Kinetics and Mechanism of Iron(III) Removal from Citrate by Desferrioxamine B and 3-Hydroxy-1,2-dimethyl-4-pyridone

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Abstract: The second-order rate constants for iron removal from citrate by the chelating agents desferrioxamine B (DFO) and 3-hydroxy-1,2-dimethyl-4-pyridone (L1) were determined. The overall rate constant k_{on} for the transfer of Fe from citrate to the chelator is 4 M^{-1} s^{-1} for DFO and 43 M^{-1} s^{-1} for L1 at pH 7.4 and 37 °C. The kinetics of transfer of iron from citrate to the two chelators was examined in detail, and a possible mechanism is proposed. Both DFO and L1 form a mixed complex with citrate–iron prior to the transfer of the metal ion. The dissociation equilibrium constant of this initial complex is 10 and 0.45 mM for DFO and L1, respectively. The first-order rate constant for its dissociation into free citrate and chelator–Fe is 0.04 and 0.02 s^{-1} for DFO and L1, respectively. These results indicate that, at concentrations close to what is achieved *in vivo* during chelation therapy (10–100 μM), although DFO has a thermodynamic advantage over L1, the latter acts 10 times faster than DFO in mobilizing citrate-bound iron. This large difference in the kinetics of iron removal is mainly due to the difference in the equilibrium dissociation constant of the initial complex between the chelator and Fe–citrate. The ability of a chelating agent to make a transitory complex with the iron bound to its biological carrier seems to be a major determinant in the kinetics of transfer. Our data show that the reaction pathway for the removal of Fe from citrate by DFO and L1 is different from what was observed with transferrin. Unlike transferrin, L1 (and to a lesser extent DFO) is able to directly interact with the polymeric iron–citrate; thus, depolymerization of Fe–citrate is not the rate-limiting step in the kinetics of transfer.

Introduction

Desferrioxamine (Desferal) is a trihydroxamic acid with a high affinity for ferric iron. It is the most widely used chelating agent for the treatment of Fe-overload diseases such as β-thalassemia. However, desferrioxamine (DFO) suffers from some disadvantages (requires subcutaneous administration, short plasma half-life), and considerable effort is being expanded toward developing orally active substitutes for this compound. A lot of attention was focused on hydroxypyridone derivatives due to their ability to form strong complexes with Fe^{3+} and their good oral bioavailability. Among this series of compounds, 3-hydroxy-1,2-dimethyl-4-pyridone (L1, CP 20, DMHP) has received the most attention, and numerous studies aimed at comparing the decorporation of Fe by DFO and L1 have been undertaken. The structures of DFO and L1 are presented in Figure 1.

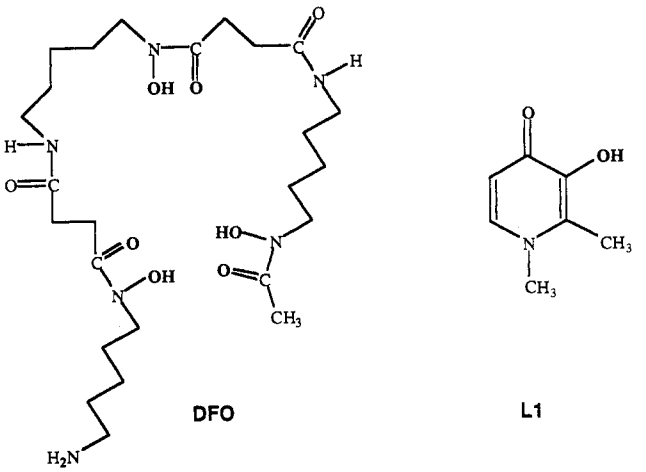

Figure 1. Structures of DFO and L1.

Citrate is present in blood plasma at a concentration of 100 μM (and even higher in certain Fe-overloaded patients) and is believed to be one of the major components of the non-transferrin-bound iron (NTBI) pool that binds Fe when transferrin is fully saturated.[1,2] NTBI is generally considered a harmful source of Fe because the molecules which Fe is bound to are not able to completely neutralize Fe. Such incomplete chelates may promote the formation of free radicals *via* Fenton reactions, leading to tissue damage.[3,4] When Fe^{3+} and citrate are reacted *in vitro* at physiologic pH, a spherical polymer of MW 2.1×10^5 with a diameter of about 75 Å is formed.[5] This polymer consists of hydroxy–iron citrate, with one sphere containing about 1.5×10^3 atoms of Fe. The presence of hydroxy–iron citrate at neutral pH was later confirmed in 1986 by R. B. Martin,[6] who described the species distribution of Fe–citrate *vs* pH. It has been proposed that this ferric-citrate has similarities in structure with Fe in the ferritin core, the major Fe storage protein in biological systems.[5] Ferritin consists of a protein shell of external diameter 100–120 Å, made of 24 subunits (H and L chains), which encloses an internal cavity of 80 Å containing a maximum of 4500 atoms of Fe as ferrihydrite associated with variable amounts of phosphate.[7]

