Kinetics and Mechanism of Iron Exchange in a Hydroxamate Siderophore Analogue Complex

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The kinetics of iron release from the dinuclear iron(III) complex of piperazine-1,4-bis(*N*-methylacetohydroxamic acid), upon treatment with ethylenediaminetetraacetate (edta) has been studied using UV/VIS absorption spectrophotometry. The absorbance *vs.* time evolution of the complex is well described by a consecutive two-step reaction mechanism. The rate constants for removal of the first and the second iron(III) ions have different dependences on the edta concentration. This is attributed to different conformations of the ligand involved in the co-ordination of di-and mono-iron(III) complexes. The increase in the rate constants with the acidity of the medium probably results from changes in conformation and the degree of co-ordination of the complexes. The results are compared with published studies for iron release from transferrins.

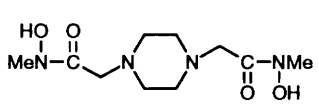
The siderophores are low-molecular-weight chelating agents produced by microbes to solubilize and transport iron(III) ion to cells.[1] We prepared a new dihydroxamate-type siderophore, piperazine-1,4-bis(*N*-methylacetohydroxamic acid) (H_2L).[2] Its complexing properties with Fe^{III}, electron-transfer mechanism and biological activity indicate that it can be considered a reasonable model of the natural occurring siderophore rhodotorulic acid.[2]

This compound forms a very stable complex (log β = 61.7 ± 0.3) of type $[Fe_2L_3]$. Electrochemical studies show that both iron binding sites are identical as regards the electron-transfer mechanism, which involves reduction of the iron(III) to the iron(II) complex followed by dissociation of the latter.[2]

The exchange of iron with chelating agents is one of the most important aspects of siderophores because of their role as microbial iron transport agents. Leong and Neilands[3b] suggest that for ferrichrome and ferrioxamine siderophores the uptake of iron by micro-organisms should be favourable through a mechanism involving reduction of iron(III) to iron(II) siderophores by the cell with subsequent re-excretion of the free ligand. A different mechanism seems to be used by *Rhodotorula pilimanae* for the uptake of the iron(III) rhodotorulate,[4] which involves release of iron at the cell membrane without penetration of the iron complex or the ligand into the cell.

Biological activity of H_2L was found in *Erwinia herbicola*,[5] which is also able to utilize ferrioxamines, as recently described by Winkelmann and co-workers,[6,7] although this new compound is biologically inactive for *Escherichia coli* which utilizes iron rhodotorulate. Further studies of its biological activity are underway in order to clarify the mechanism involved.

In an attempt to understand the mechanism of iron exchange involving this compound we have examined and describe therein the kinetics and mechanism of iron release from $[Fe_2L_3]$ to ethylenediaminetetraacetic acid (H_4edta) under several competing concentrations and physiological conditions. Preliminary results are presented on the effect of pH. This is of considerable interest, since protonation of the ligand must occur during the overall iron release.



H_2L

Experimental

The compound H_2L was synthesized through an easy two-step process involving the attachment of two hydroxamic acid arms to the cyclic diamine (piperazine), through the nitrogen atoms, as previously reported.[2]

Kinetic studies of iron removal from $[Fe_2L_3]$ by edta were performed in 1 cm quartz cells, maintained at constant temperature (25.0 ± 0.1 °C). The absorbance was monitored at λ_{max} = 425 nm using a Lambda 9 Perkin Elmer UV/VIS spectrophotometer. The absorbances at several times were collected in a database recorder system interfaced to an IBM personal computer. Absorbance *versus* time curves were analysed by a standard non-linear least-squares fitting program, based on the Marquardt algorithm.[8]

Potassium nitrate was used as the supporting electrolyte for maintaining constant the ionic strength (I = 0.1 mol dm^{-3}). Typically, a ten-fold excess of ligand was used to ensure complete complexation of the iron(III) ion. Stock solutions of iron(III) ion (0.01 mol dm^{-3}) were freshly prepared from iron(III) nitrate in nitric acid solution (0.1 mol dm^{-3}). Stock solutions of edta were prepared by dissolving the appropriate amount of Na_2H_2edta in about 40 cm^3 of the buffer [I = 0.1 mol dm^{-3}; acetate, pH 4.5–5.5; phosphate, pH 6–6.5; tris(hydroxymethyl)-methylamine (Tris)–HCl, pH 7.0], adjusting the pH with concentrated HCl or NaOH and making up to 50 cm^3 with further buffer.

