DOI: 10.1002/ejic.200500821

Dissociation of CP20 from Iron(II)(cp20)$_3$: A Pulse Radiolysis Study

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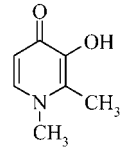
Keywords: Iron / Chelates / Reduction / Electron transfer / Kinetics

Deferiprone (CP20), a hydroxypyridine-4-one, is used in iron-chelation therapy. For the iron(III)-/(II)(cp20)$_3$ couple, a standard electrode potential of –620 mV was measured by cyclic voltammetry [Templeton et al., *Inorg. Chim. Acta* **1996**, *245*, 199; Merkofer et al., *Helv. Chim. Acta* **2004**, *87*, 3021]. On the basis of this value and the overall stability constant for the equilibrium of iron(III) and three CP20 molecules, ligand dissociation ought to take place after reduction of the iron(III)(cp20)$_3$ complex. By pulse radiolysis, we show that hydrated electrons reduce the iron(III)(cp20)$_3$ complex with a rate constant of $k = (6.4 \pm 0.3) \times 10^{10}$ M^{-1} s^{-1}, and that the iron(II)(cp20)$_3$ complex aquates rapidly. The dissociation of the first two CP20 molecules takes place within a few μs after the pulse. The dissociation rate constant for the last CP20 ligand is $(8 \pm 1) \times 10^3$ s^{-1}. The observation of a dissociation illustrates that, under dilute conditions, iron(II) is not fully complexed by CP20 and could potentially participate in redox cycling to produce oxyradicals. The $CO_2^{\bullet-}$ radical does not reduce iron(III)(cp20)$_3$, which indicates that this complex reacts, as expected, very slowly with inner-sphere reductants.

Introduction

Iron is essential to life. It is used, transported and stored by specific proteins such as haem proteins, transferrin and ferritin. When, for various reasons, iron is released from these proteins or excessively absorbed from food, it can participate in redox cycling that generates toxic oxyradicals.[1,2]

The bidentate chelating agent CP20 (1,2-dimethyl-3-hydroxypyrid-4(1*H*)-one, also known as deferiprone or L1, Scheme 1) is effective at facilitating iron removal from iron-overload patients when administered orally.[3] It forms five-membered chelate rings in which the metal is coordinated by two vicinal oxygen atoms. This chelating agent is an alternative to the well-established desferrioxamine, which requires intravenous administration. Another orally active drug, tridentate ICL670 (4-[3,5-bis(2-hydroxyphenyl)-1*H*-1,2,4-triazol-1-yl]benzoic acid), is under development.[4] It is generally assumed that these chelating agents are not toxic when the standard electrode potentials of the iron(III)/iron(II) couples are such that they cannot be reduced by ascorbate or oxidised by hydrogen peroxide, that is, below $E°'(Asc^{\bullet-},H^+/HAsc^-) = +282$ mV[5] or above $E°'(H_2O_2,H^+/HO^{\bullet},H_2O) = +370$ mV, respectively. The latter value is based on Gibbs energies of formation of the hydroxyl radical and hydrogen peroxide determined by Schwarz and Dodson[6] and Kern,[7] respectively. This is the case for the electrode potential of the iron(III)-/iron(II)(cp20)$_3$ couple, –620 mV at pH = 11. However, the in vivo conditions are far removed from those of the standard state: the concentration of iron–CP20 complexes is only in the micromolar range, and the pH is much lower than 11. By cyclic voltammetry, we could show that, under dilute conditions, the electrode potentials shift to more positive values, which may occur as a result of dissociation of ligands from iron(II).[8] Indeed, binding data derived from electrode potentials indicate that iron(II) complexes of CP20 are not fully formed at physiological pH and low iron concentration, even when CP20 is present in fivefold excess. We show here by pulse radiolysis that, indeed, the proposed ligand dissociation[8] occurs after rapid reduction of iron(III)(cp20)$_3$ by hydrated electrons.



CP20 (L1)

Scheme 1.