A continuous infusion of large amounts of Desferal is necessary to reach a negative Fe balance in Fe-overaloaded patients.[8] To efficiently induce the excretion of Fe, a chelator has to overcome not only a thermodynamic barrier (competition with other ligands for the binding of Fe) but also a pharmacokinetic barrier (chelator–Fe complex has to be formed before the elimination or

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Scheme 1

$$\text{Cit–Fe} + \text{DFO} \xrightarrow{k_{on}} \text{DFO–Fe} + \text{Cit}$$

degradation of the chelating agent). It has been suggested that part of the relatively poor efficiency of the drug stems from its slow kinetics of Fe removal.[9,10] However, no correlation has been found between the affinity of ligands and their kinetic ability to remove Fe. It has been shown that L1 mobilizes ferritin–Fe faster than DFO[11,12] despite the superior affinity for Fe of DFO over L1 under physiological conditions.[13] In the present study we compared the kinetic pathways for Fe removal from citrate by DFO and L1 and determined which step is rate-limiting in the kinetics of transfer.

Experimental Section

DFO and L1 were produced at Ciba. Citric acid and ferric chloride were purchased from Merck. All experiments were performed in HEPES 50 mM, 100 mM NaCl, pH 7.4.

Kinetics were recorded with a Uvikon 820 spectrophotometer (Kontron Instruments) equipped with a thermostated cell holder and a circulating water bath. Absorbance *vs* time pairs were automatically transferred to a microcomputer (Macintosh, Apple). Nonlinear regression analyses were performed using Ultrafit v 2.0 (Biosoft). Spectra were recorded with a Beckman DU-7400 diode array spectrophotometer. Fast kinetics were recorded with an SF/PQ 53 stopped-flow apparatus (Hi-Tech Scientific) on line with a Hewlett-Packard microcomputer Series 9000 Model 300.

Preparation of Fe–Citrate. Ferric chloride (2 mM, final concentration) was added to a 10 mM solution of citric acid in nanopure water. This solution was freshly prepared before each set of experiments. An aliquot (10–50 μL) was then transferred to 1 mL (final volume) of a buffered solution (pH 7.4) and incubated for 30 min at 37 °C prior to the addition of the chelator.

Results

Kinetics of Transfer of Fe from Citrate to the Hexadentate Chelator DFO. The formation of the DFO–Fe complex was monitored at 425 nm.[14,15] Within the range of concentrations used in our experiments, Fe–citrate does not significantly absorb at wavelengths higher than 420 nm. Change of absorbance at 425 nm with time thus directly measures the formation of DFO–Fe complexes. Scheme 1 shows the kinetics of transfer of citrate-bound Fe to DFO, with k_{on} being the second-order rate constant for the transfer of Fe.

In a first set of experiments Fe–citrate (1:5 mol/mol, 0.10 mM in Fe) was reacted with a 10-fold molar excess of DFO over Fe. After an initial, fast absorbance jump, which accounted for 10–15% of the total absorbance change, the reaction followed first-order kinetics, as depicted in Figure 2A. The fractional contribution of each phase could be accurately determined by extrapolating the second phase back to zero time. The presence of an initial, rapid absorbance jump was already reported in the kinetics of exchange between transferrin and (i) Fe-citrate[16] and (ii) Fe–NTA (nitrilotriacetic acid).[17] The simplest explanation for this phenomenon lies in the complex equilibria between monomeric and polymeric Fe^{3+}–citrate species. The initial burst in absorbance probably results from the presence of small amounts