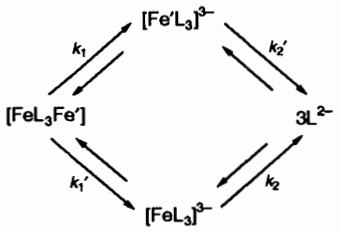
Typically, for experiments at pH 7.0, 0.1 mol dm^{-3} supporting electrolyte solution (3.0 cm^3), 0.01 mol dm^{-3} H_2L solution (300 μl) and 0.01 mol dm^{-3} iron(III) solution (30 μl) were added to a cell, the final pH being adjusted with a concentrated solution of NaOH. Known amounts of buffered edta solution were then added to make the ratio c_{edta}/c_L range from 8.0 to 0.2:1, the total volume being adjusted with small volumes of supporting electrolyte solution. The final concentrations were $c_L = 7.94 \times 10^{-4}$, $c_{Fe} = 7.94 \times 10^{-5}$ and $c_{edta} = 1.59 \times 10^{-4}$–$6.35 \times 10^{-3}$ mol dm^{-3}.

Absorbance readings were started around 10 s after the edta addition and were stopped when the value was about 20% of the initial absorbance. Studies involving variations in pH were

carried out through an identical procedure, using appropriate buffer, maintaining the concentrations of ligand and iron(III) ion and $c_{edta} = 0.1\ c_L$. The pH was measured with a 420 A Orion pH meter, equipped with an Orion 91-03 glass calomel combination electrode.

Data and Data Analysis

The compound H_2L forms a very stable dinuclear iron(III) complex $[Fe_2L_3]$ at neutral pH. The UV/VIS absorption spectrum of the latter (Fig. 1) displays a band centred at 425 nm, characteristic of iron(III) complexes co-ordinated to three hydroxamate groups.[2] Addition of edta to $[Fe_2L_3]$ solutions leads to a decrease in absorbance with time. The spectra obtained at different times after the edta addition show an isosbestic point around 350 nm, indicating that another species absorbing in the UV region (complexes of Fe with edta[9]) is produced as the equilibrium is approached. This behaviour is similar to that observed on release of Fe^{3+} from di-iron(III) transferrin upon reaction with chelating agents.[10-12] The kinetic results were analysed using a kinetic model which incorporates two distinct iron(III) centres (see Scheme 1).



Scheme 1

This scheme considers the forward steps to be described by pseudo-first-order rate constants and the backward reactions to be negligible. The time evolutions of the concentrations of the iron(III) complexes are given by[8] equations (1)–(3). The total

$$[Fe_2L_3] = [Fe_2L_3]_0 \exp[-(k_1 + k_1')t] \quad (1)$$

$$[FeL_3{}^{3-}] = \frac{[Fe_2L_3]_0 k_1}{k_1 + k_1' - k_2'} \{\exp(-k_2't) - \exp[-(k_1 + k_1')t]\} \quad (2)$$