Results

Distribution Diagram of the Iron(II)–(CP20) and Iron(III)–(CP20) Complexes

Speciation diagrams for the iron(II)–(CP20) and iron(III)–(CP20) complexes at 20 μM iron, 100 μM CP20 and pH = 7.2 are shown in Figure 1A and Figure 1B, respectively. As the binding constants β_1 and β_2 are not known for iron(II), we assumed that the ratios β_3/β_2 and β_3/β_1 for iron(II) are

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similar to those of iron(III). The data for Figure 1A and Figure 1B were obtained from the literature.[8] While iron(III) tightly binds three CP20 ligands at pH = 7 and higher, iron(II) does not. In fact, not even the 1:1 complex of iron(II) and CP20 is fully formed at pH = 7.

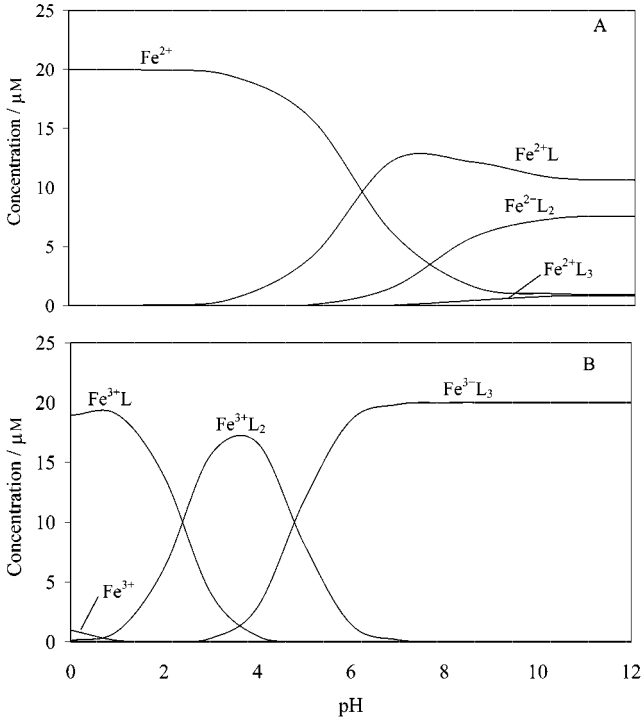

Figure 1. Iron(II) (**A**) and iron(III) (**B**) speciation in the presence of CP20. [CP20] = 100 μM, [Fe^{2+}] and [Fe^{3+}] = 20 μM. While iron(III) tightly binds three CP20 ligands at pH = 7 and higher, iron(II) does not. The pK_a value used for CP20 is 9.76.[20]

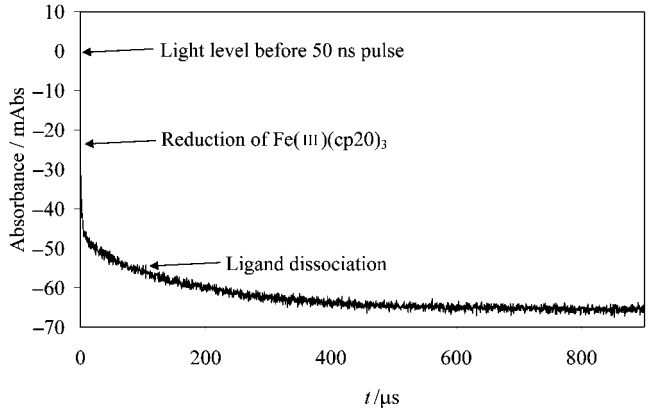

Figure 2. Reduction of $Fe^{III}(cp20)_3$ (100 μM) with hydrated electrons (14 μM) at 450 nm. Reaction conditions: phosphate buffer (5 mM) with *tert*-butyl alcohol (0.1 M) at pH = 7.2 and room temperature. Following rapid reduction, a slower process is observed that is ascribed to ligand dissociation. The reduction with hydrated electrons proceeds quantitatively.

