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Complexation of desferricoprogen with trivalent Fe, Al, Ga, In and divalent Fe, Ni, Cu, Zn metal ions: effects of the linking chain structure on the metal binding ability of hydroxamate based siderophores

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Abstract

Complexes of the natural siderophore, desferricoprogen (DFC), with several trivalent and divalent metal ions in aqueous solution were studied by pH-potentiometry, UV–Vis spectrophotometry and cyclic voltammetry. DFC was found to be an effective metal binding ligand, which, in addition to Fe(III), forms complexes of high stability with Ga(III), Al(III), In(III), Cu(II), Ni(II) and Zn(II). Fe(II), however, is oxidized by DFC under anaerobic conditions and Fe(III) complexes are formed. By comparing the results with those of desferrioxamine B (DFB), it can be concluded that the conjugated β-double bond slightly increases the stability of the hydroxamate chelates, consequently increases the stability of mono-chelated complexes of DFC. Any steric effect by the connecting chains arises only in the bis- and tris-chelated complexes. With metal ions possessing a relatively big ionic radius (Cu(II), Ni(II), Zn(II), In(III)) DFC, containing a bit longer chains than DFB, forms slightly more stable complexes. With smaller metal ions the trend is the opposite. Also a notable difference is that stable trinuclear complex, $[Cu_3L_2]$, is formed with DFC but not with DFB. Possible bio-relevance of the Fe(II)/Fe(III) results is also discussed in the paper.

Keywords: Desferricoprogen; Siderophore; Solution study; Metal complexes

1. Introduction

Microbial siderophores are low-molecular-weight compounds with high iron(III) chelating affinity [1,2] that are responsible for the solubilization and transport of iron(III) into bacterial cells. Some bacteria produce hydroxamate-type siderophores, while others produce catecholate-type ones. Fungi produce mainly hydroxamate-based compounds [1]. These oxygen-donor ligands usually contain three bidentate metal-binding groups and are able to complete the octahedral coordination sphere of iron(III) to form a highly stable complex. The first step of the iron uptake is the formation of the siderophore complex outside the cell. Then, the complex is transported into a cell and finally, iron is released by one of several different mechanisms such as reduction of the central iron(III) or/and hydrolysis of the ligand. As a result of the reduction of the iron(III), a less stable and more labile iron(II) complex is formed [1].

Useful information relating the above mentioned biological process might be obtained by solution equilibrium studies of the iron(III)/iron(II) – siderophore systems. For example studies of the interaction between siderophores and iron(II) might help to understand the reduction step of the iron release mechanism. In our

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previous investigations the interaction between iron(III)/iron(II) and desferrioxamine B (DFB) was studied [3,4] (DFB, which is used as a drug in the treatment of thalassemia, is perhaps the best-known trihydroxamic acid-based siderophore [1,2]). In addition to the iron-DFB, our work was also extended to some other metal ion–DFB systems [5–7].

In the present study the complexation of another natural siderophore, desferricoprogen (DFC), [1] produced by *Penicillium chrysogenum* and *Neurospora crassa* has been investigated. This ligand is a linear hexadentate trihydroxamic acid containing a double bond at the β-position conjugated to each hydroxamate group and one diketopiperazine ring. Only one previous work has been published for the complex formation of DFC with Fe(III) [8], and its complexes with other metal ions have not yet been studied. Therefore, Fe(II), Ga(III), Al(III), In(III), Mo(VI), Cu(II), Ni(II) and Zn(II) have also been involved in this work. In order to have data at our experimental conditions, measurements were also performed on Fe(III)–DFC, In(III)–DFB and Ga(III)–DFB systems. To evaluate the influence of the structure of the linkers situated between the two hydroxamic functions on the stability and stochiometry of the metal complexes, the results obtained were compared to those of DFB [3–7] and in a few cases to those of model dihydroxamic acids [9,10].

2. Experimental

2.1. Chemicals

Purification of DFC from *P. chrysogenum* and *N. crassa* cultures: Siderophore–iron(III) complexes were purified from cultures of *P. chrysogenum* NCAIM 00237 [11,12] and *N. crassa* 74A [8] cultivated in defined low-iron minimal media for 5–6 days. The applied purification procedure included Amberlite XAD-2 (Supelco, USA) Kieselgur G (Merck, Germany) chromatographies and preparative high performance liquid chromatography (HPLC) on a Supercosil SPLC-Si (250×10 mm) matrix [2,11]. Typical yield was 35 mg/L culture medium (*P. chrysogenum*) and 65 mg/L culture medium (*N. crassa*). The purity of the siderophore was analysed by HPLC using a C-18 reversed phase column [13] and by thin layer chromatography (TLC) (Kieselgel $60F_{254}$, Merck, Germany) in chloroform–methanol–water (35:12:2) [2]. DFC was desferriated using methanolic quinolin-8-olate [8]. The structure of the desferriated DFC was checked by ^{1}H NMR and the spectra are in good agreement with that published in the literature [2]. (Anal.: 1.60 ppm (12H, CH_2), 1.84 ppm (6H, CH_3–C═), 1.97 ppm (3H, CH_3–C(CH_2–O)═), 2.03 ppm (3H, CH_3–C═O), 2.38 ppm (4H, CH_2–C═) 3.45 ppm (6H, CH_2–N), 3.83 ppm (2H, CH_{ring}), 4.18 ppm (H, CH–N), 4.37 ppm (6H, CH_2–O), 5.89 ppm (H, CH═), 6.18 ppm (2H, CH═) in D_2O). ^{1}H NMR spectra were recorded on a Brucker AM 360 spectrometer. Trimethylsililpropane sulfonate (TSP) was used as reference. DFB was obtained from CIBA Geigy.

