Biochemistry

 Volume 31, Number 46 November 24, 1992

Perspectives in Biochemistry

Iron Metabolism—New Perspectives in View

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Received February 18, 1992; Revised Manuscript Received August 14, 1992

Iron is one of the most closely studied and important of the inorganic elements necessary for life, and yet in many ways the precarious state of its metabolism in man is relatively poorly appreciated. We walk on a knife edge essentially because human dietary intake is carefully controlled such that we absorb about 1–2 mg from our diet and we excrete 1–2 mg of iron per day [reviewed in Bothwell et al. (1979)]. Slight alterations in either of these figures can elicit depletion of iron (one-third of the world population is anaemic), while excessive intake may cause iron overload.

It is in this context that the present Perspective in Biochemistry relates to our current awareness of the way in which progress has been made to our understanding of iron metabolism during the last decade and to the new perspectives that are beginning to open.

Iron is an essential element for the growth and well-being of almost all living organisms. It is involved in a great many biological functions and is an almost ubiquitous component of ribonucleotide reductases. By varying the ligands to which it is coordinated, iron has access to a wide range of redox potentials and can participate in a wide range of electron transfer reactions, spanning the standard redox potential range from +300 to −500 mV. It is also involved in oxygen transport, activation, and detoxification, in nitrogen fixation, and in many of the reactions of photosynthesis.

We commence this perspective by discussing how our understanding of microbial systems has changed rapidly in the last few years and how it may influence our understanding of iron metabolism in mammalian systems. Thereafter, we discuss recent progress in our understanding of the biochemistry and molecular biology of iron-binding proteins. The elegant translational regulation of the expression of mRNA's for transferrin receptor, ferritin, and δ-amino laevulinate synthetase by iron regulatory element binding proteins allows us to begin to understand the mechanism of cellular iron homeostasis. Finally, we review the current status of the low molecular weight intracellular iron pool and its possible role in mediating oxidative damage.

NEW PERSPECTIVES IN MICROBIAL IRON UPTAKE

Microbial systems confronted by the insolubility of iron had to set about developing systems for iron assimilation from a milieu in which iron was present in a poorly available insoluble form. In contrast to the regulation of iron uptake by mammallian cells, it is not iron as such, but rather the lack of iron, that turns on the expression of this impressive panoply of genes organized for the major part in operons but also scattered along the chromosome and in plasmids (Figure 1).

When bacteria such as *Escherichia coli* are placed under iron-deficient conditions, siderophores (low molecular weight iron-binding chelators) are synthesized and released into the extracellular medium, where they tightly bind free Fe(III) in a strong Fe(III)–siderophore complex. In parallel with the synthesis of siderophores, a number of other proteins involved in ferrisiderophore uptake are also synthesized.

Thereafter, iron uptake (Figure 2) involves the following steps [reviewed in Braun and Hantke (1991)]: (i) binding of the ferrisiderophore complex to the outer membrane via a specific receptor, located in the outer-membrane, (ii) transfer of the ferrisiderophore from its outer membrane receptor complex to a carrier protein, (iii) release of the ferrisiderophore from its complex with the periplasmic carrier protein to a cytoplasmic membrane protein complex, which, in an energy-dependent process, translocates the ferrisiderophore across the cytoplasmic membrane, and (iv) release of the ferrisiderophore complex into the cell where the iron will be released, most likely by a reductive mechanism, involving either a membrane-bound or a soluble ferrireductase (Fischer et al., 1990). In the case of ferrienterobactin, this may involve initial hydrolysis of ester bonds in the iron–siderophore complex to bring the Fe(II)/Fe(III) redox couple within the range of physiological reductants (Raymond et al., 1987).

The genes of *E. coli* which are involved in iron uptake are known to be regulated by the Fur repressor, the protein product of the *fur* gene locus (*f*erric iron *u*ptake *r*egulation) as indicated

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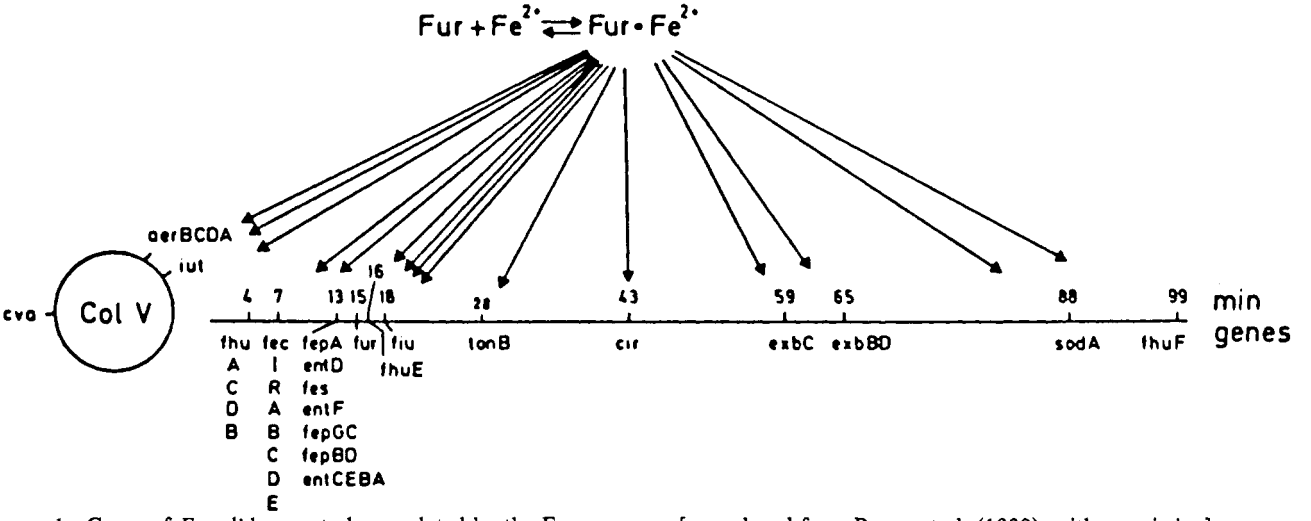


FIGURE 1: Genes of *E. coli* known to be regulated by the Fur repressor [reproduced from Braun et al. (1990), with permission].

