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Metals in Brain, Metal Transport, Storage and Homeostasis

1.1 INTRODUCTION – THE IMPORTANCE OF METAL IONS IN BRAIN FUNCTION

There can be little doubt that purely organic molecules, such as amino acids, fatty acids and lipids, carbohydrates, purines and pyrimidine bases and the polymers made out of them, proteins, polysaccharides and the nucleic acids RNA and DNA, are extremely important for living organisms. However, in order to fulfil a series of important functions, such as energy production, and, in higher organisms, for example, nerve transmission, muscle contraction and oxygen transport, metal ions are also absolutely essential. They include spectroscopically silent metal ions like potassium, sodium, calcium, magnesium and zinc together with the more spectroscopically accessible iron, copper, manganese, cobalt, molybdenum, vanadium and a few others. The role of some of these metal ion in brain function is particularly important, and has led to the introduction of the term metalloneurochemistry to describe the study of metal ions in the brain and the nervous system at the molecular level (Burdette and Lippard, 2003).

The alkali metal ions Na^+ and K^+ play a crucial role in the transmission of nervous impulses both within the brain and from the brain to other parts of the body. The opening and closing of ion channels generates electrochemical gradients across the plasma membranes of neurons. A nerve impulse is constituted by a wave of transient depolarization/ re-polarization of membranes which traverses the nerve cell, and is designated as an action potential. As was shown by Hodgkin and Huxley (1953) a microelectrode implanted into an axon (the long process emanating from the body of a nerve cell) will record an action potential (**Figure 1.1a**). This results from a rapid and transient increase in Na^+ permeability followed by a more prolonged increase in K^+ permeability (**Figure 1.1b**). The opening and closing of ion channels across cellular membranes creates the electrochemical gradients (action potentials) across these membranes, which transmits information and also regulates cellular function. The determination of the X-ray structure of the K^+ ion channel has allowed us to understand how it selectively filters completely dehydrated K^+ ions, but not the smaller Na^+ ions. Not only does this molecular filter select the ions to be transported, but the

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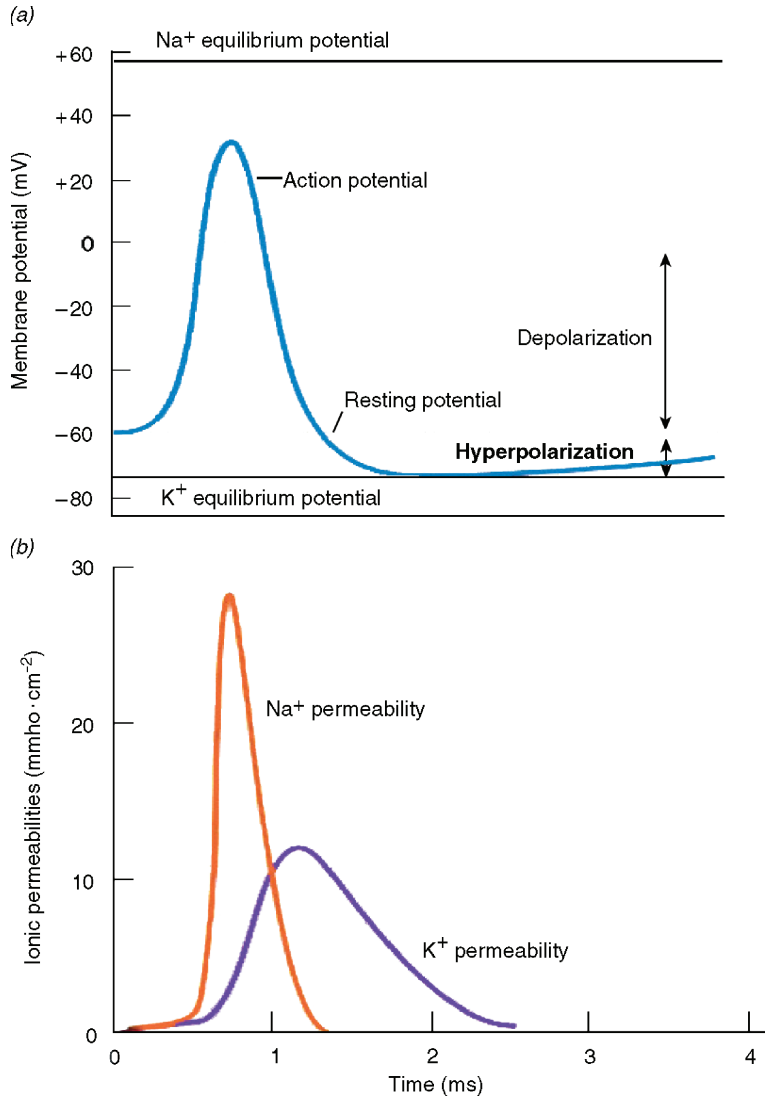


Figure 1.1: The time course of an action potential. (**a**) The axon membrane undergoes rapid depolarization followed by a nearly as rapid hyperpolarization and then a slow recovery to its resting potential. (**b**) The depolarization is caused by a transient increase in Na^+ permeability (conductance), whereas the hyperpolarization results from a more prolonged increase in K^+ permeability that begins a fraction of a millisecond later. From Voet and Voet, 1995.

electrostatic repulsion between K^+ ions, which pass through this molecular filter in Indian file provides the force to drive the K^+ ions rapidly through the channel at a rate of 10^7 to 10^8 per second.

