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Transferrins: Iron Release From Lactoferrin

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Iron loss *in vitro* by the iron scavenger bovine lactoferrin was investigated in acidic media in the presence of three different monoanions (NO_3^-, Cl^- and Br^-) and one dianion (SO_4^{2-}). Holo and monoferric C-site lactoferrins lose iron in acidic media (pH $\leqslant$ 3.5) by a four-step mechanism. The first two steps describe modifications in the conformation affecting the whole protein, which occur also with apolactoferrin. These two processes are independent of iron load and are followed by a third step consisting of the gain of two protons. This third step is kinetically controlled by the interaction with two Cl^-, Br^- and NO_3^- or one SO_4^{2-}. In the fourth step, iron loss is under the kinetic control of a slow gain of two protons; third-order rate-constants k_2, $4.3(\pm 0.2) \times 10^3$, $3.4(\pm 0.5) \times 10^3$, $3.3(\pm 0.5) \times 10^3$ and $1.5(\pm 0.5) \times 10^3$ M^{-2} s^{-1} when the protein is in interaction with SO_4^{2-}, NO_3^-, Cl^- or Br^-, respectively. This step is accompanied by the loss of the interaction with the anions; equilibrium constant K_2, 20 ± 5 mM, $1.0(\pm 0.2) \times 10^{-1}$, $1.5(\pm 0.5) \times 10^{-1}$ and $1.0(\pm 0.3) \times 10^{-1}$ M^2, for SO_4^-, NO_3^-, Cl^- and Br^-, respectively. This mechanism is very different from that determined in mildly acidic media at low ionic strength ($\mu < 0.5$) for the iron transport proteins, serum transferrin and ovotransferrin, with which no prior change in conformation or interaction with anions is required. These differences may result from the fact that in the transport proteins, the interdomain hydrogen bonds that consolidate the closed conformation of the iron-binding cleft occur between amino acid side-chain residues that can protonate in mildly acidic media. With bovine lactoferrin, most of the interdomain hydrogen bonds involved in the C-site and one of those involved in the N-site occur between amino acid side-chain residues that cannot protonate. The breaking of the interdomain H-bond upon protonation can trigger the opening of the iron cleft, facilitating iron loss in serum transferrin and ovotransferrin. This situation is, however, different in lactoferrin, where iron loss requires a prior change in conformation. This can explain why lactoferrin does not lose its iron load in acidic media and why it is not involved in iron transport in acidic endosomes.

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Abbreviations used: $LT''Fe_2$, bovine hololactoferrin in an unknown state of charge and protonation; $LT'Fe_2$, another hololactoferrin species in an unknown state of charge and protonation; $LTFe_2$, hololactoferrin in a different conformation; LTS_nFe_2, hololactoferrin in interaction with anions; $LTS_nH_lFe_2$, protonated lactoferrin in interaction with anions; $LTS_nH_{(m+2)}$, protonated apolactoferrin in interaction with anions; $LTH_{(m+2)}$, protonated apolactoferrin in its final state of conformation in acidic media; PAGE-U, polyacrylamide-urea gel electrophoresis; c_1, analytical lactoferrin concentration; S, salt; KISAB, kinetically active binding site.

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Introduction

Transferrins are the most important iron regulation systems in vertebrates. They are found also in invertebrates and bacteria (Aisen, 1998; Bruns *et al.*, 1997). Transferrins share similar polypeptide topology with several periplasmic transport proteins, which makes all these proteins members of a new superfamily, that of transferrins (Bruns *et al.*, 1997). The most representative transferrins in vertebrates are serum transferrin and lactoferrin in mammals, and ovotransferrin in birds (Aisen, 1998). Serum transferrin and ovotransferrin are responsible for Fe(III) transport from biological fluids to cytosol by receptor-mediated endocytosis

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(Dautry-Varsat *et al.*, 1982; Kurokawa *et al.*, 1995), whereas lactoferrin is considered as an iron scavenger (Anderson *et al.*, 1990). All these transferrins consist of a single polypeptide chain of about 700 amino acid residues organised in two lobes (C and N) linked by an interlobe chain of about ten to 12 residues. Each lobe consists of two domains containing four protein ligands to which iron is co-ordinated. Iron is co-ordinated also to a synergistic carbonate anion, without which the protein loses its affinity for the metal (Anderson *et al.*, 1989, 1990; Zuccola, 1992; Kurokawa *et al.*, 1995; Bou Abdallah & El Hage Chahine, 1998, 1999). When the protein is in the iron-free state, the two lobes are mostly in an open conformation, whereas they are in a closed conformation when the protein is iron loaded (Anderson *et al.*, 1990; Moore *et al.*, 1997; Kurokawa *et al.*, 1995, 1999; Sharma *et al.*, 1999). Moreover, in each lobe, interdomain hydrogen bonds occur upon complex formation with iron. These hydrogen bonds differ from one transferrin to another (Dewan *et al.*, 1993; Moore *et al.*, 1997). They can be situated at the lips of the binding cleft controlling, therefore, the access of water to the iron co-ordination cavity (Dewan *et al.*, 1993; Bou Abdallah & El Hage Chahine, 1998, 1999; Pakdaman *et al.*, 1999).

In a recent series of articles, we established the mechanisms of iron uptake by the three major transferrins, serum transferrin, lactoferrin and ovotransferrin, and that of iron release by serum transferrin and ovotransferrin (El Hage Chahine & Pakdaman, 1995; Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998, 1999; Bou Abdallah & El Hage Chahine, 1998, 1999). We showed that iron release from serum transferrin and ovotransferrin occurs in mildly acidic media by similar mechanisms with, however, some differences in the rates and in the number of proton transfer reactions involved (El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999).

With lactoferrin, iron release is practically impossible in mildly acidic media (pH $\approx$ 5.5). This protein is not involved in Fe(III) transport by receptor-mediated endocytosis and starts losing iron in acidic media (pH $\leqslant$ 3.5; Montreuil *et al.*, 1960; Leveugle *et al.*, 1993). It has a bacteriostatic effect based on its faculty of scavenging iron, is a bactericide, and seems to inhibit free radical formation and tumour cell proliferation (Bellamy *et al.*, 1992). Besides differences in the number of protons lost and in reaction rates, the behaviours of the iron transport proteins serum transferrin and ovotransferrin, and that of the iron-scavenger lactoferrin, towards iron uptake in neutral media are similar (El Hage Chahine & Fain, 1993; Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998, 1999; Bou Abdallah & El Hage Chahine, 1998). This seems logical in view of the topological identity of the three transferrins. Why then, despite this high level of topological similarity, does lactoferrin behave differently towards iron release in acidic media?