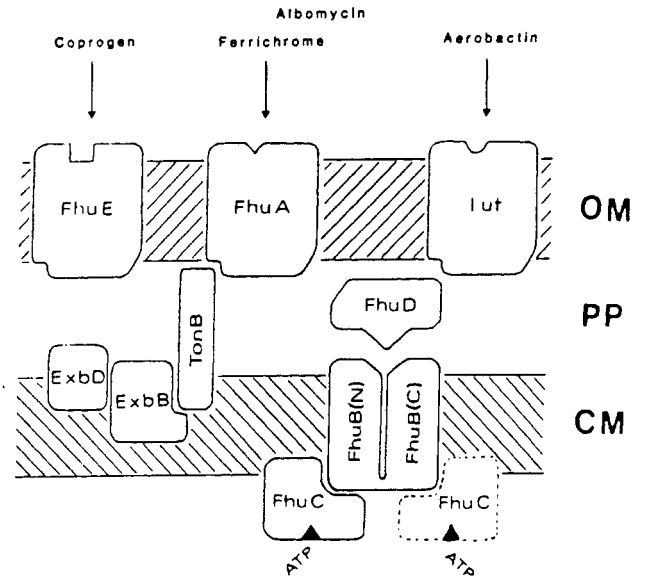


FIGURE 2: Model describing iron(III) hydroxamate transport systems in *E. coli*. The outer membrane (OM) receptor proteins FhuA, Fhu E, and Iut bind the different iron(III) siderophores which are transported across the OM through the activity of the TonB protein, itself influenced by the ExbB and ExbD proteins and by the energized state of the cytoplasmic membrane. All the iron(III) hydroxamate compounds require the FhuD protein in the periplasmic space (PP) and the FhuB and FhuC proteins in the cytoplasmic membrane (CM). It is proposed that Ton B fluctuates between an energized and a nonenergized conformation: the energized conformation triggers the release conformation in the FhuA protein. Transport across the cytoplasmic membrane is TonB-independent and involves the FhuD protein for transport of ferrichrome across the periplasmic space between the outer membrane (OM) and the cytoplasmic membrane (CM), where FhuD, FhuB, and FhuC catalyze transport of the ferrisiderophore across the cytoplasmic membrane. FhuC contains a potential nucleotide-binding domain (indicated as an ATP-binding site in the figure). FhuB(N) and FhuB(C) indicate that the FhuB protein has an internal homology. [Reproduced from Braun and Hantke (1991), with permission.]

in Figure 1. The Fur protein is a 17-kDa protein, rich in histidine, which can be converted to a transcriptional repressor by binding of its corepressor, Fe^{2+}, forming a dimeric metalloprotein. This then binds to specific DNA sequences in the promotor regions of *fur*-controlled genes, acting as a repressor of transcription. When the cellular iron concentration decreases below a critical level, this repression is released, and the synthesis of iron uptake systems can recommence. The DNA-binding regions of the Fur protein have been identified in *E. coli* as a 19 base pair consensus sequence [reviewed in Braun and Hantke (1991)], namely, 5′-GATAATGATAATCATTATC-3′, which is found in the promotor region of all genes that are negatively regulated by iron. Similar sequences are found in the promotor regions of iron-regulated genes from a number of other organisms not closely related to *E. coli*.

The combination of genetics coupled with the identification of gene products and their subcellular localization has enhanced our understanding of how iron is taken up by Gram-negative bacteria and how this uptake is regulated.

STRUCTURE AND MOLECULAR BIOLOGY OF IRON TRANSPORT AND STORAGE PROTEINS

Transferrins—Proteins of Iron Transport and Sequestration. Transferrins are a class of iron-binding proteins found in the physiological fluids of many vertebrates: the recent isolation and cloning of a transferrin from the sphinx moth *Manduca sexta* (Bartfield & Law, 1990) extends this to nonvertebrate species. The principal transferrins are (i) serotransferrin, found in serum and in other extracellular secretions, which transports iron between cells, (ii) lactotransferrin, found in milk, in neutrophils, and tears, (iii) ovotransferrin in egg white. Both lactotransferrin and ovotransferrin function as antibacterial agents. A cell-surface glycoprotein present on most human melanomas has been identified as a member of the transferrin family by sequence homology and is designated melanotransferrin (Rose et al., 1986).

The primary, secondary, and tertiary structures of these different transferrins have been elucidated and extensively reviewed [see Crichton (1991)]. Each transferrin gene which has been sequenced contains 17 exons separated by 16 introns; of these, 14 constitute seven homologous pairs which code for corresponding regions in the N- and C-lobes of the protein. The first exon codes for a signal peptide, necessary for the secretion of the transferrin molecule, while the last two exons encode a sequence which is unique to the C-terminal lobe. The exon–intron arrangements in the corresponding DNA are also homologous in the two halves of the molecule, confirming that transferrins originated by a gene duplication event, which is thought to have occurred some 500 million years ago [reviewed in Bowman et al. (1988)].

The two iron-binding sites in the lactotransferrin molecule are buried at the inner end of a deep interdomain cleft within the two lobes of the molecule. The coordination of the iron atoms is identical, involving four ligands from the protein, one carboxylate oxygen, two phenolate oxygens and one

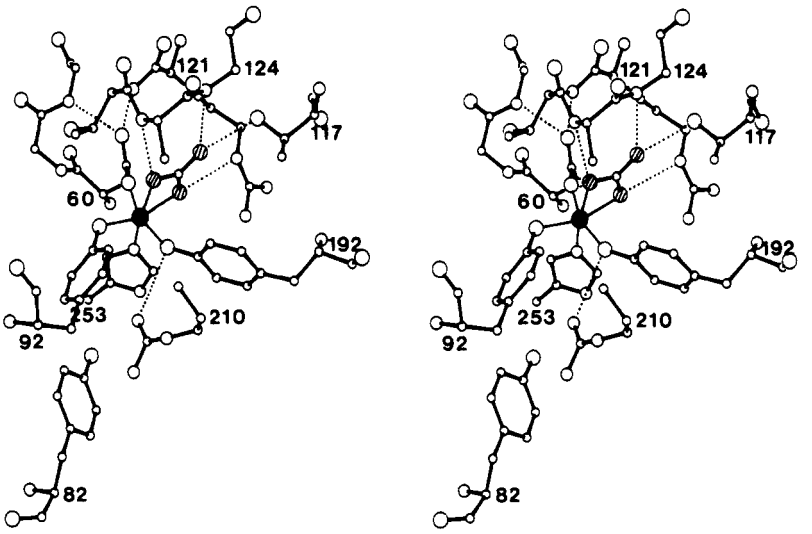


FIGURE 3: Stereodiagram of the metal- and anion-binding site of the N-lobe of human lactotransferrin. The iron atom is represented by a filled circle and the carbonate ion by hatched circles. [Reproduced from Anderson et al. (1989), with permission.]

