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Review

Chemistry for an essential biological process : the reduction of ferric iron

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Abstract

In biological systems, the predominant form of iron is the trivalent Fe(III) form, which is potentially not readily bioavailable because of its hydrolysis and polymerization to insoluble forms. It is also the easiest of the two predominant forms of iron to chelate selectively. In a short overview of iron chemistry, we point out some of the pitfalls using standard redox potentials, comment on the interaction of ferric complexes with hydrogen peroxide to give hydroxyl radicals and address the release of iron from ferrisiderophores. In biological systems there are two classes of ferric reductases, the soluble flavin reductases found in prokaryotes, and the membrane-bound cytochrome b-like reductases found in eukaryotes. Finally the role of dissimilatory ferric reduction in microbial respiration and biomineralization is discussed.

Introduction

Iron fulfils a vital role in virtually all living organisms (Crichton 2001) (the only organisms which do not require iron are the well-studied genus *Lactobacillus* and some strains of *Bacillus* (Archibald 1983). Among the significant roles of iron in biology are the transport, storage and activation of molecular oxygen, reduction of ribonucleotides and dinitrogen, activation and decomposition of peroxides and electron transport via a wide variety of electron carriers spanning a range of redox potential of essentially 1 V. The unique suitability of iron comes from:
(i) its abundance in the earth's crust: it is the second most common metal after aluminium,
(ii) the solubility of ferrous salts,
(iii) the extreme variability of the Fe^{2+}/Fe^{3+} redox potential which can be finely tuned by well-chosen ligands, so that iron sites can encompass almost the entire biologically significant range of redox potentials, from about −0.5V to 0.6V (copper, either alone or together with iron, can handle somewhat more positive redox potentials).

Life appeared in an oxygen-free environment. Large concentrations of soluble ferrous iron were present during prebiotic times and was the form used for the first stages of life: *the early chemistry of life used iron(II)*. When photosynthesis, converting water into oxygen, became efficient, soluble bioavailable iron disappeared from the surface of the Earth, as the result of the irreversible oxidation of ferrous iron and precipitation of ferric iron in the form of oxides and hydroxides. The advent of O_2 was a dreadful event for most living organisms and can be considered as the first general irreversible gaseous pollution of the Earth. From then on, the reduction of ferric to ferrous iron became an essential process for life.

The basic chemistry of iron

Its position in the middle of the first transition series implies that iron has the possibility to gain access to various oxidation states (from −II to +VI), the most common being II (d^6)and III (d^5). Fe^{3+} is water insoluble ($K_{sp} = 10^{-39}$ M and at pH 7.0 , $[Fe^{3+}] = 10^{-18}$ M) and significant concentrations of water-soluble Fe^{3+} species can be obtained only by complexation with strong chelators. Iron(III) is a hard acid which prefers hard oxygen ligands while iron(II) is on the borderline between hard and soft acids, favouring nitrogen and sulfur ligands. The coordination number 6 is the most frequent (octahedral complexes), although four (tetrahedral) and particularly five-coordinate complexes (trigonal bipyramidal or square pyramidal) are also encountered. For octahedral complexes, two different spin states (low-spin complexes with strong-field ligands and high-spin complexes with low-field ligands) can be observed. High spin complexes are kinetically labile, while low-spin complexes are exchange inert. Both oxidation states are Lewis acids, the ferric state being stronger.

Redox potential: uses and abuses

The standard redox potential of the Fe^{3+}/Fe^{2+} couple in water is +0.77 V. Since on the one hand iron complexes can display a great variety of stability constants, and on the other, for a given ligand L, the stability constant for ferric iron can be greatly different from that of ferrous iron, the redox potential of the LFe^{3+}/LFe^{2+} couple can vary from −1 V to + 1 V. Some relevant redox potentials (at physiological pH) are summarized in Figure 1 (Pierre & Fontecave 1999).