Here, by means of the techniques and methods of chemical relaxation (Eigen & DeMayer, 1963; Bernasconi, 1976; Brouillard, 1980; El Hage Chahine & Fain, 1993), we shall try to give at least a partial answer to this question.

Results

For reasons of simplicity and lack of knowledge of the state of the charge in the binding sites of lactoferrin, the charges of the protein species involved are not written. The fact that modifications in the conformation of transferrins are usually accompanied by variations in their emission and absorption spectra led us to the use of spectrophotometric detection in emission and absorption for our kinetic runs (Lehrer, 1969; Bellounis *et al.*, 1996; Pakdaman & El Hage Chahine, 1997; Muralidhara & Hirose, 2000). The excitation wavelength λ_{ex} was 280 nm and the emission was monitored between 300 and 400 nm (data not shown, Pakdaman *et al.*, 1998).

When a solution of hololactoferrin or monoferric C-site iron-loaded lactoferrin is submitted to a rapid pH-jump from neutral to acidic ($\leqslant$3.5) four kinetic processes are detected. They all occur as exponential decreases in the absorption in the 400 to 500 nm range (Figure 1) or as increases in the fluorescence emission intensity in the 300 to 400 nm range (not shown). These experiments were performed at fixed concentrations [S] with S = KCl, KBr, KNO$_3$ or K$_2$SO$_4$, where 50 mM $\leqslant$ [S] $\leqslant$ 500 mM. The four kinetic processes of Figure 1 lead to the accumulation of three kinetic products (Figure 1). Since iron uptake or release by lactoferrin is very slow near neutrality (Pakdaman *et al.*, 1998), a fast pH-jump from acidic to neutral allowed us to trap two of these intermediates with their iron load intact.

The first two processes (Figure 1(a)) occur in the 1 ms to 100 ms range and lead to the accumulation of a kinetic product. Polyacrylamide-urea gel electrophoresis (PAGE-U) performed on this kinetic product, after trapping near neutrality, clearly shows that hololactoferrin and the C-site of monoferric lactoferrin keep their load of iron unchanged. These two processes are observed with apolactoferrin. The experimental reciprocal relaxation times related to these fast phenomena are invariable and do not depend on any of our experimental parameters. The third kinetic process (Figure 1(b)) occurs in the 10 second range and leads to the accumulation of another kinetic product. Here too PAGE-U indicates, after trapping the intermediate in neutral media, that the protein iron load is still intact. This process is not observed with apolactoferrin. The experimental relaxation times measured for this phenomenon depend on the pH, on the ionic strength, and on the nature of the anion component of this ionic strength. The fourth process occurs in the 1000 second range (Figure 1(c)). The spectrum of the final protein species is identical
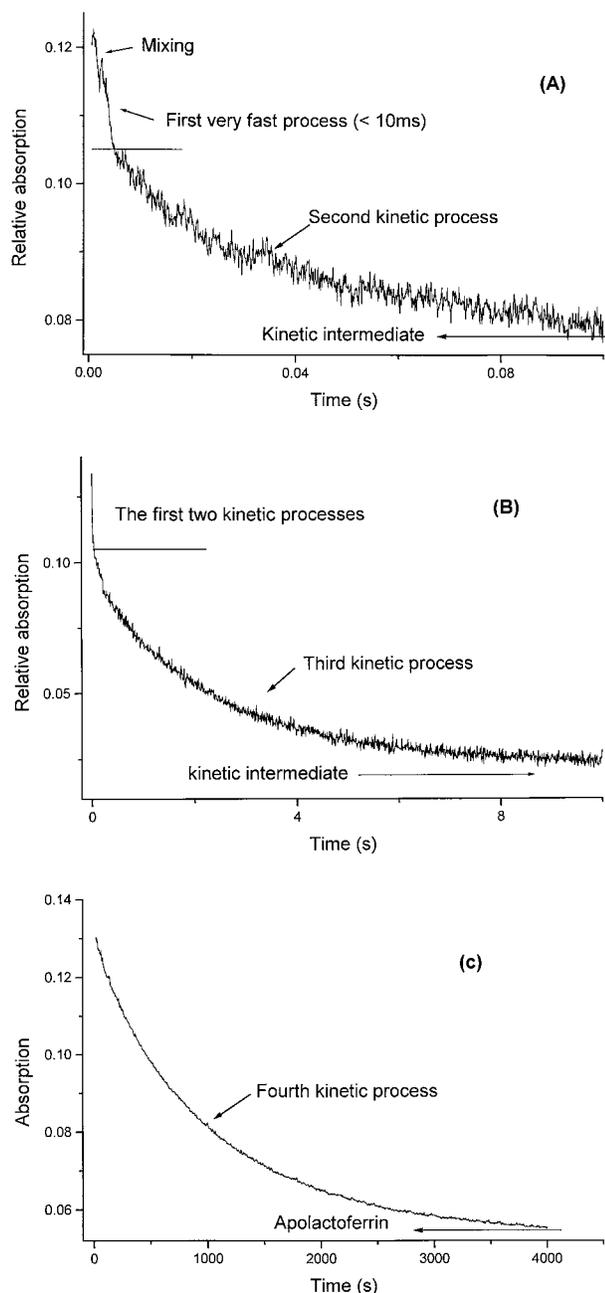
Figure 1. Fluorescence and absorption variation with time, when a solution of bovine hololactoferrin is submitted to a fast pH-jump from neutral to acidic media with $\lambda = 465$ nm for absorption and an excitation wavelength $\lambda_{ex} = 280$ nm for emission at $25(\pm 0.5)\,°C$. (a) to (c) Multiexponential decreases of absorption recorded on three different time-scales in the presence of (a) 180 mM KBr at pH 2.54, (b) 30 mM NaBr at pH 2.14 and (c) 150 mM KCl at pH 3.16, with $c_1 = 50\ \mu M$.

with that of an apolactoferrin partially unfolded in 4 M urea in neutral media (data not shown; Kilar & Hjerten, 1993). Here also, the experimental relaxation times depend on pH, ionic strength, and the nature and concentrations of the anions present.

All rates were independent of protein concentration c_1 and of the nature of the used cation components of the ionic strength, K^+ or Na^+.