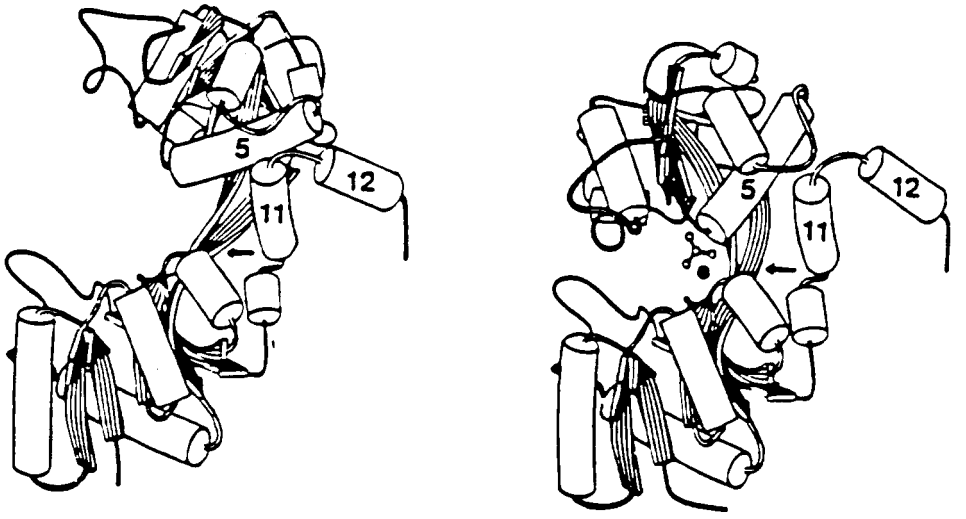


FIGURE 4: Open and closed conformations of the N-lobe of human lactotransferrin. [Reproduced from Anderson et al. (1990), with permission.]

imidazole nitrogen, and two bidentate oxygen ligands from the carbonate anion (Figure 3). The four protein ligands in the N-lobe are Asp 60 and Tyr 192, trans to one another with Tyr 92 and His 253 in cis positions. The two remaining cis-octahedral positions are left vacant for the two nonprotein carboxylate ligands (Anderson et al., 1989). The protein ligands are widely spaced along the polypeptide backbone, with Asp 60 in the main part of doman N1, Tyr 192 in domain N2, and Tyr 92 and His 253 coming from the interconnecting backbone strands which cross between the two domains at the back of the iron site. Unlike many other metal-binding proteins, the essential metal-binding ligands are distributed among several exons, reflecting the fact that iron is not necessary for the correct folding of the apotransferrin polypeptide chain.

The anion site appears to involve carbonate, which binds as a bidentate ligand to the iron atom fitting perfectly between the iron atom, the side chain of Arg 121, and the N-terminus of helix 5, residues 121–136. One oxygen is bound to iron and forms a hydrogen bond with Arg 121, the second also binds to iron and forms a hydrogen bond with the NH of residue 123, while the third oxygen makes two hydrogen bonds with NH(124) and the side chain of Thr 117. The importance of helix 5 in defining this anion-binding site in the pocket created by the side chain of Arg 121 and the main-chain atoms of residues 122–125 (and of the corresponding residues 465–469 in the C-lobe) is underlined by their invariance in all transferrins so far sequenced (except for the C-lobe of melanotransferrin, which lacks several other residues necessary for iron binding). Residues 121, 123, 124, and 125 are totally invariant in both halves of each transferrin, and 122 is always either Thr or Ser.

In apotransferrin (Anderson et al., 1990) the N-lobe is in an open configuration, with its two domains N1 and N2 well separated from each other (Figure 4). This open configuration of the binding cleft exposes two basic amino acid residues which were buried in the closed form, namely, Arg 121 and Arg 210. If we assume that carbonate binds first, it would be attracted into the bottom of the open interdomain cleft by these positive charges. Four of the iron-binding ligands are now in place (Tyr 92, Tyr 192, and the two carbonate oxygens), and the iron could then bind to the N2 domain. Binding would be completed by rotation of N2, closing the cleft with Asp 60 and His 253 completing the iron coordination and Asp 60 further linking the two domains by hydrogen bonding (Anderson et al., 1990).

Transferrin Receptors—Plasma Membrane Recognition and Uptake Systems. Most, if not all, mammalian cells have transferrin receptors (TfR) located on their plasma membranes. Iron uptake by a transferrin to cell cycle is by now

