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Iron metabolism in aerobes: managing ferric iron hydrolysis and ferrous iron autoxidation

Daniel J. Kosman¹

University at Buffalo, School of Medicine and Biomedical Sciences, Buffalo, NY, USA

Abstract

Aerobes and anaerobes alike express a plethora of essential iron enzymes; in the resting state, the iron atom(s) in these proteins are in the ferrous state. For aerobes, ferric iron is the predominant environmental valence form which, given ferric iron's aqueous chemistry, occurs as 'rust', insoluble, bio-inert polymeric ferric oxide that results from the hydrolysis of $[\text{Fe}(\text{H}_2\text{O})_6]^{3+}$. Mobilizing this iron requires bio-ferrereduction which in turn requires managing the rapid autoxidation of the resulting Fe^{II} which occurs at $\text{pH} > 6$. This review examines the aqueous redox chemistry of iron and the mechanisms evolved in aerobes to suppress the 'rusting out' of Fe^{III} and the ROS-generating autoxidation of Fe^{II} so as to make this metal ion available as the most ubiquitous prosthetic group in metallobiology.

Keywords

Electrochemistry; Electron transfer; Ferrereduction; Ferrooxidation; Iron metabolism; Iron trafficking; Iron transport; Multicopper oxidase; Siderophore; Transferrin

1. Introduction [1-7]

Aerobes and anaerobes alike express a broad array of enzymes and other proteins whose structure and function depend on iron as a prosthetic group. These prosthetic groups include mono- and dinuclear iron; Fe-S clusters; and heme. Irrespective of the prosthetic group, the assembly of all of them requires ferrous iron, Fe^{II} . Although the functions supported by these enzymes, in particular, are broad, it is their roles in energy metabolism which allows one to say that nearly all organisms 'run' on enzymes with active sites metallated by ferrous iron (Fig. 1).

That this is true of both anaerobes and aerobes underscores the fact that these enzymes evolved along with the proliferation of anaerobic life in the Archean during which time the environmental redox potential was <0 relative to the potential of the standard hydrogen electrode (SHE). Indeed, this potential is estimated to have been *ca* -400 mV [8]. Under these geochemical conditions, the stable form of Fe was Fe^{II} ; in the Archean, iron was soluble and biologically freely available. This fact simply reflects the speciation of iron as a function of solution *E* and pH which is illustrated by iron's Pourbaix diagram (Fig. 2) [9].

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¹Corresponding author at: Department of Biochemistry, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214 USA. Tel.: +17168292842; fax: +17168292661 camkos@buffalo.edu.

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Given the relative availability of aqueous iron (the element which constitutes ~5% of the earth's crust) one is not surprised that iron found its essential niche as the most common prosthetic element in biology. However, iron's promiscuous coordination and redox chemistry truly explains why it was the metal of choice upon which to design broad-based intermediary metabolism and energy transduction pathways in evolving anaerobes. These two chemical characteristics are tightly coupled: as goes iron's ligand sphere, so goes its redox chemistry and bioavailability. The Proposal presented here is that tuning of Fe electrode potential, E , by the metal's coordination is a defining feature of not only iron's function, but of its metabolism as well.

2. The aqueous chemistry of iron under air

Underlying iron's Pourbaix diagram (Fig.2) are the details of the aqueous redox and hydrolysis equilibria associated with Fe^{II} and Fe^{III} , equilibria which, naturally, are thermodynamically tightly linked. Fig. 3 summarizes two features of the solution behavior of these two ions (Fig. 3A) and the hydrolysis reaction involving Fe^{III} in H_2O that is associated with these behaviors (Fig. 3B) [10]. Thus, aquo Fe^{II} is relatively stable and soluble, in comparison to Fe^{III} . The reason for this difference, and for the pH-dependent instability of Fe^{III} in water (Fig. 2) is the rapid and pH-dependent rate of hydrolysis of $[\text{Fe}(\text{H}_2\text{O})_6]^{3+}$ which results in formation of polymeric ferric hydroxide species (Fig. 3B), known colloquially as rust [11]. The propensity for Fe^{III} -bound H_2O to lose H^+ (hydrolyze) rather than for aqua- Fe^{III} to lose H_2O is reflected in the 10^4 -fold greater rate of water exchange from ferrous in comparison to ferric iron (Fig. 3A). That is, water as a ligand to Fe^{II} is far more labile; this kinetic difference is, not surprisingly, seen with other ligand types and reflects the kinetic basis for the relative stability of ferric *versus* ferrous complexes involving identical ligands. The ratio of this difference in stability can be represented by $\log \beta_{\text{III}}/\beta_{\text{II}}$ where β is the overall stability constant of a given complex. Of course, this ratio can be greater *or* less than one; broadly, for 'hard', oxygen-rich ligands, this ratio is >1 , while for nitrogenous ligands, it is typically <1 .