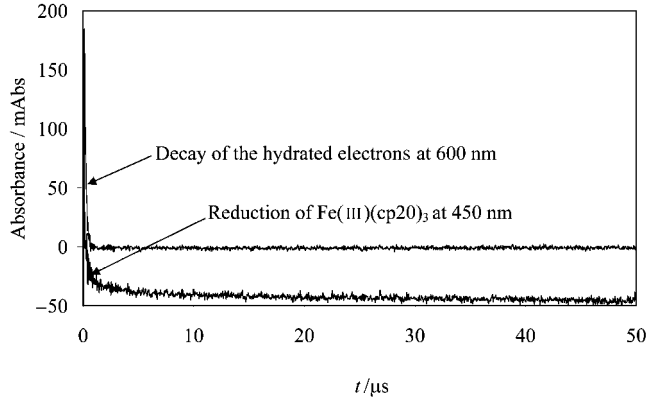

Figure 3. Reduction of $Fe^{III}(cp20)_3$ (100 μM) with hydrated electrons (12 μM) at 450 nm and decay of the hydrated electrons at 600 nm. Reaction conditions: phosphate buffer (5 mM) with *tert*-butyl alcohol (0.1 M) at pH = 7.2 and room temperature. The decay of the hydrated electrons at 600 nm shows the same time-dependence as the fast decay at 450 nm.

Reduction of the Iron(III)(cp20)$_3$ Complex by e^-_{aq} and $CO_2^{\cdot-}$

Rapid reduction of iron(III)(cp20)$_3$ (100 μM) by hydrated electrons (14 μM) leads to decreases in absorption at 450 nm. The fastest is finished within one μs and the slowest within one ms (Figure 2 and Figure 3). Reduction proceeds quantitatively (Figure 2), based on an extinction coefficient of 4600 $M^{-1}cm^{-1}$ for the iron(III)(cp20)$_3$ complex at 450 nm.[8] These processes are not caused by the presence of *tert*-butyl alcohol (data not shown). The rate of decay of hydrated electrons measured at 600 nm is identical to the rate of the fast process at 450 nm (Figure 3). This rate is first-order in iron(III)(cp20)$_3$ (Figure 4), from which a rate constant of $(6.4 \pm 0.3) \times 10^{10}$ $M^{-1}s^{-1}$ is calculated. The rate of decay of the hydrated electrons measured at 600 nm indicates that the reduction is over within one μs. The change in absorbance at 450 nm during 1 and 3 μs is interpreted as a result of the sequential dissociation of two CP20 ligands. The slow absorbance change after that process, which fits to a single exponential, is attributed to the dissociation of the last ligand, as described in Equation (1). The reaction is strictly first-order, with a reaction rate constant of $(8 \pm 1) \times 10^3$ s^{-1}.

$$[Fe^{II}(cp20)]^+ \rightarrow Fe^{2+} + CP20^- \quad (1)$$

Mechanistically, the observed first-order reaction might also be represented by Equation (2) at constant pH.

$$H^+ + [Fe^{II}(cp20)]^+ \rightarrow [Fe^{II}(Hcp20)]^{2+} \rightarrow Fe^{2+} + Hcp20 \quad (2)$$

However, control experiments at pH = 8.6 showed that the rate of reaction is independent of pH; therefore, reaction 2 is not relevant.

Identical results to those with *tert*-butyl alcohol were obtained when methanol was used as a hydroxyl-radical scavenger. Interestingly, dioxocarbonate(·1–), a strong reductant $E°$ ($CO_2/CO_2^{\cdot-}$) = –1.9 V,[9] did not reduce iron(III)(cp20)$_3$ to any detectable degree.

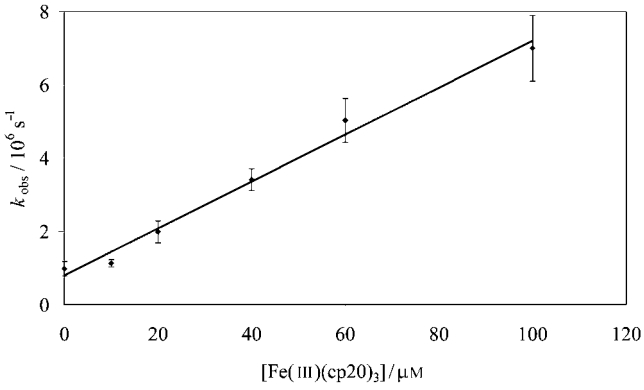

Figure 4. First-order rate constants at various concentrations of $Fe^{III}(cp20)_3$. The reactions with hydrated electrons (5 μM) were carried out at pH = 7.2 and room temperature. The reduction of iron(III)(cp20)$_3$ is linearly dependent on the complex concentration. The bimolecular rate constant is $k = (6.4 \pm 0.3) \times 10^{10}$ $M^{-1} s^{-1}$.

