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AN IRON-MESSENGER SYSTEM — A HYPOTHESIS

T. RAMASARMA

Department of Biochemistry Indian Institute of Science Bangalore 560 012, India

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Iron in the free ionic state is very reactive as it generates and reacts with free radicals of the type $HO^{\cdot}$, $LO^{\cdot}$, $LOO^{\cdot}$, $O_2^{\cdot-}$ and $C^{\cdot}$. These are highly reactive species and participate in reactions that are mostly deleterious to cell components, including DNA, proteins and lipids. In association with a complexing agent such as bleomycin, Fe^{2+} degrades DNA into soluble nucleotides[1]. In the presence of oxygen and a reducing agent, DTT, Fe^{2+} inacativates enzymes by peptide bond cleavage[2]. Chelated to ADP, Fe^{2+} promotes peroxidation of polyunsaturated fatty acids and breakdown of membranes[3]. These do not occur in normal cells as they are kept under check by antioxidant devices, and more importantly by keeping free iron concentration low.

Cellular processes dependent on iron

Besides the well-known functions in transfer of oxygen and electrons by hemoproteins and cytochromes, iron plays an essential role in many cellular processes and is involved in some way in cell proliferation[4]. Deficiency of iron impairs a number of metabolic processes[5] particularly concerning synthesis of DNA, collagen and cholesterol. An overview of some iron-dependent cellular processes is given in Table 1.

Enzymes dependent on Iron for activity

Implicit in the gross metabolic effects of iron is the metal-dependent regulation of activities of specific enzymes. Over the past 15 years a significant number of enzymes, distinct from heme-proteins, were found to have their activities modified in presence of ionic form of iron (Table 2). In most of these cases addition of an iron salt to the reaction mixture is needed to show the activity of the enzyme. In some cases, iron is present in the protein in a loosely bound form, removal of which decreases the activity. The types of reactions affected are generally hydroxylations, oxygenations, oxidations and dehydration/rehydration reactions. In a majority of these cases, molecular oxygen is involved or is necessary for the reaction to occur. Thus, some "active iron species" utilizes an aspect of oxygen-activation mechanism in converting the ground state triplet into a reactive complex form, such as perferryl radicals. It is likely that these reactive complexes at the enzyme active site abstract a hydrogen atom from carbon (or sulfur), which forms the "core reaction".

Protein factors regulate Fe effects

The first example of this was the liver cytosolic proteins, identified and named "Ferro-activators" by Lardy and coworkers[36], which enhanced the Fe^{2+}-activating

TABLE I
Some Cellular Processes Involving Iron

Process	Effect/modification
1. Mitotic cell division	Fe bound to polysaccharide needed; Fe transferred to chromosomes[6]
2. Proliferation of bone marrow and epithelial cells	Reduced in Fe-deficiency[7]; possibly due to decreased DNA synthesis[8]
3. Regeneration of liver	Impaired in Fe deficiency[9]
4. Stimulation of lymphocytes by phytohemoagglutinin	DNA synthesis enhanced by Fe[10]
5. Infection and neoplasia	Withholding Fe a defence against infection and tumor cell cytotoxicity; Fe increased risk of infection and carcinogenesis; serum Fe concentration inversely correlated with fever index during incubation phase of infection (see ref 4, 11, 12 for reviews)
6. Phagocytosis	Excess iron suppresses respiratory burst[13]
7. Cellular immune functions	Cell-mediated immunity[14] and immuno-competence[15] defective in Fe-deficiency; imflamatory response requires iron[16]
8. Haemochromatosis	Increased free Fe concentration probably responsible for pathology of disease[17]
9. Thermoregulation in cold exposure	Despite increased noradrenaline in cold exposure Fe-deficient animals cannot maintain body temperature[18]
10. Collagen synthesis	Impaired in Fe-deficiency[19]
11. Serum components	Triglycerides, phospholipids, cholesterol[20] and phenylalanine[21] increased in Fe-deficiency