The Pourbaix diagram in Fig. 2 is a direct consequence of this ratio and its dependence on $[\text{H}^+]$; as given by the Nernst equation, this corresponds to a decrease of 60 mV per each pH unit above the standard state of 1M H^+ ; at pH = 0 E° for the $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ couple is +770 mV, *i.e.*, Fe^{II} is a poor reductant. This diagram also reflects the solubility products of ferrous *versus* ferric hydroxide which favors ferrous iron by a factor of 10^{22} . Thus, when $[\text{OH}^-] = 1\text{M}$, $E^\circ = -530\text{ mV}$, *i.e.* ferrous iron becomes a very strong reductant because the ratio, $[\text{Fe}^{\text{III}}]/[\text{Fe}^{\text{II}}] = 10^{-22}$, favors Fe^{III} by mass action. This behavior is simply a reflection of the relative stability of the ferrous *versus* ferric state within the context of the iron's ligand sphere, whether contributed solely by water or hydroxide; a ligand set composed entirely of atoms from non-solvent ligands; or a mixed ligand set composed of both water and solute- (or protein) derived atoms. Again, this relative stability is given by the ratio $\log \beta_{\text{III}}/\beta_{\text{II}}$ which functions quantitatively in the Nernst equation similar to $\log[\text{H}^+]$ or $\log[\text{Ox}]/[\text{Red}]$.

This equilibrium picture of iron's aqueous redox chemistry is complemented by the kinetic one: the rate of Fe^{II} oxidation – electron transfer – to dioxygen (autoxidation) strongly correlates to the solution pH, or to $\log \beta_{\text{III}}/\beta_{\text{II}}$: stabilizing Fe^{III} relative to Fe^{II} 'catalyzes' ferrous iron autoxidation. In other words, complementing iron's Pourbaix diagram which is a reflection of equilibrium thermodynamics is electron transfer from Fe^{II} to O_2 . This pH-dependence of autoxidation is illustrated in Fig. 4; here the rate of Fe^{II} oxidation is quantified by the rate of O_2 -reduction using a Clark-type O_2 electrode. Corresponding to the Pourbaix diagram, above pH 7, the half-life of Fe^{II} under air ($p\text{O}_2 = 160\text{ mmHg}$ or $240\text{ }\mu\text{M}$) $<1\text{ min}$ (Fig. 4). Given the rate of hydrolysis of $[\text{Fe}(\text{H}_2\text{O})_6]^{3+}$ which is $\sim 3 \times 10^7\text{ s}^{-1}$ [11] aqueous ferric iron will precipitate as fast as it is formed by autoxidation at neutral pH and

[Fe]_{total} > 1 μM or so [12]. Put another way, 10 μM solutions of ferrous ammonium sulfate at pH 7 under air can't be stored for later use.

3. Ligand modulation of iron's redox chemistry

The $\log\beta_{\text{III}}/\beta_{\text{II}}$ term above reflected the solubility product constants of Fe^{III} versus Fe^{II} when liganded to water molecules. Of course, it applies irrespective of the coordinating ligands. As noted, $\log\beta_{\text{III}}/\beta_{\text{II}}$ will be positive for a ligand set that includes 'hard' ligand atoms (primarily containing -O) and negative for a set that includes 'soft' atoms such as -N. Biologically, the first ligand set would include carboxylate and hydroxamate groups; the second would encompass imidazole or pyrrole (N) and, less commonly, methionine or cysteine side chains (S). A common ligand set is represented by the carboxylate-containing ones that are minimally bi-dentate, starting with citrate, ending with EDTA. For this series, although the log stability constants (for the ferric iron complexes) vary from 11.5 to 25.1, the $\log\beta_{\text{III}}/\beta_{\text{II}}$ values vary over a much smaller range, from 8 to 10.7, although always favoring Fe^{III}. If considered in terms of a Brønsted-type relationship between stability of the Fe^{III} complex and the $\log\beta_{\text{III}}/\beta_{\text{II}}$ value, the Brønsted β value is fractional. Nonetheless, the difference in this ratio does correlate with a difference in the rate of autoxidation of the corresponding ferrous iron complexes. This is illustrated in Fig. 5. The associated rates can be plotted as a function of the $\log\beta_{\text{III}}/\beta_{\text{II}}$ values which demonstrates that a linear, rate-equilibrium free energy relationship governs the kinetic stability of ferrous iron under air in relation to the coordination sphere of the metal.

