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Mini-review

Iron and citric acid: A fuzzy chemistry of ubiquitous biological relevance

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Abstract

This paper briefly presents a review concerning the species which can arise when iron salts and citric acid are mixed together. The data commented on are required for a correct interpretation of the chemical processes which play a paramount role in biology and in the biological studies involving iron-citrate complexes.

Introduction

Citric acid is ubiquitous in nature (it accounts for 5% by weight of lemon juice and 0.3% by weight of teeth and bone). Its basic chemistry and its chelating properties have been reviewed some years ago (Glusker 1980; Milewska 1988). There are four ionizable groups in citric acid (Figure 1): pK_1 3.13; pK_2 4.76; pK_3 6.40 and $pK_4 \sim 11$ (hydroxyl group).

Ferric citrate plays a paramount role in iron metabolism in living systems. In plants, citric acid is present in roots exsudates which depolymerizes and solubilizes the ferric hydroxide of the soils and mobilize the iron to the membranes of the root cells (Tiffin 1966). Citrate is also used to transport ferric iron to leaves by the xylem sap and the link between iron metabolism and citrate concentration in plants has been established (Pitch *et al.* 1991). *E. coli* possesses a specific transport system for ferric citrate; citrate acts as an exogenous siderophore (Ochs *et al.* 1995). It has been shown that *Bradyrhizobium japonicum* releases citric acid under iron-deficient growth conditions (Guerinot *et al.* 1990). Citrate is present in blood plasma (∼0.1 mM) and is believed to be one of the major components of a pool of iron(III) that is not bound to transferrin (Parkes *et al.* 1991). It has been proposed that non-transferrin-bound iron in the plasma of iron-overloaded patients exists largely as complexes with citrate (Grootveld *et al.* 1989). Citrate has also been proposed to be a constituent of the low molecular weight cytosolic iron pool (Bakkeren *et al.* 1985).

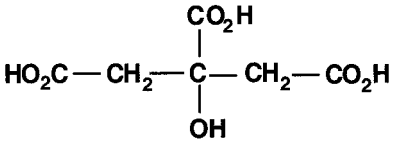

Figure 1. Citric acid.

The presence of Fe(II) and a reducing agent is required for activation of the Krebs cycle enzyme aconitase. The iron A ternary enzyme-Fe(II)-citrate complex has been detected at the active site of aconitase (Villafranca & Mildvan 1972): one iron of the iron-sulfur cluster expands its coordination number from four to six through the formation of a chelate ring with the citrate.

Taking into account the biological importance of the iron-citrate system, a clear description of its chemistry is required for a correct interpretation of the biological processes involving iron and citric acid. Unfortunately, a survey of the literature reveals that the coordination chemistry of iron citrate remains fuzzy. The different experimental conditions used for the preparation of the so-called ferric citrates (stoechiometry, solvent, pH, added ligands or bases) lead to different species, which, moreover, can be in equilibrium in solution. The biological relevances of the

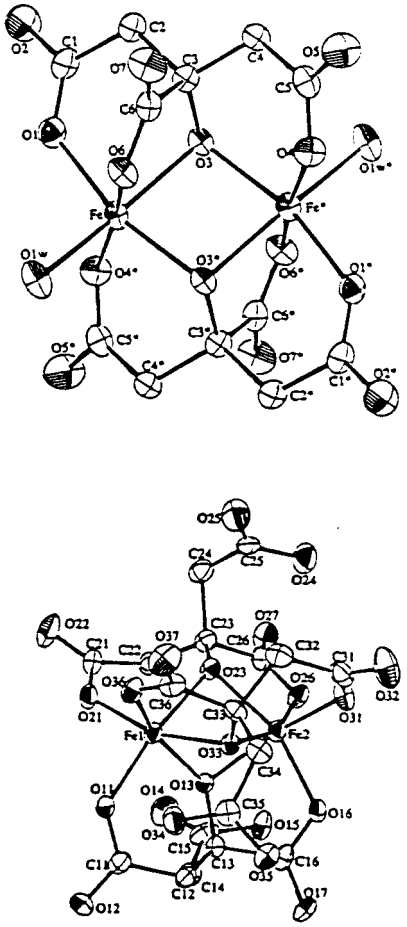

Figure 2. $[Fe_2(cit)_2\ (H_2O)_2]^{2-}$ and $[Fe_2(citH)_3]^{3-}$ from Shweky *et al.* 1994).

complexes are also unclear. Ferrous citrate complexes are easily oxidized and difficult to study.