The first two kinetic processes

The first fast step ends within 8 to 10 ms (Figure 1(a)). The mixing time of our stopped-flow apparatus is typically in the 3 to 5 ms range. Therefore, the experimental reciprocal relaxation times $(\tau_1)^{-1}$ related to the first process cannot be analysed accurately. Nevertheless, within the experimental uncertainty, $(\tau_1)^{-1}$ can be considered as independent of our experimental parameters and of the protein iron load. Furthermore, the reciprocal relaxation times $(\tau_2)^{-1}$ related to the second kinetic process (Figure 1(a)) are independent of our experimental parameters and iron load $((\tau_2)^{-1} = 22(\pm 3)\ s^{-1})$. This independence of the experimental parameters implies both $(\tau_1)^{-1}$ and $(\tau_2)^{-1}$ are associated with first-order kinetic processes (reactions 1 and 2, Eigen & DeMaeyer, 1963; Bernasconi, 1976; Pakdaman & El Hage Chahine, 1996). This led us to ascribe these two processes to a phenomenon involving the entire protein, such as a modification in the conformation independent of iron load. The fluorescence emission spectrum of apolactoferrin measured at the end of these two phenomena, that measured at the end of the fourth process after performing a pH-jump from neutral to acidic on hololactoferrin and that measured in neutral media for apolactoferrin in the presence of 4 M urea, where the protein is partially unfolded (Kilar & Hjerten, 1993), are almost identical (data not shown). Moreover, these spectra reveal a 10 nm red shift in the fluorescence emission maximum as compared to that of an apolactoferrin in neutral media (data not shown). This can be typical of a partial unfolding of the protein (Harrington, 1992). However, we cannot at this stage confirm this hypothesis. Hololactoferrin ($LT''Fe_2$) is, therefore, assumed to undergo conformational change in two steps leading to a new diferric or monoferric species ($LTFe_2$):

$$LT''Fe_2 \rightleftharpoons LT'Fe_2 \tag{1}$$

$$LT'\ Fe_2 \rightleftharpoons LTFe_2 \tag{2}$$
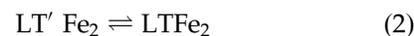
Whether the first step in Figure 1(a) involves other processes such as interactions with salt, proton transfers or carbonate loss, cannot be established under our experimental conditions.

Third kinetic process

During the third process (Figure 1(b)), the kinetic intermediate $LTFe_2$ is transformed into another intermediate in which the apolactoferrin is still iron-loaded. An experimental linear relationship is obtained between the reciprocal relaxation times $(\tau_3)^{-1}$ and $[H^+]^2[S]^n$ with $n = 2$ when S is Cl^-, Br^- or NO_3^-, and with $n = 1$ when S is SO_4^{2-} (as shown

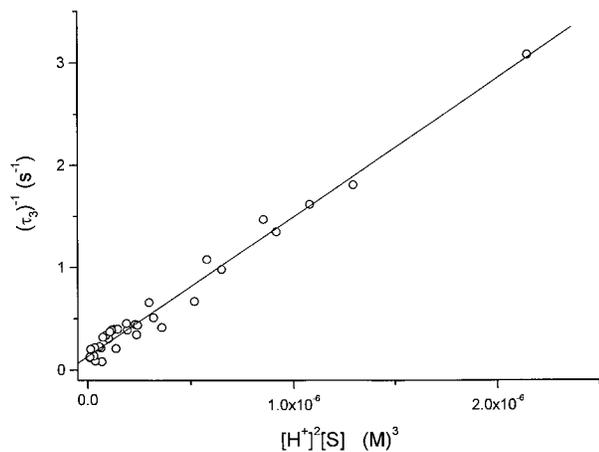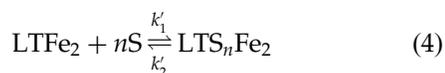**Figure 2.** Plot of $(\tau_3)^{-1}$ against $[H^+]^2[S]^n$ at $25(\pm0.5)\,^\circ$C for $2\,\mu M \leqslant c_1 \leqslant 50\,\mu M$, with $n = 1$ and $18.75\,mM \leqslant [S] = [SO_4^{2-}] \leqslant 120\,mM$; intercept, $0.13(\pm0.03)\,s^{-1}$; slope, $1.35(\pm0.15) \times 10^6\,M^{-3}\,s^{-1}$ and $r = 0.98964$.

for SO_4^{2-} in Figure 2). This linear relationship can be expressed as:

$$(\tau_3)^{-1} = k_{-1(obs)} + k_{1(obs)}[H^+]^2[S]^n \qquad (3)$$

in which $k_{-1(obs)}$ and $k_{1(obs)}$ are the intercepts and slopes of the regression line (Figure 2).

The single exponential process detected for the third step (Figure 1(b)) can be treated as a relaxation mode (Eigen & DeMaeyer, 1963; Brouillard, 1980). If we assume that in this step the protein undergoes an interaction with the anions of the medium and two classically diffusion controlled proton gains (Eigen & DeMayer, 1963), two possible mechanisms can be envisaged, option I:

$$LTFe_2 + nS \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} LTS_nFe_2 \qquad (4)$$

$$LTFe_2S_n + lH^+ \rightleftharpoons LTS_nH_lFe_2 \qquad (5)$$

and option II:

$$LTFe_2 + lH^+ \rightleftharpoons LTH_lFe_2 \qquad (6)$$

$$LTH_lFe_2 + nS \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LTS_nH_lFe_2 \qquad (7)$$

with $K_{1a}' = [LTFe_2S_n][H^+]^l/[LTS_nH_lFe_2]$, $K_{1S}' = [LTFe_2][S]^n/[LTS_nFe_2]$, $K_{1a} = [LTFe_2][H^+]^l/[LTH_lFe_2]$ and $K_{1S} = [LTH_lFe_2][S]^n/[LTS_nH_lFe_2]$.

In option I, the first step would be that of the interaction of the protein with anions (equation (4)) followed by the gain of l protons (equation (5)). Whereas, in option II, the first step would be that of the gain of l protons followed by the interaction with the anions.

