
1.5	0.119 ± 0.003	0.14	0.061 ± 0.002
3.0	0.137 ± 0.002	0.20	0.050 ± 0.001
5.0	0.151 ± 0.001	0.30	0.041 ± 0.001
8.0	0.161 ± 0.002	0.40	0.036 ± 0.003
		0.50	0.029 ± 0.002
		0.70	0.024 ± 0.001
		1.0	0.018 ± 0.003

Another relevant peak at 240 nm could not be distinguished from the strong absorption background. Also, no absorbance band at 520 nm, deriving from the ternary complex of transferrin-Fe-EDTA [17], was found during the course of the reaction. This observation is consistent with that of Baldwin [5].

Spectra recorded at 2.5 min intervals after the addition of EDTA were made with a solution of the wild-type Fe-hTF/2N at pH 6.5 (Figure 1A). Figure 1(B) shows the time-dependence of both the A_{295} and A_{470} . A pseudo-first-order kinetic equation:

$$A = a + b \cdot e^{-kt}$$

where $a = A_0$, $b = A_{\infty} - A_0$ and $k = k_{\text{obs}}$, was used to fit the curves and gave satisfactory results. Similar reaction rate constants were obtained from either 295 nm or λ_{max} , suggesting that either can be used to monitor iron release.

Experiments measuring iron release from the protein with EDTA concentrations from 0.4 to 8 mM (about 10–200-fold higher than the concentration of Fe-hTF/2N) at pH 6.5 were performed. The pseudo-first-order rate constants at six different EDTA concentrations are listed in Table 1. When these data, together with the zero point, were fitted to the kinetic model:

$$k_{\text{obs}} = k_1[L]/(1 + k_2[L]) \quad [7]$$

a simple saturation kinetic behaviour with respect to the free-ligand (L) concentration was found. As shown in Figure 2(A), there is an excellent fit to the data.

Chloride effect on iron release from hTF/2N at different pH values

Experiments were conducted to determine the influence of KCl on iron release. The results with KCl concentrations up to 1 M are given in Table 1 and Figure 2(B). The retarding effect of chloride on iron release shows a saturation profile: the effect was initially sharp and then levelled off. Besides the negative effect observed above, chloride is capable of accelerating iron release from hTF/2N at lower pH. The pH-dependence of this effect was determined by a series of studies of iron release from hTF/2N by EDTA at different pH values. Mes buffer, which has a useful pH range of 5.6–6.7, was used for supporting solutions. The ionic strength of the buffer solutions, the concentration of EDTA (0.8 mM, $\approx$ 20-fold excess over the protein concentration), and the chloride concentration ([KCl] = 0 or 0.14 M) were kept constant to eliminate these as variable influences.

Iron release from Fe-hTF/2N by EDTA at pH 5.9–6.7 showed a first-order kinetic behaviour similar to that observed at pH 6.5.

Figure 2 (A) Plot of k_{obs} for hTF/2N (WT) as a function of increasing concentrations of EDTA (50 mM Mes, pH 6.5, 25 °C) and (B) effect of KCl on iron release from hTF/2N (WT) with EDTA (0.8 mM; 50 mM Mes, pH 6.5, 25 °C).**Table 2** Rate constants for iron release from wild-type hTF/2N at various pH values

The conductivity of the Mes buffer was 0.88 ± 0.01 mS/cm and the temperature was 25.0 ± 0.1 °C. Values are means $\pm$ S.D. k : [EDTA] = 0.8 mM, [KCl] = 0; k_{Cl} : [EDTA] = 0.8 mM, [KCl] = 0.14 M; k_0 : [EDTA] = 0, [KCl] = 0.14 M.

pH	k (min ⁻¹)	k_{Cl} (min ⁻¹)	$(k_{\text{Cl}} - k)/k \times 100$	k_0 (min ⁻¹)
5.93	1.140 ± 0.010	2.273 ± 0.051	99.4	0.014 ± 0.003
6.14	0.498 ± 0.008	0.669 ± 0.006	34.2	0.005 ± 0.001
6.25	0.374 ± 0.015	0.387 ± 0.005	3.4	
6.40	0.199 ± 0.014	0.144 ± 0.003	-27.4	
6.55	0.105 ± 0.005	0.061 ± 0.002	-41.8	
6.69	0.055 ± 0.010	0.027 ± 0.004	-50.5	