In the following we want, with some well-chosen examples, to show how careful one must be in the utilization of these thermodynamic data.

a- Iron and H_2O_2. A given ferric complex can catalyze the one-electron reduction of H_2O_2 to HO^- and the toxic hydroxyl radical ($H_2O_2 \rightarrow HO^- + HO^\bullet$) only if the redox potential of the LFe^{3+}/LFe^{2+} couple is such that: (i) LFe^{3+} can be reduced by the reducing agent; (ii) the resulting LFe^{2+} can transfer one electron to H_2O_2. This is thermodynamically allowed for the complexes of Figure 1 whose redox potentials are in the range −324 mV (for NADPH as the electron donor) to +460 mV. However, these numbers correspond to standard *equilibrium conditions*, which obviously are rarely fulfilled within cells. Incorrect uses of these redox potential have led to misinterpretations often encountered in the literature. For example, superoxide has often been proposed as an important ferric reducing agent and this reaction used to explain the toxicity of superoxide. The thermodynamic condition for the reducibility of iron(III) is : E^o (LFe^{3+}/LFe^{2+})> −160 mV, this last value corresponding to E^o O_2/O_2^- (see Figure 1). This is effective only if $[O_2] = [O_2^-]$. However, in the living cell (in the absence of oxidative stress), the actual concentration can be estimated at $[O_2] = 3.5 \times 10^{-5}$ M and $[O_2^-] = 10^{-11}$M. Therefore, the reducing power of superoxide in a living cell is in fact much weaker, as shown from the Nernst equation : $E = -160 + 0.06 \log[O_2]/[O_2^-]$, i.e. +230 mV !

There are many fewer ferric complexes that can be reduced by superoxide than predicted by the usual thermodynamic data. It is therefore unlikely that the toxicity of superoxide is mostly dependent on its reducing power with regard to iron, as is sometimes proposed.

b-Iron release from ferrisiderophores. By far, the most common mechanism of iron acquisition by microorganisms involves the chelation of ferric iron by siderophores which are low molecular weight, ferric ion specific, chelating agents elaborated by bacteria and fungi growing under low iron stress (Neilands 1995). The redox potentials depicted in Figure 1 for ferrisiderophores (ferrioxamine B, ferrienterobactin) and for all the known ferrisiderophores suggest that the reduction of these iron complexes by biological reducing agents are thermodynamically unfavorable. However, flavin reductases providing, at the expense of NADPH, free reduced flavins working only at −200 to −400 mV, exhibit ferrisiderophore reductase activity (Coves & Fontecave 1993). This is easily understandable if one considers that a rather strong Fe^{2+} complexing agent L is present in the medium. Then, L is able to take Fe^{2+} from the resulting Fe^{2+}-siderophore complex since siderophores usually have weak affinities for Fe^{2+}. The reduction of ferrisiderophores can thus be driven to completion by the coupled thermodynamically favorable complexation of Fe^{2+} by L:

$$Fe^{3+}\text{-siderophore} \rightleftarrows Fe^{2+}\text{-siderophore}$$

$$Fe^{2+}\text{-siderophore} + L \leftrightarrows Fe^{2+}\text{-L} + \text{siderophore}$$

Apoproteins or porphyrins (which incorporate iron in the ferrous state), can function as the Fe^{2+}