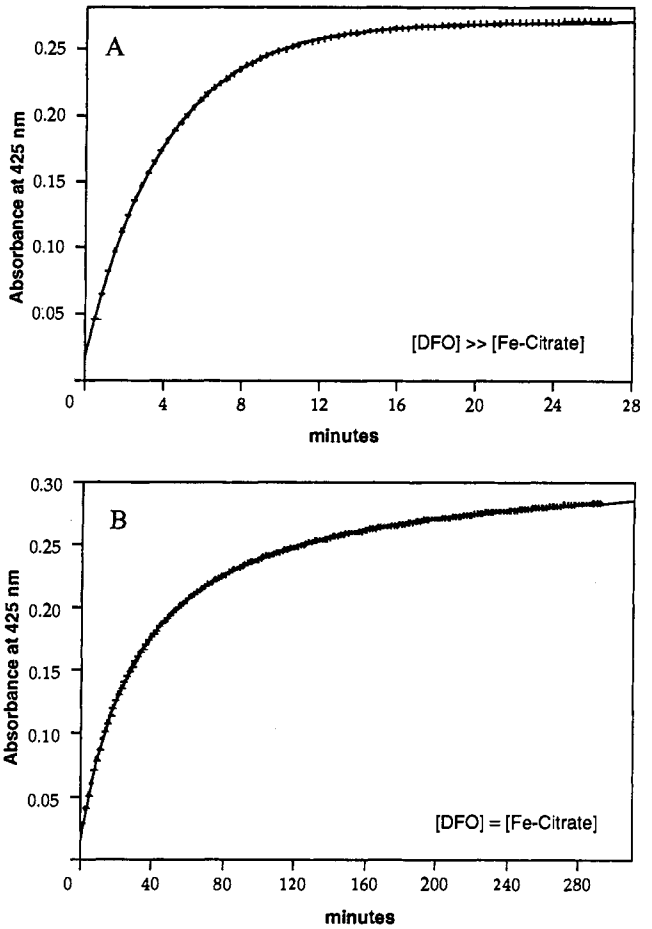

Figure 2. Kinetics of transfer of citrate-bound Fe to DFO. (A) [DFO] ≫ [Fe–citrate]. DFO (1 mM, final concentration) was added to a buffered solution of Fe–citrate (0.1:0.5 mM), and the increase of absorbance at 425 nm was recorded with time. The crosses indicate the experimental absorbance/time pairs. The solid line is the fitted curve generated using a nonlinear regression analysis procedure based on eq 1 with $k = 4 \times 10^{-3}$ s^{-1}. (B) [DFO] = [Fe–citrate]. The kinetics of transfer were measured using the same conditions as in A except that equal concentrations of DFO and Fe–citrate were used (0.1 mM). The best fit (solid line) to the experimental data (crosses) was obtained using eq 2 and $k_{on} = 1.58 \times 10^{-3}$ OD^{-1} s^{-1}.

of highly reactive mononuclear species having coordination sites available for chelation. The theoretical curve used to fit the second phase is shown in Figure 2A and was generated using the following equation:

$$(OD)_t = (OD_{eq} - OD_0)(1 - \exp^{(-kt)}) + C \qquad (1)$$

where k is the pseudo-first-order rate constant ($k = k_{on}[DFO]_0$), OD_0 initial absorbance, OD_{eq} the absorbance at equilibrium, and C the offset. With a DFO concentration of 1 mM the best fit was obtained for $k = 4 \times 10^{-3}$ s^{-1}. In contrast to what was observed in the exchange of Fe between citrate and transferrin[16] the citrate: Fe ratio did not affect the kinetics of transfer (Table 1).

In a second set of experiments, Fe–citrate (0.1 mM) was reacted with an equimolar concentration of DFO. In this case, second-order kinetics were obeyed (Figure 2B), and the experimental data were well described by the following equation:

$$(OD)_t = \frac{(OD_{eq} - OD_0)^2 k_{on} t}{1 + (OD_{eq} - OD_0) k_{on} t} + C \qquad (2)$$

where k_{on} is the second-order rate constant for the transfer (Scheme 1) and C the offset. The best fit was obtained for $k_{on} = 1.58 \times 10^{-3}$ OD^{-1} s^{-1}. The shift from first-order to second-

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Table 1. Influence of Citrate:Fe Ratio on the Kinetics of Transfer[a]

	cit:Fe ratio				
	2	5 (*)	10	20	40
			DFO		
k (s^{-1})	5.8×10^{-3}	6.4×10^{-3}	6.2×10^{-3}	5.7×10^{-3}	5.05×10^{-3}
amp (%)	4.5	10.1	11.4	17.1	28.4
			L1		
k (s^{-1})	1.25×10^{-2}	1.29×10^{-2}	1.22×10^{-2}	1.18×10^{-2}	1.15×10^{-2}
amp (%)	17	18.3	21.5	26.5	35.6

[a] [Fe] was kept constant (0.02 mM) while the citrate concentration was varied (0.04–0.8 mM). Chelator concentration was fixed at 2 mM (DFO series) and 1 mM (L1 series). The asterisk indicates the citrate:Fe ratio used in the kinetic experiments presented in this work. Amp (%) refers to the amplitude of the initial OD jump as a percentage of the total absorbance change. The numbers presented in this table are the average of three experiments.

order kinetics when $[\text{Fe–citrate}]_0 = [\text{DFO}]_0$ indicates that the transfer of Fe from citrate to DFO is not governed by the dissociation of Fe–citrate. Using a molar extinction coefficient of 2700 M^{-1} cm^{-1} (at 425 nm), a second-order rate constant (k_{on}) of 4.3 M^{-1} s^{-1} was calculated. This value compares favorably with the second-order rate constant obtained from the previous experiment performed under pseudo-first-order conditions (k was 4.10^{-3} s^{-1} for 1 mM DFO), from which $k_{on} = k/[\text{DFO}]_0 = 4$ M^{-1} s^{-1}.