Within cells, including nerve cells, fluxes of Ca^{2+} ions play an important role in signal transduction*. When intracellular Ca^{2+} increases, the ubiquitous eukaryotic Ca^{2+}-binding

* For a superb and entertaining review of calcium signalling see Carafoli, 2002.

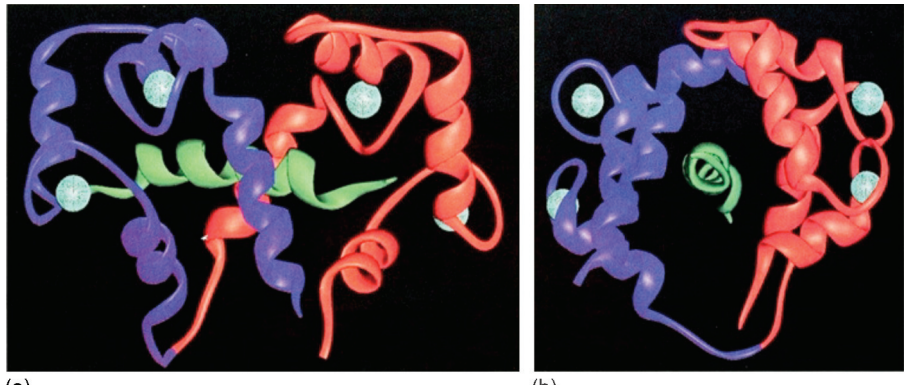


Figure 1.2: Ribbon diagrams showing the NMR structure of $(Ca^{2+})_4$-calmodulin from the fruit fly *Drosophila melanogaster* in complex with its 26-residue target polypeptide from rabbit sketelal muscle myosin light chain kinase. The N-terminal domain of calmodulin is on the left, the C-terminal domain on the right, the target peptide in the middle and the Ca^{2+} ions represented as spheres. (**a**) A view of the complex with the N-terminus of the target peptide on the right. (**b**) A perpendicular view as seen from the right side of (a). From Voet and Voet, 1995.

protein calmodulin binds Ca^{2+} ions, thereby exposing a previously buried hydrophobic patch, which in its turn can bind to a large number of target enzymes and modify their activity. The structure of the complex between $(Ca^{2+})_4$-calmodulin and a target polypeptide is shown in **Figure 1.2**. Evidence has been presented for a role for Ca^{2+}-dependent translocation of calmodulin in neuronal nuclei in both rapid signalling and memory formation (Mermelstein et al., 2001). A target protein for calmodulin in mammalian brain is calcineurin, a heterodimeric phosphatase, which has both a calmodulin binding domain in its catalytic subunit and a subunit which binds Ca^{2+} (Olson and Williams, 2000). Calcineurin seems to be involved in some way in synaptic plasticity (Arambura et al., 2001). It may be involved in an oestrogen-dependent pathway in the hippocampus, the memory and learning centre of the brain (Sharrow et al., 2002), which could have implications in hormone replacement therapy for improving memory. Another family of Ca^{2+}-binding proteins, called synaptotagmins, have been localized on the membranes of synaptic vesicles (Sudhoff, 2002), where they seem to be involved in the release of neurotransmitters (Augustine, 2001; Koh and Bellen, 2003). While the mechanism by which synaptotagmins are involved in Ca^{2+}-mediated synaptic transmission remains unclear, it appears likely that the neurotoxicity of heavy metals such as Pb may be due to a higher affinity of synaptotagmins for Pb^{2+} than for Ca^{2+} (Godwin, 2001).

Highly enriched in brain tissue, and present throughout the body, the Ca^{2+}/calmodulin-dependent protein kinase CaMKII plays a central role in Ca^{2+} signal transduction (Hudmon and Schulman, 2002). CaMKII accounts for about 2% of total hippocampal protein and around 0.25% of total brain protein (Erunda and Kennedy, 1985), and is the most abundant protein in the postsynaptic density. This is a region of the postsynaptic membrane which is physically connected to the ion channels which mediate synaptic transmission. The structural modification of synaptic proteins is thought to be the molecular event which is involved in the memory storage process. The unique biochemical properties of CaMKII have made it one of the paradigms of the long sought after '**memory molecule**'. Binding of Ca^{2+}/calmodulin to CaMKII allows autophosphorylation together with complex changes in

CaMKII's sensitivity to Ca^{2+}/calmodulin, which confer a kind of molecular memory to its autoregulation and activity (Hudmon and Schulman, 2002; Cammarota et al., 2002). In addition, the central role of CaMKII as a sensor of Ca^{2+} signals generated by activation of NMDA receptors after the induction of long-term plastic changes, has underlined its role in synaptic plasticity (Cammarota et al., 2002).