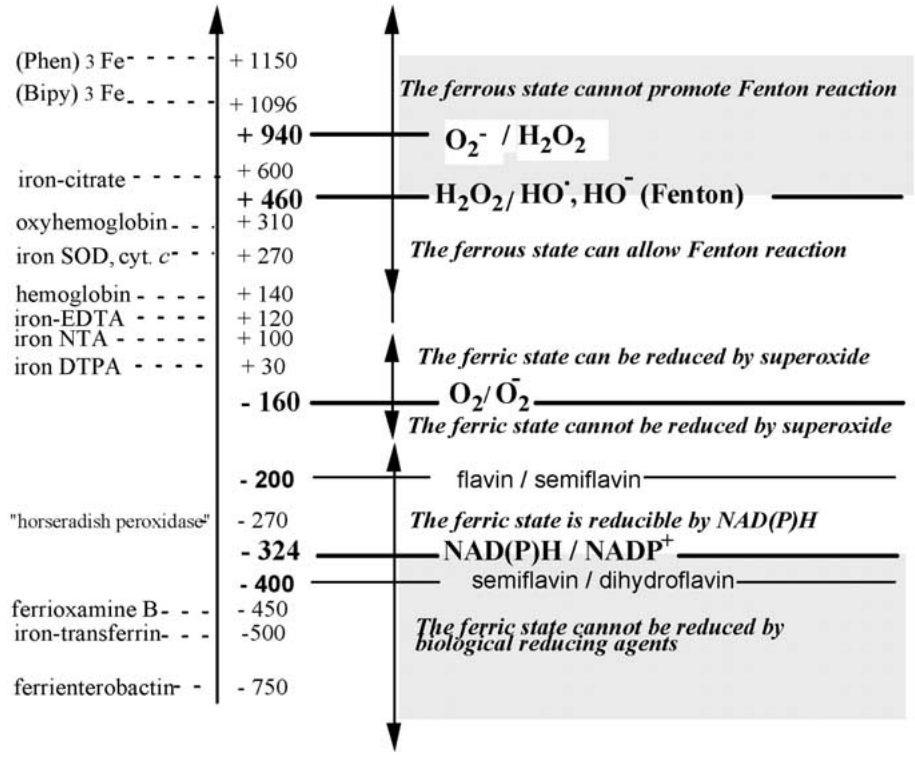

Fig. 1. Fe^{3+} / Fe^{2+} redox potentials at pH 7 (mV/NHE).

complexing agent. Another way to look at this is the following: if we apply the Nernst equation to the iron-siderophore system $E = E^o + 0.06 \log[Fe^{3+}\text{siderophore}] / [Fe^{2+}\text{siderophore}]$. It is apparent that the sequestration of Fe^{2+} by a strong ferrous chelator L results in the decrease of [Fe^{2+} siderophore] and thus in a drastic increase in the potential E, allowing reduction of Fe^{3+}-siderophore by usual biological reducing agents, such as reduced flavins. When strong ferrous chelating agents, resulting in complexes with high redox potentials (such as ferrozine or phenantroline), are used *in vitro* as tools to monitor a biological ferric reduction reaction, one has to be very cautious : the observed reduction may have been the result of this chemical coupling process, and not a true biologically relevant reaction.

c-The problem of the medium. Another cause of misinterpretations may result from the use of the data obtained in aqueous solutions for processes occuring in hydrophobic media (membranes, receptor proteins) . As we will develop further, ferric reductases are often located in membranes and it is well-known that redox potentials greatly vary with the solvent. Redox potentials are also very dependent on pH. Some biological reductions take place in compartments (such as vacuoles) in which the pH can be far from neutrality. As an example, the reduction potential of ferrioxamine B increases by more than 300 mV with a decrease in pH to 3.5, moving the midpoint potential for reduction into the range of biological reductants.

Reduction and uptake of iron in biological systems

Reduction of ferric iron is a key biological process during cellular iron uptake. It is mainly required for the two following purposes. On the one hand, it provides ferrous iron from exogenous ferric iron at the surface of the cell for the uptake and transport systems by reduction with transmembrane electron transfer enzymes. This is examplified by the well-characterized ferric reductases of yeast described below. On the other hand, it provides a mechanism to make intracellular iron, sequestered either within compartments or by strong chelators, available to target enzymes and proteins. One well studied example is the reduction of ferrisiderophores in bacteria.

Considering the importance of iron reduction for living organisms, it is amazing how little we know about enzymatic systems involved in this process in terms of structure, mechanism and regulation. It is very likely that an increased knowledge of this reaction would be extremely beneficial in many different applications : in therapy, in cases of human diseases linked to altered iron absorption and metabolism and to oxidative stress; in agriculture for example for improving plant growth in iron-deficient media; in biotechnology for example with the possibility to create iron biosensors. . . .In the following we discuss briefly the two different reductase systems known so far. We exclude from the list all of the cellular reductases, which function as electron donors to specific iron enzymes at a controlled potential such as the cytochrome P-450 reductase for cytochrome P-450 and many other similar systems.

Ferric reductases

So far only two classes of ferric reductases are known: the soluble prokaryotic flavin reductases and the membrane cytochrome b-like reductases, found in eukaryotes.