TABLE II
Enzymes dependent on Iron for activity

Enzyme	Conc. of Fe^{2+}	Reaction
1. Ribonucleotide reductase[22]	10 μM	NDP $\xrightarrow{O_2}$ deoxy NDP ($\longrightarrow$ DNA)
2. myo-Inositol oxygenase[23]	250 μM (Km)	myo-Inositol $\xrightarrow{O_2}$ D-glucuronate ($\longrightarrow$ xylulose)
3. γ-Butyrobetaine hydroxylase[24]	–	γ-Butyrobetaine $\xrightarrow{O_2}$ carnitine (fatty acid transport)
4. Lysine/Proline oxidases[25] (protein-bound substrates only)	–	Lysine/proline $\xrightarrow{O_2}$ OH-lysine/proline ($\longrightarrow$ collagen)
5. Phenylalanine hydroxylase[26]	100 μM	Phenylalanine $\xrightarrow{O_2}$ tyrosine ($\longrightarrow$ phenolic products)
6. Tyrosine hydroxylase[27]	–	Tyrosine $\xrightarrow{O_2}$ DOPA (neurotransmitter)
7. Tryptophan hydroxylase[28]	800 μM	Tryptophan $\xrightarrow{O_2}$ 5–OH tryptophan ($\longrightarrow$ serotonin)
8. Lipoxygenase/Cyclooxygenase[29]	–	Arachidonate (A) $\xrightarrow{O_2}$ A–OOH (prostaglandins, thromboxanes, leukotrienes; regulation of hormone action)
9. Oxalate oxidase[30] (only plants)	500 μM	Oxalate $\xrightarrow{O_2}$ CO_2
10. Lipid peroxidation[31] (cytosolic protein inhibitor[32])	5–20 μM	Polyunsaturated fatty acids (L) $\xrightarrow{O_2}$ L.OO$^{\cdot}$ ($\longrightarrow$ aldehydes)
11. HMGCoA reductase (cytosolic protein inhibition[33])	(100 μM)	HMGCoA $\longrightarrow$ mevalonate ($\longrightarrow$ cholesterol)
12. Aconitase[34]	500 μM	Citrate $\longrightarrow$ isocitrate (Krebs cycle)
13. Phosphoenolpyruvate carboxykinase[35]	30 μM	Phosphoenolpyruvate $\longrightarrow$ oxaloacetate (gluconeogenesis) (cytosolic proteins, ferroacativators, needed to keep H_2O_2 low)

effect of PEP carboxykinase. It now appears that these proteins are H_2O_2-utilizing enzymes, such as catalase[37]. They show apparent activation by destroying H_2O_2 which otherwise inactivates the enzyme, probably by the mechanism proposed by Stadtman[2], in generating hydroxyl radicals.

The regulatory enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase, is rapidly inactivated in the combined presence of Fe^{2+} and a cytosolic protein[33]. The maximum inhibition was dependent on concentration of this protein and appeared to be due to a physical association rather than a catalysis[38]. This Fe-dependent inhibition was not obtained or affected by bovine serum albumin, transferrin, apoferritin, superoxide dismutase, horseradish peroxidase or catalase. The cytosolic protein was purified to homogeneity (Mr 58000) and was found to bind two Fe ions per mole[39]. Many properties of this Fe-dependent modulating protein are parallel to the calcium-calmodulin system. This prompted a naming of the protein as "Fermodulin".

If significant concentrations of iron required for realizing the activities of Fe-dependent enzymes are released, lipid peroxidation (promoted by trace quantities of Fe in presence of ADP), must be suppressed to prevent membrane damage. This is achieved by cytosolic proteins — one requires glutathione[40], another high concentration of Fe and ADP[32]and a third one appears to be lactoferrin[41].

Distribution and mobility of Iron

The total amount of iron is about 60 mg Fe/kg body wt. in man. It is distributed in four distinct pools — storage, transport, oxygen-carrying and functional — represented by ferritin, transferrin, hemoglobin and Fe-dependent enzymes, respectively. It may be noted that in all cases Fe is bound to proteins. The last to be depleted of iron is the functional pool during iron-deficiency states[42] (Figure 1). This underscores the importance of the functional pool on which rests the performance of Fe-dependent metabolic reactions.

The iron content of the body is regulated not by its excretion, but by absorption. Absorption of iron by intestinal mucosal cell is facilitated by an intracellular transferrin-like Fe^{3+}-binding protein[43] and by xanthine oxidase[44] which promotes oxidation and incorporation of iron into the mucosal transferrin. Similar process occurs in the mobilization of iron from tissues through plasma transferrin and ceruloplasmin, possessing ferroxidase activity[45]. Iron in a free state does not appear in any of these steps of its entry into cells. After binding of Fe^{3+} to transferrin this protein is recognized by a specific receptor[46] on the cell membrane and then internalized by receptor-mediated endocytosis. Both transferrin and its receptor are returned undegraded[47] to the plasma membrane after unloading iron. The process of this transfer of iron remains unknown but it reaches the bound form in membranes and the storage form, ferritin.