Contributing to the autoxidation rate and the underlying chemical basis for it is the iron speciation with regards to bound waters whose hydrolysis will modulate the iron's reduction potential and thus driving force for the electron transfer to O₂. Hydrolysis of bound waters increases the stability of the ferric form and thus drives down the reduction potential. An example of this is the iron-NTA system in which, depending on the number of NTA and water molecules (and their hydrolysis) in the coordination sphere $\log\beta_{\text{III}}$ varies from 24 to 52 with a corresponding decrease in reduction potential from -90 to -550 mV [13]. The experiments in Fig. 5 were carried out at pH 5 to suppress this complex speciation which follows from hydrolysis of bound waters.

Of importance to biologic iron's redox chemistry is how the protein scaffold manages this water hydrolysis (pH) modulation of the prosthetic group's *E*. At one extreme is a biologic ligand sphere that excludes H₂O entirely by being fully 6-coordinate. With no inner sphere substrate ligand access, such a group would serve only in strictly outer-sphere (OS) electron transfer (ET) reactions, *e.g.* cytochrome *c*. In 5-coordinate Fe^{II} heme, *e.g.* the heme *b* in hemoglobin, the ligand sphere modulates O₂ redox behavior in oxy-HbA *via* control of spin state and bonding so as to suppress Fe^{II}→O₂ charge (electron) transfer [14] With an *E*' = 140 mV, the Fe^{II} in HbA would be a good candidate thermodynamically for autoxidation. This represents a common theme in biology: kinetic control of an otherwise thermodynamically favorable process.

The discussion above is provided to state the obvious, namely that the Fe^{III}/Fe^{II} reduction potential can vary by well over a volt within the context of reasonably physiologic conditions of pH and coordination sphere (Fig. 6) [15]. As the strength of the ligand field increases, Fe^{II} becomes a better reductant because Fe^{III} is relatively better stabilized; simply, Fig. 6 illustrates the $\log\beta_{\text{III}}/\beta_{\text{II}}$ relationship discussed above. The diagram also illustrates the fact that the typical iron complex under air is in the ferric form. The diagram and this fact raise the two questions fundamental to iron metabolism: 1) what are the characteristics of these complexes that prevent the ferric iron from 'rusting out'; and 2) how does an organism

labilize this seemingly redox inert and coordinately stable ferric iron so as to make it a substrate for its iron metabolic pathways?

The answer to the first question is simple: provide a ligand sphere that prevents ferric iron hydrolysis. This can be achieved by excluding solvent water altogether, or by sterically restricting the oxo-bridging of Fe^{III} atoms that leads to polymerization. Thus, many hydroxamate and catecholate siderophores are 6-coordinate [15, 16]. In transferrin (Tf), while there are two 'open' coordination sites, the metal ion is buried in a clam-shell fold in the protein making it sterically impossible for two Tf molecules to dimerize *via* the oxo-bridging of their Fe-atoms [17]. Answering the second question will be the focus of the remainder of this brief review.

4. Ferric iron binding proteins: stabilizing Fe^{III}

Free-living unicellular aerobes must scavenge ferric iron and must do so either from the small pool of soluble ferric iron complexes, *e.g.*, ferric citrate, or *via* the intermediary of catecholate and/or hydroxamate siderophores (among others) [18-24]. With affinities which correlate with $\log\beta_{\text{III}}$ values as great as 50, siderophores compete efficiently with HO^- for Fe^{III} . Siderophores are synthesized and secreted by some, but not all, bacteria and fungi; however, irrespective of whether a given microorganism synthesizes one or more of these extracellular ferric iron chelating agents, the vast majority express the exocyttoplasmic protein components required for their sequestration and metabolism leading to ionic Fe-accumulation. Thus, some microbes 'cannibalize' siderophore iron targeted for competitor free-living organisms much as pathogens use those same siderophores to cannibalize host iron in the context of an infection [18, 19, 25, 26]. Among multi-cellular organisms, metazoa obtain iron *via* transcellular trafficking of ingested iron into and across the epithelial cells of the gut. The iron species presented to these cells at their luminal (apical) side is complex, reflecting both inorganic (ferric citrate) and bioinorganic forms (siderophore, heme). The primary uptake route of this iron into the intestinal epithelium is *via* the divalent metal ion transporter, DMT1 [27-29].