The reciprocal relaxation time equations associated with equations (4) and (7) when each is con-

Table 1. Values of k_1/K_{1a} and k_{-1} at $25(\pm0.5)\,^\circ$C

Anion	k_1/K_{1a}	k_{-1}
SO_4^{2-}	$1.35(\pm0.15) \times 10^6\,M^{-3}\,s^{-1}$	$0.13(\pm0.03)\,s^{-1}$
NO_3^-	$2.65(\pm0.25) \times 10^5\,M^{-4}\,s^{-1}$	$0.13(\pm0.05)\,s^{-1}$
Cl^-	$1.50(\pm0.25) \times 10^5\,M^{-4}\,s^{-1}$	$0.08(\pm0.02)\,s^{-1}$
Br^-	$8.0(\pm0.6) \times 10^4\,M^{-4}\,s^{-1}$	$0.09(\pm0.02)\,s^{-1}$

sidered rate-limiting are expressed as equations (8) and (9), respectively (see Appendix):

$$(\tau_3')^{-1} = K_{1a}k_{-1}'/([H^+]^l + K_{1a}) + k_1'[S]^n \qquad (8)$$

$$(\tau_3)^{-1} = k_{-1} + k_1[H^+]^l[S]^n/K_{1a} \qquad (9)$$

Only rate-limiting equation (7) in option I with $l = 2$, $n = 2$ for Cl^-, Br^-, NO_3^- and $n = 1$ for SO_4^{2-} (as shown in Figure 2) permits the interpretation of the experimental data. Indeed, the reciprocal relaxation time equation associated with equation (7) (equation (9)) with $k_{1(obs)} = k_1/K_{1a}$ and $k_{-1(obs)} = k_{-1}$) is identical with linear relationship 3 only when $l = 2$. For each salt, k_1/K_{1a} and k_{-1} are determined from the slopes and intercepts of the best regression line (equation (3)) as shown for SO_4^{2-} in Figure 2 (and see Table 1). This implies that the intermediate $LTFe_2$ gains two protons and then interacts with one dianion or two monoanions to yield the third kinetic intermediate $LTS_nH_2Fe_2$.

Fourth kinetic process

The fourth kinetic process (Figure 1(c)) describes iron loss from the lactoferrin $LTS_nH_2Fe_2$ intermediate. At fixed [S] when S is Cl^-, Br^-, NO_3^- or SO_4^{2-}, the plots of the experimental reciprocal relaxation times related to this kinetic process $(\tau_4)^{-1}$ are linear with $[H^+]^2$ (as shown for Cl^- in Figure 3). Furthermore, the plots of the slopes of the best regression lines in Figure 3 are linear with $[S]^n$ with $n = 2$
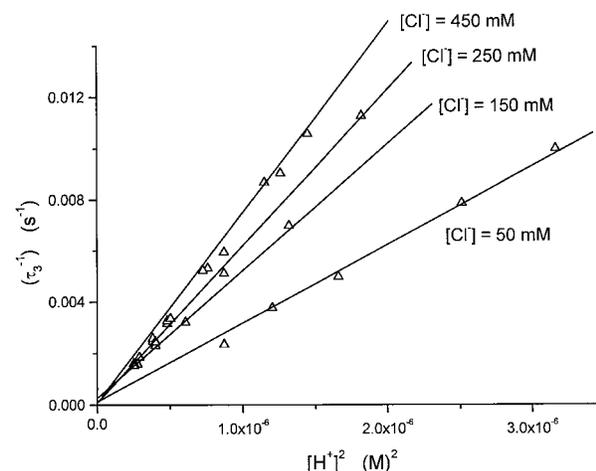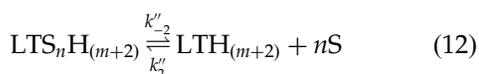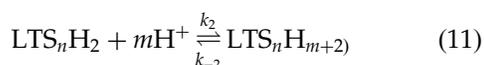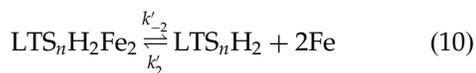

Figure 3. Plot of $(\tau_4)^{-1}$ against $[H^+]^2$ at fixed $[Cl^-]$ at $25(\pm0.5)\,^\circ$C for $1\,\mu M \leqslant c_1 \leqslant 50\,\mu M$.

where S is Cl^-, Br^- or NO_3^- and $n = 1$ when S is SO_4^{2-} (as shown for NO_3^- in Figure 4). However, there is no linear relationship between $(\tau_4)^{-1}$ and $[H^+]^2[S]^2$ as observed for the third step. We have to deal here with all the possible mechanisms in which the intermediate $LTS_nH_2Fe_2$ can protonate, interact with anions and lose Fe(III). Fortunately enough, only the following possibility can be validated by our experimental observations:

$$LTS_nH_2Fe_2 \underset{k'_2}{\overset{k'_{-2}}{\rightleftharpoons}} LTS_nH_2 + 2Fe \qquad (10)$$

$$LTS_nH_2 + mH^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} LTS_nH_{m+2)} \qquad (11)$$

$$LTS_nH_{(m+2)} \underset{k''_2}{\overset{k''_{-2}}{\rightleftharpoons}} LTH_{(m+2)} + nS \qquad (12)$$

with:

$$K_{Fe} = [LTS_nH_2][Fe]^2/[LTS_nH_2Fe_2]$$

$$K_{2a} = [LTS_nH_2][H^+]^m/[LTS_nH_{(m+2)}]$$

$$K_2 = [LTH_{(m+2)}][S]^n/[LTS_nH_{(m+2)}]$$

The $LTS_nH_2Fe_2$ intermediate loses iron and undergoes a gain of m protons accompanied by the loss of the anion(s) with which the protein is in interaction. The reciprocal relaxation time equations associated with equations (10), (11) and (12) when each is considered rate-limiting are expressed as equations (13), (14) and (15), respectively (see Appendix):

$$(\tau'_4)^{-1} = k'_{-2} + k'_2 8(c_1)^2\{(K_{2a}[S]^n + [H^+]^m[S]^n$$
$$+ [H^+]^m K_2)/[H^+]^m K_2\} \qquad (13)$$

$$(\tau_4)^{-1} = k_{-2}[S]^n/K_2 + k_{2(obs)}[H^+]^m \qquad (14)$$

$$(\tau''_4)^{-1} = k''_{-2}[H^+]^m/(K_{2a} + [H^+]^m) + k''_2[S]^n \qquad (15)$$

with under our experimental conditions (pH $\leqslant 3.5$):

$$k_{2(obs)} = k_2 + k_2[S]^n/K_2 \qquad (16)$$

The experimental reciprocal relaxation times associated with the fourth step (Figure 1(c)) are independent of the protein concentration c_1, which discards equation (13). Moreover, equation (15) will not be obeyed by experimental data, unless $K_{2a} \gg [H^+]^m$. This would imply that this proton gain would be extremely acidic with an average $pK_{2a}/m < 1.5$. In contrast, equation (14) is obeyed by experimental data for $m = 2$. $k_{2(obs)}$ (equation

(16)) are determined for each [S] from the slopes of regression lines 14 at fixed [S] (Figure 3), and k_2 and K_2 are determined from the slopes and intercepts of regression line 16 (Figure 4, Table 2). This implies that iron loss from $LTS_nH_2Fe_2$ is controlled by a slow gain of two protons according to equations (10) to (12).

