Fe^{2+}-DEPENDENT METABOLIC MODULATION

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IN growth and maintenance of living cells metal ions have a number of functions, usually electrochemical, structural or catalytic in nature. Metalloenzymes depend on the presence of firmly bound metal, commonly Zn, Cu, Mn, Co, Se, Mo and Fe as part of their protein structure, for their activity. These metals either directly or in the form of their chelates bind strongly to the ligands in proteins and therefore do not exchange with the medium. Some metals bind reversibly to enzyme proteins and regulate their activity and these are referred to as metal-activated enzymes. This class utilizes metals of intermediate binding strength such as Mg, Mn, Ca, V and Fe. Of these the most commonly occurring metal is Mg which is invariably used as a cofactor by a large number of enzymes that have phosphate compounds as substrates. Mg is required and occurs in mM concentrations in cells. Other metals, such as Ca, Mn and Fe, do not occur in high concentrations in the free ionic state. However, calcium in mitochondria and iron in ferritin are found in high concentrations but are sequestered. If their concentrations increase transiently, they exhibit profound effects on a number of enzymes. Thus a change in the metal ion concentration can be an effective way of modulation of enzyme activities and thereby the metabolic flux.

CALCIUM AS MESSENGER SYSTEM

The discovery in 1958 of cyclic AMP[1] (cyclic 3,5 adenosine monophosphate) in epinephrine-induced increase in hepatic phosphorylase system had irrevocably committed the mechanism of hormone action to the concept of secondary intracellular chemical messengers[2]. This was reinforced by the discovery that cyclic AMP produced its effects by controlling a single class of enzymes, protein kinases[3]. These in turn control the activities of enzymes through a phosphorylation-dephosphorylation mechanism. Of these enzymes, the most widely-studied phosphorylase *b* kinase was shown to be sensitive equally to cyclic AMP and calcium. That calcium ions played a critical role in excitation-contraction coupling was known during the same period and in this process the skeletal muscle protein, troponin C, was found to act as a calcium-receptor[4]. Calcium was also required for the release of acetylcholine from nerve endings on electrical stimulation, and for the acetylcholine-induced secretion of catecholamines from adrenal medulla[5].

CALMODULIN

The importance of calcium in metabolic regulation was enhanced by a key discovery made in 1970. Cheung[6] and Kakiuchi & Yamazaki[7] independently found that phosphodiesterase, the enzyme responsible for hydrolysis of cyclic AMP, required a protein in addition to calcium for showing maximum activity. This protein was later purified and named "Calmodulin" (see ref. 8 for a review).

Calmodulin (CaM) is a calcium-binding protein. It is present in a variety of tissues of animals and in many animals and plants. The protein is heat-stable and is of small molecular weight of about 17000 daltons. It is an acidic protein (isoelectric pH 4.1). Its sequence of amino acids is conserved during evolution to a large extent. Both tryptophan and cysteine are absent and the ratio of phenylalanine/tyrosine is high. These characteristics are responsible for the atypical ultraviolet spectrum with peaks at 253, 259, 265 and 276, but not at 280 nm unlike other proteins.

Calmodulin has 4 binding sites for Ca. With two calciums bound per molecule of calmodulin, confirmational change occurs which allows it to interact with specific enzymes. The complex of Ca-CaM-phosphodiesterase is formed when free calcium concentration is increased and the

enzyme is activated. Other calcium-binding proteins, troponin C or parvalbumin, do not show this activation effect. Another example is phosphorylase *b* kinase which has CaM as one of its four subunits and is activated on binding Ca to it.

Several enzyme systems are now shown to be responding to Ca-calmodulin. Among these are: phosphodiesterase, adenylate cyclase, calcium pumps, protein kinases, phosphorylase *b* kinase, platelet phospholipase A_2, myosin lightchain kinase, tryptophan 5-monooxygenase, guanylate cyclase, microtubule assembly and NAD kinase (see ref. 9 for details). It is now clear that both cyclic AMP and calcium act as messengers and serve as interdependent coupling mechanisms in excitable and non-excitable tissues and thereby integrate the information transfer process in the actions of hormones and neurotransmitters.