Kinetics of Transfer of Fe from Citrate to the Bidentate Chelator L1. Fe–$(L1)_3$ is the only iron–ligand complex present at physiological pH within the concentration range used,[13] The transfer of citrate-bound Fe to L1 was monitored at 450 nm, where the Fe–$(L1)_3$ complex can be detected spectrophotometrically.[18]

As already observed with DFO, the kinetics of transfer of citrate-bound Fe to L1 decomposes into an initial rapid increase of absorbance, followed by a second phase with a larger amplitude (Figure 3). When Fe–citrate and L1 were reacted under pseudo-first-order conditions (at least a 10-fold excess of L1 over Fe–citrate), the second phase followed a simple exponential, as depicted in Figure 3A. The absorbance *vs* time curve was analyzed using eq 1, and the best theoretical curve was obtained for $k = 8.15 \times 10^{-3}$ s^{-1} (for L1 = 0.3 mM), from which a second-order rate constant (k_{on}, Scheme 2) of 27.15 M^{-1} s^{-1} was calculated. The reason we expressed k_{on} in M^{-1} s^{-1} despite the 3:1 stoichiometry of association is explained further in the text (see Scheme 4). As with DFO, variation of the citrate:Fe ratio did not significantly affect the kinetics of transfer (Table 1).

In a second set of experiments, the L1 concentration was lowered to 0.06 mM and reacted with an equimolar concentration (in iron-binding sites) of Fe–citrate (0.02 mM in Fe). Under these conditions, the kinetics deviated from first-order but could not be properly described as a simple second-order reaction (eq 2). Species distribution was calculated according to the stability constants measured by Motekaitis and Martell.[18] It showed that the displacement of citrate-bound Fe by 0.06 mM L1 cannot be expected to be complete and that about 5% of the total Fe remains as Fe–citrate. Eq 2, which describes irreversible (or pseudoirreversible) second-order kinetics, therefore does not apply in this situation. The simplest reaction scheme that best accounts for this exchange process is shown in Scheme 2. The data were reanalyzed using eq 3, which describes a second-order reaction in the forward direction and a first-order process in the reverse direction (the reverse reaction is expected to be pseudo-first-order since [free citrate] ≫ $[(L1)_3\text{–Fe}]$).

$$OD_t = \frac{E(\exp^{Ft} - 1)}{\exp^{Ft} - E^2/A^2} + C \qquad (3)$$

with

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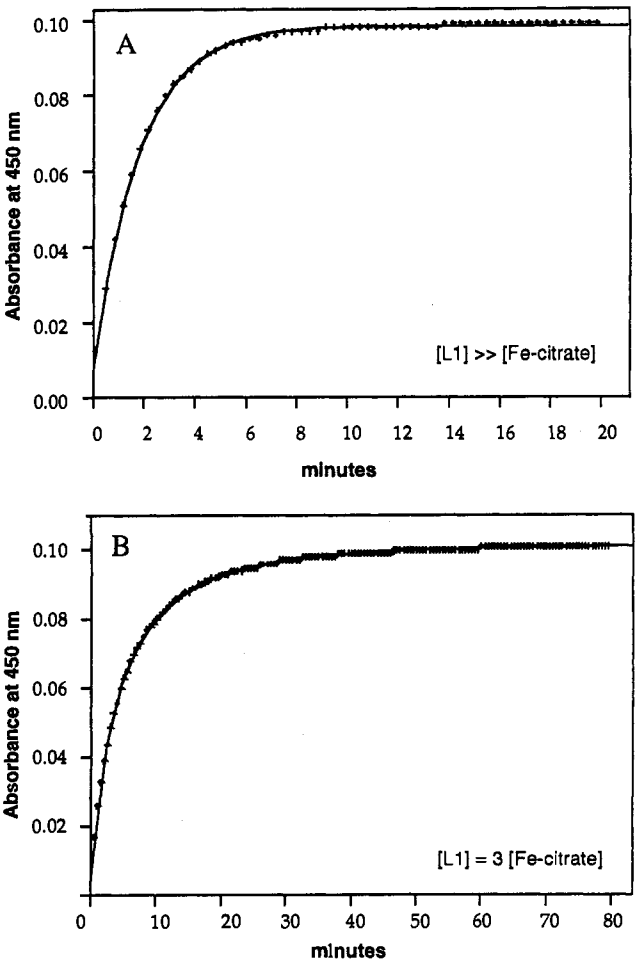

Figure 3. Kinetics of transfer of citrate-bound Fe to L1. (A) [L1] ≫ [Fe–citrate]. L1 (0.3 mM, final concentration) was added to a buffered solution of Fe–citrate (0.02/0.1 mM), and the increase of absorbance at 450 nm was recorded with time. The best fit (solid line) to the experimental data (crosses) was obtained using eq 1 with $k = 8.1 \times 10^{-3}$ s^{-1}. (B) [L1] = [Fe–citrate]. The kinetics of transfer were measured under conditions similar to A except that the end concentrations of Fe–citrate and L1 were equal in iron-binding sites (0.02 mM Fe–citrate and 0.06 mM L1). The best fit (solid line) to the experimental data (crosses) was obtained using eq 3 with $A = 0.11$, $E = 0.095$, and $k_{on} = 3.38 \times 10^{-2}$ OD^{-1} s^{-1}.