Discussion

Iron uptake by the iron-conveyors serum transferrin and ovotransferrin takes place by the same mechanisms as those observed for lactoferrin. With the three transferrins, Fe(III) uptake first occurs by the C-site. This activates the N-site, which then becomes capable of acquiring Fe(III) (Pakdaman & El Hage Chahine, 1996; Pakdaman et al., 1999; Bou Abdallah & El Hage Chahine, 1998). This cooperativity towards iron uptake was considered as responsible for the iron-binding stability of lactoferrin over a wide range of pH values (Ward et al., 1996). Moreover, in these three transferrins, the affinity of the C-site for iron was always considered higher than that of the N-site (Aisen, 1998; Bou Abdallah & El Hage Chahine, 1998). With serum transferrin and ovotransferrin, after decarbonation, loss of Fe(III) occurs first from the N-site and then from the C-site (Figure 5). It involves ternary complex formation with external competing ligands in the presence of iron chelators such as citrate. In contrast, no ternary complex is detected in the absence of external competing ligands. These processes are always controlled by slow proton transfers reflecting changes in the conformation of the proteins (El Hage Chahine & Fain, 1994; El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999). In view of the topological similarities between the three transferrins and their identical behaviour toward iron uptake, iron release from lactoferrin would be expected to occur sequentially from the N-site and then from the C-site (El Hage Chahine & Pakda-
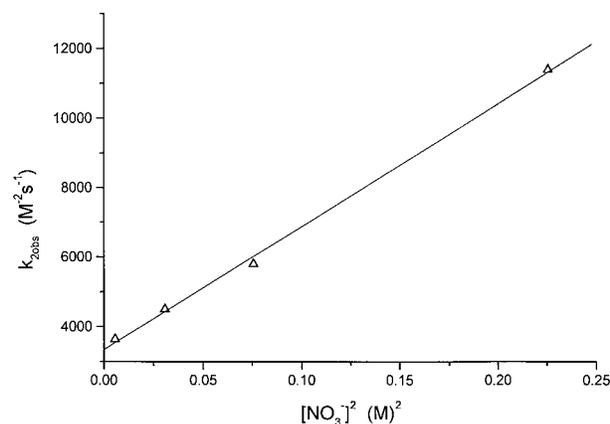

Figure 4. Plot of k_{obs} against $[NO_3^-]^2$; intercept, $3.4(\pm 0.5) \times 10^3$ M^{-2} s^{-1}; slope, $3.5(\pm 0.5) \times 10^4$ M^{-4} s^{-1}; $r = 0.99907$.

Table 2. Values of second-order rate constants k_2 and equilibrium constant K_2 at 25($\pm$0.5) °C

Anion	Rate constant k_2 (M^{-2} s^{-1})	Interaction constant K_2	Average affinity constant (M^{-1})
SO_4^{2-}	4.3($\pm$0.2) $\times 10^3$	2($\pm$0.5) $\times 10^{-2}$ M	50 $\pm$ 12
NO_3^-	3.4($\pm$0.5) $\times 10^3$	1($\pm$0.2) $\times 10^{-1}$ M^2	3.2 $\pm$ 0.7
Cl^-	3.3($\pm$0.5) $\times 10^3$	1.5($\pm$0.5) $\times 10^{-1}$ M^2	2.6 $\pm$ 0.9
Br^-	1.5($\pm$0.5) $\times 10^3$	1($\pm$0.3) $\times 10^{-1}$ M^2	3.2 $\pm$ 1.3

man, 1995; Bou Abdallah & El Hage Chahine, 1999). Nevertheless, our observations and results indicate that this is not the case. At low ionic strength ($\mu \leqslant 0.5$), loss of Fe(III) occurs with completely different mechanisms, depending on whether the proteins involved are the iron-conveyors, serum transferrin and ovotransferrin, or the iron-scavenger lactoferrin (Figure 5). With lactoferrin, iron loss occurs from both sites and is similar for hololactoferrin and the monoferric C-site iron-loaded lactoferrin (Table 3, Figure 5). This Fe(III) loss occurs at pH < 3.5 and is always preceded by two very fast processes, which are independent of the iron charge as they occur uniformly with the apo and holoprotein. This reflects a common change in conformation, which may be a partial unfolding of lactoferrin. Folding and unfolding of transferrins are not well known and, to our knowledge, the kinetics of unfolding of these proteins have not been investigated (Morgan & Peters, 1985; Harrington *et al.*, 1987; Harrington, 1992; Lodish & Kong, 1991; Kilar & Hjerten, 1993; Ou *et al.*, 1993; Wada *et al.*, 1997). This change in conformation affects both apo- and holotransferrin. With the apotransferrin, the thermodynamic equilibrium is reached very rapidly, whereas with hololactoferrin the final equilibrated state is attained a few thousand seconds later after iron loss (Figure 1).

This implies, as already reported, that the iron load protects the transferrin from denaturation (Harrington *et al.*, 1987; Harrington, 1992). Indeed, hololactoferrin keeps its iron-binding capacities even after mild tryptic digestion (Legrand *et al.*, 1984). Whether synergistic carbonate loss occurs during these two rapid processes is impossible to detect. However, since decarbonation is essential for iron loss from serum transferrin and ovotransferrin (El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999; MacGillivray *et al.*, 1998), it can occur during these two first steps. The rapid change of conformation of monoferric or iron-saturated lactoferrin is followed by two proton transfers and accompanied by the apparent unveiling of two specific sites of interaction with anions (Table 3, Figure 5). The protein interacts then with two monoanions or one dianion, which indicates two specific sites of electrostatic interaction. These slow interactions point to a kinetic control by conformational changes affecting the entire protein. The kinetic product generated at the end of this process from the holo- or the monoferric protein loses the load of Fe(III), undergoes two rate-limiting proton transfers and finally loses its interaction with the anions. Classical proton transfers are usually diffusion-controlled and occur in the microsecond range (Eigen & DeMayer, 1963;
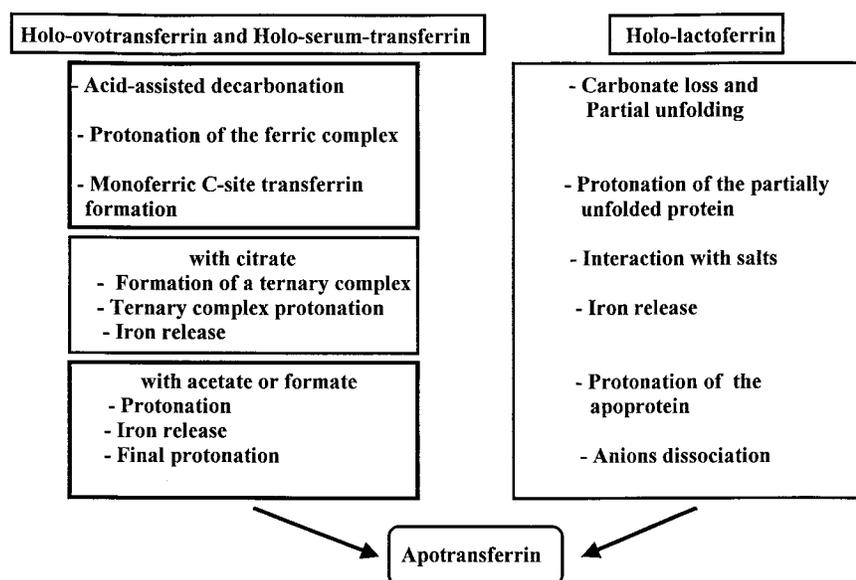