All the curves were fitted with a single exponential equation giving R^2 factors > 0.99 . Table 2 lists the rate constants of the reactions in the absence and presence of KCl (k and k_{Cl}). Figure 3(A) shows the pH-dependence of k and k_{Cl} values. Both curves for k and k_{Cl} have a similar trend in that the reactions slow down as the pH increases. With the k curve as a reference, an interesting observation can be made: below pH 6.26 (the cross-over point of the two curves) chloride accelerated iron release, and, above this pH, chloride inhibited iron release. When the k and k_{Cl} data are taken in the form of $100 \times (k_{\text{Cl}} - k)/k$, the resulting positive

Figure 3 (A) pH effect on iron release from hTF/2N(WT) with EDTA (0.8 mM) in the absence (○) and presence (△) of KCl (Mes buffer, conductivity 0.88 ± 0.01 mS/cm, 25°C) and (B) the percentage of chloride enhancing (positive) and retarding (negative) iron release from hTF/2N(WT) by EDTA (0.8 mM) at various pH values

values are the percentages of the extent of the acceleration by chloride and the negative values are the percentage of the extent of the inhibition by chloride. A plot of $100 \times (k_{\text{ci}} - k)/k$ as a function of pH is shown in Figure 3(B), where the biphasic behaviour is more obvious: chloride enhanced the reaction with a linear mode at lower pH and retarded the reaction with a hyperbolic mode at higher pH, with the null point at pH 6.26.

To test the effect of chloride alone on iron release at lower pH, two reference experiments were carried out in the absence of EDTA with $[\text{KCl}] = 0.14$ M at pH 5.93 and 6.14 respectively. The resulting rate constants (k_0 , Table 2) are very small, ≈ 100 -fold lower than the corresponding k values (Table 2), showing that the effect from chloride alone is limited.

Iron release from D63 mutants

EDTA removes iron from the D63 mutants, also resulting in two negative peaks at around 294 nm and at λ_{max} in the visible region. This reaction is much faster than that for wild-type hTF/2N, even at pH 7.4. Iron removal from the mutants by EDTA follows biphasic kinetics. Typical absorbance-versus-time data for iron release from D63S at two KCl concentrations, together with their best-fit curves, are given in Figure 4. Obviously, the double-exponential model:

$$A = a + b \cdot e^{-kt} + c \cdot e^{-k't} \quad (\text{continuous lines})$$

Figure 4 Iron release from D63S by EDTA (0.8 mM) in Hepes (50 mM), KCl (0 and 0.14 M) at pH 7.4 and 25°C

—, double-exponential fit; ---, single-exponential fit.

Table 3 k and k' values for iron release from the D63 mutants of hTF/2N by EDTA (0.8 mM) in the absence or presence of KCl

Hepes was present at 50 mM. The pH was 7.4 and the temperature was $25.0 \pm 0.1^\circ\text{C}$. Values are means $\pm$ S.D.

Mutant	k (min^{-1})		k' (min^{-1})	
	$[\text{KCl}] = 0$	$[\text{KCl}] = 0.14$ M	$[\text{KCl}] = 0$	$[\text{KCl}] = 0.14$ M
D63S	2.11 ± 0.19	3.53 ± 0.32	0.46 ± 0.04	0.51 ± 0.02
D63E	2.08 ± 0.30	3.89 ± 0.17	0.48 ± 0.06	0.58 ± 0.02
D63N	4.12 ± 0.28	5.47 ± 0.28	0.53 ± 0.05	0.61 ± 0.06
D63A	4.29 ± 0.21	4.82 ± 0.57	0.54 ± 0.02	0.59 ± 0.03

has a much better fit to the data than has the single-exponential model:

$$A = a + b \cdot e^{-kt} \quad (\text{broken lines})$$

This biphasic kinetic behaviour may be explained by proposing that EDTA removes iron by two concurrent pathways (see the Discussion). The iron-release curves for the other D63 mutants feature similar biphasic kinetic behaviour. Table 3 summarizes the resulting k and k' values from the double-exponential fitting for the iron-release reactions of the mutant proteins in the presence or absence of KCl. Roughly, these observed rates increase in the following order:

$$\text{D63S} \approx \text{D63E} < \text{D63N} \approx \text{D63A}$$

It must be pointed out that chloride itself is able to affect iron-release from D63S (results not shown). This action becomes considerable with KCl concentrations above 1.0 M. Therefore the experiments to test the effect of chloride were limited to a range of ≤ 0.5 M, where, except for a small initial decrease, the λ_{max} absorbance essentially remained unchanged before the measurements.