Oxidation of Iron(II) by Hydrogen Peroxide in the Presence of CP20

The absorbance change of this oxidation, per reducing equivalent, should equal that for the reduction of iron(III)-(cp20)$_3$ by hydrated electrons. To calculate a difference spectrum for the iron(II)- and the iron(III)(cp20) complexes, we mixed a solution of iron(II) (20 μM) and CP20 (100 μM) with hydrogen peroxide (200 μM) at pH = 7.2 and 15 °C (Figure 5). At this temperature, the reaction becomes slower, and the signal-to-noise ratio of the time-dependent spectrum improves. Between 350 and 550 nm, the absorption increase per hydrogen peroxide matches the absorption decrease per hydrated electron in this range, as shown in Figure 6. The hydroxyl radical or oxoiron(IV) formed in the Fenton reaction is scavenged by the Tris buffer. The resulting Tris radical is not expected to re-reduce iron(III)-(cp20)$_3$. Because the iron(II)(cp20) complex can reduce water, it is impossible to prepare solutions of the pure complex: Under anaerobic conditions, in gas-tight syringes as well as in a flask purged with argon, the colour of the iron(II)(cp20) solution changes from light green to light orange within several minutes, indicating the formation of

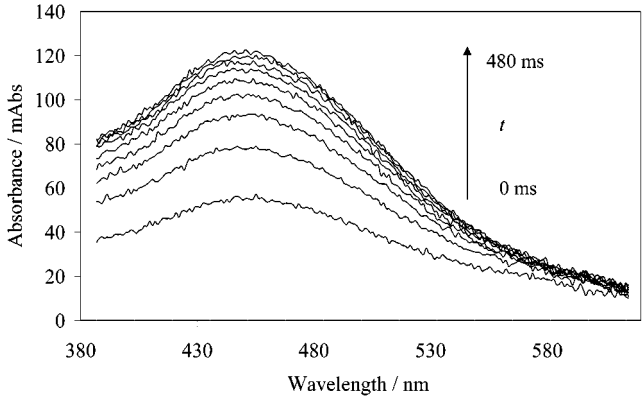

Figure 5. Time-dependent spectrum of the reaction of iron(II) (20 μM) and CP20 (100 μM) with hydrogen peroxide (200 μM) at pH = 7.2 and 15 °C. Formation of the iron(III)(cp20)$_3$ complex.

the iron(III) complex. At high pH values, this process is slower. Hegetschweiler and coworkers[10] reported that the iron(II)–ICL670 complex behaves similarly.

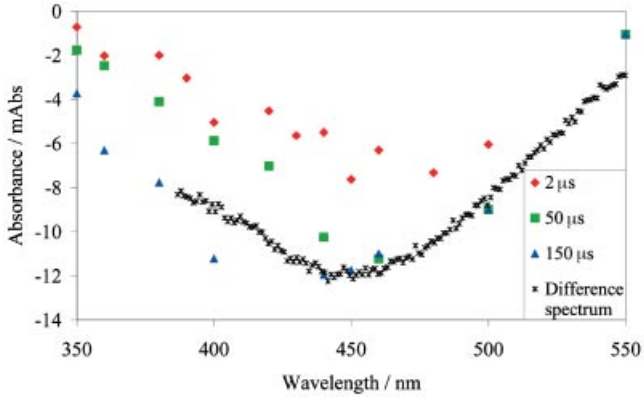

Figure 6. Time-dependent spectra measured by pulse radiolysis (diamonds, squares and triangles) of the reaction of iron(III) (33 μM) and CP20 (100 μM) with hydrated electrons (3.4 μM) at pH = 7.2 and room temperature. The small crosses indicate the difference spectrum of the iron(II)- and the iron(III)(cp20) complex measured by stopped-flow spectroscopy (see text). Per redox equivalent, the final difference spectrum from the pulse radiolysis is equal to that obtained by the stopped-flow technique.