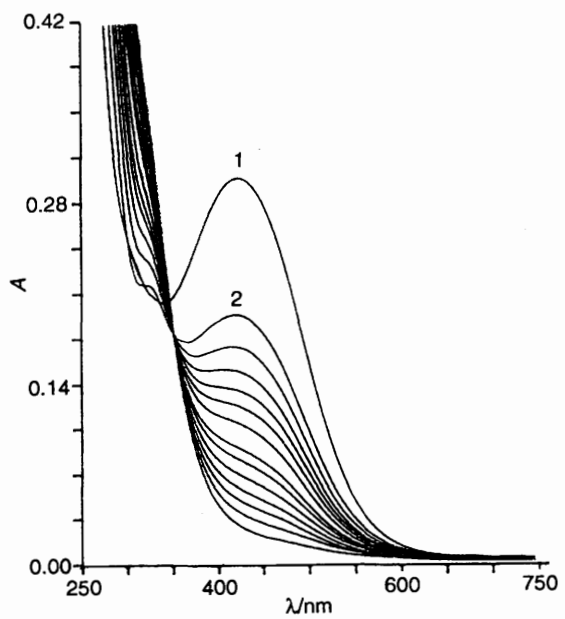


Fig. 1 Absorbance spectra of $[Fe_2L_3]$ ($c_L/c_{Fe} = 10:1$; $c_L = 9.0 \times 10^{-4}$ mol dm^{-3}; pH 7.0) before (1) and after (2) the addition of edta ($c_{edta}/c_L = 1.5:1$) at different times

$$[Fe'L_3{}^{3-}] = \frac{[Fe_2L_3]_0 k_1'}{k_1 + k_1' - k_2'} \{\exp(-k_2't) - \exp[-(k_1 + k_1')t]\} \quad (3)$$

absorbance at time t is given by equation (4) where A_∞ is the

$$A_t = A_\infty + 2\varepsilon l[Fe_2L_3] + \varepsilon l[FeL_3{}^{3-}] + \varepsilon l[Fe'L_3{}^{3-}] \quad (4)$$

absorbance at infinite time, ε (dm^3 mol^{-1} cm^{-1}) the molar absorption coefficient per iron(III) centre at the observation wavelength and l (cm) the optical path length. The value of ε is considered equal for all complexes, since each iron(III) ion is co-ordinated by three hydroxamate groups.[13] The time evolution of the absorbance is obtained from equation (4) by substituting the expressions (1)–(3) of the concentrations.

In our case this scheme is simplified since both iron(III) centres should be kinetically equivalent. This is supported by the redox properties of the complex which indicate the existence of two identical binding sites.[2] Accordingly, the kinetic scheme simplifies to a consecutive two-step reaction, the absorbance

$$A_t = A_\infty + \varepsilon l[Fe_2L_3]_0 \left[\left(2 - \frac{k_a}{k_a - k_b}\right)\exp(-k_at) + \frac{k_a}{k_a - k_b}\exp(-k_bt)\right] \quad (5)$$

being given by equation (5) where $k_a = 2k_1 = 2k_1'$, $k_b = k_2 = k_2'$. A further simplification can be obtained if $k_a = k_b = k$ [the release of the first iron(III) ion does not disturb the removal of the second one], which simplifies equation (5) to (6).

$$A_t = A_\infty + 2\varepsilon l[Fe_2L_3]_0 \exp(-kt) \quad (6)$$

Fig. 2 shows the time dependence of the absorbance measured at 425 nm for two edta concentrations. The curves are dependent on the edta concentration, implying that k_a and/or k_b are pseudo-first-order rate constants. When the absorbances were fitted by equation (6) deviations at early times were detected (see Fig. 2). These deviations persisted even when a