In either system, the environmental iron form is Fe^{III} , stabilized at neutral pH by chelation so as to prevent water hydrolysis as noted above. Obviously, to access this iron for uptake, the bacterial, fungal or epithelial cell must mobilize this tightly bound iron. This mobilization must, to some extent, be kinetically-controlled; simply having an iron mousetrap which binds Fe^{III} more tightly doesn't guarantee the mouse-trap will work fast enough to meet metabolic needs. Catabolism provides one pathway: the chelating agent is degraded. This occurs in some cases of siderophore iron accumulation [30], and in the recycling of iron from heme in mammals [31]. The second pathway spares the carrier, labilizing the iron by reductive release. Taking advantage of the $\log\beta_{\text{III}}/\beta_{\text{II}}$ values of these chelates which favor Fe^{III} , reductase-dependent $1e^-$ transfer converts the bound Fe^{III} to Fe^{II} which is, of course, more weakly bound, and which exhibits much faster rates of ligand exchange. In short, the reductive pathway acts on both the driving force for and barrier to iron release from the bio-chelate. This Fe^{II} becomes the substrate for subsequent trafficking, *e.g.* transport into the cell *via* DMT1. The latter, reductase-dependent pathway is by far the most prevalent. Being so ubiquitously expressed, it must function efficiently, but considering the electro-chemistry involved, one wonders how this is so.

This puzzle is inherent in the redox scale illustrated in Fig. 6. That is, ferric iron – predominant under air – is made bioavailable by chelation so as to prevent hydrolysis. This requires coordination that ensures large $\log\beta_{\text{III}}/\beta_{\text{II}}$ values which, of course, correlate with large, *negative* values of iron E , *e.g.* Tf and siderophore Fe^{III} with $E^\circ \cong -500$ mV [15, 23, 32]. To reduce this ferric iron requires a reducing agent with a significantly *lower*, more

negative potential: there simply aren't such bio-reductants. The 'reducing potential' of the cytoplasm of a mammalian cell can be estimated at -250 mV using the GSH/GSSG ratio of *ca* 20 as representing the dominant redox couple in this compartment. E° for pyridine nucleotides is lower, -315 mV, but their concentration is $\sim 1/50$ that of glutathione which is in the low millimolar range [33-35]. In short, there are no cytoplasmic reducing equivalents which can drive the $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ couple towards Fe^{II} . Thus, if -300 mV is the lower limit of a cell's reducing potential, to make reductase-dependent uptake possible the potential of the bound Fe^{III} will have to be 'tuned' *up*, making the iron a better oxidant. The mammalian transferrin (Tf) and bacterial ferric iron binding protein (FbpA) exemplify how nature both protects Fe^{III} from hydrolysis while tuning its potential to less negative values thus making it a substrate for ferri-reduction.

The Fe^{III} coordination sites in the two proteins are illustrated in Fig. 7. They are identical in protein lig and set and configuration: 1) phenol oxygens in *cis*; 2) carboxylate and phenol oxygens in *trans*; and 3) one solute ligand site each in *trans* to phenol oxygen and imidazole nitrogen. Importantly, these two open coordination sites are in *cis* allowing for binding of both mono- and bidentate ligands. This ligand (or ligands) is generally referred to as "synergistic" since the nature of the ligand(s) modulates the overall stability of the complex and, as we will see, the iron's reduction potential. The Tf site includes an additional feature that is linked to the modulation of ferric iron binding by pH, at the least; this is a water-bridged, H-bond network between E93 and N81 in H249. DFT calculations are consistent with, although do not prove, that this network promotes the ionization of the proton from this nitrogen [36, 37]; thus, this ligand may be imidazolate and could participate in strong $\text{Im} \rightarrow \text{Fe}^{\text{III}}$ CT, contributing to the highly negative E° exhibited by Tf- Fe^{III} (Fig. 6).

The thermodynamic and kinetic properties of iron binding to both complexes are strongly dependent on solute-derived ligands; of interest here is how such modulation is reflected in the Fe^{III} reduction potential. This is certainly the case in FbpA [38-41]. Crumbliss and his co-workers have demonstrated a linear relationship between stability ($\log K_{\text{eff}}$) and $E_{1/2}$ as a function of this ligand (Fig. 8); the negative ' β ' value is expected, of course, as it merely reflects the relation between $\log \beta_{\text{III}}/\beta_{\text{II}}$ and E° discussed above. These data suggest the hypothesis that solvent ligand composition can play a physiologic role in regulating the binding and release of Fe in the FbpA system.

In the Fe-Tf system, the exogenous ligand does modulate Fe^{III} release (reflecting a change in K_{eff}) but a correlation with E° has not been reported. This brings the discussion back to the notion that Tf Fe^{III} can be made a substrate for cellular reducing equivalents only if it can be made a better oxidant as, for example, citrate does in the FbpA system (Fig. 8). In fact, citrate *does* modulate Tf Fe^{III} as substrate for ferri-reduction and, consequently, for Fe-uptake by both mammalian cells and yeast. We will use the yeast Fe-uptake system as a model for how ligand sphere modulates the thermodynamics and kinetics of iron mobilization and trafficking.