Another metal ion that has been implicated in brain function is Zn^{2+}. Around 10% of total brain zinc is found distributed thoughout the CNS in synaptic vesicles of glutamatergic neurones, although the function of vesicular zinc is poorly understood. It has been suggested from microdialysis studies that zinc enhances GABA release via potentiation of α-amino-3-hydroxy-5-methyl-4-isoxaloleproprionate (AMPA)/kainate receptors in the CA3 region of the hippocampus, followed by a decrease in presynaptic glutamate release in the same region (Takeda et al., 2004). In both the CA1 and CA3 regions of the hippocampus, zinc seems to be an inhibitory neuromodulator of glutamate release (Takeda et al., 2003, 2004). When hippocampal fibres are stimulated, large amounts of Zn are co-released with glutamate, and it has been suggested that this synaptic pool of Zn functions as a neuromodulator. However, *Znt-3*-null mice exhibit no characteristic phenotype, which would indicate either that Zn does not play an important role, or that the lack of vesicular Zn is compensated in another way.

We have, clearly not by accident, left until the last in this overview the redox metal ions iron, copper, manganese. Metalloenzymes containing these metals play extremely important roles in a number of key metabolic pathways within nervous tissues, They are, for example, involved in neurotransmitter synthesis (the Fe enzyme, tyrosine hydroxylase in the formation of dopa from tyrosine, the Cu enzyme dopamine β-hydroxylase which transforms dopamine to nor-adrenaline) and in neuroprotection (the Cu/Zn superoxide dismutase in cytosol and the Mn superoxide dismutase in mitochondria). However, as we will see in later chapters, the presence of any of these redox-active metals in excess within localized regions of the CNS frequently spells disaster, with associated neurodegeneration.

1.2 METAL ION TRANSPORT AND STORAGE – IRON

Iron is normally transported in plasma and other extracellular fluids bound to transferrin, a bilobal protein which can bind two Fe^{3+} ions, in octahedral coordination, with four protein ligands and a bidentate carbonate anion (Crichton, 2001). It is taken up by cells via the transferrin-to cell cycle (**Figure 1.3**), which begins with the binding of the holo(diferric)-transferrin molecule (HOLO-TF) to transferrin receptors at the cell surface. The complexes localize to clathrin-coated pits, which pinch off from the membrane to form coated vesicles, initiating the process of endocytosis. After budding is complete, the clathrin coat is removed, and smooth-surfaced endosomes are formed. The pH of the endosome is acidified by the action of an ATP-dependent proton pump. At the acidic pH, the holotransferrin undergoes a conformational change, releasing iron from transferrin as Fe^{3+}, presumably accompanied by protonation of the bound carbonate. Acidification facilitates proton-coupled transport of iron transport out of the endosomes through the action of the divalent metal transporter, DMT1, a member of the SLC11 family of H^+-coupled metal ion transporters (McKenzie and Hediger, 2004). However, the ferric reductase, which is assumed to reduce the iron, prior to its transport out of the endosome by DMT1, has not yet been identified. Apotransferrin (APO-TF) bound to its receptor, returns to the plasma membrane, where, at neutral pH, the complex dissociates. The two proteins can then participate in further

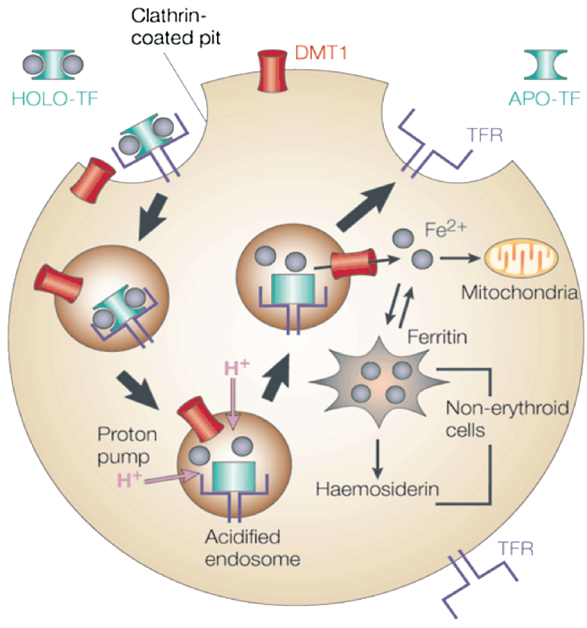


Figure 1.3: The transferrin cycle. Holotransferrin (HOLO-TF) binds to transferrin receptors (TFR) at the cell surface. The complexes localize to clathrin-coated pits, which invaginate to initiate endocytosis. Specialized endosomes form, and are acidified by a proton pump. At the acidic pH, iron is released from transferrin and is co-transported with protons out of the endosomes by the divalent metal ion transporter DCT1. Apotransferrin (APO-TF) bound to TFR is returned to the cell membrane, where, at neutral pH they dissociate to participate in further rounds of iron delivery. In non-erythroid cells, iron is stored as ferritin and haemosiderin. From Zecca et al., 2004. Reproduced by permission of Nature Reviews Neuroscience.

rounds of iron delivery. This transferrin to cell cycle ensures iron uptake by cells that have transferrin receptors.