Iron(III)-citrate systems

Discrete iron(III) citrate compounds have been structurally characterized only since 1994. Lippard *et al.* (Shweky *et al.* 1994) have prepared the two dinuclear complexes $[Fe_2(cit)_2\ (H_2O)_2]^{2-}$ and $[Fe_2(citH)_3]^{3-}$ depicted on Figure 2.

In the former, the citrate ligands are fully deprotonated, while in the latter, each citrate ligand, which uses its alkoxide functionality to link the two ferric iron, has a noncoordinating protonated carboxylate group. The first complex exhibits antiferromagnetic exchange coupling between the iron atoms, while in the second complex the two iron atoms are ferromagnetically coupled. In the presence of excess citric acid, the two complexes are in equilibrium in aqueous solution. To date, the biological relevance of these two complexes is unknown.

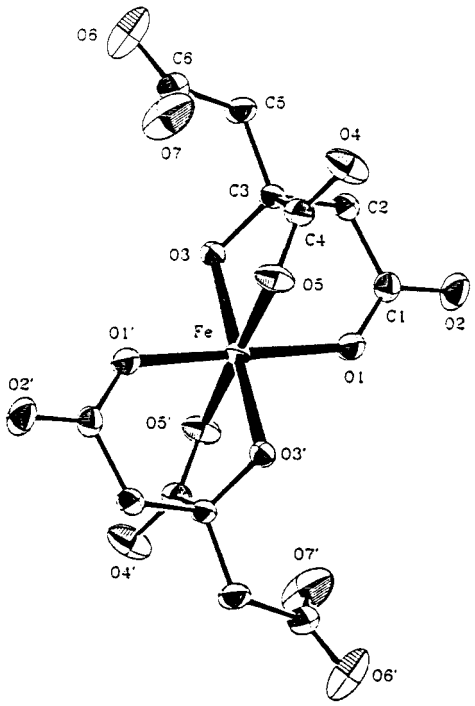

Figure 3. $[Fe\ (cit)_2]^{5-}$ from Matzapetakis *et al.* (1998).

Very recently (Matzapetakis *et al.* 1998), described the first mononuclear anionic iron(III)-citrate complex $[Fe\ (cit)_2]^{5-}$ (Figure 3), in which both citrate ligands are tetraionized with one terminal and the central carboxylate groups and hydroxyl moieties utilized to bind in a monodentate fashion the octahedral iron(III). The third terminal ionized carboxylate group is not coordinated to the metal. Spectroscopic data are consistent with the presence of a high-spin iron(III).

Two polynuclear iron (III) anionic complexes have been recently structurally characterized, a nonairon complex $[Fe_9O(cit)_8(H_2O)_3]^{7-}$ (Bino *et al.* 1998) and an octairon complex $[Fe_8(\mu_3\text{-}O)_2(\mu_2\text{-}OH)_2(cit)_6(OAc)_2(im)_2]^{8-}$ (Gautier-Luneau *et al.* 1999). The nonairon complex consists of three parallel triangular Fe_3 units. The outer two units are connected by three bridging tetradentate cit^{4-} ligands to the central one, and each of the terminal Fe_3 units is capped by another cit^{4-} ligand. The central Fe_3 unit has the oxo-bridged basic iron carboxylate structure, whereas the two terminal ones have a structure with an $[Fe_3O_4]$ core. Mössbauer spectra reflects the existence of two

inequivalent trivalent iron sites. The octairon complex involves six fully deprotonated citrates. It consists of two equivalent tetrairon subunits ($[Fe_4O_5]$ core) connected by two citrates ligands.

The five iron(III) citrate complexes structurally characterized show five different modes of coordination of the citate ligand which are depicted on Figure 4.