Flavin reductases are chromophore-less enzymes which catalyze the reduction of free flavins, FMN, FAD and riboflavin, by NADPH or NADH. They are thus not flavoproteins but rather they use flavins as substrates. The enzyme provides a site which can accomodate both NAD(P)H and flavins so that a greatly accelerated hydride transfer can occur. The prototype for this class of enzymes is Fre, from Escherichia coli, whose three-dimensional structure has been recently determined (Ingelman *et al.* 1999). The products of the reaction are the corresponding reduced flavins which are then released in solution. A large number of studies have associated flavin reductases to ferrisiderophore reduction in bacteria. This has been discussed by us in a review article in 1994, which is still valid (Fontecave *et al.* 1994). More examples of flavin reductases involved in iron metabolism have been described but the general concepts described in this paper still operate. It is generally assumed that iron is liberated from ferrisiderophores in the form of ferrous iron by reaction with free enzymatically reduced flavins (Figure 2).

In graminaceous plants iron can also be taken up with the help of phytosiderophores. However there is no evidence so far that flavin reductases participate in the release of iron. To date there are only preliminary characterizations of NADPH-dependent ferric chelate

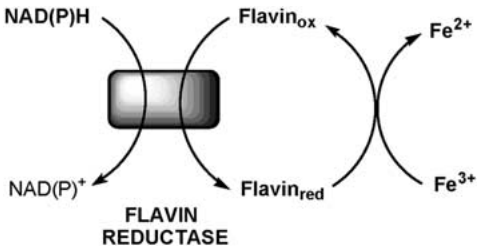

Fig. 2. Reduction of iron by flavin reductases.

reductases from maize (Bagnaresi & Pupillo 1995)) and tomato (Bagnaresi *et al.* 1997), which seem to be more related to the cytochrome *b* reductase family described below.

Cytochrome b-like reductases have been found and extensively studied in yeast (Radisky & Kaplan 1999). In yeast the transport sytems (FET3-FET4-FTR1) require soluble ferrous iron as a substrate and thus the first step in iron transport is the reduction of exogenous ferric iron and the release of iron from extracellular ferric chelates (Figure 3). This reaction is mediated by a transmembrane electron transporter system encoded by the *fre1* and *fre2* genes. FRE proteins have many similarities (primary sequences, spectroscopic properties,. . .) to b-type cytochromes such as flavocytochrome b558 of human neutrophiles or human phagocyte NADPH oxidase, which contain flavin prosthetic groups and NAD(P)H binding sites (Shatwell *et al.* 1996; Finegold *et al.* 1996; Lesuisse *et al.* 1996). A bis-haem structure within the membrane is supposed to play a role in transmembrane electron transport from intracellular reduced pyridine nucleotides and extracellular ferric iron. Furthermore FRE1 is regulated by both iron deprivation, through the action of the iron transcription factor AFT1, and copper deprivation, through the action of the Cu transcription factor MAC1. FRE2 is regulated only by Fe deprivation through AFT1. In fact, in yeast there is a whole family of FRE proteins with an apparent redundancy which confers the capacity to utilize iron from a variety of sources including ferrisiderophores (Yun *et al.* 2001). Only one such mammalian reductase has been reported so far. This is the iron-regulated ferric reductase Dcytb associated with the absorption of dietary iron from mouse duodenal mucosa (Mc Kie *et al.* 2001). The function of this reductase is to convert dietary non haem iron, mostly in the ferric form, to ferrous iron so that divalent cation transporters such as DCT1 (also named Nramp2 or DMT1) can bind it and transport it. This enzyme has homolo-

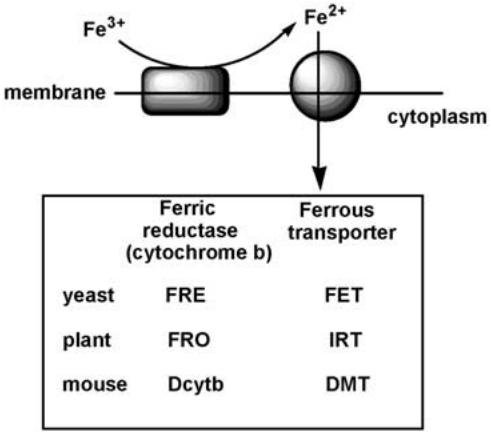

Fig. 3. Cytochrome b-like ferric reductases provide ferrous iron for uptake.

gies to cytochrome b561 and might receive electrons from ascorbate. Finally, a ferric reductase, FRO2, involved in iron uptake from soils by roots of *Arabidopsis thaliana*, has been shown to also belong to the superfamily of flavocytochromes (Robinson *et al.* 1999). FRO2 possesses intramembranous binding sites for haem and cytoplasmic binding sites for nucleotide cofactors that donate electrons. In this case it is also assumed that the resulting ferrous iron is then transported by IRT1.