Scheme 2

$$\text{Fe–Cit} + 3\text{Li} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} (L1)_3\text{–Fe} + \text{Cit}$$

$$F = \frac{k_{on}(A^2 - E^2)t}{E}$$

E being $OD_{eq} - OD_0$, A the maximum absorbance change for a complete transfer of Fe from citrate to L1, and C the offset.

k_{off} can be calculated from k_{on}, A, and E using the following relation:

$$k_{off} = \frac{k_{on}(A - E)^2}{E} \qquad (4)$$

The best fit was obtained for $k_{on} = 3.38$ 10^{-2} $OD^{-1} \times s^{-1}$, $A = 0.11$, and $E = 0.095$ (Figure 3B). A k_{off} value of 7.5×10^{-5} s^{-1} was calculated using eq 4. Using a molar extinction coefficient of 5000 M^{-1} cm^{-1}, a second-order rate constant of 55 M^{-1} s^{-1} (k_{on}) was calculated for the forward reaction. This value is somewhat higher than the one previously obtained under pseudo-first-order conditions (27.15 M^{-1} s^{-1}). A second-order rate constant for the reverse reaction (k_{off}) of 0.75 M^{-1} s^{-1} was calculated from the pseudo-first-order rate constant.

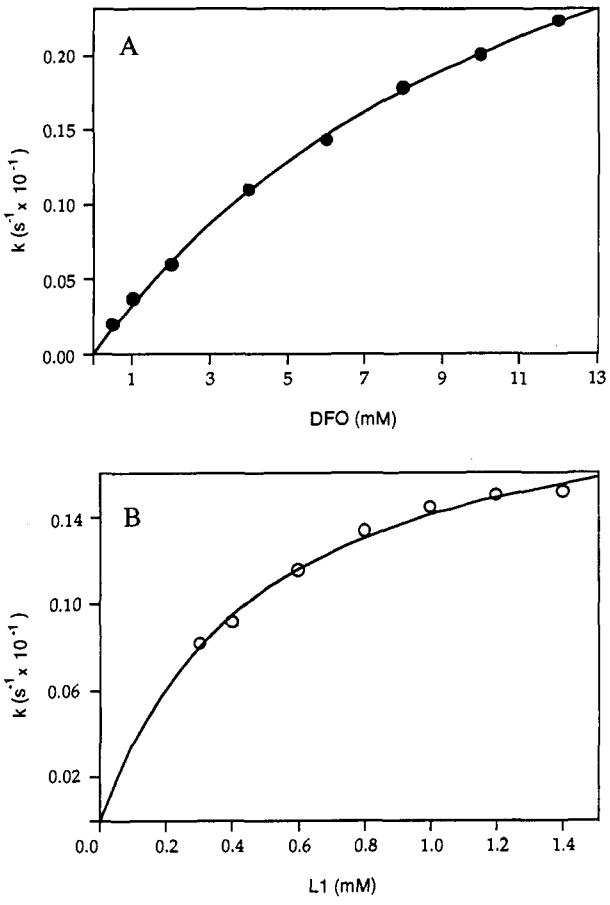

Figure 4. Concentration dependence of the kinetics of transfer. The variation of k, the pseudo-first order rate constant for the exchange reaction, was studied over a broad range of DFO (A) and L1 (B) concentrations. The solid line represents the best fit to the experimental data which were obtained using eq 5 and the values summarized in Table 2.

Concentration Dependence of the Kinetics of Transfer. The kinetics of transfer of Fe from citrate to DFO and to L1 were studied under pseudo-first-order conditions over a broad range of chelator concentrations (from 0.3 to 1.4 mM for L1 and from 0.5 to 12 mM for DFO). At all chelator concentrations first-order kinetics were observed (eq 1) and a pseudo-first-order rate constant k could be calculated. Variation of k with [DFO] and [L1] is presented in Figure 4, parts A and B, respectively. In both cases k increases with increasing chelator concentration, indicating that the rate of transfer of Fe from citrate to the chelator is concentration dependent. A similar observation was already reported for the kinetics of exchange of Fe between transferrin and (i) citrate and nitrilotriacetic acid,[16] (ii) EDTA,[19] (iii) tricatechols,[20] (iv) acetohydroxamic acid,[21] and (v) pyrophosphate.[22]