Figure 5. A simplified approach to the mechanisms of iron release by serum transferrin, ovotransferrin and lactoferrin at $\mu \leqslant 0.5$.

Table 3. The mechanism of iron release by hololactoferrin

Reaction		Description
$LT''Fe_2 \rightleftharpoons LT'Fe_2$	(1)	Rapid change in conformation
$LT'Fe_2 \rightleftharpoons LTFe_2$	(2)	Rapid change in conformation
$LTFe_2 + 2H^+ \rightleftharpoons LTH_2Fe_2$	(5)	Gain of two protons
$LTH_2Fe_2 + nS \rightleftharpoons LTS_nH_2Fe_2$	(6)	Interaction with two negative charges
$LTS_nH_2 + 2Fe \rightleftharpoons LTS_nH_2Fe_2$	(7)	Iron loss
$LTS_nH_2 + 2H^+ \rightleftharpoons LTS_nH_4$	(8)	New gain of two protons
$LTH_4 + nS \rightleftharpoons LTS_nH_4$	(9)	Loss of interaction with anions

Bernasconi, 1976). We assume, therefore, that the observed slow proton transfers are under the kinetic control of conformational changes in the protein. This was already proposed for the slow proton transfers observed during Fe(III) loss from serum transferrin and ovotransferrin (El Hage Chahine & Fain, 1994; El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999).

In neutral and mildly acidic media, iron release from serum transferrin and ovotransferrin is independent of ionic strength for $\mu \leqslant 0.5$ (El Hage Chahine & Fain, 1994; El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999). However, for the separate N-lobe of ovotransferrin, and when it occurs with the presence of competing ligand in neutral media at higher ionic strength, this release becomes dependent on ionic strength and on the nature of the salt involved (Muralidhara & Hirose, 2000; Zak et al., 1997; Kretchmar & Raymond, 1988; Foley & Bates, 1988; Williams et al., 1982). In the case of lactoferrin, iron release is more dependent on the charge of the inorganic anion than on its nature. Indeed, the affinities of the protein for the three monoanions Cl^-, Br^- and NO_3^- are of the same order of magnitude, whereas the affinity for the dianion is about one order of magnitude higher (Table 2). With K^+ and Na^+, the salt effect is independent of the nature of the cation and occurs on two specific sites. We are not, therefore, dealing here with a non-specific chaotropic effect of ions such as that of Hoffmeister (Cacace et al., 1997). With serum transferrin and ovotransferrin at high ionic strength, the nature of the anion plays a role in enhancing iron release from one of the sites while not affecting or inhibiting this release from the other site (Williams et al., 1982; Zak et al., 1997). We did not observe this with lactoferrin, probably because the fast change in conformation of the protein can render the two anion-binding sites equally accessible. Two anion-binding sites in serum transferrin, ovotransferrin and lactoferrin were reported (Chasteen et al., 1994). Moreover, with lactoferrin, the affinity of these sites for Cl^- is very similar to that reported in Table 2 for the same anion (Chasteen et al., 1994). Does this imply that these anion-binding sites are common to all transferrins? This question cannot be answered at this stage. With lactoferrin, these anion-binding sites promote the release of iron. These kinetically active binding sites (KISAB)

were proposed for iron release from serum transferrin at pH 5.6 (Zak et al., 1997). In all this, however, there is still a missing link. This link can be provided by the fact that at high ionic strength, proteins can undergo modifications in their conformation that can be strongly affected by ionic strength (Fless et al., 2000; Stevens et al., 2000). The salt effect on iron release by serum transferrin is observed at high ionic strength (Williams et al., 1982), where we believe that the conformation of the protein is probably altered, partially unveiling the anion-binding sites. This situation is not normally required for iron release from serum transferrin and ovotransferrin at $\mu \leqslant 0.5$ in mildly acidic media (El Hage Chahine & Fain, 1994; El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999). It is imperative, however, for iron release from lactoferrin. This protein is not involved in iron transport and does not lose its iron charge under the same conditions where the iron is lost from ovotransferrin and serum transferrin. We believe, therefore, that iron loss from transferrins can occur by two different mechanisms, that established without the obvious intervention of KISAB (El Hage Chahine & Fain, 1994; El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999) and what we report here for lactoferrin (Figure 5). This mechanism would occur with the other transferrins under extreme conditions of ionic strength and also at very low pH values. This, of course, remains to be investigated.

Transferrins possess extremely high topological similarities and identical iron-binding sites (Jeffrey et al., 1998; Moore et al., 1997; Kurokawa et al., 1995; Zuccola, 1992). Why then does lactoferrin need to rapidly change its conformation and to interact with ions in order to lose iron while ovotransferrin and serum transferrin can behave otherwise? This may be the consequence of the three main slight differences between these three transferrins. The first concerns the structures of the amino acid chain linking the C and the N-lobes of the protein. These chains are α-helix in lactoferrins and β-sheets in serum transferrins and ovotransferrins (Zuccola, 1992; Moore et al., 1997; Kurokawa et al., 1999). We showed that this interlobe chain might play a role in transmitting the cooperativity between the two iron-binding sites upon iron uptake (Pakdaman et al., 1998). The second con-
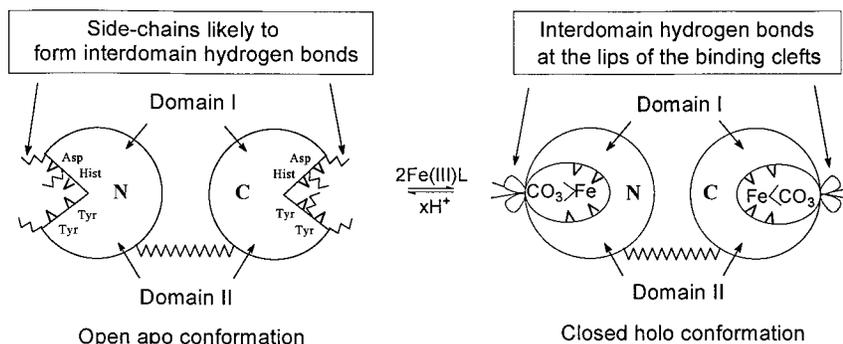
Figure 6. Schematic view of the modifications in the conformation of transferrin when it goes from an open apotransferrin to a closed holotransferrin with the occurrence of interdomain H-bonds at the lips of the Fe(III) binding clefts. x is the number of protons lost during iron uptake and Fe(III)L is the iron donor chelate (Bou Abdallah & El Hage Chahine, 1998).