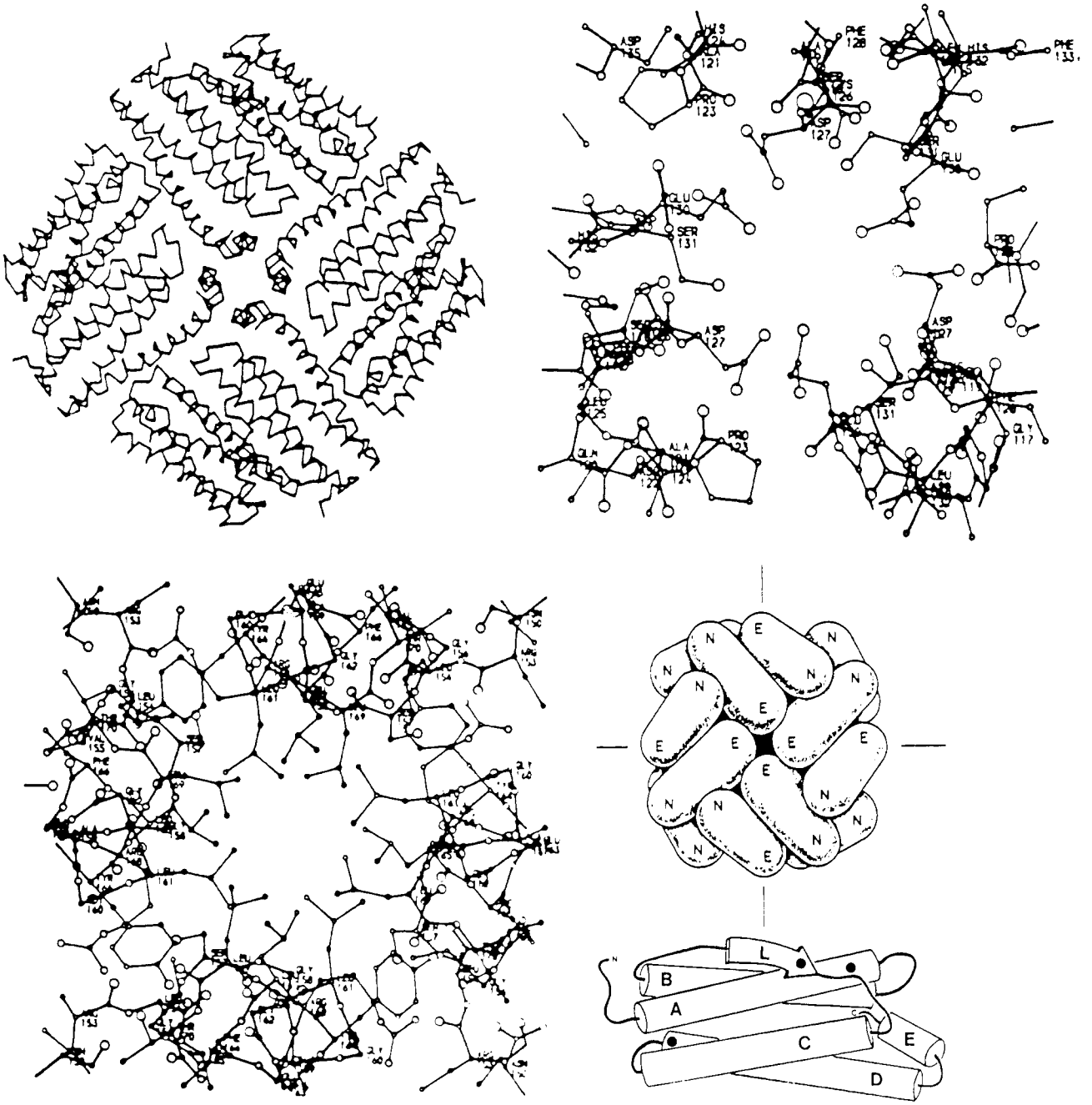


FIGURE 5: Several views of horse spleen apoferritin: (a, right center) schematic diagram of the apoferritin molecule viewed down a 4-fold axis; (b, upper left) α-carbon backbones of eight subunits, in the same orientation as in panel a; note that they constitute four antiparallel subunit dimers; (c, bottom right) a ribbon diagram of the subunit showing the four long helices, A (residues 9–39), B (44–73), C (91–121), and D (122–154), the short helix E (158–172), and the loop L (74–90). Intron positions are indicated by filled circles: (d, lower left) region near the hydrophobic 4-fold channel; (e, upper right) region near the hydrophilic 3-fold channel. [Reproduced from Rice et al. (1983) and Harrison et al. (1989), with permission.]

well established (Thiel & Aisen, 1987). The TfRs are disulfide-linked dimers, each of molecular mass 90–95 kDa, corresponding to about 760 amino acid residues [reviewed in Trowbridge and Shackelford (1986)]. The receptor is anchored in the plasma membrane by a transmembrane helix with an internal N-terminal domain of 61 residues and an external domain for the human transferrin receptor of 671 amino acid residues. It is proposed (Collawn et al., 1990) that the internalization signal structure of TfR consists of a tetrapeptide sequence Tyr-X-Arg-Phe forming an exposed tight turn separated from the transmembrane region of the receptor by a sequence of at least seven residues.

Iron Storage in Ferritins and Hemosiderins. Intracellular iron storage involves two proteins: ferritin, occurring principally in normal cellular metabolism, while significant quantities of hemosiderin are found in iron-loading syndromes. Ferritins are constituted by soluble protein shells enclosing a roughly sperical internal cavity within which a ferrihydrite core of variable iron content is deposited. This contrasts with hemosiderins, which are water-insoluble protein complexes, characterized by a higher iron to protein ratio than ferritins.

Ferritin Structure. As indicated above, ferritins consist of a hollow protein shell, generally of external diameter 12–13 nm, which encloses an internal cavity of some 7–8 nm diameter (Figure 5). The occurrence of ferritins is much more extensive than transferrins, encompassing not only mammals but also invertebrates, fungi, and prokaryotes (it remains to be established if it plays the same role in all of these different organisms). The apoferritin molecule (the iron-free protein) is made up of 24 subunits. There are two subunit types, H

(22–24 kDa) and L (20–22 kDa); the apoferritin icosatetramer thus consists of a mosaic of heteropolymers, ranging from all-H to all-L. The amino acid sequences of H and L subunits from a number of different species have been established [reviewed in Crichton (1991) and Harrison et al. (1991)]. The two subunit types are very homologous to one another within one subunit class, whereas, within one single species, H and L subunits are very different from one another.

The three-dimensional structures of apoferritins from horse spleen (essentially 90–95% L), rat liver (66% L), recombinant human H, and recombinant L (both of the latter 100% of the subunit in question) have been determined by the group of Pauline Harrison at the University of Sheffield (Lawson et al., 1991). A schematic representation of the apoferritin molecule viewed down the 4-fold axis as well as the α-carbon backbones of eight subunits in the same orientation are presented in Figure 5a,b.

The subunit, which is represented as a ribbon diagram in Figure 5c, presents a bundle of four helices A–D, together with N- and C-terminal appendages. The antiparallel helix pairs A–B and C–D are connected by a loop L, which, together with its 2-fold related counterpart in a second subunit, forms a section of antiparallel β-sheet within the dimer at the external surface of the apoferritin protein shell. The N-terminal sequence projects toward the outside of the multimeric protein shell, whereas the C-terminal sequence forms a short helix E (residues 158–172) which lies almost perpendicular to the principal helix bundle and which terminates in a short peptide oriented toward the internal cavity of the protein shell. The approximate positions of genomic introns are marked by black circles. The E helix exposes a collection of hydrophobic residues which are buried in the interior of the four channels (Figure 5d) which lie along the 4-fold symmetry axes. In contrast, the eight 3-fold funnel-shaped channels (Figure 5e) are extremely polar, with three Glu 130 residues situated toward the wider end of the channel on the outer surface of the protein shell and three Asp 127 residues toward the narrower inner surface. These two types of channels may provide access routes to and from the interior of the hollow protein shell.

The central iron-rich core of mammalian ferritins consists of a ferrihydrite biomineral phase, containing up to a maximum of 4500 atoms of iron (Fischbach & Anderegg, 1967) together with additional variable amounts of phosphate. Ferrihydrite is the name proposed and approved by the International Mineralogical Association [see Murray (1979)] for the brown X-ray amorphous hydrous iron oxide of composition $5Fe_2O_3 \cdot 9H_2O$ (Towe & Bradley, 1967). While many mammalian ferritin cores consist of well-ordered single crystals, which are very similar to ferrihydrite as judged by their electron diffraction (St. Pierre et al., 1989), others contain mixtures of small crystallites and poorly crystalline or amorphous regions.