The mode (a) is observed in the mononuclear complex $[Fe(cit)_2]^{5-}$. The coordination of one terminal and the central carboxylate groups and the alkoxide fonction on the same iron involves the formation of one seven, one six and one five-membered coordination rings. These three type of chelate rings are also observed in the mode of coordination (b), in which the alkoxide bridges two iron ions, leading to a new six-membered ring. The mode (b) is observed in the dinuclear complex $[Fe_2(cit)_2(H_2O)_2]^{2-}$ and in the octanuclear complex $[Fe_8(\mu_3\text{-}O)_2(\mu_2\text{-}OH)_2(cit)_6(OAc)_2(im)_2]^{8-}$. In the mode (c), which is observed in the dinuclear complex $[Fe_2(citH)_3]^{3-}$, one terminal carboxylate group remains protonated and is not bound to the metal center. In the mode (d), this terminal carboxylate bridges in bis-monodentate fashion two other iron ions: so, the citrate ligand is bound to four iron atoms in the octa and in the nonanuclear complexes. In the last mode (e), observed only in the nonanuclear complex, the citrate ligand caps three iron by a μ3-alkoxo bridge and each of the three carboxylato groups is coordinated to one of the iron ions. These different structures show the versatility of the citrate ligand, which is able to coordinate one, two, three or four iron ions. The L/Fe ratio varies between 2 and 0.75.

The solution chemistry of iron citrate systems is not obvious. Among the complexes described in the solid state, few of them have been the purpose of solution studies. The nomenclature used varies from one to another paper! We have converted the data cited herein in a unique formalism, with $citH_4$ representing the unionized citric acid. At physiological pH, Fe^{3+} and citrate combine to form a polymer in the form of large red-brown spheres (Mw 2.1×10^5; diameter 72) (Spiro *et al.* 1967). This polymer is often considered as a good model for the iron storage protein, ferritin, constituted of an iron hydroxide core with citrate bound to the surface. Addition of bases to iron (III) solutions containing excess citrate ion leads to the formation of an anionic chelate which contains two citrates, each with the hydroxyl proton removed (probably $[Fe(cit)_2]^{5-}$); this reaction competes kinetically

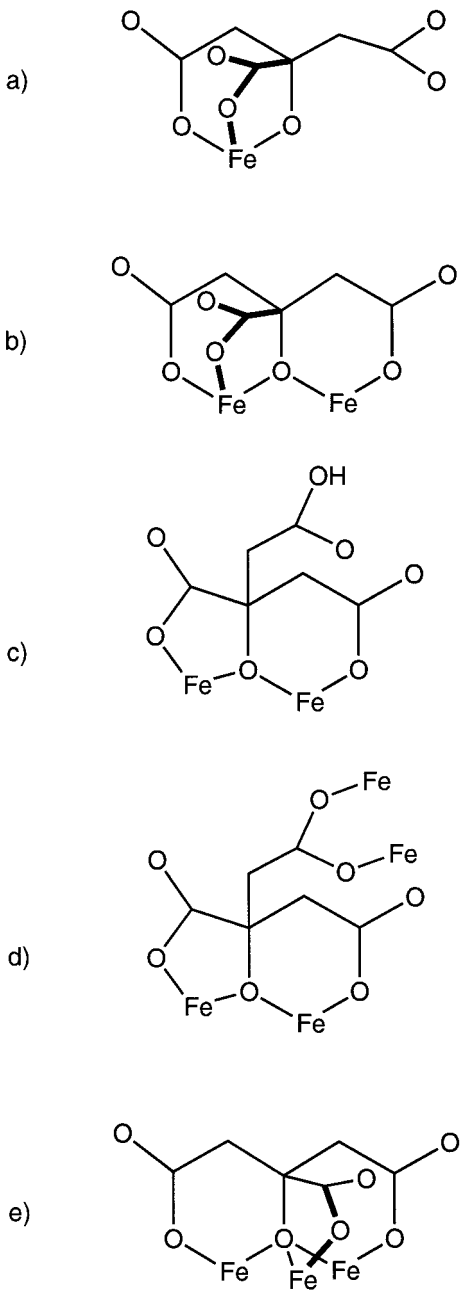

Figure 4. Modes of coordination encountered in iron-citrate complexes.

with polymerization of a 1:1 chelate $[Fe(cit)]^-$ stable at low pH (Spiro *et al.* 1967). For 10^{-3}M iron solutions, the presence of 0.02 M citrate is sufficent to prevent detectable polymerization. Potentiometric studies at pH < 3 revealed an uncharged monomer [Fe(citH)] and an anionic dimer $[Fe_2(cit)_2]^{2-}$ (Timberlake 1964), species not cited by Spiro. In the same range of pH