cerns small discrepancies in the number of disulfide bridges involved in each protein (Aisen, 1998). Finally, the third difference concerns the interdomain hydrogen-bonding network in holotransferrins (Moore *et al.*, 1998; Kurokawa *et al.*, 1995; Dewan *et al.*, 1993). Each lobe of a transferrin consists of two domains (Figure 6). When the proteins are in the apo form, the lobes can be in an open conformation, rendering the iron-binding ligands accessible to the outside medium (Kurokawa *et al.*, 1999). When the protein interacts with iron, the conformation of the lobes goes from open to closed and iron becomes inaccessible to the outside medium at about 10 Å from the protein surface (Kurokawa *et al.*, 1995; Jeffrey *et al.*, 1998; Moore *et al.*, 1997; Zuccola, 1992). During this modification in conformation, an interdomain hydrogen bonding network occurs between the two domains of each lobe, further consolidating the conformation of the holoprotein (Figure 6). A hydrogen bond is usually weak (Zundel, 1976), whereas metal co-ordination bonds can be very strong (Wilkins, 1976). Therefore, stabilisation of the main conformation in transferrins is almost certainly achieved by the metal co-ordination bonds. Nevertheless, the interdomain hydrogen bonds, especially when situated at the lips of the iron-binding cleft, can control the access of water from the outside medium and constitute a trigger for the cleft opening (Figure 6, Dewan *et al.*, 1993). Breaking these weak hydrogen bonds may, therefore, be necessary for iron release (Bou Abdallah & El Hage Chahine, 1998, 1999). In the iron-conveyors ovotransferrin and serum transferrin, one of the constituents involved in each of the interdomain hydrogen bonds, such as Asp, Glu and Lys, can be easily protonated. On the other hand, in bovine lactoferrin, at least one of the interdomain hydrogen bonds in the N-site and three in the C-site occur between two amino acid side-chains that cannot be protonated, such as Ser, Asn, Gln and Thr (Dewan *et al.*, 1993; Moore *et al.*, 1997; Kurokawa *et al.*, 1995). We can, therefore, cautiously imagine that in ovotransferrin and serum transferrin, lowering the pH leads to the protonation of the side-chains involved in the interdomain hydrogen bonding network. Such a protonation would very probably break these bonds (Zundel,

1976). This gives the outside medium access to the binding cleft, allowing iron to be released according to the mechanism reported for ovotransferrin and serum transferrin (Figure 5, El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999). In contrast, with the iron-scavenger bovine lactoferrin, lowering the pH may have less effect on the interdomain hydrogen bonding networks. The holoprotein would undergo, then, a prior change in conformation to allow iron to be released by the mechanism we are proposing here (Figure 5). This mechanism does not at all exclude the opening of the binding cleft to allow metal release. It may, however, involve different changes in conformation than those already proposed for serum transferrin and ovotransferrin (El Hage Chahine & Pakdaman, 1995; Bou Abdallah & El Hage Chahine, 1999).

Conclusion

In vitro, iron loss from hololactoferrin occurs at pH < 3.5, involves a fast change in the conformation of the protein and requires two specific interactions with the anions of the medium. At high salt concentration ($\mu > 0.5$), the iron-transport proteins ovotransferrin and serum transferrin can lose iron by a similar mechanism, involving interaction with the salt. However, under milder conditions with low salt concentration ($\mu < 0.5$), the loss of iron from these two proteins involves proton-assisted decarbonation of the binding clefts, and protonation of the interdomain hydrogen bonds and that of the protein ligands. Under these mild conditions of acidity and ionic strength, lactoferrin retains its iron load. The affinities of ovotransferrin, lactoferrin and serum transferrin for iron are of the same order of magnitude (Pakdaman & El Hage Chahine, 1996; Pakdaman *et al.*, 1998; Bou Abdallah & El Hage Chahine, 1999). Since the 1940s, these proteins are known to be bacteriostatic in neutral media (Schade & Caroline, 1944, 1946). The bacteriostatic effect is related to their capacity to bind iron and to keep it (Aisen, 1998). However, ovotransferrin and serum transferrin start losing their iron in the vicinity of pH 6.5. This seems logical, since these proteins are destined to deliver iron by receptor-mediated endocy-

tosis in mildly acidic endosomes (Dautry-Varsat *et al.*, 1982). Therefore, at the pH of milk (pH 6.5) where lactoferrin was first isolated (Montreuil *et al.*, 1960; Johanson, 1960; Groves, 1960), the affinities of ovotransferrin and serum transferrin for iron are decreased, thereby diminishing the bacteriostatic effect. In contrast, lactoferrin keeps its iron load in acidic media and thereby keeps its bacteriostatic capacity.

Materials and Methods

KCl, NaCl, KNO$_3$, and NaBr, (Merck extra pure), NaOH, and HCl (Merck Titrisol), EDTA (Merck Titriplex), FeCl$_3$, sodium carbonate (Normapur) and acetic acid (100 %, Merck) K$_2$SO$_4$, Na$_2$SO$_4$, nitrilotriacetic acid and Hepes (Aldrich), glycerol, urea, SDS and boric acid (Sigma), acrylamide and APS (Boehringer Mannheim), bromophenol blue (BioRad), Coomassie blue (Biowittaker, France) and TEMED (Promega) were used without further purification. Water and glassware were prepared as described (El Hage Chahine & Fain, 1993).