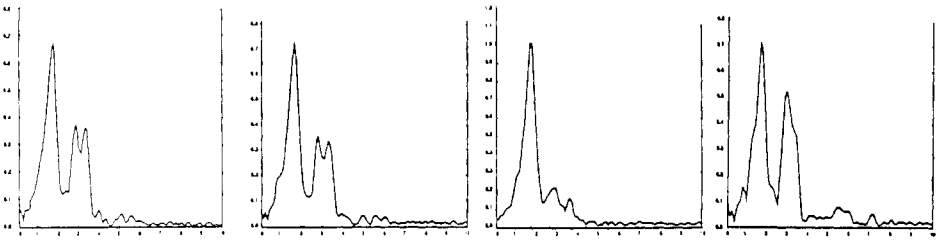
Hemosiderin. Although hemosiderin was identified over 100 years ago by Perls (1867), little was known until recently in biochemical terms of its composition, structure, biosynthesis, and metabolism or indeed of its role in causing cell damage in iron-loading syndromes. It is present in relatively small amounts in normal tissues but accumulates during iron overload (Selden et al., 1980). Hemosiderin is a water-insoluble protein with a high iron to protein ratio and a molecular mass greater than 4000 kDa, but of ill-defined nature. It is probably derived from ferritin after lysosomal degradation of its protein shell (Iancu & Neustein, 1977) although it could also be formed independently of ferritin (Crichton & Ward, 1992). Such a process may occur when high circulating levels of non-transferrin bound iron are present, as in hemochromatosis, which will be internalized into the cell by simple pinocytosis and be directly taken up by lysosomes. The existence of at least three different types of iron cores has been confirmed by a variety of techniques, both biophysical, i.e., Mössbauer spectroscopy (Dickson et al., 1988), EXAFS (Mackle et al., 1992), and electron diffraction and electron microscopy (Mann et al., 1988) and biochemical, i.e., peptide content (Ward et al., 1992), iron release (O'Connell et al., 1989; Ward et al., 1989), and elemental analysis (Ward et al., 1992) (Table I). Hemosiderin with iron cores similar to the ferrihydrite cores of ferritin has been identified in hemosiderins isolated from alcoholic livers, in livers from untreated genetic hemochromatotis patients, and in the livers of animals with either naturally occurring or artificially induced iron overload, with a major peptide on SDS–PAGE on 20 kDa (Ward et al., 1991) and occasionally a band at 14.5 kDa in untreated hemochromatosis hemosiderins. Secondly, hemosiderin isolated from tissues from patients with genetic hemochromatosis who have undergone venesection has a primarily amorphous Fe(III) oxide iron core with some partial ordering based on the ferrihydrite structure and a major peptide band at 20 kDa after SDS–PAGE. Thirdly, the hemosiderin identified in a chelated secondary hemochromatosis tissues identified initially by Weir et al. (1984) has a goethite-like iron core and predominantly a band at 14.5 kDa after SDS–PAGE: goethite (α-FeOOH) is the most common form of the Fe(III) oxyhydroxides and is the polymorph to which most other FeOOH phases eventually revert to upon aging (Murray, 1979).

It is suggested that there may be a biological advantage to the cell by the formation of hemosiderin as iron is sequestered into a protein from which it is less available to participate in iron-catalyzed lipid peroxidation (O'Connell et al., 1985). Indeed, in vitro experiments clearly show that the release of iron to both weak, i.e., oxalate (Ward et al., 1989), and strong chelators, i.e., desferrioxamine (O'Connell et al., 1989), is significantly reduced from both SH hemosiderin (goethite) and horse hemosiderin (ferrihydrite) by comparison to ferritin.

Ferritin Iron Deposition. It is clearly of paramount interest to understand how iron is deposited in ferritin and how it is released from the ferrihydrite iron core. Apoferritin catalyzes the oxidation of ferrous iron to the ferric state. Although oxidation at specific site on the protein is involved in the initial phase of core formation, it is generally accepted that once a critical nucleus of Fe(III) ions has been formed, and has begun to hydrolyze, it will then act in an autocatalytic manner to promote crystal growth on the surface of the initial biomineral core; in other words, once the protein has initiated the nucleation of the iron core, it thereafter plays a less important role, since thereafter iron oxidation and biomineralization take place essentially on the surface of the core crystallite, forming the ferrihydrite single crystal cores, albeit with extensive structural and stoichiometric irregularities [reviewed in Crichton (1991), Harrison et al. (1991), and St. Pierre et al. (1989)].

There are three questions with regard to iron deposition which merit attention. By what route does Fe(II) traverse the apoferritin protein shell to gain access to the ferroxidase sites? Where are the ferroxidase sites, and what are the protein ligands which are involved? And how does the oxidized Fe(III) initiate the nucleation of the core crystallite? It seems highly likely that the route of iron penetration is via the 3-fold channels. The binding stoichiometries of Mn(II), VO(II),

Table I: Biophysical and Biochemical Properties of the Three Hemosiderin Types[a]

	ferritin	Hd1	Hd2	Hd3
Fe/protein ratio	0.11	0.25	0.43	0.40
size of iron core (nm)	7.22 ± 0.44	6.12 ± 0.51	5.36[b]	5.49 ± 0.55
Mossbaüer blocking temp (K)	40	28	<3	63
electron diffraction lines (Å)				4.25
				2.68
	2.47	2.50	2.49	2.45
	2.22		2.12	2.20
	1.93	1.99		1.99
	1.71	1.71		1.71
	1.51	1.51	1.53	1.54
	1.46	1.46		1.47
EXAFS Fourier transformation				
elemental composition (mmol element/ mol Fe) principal elements detected	P 115	306	1454	98
	Cu 0.15	0.88	50.4	0.07
	Ca 3.2	13.6		1.8
	Zn 12.4	1.39	45.8	0.17
crystallinity	good	good	poor	good
peptides identified after SDS–PAGE (kDa)	20	20	20.0	14.5, 20.0
mineralization	ferrihydrite	ferrihydrite	amorphous ferric oxide	goethite

[a] Hd1, hemosiderin isolated from the liver of untreated primary hemochromatosis and liver and spleen of animals with naturally occurring or induced iron-overload; Hd2, hemosiderin isolated from the liver of treated primary haemochromatosis; Hd3, hemosiderin isolated from the liver and spleen of treated seconary hemochromatosis patients. [b] Too few cores to obtain a statistical analysis. The result is an average of the iron cores counted.

and Cd(II) are consistent with their binding in the 3-fold channels (Wardeska et al., 1986). X-ray crystallographic studies on apoferritin crystals grown from $CdSO_4$ and $ZnSO_4$ have identified two binding sites for both of these metal ions; the ligands are the three residues of Glu 130 on the wider outer face of the funnel-like hydrophilic 3-fold channels and the three Asp 127 at the narrower inner face of the same channels [reviewed in Harrison et al. (1989)]. Chemical modification and ^{113}Cd NMR studies also suggest that iron enters by the 3-fold channels (Stefanini et al., 1989). However, mutation of the conserved Glu 130 and Asp 127 in the channels does not prevent iron uptake or the manifestation of ferroxidase activity (Levi et al., 1989). Taken together, these results would suggest that while the 3-fold channels may be the sites of iron entry, they are not the primary site of Fe^{2+} oxidation.