The Iron Regulatory Protein 1, IREG1, (also known as ferroportin) is thought to be involved in iron export at the plasma membrane in macrophages: mutations in this protein are associated with iron accumulation within macrophages. IREG1 is also implicated as the iron transporter at the basolateral membrane of gastrointestinal enterocytes.

During evolution, mammalian species appear to have developed extensive mechanisms for iron uptake into parenchymal cells but have not developed corresponding pathways for its removal. This is in marked contrast to reticuloendothelial cells which can both take up iron and release it by utilization of IREG1.

Within the cell, the 'labile iron pool' (LIP) in the cytoplasm is used for the synthesis of haem and Fe-S cluster iron-containing proteins within the mitochondria, or is stored in the cytosol as ferritin. When large amounts of iron accumulate, haemosiderin is formed, due to the breakdown of ferritin within lysosomes. Haemosiderin was in fact the first iron-containing protein to be detected histologically by the intense Prussian blue reaction that it gave with potassium ferrocyanide by Perls (1867), a detection technique that is still used today. Haemosiderin is present in tissues when iron concentration increases 10 to 20 fold, e.g. liver and spleen, and has been characterized by a variety of biophysical and electron microscopic techniques, e.g. Mossbauer, EXAFS (Ward et al., 1994). However brain haemosiderin has not been characterized, and may not actually occur, since L-ferritin, its

precursor, is not necessarily increased in many of the neurodegenerative diseases, as we shall discuss. Ferritin is a hollow protein shell made up of 24-subunits of two types, namely H (heavy) and L (light) within which substantial amounts of iron (up to 4,500 atoms/molecule) can be stored in a soluble, non-toxic yet bio-available form. In mammals this iron core is relatively well ordered and corresponds to the mineral phase ferrihydrite (Crichton, 2001). The two ferritin subunits have different functions and are encoded on different chromosomes. The H subunit catalyses the oxidation of Fe^{2+} to Fe^{3+} at di-iron sites known as ferroxidase centres. Indeed, H-chain ferritins are members of a superfamily of (μ-carboxylato)diiron proteins, which include methane monooxygenase, ribonucleotide reductase, stearoyl-acyl carrier protein desaturase and hemerythrin. In contrast, the L subunit contains a site within the protein shell to which iron from the ferroxidase site migrates, which allows nucleation of the ferrihydrite core.

In mitochondria, iron is utilized by ferrochelatase for haem synthesis, and for the assembly of Fe-S clusters. It has become apparent recently that the mitochondrial protein frataxin may function as a kind of iron chaperone which delivers iron both to ferrochelatase (Lesuisse et al., 2003; He et al., 2004) and to the scaffold proteins which assemble the Fe-S clusters (Muhlenhoff et al., 2002). Indeed, as we will see later in Chapter 5, defective production of frataxin is the cause of Friedreich's ataxia, and results in massive mitochondrial iron accumulation.

The brain is unique among all the organs of the body, hidden behind a relatively poorly permeable vascular barrier, which limits its access to plasma nutrients, such as iron (**Figure 1.4**). Iron transport into the brain mostly involves the transferrin to cell cycle, using

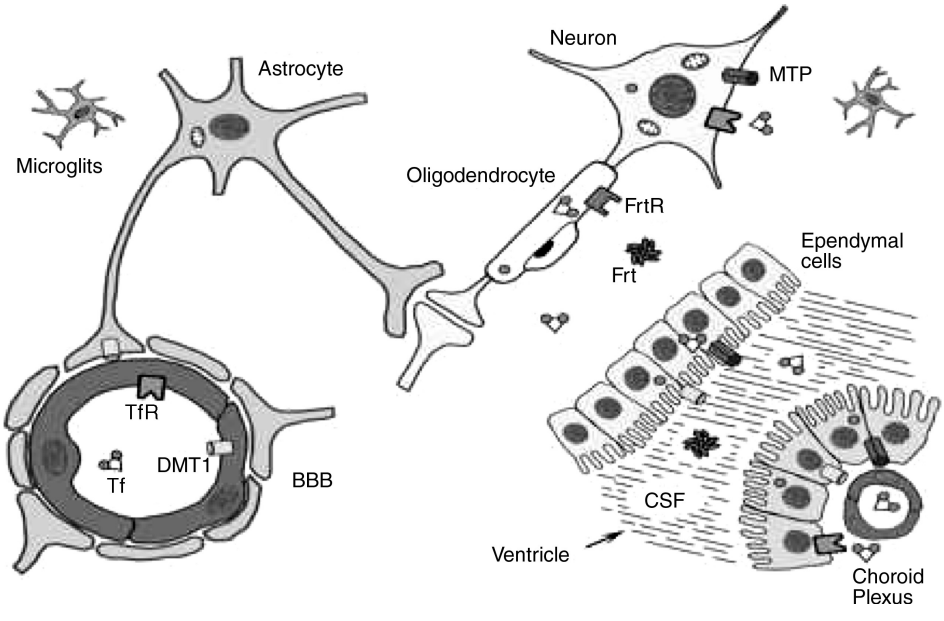


Figure 1.4: Diagrammatic portrayal of the blood-brain barrier and the iron transport proteins thought to be involved in iron movement into brain. DMT1, divalent metal transporter; Tf, transferrin; TfR, transferrin receptor; Frt, ferritin; FrtR, ferritin receptor; MTP, metal transport protein (or IREG1, or ferroportin) BBB Blood-Brain Barrier. From Beard, 2003. Reproduced by permission of the American Society for Nutritional Sciences.