Possible use of Fe and Mn in regulation

Even before the calcium concept burst on the scene, it is increasingly realized that multiple regulatory mechanisms are needed to support the diverse needs of metabolic modulation. The excellent way the cells used calcium-calmodulin system prompted consideration of other similar systems. Proposing a thoughtful hypothesis of Fe(II) and Mn(II) acting as intracellular controls R. J. P. Williams[10] stated: "The intermediate strength of binding of metal ions such as Fe(II) and Mn(II) and the consequent intermediate concentrations of free ions suggest that these two metals could act simultaneously both in catalytic and control roles". We are poised for developing new information on the potential use of Fe as a metabolic modulator.

Iron-dependent cellular activities

Iron is required for synthesising hemoproteins needed in oxygen transport and oxidations. Iron is absorbed, transported bound to transferrin and stored in the core of protein shell of ferritin. Deficiency of iron affects metabolic processes much before the stage of *hematopoiesis* and many Fe-dependent proteins/enzymes are decreased. These studies pointed the involvement of iron in a variety of cellular functions. Some of these are: mitotic process[11], stimulation of lymphocytes by phytohemoagglutinin[12], cell-mediated immunity[13], and synthesis of DNA[14] and collagen[15]. It is now apparent that iron plays an important role in many aspects of metabolism besides electron and oxygen transfer.

Enzymes dependent on Fe for activity

A number of enzymes are affected by iron: aconitase[16], gammabutyrobetaine hydroxylase[17], myoinositol oxygenase[18], tyrosine hydroxylase[19], tryptophan hydroxylase[20], phenylalanine hydroxylase[21], ribonucleotide reductase[22], lipoxygenase[23], lipid peroxidation[24] and phosphoenolpyruvate carboxy kinase[25], and oxidases of protein bound lysine and proline in collagen[26]. All these enzymes require addition of iron in the range of 10–200 μM concentration for expressing the full activity and are distinguished from Fe-containing enzymes. In the case of aconitase additional Fe^{3+}-binding subunit is present[16]. Fe is bound to the minor subunit (mol. wt. 58000), M_2, of ribonucleotide reductase[27]. So far it is not clear whether other proteins which can act as receptors for Fe are required or would potentiate the Fe effect. Phosphoenolpyruvate carboxykinase seems to require, proteins isolated from the cytosol, named ferroactivators[28], for its activation by Fe^{2+}. It now appears that these proteins are H_2O_2-utilizing enzymes like catalase or glutathione peroxidase and show apparent activation by destroying H_2O_2 which otherwise inactivates the enzyme.

Iron-dependent inhibition of HMG CoA reductase

The key regulatory enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase, located in microsomes, was rapidly inactivated by Fe^{2+} in the presence of liver cytosol[29]. The observation introduced a new concept in the regulation of the reductase. The inhibition was dependent on the combined presence of iron and a cytosolic protein and seemed to be rapid, irreversible and nearly complete at high concentrations of the cytosolic protein. The inhibitory effect was not due to

artificial loss of substrates, co-factors or the product[30]. The extent of inhibition was dependent on the concentration of cytosolic protein and not on time or temperature of incubation. The effect seems to be due to physical association of the components and is not catalytic in nature[31]. The iron-dependent inhibition was not obtained with bovine serum albumin, apoferritin, transferrin, superoxide dismutase, horseradish peroxidase or catalase. It was not due to oxygen radicals, H_2O_2 or lipid peroxidation. The protein was purified to homogeneity and was found to have a molecular weight of about 58000 daltons with two non-identical subunits (mol.wt. 43000, 28000)[32]. It showed ultraviolet absorption spectrum typical of a tryptophan-containing protein. It was able to bind about two atoms of Fe^{2+} per mole. Many of the properties described above are parallel to those of calcium-calmodulin system prompting the naming the inhibitor protein as "Fermodulin".