With regard to our second question, which concerns the role of the apoferritin protein in the initiation of iron oxidation, as has been pointed out recently (Hanna et al., 1991a), it appears "from kinetic studies, that the process of iron oxidation in apoferritin involves a binuclear centre bridging two polypeptide chains" [reviewed in Crichton and Charloteaux-Wauters (1987) and detailed in Crichton and Roman (1978) and Paques et al. (1979)]. This appears to involve the binding of Fe^{2+} at specific sites within the protein shell where a "ferroxidase" activity of the protein catalyzes the oxidation of Fe^{2+} to Fe^{3+}. Chemical modification studies (Wetz & Crichton, 1976) as well as spectroscopic data (Chasteen & Theil, 1982; Sayers et al., 1983) indicate that carboxyl groups are most likely involved in iron(II) oxidation and perhaps as well in nucleation of the core crystallite [reviewed in St. Pierre et al. (1989)].

It has been suggested (Lawson et al., 1989, 1991) that the ferroxidase center is located exclusively in H subunits and comprises Glu 23, Glu 58, and His 61 (L-subunit numbering). In most L subunits these are replaced by Tyr or His 23, Lys 58 and Gly, or Ala 61. Mutation of all three of these residues in human recombinant H ferritin to Ala results in a considerable decrease in the rates of iron uptake, although both the triple mutant as well as human rat recombinant L-chain homopolymers are able to make up iron, albeit slowly, and to form iron cores (Lawson et al., 1989). We would conclude that the L-chain homopolymers can still initiate iron core formation. Recent studies (Hanna et al., 1991b) indicate that VO^{2+} competes 1:1 with Fe^{2+} for binding to the same site on horse spleen apoferritin (96% L subunits), and electron nuclear double resonance (ENDOR) measurements on the VO^{2+}–apoferritin complex provide evidence for an iron(II)-binding site with nitrogen coordination. Evidence has also been presented, from both EPR and Mössbauer spectroscopy, for the presence of mononuclear Fe^{3+}–apoferritin complexes which were thought initially to be intermediates in an early stage of core formation (Chasteen et al., 1985; Bauminger et al., 1989), while the presence of a mixed valence Fe^{2+}–Fe^{3+}–protein complex with a net electron spin of $^1/_2$ has also been reported in early stages of core formation (Chasteen et al., 1985). Recent studies suggest that the mixed valence complex is formed in the interior of the apoferritin protein shell in the vicinity of the 2-fold symmetry axis of the subunit dimer and that it may contain a μ-oxo bridge (Hanna et al., 1991a). It was concluded that the mononuclear Fe^{3+}–complex is not a precursor for formation of the mixed valence complex. The Mössbauer studies referred to above (Bauminger et al., 1989) indicated the presence of an oxo-bridged Fe(III) dimer as an early intermediate in iron core formation. It has very recently been proposed (Treffry et al., 1992) that a second iron-binding site constituted by the second carboxyl oxygen of Glu 58, and Glu 57 and Glu 103 (L-chain numbering), might permit the formation of a μ-oxo-bridged Fe(II) dimer to which dioxygen could bind. Electron transfer with formation of the oxo-bridged Fe(III) dimer would be accompanied by release of

hydrogen peroxide.

This brings us to the last phase of core formation. Oxidation of Fe^{2+} at specific sites at the subunit dimer interface creates a critical nucleus of Fe(III) species, which becomes the focus for subsequent Fe^{2+} diffusion and oxidation. Recent studies suggest that such a nucleation site might be constituted by Glu 57, Glu 60, and Glu 63, which are conserved in all vertebrate vertebrate H and L chains [reviewed in Harrison et al. (1991)]. Replacement by site-directed mutagenesis of both ferroxidase and "nucleation" site residues by Ala resulted in complete inhibition of iron uptake and deposition (Wade et al., 1991). When the number of localized Fe(III) species reaches a critical size at the interface, the activation energy for nucleation is overcome (St. Pierre et al., 1989), and growth of the core thereafter continues by further addition, oxidation, and hydrolysis of Fe^{2+} to the nucleation zone. What happens thereafter will be determined by the mineral phase itself, with iron oxidation and polymerization taking place on the surface of the iron core mineral: this is the crystal growth phase of ferritin iron core biomineralization. If there are few counterions, such as phosphate, the iron core will grow in a more or less structured ferrihydrite form, as in mammalian ferritins; if there is a high concentration of anions, the structure of the core will be amorphous [reviewed in St. Pierre et al. (1989)].

CELLULAR IRON UPTAKE AND RELEASE

The iron uptake of a great many mammalian cell types seems to be mediated by the transferrin receptor (TfR). It is present on virtually all dividing cells, suggesting that its expression is coordinated with cell proliferation. Both iron uptake and cellular proliferation can be blocked by specific monoclonal antibodies to TfR [reviewed in Trowbridge and Shackelford (1986)]. The rate of iron uptake during the development of erythroid cells will be dependent on the level of TfR in cultured cells (Iacopetta et al., 1982), while the expression of TfR is regulated by the availability of iron. TfR expression in cultured cells increases 2–5-fold in the presence of desferrioxamine B, a cell-permeable iron chelator (Mattia et al., 1984; Bridges & Cudkowitz, 1984; Ward et al., 1984), and decreases by the same order of magnitude in the presence of sources of exogenous iron such as haemin or ferric salts (Ward et al., 1982, 1984; Pelicci et al., 1982; Rao et al., 1985). The concentration of transferrin in the circulation is 30–40 μM with an iron saturation level in normal humans of 30%, and the dissociation constant of the receptor for diferric transferrin is typically 1 nM. Thus there is always a saturating concentration of transferrin with regard to its receptor, and so if a particular cell wishes to increase its iron uptake, it must increase the number of transferrin receptors that it expresses, which is precisely what tumor cells do [reviewed in Trowbridge and Shackelford (1986)].