Figure 5 (A) Plot of k (●) and k' (○) for D63S as a function of increasing concentrations of EDTA (50 mM Hepes, pH 7.4, 25 °C, monitored at 426 nm) and (B) plot of k (▲) and k' (△) for D63S as a function of increasing concentrations of KCl (0.8 mM EDTA, 50 mM Hepes, pH 7.4, 25 °C; monitored at 426 nm).

A noticeable accelerating effect of KCl on iron release is observed from the k data. To investigate further the mechanism of this effect from ligand and chloride, detailed studies were performed with D63S, as a representative of the mutants. Figure 5 shows the k and k' data for D63S at various EDTA and KCl concentrations respectively. Iron release from D63S exhibited a saturation kinetic behaviour with respect to the ligand concentration, and a linear dependence with increasing chloride concentration. Interestingly, the k' values were low and only increased slightly in the reactions with different ligand and KCl concentrations.

DISCUSSION

Iron release from wild-type hTF/2N

Because of the inequivalent properties of the two metal-binding sites of the protein, iron release from native transferrin generally displays biphasic kinetic behaviour, corresponding to the N- and C-lobe binding sites respectively [5,8,18]. The single first-order kinetic mode exhibited in iron removal from hTF/2N by EDTA is logically accounted for by the single binding site, similar to those found in kinetic studies for the monoferric transferrins [6,7,19,20]. A recent study by Zak et al. also showed that single-exponential functions have a good fit ($R^2 > 0.99$) to the data of iron release from wild-type hTF/2N to pyrophosphate [9]. Furthermore, as for most studies with native transferrins [8,18], iron release from hTF/2N by EDTA showed a simple hyperbolic dependence with respect to the free-ligand concentration, which could be explained by a mechanism involving a rate-determining

conformational change [21–23]. These facts, along with our earlier studies with Tiron, suggest that recombinant hTF/2N does not show any pronounced differences in terms of its iron-binding and -release behaviours. The isolated N-lobe, of course, has the advantage that the mechanism and functional studies can be explored in the context of a molecule with a single binding site.

Chloride effect and the mechanism of iron release

The influence of the simple inorganic anion, chloride, on the iron release from transferrin has attracted much attention; results by other investigators show that this chloride effect is not simple. Discordant results on the chloride effect on iron release from hTF/2N have been reported by several groups. Chloride showed an accelerating effect on iron removal with the tricatechol synthetic chelator *N,N',N''*-tris(5-sulpho-2,3-dihydroxybenzoyl)-1,5,10-triazadecane ('3,4-LICAMS') [19] and an inhibiting action with pyrophosphate as sequestering agent [9,24]. It is also reported that chloride retarded iron release by pyrophosphate at 5 mM chelator, but accelerated the release at 100 mM chelator [9]. Besides chloride, other factors such as pH, ionic strength and mutation of a side chain can promote or retard iron release. Several models have been proposed to explain the influence of these factors. The pH could induce conformational change by means of protonation or deprotonation of bound carbonate and of amino acid side chains [25,26]. A variety of anions accelerate iron release, which may be attributed to the presence of a specific effector site near the metal-binding centre [27,28] or the existence of a kinetically significant anion-binding (KISAB) site [6,20]. The negative chloride effect may result from anion competition between chloride and chelator for binding to an active site where iron release initiates [9]. On the basis of our results for iron release from hTF/2N and the D63 mutants (see next subsection), we believe that these pathways operate together, with more or less impact, depending on the different conditions. With the conformational change as a key feature for initiating iron removal, these factors could play a part in promoting this change upon attack by a chelator, in terms of a 'pH trigger' [29], a 'salt trigger' [19,30] and a 'dilysine trigger' [26] etc. Three different conditions are used to discuss the chloride effect as follows.