The purity of the ligands and the concentrations of the ligand stock solutions were determined by *Gran*'s method [14]. The metal ion stock solutions were prepared from $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, $AlCl_3 \cdot 6H_2O$, $Na_2MoO_4 \cdot H_2O$ (Reanal) dissolved in doubly distilled water. ZnO, $FeCl_3$, iron wire and In (Reanal) were dissolved in a known amount of HCl solution and Ga in $HClO_4$. The dissolution of indium metal took 3 weeks at 80–90 °C. Dissolution of iron was made under purified, strictly oxygen-free argon atmosphere (For deoxygenation the argon was passed through acidic solution of $CrCl_2$). The $FeCl_2$ solution obtained was then filtered and stored in a tightly closed vessel under argon atmosphere. KSCN solution was used to test the absence of Fe(III) traces in this stock solution. Argon overpressure was used when Fe(II) was added to the samples, which were previously completely deoxygenated by bubbling a stream of argon through them for ca. 20 min. Basic solution of 1,2,3-trihydroxy-benzene (pyrogallol) was used to test whether oxygen traces had got into the vessels used during titration of Fe(II)-containing samples.

The concentrations of the metal ion stock solutions except for Fe(II), In(III), Ga(III) were determined gravimetrically via precipitation of quinolin-8-olates, while the concentration of the Fe(II) solution was determined by titrimetry using $KMnO_4$ as titrant under acidic conditions. For the measurement of the concentration of Ga(III) or In(III) solutions, known amount of ethylenediaminetetraacetate (EDTA) was added to them and the excess of EDTA was determined with $ZnCl_2$ stock solution. The HCl concentration of the Fe(II), Fe(III), Ga(III), In(III) and Zn(II) solutions were determined by pH-potentiometry.

2.2. pH-potentiometric studies

The exact concentration of the carbonate-free KOH titrant was determined by pH-potentiometry. The pH-metric titrations were performed in the pH range 2.0–10.5 or until precipitation on samples of 4.00 or 10.00 mL, at an ionic strength of 0.2 M (KCl) and at 25.0 ± 0.1 °C. During the titrations purified, strictly oxygen-free argon was continuously bubbled through the samples. Samples were always freshly prepared and titrations were completed within 2 h to avoid any measurable hydrolysis of DFC. The ligand concentrations were varied in the range 1×10^{-3}–4×10^{-3} M and metal to ligand ratios were in the range of 1:1–1:2, except the

[1] In some papers, e.g., in [1], the iron-free siderophore is named coprogen.

Cu(II)-containing system, where the ratio was varied in the range of 1.5:1–1:2. Samples at two or four different ratios were measured. The pH-metric titrations were made with a Radiometer pHM84 instrument equipped with a Metrohm 62104130 combined electrode. The titrant was added from a Metrohm 715 Dosimat autoburette. The electrode system was calibrated by the method of Irving et al. [15] so that the pH-meter readings could be converted into hydrogen ion concentration. The equilibrium position determined during the titrations was 0.001 ΔpH/min. The water ionization constant (pK_w) obtained was 13.75 ± 0.01.

The pH-metric results were utilised to find the stoichiometry of species and to calculate the stability constants. If any precipitation in a certain sample occurred, calculations were always made from the experimental results obtained before the precipitation. [2] The calculations were made with the help of the computer program PSEQUAD [16] using the literature data for Fe(III) [17], Ga(III) [18], Al(III) [19] and In(III) [20] hydroxo complexes. Since In(III) shows measurable interaction with the chloride anion, during the calculation the known stability constants of the chloro-complexes, $[InCl]^{2+}$, $[InCl_2]^{+}$ and $[InCl_3]$ [21], were also involved in the equilibrium model. (The literature data of hydroxo and chloro complexes used during the calculations are summarized in a Supplementary table, Table S1). Volumes of titrant were fitted and the accepted fittings were always below 1×10^{-2} mL.

2.3. Spectrophotometric studies

UV–Vis measurements on systems containing Cu(II), Fe(III), Fe(II) were performed. The metal ion to ligand ratios were varied from 1:1 to 1:1.5, at 1.5×10^{-3} M Cu(II), 5×10^{-4} M Fe(III) and 6×10^{-2} M Fe(II) concentrations. Measurements for iron(III)-containing systems were also carried out on individual samples in which the 0.2 M KCl was partially or completely replaced by HCl. In these samples the pH values varied in the range of 0.7–1.4 and therefore were calculated from the HCl content. A HP 8453 spectrophotometer was used to record the spectra in the region of 250–950 nm. Path length was 1 cm. Investigation on Fe(II)-containing samples were carried out in the region of 300–700 nm by using a special tightly closed tandem cuvette (Tandem Cell 236 HELLMA). Both isolated pockets of the tandem cell were completely deoxygenated by bubbling a stream of argon for 10–10 min before $FeCl_2$ was added. The spectra were recorded immediately after mixing the reactants at pH 6. The pH was adjusted by MES (2-morpholine-ethanesulphonic acid, $pK = 6.06$). In the samples the metal to ligand ratio was 1:5 or 1:1.9 at 1.3×10^{-4} M iron(II) concentration. The absorbance values were recorded at 435 nm. During the calculations the absorbance-time curves were fitted using the software of the HP Instrument.

2.4. Cyclic voltammetry

Cyclic voltammetric measurements were performed on a Metrohm 746 VA Trace Analyser. A Pt-wire auxiliary electrode and a glassy-carbon working electrode polished with alumina and sonicated in deionised water after measurements were used. The reference electrode was Ag/AgCl/3 M KCl ($E_{1/2} = 209$ mV vs. NHE (normal hydrogen electrode)) [22]. Aqueous solution of $K_3[Fe(CN)_6]$ was used to calibrate the system. ($E_{1/2} = 458$ mV vs. NHE in 0.5 M KCl) [22]. Redox potentials shown in the paper were obtained at 5 mV/s of scan rate. Concentration of Fe(III) was 8×10^{-3} M and the metal to ligand ratio was varied in the range of 1–1.2, where the ionic strength was 0.05 M (KNO_3) and the volume of the sample was 5.0 mL. Aqueous samples were measured at 25 °C, 1 bar Ar-overpressure and were purged for 10 min with argon gas before scanning.