Although the transferrin to cell cycle has been well established in erythroid cells and in many transformed cell lines, we may now pose the question of the mechanism of iron release from ferritransferrin, which we assume takes place in the endosome or CURL at a pH value of 5.0–5.5. We know from in vitro studies that the rate of spontaneous dissociation of iron from transferrin at this pH is much too slow to account for the observed rate of iron release, suggesting that some other factors such as chelation and/or reduction are necessary to ensure that iron is effectively released during the relatively short dwell time that the ferritransferrin molecule remains within the endosome/CURL (a matter of a few minutes at most). Recent in vitro results indicate that iron release from ferritransferrin at mildly acidic pH values (5.6–6.0) is substantially increased when it is bound to its receptor compared to release from free ferritransferrin (Bali et al., 1991; Sipe & Murphy, 1991), suggesting that within the endosome/CURL the transferrin receptor may facilitate iron release from transferrin. Further, the receptor could minimize nonspecific release of iron from the diferritransferrin–TfR complex at the neutral or weakly alkaline pH of the cell surface (Bali et al., 1991). It has also been argued, from studies on preparations of endocytic vesicles from reticulocytes enriched in transferrin–transferrin receptor complexes, that an NADH–ferricyanide reductase activity might be involved in iron release from ferritransferritin, in a manner analogous to iron release from microbial ferrisiderophore complexes (Nunez et al., 1990). The authors propose, in addition, a translocation system that would move Fe(II) to the trans side of the vesicular membrane and that would subsequently deliver the reduced iron to the low molecular weight iron pool. The current concensus would be that, by whatever mechanism iron is released from the diferritransferrin–TfRA complex, transferrin iron is rapidly transferred thereafter either to the cytoplasmic iron storage protein ferritin or to mitochondria, possibly by the low molecular weight ligand pool, where it can be used for heme synthesis [reviewed in Crichton (1991)].

It has been suggested that mechanisms other than the transferrin/transferrin receptor cell cycle described above might be involved in iron uptake by mammalian cells [reviewed in Thorstensen and Romslo (1990)]. The case of the hepatocyte is particularly interesting, since the number of transferrin receptors on hepatocyte plasma membranes is relatively small, and in vitro studies show that at 37 °C cellular uptake of iron increases continuously when the extracellular concentration of transferrin is increased to levels far above that needed to saturate hepatocyte TfR receptors. It has been concluded that adsorptive or fluid phase endocytosis is the main pathway of hepatocyte iron uptake from transferrin, although no evidence was found for the release of degradation products of transferrin into the extracellular medium [reviewed in Sibille et al. (1986), Morgan et al. (1986), and Thorstensen and Romslo (1990)]. This might be due to fusion of pinocytic vesicles containing transferrin with endocytic vesicles containing unoccupied transferrin receptors (Quintart et al., 1989), which would ensure that the reconstituted apotransferrin–transferrin receptor complex escapes lysosomal degradation by virtue of its high affinity and that the apotransferrin is then returned intact to the plasma membrane. The uptake of iron by hepatocytes from non-transferrin sources, particularly in iron overload, where the iron-binding capacity of serotransferrin is saturated, may represent an additional example of non-TfR-mediated cellular iron uptake [reviewed in Crichton (1991) and Crichton and Ward (1992)]. It has recently been reported from in vivo studies that iron uptake by rat liver is largely dependent on transferrin–receptor interaction (Morgan, 1991), although it should be pointed out that in this study chicken ovotransferrin resulted in a higher uptake of iron than with any other species of transferrin.

When macrophages, whether peritoneal, bone marrow, or liver Kupffer cells, are exposed to IgG-coated red cells, they assimilate them (Saito et al., 1986; Rama et al., 1988; Kondo et al., 1988). They then begin to secrete neosynthesized macrophage ferritin into the extracellular medium. If apotransferrin is present in the medium, it will take up iron from ferritin (Saito et al., 1986; Rama et al., 1988; Kondo et al., 1988); substances from serum such as ascorbate, citrate, bicarbonate, and lactate seem to be involved (Jin & Crichton, 1987).

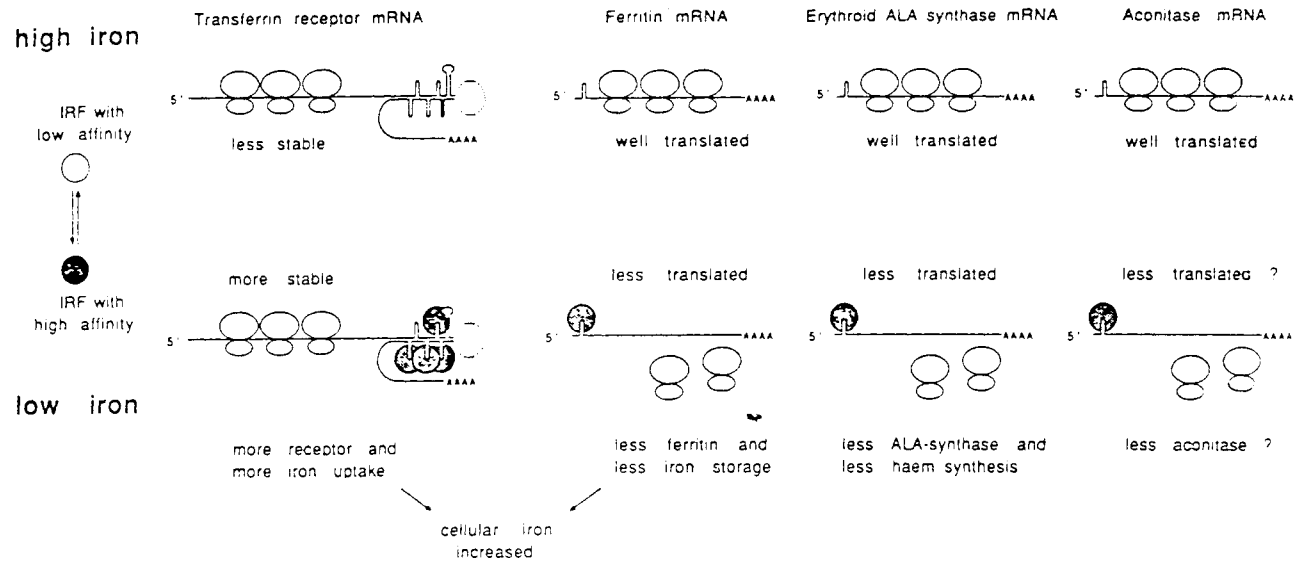


FIGURE 6: Coordinate control of central pathways in cellular iron metabolism. [Reproduced from Kühn (1991), with permission.]

However, if the Kupffer cells are cocultured with hepatocytes, the presence of a specific ferritin receptor on the hepatocyte plasma membrane (Mack et al., 1983) will ensure that the hepatocytes will take up the macrophage ferritin iron and protein (Sibille et al., 1988). The ferritin is internalized by the hepatocytes and follows the endocytic pathway to the CURL and thence to the lysosomal compartment where the protein is degraded and the iron released, either for incorporation into lysosomal hemosiderin or into the cytosolic ferritin pool (Sibille et al., 1989). Whereas the rate of ferritin endocytosis is modest compared with that of transferrin, the amount of iron delivered per ferritin molecule (the Kupffer cell ferritin contains about 2500 atoms of iron per molecule compared with the two ferric iron atoms per transferrin molecule) is much greater and might represent an important pathway of hepatocyte iron supply.