Discussion

As revealed by the speciation diagrams and as substantiated by kinetics experiments, CP20 stabilises iron(III), but not iron(II), very well. The first-order rate constant for the dissociation of the last CP20 molecule from iron(II) is of the same order of magnitude, 10^4 s^{-1}, as that for the dissociation of a pyridine ligand from a ferriprotoporphyrin in chloroform.[11] CP20 is a bidentate ligand with two hard, oxygen coordination atoms, whereas pyridine is a monodentate ligand with a soft donor atom that stabilises iron(II), which leads to similar stability constants and similar dissociation rate constants. To the best of our knowledge, no other dissociation rates of ligands similar to hydroxypyridinones have been reported in the literature. The solvent-exchange rates for water ($k = 4.4 \times 10^6$ s^{-1}),[12] methanol ($k = 5 \times 10^4$ s^{-1})[13] and acetonitrile ($k = 6.6 \times 10^5$ s^{-1})[14] on iron(II) are higher. Given the speciation diagram (Figure 1A), the observed dissociation process involves all three ligands. After dissociation of CP20 from iron(II), monodentate ligands, such as water, bind to the metal ion and exchange much faster than CP20. Consequently, the probability that hydrogen peroxide coordinates to the metal centre and undergoes a Fenton reaction is increased.[15]

It is to be emphasised that the dissociation rate constant is independent of pH, and, therefore, does not result from protonation of the coordinated CP20 ligands. The spectral changes underline that the observed process is also not a dissociation of CP20 from iron(III), because this would result in a red shift, rather than the observed blue shift, of the spectrum.[8]

The reduction of the iron(III)(cp20)$_3$ complex by hydrated electrons is most likely a diffusion-controlled process. Reduction is possible with hydrated electrons, but not with dioxocarbonate(·1–) radicals, although from a comparison of the electrode potential of the iron(III)-/iron(II)(cp20)$_3$ couple (–0.62 V)[8] with that of the carbon dioxide/dioxocarbonate(·1–) couple (–1.90 V), it is clear that reduction is thermodynamically very favourable. This result shows that it is difficult to reduce the iron(III)(cp20)$_3$ complex with an inner-sphere reductant, because accessibility of the metal centre is sterically hindered by the large CP20 ligands. In a pulse radiolysis study of 2-pyridone, Moorthy et al.[16] could reduce the ligand with hydrated electrons, but not with dioxocarbonate(·1–) radicals. They concluded that an electrode potential smaller than –1.90 V is required to reduce 2-pyridone. On the basis of these and our observations, we conclude that the iron(III) is not directly accessible and is reduced via the ligand.

Reoxidation of iron(II) by hydrogen peroxide formed during the irradiation was not detected. This is understandable: if 10 μM iron(II) is produced, 2.5 μM hydrogen peroxide is also formed. Given a reaction rate constant of 10^4 M^{-1} s^{-1} for the Fenton reaction, the pseudo-first-order rate constant would be ca. 10^{-2} s^{-1}.

We have shown that reduction by hydrated electrons is followed by ligand dissociation. One might now ask what would happen with more physiologically relevant reductants. El-Jammal and Templeton[17] proposed on the basis of their electrochemical measurements that reduction of iron(III) takes place after dissociation of the complex. We assess here the thermodynamics of two scenarios: first reduction followed by dissociation, and then the opposite. The standard electrode potential of the iron(III)-/iron(II)-(cp20)$_3$ couple is –0.62 V, and reduction by an electron donor with an electrode potential near 0 V "costs" ca. 58 kJ mol^{-1}. This process is then followed by favourable ligand dissociation. The dissociation of one CP20 ligand from the iron(III)(cp20)$_3$ complex requires less energy, about 53 kJ mol^{-1}, based on the difference between β_3 and β_2. In dilute solutions, this Gibbs energy decreases considerably, which makes the "ligand dissociation first" scenario more favourable. Therefore, for biologically relevant conditions, we agree with the proposal of El-Jammal and Templeton.[17]

Iron(II) complexes of other hydroxypyridinones and of ICL670 are also unlikely to be formed, given the similarly low electrode potentials.[8] Previously, a chelating agent with an electrode potential for its iron(III)/iron(II) complex of ca. –470 mV or lower, such as that of iron(III)-/iron(II) desferrioxamine,[18] was considered nontoxic. This hexadentate ligand binds iron(II) well at high dilution, in contrast to bi- and tridentate chelating agents. Iron complexes with bidentate chelating agents are more sensitive to dissociation upon dilution, which causes their electrode potentials to shift to more positive values.[8] Considering that the "free" iron concentration in vivo is about 10 times lower than that used here, one can conclude that CP20 does not bind iron(II) under physiological conditions. Recently, we showed that the electrode potentials of the iron(III)/iron(II) complexes with CP20 and ICL670 are near –150 mV under physiological conditions, and that superoxide is unable to reduce these complexes when they are fully formed. For complexes with less than three CP20 or two ICL670 molecules, reduction is thermodynamically feasible.[8] Electrode potentials of other reducing agents will be discussed in a future communication.