(acidic medium), only binuclear species of 1:1 stoechiometry have been detected (Field *et al.* 1974). The most reliable results in aqueous solution 1 μM Fe^{3+} and 0.1 mM citrate (Martin 1986) concludes that binding of Fe^{3+} by excess citrate is well described in terms of $[Fe(citH_{2)}]^{+}$, [Fe(citH)], and $[Fe(cit)]^{-}$ at low pH. The $[Fe(citH)_2]^{3-}$ complex (40%) and the $[Fe(cit)]^{-}$ complex (60%) are the only species at pH 5-6 and at pH 7-8 only the latter is present. More recently, complex formation between iron(III) and citric acid in pH range 1 to 13 has been studied by magnetic susceptibility measurements (Yokoi *et al.* 1994). The solutions were 10 mM Fe^{3+} and 30 mM in citric acid. The major species (not clearly depicted) at physiological pH would involve two iron and one citrate.

To date, it is difficult to correlate the structural studies (solid state) and the solution studies. Only the $[Fe\ (cit)_2]^{5-}$ seems to be stable in solution, may be in a form $[Fe\ (citH)_2]^{3-}$ (see Martin 1986) or [Fe (cit)(citH)]$^{4-}$ (non cited) with protonation of the uncoordinated carboxyl groups according to the precise pH. This complex is probably the so-called dicitrate complex which is 'recognized' by the citrate transport system of *E. coli* , and which exists in the human plasma.

The complexation of iron(III) by citric acid has been studied in DMF (Escot *et al.* 1989). Using a tenfold excess of citric acid (versus iron) and the base $(CH_3)_4NOH$, only a partial complexation of iron is observed with the formation of a unique complex for which the formula $[Fe(citH)_2]^{3-}$ is proposed. This complex is the dicitrate complex with one unionized carboxylate group on each citrate moiety.

Ligand exchange reactions at iron of citrate complexes have been few studied. Fe(III) removal from its citrate complex is useful in assessing the ability of a siderophore to mobilize Fe(III) in humans. The kinetics and mechanism of direct iron removal from its citrate polymer complex by desferrioxamine B (therapeutic agent for iron-overloaded patients marketed as the methane sulfonate under the name of Desferal) have been investigated (Faller & Nick 1994). Kinetics data at pH 7.4 and 37 °C are consistent with the formation of a ternary complex (Desferal-iron-citrate), followed by its rate limiting dissociation into free citrate and Desferal-Fe. The depolymerization of Fe-citrate is not the rate-limiting step in the kinetics of transfer. The transfer of citrate-bound Fe to transferrin proceeds through a different route in which the dissociation of the polymeric Fe-citrate to monomeric reactives units, is the rate-limiting step.

Iron(II)-citrate systems

When citrate ions are coordinated to Fe(II) ions, the hydroxyl groups are predominantly ionized in neutral or alkaline aqueous solution. ^{13}C NMR studies of the paramagnetic 1:1 Fe(II)-citrate system has been carried out as a function of pH, temperature and concentration (Strouse 1977). The data revealed equilibria involving several forms of the complex. The molecular weight determinations indicated that studies of these complexes in solution should be interpreted in terms of monomeric complexes of triionized citrate and tetrameric complexes of tetraionized citrate. A tetrameric complex of cit^{-4} of *S*4 symmetry based on a tetrahedral cluster of non- bonded Fe(II) ions was proposed as the predominant form in alkaline solution. X-ray structural studies of an Fe(II) citrate complex crystallized from alkaline solution, $[Fe(II)(H_2O)_6][Fe(II)(citH)(H_2O)]_2 . H_2O$, in wich the protonated hydroxyl group, the central carboxyl group and one terminal carboxyl group were coordinated to a single Fe(II) ion (Strouse *et al.* 1977). Both oxygen atoms of the other terminal carboxyl group are coordinated to two other symmetry-related Fe(II) ions. Hexaquoiron(II) is the counterion. The 1:1 complex of ferrous ion-triionized citrate forms an infinite chain of covalently linked units. Comparison of the molecular weight data with the results of X-ray crystallographic studies suggests that the coordination of both oxygen atoms of a citrate carboxyl group to two different iron ions is easily disrupted in solution: the replacement of these carboxyl oxygen atoms by water molecules in the coordination sphere of the chelated Fe(II) ions would result in a monomeric complex of Fe(II) with triionized citrate. A mononuclear monomeric complex had been suggested in acidic medium (Timberlake 1964).