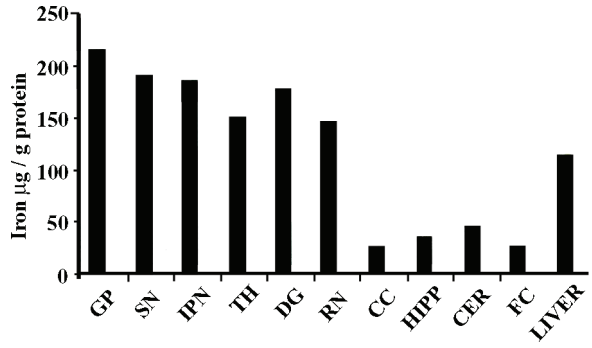


Figure 1.5: Distribution of iron in human brain. GP, globus pallidus; SN, substantia nigra; IPN, interpeduncular nucleus; TH, thalamus; DG, dentate gyrus; RN red nucleus; CC, cerebral cortex; HIPP, hippocampus; CER, cerebellum; FC, frontal cortex. From Götz et al., 2004.

transferrin receptors within epithelial cells lining the blood-brain barrier, although the precise mechanism of iron transfer is still uncertain (Connor, 2003). Iron content within the brain varies greatly from one region to another. Significantly greater iron concentrations, as high as 150–200 µg/g protein, are found in the substantia nigra and the globus palidus than in liver (Götz et al., 2004), and other brain regions with high concentrations are the dentate gyrus, interpeduncular nucleus, thalamus, ventral pallidus, nucleus basilis and red nucleus (**Figure 1.5**). Regions of the brain associated with motor functions tend to have more iron than non-motor related regions (Koeppen, 1995). This may explain why movement disorders are often associated with iron loading. In early studies (Connor et al., 1994) the iron in oligodendrocytes was reported to be bound to both H- and L-chain ferritin, in microglia to L-ferritin, while neurons had mostly neuromelanin with a little H-ferritin, and astrocytes contained hardly any ferritin. Since H-ferritin and L-ferritin have different functions their specific locations might indicate specific biological roles, one predominantly involved with iron storage (L-ferritin) and the other associated with responses to stress as well as catalysing the oxidation of Fe^{2+} to Fe^{3+} via the ferroxidase centre (H-ferritin). Large amounts of iron are sequestered in substantia nigra and in locus coerulus in neuromelanin, particularly in dopaminergic neurons (Zecca et al., 2003). Transferrin is thought to transport iron within the brain, although there is some evidence to suggest that other transport pathways may also be present, involving NTBI, non-transferrin-bound iron (Qian and Shen, 2001). The latter may include various forms of iron, as ferritin, and/or low-molecular weight iron. There appear to be at least two receptors in the brain for iron uptake, namely transferrin receptors, expressed exclusively in grey matter, and ferritin receptors present only in white matter (Hulet et al., 1999).

Evidence has been recently found for the presence of DMT1 and IREG1 in brain cells (Siddappa et al., 2002; Jiang et al., 2002), suggesting that they play an active role in both iron uptake and iron export pathways within the CNS.

1.3 METAL ION TRANSPORT AND STORAGE – COPPER

Ceruloplasmin contains 95% of the copper found in serum (Harris and Gitlin, 1996), and was assumed for many years to be the principal copper transport protein, although this