There are many crucial steps in iron metabolism involving electron transfers which have not been associated with a specific enzyme. The most notable example is that of ferric transferrin reduction. In mammals, iron is transported in the plasma by transferrin (Figure 4). The complex binds to the transferrin receptor at the surface of the cells and is internalized within an acidic endosomal compartment. Although the iron can be liberated from the transferrin-transferrin receptor complex at the acid pH (around 5.5) within the endosome, subsequent reduction of ferric iron is required for its transport out of the endosome and into the cytoplasm. Iron transport out of the endosome is mediated by DCT1 (the divalent cation transporter protein), and perhaps also by SFT (stimulator of Fe transport), both of which transport ferrous iron (Gunshin *et al.* 1997) (Inman & ResWessling-Resnick 1993). It is remarkable that to date, endosomal transport-associated ferric reductases remain to be identified!

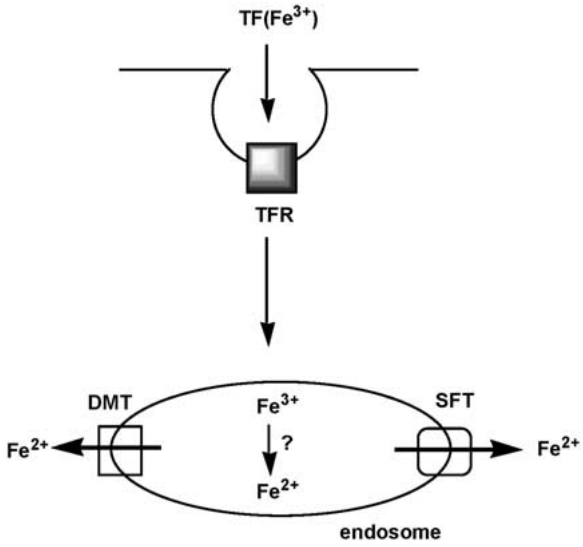

Fig. 4. Transferrin-dependent transport of iron. TF – transferrin, TFR – Transferrin receptor.

Reduction of iron and dissimilation

Dissimilatory ferric reduction is proposed to be an early form of microbial respiration. Vargas *et al.* 1998 have shown that Archaea and Bacteria, that are most closely related to the last common ancestor, can reduce Fe(III) to Fe(II) and generate energy from this respiration to support growth. Some anaerobic or thermophilic bacteria have been recently discovered that reduce extracellular amorphous Fe(III)-oxyhydroxide as the terminal electron acceptor for the oxidation of organic matter to magnetic iron oxides under anaerobic conditions (Lovely *et al.* 1987). This is an interesting finding since this microbial metabolism might have played an important role in the magnetization of anaerobic sediments and could account for the accumulation of magnetite in ancient iron formations. Magnetite accumulation has also been used as an indication that Fe(III) reduction was an important respiratory process on early Earth (Walker 1987) and is significant in modern hot deep terrestrial biospheres, allowing a life in the absence of solar energy (Gold 1992, Liu *et al.* 1997). Whether the presence of magnetite on Mars is a sign of extraterrestrial life remains a matter of discussion (Mc Kay *et al.* 1996).

From a chemical point of view, it would be highly interesting to identify and characterize the reductases involved in this dissimilatory process. Nothing is known on the subject so far.

Conclusion

In this brief review we have underlined the importance in biology of the reduction of ferric iron. In recent years we have gained considerable information on the structures and mechanisms of some of the reductases that are involved in iron reduction in bacteria and yeast. However, although the ferrous iron transporter involved in moving iron across the apical pole of the enterocyte and out of the endosomal compartment within mammalian cells, DCT1, is well characterized, we still lack details about the reductases. While the Dcytb ferric reductase, analogous to the family of membrane bound cytochrome b-like reductases, has been cloned from mouse, we still know little about its biochemistry. As for the reductase in endosomes, which is required to reduce ferric iron prior to its transport into the cytoplasm by DCT1, it so far remains conspicuous by its absence. Nevertheless we can confidently anticipate that the joint efforts of current molecular biological techniques together with good old-fashioned biochemistry will result in an increasing understanding of the role of ferric reductases in the future.

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