The redox chemistry of iron-citrate complexes

The redox chemistry of iron citrate complexes remains virtually unknown. In plants, iron(III) citrate complexes are photoreduced, releasing the iron in the leaves (Bienfait & Scheffers 1992). The photoreaction, studied in acidic medium and with citrate excess, involves reduction of Fe(III) to Fe(II) and the concomitant oxidation of a carboxylic group, accompanied by decarboxylation (Abrahamson *et al*. 1994). A superoxide dismutase like activity has been reported for iron (Minotti & Aust 1987). The ferric citrate

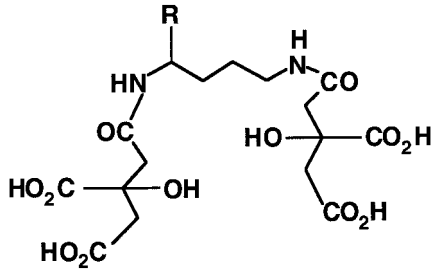

Figure 5. Rhizoferrin (R=H) and staphyloferrin (R=CO_2H).

was prepared by using equimolar amounts of iron and citrate (10 mM solutions) at pH adjusted to 7. This system was found to catalyze the dismutation of superoxide ion. The iron citrate species involved has not been characterized. Auto reduction of ferric citrate (unprecised species) has been studied (Gutteridge 1991), giving Fenton chemistry. The interpretation of the data are not sound since light and the strong ferrous chelator bipyridine are present.

The 'biscitrate' siderophores, rhizoferrin and staphyloferrin A

Rhizoferrin and staphyloferrin A are composed of putrescine or D-ornithine conjugated to two molecules of citric acid via amide bonds (Figure 5). Rhizoferrin is a fungal siderophore (Drechsel *et al.* 1991, 1992) which is the common siderophore of zygomycetes. The transport system of rhizoferrin in *Morganella morganii* involves two genes; they encode an outer membrane protein and a periplasmic protein (RumA and RumB) (Kühn *et al.* 1996).

CD measurements revealed that the quaternary carbon atoms of the two citric acid residues possess an *R, R* configuration and that the 1:1 iron complex of rhizoferrin adopts a Λ configuration around the metal center (Drechsel *et al.* 1992). No structural data are available but the solution coordination chemistry of rhizoferrin have been studied (Carrano et al. 1996). The preorganization due to the linkage between the two citrate subunits induces the formation of only 1:1 high spin iron complexes varying only by their number of protons depending on the pH, with pFe=19.7 at pH 7.4 while the pFe value of citrate is 17.7. Six ligand protonation constants have been determined at 25 °C: log K = 11.3, 10.05, 5.25, 4.21, 3.05 and 2.86, the first two being assigned to the hydroxyl groups. If the fully deprotonated form of rhizoferrin is denoted L^{6-}, only $[FeL]^{3-}$ is observed at pH $\geq$7. $[FeLH]^{2-}$ and $[FeLH_2]^{-}$ are the major species at pH 5 and 3 respectively. It has to be emphasized that fungi are known to acidify their growth environment (pH near 5) and that the complex $[FeLH]^{2-}$ has probably the best biological relevance. The redox potential (-0.082 mV/NHE at pH=7) is high compared to usual siderophores.

Rhizoferrin was found to be an efficient source of iron for barley and maize (Yehuda *et al.* 1996). It was suggested that Fe(III)-rhizoferrin is taken up by strategy II plants via an indirect mechanism in which there is ligand exchange between the microbiol siderophore and phytosiderophores of the plant. To date, ligand exchange reactions at iron of rhizoferrin complexes have not been studied.

S, S -rhizoferrin, the optical antipode of rhizoferrin, has been recently isolated from the culture medium of *Ralstonia (Pseudomonas) pickettii* DSM 6297 grown under iron-limited conditions (Munzinger *et al.* 1999). Transport experiments with radiolabelled iron using rizhoferrin and enantio-rhizoferrin have shown no differences in the bacterial *Ralstonia* strain, while transport of rhizoferrin was superior in the producing fungal *Rhizopus* strain, suggesting stereoselective recognition in the fungus.

Conclusion

Despite the fact that numerous chemical studies of iron-citrate systems are described in literature, the coordination chemistry of these system remains fuzzy. Structural studies are still required and the behavior in solution of the characterized species has to be precised. Moreover, the biological relevance of each characterized complex will have to be studied. The chemistry and the biochemistry of iron-citrate remains an open and interesting challenge for researchers in the field of iron metabolism.

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