INTRACELLULAR IRON HOMEOSTASIS

One of the most important questions in iron metabolism is how cellular iron homeostasis is achieved, i.e., how does a cell regulate iron uptake with intracellular utilization? Considerable progress has been made on this front in the last few years, which confirms that ferritin synthesis is regulated essentially at the translational level (Drysdale & Munro, 1966). It had been proposed by Spirin (1969) that the translational control of specific mRNA's might be mediated by specific proteins. It was established that a highly conserved stem–loop sequence designated "iron regulatory element" or IRE was present both at the 5′-untranslated region of ferritin mRNA's and at the 3′-untranslated region of transferrin receptor mRNA's (the latter in five copies). An IRE-binding protein (designated "iron regulatory factor" or IRF) was then found, and it was suggested that the IRF acts as an iron sensor within the cell (in a way which can be compared to the Fur protein in microbial cells, although Fur acts at the level of gene expression, whereas IRF acts at the level of translation of mRNA). IRF can oscillate between a low-affinity conformation when iron levels within the cell are adequate, thus stimulating the synthesis of ferritin, and a high-affinity form when iron is in short supply by stimulating the synthesis of transferrin receptors, enhancing cellular iron uptake, and shutting down intracellular iron utilization (Figure 6). Thus, when the cell is iron-replete, the iron sensor activates iron assimilation systems and downregulates the iron assimilation pathway. When the cell requires iron, it downregulates the intracellular expression of mRNA's required for iron utilization and promotes iron uptake by protecting the mRNA of the transferrin receptor against nuclease degradation. It remains to be proved that the IRF can switch its conformation from the high- to the low-affinity form in the presence of Fe^{2+}, but it is nonetheless a very attractive hypothesis.

The sequencing of the cDNA for the IRF reveals that it has extensive homologies with the mitochondrial Kreb's cycle enzyme aconitase (Roualt et al., 1991). This is an interesting coincidence, because the iron–sulfur protein aconitase exists in two forms, one which has a 3Fe/4S cluster and is catalytically inactive, and a second form, with a 4Fe/4S cluster, which is active in transforming citrate to isocitrate and which can be reconstituted from the inactive form by incubation of the 3Fe/4S form with Fe^{2+} in a reducing medium [reviewed in Beinert and Kennedy (1989)]. The IRF could then be envisaged to be a cytoplasmic iron–sulfur protein analogous to aconitase, which can exist in two conformational states, one with low affinity for IRE's (presumably the 4Fe/4S form) and the other with high affinity for IRE's (presumably the 3Fe/4S form), which in the presence of intracellular concentrations of Fe^{2+} above a certain level would shift to the low-affinity conformation. If it can be established that the IRF responds to intracellular iron levels, not only in tumor cells but in normal cells, we might begin to hope that we have at last found a molecular mechanism of cellular metal homeostasis. One additional recent observation which makes perfect biochemical sense is that the gene for the first committed enzyme on the heme biosynthetic pathway, δ-aminolaevulinate synthase, also contains an IRE in its 5′-UTR (Cox et al., 1991). It seems likely that this IRE functions in the same way as does the ferritin mRNA IRE, i.e., binds the high-affinity form of IRF and is not translated when iron is in short supply but allows heme synthesis to proceed when iron is abundant within the cell. This would be an additional and logical correlate for cellular iron homeostasis, namely, that in iron penury cellular iron utilization would be downregulated in order to favor iron uptake, whereas when iron is available, intracellular pathways of iron utilization would be upregulated. An IRE has also been observed in the 5′-UTR of aconitase mRNA (Dandekar et al., 1991).

LOW MOLECULAR WEIGHT IRON POOLS IN NORMAL AND PATHOLOGICAL CONDITIONS

Despite many in vivo and in vitro studies, neither the speciation nor concentration of this pool is known. It is highly probable that such a pool exist within the cell, in low concentrations (0.5–1.0 μmol/L) which would be readily available to participate in cellular iron metabolism. Despite earlier suggestions that this low molecular weight iron pool is increased in iron-loaded cells, and thus might provide the necessary catalyst for Fenton-type chemistry to be initiated, studies to substantiate this statement have not yielded conclusive results. Indeed, the study of Mulligan et al. (1986) was unable to determine any change in its cellular concentration when either loaded or deficient in iron. We would therefore suggest that the essential difference between these two extremes of iron loading in cells is due to an alteration in the flux of iron through the cell, which will be increased in iron-loaded cells such that more iron would be available to the chelator at a given time (Crichton, 1985).

As to its speciation, various ligands have been proposed, i.e., AMP, ATP, pyrophosphates, various amino acids, polypeptides, proteins, and uncharacterized growth factors. None have, as yet, been unequivably identified as being the predominant ligand of the low molecular weight iron pool. Its identity will remain unknown until new sensitive nondisruptive techniques are available, e.g., scanning imaging mass spectrometry (to minimize decompartmentalization of iron), which will facilitate its identification within intact cells during both normal and pathological states of iron metabolism.

The participation of low molecular weight iron pools in catalyzing the production of reactive oxygen species in various pathological conditions has been the subject of an ever increasing literature in the past 20 years. The production of such a species in vivo would rely upon their being some decompartmentalization of iron provoking oxidative trauma to the cell. Such iron-provoked oxidative damage seems to be implicated in human disorders such as carcinogenesis, liver cirrhosis, hepatitis and fibrosis, ischaemia–reperfusion damage, inflammatory-immune injury, alcohol abuse, neurological disorders, and atherosclerosis (reviewed in Crichton (1991)).

CONCLUSIONS

We have tried to draw attention in this all too short perspective to the probable areas which are expanding and will be better understood to the next few years. There can be no doubt that the combined approach of molecular biology and structure/function analysis will lead to important developments in our understanding of the ways in which the inexorable requirements of dividing cells for iron are regulated and will lead to a better understanding of global homeostasis of iron metabolism. It remains to be established, for example, that the elegant mechanism of iron homeostasis which has been established in K-562 cells will be operative in normal cell lines and indeed whether the IRE-BP is really an iron–sulfur protein. We can also ask whether what is relevant for the control of iron uptake in *E. coli* is representative for other microbes or whether the transferrin to cell cycle is the most quantitatively important iron uptake pathway in mammalian hepatocytes, or for that matter in insects, where transferrin has been recently identified. Furthermore, it is pertinent to investigate the different biomineralization states in both ferritin and hemosiderin iron cores in different iron-loading syndromes to understand their derivation and pathogenesis. These, and many other questions will surely be answered in the next few years. It is our profound hope that as our understanding of iron metabolism at a molecular level grows so too will our capacity to intervene in the treatment of the many disorders of iron metabolism which continue to afflict mankind.

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