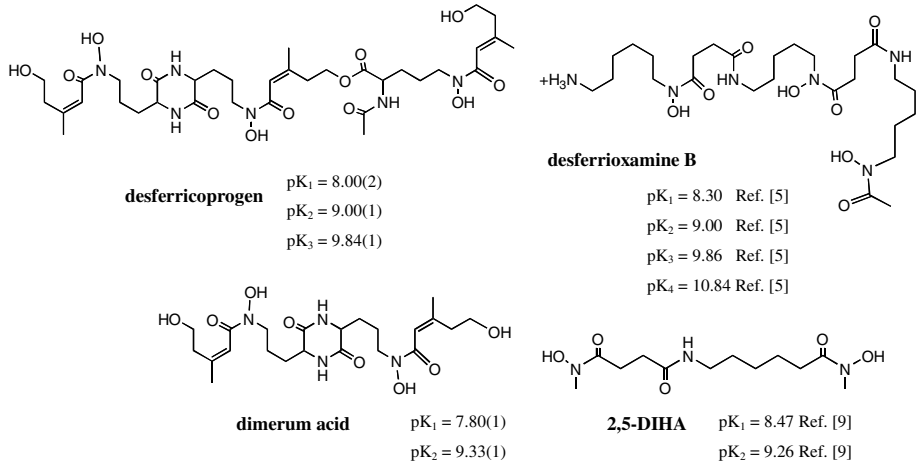
3. Results and discussion

3.1. Proton complexes

Formulae and the corresponding dissociation constants of DFC and DFB together with those of their dihydroxamic acid models, dimerum acid and 2,5-DIHA (*N*-methyl-*N*-hydroxy-*N*′-[3-(*N*-methyl-*N*-hyd-roxycabarmoyl)-propyl]-heptane-1,7-dicarboxamide), res- pectively, can be seen in the Scheme 1. The protonated forms of these hydroxamic acids have three (H_3L = DFC), four (H_4L^+ = DFB) or two (H_2L = dimerum acid, 2,5-DIHA) dissociable protons. Dissociation constants of DFB and 2,5-DIHA were determined in our previous work [5,9] and those for the DFC and dimerum acid were determined in the present work. Most probably the differences in the experimental conditions used in [8] and this work are responsible for the somewhat different dissociation constants determined for the DFC in these two cases.

Since DFC has an ester linkage, which could decompose especially at basic pH, the hydrolytic stability of this compound was monitored in the following way. After the titration of a DFC sample with KOH titrant up to pH ca. 11.5, HCl was added to it to set the pH back to ca. 2, and the same sample was titrated with KOH again. The two registered titration-curves being almost completely superimposed are shown in Fig. 1 (The small difference between the two curves can be attributed to the dilution).

[2] If precipitation starts in a solution, even if it is not visible yet, a continuous pH-decrease indicates the situation immediately.



Scheme 1.

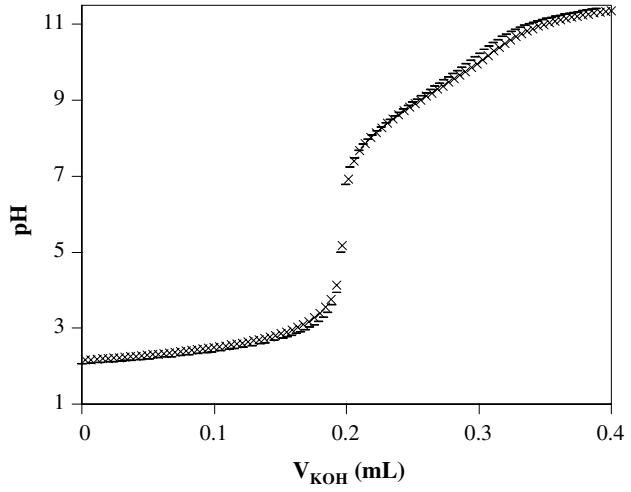


Fig. 1. pH-potentiometric titration curves of DFC (−) and of the same sample after the acidification (×); $c_{coprogen} = 1.0 \times 10^{-3}$ M.

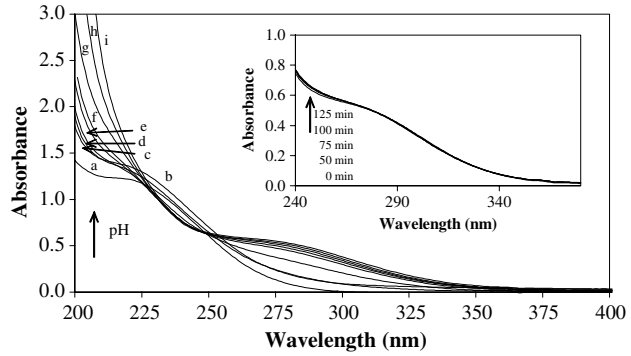


Fig. 2. UV–Vis spectra of DFC registered within the region of 200–400 nm, at different pH values; (a) 6.61, (b) 7.13, (c) 8.17, (d) 9.09, (e) 9.94, (f) 10.15, (g) 10.47, (h) 11.11, (i) 11.56; $c_{coprogen} = 4.0 \times 10^{-5}$ M *Inset*: UV–Vis spectra registered at pH 10.47 as a function of time.

The hydrolytic stability of this compound was also checked by spectrophotometry. Fig. 2 shows selected UV spectra registered at various pH values (The different curves do not go through isosbestic points, because the three protons are released in overlapping processes). According to these spectra, the completely protonated form of DFC has a characteristic band with λ_{max} at 219 nm and $\varepsilon^0 = 3.06 \times 10^4$ M^{-1} cm^{-1}, while the values for the completely deprotonated form are 267 nm and 1.39×10^4 M^{-1} cm^{-1}, respectively. Time dependant spectra of DFC at pH 10.47 were monitored over 125 min. The result, which does not show any noticeable change during the measuring time, can be seen in the inset in Fig. 2. Conclusively, both the pH-potentiometric and spectrophotometric results indicate that DFC does not hydrolyse in aqueous solution in measurable extent during the period which is usually necessary for one titration (ca. few hours). [3]

3.2. Metal complexes of DFC

Representative titration curves registered for the Fe(III)–, Al(III)–, Ga(III)–, In(III)–, Cu(II)–, Ni(II)–, and Zn(II)–DFC systems at 1:1 metal to ligand ratio are shown in Fig. 3. The reason for the absence of a titration curve for the Fe(II)–DFC will be discussed below. In the case of Mo(VI), precipitation occurring below pH 2 and remaining in the whole pH-range studied, hindered any equilibrium measurements.