Ferritin, occurring predominantly in liver, spleen, intestines and bone marrow, is a large protein (Mr 445,000) with its core filled with hydrated ferric iron oxide/phosphate[48]. Exchange of iron between transferrin and ferritin[49] or mitochondria[50] occurs to some extent. A reducing agent, to weaken iron binding, and chelating agents such as citric acid, amino acids, sugars or nucleotides to act as carriers were found to promote this process[51]. On the other hand, reducing agents themselves were found to release iron from ferritin[52]. Of several physiological agents tested, reduced flavins[53] were most effective. This may be related to the ability of xanthine oxidase[54] or

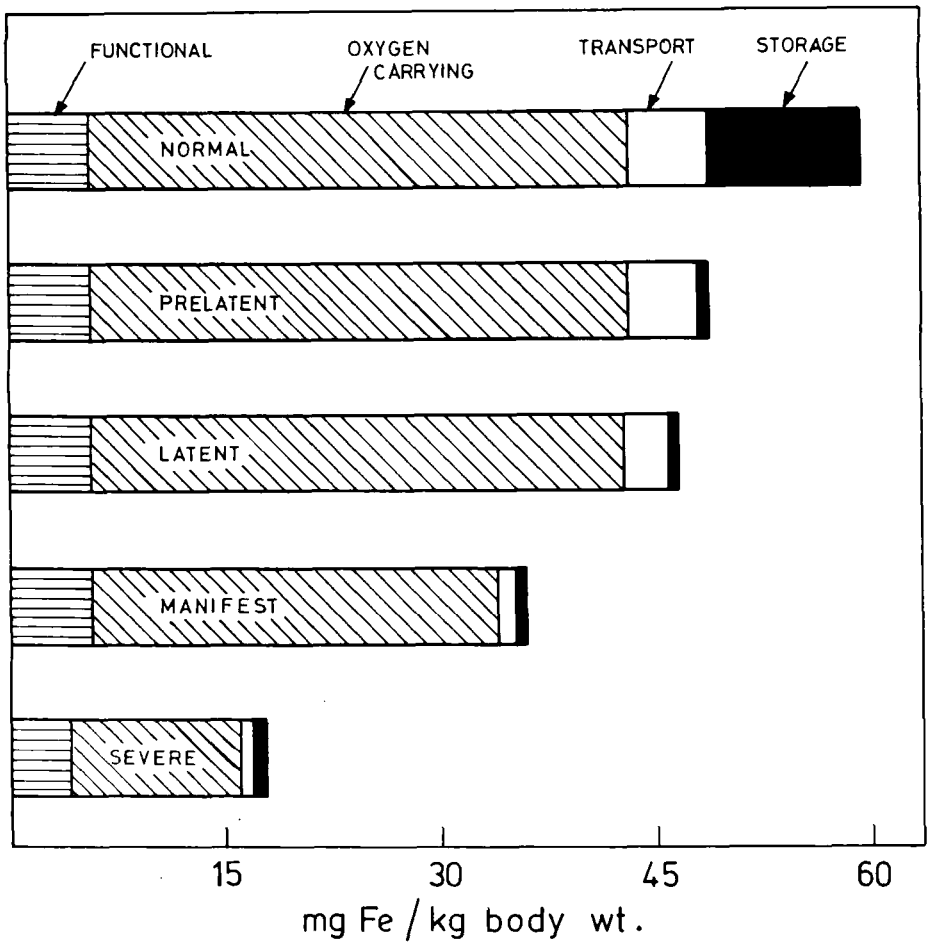

FIGURE 1 Changes in the four pools of iron during stages of iron deficiency. Note the functional pool is retained to a large extent (adapted from ref. 42).

NADH-dependent xanthine dehydrogenase for such mobilization. In all these cases of demonstrated iron transfer, free Fe was not detected.

Concentration of ionic form of iron

Cellular concentrations of free ionic iron appear to be extremely small. It has not been possible to measure them with any accuracy. Direct experimental evidence was obtained for the presence of free Fe within liver cells[56]. By the bleomycin-dependent DNA degradation assay, Gutteridge et al.[1] found that Fe occurred in micromolar concentration in the non-protein bound form in some extracellular tissue fluids. None was found in normal human serum but upto 20 μM Fe was detected in iron-overload patients[17]. From the solubility product of $Fe(OH)_3$ (10^{-38}), redox potential (about

0.0 V) and pH (about 7.0), free Fe^{2+} in equilibrium with ferritin-$Fe(OH)_3$ was calculated by Williams[37] to be about 1 μM, in the same range as free Ca^{2+} concentration[60]. By simply lowering pH, free iron concentrations can be increased (R.J.P. Williams, personal communication). This can be used only in special vesicles.