(a) Chloride-dependent kinetics at pH 6.5

At pH 6.5, as KCl concentration increased, chloride steadily slowed iron release from hTF/2N by EDTA with a saturation mode, akin to the early phase with Tiron at pH 7.4 [12]. This implies that, at pH 6.5, the protein may maintain a closed conformation around the binding site, similar to that at pH 7.4. Potential protonation at this pH would be less and might not be the major driving force to induce the conformational change which promotes iron release. In contrast, another factor, binding competition between chloride and chelate for the active site of iron release, may play a major role. Therefore, a retarding action of chloride is observed and a saturation mode reflects the chloride binding reaching its maximum (Figure 2B).

(b) Kinetics at pH 5.93–6.26 with $[\text{KCl}] = 0.14 \text{ M}$

As the environment becomes more acidic, the 'pH trigger' becomes more important for enhancing the conformational change of the protein. The evidence for this change has been found in structural studies [26]. Although the negative effect derived from the binding competition of chloride and chelate may exist, at decreased pH, this effect contributes less and less to the observed rate. Chloride along with EDTA could enter the

'open' cleft more easily to bind iron; the initially sharp curves shown in Figure 3(A) may reflect this action. Chloride itself can remove iron from transferrin at low pH [31], showing the iron centre in the 'open' cleft to be easily approached. However, the effect from chloride alone on iron release is so minor (Table 2), the significant difference between k_{Cl} and k at the low pH values means that EDTA and Cl^- act to remove iron synergistically rather than additively. The co-ordinated iron removal by both EDTA and chloride may contribute to the linear positive effect at $\text{pH} < 6.26$ (Figure 3B). This conformational change induced by pH could be stimulated by means of protonation of the carbonate and/or other residues in the cleft such as lysine residues [25,26], as stated above. The former could result in a cleavage of the hydrogen bond with Arg^{124} or promote a change to monodentate bicarbonate, either of which could lead to the break-up of the Fe-hTF/2N complex [25]. The latter may split the hydrogen bond between the pair of lysine residues (Lys^{206} and Lys^{296}) linking the two domains and thus destabilize the closed structure.

(c) Kinetics at pH 6.26–6.69 with $[\text{KCl}] = 0.14 \text{ M}$

Kinetic studies in this pH region are an expansion from that at pH 6.5, but with the chloride concentration constant. The protein may essentially maintain a normal stable conformation at $\text{pH} > 6.26$, leading the potential negative effect of chloride to be more pronounced. During the course of increasing pH from 6.26 to 6.69, the deprotonation of some amino acid side chains could result in a more negative microenvironment and less of the KISAB site [6,20] in the protein. Still, chloride could compete with EDTA for binding to these sites where iron release may initiate. These combined factors may cause an inhibitory behaviour with a hyperbolic mode at pH 6.26–6.69 (Figure 3B). The zero point (Figure 3B) at pH 6.26 is the equilibrium point for the opposite effects of these various factors. An inference is that at $\text{pH} \leq 6.26$ Fe-hTF/2N has an intermediate state where protonation (at least in part) of the binding carbonate leads Arg^{124} to dissociate a hydrogen-bond to the carbonate, so that a disordered ligation of the iron centre occurs prior to the breakup of a 'closed' conformation.

Iron release from D63 mutants

A recent crystallographic analysis of the D60S mutant of lactoferrin showed that the mutation perturbed both the iron site and domain closure [13]. The introduced serine residue does not bind to the iron atom, nor does it engage in any interdomain interaction. A water molecule fills the binding position of the serine residue to complete the iron co-ordination. The weaker hydrogen bonds mediated by the iron-bound water molecule replace the direct $\text{Asp}^{60} \cdots \text{Thr}^{122}$ interdomain hydrogen bond found in the parent N-lobe of lactoferrin. The replacement of aspartic acid with serine and an associated water molecule clearly weakens iron-binding and interdomain contacts. As closely related mutants, the D63 series may have a similar complexation pattern around the binding site. For iron release from these mutants by EDTA, the structural factor (conformational change) dominates over those potential negative effects, since the loose interdomain closure opens more easily, although it may look closed at pH 7.4, similar to the D60S of hLF. It is not surprising, therefore, that iron removal from D63S by EDTA is much more facile than that for wild-type hTF/2N even at pH 7.4. Especially in the D63A mutant, the complete loss of a key linking component of the interface makes the binding site more unstable. For the D63N mutant, where the substituted

side-chain asparagine residue has a similar size to aspartic acid, the easy release of iron may be due to the loss of a negative charge. This loss quenches a presumably strong interaction given the positive charge on the helix 5N terminus and the negative carboxylate group [13] that results in a labile closure of the binding site. Mutation from aspartic acid to a homologous amino acid, glutamic acid, also led to a dramatic decrease in iron-binding stability, probably due to steric strain. Perhaps E63 could directly co-ordinate to the iron centre rather than via water, as there is minor change in λ_{max} compared with the wild-type protein [12], but the steric strain derived from the difference of the single methylene group from D63 may cause some degree of cleft opening, which would make the bound metal easily approached and removed by chelator.