[3] To demonstrate that the results shown in Fig. 1 are convincing of the absence of measurable hydrolysis of DFC within the experimental time, titration curves for DFC and dimerum acid (one of the two products of the hydrolysis of DFC) are plotted together in a Supplementary figure (Figure S1).

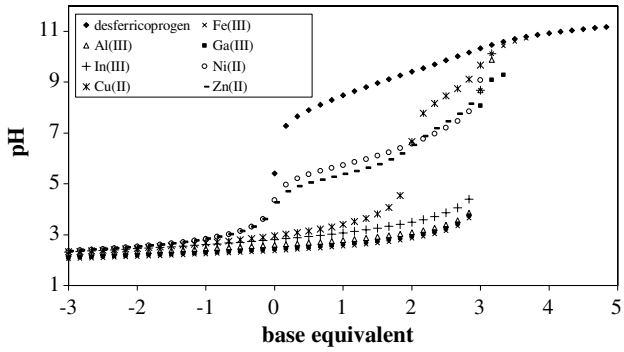


Fig. 3. Selected pH-potentiometric titration curves for H^+–DFC and the metal ion – DFC systems; $c_{desferricoprogen} = 1.5 \times 10^{-3}$ M, metal to ligand ratio = 1:1) (Negative base equivalent values mean acid excess).

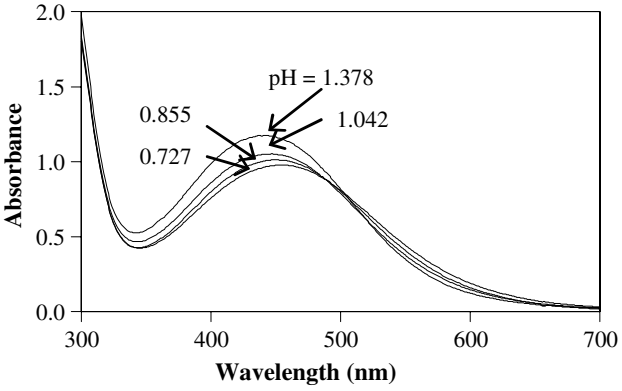
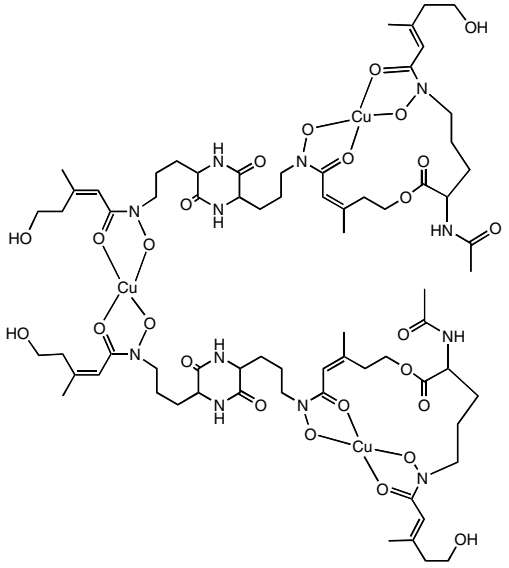


Fig. 4. Absorbance spectra registered for Fe(III)–DFC system in the pH-range 0.72–1.38; $c_{Fe(III)} = 5.0 \times 10^{-4}$ M, metal to ligand ratio = 1:1.5.

The titration curves in Fig. 3 shows different pH effects for the different metal ions studied, indicating different stability of the complexes formed. The complex formation with Fe(III), Ga(III), Al(III) and In(III) starts below pH 2 and seems to be completed by pH ca. 2–3 with the former three metal ions and by ca. pH 4 with In(III). The interaction of DFC with Ni(II) and Zn(II) starts above pH 3 and completes somewhat above pH 7. It is also shown in Fig. 3 that all the already mentioned metal ions displace all the three protons of DFC. The Cu(II) ion is the only exception, since one Cu(II) ion displaces only two protons of three of a siderophore molecule by pH ca. 4. Because there was no metal ion excess in the sample, for what the titration curve in Fig. 3 is presented, here the third hydroxamic proton is released in the same pH-range as in the free ligand However, the third proton can also be displaced by excess of Cu(II) ion. If 1.5:1 metal to DFC ratio was used all three protons were completely released by pH 6 and up to 1.5:1 copper(II) to ligand ratio any copper(II)-hydroxide precipitate did not form in the samples even at pH 10. This result is different from that obtained previously with DFB, where precipitation at approximately pH 6 occurred in the samples containing excess Cu(II) [5].

When the pH-metric experimental data were fit, it turned out that the complex formation between Fe(III) and DFC was completed by pH 2. To calculate stability constant for any Fe(III)–DFC complex, spectrophotometry was used to extend the studied pH-region down pH ca. 0.7 (see Section 2). The charge-transfer band (well-known for Fe(III)–hydroxamate complexes [1–4,8]) at various pH was registered (Fig. 4). At pH 0.72 a spectrum showing λ_{max} at 455 nm ($\varepsilon = 2440$ $M^{-1}\,cm^{-1}$) was obtained. This λ_{max} shifted to 435 nm ($\varepsilon = 2900$ $M^{-1}\,cm^{-1}$) by pH 1.38 and did not change if the pH was further increased. At the same time the λ_{max} and $\varepsilon_{435\ nm}$ obtained at this latter pH are almost equal to the previously published ones, 434 nm and 2820 $M^{-1}\,cm^{-1}$, respectively, for the tris-chelated Fe(III)–DFC complex, [8].