Experimental Section

All chemicals used were of analytical reagent grade or higher. $Fe(NO_3)_3 \cdot 9H_2O$ was the source of Fe^{III}. Hydrogen peroxide stock solutions were prepared from Merck Analyzed Reagent 30% hydrogen peroxide. Water was purified with a Millipore Milli-Q unit fed with deionised water.

For pulse radiolysis, a Febetron 705 (Titan Systems Corp., San Leandro, CA, USA) 2.3-MeV accelerator with a pulse width (full width at half maximum) of <50 ns was used as the radiation source. The optical system consisted of a 75W Xe arc lamp (Hamamatsu, Schüpfen, Switzerland), a 1-cm quartz cell (Hellma GmbH & Co KG, Müllheim, Germany) and an Acton SP300 monochromator (Roper Scientific, Ottobrunn, Germany). For signal detection, there were two options: (1) a R928 photomultiplier (Hamamatsu, Japan) with a DHPCA-200 amplifier (Femto Messtechnik GmbH, Berlin, Germany) and a DL7100 digital storage oscilloscope (Yokogawa Electric Corporation, Tokyo, Japan) for kinetics traces or (2) a Princeton Instruments PI-MAX 512T gateable ICCD-camera (Roper Scientific) for time-resolved spectra. The dose per pulse used was 7–65 Gy, which corresponds to hydrated electron concentrations of 1.8–17 μM.[19] Dosimetry was carried out with thiocyanate.

The primary products from the radiolysis of water are H^+_{aq}, e^-_{aq}, $HO^\cdot$, $H^\cdot$, H_2 and H_2O_2. We used *tert*-butyl alcohol (0.1 M) or methanol (0.1 M) as scavengers for $HO^\cdot$ and $H^\cdot$ radicals to produce the fairly unreactive 2-hydroxy-2-methylpropyl- and hydroxymethyl radicals.[19] Solutions for experiments with hydrated electrons contained phosphate buffer (5 mM) and *tert*-butyl alcohol (0.1 M) or methanol (0.1 M), and were evacuated and saturated with argon at least five times.

The dioxocarbonate(·1–) radical was generated by reactions represented by Equation (3) and Equation (4). These solutions were saturated with dinitrogen monoxide (24 mM) and contained formate (50 mM, pH = 8.6).

$$e^-_{aq} + N_2O \rightarrow HO^\cdot + HO^- + N_2 \quad (3)$$

$$HCOO^- + HO^\cdot \rightarrow CO_2^{\cdot-} + H_2O \quad (4)$$

The kinetics of the reduction of the iron(III)(cp20)$_3$ complex and the ligand dissociation were recorded at 450 nm. The decay of the hydrated electrons was followed at 600 nm.

Stopped-flow experiments were obtained with an OLIS stopped-flow instrument (Bogart, GA, USA) equipped with an OLIS RSM 1000 rapid-scanning monochromator set to collect up to 1000 spectra per second. Solutions for the stopped-flow experiments were prepared in Tris buffer (0.1 M), and evacuated and saturated with argon at least five times before addition of iron(II). Hydrochloric acid was used to adjust the pH. The hydrogen peroxide solution was purged with argon for at least 30 min.

Species-distribution diagrams were calculated with the SPEC program (R. Kissner, ETHZ). In SPEC, which was designed to calculate the species distribution in solution equilibria from known formation constants and total concentrations, the Newton–Raphson algorithm is used to solve the system of nonlinear partition equations ("tangential method").

Acknowledgments

This work was supported by the ETH Zürich, Swiss National Science Foundation, and the BBW (01.0397), in support of EU (QLK1-CT-2002–00444).

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Received: September 15, 2005
Published Online: December 20, 2005