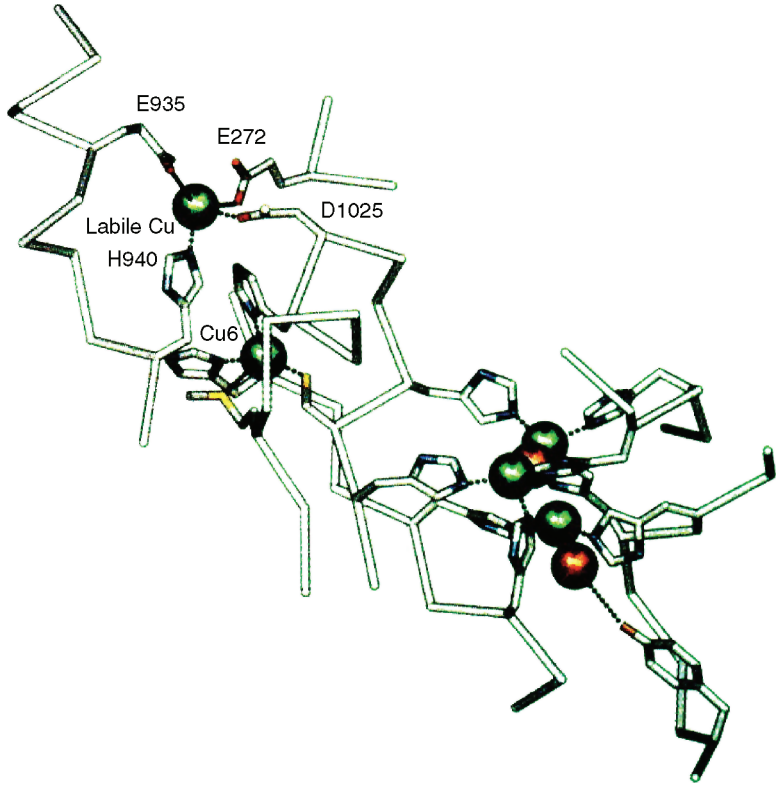


Figure 1.6: The putative metal binding site in the vicinity of the domain 6 copper atom (Cu_6) of human ceruloplasmin. The trinuclear copper cluster is located at the interface between domains 1 and 6 (bottom right of figure, copper atoms in green, water molecules in red). The green sphere at the top left of the figure represents a labile metal ion binding site (copper or iron), surrounded by His 940 and three negatively charged residues, E272 from domain 1 and E935 and D1025 from domain 6: the distance from the mononuclear copper centre is about 0.75 nm (centre of figure). The negatively charged residues Asp 1025, Glu 272 and Glu 935 could bind to a ferrous ion prior to its oxidation to the ferric state and the transfer of an electron via His 940 and the mononuclear copper towards the trinuclear centre. Adapted from Lindley (1996); figure kindly provided by Peter Lindley.

now seems improbable (see below). There are six atoms of Cu in ceruloplasmin, three mononuclear centres and one trinuclear centre (Zaitseva et al., 1996). The latter, together with the closest mononuclear site, is organized in a similar manner to that in other multi-copper oxidases (**Figure 1.6**), like ascorbate oxidase (Messerschmidt et al., 1992), suggesting that ceruloplasmin could also have oxidase activity. There is a labile metal binding site close to the mononuclear site, which could be a site for binding and oxidation of Fe^{2+} to Fe^{3+}. Ceruloplasmin has long been thought to be a ferroxidase (Osaki and Johnson, 1969), and it has been proposed (Lindley, 1996) that ceruloplasmin has a custodial role *in vivo*, ensuring that Fe^{2+} released from cells is oxidized to the potentially less toxic Fe^{3+} prior to its incorporation into apotransferrin. The neurodegenerative disease aceruloplasminaemia (Chapter 9) is associated with the absence of Cu-ceruloplasmin due to the presence of inherited mutations within the ceruloplasmin gene. This condition results in disruption of iron homeostasis, with extensive iron accumulation in a number of tissues such as brain and

liver. However, in these patients, as in aceruloplasminaemic mice (Meyer et al., 2001), both copper transport and metabolism are normal, providing strong evidence against the role of ceruloplasmin as a major copper transporter (for a review see Nittis and Gitlin, 2002). It is most likely that copper is transported in serum bound to albumin.

Much of what we know about many of the component proteins which play a role in copper metabolism has been gleaned from studies in the baker's yeast, *Saccharomyces cerevisiae* (De Freitas et al., 2003) and from our increased understanding of the molecular basis of Cu-related genetic diseases (Shim and Harris, 2003). Mutations in one of two genes encoding Cu transporting P-type ATPases are responsible for Menkes and Wilson diseases in humans (Di Donato and Sarkar, 1997; Schaeffer and Gitlin, 1999). Menkes patients have a defect in intestinal Cu absorption, accumulate Cu in intestinal cells, and have Cu deficiency reflected in defects in the activity of Cu-containing enzymes. In Wilson's disease, Cu accumulates in the liver, resulting in liver cirrhosis and subsequent 'copper leakage' from the liver results in Cu deposition in the brain, provoking neurodegeneration. Characteristic Kayser-Fleischer rings arise in a similar manner from Cu deposition in the cornea of the eye.

Cu is imported across the plasma membrane of mammalian cells by a transport system that certainly requires the product of the Ctr1 gene (**Figure 1.7**), which was first identified in yeast as one of a number of high affinity Cu transporters. Yeast cells lacking high-affinity Cu transporters have defective Cu and Fe uptake, which can be restored by complementation of yeast Ctr mutants by the human Ctr1 gene (Zhou and Gitschier, 1997). Mammalian Ctr1 mRNA is expressed in all tissues examined with higher levels in liver and kidney and lower levels in brain and spleen. Ctr1 is a 190 amino acid residue protein predicted to have three