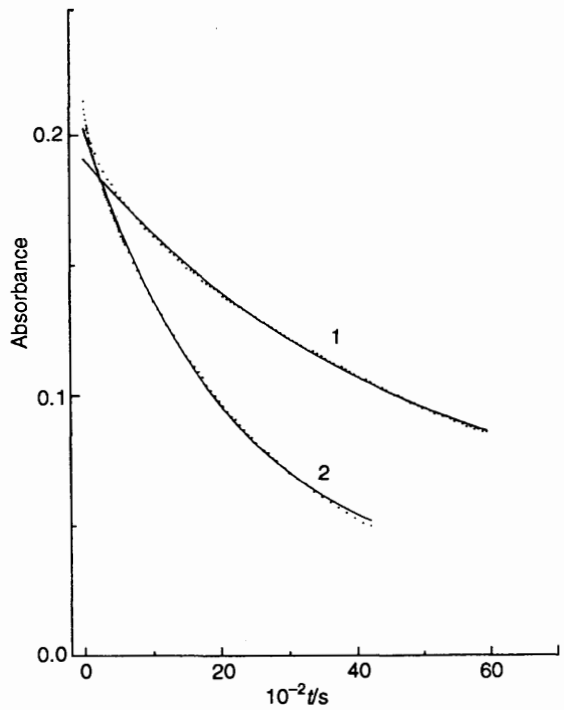


Fig. 2 Decay of the absorbance of a solution of $[Fe_2L_3]$ ($c_L/c_{Fe} = 10:1$, $c_{Fe} = 7.94 \times 10^{-5}$ mol dm^{-3}; $I = 0.1$ mol dm^{-3}, KNO_3; pH 7.0) after the addition of 3.97×10^{-4} (1) and 1.59×10^{-3} mol dm^{-3} edta (2). (–), Fit of the absorbance by equation (6)

delay time ($\leqslant$0.2 min), owing to the mixing of the reactants, was included in the fitting. The decay curves were then fitted to equation (5), using a non-linear least-squares fitting program,[8] with a constant delay time of 0.2 min. Small variations in the delay time did not introduce significant differences in the resulting parameters. Fig. 3 shows one of the experimental decays (c_{edta} = 5.56 × 10^{-3} mol dm^{-3}), the best-fit curve and a plot of the residuals. The residuals are randomly distributed indicating that the decay curve follows equation (5). The absorbances can also be fitted to a double-exponential function plus offset. This procedure is only appropriate if an additional constraint is introduced, owing to the dependence of the pre-exponential factors on the rate constants. For all edta concentrations it was found that the absorbance at infinite time, A_∞, is zero within the experimental error. This is reasonable since at 425 nm the molar absorption coefficient of the ligand is practically zero and the absorbances of the edta complexes at neutral pH are negligible.

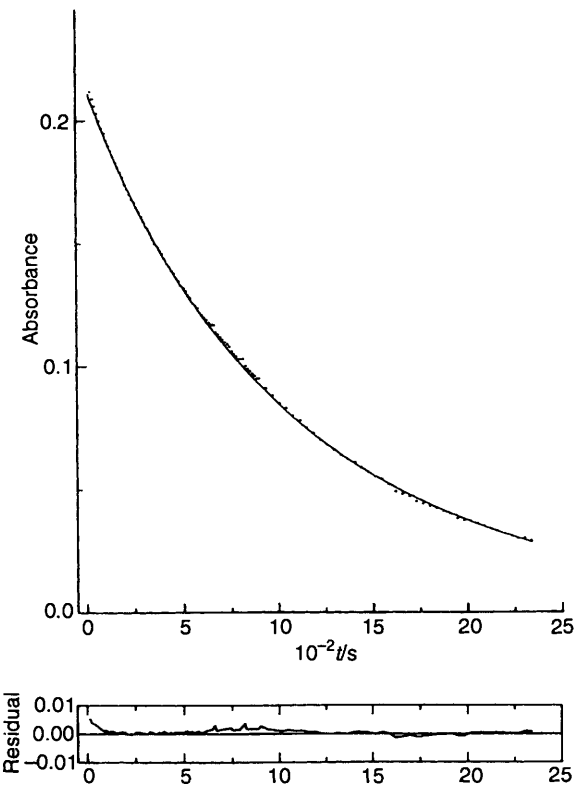


Fig. 3 Decay of the absorbance of a solution of $[Fe_2L_3]$ (c_L/c_{Fe} = 10:1, c_{Fe} = 7.94 × 10^{-5} mol dm^{-3}; I = 0.1 mol dm^{-3}, KNO_3; pH 7.0) after the addition of 5.56 × 10^{-3} mol dm^{-3} edta. (−), Fit of the absorbance by equation (6)