In a system containing a certain metal ion (M) and DFC (H_3L), theoretically the species [MLH_2], [MLH] and [ML] involving one, two and three coordinated hydroxamates, respectively, can be formed, where the non-coordinated hydroxamic function(s) is(are) still protonated in the various protonated complexes. The best fitting of the pH-metric data of Al(III)–, Ga(III)– and In(III)–DFC samples was obtained with the assumption of [MLH] and [ML] formation ([MLH_2] is formed with these three metal ions below the measurable pH region), while all three expected species, [MLH_2], [MLH] and [ML], were found with Cu(II), Ni(II) and Zn(II). Cu(II) was the only metal ion which allowed the formation of a polynuclear species, [Cu_3L_2]. Good fitting of the experimental results (four titration curves, 473 experimental points) was obtained only by involving [Cu_3L_2] into the equilibrium model. A possible bonding mode for this trinuclear species is shown in Scheme 2.



Scheme 2.

By fitting the spectra recorded between pH 0.72 and 1.38 for the Fe(III)–DFC, the stability constant for [FeL] could be calculated.

The equilibrium models obtained for the studied systems and the calculated stability constants are shown in Table 1. For comparison, Table 1 also contains the results of the various metal ion–DFB complexes. Complexation of DFB with Ga(III) or In(III) was studied in this work, all the other data are taken from our previous papers [5–7]. Taking into account the different experimental conditions used, the few constants published previously for the complexes of DFB with Ga(III) [24,25] and In(III) [24] and the values obtained in this work are in acceptable agreement. Representative distribution diagrams are put in the Supplementary (Figure S2).

Out of the complexes of DFC collected in Table 1, [FeL] (where L^{3-} = the completely deprotonated form of the ligand) is the only one for which a stability constant was previously published in the literature [8]. Also in this case the values obtained in the present and in the previous works, considering the condition differences, are in acceptable agreement with each other (the previously published $\log K_{[FeL]} = 30.2$) [8].

Because many of the overall constants shown in Table 1 involve the protonation constant of the still protonated function, their comparison to each other, or drawing any conclusion for the bonding mode, except in the case of the non-protonated [ML] complexes, is rather complicated. However, the stepwise dissociation constants of the metal ion – DFC protonated complexes (pK_{MLH2}, pK_{MLH}), especially if they are compared to the appropriate dissociation constants of the free ligand, provide useful information. By the use of the overall stability constants the pK values were calculated and are shown in Table 2.

As it is shown in Table 2, the dissociation constants of the protonated complexes of DFC are much smaller (except the case of [CuLH]) than the corresponding constants of the free ligand (Scheme 1). This decrease indicates that one, two and three hydroxamate protons in the [MLH_2], [MLH] and [ML] complexes, respectively, are displaced by the metal ions studied. Only in the case of Cu(II), the deprotonation of [MLH] occurs roughly in the same pH-range, where the free DFC would also release its proton. This finding supports the assumption that the deprotonation of the third hydroxamic group in the Cu(II)–DFC complex [MLH] occurs without coordination. The same conclusion can be drawn from the spectrophotometric results shown in Fig. 5(a).

In Fig. 5(a) the λ_{max} values obtained for the Cu(II)–DFC system as a function of pH are presented along with the concentration distribution curves. It is clear from this Figure that the λ_{max} decreases as the pH is increased from 2 to 4.5, but above this pH it does not change further. Moreover, the λ_{max} = 650 nm obtained in the pH-range, where the [MLH] and [ML] exist, is equal to that of the [Cu(acetohydroxamate)$_2$] (λ_{max} = 653 nm) [18]. Since maximum two hydroxamates coordinate to one Cu(II) ion, the third hydroxamate can

Table 1
Overall stability constants ($\log\beta$) for the complexes formed in Fe(III)–, Al(III)–, Ga(III)–, In(III)–, Ni(II)–, Cu(II)–, Zn(II) – desferricoprogen (DFC) and desferrioxamine (DFB) systems[a], (t = 25 °C; I = 0.20 M KCl)

$$\beta = \frac{[M_xL_yH_z]}{[M]^x[L]^y[H]^z}$$

Metal ion	Ionic radius (pm)		[MLH_3][b]	[MLH_2]	[MLH]	[ML]	[M_2LH]	[M_3L_2]
Fe(III)	64.5	DFC	–	–	–	29.35(8)[c]	–	–
		DFB[d]	–	*42.4*	*41.01*	*30.4*	–	–
Al(III)	53.5	DFC	–	–	25.05(5)	22.51(3)	–	–
		DFB[e]	–	*36.6*	*33.8*	*23.9*	–	–
Ga(III)	62	DFC	–	–	28.0(2)	25.8(2)	–	–
		DFB	–	–	36.92(7)	*27.56(9)*	–	–
In(III)	80	DFC	–	–	26.62(2)	22.88(4)	–	–
		DFB	–	*36.40(5)*	*32.48(4)*	*22.18(7)*	–	–
Ni(II)	69	DFC	–	23.74(7)	18.53(1)	11.42(2)	–	–
		DFB[f]	*33.20*	*27.66*	*19.71*	*8.89*	–	–
Cu(II)	73	DFC	–	26.89(3)	23.70(4)	15.25(8)	–	46.30(7)
		DFB[f]	*36.99*	*33.10*	*23.98*	*13.73*	*32.09*	–
Zn(II)	74	DFC	–	23.6(3)	19.23(2)	11.80(4)	–	–
		DFB[f]	*33.40*	*28.17*	*20.40*	*10.36*	–	–

[a] SD in parenthesis are shown if the values were determined in the present work.
[b] Charges of the complexes are not shown because of the their different values in the case of the trivalent and bivalent metal ions.
[c] determined by spectrophotometry.
[d] Ref. [6].
[e] Ref. [7].
[f] Ref. [5].