5. Fungal iron uptake: tuning ferric iron's E° for ferri-reduction

Both pathogenic (*Candida albicans*, *Cryptococcus neoformans*) and non-pathogenic fungi (*Saccharomyces cerevisiae*) express two primary pathways for iron uptake [30, 42-46]. One involves receptor-mediated absorption of siderophore or heme with intracellular recovery of the iron following ligand degradation or reductive iron release. In the second pathway, iron released from its environmental chelate by ferri-reduction is substrate in a subsequent ferroxidase-dependent step. The Fe^{III} product of the ferroxidase reaction is transported into the cytoplasm *via* a ferric iron transporter. This latter pathway (Fig. 9) is essential to the virulence of pathogenic fungi indicating that it plays a major role in iron acquisition [42]. As

illustrated in Fig. 9 the essential protein components of this pathway in fungi are: an NADPH-dependent, heme and flavin-containing ferrireductase (the Fre family); a multicopper ferroxidase (the Fet family); and a ferric iron-specific permease (the Ftr family). The chemical 'logic' of this iron redox cycling is outlined as follows.

Fungal pathogens that colonize humans have two primary sources of circulating Fe: non-transferrin bound iron (NTBI) and transferrin bound iron (TBI). The latter represents 90% of the total iron in the plasma and is therefore the pathogen's primary iron source [47]. Fungi do not express a receptor for Tf; therefore, Fe-uptake from Tf must rely on the ferrireductase, ferroxidase-dependent pathway for Fe-acquisition [43-45]. Thus uptake of Fe into these cells from the circulation illustrates how a serum solute like citrate, provides the 'synergy' needed for these cells to access Tf iron. This synergy is illustrated in three ways in Fig. 10 using *S. cerevisiae* as a model for how fungi utilize Tf iron.

In a buffer that lacks a potential biologic Fe^{III} chelating agent - *e.g.*, aspartic and glutamic acids; citrate; lactate, pyruvate, oxalate - *S. cerevisiae* exhibits very weak ferri-reductase activity towards Fe^{III}-Tf (Fig. 10A); a weak ⁵⁹Fe-uptake activity using ⁵⁹Fe-Tf as substrate results (Fig. 10B). Addition of 100 μM citrate stimulates ferri-reduction 10-fold with a corresponding 7-fold increase in ⁵⁹Fe-uptake. Other ligands (above) support a comparable stimulation. In plasma, [Cit] varies from 100-200 μM. The molecular basis for this stimulation in ferri-reduction of Tf-Fe^{III} is indicated by the fact that 100 μM citrate increases the reduction potential of Tf iron from -410 to -285 mV (Fig. 10C). This +135 mV increase makes the Fe^{III} a substrate for the ferri-reductase (Fre1, Fig. 9); in yeast the reducing equivalents which drive this reduction come from NADPH. Thus, citrate as the synergistic anion has a comparable effect in Tf as documented in FbpA [38, 41]; in both cases, citrate binding shifts the Fe^{III}/Fe^{II} *E*° up into what can be thought of as iron's 'metabolic' potential range which at the negative end is set by the -330 mV of the NADP/NADPH couple. This range was indicated in Fig. 6 (symbols **R** and **O**).

As indicated in Fig. 9, fungi (and alga) do not transport this Fe^{II} *via* a divalent metal ion transporter as in plants and metazoa. These unicellular organisms couple ferri-reduction to ferro-oxidation; it is the product of the latter reaction which is substrate for a *ferric iron* transporter, the Ftr protein. The ferroxidase, Fet3, is a multicopper oxidase. A summary of how this enzyme makes the Fe^{II} product of the ferri-reductase a substrate for ferrooxidation concludes this review [30, 48-52].

6. Fungal iron uptake: tuning ferrous iron's *E*° for ferri-oxidation

As indicated in Fig. 6, at pH 6 the *E*° of the Fe^{II} product of the ferri-reductase is ~420 mV. To be substrate in a ferroxidase reaction, its redox partner must be a better oxidant, that is have a 1e⁻ potential of at least 60 mV higher to ensure a reasonable driving force, both thermodynamically and kinetically. With respect to the latter, however, electron transfer rate is more strongly dependent on the additional factors included in the Marcus equation which quantifies outersphere electron transfer (OS ET). The Marcus relationship is given in Eq. 1 [53-56].

$$k_{\text{ET}} = S K_A \left(4\pi^3 / h^2 \lambda kT \right)^{1/2} (H_{\text{DA}})^2 \exp \left[-(\Delta G^\circ + \lambda)^2 / 4\lambda kT \right] \quad \text{Eq. 1}$$