Table 1 Parameters obtained from fitting the time evolution of the absorbances of $[Fe_2L_3]$ solutions (c_{Fe} = 7.94 × 10^{-5} mol dm^{-3}; c_L/c_{Fe} = 10:1; I = 0.1 mol dm^{-3}, KNO_3; pH 7.0) after adding various concentrations of edta

10^3 [edta]/mol dm^{-3}	$\varepsilon[Fe_2L_3]_0l$	$10^3 k_a/s^{-1}$	$10^4 k_b/s^{-1}$
6.35	0.106	2.10	8.40
5.56	0.106	2.02	7.80
4.76	0.105	1.85	5.58
3.97	0.104	1.54	5.22
3.18	0.105	1.46	4.12
2.38	0.106	1.44	3.67
1.59	0.103	0.93	2.47
1.19	0.109	1.00	2.32
0.74	0.105	0.67	1.40
0.66	0.105	0.73	1.24
0.40	0.098	0.39	0.68
0.32	0.102	0.30	0.47
0.24	0.104	0.24	0.36
0.16	0.104	0.12	0.06

Table 1 shows the fitting parameters at pH 7 obtained for several edta concentrations. The absorbance at time zero is constant within the experimental error for all concentrations, allowing the calculation of ε per site (2640 ± 100 dm^3 mol^{-1} cm^{-1}) of the initial iron(III) complex. This value agrees with published values for similar complexes with an iron(III) ion coordinated by three hydroxamate groups.[13] On the other hand the rate constants are strongly dependent on the edta concentration as was already observed by several authors[10–12,14] for removal of iron from mono- and di-iron(III) transferrins by chelating agents. Notwithstanding some similarities between these results, they are not directly comparable. Indeed, we consider two kinetically equivalent sites, while in transferrins two distinct iron(III) centres exist.[10–12] The values for the pseudo-first-order rate constants k_a and k_b show that the first iron is removed at a faster rate than is the second. As both centres are kinetically equivalent in the original complex $[Fe_2L_3]$, the removal of one disturbs the kinetic behaviour of the other.

Fig. 4 shows the hyperbolic dependence of the pseudo-first-order rate constant k_a on the edta concentration. Such a dependence was also observed for iron removal from transferrins by chelating agents and it was interpreted through the empirical expression (7).[12] The best-fit curves to equation (7),

$$k_{obs} = \frac{k'[edta]}{1 + k''[edta]} + k'''[edta] \qquad (7)$$

considering only the first term or both terms, are shown in Fig. 4. A reasonable fit can be achieved only when both terms are considered. The calculated rate constants are k_a' = 1.3 ± 0.2 dm^3 mol^{-1} s^{-1}, k_a'' = 15 ± 5 dm^3 mol^{-1} and k_a''' = (1.4 ± 0.6) × 10^{-1} dm^3 mol^{-1} s^{-1}. In order to explain the hyperbolic dependence of k_a on edta concentration, the existence of two distinct conformers in dynamic equilibrium (Scheme 2) is proposed for the $[Fe_2L_3]$ complex. One conformation (A) reacts directly with edta through a pseudo-first-order step, while the other (B) reacts *via* an intermediate. For simplification, in Schemes 1 and 2 the last step involving protonation of the hydroxamate moieties of the free ligand (at pH 7) has been omitted since it should be very rapid. Assuming that reactions of iron removal do not disturb significantly the equilibrium and