It is obvious that this low concentration of Fe^{2+} is an advantage to the cell in avoiding damaging effects on Fe-derived radicals. But the question to answer is how the Fe-dependent enzymes mentioned above, which require Fe^{2+} in the range of 10–200 μM for showing full activity (Table 2) will ever be functional if free iron concentration is always kept low. Mechanisms must exist for release of iron from the stored forms of ferritin, Fe-S membrane proteins or other Fe-binding compounds, only when increased concentration is required. Even if iron is released from a store, it must exist in an intermediate pool with Fe^{2+} bound to complexing agents or proteins such as fermodulin, but available to the enzyme-proteins by exchange.

Alterations in enzyme activities traceable to iron

It is instructive to compare information in Table 1 and 2 to obtain correlations between changes in iron concentration and enzyme activity and Fe-dependent cellular processes. Most information can be obtained in iron-deficiency and iron-overload states which represent the far-ends of Fe-dependent metabolic regulation. It can be seen that a number of processes dependent on cell proliferation are controlled by iron through ribonucleotide reductase and DNA synthesis. Excess iron increases the risk from infection by suppressing phagocytosis through blocking the respiratory burst. This may be due to inhibition of H_2O_2 generation by iron, an effect we found in three systems, mitochondria[58], plasma membrane and microsomal membranes (S. Usha Devi, unpublished data). Iron overload effect in haemochromatosis may be obtained primarily through production of toxic oxygen radicals, lipid peroxidation and membrane damage. In a controlled measure, iron and polyunsaturated-fatty-acid-dependent microsomal NADPH oxidase may become a thermogenic reaction to produce heat[59] needed to keep body temperature in cold-exposed animals. This could explain why the animals failed to maintain body temperature on diets deficient in iron[18] or polyunsaturated fatty acids (B.S. Sekhar, unpublished data). Collagen synthesis will be regulated through the activity of lysine/proline hydroxylases by iron released from stored form of iron. That ferritin can become a source of iron was shown in the presence of superoxide-generating xanthine oxidase system[60] as revealed by acceleration of lipid peroxidation. Iron can also be released from mitochondrial membranes on adding Ca^{2+} and this was detected by increased activity of PEP carboxykinase[61].

Phenylalanine hydroxylase is regulated in a functionally reversible fashion by iron concentration. In the Fe-deficiency condition, increase in circulating phenylalanine[21] was observed, presumably due to low phenylalanine hydroxylase activity. This activity increased on perfusing the liver with Krebs-Henseleit bicarbonate containing glucose, serum albumin and washed erythrocytes. Such an activated enzyme was indistinguishable from the Fe^{2+}-activated enzyme . This activation was abolished when transferrin, which sequesters iron, was present in the perfusion medium[62].

It is known that cytosolic PEP carboxykinase activity[63] (and therefore gluconeogenesis) increases 2.5 fold, and that of microsomal HMGCoA reductase[64] decreases to about 10% of the controls in the livers of rats deprived of food. A brief report indicates that the iron content of starved liver increases[65]. We have found that the

total iron content in liver does not significantly increase, but in view of depletion of protein from the liver tissue, the iron per unit protein shows four- and two-fold increased concentration, respectively, in cytosol and microsomes (S. Usha Devi, unpublished data). The storage protein apparently decreases on starvation and thereby increasing the free iron concentration. The increased serum cholesterol concentration[20] observed in iron-deficiency might be due to increased HMGCoA reductase, by relieving it from inhibition by iron and fermodulin[38]. It has now been possible to show a decrease of HMGCoA reductase activity by 50% on giving excess iron to rats intraperitoneally, which indicates that the free iron concentration may have increased through this mode of administration so as to inhibit the enzyme, as sufficient fermodulin is always present in the cytosol (S. Usha Devi, unpublished data). It is possible that in cases of unexplained rapid decrease in activity of HMGCoA reductase such as the early phase of cholesterol feeding[66] and the LDL internalization[67], the effects may have been mediated by release of iron.

The dependence of tyrosine and tryptophan hydroxylases, and of lipoxygenase/cyclooxygenase, on the availability of iron will affect neuro-transmitter and hormone actions and, indirectly adenylate cyclase and cyclic AMP-mediated actvities in the tissue. Moreover, addition of ferric iron-gluconate to mitochondria caused Ca^{2+} efflux[68] by a selective pathway of release. Addition of 5 μM $FeSO_4$, but not $FeCl_3$, resulted in a decrease in Ca^{2+}-induced respiration in coupled mitochondria without a change in ^{45}Calcium uptake, thus increasing Ca^{2+}/O ratio (Vidya Shivaswamy, unpublished data). It appears possible that interlocking effluxes of Ca^{2+} and Fe^{2+} may form a basis of expanding the regulatory potential of these metal ions. A great deal of work is needed to understand the mechanisms of release and uptake to terminate the effects of iron on enzymes.