With both DFO and L1 a hyperbolic relationship between k and the chelator concentration was observed, indicating that the kinetics of exchange is a two-step process in which a second-order step is followed by a first-order step. This is well described by Scheme 3, assuming that the first step is rapid compared to the second (fast preequilibrium), where Cit–Fe is the initial Fe–citrate complex and C the incoming chelator. According to Scheme 3, the variation of k *vs* DFO and L1 can be described

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Scheme 3

$$\text{Cit–Fe} + \text{C} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Cit–Fe–C} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Cit} + \text{Fe–C}$$

Table 2. Kinetic Constants for the Mobilization of Citrate-Bound Fe by DFO and L1

	K^* (mM)	k_2 (s^{-1})	k_2/K^* (M^{-1} s^{-1})
DFO	10	0.040	4
L1	0.45	0.019	43

Scheme 4

$$2\text{L1} + \text{Fe–Cit} \rightleftharpoons \text{(fast)}$$

$$\text{L1–Fe–Cit} + \text{L1} \underset{k_{-1}\ \text{fast}}{\overset{k_1}{\rightleftharpoons}} \text{L1–Fe–Cit–L1} \underset{k_{-2}\ \text{slow}}{\overset{k_2}{\rightleftharpoons}} \text{L1–Fe–L1} + \text{Cit} \quad (a)$$

$$\text{L1} + \text{Fe–Cit} \underset{k_{-1}\ \text{fast}}{\overset{k_1}{\rightleftharpoons}} \text{L1–Fe–Cit–L1} \underset{k_{-2}\ \text{slow}}{\overset{k_2}{\rightleftharpoons}} \text{L1–Fe} + \text{Cit} + \text{2L1} \overset{\text{fast}}{\rightleftharpoons} \text{L1–Fe–L1} \quad (b)$$

by the following equation:

$$k = \frac{k_2\,[\text{C}]_0}{[\text{C}]_0 + K^*} + k_{-2} \quad \text{with} \quad K^* = k_{-1}/k_1 \qquad (5)$$

where $[\text{C}]_0$ is the initial chelator concentration and $k_{1,-1,2,-2}$ the rate constants according to Scheme 3. These constants are related to the overall constants k_{on} and k_{off} (Schemes 1 and 2) as follows: $k_{on} = k_2/K^*$ and $k_{off} = k_{-2}$. The solid lines shown in Figure 4 were obtained using a nonlinear regression analysis procedure based on eq 5. With both DFO and L1 the theoretical lines intercept at the origin, indicating that k_{-2} is small compared to $k_2\,[\text{C}]_0/K^*$ and can be neglected in eq 5 or that the transfer of Fe from citrate to the chelators was essentially pseudoirreversible under the conditions used in this set of experiments. The values obtained for k_2 and K^* are summarized in Table 2. Scheme 3 is qualitatively similar to that of Michaelis–Menten kinetics. The fact that the variation of k with [L1] obeyed such a reaction scheme indicates that the accumulation of the intermediate complex upon increasing chelator concentrations results from a stoichiometric (1:1) reaction between L1 and Fe–citrate.

However, based on our kinetic data we cannot distinguish between Scheme 4, parts a and b. The k_{on} value measured is indicated by the bold arrow. At high ligand concentration, for both DFO and L1 the rate-limiting step is the dissociation of citrate from the citrate–Fe–chelator ternary complex.

Stopped-Flow Trace for the Transfer of Citrate-Bound Fe to L1. A stopped-flow experiment was performed to measure the kinetics of transfer of citrate-bound Fe in the presence of a high ($>K^*$) concentration of L1 (5 mM). Here too, the reaction decomposes into two phases. The use of stopped-flow technology allows the detection of the fast phase (Figure 5), which was completed after 4 s. This explains why an offset occurred when manual mixing was employed (Figure 3). The amplitude of the first fast phase corresponds to the offset value previously obtained in Figure 3A. The second phase followed first-order kinetics, and the analysis of the data with eq 1 gave a rate constant k of