Protein preparation and purification

Only one bovine lactoferrin lot from Sigma (75H3817) was used in all the reported experiments. The holo- and the *C*-site iron-loaded protein were prepared and purified according to published procedures (Princiotto & Zapolski, 1975; Makey & Seal, 1976; Chasteen & Williams, 1981; El Hage Chahine & Pakdaman, 1995). The protein was purified and its iron load and purity checked by spectrophotometric analysis and by urea- and SDS/polyacrylamide gel electrophoresis as described (Pakdaman *et al.*, 1998; Makey & Seal, 1976).

Stock solutions

All stock solutions were used fresh. The sodium chloride, sodium bromide and sodium nitrate, and the potassium chloride and potassium nitrate concentrations varied from 20 to 500 mM, whereas those of sodium or potassium sulfate ranged from 20 to 120 mM. For acidic solutions (pH < 3.5), the use of a buffering salt was not required, whereas neutral solutions (pH ≈ 7.4) were slightly buffered with 3 mM Hepes. The pH was adjusted with microquantities of concentrated HCl and NaOH. The protein concentrations (c_1) ranged from 0.8 to 125 μM.

pH measurements

pH values were measured as described at 25($\pm$0.1) °C with a Jenco pH meter equipped with an Ingold calomel/glass combined microelectrode (Pakdaman *et al.*, 1998). Buffers used for pH standardisation were pH 7.00 and 10.01 (Sigma).

Spectrophotometric measurements

Spectrophotometric measurements were performed on Perkin Elmer lambda 2 and Aminco-Bowman series 2 luminescence spectrometers. Both apparatuses were equipped with a thermostatically controlled cell-carrier with the sample-cell temperature maintained at 25($\pm$0.1) °C.

Stopped-flow measurements

Fast kinetic measurements were performed on an SF 3L Hi-Tech stopped-flow spectrophotometer equipped with a thermostatically controlled bath at 25($\pm$0.5) °C, by mixing apo, iron-saturated or unsaturated lactoferrin in the neutral salt solutions with acidic solution of identical salt concentrations and ionic strength. The apparatus was equipped with two independent light sources and monochromators as described (Bou Abdallah & El Hage Chahine, 1999).

Mathematical formalism and signal analysis

The experimental conditions were set so as to permit the use of chemical relaxation formalism (Brouillard, 1980). All experimental signals were analysed as described (El Hage Chahine & Fain, 1993, 1994). They all were pure mono- or multiexponentials and were dealt with as relaxation modes (Eigen & DeMaeyer, 1993; Bernasconi, 1976; Brouillard, 1980).

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Appendix

All the reciprocal relaxation time equations used here were derived by the substitutions method (Eigen & DeMayer, 1963; Bernasconi, 1976).

Under our experimental conditions, [S] and $[H^+] \gg c_1$. They can, therefore, be considered constant during each kinetic run. This implies that their variation $\Delta[S]$ and $\Delta[H^+] \approx 0$.

Derivation of equation (8)

The reciprocal relaxation kinetic equation associated with equation (4) when considered rate-limiting is expressed as:

$$-\mathrm{d}\Delta[LTFe_2]/\mathrm{d}t = k_1'[S]^n\Delta[LTFe_2] - k_{-1}'\Delta[LTS_nFe_2]$$
(17)

The conservation of mass allows us to write:

$$\Delta[LTFe_2] + \Delta[LTS_nFe_2] + \Delta[LTS_nH_lFe_2] = 0 \quad (18)$$

The constant state of equilibrium of equation (5) can be expressed as:

$$\Delta[LTS_nH_lFe_2] = [H^+]^l\Delta[LTS_nFe_2]/K_{1a}' \quad (19)$$

Equation (8) is determined from equations (17) to (19).

Derivation of equation (9)

The reciprocal relaxation kinetic equation associated with equation (5) when considered rate-limiting is expressed as:

$$-\mathrm{d}\Delta[LTS_nH_lFe_2]/\mathrm{d}t = k_{-1}\Delta[LTS_nH_lFe_2] - k_1[S]^n\Delta[LTH_lFe_2] \quad (20)$$

The conservation of mass allows us to write:

$$\Delta[LTS_nH_lFe_2] + \Delta[LTH_lFe_2] + \Delta[LTFe_2] = 0 \quad (21)$$

The constant state of equilibrium of equation (6) can be expressed as:

$$\Delta[LTH_lFe_2] = [H^+]^l\Delta[LTFe_2]/K_{1a} \quad (22)$$

Equation (9) is determined from equations (20) to (22).

Derivation of equations (13) to (16)

The reciprocal relaxation times equations associated with equations (10), (11) and (12) when each is assumed rate-limiting can be expressed as, respectively:

$$-\mathrm{d}\Delta[LTSH_2Fe]/\mathrm{d}t = k_{-2}'\Delta[LTS_nH_2Fe_2] - k_2'(2[Fe][LTS_nH_2]\Delta[Fe] + [Fe]^2\Delta[LTS_nH_2]) \quad (23)$$

$$-\mathrm{d}\Delta[LTH_{(m+2)}]/\mathrm{d}t = k_{-2}\Delta[LTS_nH_{(m+2)}] - k_2[H^+]^m\Delta[LTS_nH_2] \quad (24)$$

$$-\mathrm{d}\Delta[LTH_{(m+2)}]/\mathrm{d}t = -k_{-2}''\Delta[LTS_nH_{(m+2)}] + k_2''[S]^n\Delta[LTH_{(m+2)}] \quad (25)$$

The conservation of mass allows us to write:

$$\Delta[LTS_nH_2Fe_2] + \Delta[LTS_nH_2] + \Delta[LTS_nH_{(m+2)}] + \Delta[LTH_{(m+2)}] \quad (26)$$

The constant states of equilibrium of equations (7) and (9) allow us to write:

$$\Delta[\text{LTS}_n\text{H}_2\text{Fe}_2] = 2[\text{LTS}_n\text{H}_2][\text{Fe}]\Delta[\text{Fe}^{3+}]/K_{\text{Fe}}$$
$$+ [\text{Fe}^{3+}]^2\Delta[\text{LTS}_n\text{H}_2]/K_{\text{Fe}} \qquad (27)$$

$$\Delta[\text{LTS}_n\text{H}_{(m+2)}] = [\text{H}^+]^m\Delta[\text{LTS}_n\text{H}_2]/K_{2a} \qquad (28)$$

$$\Delta[\text{LTS}_n\text{H}_{(m+2)}] = [\text{S}]^n\Delta[\text{LTH}_{(m+2)}]/K_2 \qquad (29)$$

Equation (13) is determined from equations (23), (26), (28) and (29), equation (14) is determined from equations (24), (26), (27) and (29), and finally equation (15) is determined from equations (25) to (28).

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