Table 2
Stepwise dissociation constants (pK) of Fe(III)–, Al(III)–, Ga(III)–, In(III)–, Ni(II)–, Cu(II)–, Zn(II)–desferricoprogen (DFC) protonated complexes

	Fe(III)	Al(III)	Ga(III)	In(III)	Ni(II)	Cu(II)	Zn(II)
$[MLH_2] = [MLH] + H$ p$K = -\log(\beta_{MLH}/\beta_{MLH2})$	–	–	–	–	5.21	3.19	4.37
$[MLH] = [ML] + H$ p$K = -\log(\beta_{ML}/\beta_{MLH})$	–	2.54	2.2	3.74	7.11	8.45	7.43

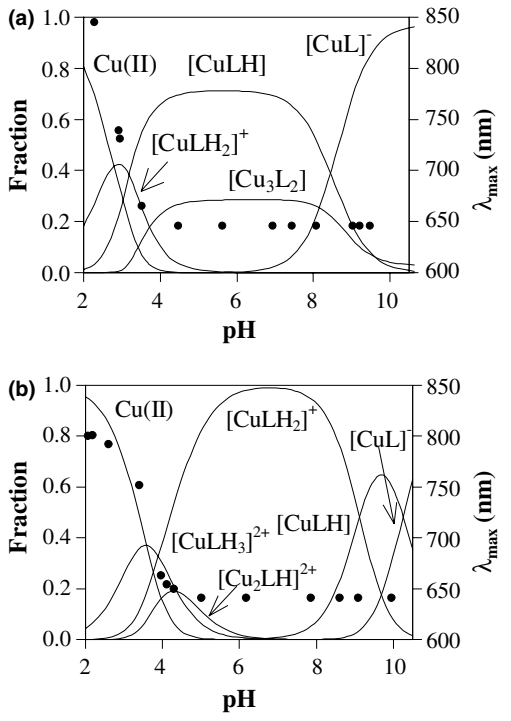


Fig. 5. Concentration distribution curves of Cu(II)–DFC (a) and Cu(II)–DFB (b) complexes plotted together with λ_{max} values in dependence of pH (●); $c_{Cu(II)} = 1.5 \times 10^{-3}$ M, metal to ligand ratio = 1:1.5).

bind another Cu(II) ion. Indeed, Cu(II)-containing samples up to 3:2 metal to ligand ratio and up to pH 10 could be titrated without any precipitation and the experimental results could be fitted only with involvement of $[Cu_3L_2]$ into the equilibrium model.

In the Fe(II)–DFC system, by using strictly anaerobic conditions, the ligand–metal interaction was found to start somewhere above pH 4. At this pH, however, a continuous pH decrease was observed and parallel with this, the characteristic deep-red colour of the tris-chelated Fe(III)–DFC complex appeared. These experimental results are exactly the same as those of Fe(II)–DFB [3], where the oxidation of Fe(II) by DFB was proved by different methods. The above experimental results strongly suggest that Fe(II) is oxidized to Fe(III) under anaerobic condition also by DFC. The formation of the Fe(III)–DFC complex was proved by spectrophotometry when the spectra as a function of time (at pH 6, buffered with MES, by the use of tandem cuvette) were recorded (Fig. 6).

Although, the kinetic study on the redox reaction occurring between Fe(II) and DFC is still under progress in our laboratory, but all the results obtained up to this point are in complete agreement with the following stoichiometry (the same as with DFB in [3]):

$$2Fe^{2+} + 3H_3DFC = 2[Fe(III)(DFC)] + H_2DFC - \text{monoamide} + H_2O + 4H^+ \quad (1)$$

The above reaction reveals that two moles of Fe(II) are oxidised by one of the three functional groups of one DFC, while the other two DFC molecules are required to bind Fe(III) in the high stability tris–hydroxamato complex.

Since the redox reaction (1) hinders the determination of the stability constant for any Fe(II)–DFC complex, cyclic voltammetry was used to obtain more information about this system. Cyclic voltammograms (some of them are shown in Fig. 7) were registered for the Fe(III)–DFC system at different pH values.

The voltammograms, although the peak-to-peak separations were somewhat higher than expected (ca. 90 mV) indicate nearly reversible electrochemical processes under the conditions used in the pH range 4–11. The reversible voltammograms formal redox potential ($E_{1/2}$) of −432.3 mV vs. NHE was calculated which reasonably agrees with the literature data of −447 mV [8]. Decreasing the pH below 4 causes an increase of the formal potential and the processes become more and more irreversible. The formal potential obtained in the pH range 4–11 is a bit higher than that of DFB (−481 mV) showing a small influence of the different ligand

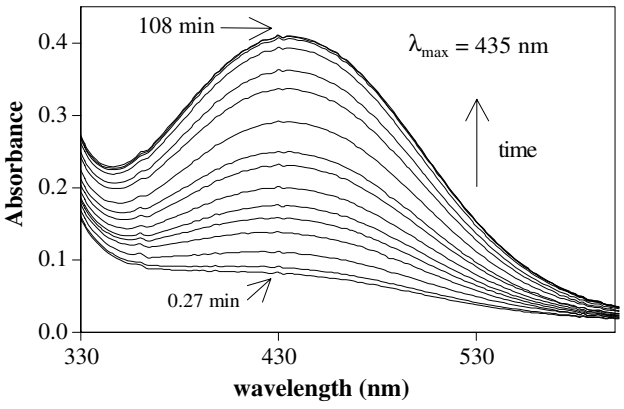


Fig. 6. Absorbance spectra registered for Fe(II)–DFC system as a function of time; $c_{Fe(II)} = 1.3 \times 10^{-4}$ M, metal to ligand ratio = 1:5, pH 6.0 (MES).