Fermodulin

Fermodulin (FeM) appears to be a new intracellular iron-binding protein requiring about 5 μM Fe^{2+} for half-saturation. Unlike transferrin this seems to bind both Fe^{2+} and Fe^{3+}, albeit weakly. This may in fact make fermodulin useful in intracellular iron transfer. There is a need for such an intracellular macromolecule in the transfer of iron bound to transferrin and internalized by transferrin receptor[33], and this should be able to pick up Fe^{2+} in the vesicle before returning the transferrin and its receptor to plasma membrane (figure 1). It will be of interest to see participation of fermodulin in the transfer of Fe into and out of ferritin. Also, it is not yet known whether fermodulin affects other enzymes and whether it can regulate the availability of Fe to the enzymes dependent on iron.

Inhibitor protein of lipid peroxidation

Peroxidation of lipids, a primary event in

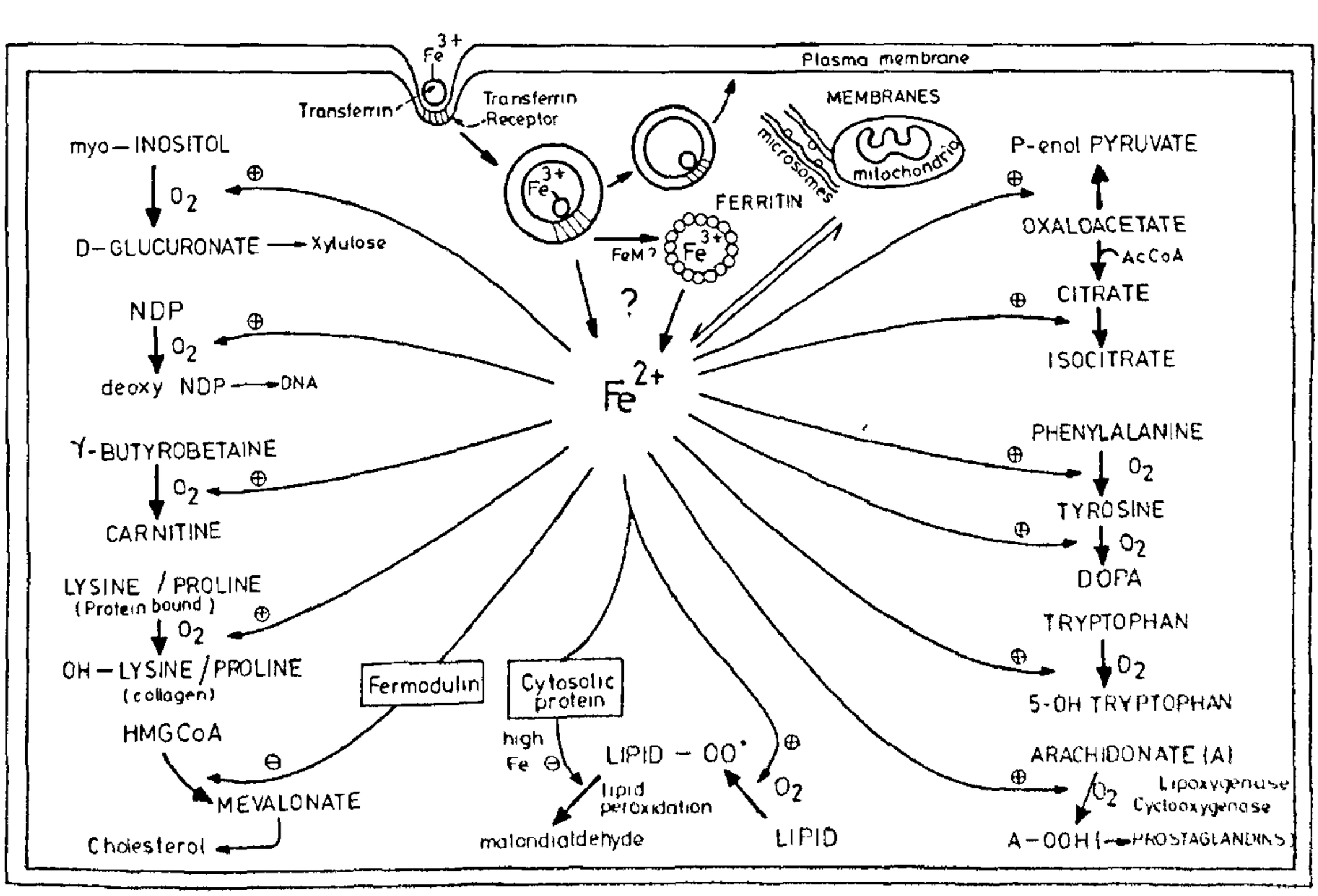

Figure 1. Some enzymes affected by irons.

cellular "oxidant damage", plays an important role in physiological processes of phagocytosis, prostaglandin biosynthesis and aging. Lipid peroxidation, lipoxygenase and cyclooxygenase require trace quantities of Fe for generating peroxy- and hydroxy-derivatives of unsaturated fatty acids. NADPH-dependent lipid peroxidation of rat liver microsomes is associated with uptake of oxygen several fold higher than the oxidation product, malondialdehyde[34]. This activity was inhibited by high concentrations of ADP and Fe^{2+} in presence of liver cytosol[35]. The factor responsible for inhibition was found to be a protein, unstable above 60°C and is not related to Z-protein, sterol-carrier protein, fermodulin and other heat-stable protein factors known before. The inhibition was limited only to the formation of thiobarbituric acid-reactive material (malondialdehyde) but not of disappearance of NADPH or accompanying oxygen uptake. Thus, the Fe-ADP-cytosol protein complex converts NADPH-cytochrome P-450 reductase into a thermogenic NADPH oxidase while keeping the turnover of polyunsaturated fatty acids at the minimum under conditions of increased Fe concentration. These studies are indicative of existence of proteins which act in conjunction with Fe to modulate metabolic activities.