OS ET depends on four factors: **1)** probability of a productive ET complex (*SK_a*); **2)** electronic coupling between donor and acceptor [*H_{DA}*, modeled as the lowest unoccupied molecular orbital (LUMO) including both centers]; **3)** the driving force, Δ*G*° for the redox pair; and **4)** the reorganization energy, λ. Clearly, the first two factors make a proportionally

greater contribution to k_{ET} than does ΔG° . Nonetheless, data suggest that the structure of the Fet3 ferroxidase site, which is representative of all multicopper ferroxidases, includes features that maximize all four factors in making Fe^{II} the specific substrate for this class of enzymes.

The structure of this active site is given in Fig. 11 [48]. Two carboxylate side chains contribute to a favorable SK_a that is represented by the reciprocal of the Michaelis constant for Fe^{II} , $1/K_M$ [49]. In addition, these carboxylates, *via* their H-bonds to the T1 Cu^{II} ligands, the imidazoles of H413 and H483, contribute to the electronic matrix coupling, H_{DA} . Last, data indicate that in binding to these two oxygenous ligands, the iron is made a better reductant, with a shift in E° from its potential at pH = 6 of 420 mV, to a potential that could be as low as 190 mV [50, 52]. These data are as follows.

Anaerobic titration of Fet3 with Fe^{II} is stoichiometric, that is, the 4 Cu^{II} in the protein are completely reduced with 4 equivalents of Fe^{II} . This result indicates that the E for the ‘cell’ $Fe^{II} + Cu^{II} \leftrightarrow Fe^{III} + Cu^I$ is greater than 120 mV corresponding to a $K = 100$ favoring ET from $Fe^{II} \rightarrow Cu^{II}$. Since the T1 Cu^{II} E° is ~ 430 mV, the E° of the Fe^{II} *bound as substrate* would be no more than 310 mV to account for the observed redox equilibrium. In relation to iron’s reduction potential scale illustrated in Fig. 6, this shift towards lower reduction potential for bound Fe^{II} is equivalent to an $\log \beta_{III}/\beta_{II} = 2$ term in addition to the factor of 6 associated with the $[H^+]$ -dependent $\log \beta_{III}/\beta$. In effect, moving the Fe^{II} II from pH = 6 water into the carboxylate-rich ‘solvent’ at the T1 Cu site in Fet3 is equivalent to increasing $[HO^-]$ 100-fold. Relative to its stability in bulk solvent, bound Fe^{II} is poised in a redox ‘entatic’ state [57-61].

This entasis is not functionally different than the one associated with Fe^{II} coordination by citrate in relation to Fe^{II} autoxidation. $FeCit^{1-}$ is the dominant species at pH 6 (Fig. 12) [62-64]. As in Fet3, the Fe^{II} ligand sphere in the monocitrate complex includes two carboxylates; these two carboxylates are sufficient to support an 5×10^3 -fold increase in Fe^{II} autoxidation rate (at pH 6) [62]. One can view this increase as abiologic, *forward* genetics in that ‘mutation’ from water to citrate increases function. In comparison, *reverse* genetics in Fet3, in which the corresponding two carboxylates (E185 and D409) are eliminated by substitution with alanine, results in an 800-fold *decrease* in electron transfer from Fe^{II} to the enzyme’s T1 Cu^{II} [49]. Given the multiple factors which contribute to k_{ET} (Eq. 1), one can’t take this similar effect to be of any more significance than support for the model that moving Fe^{II} into a ligand sphere dominated by oxygenous ligands increases the driving force for subsequent electron transfer. This ‘tuning’ concept has been the point of this review.

7. Summary

Iron metabolism involves cycling of iron between the metal ion’s two redox states which are thermodynamically accessible at pO_2 levels between 20-160 mmHg, and in the reduction potential range set by common biologic redox couples, +400 to –300 mV, and at neutral pH (Fig. 6). In general, ferric iron is the more favored at neutral pH due to the stronger water coordination to Fe^{III} in comparison to Fe^{II} . With hydrolysis, the solubility constant for $Fe^{III}(H_2O)_n$ is $1/10^{22}$ that of Fe^{II} . Organisms and cells keep this ferric iron in solution by secretion of chelating agents that bind Fe^{III} more tightly than HO^- and by sterically preventing the oxo-bridging of Fe^{III} species that leads to ‘rust’. However, in stabilizing this ferric iron state, the chelating agents set the Fe^{III} reduction potential too low for participation as oxidant in reaction with a biologic reducing agent; this ‘stable’ ferric iron is inert in regards to the ferri-reduction ‘half-reaction’ associated with normal iron metabolism.