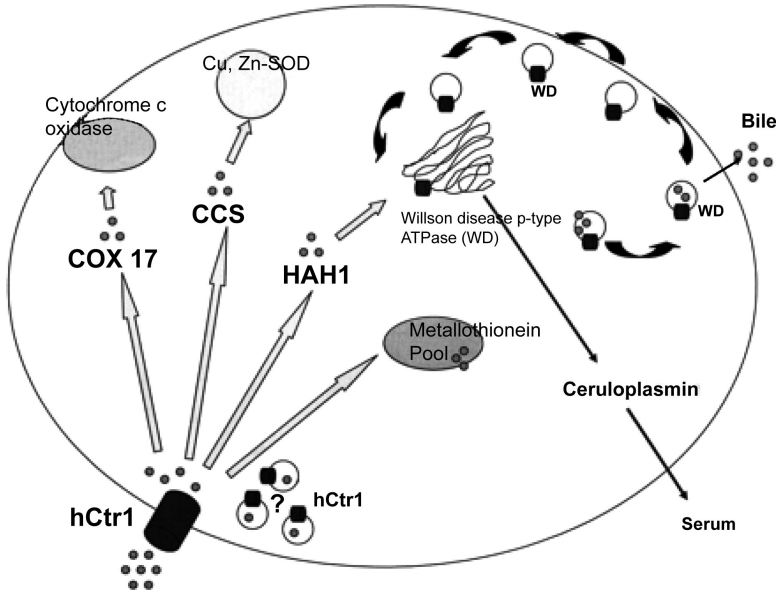


Figure 1.7: Model of copper trafficking in a hepatocyte; Cu, bound either to albumin or histidine, enters the cell via the copper transport protein Ctr1, which can be localized to the plasma membrane and to an intracellular vesicular compartment. Once inside the hepatocyte, Cu can be incorporated into the Cu/metallothionein pool, transfer to the mitochondria bound to COX17, to the cyosol bound to CCS, transfer to the Golgi compartment bound to HAH1. COX17, CCS and HAH1 are all members of the copper chaperone family. Reproduced with permission from Shim and Harris, 2003. Copyright (2003) American Society for Nutritional Sciences.

transmembrane-spanning domains (Lee et al., 2002), which can be localized to the plasma membrane and, in certain cell lines, to an intracellular vesicular compartment (Klomp et al., 2002). The role of mouse Ctr1 protein has been studied using Ctr1 gene knock-out mice (Kuo et al., 2001; Lee et al., 2001). Homozygotes die *in utero*, while heterozygotes are indistinguishable from wild-type littermates, but have a severely reduced brain Cu content. This underlines the crucial role for Ctr1 for Cu uptake, notably in brain, as well as for Cu homeostasis and embryonic development. An additional human gene, hCTR2, similar to hCTR1 and like it located in chromosome 9q31/32 has been identified in a data-base search (Zhou and Gitschier, 1997), but its role in Cu transport remains unknown. However, it shows greater homology to the yeast Ctr2 protein, which is located in the vacuole, suggesting that its role in human cells may be the release of Cu from internal compartments (Petris, 2004).

Once inside the cell (**Figure 1.7**) copper can suffer one of four possible fates: (i) it can directly enter the Cu-metallothionein storage pool, (ii) it can be transported to the mitochondria for incorporation into the terminal oxidase of the respiratory chain, cytochrome c oxidase, (iii) it can be incorporated into cytoplasmic Cu/Zn superoxide dismutase or (iv) it can be transported to the Wilson disease P-type ATPase in the *trans*-Golgi network for subsequent incorporation into the multicopper oxidase of serum, ceruloplasmin. The last three of these processes require the Cu to be transported to the target acceptor protein by specific copper chaperones (for a review see Huffman and O'Halloran, 2001). Cox17 transports copper to its site of incorporation into cytochrome c oxidase in the mitochondrion, CCS to superoxide dismutase in the cytosol, and HAH1 to the P-type ATPase and to the key enzyme in neurotransmitter synthesis, tyrosinase, in the secretory pathway (Petris, 2004). The copper chaperones are a ubiquitous family of proteins which have been identified in species from bacteria to man. They have very similar protein folds, an open-faced β-sandwich consisting of four β-strands forming an antiparallel β-sheet, situated below two α-helices, illustrated for CopZp in **Figure 1.8**. The metal-binding sequence motif –CxxxC–, occurs on the mobile loop between the first β-strand and the first α-helix. Like the Cu transporter Ctr1, they are adapted to bind Cu^{+} rather than Cu^{2+}. Since Cu is transported in extracellular fluids bound to albumin or to histidine (most likely as Cu^{2+}), the question which remains is whether Cu is transported across the plasma membrane in mammals in this form by the divalent cation transporter DMT1, or whether a reductase system coupled to the Ctr1 gene product, is involved. It also remains to be established whether the transport of Cu across the intestinal tract and within brain cells may also involve the divalent metal ion transporter DMT1 or a reductase associated with an apical membrane Ctr1 protein.

Metallothioneins are ubiquitous, low molecular weight, thiol-rich proteins which have a selective capacity to bind metal ions, such as zinc, cadmium, mercury and copper. Mammalian metallothioneins are made up of 4 major isoforms designated MT-1 to MT-4. MT-1 and MT-2 are expressed in most tissues, including brain while MT-3 is predominantly expressed in the CNS, and is thought to be involved in neuromodulatory events (Hidalgo et al., 2001).