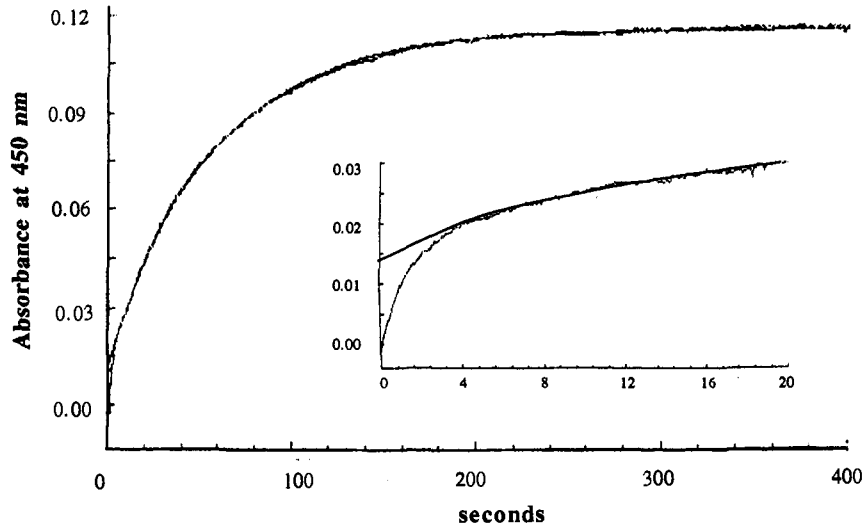


Figure 5. Stopped-flow trace for the transfer of citrate-bound Fe to L1. Syringes A and B were filled with Fe–citrate (0.04:0.2 mM) and 10 mM L1, respectively. For one run, each syringe delivered 100 μL. The variation of absorbance at 450 nm was monitored with time; 800 OD/time pairs were recorded per run. The reaction decomposes into two phases: a first fast phase (see inset) involving 5–10% of the total OD change, followed by a slower second phase of larger amplitude. The slow phase obeyed first-order kinetics, and the best fit was obtained using eq 1 with $k = 0.0177\ s^{-1}$.

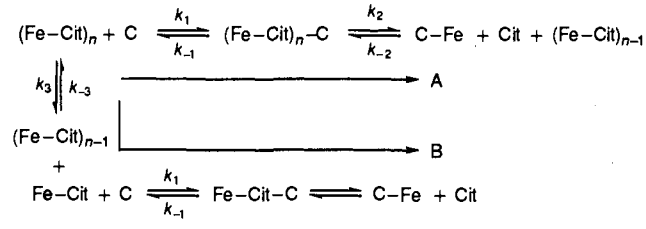
$0.0177\ s^{-1}$. This value is in good agreement with the value of k_2 calculated from the best fit of the k *vs* [L1] curve presented in Figure 3B ($0.019\ s^{-1}$).

Discussion

This work focused on kinetic aspects of Fe-chelation and is an attempt to understand the mechanism of removal of citrate-bound Fe by DFO and L1. The shift from first-order (excess of chelator over Fe–citrate) to second-order kinetics when equal concentrations (in Fe-binding sites) were reacted indicates that the reaction is not controlled by a dissociation (first-order) step. This finding was strengthened by the variation of k, the pseudo-first-order rate constant for the transfer of Fe from citrate to the competing ligand upon increasing chelator concentrations (Figure 4). While the reaction between 0.02 mM DFO and an equivalent concentration of Fe–citrate could be treated as a pseudoirreversible reaction, this was not true when an equivalent concentration (in Fe-binding sites) of L1 was employed. In this latter case, a small reverse reaction occurred, thus showing that L1 has a lower kinetic affinity for Fe than DFO, despite its higher stability constant ($10^{35.92}$ as compared to $10^{30.50}$). This confirms previous studies[13,18] and highlights the usefulness of the pM value (-log [free Fe^{3+}] in the presence of 1 μM Fe and 10 μM ligand at pH 7.4, although pM values can be calculated for other pH values and ligand concentrations). The pM concept was introduced some years ago by K. N. Raymond[23] to characterize and compare the affinity of chelating agents of different acidities and denticity under near physiological conditions. When DFO and L1 are compared with respect to their pM value (26.8 and 19.4, respectively), it becomes clear that DFO has a higher affinity for Fe, in agreement with what we report in the present paper.

Possible mechanisms of chelate exchange reactions are described by R. G. Wilkins.[24] The saturation kinetics shown in Figure 4 indicate that the kinetics of transfer of Fe from citrate to either DFO or L1 proceed through an intermediate step. Both DFO and L1 show second-order kinetics at low ligand concentration and first-order kinetics at high ligand concentration. Ternary complexes in the exchange of Fe between ligands have already been reported in the literature. Kinetic evidence for such intermediate complexes were observed in the exchange of Fe–transferrin with citrate and tricatechols.[16,19,20] In other cases,

Scheme 5



direct evidence for the presence of a two-step mechanism was obtained. Spectrophotometric evidence for a mixed complex was reported in the kinetics of Fe exchange between tranferrin and EDTA[19] and acetohydroxamic acid.[21] More recently the presence of a transitory species was detected by EPR spectroscopy in the kinetics of Fe exchange between transferrin and pyrophosphate.[22] Here we provide kinetic evidence for a two-step mechanism in the exchange of Fe between citrate and the two chelators, DFO and L1.