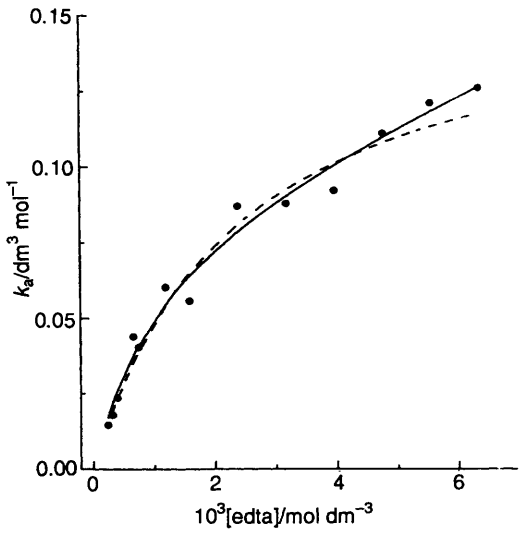
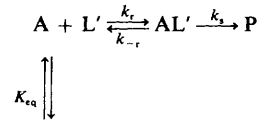
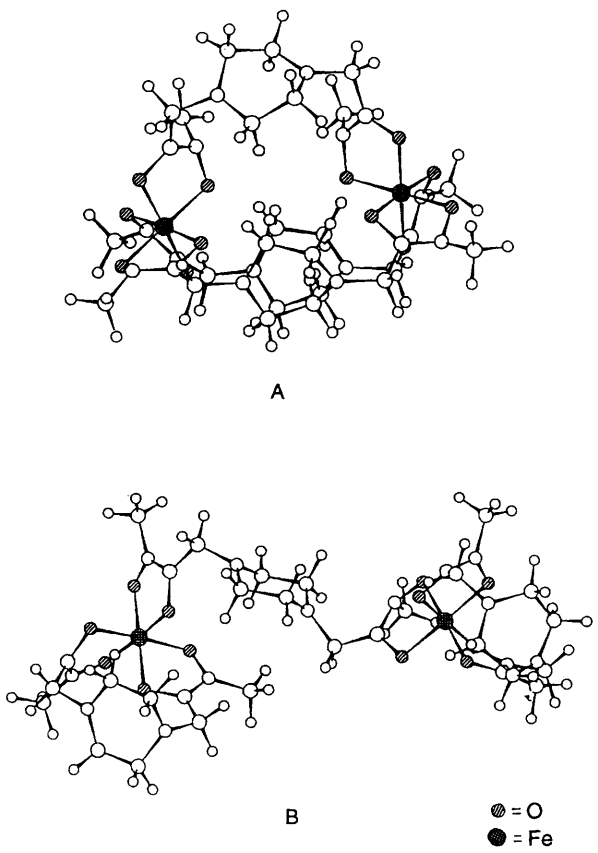


Fig. 4 Variation of the pseudo-first-order rate constant k_a(●) *versus* edta concentration. Fit of the absorbance by equation (7) using both terms (——) and only the non-linear term (– – – –)



Scheme 2 L' = edta



Scheme 3

applying the steady-state approximation to the intermediate, equation (7) can be obtained with constants given by (8)–(10).

$$k' = \frac{1}{1 + K_{eq}} \frac{k_s k_r}{k_s + k_{-r}} \quad (8)$$

$$k'' = k_r/(k_s + k_{-r}) \quad (9)$$

$$k''' = k_t \frac{K_{eq}}{1 + K_{eq}} \quad (10)$$

Scheme 3 shows both conformations of the diiron(III) complex as drawn by the CERIUS program.[15] In one (A) the three ligands bind both iron(III) ions and adopt the chair conformation for the piperazine ring. In the other (B) one ligand connects the iron(III) ions and adopts the chair conformation, while each of the other binds an iron(III) ion and has a distorted boat conformation.* We were unable to detect the intermediate. Nevertheless, a quaternary complex was previously observed during the donation of iron to transferrin by the iron(III) chelates of pyrophosphate and acetohydroxamic acid.[16,17]

* It is impossible to predict which conformation reacts *via* the intermediate. Nevertheless, as the removal of the iron(III) ion from conformation B is more hindered by the ligands, this suggests that it is the one involved in the intermediate.