An unexpected result is the fact that iron release by EDTA exhibits biphasic kinetics in the case of the mutants, thus differing from the parent hTF/2N. Apparently, there is a second iron-removal process accompanying the major iron-release procedure. A possible explanation is that, in contrast with that for wild-type hTF/2N, iron removal from the D63 mutants by EDTA involves the formation of a low concentration of an EDTA transition-state complex in the binding site [5], which, as the second procedure, leads to some iron release from the protein. Mutations weaken interdomain contacts, cause more facile cleft opening and create more space and access for the chelator, so that some possible intermediate between EDTA and the mutants may form prior to the entire Fe-EDTA complex. Unfortunately, direct spectral evidence for the formation of the intermediate was not observed, perhaps owing to rapid decomposition.

The significant accelerating effect exhibited by chloride on the iron release from the mutants follows the explanation for the positive chloride effect in iron release from wild-type hTF/2N at low pH, since the 'loose' conformation of the mutants has an advantage for iron release similar to the 'open' conformation of hTF/2N at low pH. During the course of the release, chloride, with a much smaller size than EDTA, could enter the 'looser' binding site or replace the putative bound water molecule, assisting EDTA in the removal of the ferric ion. Although a competition between chloride and EDTA for an active site close to the iron centre could exist, as stated above, this negative factor becomes smaller than the positive effect from chloride. As shown in these experiments, chloride itself is able to remove iron from the D63 mutants, even at pH 7.4. This potential ability of chloride may make a fundamental contribution to the positive linear dependence of iron release by EDTA. Again, in the reaction, Cl^- and EDTA must behave synergistically, as for wild-type hTF/2N at low pH values.

It should be pointed out that, in their recent studies on iron release from recombinant N-lobe and mutants with pyrophosphate, Zak et al. observed a complete negative effect of chloride in all cases they tested: with wild-type and mutants of hTF/2N, at neutral and low pH values [9]. A model was proposed to explain this retarding effect as a binding competition between pyrophosphate and chloride to an active site for iron release. Obviously, when chloride appears, something happens to block or slow down the approach of pyrophosphate to the iron centre, even at pH 5.6, where hTF/2N is assumed to have an 'open' conformation. This difference from our observations is probably due to the difference in chelators. If, as suggested by Zak et al. [9], there are several classes of anion-binding sites governing the binding and release of iron from transferrin, the difference in chelators, combined with other factors, could make significant differences in iron-release behaviours. We agree that the mechanisms for iron release appear to be more complex than once believed.

Concluding remarks

Iron release from recombinant hTF/2N and four single-point Asp⁶³ (D63) mutants of hTF/2N by EDTA has been studied kinetically. The results show that iron release can be influenced by various factors such as pH, chloride and mutation of a side chain, which act together, with more or less impact, depending on the conditions. At pH > 6.26, wild-type Fe-hTF/2N appears to maintain a closed conformation; iron release from Fe-hTF/2N may follow a mechanism dictated by a rate-determining conformational change, so that simple saturation kinetics with respect to the EDTA concentration are observed. Competitive binding between chloride and EDTA to an iron-release active site may result in the negative hyperbolic chloride effect observed both at pH 6.5, with respect to the increasing chloride concentration and in the range of pH 6.26–6.69, with [KCl] = 0.14 M. With increasing acidity, the 'pH trigger' appears to become the most important factor in promoting the conformational change of the protein. Chloride, along with EDTA, may easily enter the 'open' cleft to remove iron synergistically, leading to a linear positive effect of chloride in the pH range 5.93–6.26. The opposite effect of chloride at different pH values has a cross-over point at pH 6.26. For iron release from the D63 mutants, chloride has an accelerating effect due to the 'loose' conformation resulting from the mutation of the key linking component, D63, in the interdomain contacts.

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