Concentration changes of free Fe cations

It is important to consider the concentration of free Fe available and its variability under physiological conditions. The total Fe content in the liver is very large and most of it is in the stored form in ferritin or hemo-proteins. The free Fe is normally 1 μM[10], similar to free Ca and one report claimed as high as 200 μM[36]. It is therefore necessary to look into the origin of Fe that can be released and the mechanism of its mobilization. It is obvious that those enzymes dependent on Fe for activity will be functional only when Fe concentrations become sufficiently large. Some observations are available to indicate this correlation. Phenylalanine hydroxylase was low in activity at night and high during day[37] and the converse is true for HMG CoA reductase[38]. These results are consistent with a low concentration of Fe at night and high during the day. Starvation increased phosphoenolpyruvate carboxykinase[39] and decreased HMG CoA reductase[40], as anticipated by an increased Fe concentration[41]. Using the technique of perfusion with defined media it was possible to show iron-dependent regulation of phenylalanine hydroxylase in rat liver[37]. Information is now available on the presence of Fe in mitochondria, microsomes and plasma membranes in a form distinct from hemoproteins or iron-sulfur proteins. It may be associated with proteins, carbohydrates or lipids in a weak-binding state and released to the enzyme proteins on stimulation, specific to the physiological change. Mechanisms of release of Fe from ferritin through xanthine oxidase system and from mitochondrial membranes through calcium activation are being proposed. Measurement of such small quantities of free Fe release will offer technical difficulties. It should be possible to extend the work to response to hormones of Fe release and correlate with the known changes of metabolic and enzyme activities. It is expected that iron and iron-dependent enzymes and iron-binding modulator proteins will expand our understanding of the potential of iron as a metabolic modulator (figure 1).

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