Ferroxidation is the other ‘half-reaction’ of this metabolism; the electrochemical ‘cell’ that constitutes this redox reaction involves Fe^{II} and the T1 Cu^{II} of a multicopper oxidase. There are MCOs which have T1 Cu atoms with E values >600 mV [65, 66]. One might think these would be excellent catalysts of the ferroxidase reaction given the ~ 420 mV potential of Fe^{II} at pH 6. In fact, these enzymes – laccases – react very slowly with Fe^{II} despite the apparent strong driving force for this electron transfer. The reason for this sluggish reactivity is in the SK_A and H_{DA} Marcus terms which, in laccases, are maximized for electron transfer from aromatic phenols and amines, and not for ET from Fe^{II} [67]. In contrast, ferroxidases, which include the fungal Fet and algal Fox proteins [68, 69], and mammalian ceruloplasmin [70, 71] and hephaestin maximize ET from Fe^{II} with specific contributions from SK_A and H_{DA} and by making the Fe^{II} a better reductant. All three factors are contributed by a Fe^{II} ligand sphere composed of 2 or 3 carboxylate side chains, two of which are in an H-bond network with the two imidazoles which contribute to the T1 Cu binding site in all ‘blue’ copper proteins.

Potential Fe-ligands from solvent play significant roles in this redox tuning of iron for metabolism. Citrate is one of these. Its concentration in plasma varies between 100-200 μM [72]; as demonstrated in Fig. 10, this [Cit] is sufficient to make Tf iron a substrate for ferri-reduction by shifting the metal ion’s reduction potential into the biologic range. In a similar vein, one can appreciate that the biologic ferroxidase site is functionally little different from the ‘site’ represented by citrate in its coordination of Fe^{II} , one which promotes autoxidation, or ferroxidation by another name. This raises a question: If citrate is a good ‘ferroxidase’ why, for example, are ceruloplasmin and hephaestin essential to human iron metabolism? The reason is unrelated to the iron redox chemistry *per se* but is due to the dependence on $[\text{O}_2]$ in a given ferroxidase reaction. Abiologic autoxidation is first-order in $[\text{O}_2]$; thus, at the typical $p\text{O}_2$ in arterial blood – 40 mmHg, or 50 μM O_2 – the rate of autoxidation is too sluggish to support normal iron redox cycling. In strong contrast, with a K_M for O_2 of ~ 5 μM , multicopper ferroxidases function at 90% capacity at this $p\text{O}_2$ and thus continue to support iron homeostasis even under strongly ischemic conditions [73]. Last, irrespective of the ambient $[\text{O}_2]$, the coupling of ferrous iron oxidation to reduction of dioxygen to water, bypassing intermediate, one-electron O_2 reduction products – ROS – will have selective advantage for any organism living under air. In this regard, MCO metallo-oxidases, those with specificity towards both Fe^{II} and Cu^{I} , are important components of the anti-oxidant systems required for aerobic homeostasis.

In conclusion, the iron redox cycling apparent in fungal and algal iron uptake (Fig. 9) can be understood in terms of the differing aqueous behavior of Fe^{II} and Fe^{III} under air. Iron available to free-living, unicellular organisms is ferric; as is the case for the intestinal enterocyte, which is “outside the body” topologically, to be mobilized for uptake, this ferric iron must be reduced. Fungi express Fre proteins for this [30, 74], enterocytes express Dcytb [27, 28]. This Fe^{II} is a pro-oxidant. In the gut, which is anoxic [75, 76], this abiologic chemistry is suppressed allowing for the direct uptake of this Fe^{II} by the enterocyte *via* the divalent metal ion transporter, DMT1 [27, 28]. In the microorganism under a $p\text{O}_2 = 160$ mm Hg, autooxidation is rapid (Fig. 4 and 5), thus the inherent pro-oxidant activity of Fe^{II} must be managed, a function performed by the MCO ferroxidase. The product of the ferroxidase reaction, Fe^{III} , is unstable at neutral pH; to suppress its hydrolysis, it must be shielded from solvent water. This is achieved by the direct, non-dissociative trafficking – the channeling – of Fet-produced Fe^{III} to the Ftr permease for translocation into the cell. That is, much as access to coordination sites at siderophore and transferrin ferric iron can effect modulation of Fe^{III} reduction potential, restricted water access to Fe^{III} can modulate this metal ion’s propensity to ‘rust out.’