At low ($<K^*$) chelator concentration the rate of transfer is given by k_2/K^*. Under these conditions L1 acts 10 times faster than DFO in removing citrate-bound Fe (Table 2). In contrast, at high ($>K^*$) ligand concentration, the reaction rate is governed by k_2 only the DFO proceeds 2 times faster than L1. Between these two extremes, the reaction rate is described by eq 5. While the second-order rate constant for the transfer of Fe determined under pseudo-first-order and second-order conditions fit well in the case of DFO (4.3 and 4.0 $M^{-1}\ s^{-1}$, respectively), a discrepancy was observed when L1 was tested (55 against 28 $M^{-1}\ s^{-1}$). This difference can be explained in view of Scheme 2 and eq 5. When the chelator concentration is small compared to K^*, eq 5 simplifies to $k = k_2/K^*$; hence the second-order rate constant can be obtained dividing the pseudo-first-order rate constant by the ligand concentration. The L1 concentration used in the experiment performed under pseudo-first-order conditions (0.3 mM) is already close to the K^* value (0.45 mM), which means that k no longer linearly varies with [L1] and that the use of the simplified form of eq 5 leads to an underestimation of the real second-order rate constant.

Bates et al.[16] showed that the kinetics of transfer of Fe from citrate to transferrin is governed by the dissociation of the polymeric Fe–citrate to monomeric reactive units (Scheme 5, pathway B). This conclusion was supported by the fact that (i) the kinetics were highly influenced by the Fe:citrate ratio but only poorly by [transferrin], (ii) first-order kinetics were obtained

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when equimolar concentrations of Fe–citrate and transferrin were reacted, and (iii) a linear relationship between the initial velocity and [Fe–citrate] was obtained, which indicates that the rate-limiting step is first-order with respect to Fe–citrate. This reaction pathway appears not to be valid for DFO and L1. In the present study the rate constant did not change with varying citrate:Fe ratios or with Fe–citrate concentrations (see Table 1), while the relative contribution of the initial absorbance jump to the total was increasing with citrate:Fe ratios. This result is in line with what was observed by Spiro et al.,[25] who showed that high citrate:Fe ratios tend to suppress polymerization. In addition, the transfer of Fe from citrate to DFO ($t_{1/2}$ = 20 s when [DFO] > K^*) and [L1] ($t_{1/2}$ = 40 s when L1 > K^*) is very much faster than what was reported for the transfer of Fe to transferrin ($t_{1/2}$ = 2.5 h whatever the concentration). Therefore, the rate-limiting step with DFO and L1 is not the dissociation of polymeric to monomeric ferric–citrate units. We conclude that DFO and L1, in contrast to transferrin, are able to remove Fe directly from the polymeric Fe–citrate (pathway A dominates in Scheme 5). At that point, we should emphasize that the abbreviation used in Scheme 5 for polymeric iron–citrate $(Fe–Cit)_n$ is a simplification since the ratio of citrate to Fe in the polymer is not 1:1 but close to 4:1. Therefore, $(Fe–Cit)_n$ should in fact be taken as $(Fe–(Cit)_x)_n$.

According to Scheme 5, the rate of exchange of Fe is a function of the capability of the chelator (i) to interact with the Fe–ligand complex and (ii) to drive the replacement reaction step k_2. The equilibrium constant K^* (k_{-1}/k_1) in fact measures the affinity of the chelator for Fe–citrate. The difference in the kinetics between DFO and L1 mainly stems from the large difference (20 times) in their K^* value. The inability of transferrin to remove Fe from the polymer could result from a very high K^* value, which would explain why the major reaction pathway involves the depolymerization of Fe–citrate. This high K^* value could reflect the inaccessibility of the Fe-binding sites in Fe–citrate to transferrin, i.e. the difficulty of making a ternary intermediate. Throughout this work a constant NaCl concentration of 100 mM was used. It has been shown that chloride ion can catalyze ligand exchange reactions at Fe^{3+}.[26] This has undoubtedly influenced our kinetic data, and the kinetic constants determined in this paper might differ depending on the concentration of chloride ion present. However, the NaCl concentration in human serum (~150 mM) is close to what we used in the present study, and we believe that our data have certain physiological relevance. At concentrations close to what is achieved *in vivo* during chelation therapy (10–100 μM) none of the ternary complexes (Cit–Fe–C) will significantly accumulate; therefore the kinetics of Fe removal are concentration dependent within this chelator concentration range. Under these conditions the kinetics of transfer are governed by k_2/K^* only (L1 about 10 times faster than DFO). In view of the structural similarities between polymeric citrate and the iron storage protein ferritin we can hypothesize that the lower kinetic ability of DFO to mobilize ferritin iron *in vitro* might be related to a higher K^* value.

In summary, the transfer of citrate-bound Fe to transferrin and to the chelators DFO and L1 proceeds through two different routes. Whereas dissociation of Fe–citrate is the rate-limiting step in the presence of transferrin, both DFO and L1 are able to directly interact with polymeric Fe–citrate. The difference in the kinetics of Fe removal between DFO and L1 is mainly due to the difference in their ability to make a ternary complex with Fe–citrate. This difference in ability to make a transitory species may be due to a ligand size effect; L1 is more compact.

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