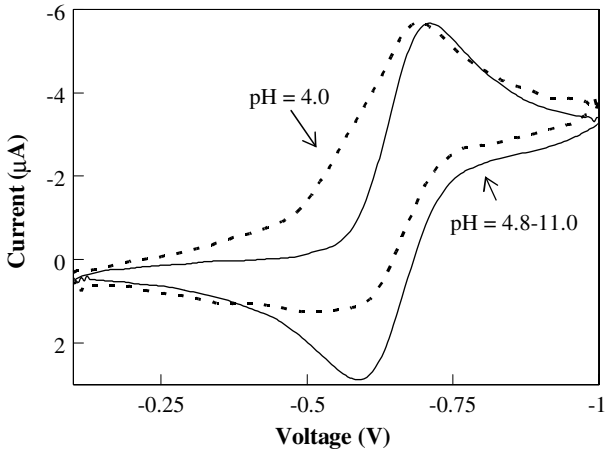


Fig. 7. Cyclic voltammograms of Fe(III)–DFC system at different pH using glassy carbon working electrode vs. Ag/AgCl (3 M KCl) reference electrode; $c_{Fe(III)} = 8.0 \times 10^{-3}$ M, metal to ligand ratio = 1:1.2; $c_{KNO3} = 0.05$ M ($E_{pa} - E_{pc}$: 158 mV at pH 4.0; 142 mV at pH 4.8–11.0).

structure. With the help of the Nernst equation (2) the stability constants of both the iron(II)–DFC and iron(III)–DFB tris-chelated ([FeL]$^-$) complexes were calculated.

$$\varepsilon^0_{[Fe(III)-L]/[Fe(II)-L]} = \varepsilon^0_{[Fe(H_2O)_6]^{3+}/[Fe(H_2O)_6]^{2+}} - 59 \log \frac{\beta_{[Fe(III)-L]}}{\beta_{[Fe(II)-L]}}, \quad (2)$$

$$\varepsilon^0_{[Fe(H_2O)_6]^{3+}/[Fe(H_2O)_6]^{2+}} = 770 \text{ mV} \quad \text{Ref. [23].}$$

The $\log \beta_{[Fe(II)-L]}$ with DFC is 8.97, with DFB is 9.20. These values are acceptable based on the Irving-William's trend [3] and on the stability constants obtained for the corresponding octahedral complexes of other $3d^{5-10}$ bivalent metal ions (Ni(II), Zn(II)) studied. Additional support for the validity of these numbers is given by the recently determined stability constant for the bis-chelated complex of the Fe(II)–dimerum acid (Scheme 1), $\log \beta_{[Fe(II)-L]} = 7.56$. [4]

Bio-relevance of the above detailed Fe(II)/Fe(III) results is also possible. As mentioned in the Introduction, there are several mechanisms of siderophore-mediated iron transport in fungi, including the uptake of intact siderophore complexes into the fungal cells with subsequent egress of the ligand molecules (''shuttle mechanism'') [25]. This mechanism is typical for the ferrichrome and coprogen families. In general, the transported siderophore–Fe(III) complexes are temporarily stored in the cytoplasm and the iron is released by ferrisiderophore reductase in the form of biologically active Fe(II) [26,27]. The intracellular localisation of iron-release has been tracked in the maize pathogen *Ustilago maydis* by using biomimetic fluorescent analogues of ferrichrome [28,29]. Since the redox reaction observed between DFC and Fe(II) in the present work occurs only above pH 4, any reduction of the ligand by free Fe(II) (especially in an acidic vesicle milieu) can not be assumed. Consequently, it seems evident that the iron-released DFC can be re-cycled to perform new transport cycles.

3.3. Comparison between the stability of complexes formed with desferricoprogen and desferrioxamine B

Although, both DFC and DFB are trihydroxamate type natural siderophores (Scheme 1), the structure of their connecting chains, each linking two hydroxamic groups, shows several differences: (1) 9-atom distance exists between two hydroxamic functions in the DFB but 10 in the DFC; (2) one peptide group is situating in a certain position in each of the connecting chains of DFB, while a diketopiperazine ring in one of the chains and a more flexible ester moiety in the another one are sitting in the chains of DFC; (3) one double bond at the β-position is conjugated to each of the hydroxamate groups in the DFC molecule. Due to these structural differences, the stability of the complexes of DFC and DFB are also expected to differ. However, a comparison of the corresponding overall stability constants of the protonated complexes (Table 1) is rather difficult, since the non-coordinated terminal primary amino group of DFB is protonated almost in the whole measurable pH-range. Consequently, the same stoichiometry of the complexes, except [ML], does not relate to the same type of bonding mode of the two siderophores. For example, [MLH] means either bis-chelated complex of DFC, or tris-chelated, amino-protonated complex of DFB. For the sake of comparison, equilibrium constants for the processes shown in Table 3 were calculated (but, when any comparison between these constants is made one has to take into account that the macroconstants for the overlapping protonation processes of these two ligands were used to calculate them).

Since only one hydroxamate of a single ligand is coordinated to the metal ion in the mono-chelated complexes, no measurable effect of the structure of the connecting chains, except the effect of the double bond one conjugating with each hydroxamate function of DFC, on the complex stability can be expected. Unfortunately, such type of complexes ([MLH_2] and [MLH_3] with DFC and DFB, respectively), are formed in the measurable pH-range only with Ni(II), Cu(II) and Zn(II). All these metal ions, however, form slightly more stable mono-chelated complexes with DFC than DFB (Table 3). The trend, which is similar to the previously observed one for monohydroxamic acids [5], is also supported

[4] E. Farkas, É.A. Enyedy, to be published.

Table 3
Derived stability constants calculated for Fe(III)–, Al(III)–, Ga(III)–, In(III)–, Ni(II)–, Cu(II), Zn(II)–DFC and – DFB complexes

		Fe(III)	Al(III)	Ga(III)	In(III)	Ni(II)	Cu(II)	Zn(II)
DFC	$M + H_2L = [MLH_2]$ $\log\beta_{MLH2}$ $(pK_3 + pK_2)$	–	–	–	–	4.90	8.05	4.76
	$M + HL = [MLH]$ $\log\beta_{MLH}$ pK_3	–	15.21	18.16	14.43	8.69	13.86	9.39
	$M + L = [ML]$ $\log\beta_{ML}$	29.35	22.51	25.8	22.88	11.42	15.25	11.80
	pM[a]	25.6	18.7	22.0	19.0	7.8	12.6	8.3
DFB	$M + H_3L = [MLH_3]$ $\log\beta_{MLH3}$ $(pK_4 + pK_3 + pK_2)$	–	–	–	–	3.50	7.29	3.70
	$M + H_2L = [MLH_2]$ $\log\beta_{MLH2}$ $(pK_4 + pK_3)$	21.7	15.9	–	13.28	6.96	12.40	7.47
	$M + LH = [MLH]$ $\log\beta_{MLH}$ pK_4	30.17	22.96	26.08	19.45	8.87	13.14	9.56
	$M + L = [ML]$ $\log\beta_{ML}$	30.4	23.9	27.56	22.18	8.89	13.73	10.36
	pM[a]	26.1	18.9	22.0	17.6	6.1	10.8	6.3