Table 2 Parameters obtained from fitting the time evolution of the absorbances of $[Fe_2L_3]$ solutions (c_{Fe} = 7.94 × 10^{-5} mol dm^{-3}; c_L/c_{Fe} = 10:1; I = 0.1 mol dm^{-3}, KNO_3) after adding a constant [edta] (7.94 × 10^{-5} mol dm^{-3}) at various pH values

pH	$\varepsilon[Fe_2L_3]_0 l$	$10^2 k_a/s^{-1}$	$10^3 k_b/s^{-1}$
6.5	0.095	0.073	0.082
6.0	0.092	0.25	0.34
5.5	0.096	0.52	0.35
5.0	0.075	1.50	1.80
4.75	0.084	1.85	3.67
4.5	0.055	0.93	2.47

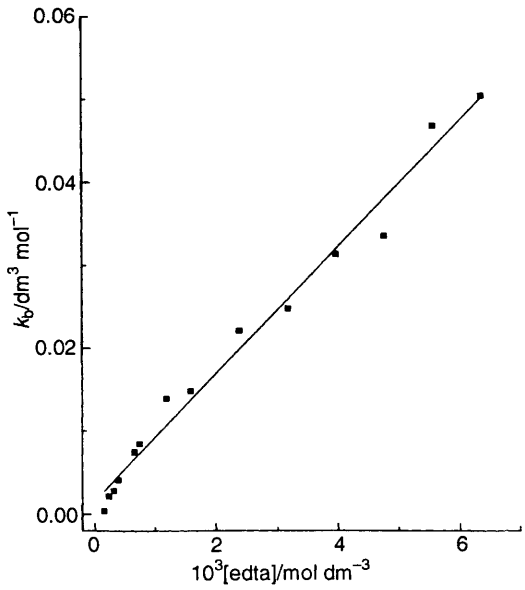


Fig. 5 Variation of the pseudo-first-order rate constant k_b(■) *versus* edta concentration. (—), Best linear fit line

Fig. 5 shows the plot of k_b *versus* edta concentration. From a linear fitting the rate constant $k_b' = (1.3 \pm 0.4) \times 10^{-1}$ dm^3 mol^{-1} s^{-1} was calculated. This dependence is due to the second term of equation (7), the first term being approximately constant (k''[edta] ≫ 1.0). A similar linear dependence was observed for the iron removal from a mono-iron(III) transferrin by tetrasodium pyrophosphate.[12] The rate constant k_b' is similar to k_a''', suggesting that the mechanism of iron removal from the $[FeL_3]^{3-}$ complex is identical to the linear pathway of iron release from the $[Fe_2L_3]$ complex.

The influence of pH on the macroscopic rate constants was studied in the range pH 4.5–7.0. At pH < 7.0 the system becomes more complex owing to the presence of more species in solution (different protonated complexes) and also a minor influence of iron(III) complexation with the buffer anions. Nevertheless, some trends can be observed, the increase in the reaction rates with decreasing pH being the most important. A similar behaviour was observed by Baldwin *et al.*[18] for the release of iron from human transferrin. The increase in the reaction rates should result mainly from the protonation of the complex which is accompanied by a decrease in co-ordination. This is supported by both the red shift of the absorption band and the diminution of the absorbance at the maximum.[2]

The absorbances continue to be well fitted with equation (5). The resulting parameters are shown in Table 2. At very low pH (< 5.0) the absorbance obtained from the fitting at t = 0 is lower than that before the addition of edta. This indicates that at early times a very fast reaction occurs. The substantial increase in the rate constants with decreasing pH is associated with a higher reactivity of the protonated complex. This overcomes the lower reactivity of the major edta species (H_2edta^{2-}) in acidic

range ($4.5 < pH < 6.0$). Indeed this species should be less reactive than the predominant species at pH 7.0, $Hedta^{3-}$, which has a higher negative charge.

Conclusion

The kinetics of iron release from the dinuclear iron(III) complex of H_2L upon reaction with edta is well described by a consecutive two-step reaction mechanism. The rate constants for the first iron release exhibit a hyperbolic dependence on the edta concentration while those for the second one have a linear dependence. The hyperbolic dependence is attributed to the presence of two conformers in equilibrium which react with edta by different mechanisms. A pH decrease in the solution increases both rate constants owing to protonation of the complex leading to a form which is more reactive than the unprotonated one. This may be attributed either to conformational changes or to a weaker metal wrapping due to protonation of the chelating groups.

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