Iron-messenger system

A metal-dependent messenger system is well worked out in the case of calcium. The requirements are that the metal must be an essential nutrient, occurring in sufficient quantities but present mostly in stored form and made available in low, micromolar concentrations in active ionic form, the process of which is controlled by hormones or neurotransmitters[69]. Metabolic control is achieved by calcium ions in activating a number of enzymes, among which are phosphodiesterase, adenylate cyclase, protein kinases, phospholipase A_2, myosin light chain kinase and calcium-dependent ATPases[67]. In this process calcium ions are bound to an ubiquitously occurring protein, called "Calmodulin" (Mr 17000) which in its complex form with calcium modulates the activity of the enzymes[70].

Williams[57] proposed that Fe^{2+} ions which occur in low concentrations and have intermediate binding strength are capable of both catalytic and regulatory roles. Iron has the potential of a metabolic modulating agent because it occurs in stored form intracellularly that can become available in free ionic state and this can affect several enzymes that depend on Fe^{2+} for expressing their activity. The number of Fe-dependent enzymes is larger than Ca^{2+}-dependent enzymes. In addition there may be other enzymes whose activities are inhibited by Fe^{2+} or by free radicals generated in its presence or by cellular Fe-binding proteins such as fermodulin.

The sequence of events in the calcium-dependent metabolic modulation, presently known as the "Calcium-messenger system"[69] is depicted in Figure 2. Fe^{2+}, like Ca^{2+}, is likely to provide regulatory potential to meet the diverse needs of the cells. From

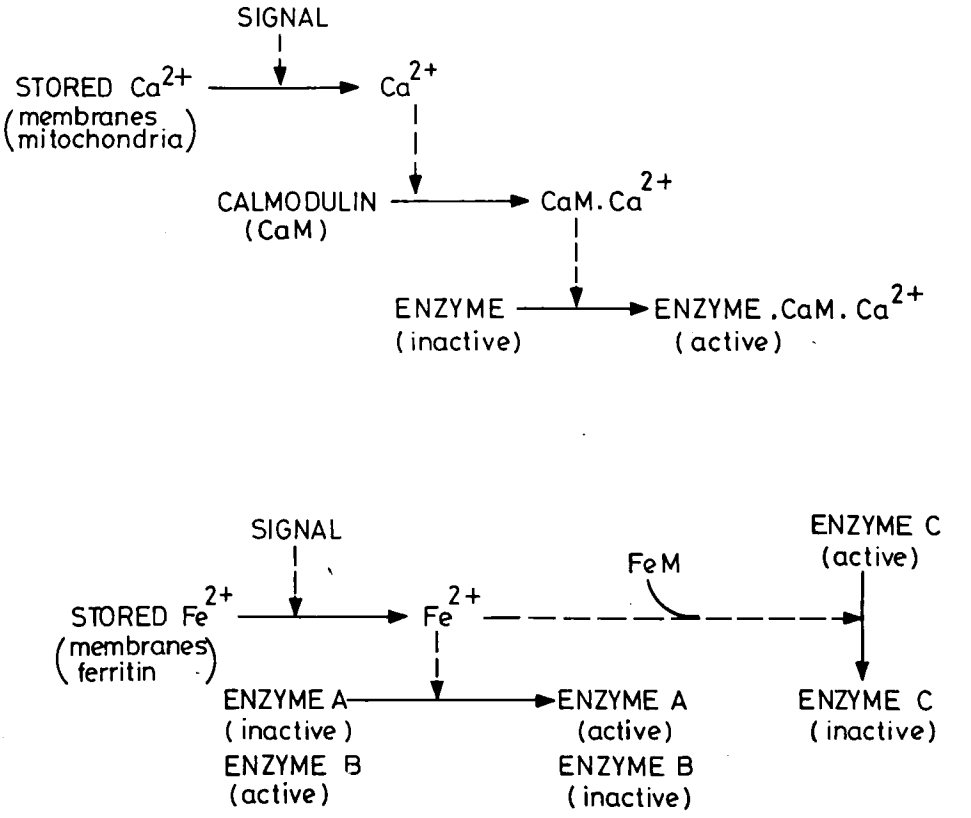

FIGURE 2 Schematic representation of Calcium-messenger system and proposed Iron-messenger system.

the known information, a model of an "iron-messenger system" has been developed as shown in Figure. 2. There is one distinction, in that Fe^{2+} can directly modify enzymes, either through direct binding or by generating free radicals. It is hoped that this working hypothesis will stimulate further work.

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