[a] pM values were calculated at pH 7.40, $c_M = 10^{-6}$ M; $c_L = 10^{-5}$ M.

by the spectrophotometric results of Cu(II)-containing systems. Namely, λ_{max} values in Fig. 4 indicate the formation of the Cu(II) complexes with DFC at lower pH (4(a)) than with DFB (4(b)). Since the basicities of the hydroxamate functions in the two ligands are similar, this difference means the higher stability of the complexes of DFC.

Significant effect of the structure and length of the connecting chains can be expected on the stability of bis- and tris-chelated complexes. The stoichiometry of the bis-chelated complexes is [MLH] and $[MLH_2]$, that of the tris-chelated complexes is [ML] and [MLH] or [ML] with DFC and DFB, respectively. Considering the corresponding stability constants and also the calculated pM values shown in Table 3, it can be concluded that the smaller Al(III), Ga(III) and Fe(III) (for ionic radius see Table 1) form a bit more stable complexes with DFB containing shorter connecting chains compared to DFC. The stability trend is just the opposite with the bigger metal ions, such as In(III), Cu(II), Ni(II) and Zn(II). Perhaps, steric reasons are first of all responsible for these differences appearing in the stability of bis- and tris-chelated complexes. The above-detailed stability differences can be demonstrated more clearly by the so called ''preference figures'' in which the ratio of the total concentrations of the DFC and DFB complexes formed in hypotetical systems containing one metal ion plus DFC and DFB as a function of pH are plotted. As representative examples, figures for the Al(III)- and Zn(II)-containing hypothetical systems are shown in Fig. 8.

As it can be seen in Fig. 8, below pH 4 a bit more Al(III) is bound by DFB than DFC (the situation is just the same with Fe(III) and Ga(III)), but the difference with Zn(II) (and also with In(III), Cu(II), Ni(II)) is just the opposite and, what is more interesting, exists also at physiological pH.

A significant difference was observed between the Cu(II)-binding ability of the two siderophores studied. While DFC binds excess of copper effectively, DFB practically not. Our results, obtained recently for the Cu(II)–2,5-DIHA system [10] (the formula of the ligand

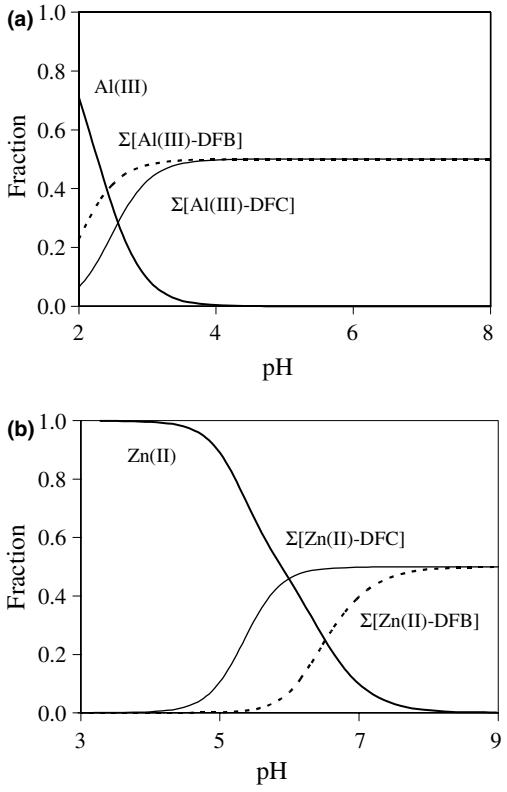
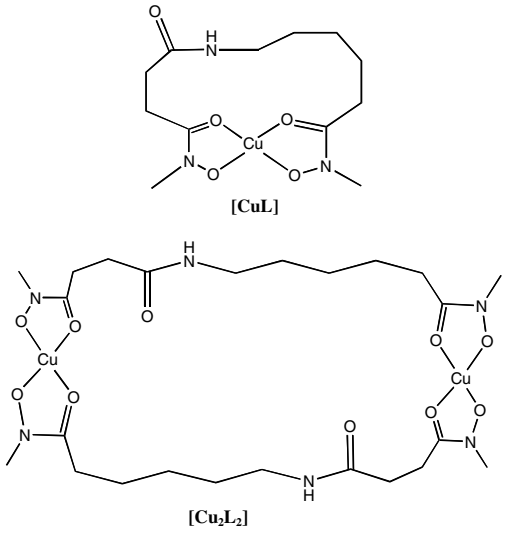


Fig. 8. Concentration ratio of the free metal ions, the total concentration of DFC (solid line) and DFB (dashed line) complexes in a hypothetical Al(III)–DFC–DFB (a) Zn(II)–DFC–DFB (b) systems ($c_M = 1.0 \times 10^{-3}$ M, metal to DFC to DFB ratio = 1:0.5:0.5).

is shown in Scheme 1), might help to understand this difference. According to ESI-MS (Electrospray Ionization Mass Spectrometry) results, the coordination of the two hydroxamates of a 2,5-DIHA molecule (having the same type of connecting chain as those of DFB) to the same Cu(II) ion is not favoured and in addition to [CuL], dimeric species $[Cu_2L_2]$ (Scheme 3) is also formed.

Perhaps the coordination of two next hydroxamates is not favoured also in the bis-chelated Cu(II)–DFB complex, what might be not the case with DFC.



Scheme 3.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2004.08.017.

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