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Abbreviations

SHE	standard hydrogen electrode
OS ET	outersphere electron transfer
Tf	transferrin
DMT1	divalent metal ion transporter 1
FbpA	ferric iron binding protein A
DFT	density functional theory
CT	charge transfer
NTBI	non-transferrin bound iron
TBI	transferrin bound iron

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Highlights (for review)

- Iron is a ubiquitous redox cofactor
- Iron's aerobic aqueous redox chemistry strongly limits its bioavailability and redox stability
- Aerobic iron metabolism involves cycles of ferrireduction and ferroxidation
- The electrode potential of the ferrous-ferric couple is 'tuned' for this bio-metabolism by specific coordination

Fig. 1.

Iron enzymes common to aerobes. Nitrogenase is included although not common in aerobes.

Adapted from unpublished image created by J. Imlay (with permission).

Fig. 2. Oxygenation of the biosphere, the environmental redox potential [8], and iron's Pourbaix diagram [9].

Fig. 3.

Aqueous chemistry of Fe^{II} and Fe^{III} . (A) Solubility product constants and H_2O exchange rates. (B) Hydrolysis reaction of $[\text{Fe}(\text{H}_2\text{O})_6]^{3+}$. Data from [10].

Fig. 4.

Autoxidation of Fe^{II}. Fe^{II}→Fe^{III} conversion is quantified by consumption of O₂ using a Clark electrode; the electron stoichiometry was 3.8:1 at all pH values. Overlapping buffers used were 100 mM acetate, MES and MOPS. Chen and Kosman, unpublished.

Fig. 5. 'Catalysis' of Fe^{II} autoxidation by chelation. **(A)** Autoxidation of Fe^{II} in the presence of oxygenous chelators of increasing ligand stability. **(B)** Rate equilibrium free energy relationship between relative stability of Fe^{III} complex and autoxidation rate. The buffer was 50 mM MES with [chelator] = 1 mM. In addition to citrate and EDTA, the chelators were: IDA, iminodiacetic acid; EDDP, ethylenediamine-N,N'-dipropionic acid; HIDA, N-(2-hydroxyethyl)iminodiacetic acid; NTA, nitrilotriacetic acid; and HEDTA, N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid. Chen and Kosman, unpublished.

Fig. 6.

Dispersion of iron redox potentials in biologically-relevant coordination complexes.

Symbols **R** and **O** denote lower and upper limits of cellular redox couples involved in iron trafficking. Consequently, to be a substrate for these metabolic pathways, the E° of biologic iron must fall within these limits. Diagram based on [15].

Fig. 7. Fe^{III} coordination in human transferrin (**A**, 1A8E) and *Neisseria gonorrhoeae* FbpA (**B**, 1D9Y). Unresolved solute ligands have been inserted, CO₃²⁻ in hTf, PO₃²⁻ and H₂O in FbpA. Note that in both proteins, solute ligation is *trans* to homologous His and Tyr ligands. In hTf, H-bond network between N61 in H249 and E93 modulates Fe^{III} binding, possibly by tuning Im→Fe CT.

Fig. 8. Modulation of holo-FbpA stability and Fe^{III} reduction potential by solute ligand. Citrate effect is specifically noted. Adapted from ref. 37.

Fig. 9.

Ferri-reductase, ferri-oxidase Fe-uptake pathway in fungi. The ligand, L, can be anything from citrate to Tf. This redox cycling ‘solves’ the recalcitrant aqueous and redox chemistry of iron at neutral pH as summarized in the text.

Fig. 10.

Citrate mobilization of Fe from Tf for Fe-uptake by yeast. Citrate is synergistic in Tf-Fe^{III} reduction (A) and in ⁵⁹Fe-uptake from Tf (B). Citrate makes Tf-Fe^{III} a better oxidant by 135 mV (C); with $E^{\circ} = -285$ mV, Tf-Fe-Cit is substrate for ferri-reduction and Fe-uptake. In both (A) and (B) yeast cells are incubated with 10 μM FeCl₃ for 30 min in absence or presence of citrate as noted. In (A), ferri-reduction by Fre1 was quantified by trapping the Fe^{II} produced with 4,4'-(3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl)bisbenzenesulfonic acid (ferrozine). In (B) Fe uptake *via* the Fet3, Ftr1 pathway was quantified using the radio-tracer, ⁵⁹Fe. The data in (C) were obtained spectroelectrochemically following the absorbance of Tf•Fe^{III} using a BASi Epsilon workstation. Ziegler and Kosman, unpublished.

Fig. 11. Fe^{II} ET site in Fet3 (1ZPU). E185 and D409 provide oxygenous ligand set that tunes down E° of bound Fe^{II} while supporting favorable Marcus SK_a and H_{DA} terms (Eq. 1).

Fig. 12.
Monomeric ferrous citrate [62].