1.4 METAL ION TRANSPORT AND STORAGE – ZINC

Zinc is a ubiquitous metal ion, which plays an important role both in catalysis and in the stabilization of the structure of many proteins, including a number of so-called zinc-finger

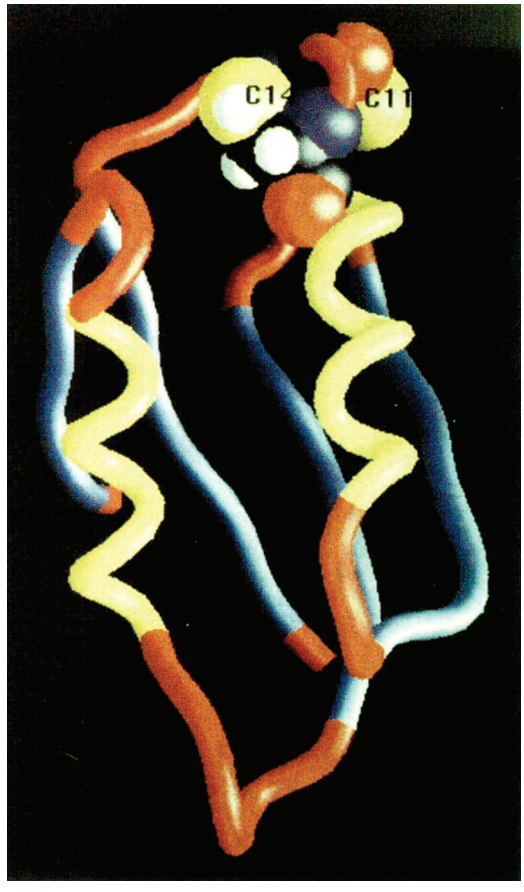


Figure 1.8: NMR structure of the reduced copper chaperone CopZ from *Enterococcus hirae*. Reprinted from Harrison et al., Copyright 2000, with permission fron Elsevier.

proteins, many of which bind to DNA promoter sites. Several mammalian Zn transporters have been identified recently (for recent reviews see Eide, 2004; Palmiter and Huang, 2004), and the number of candidate genes is growing rapidly. They can be classified into two families. The first, the SLC39 transporters (Eide, 2004), are found across the entire phylogenetic spectrum and are members of the ZIP family of metal ion transporters. This designates Zrt-, Irt-like proteins, reflecting the first members of the family to be identified, namely Zrt1 and Zrt2, the primary Zn uptake transporters in the yeast *Saccharomyces cerevisiae*, and Irt1, the major iron uptake transporter into the roots of the plant *Arabadopsis thaliana*. There are more than 90 members of the ZIP family (Gaither and Eide, 2001) which transport metal ions into the cytosol, mostly across the plasma membrane, although some, like Zrt3 in yeast, transport Zn out of intracellular compartments (the lysosome-like storage vacuole). The human genome encodes 14 SLC39-related proteins, of which so far only a few (Zip1, Zip2, Zip4) have been shown to be involved in Zn transport (**Figure 1.9**). Zip1 is widely distributed in mammalian tissues, localizes to plasma membrane and endoplasmic reticulum and seems to be regulated by Zn status. Zip2 is expressed at low levels and only in a few tissues, which however include cervical epithelium and optic nerve: this expression,

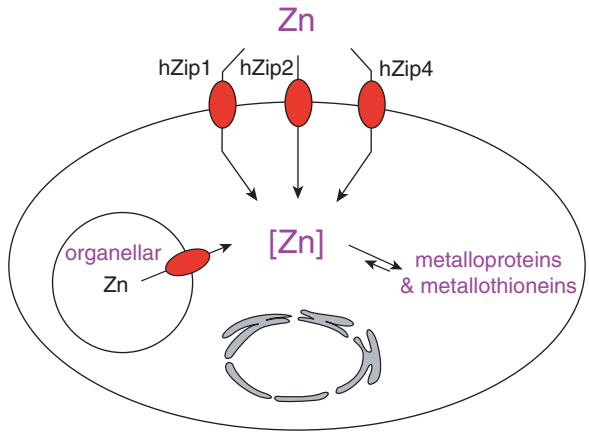


Figure 1.9: Roles of SLC39 proteins in metal metabolism. Available evidence indicates that hZip1, hZip2 and hZip4 are responsible for Zn uptake in cells where they are expressed. Based on their similarity to yeast ZIP proteins, it is possible that human SLC39 proteins transport metal ions out of intracellular compartments. Some SLC39 proteins may transport other metal ions than Zn (eg. Fe, Mn). From Eide (2004). Reproduced with kind permission of Springer Science and Business Media.

like Zip1, appears to be regulated by Zn (Cao et al., 2001). Zip4 is clearly involved in the uptake of dietary Zn into intestinal enterocytes, and mutations in Zip4 have been found in patients with acrodermatitis enteropathica, a recessive disorder of Zn absorption which results in Zn deficiency.

The second, the SLC30 family of transporters (Palmiter and Huang, 2004), was previously called the CDF (cation diffusion facilitator) family and all of the members are thought to be involved in the efflux of Zn from the cytosol, either out of the cell across the plasma membrane or into intracellular compartments, like endosomes, secretory granules, synaptic vesicles, or the Golgi apparatus. Nine members of the ZnT family have been identified in mammals (**Figure 1.10**). ZnT-1 is widely expressed in tissues, including brain, and is the only member of the family to locate to the plasma membrane where it is assumed to function as a Zn efflux transporter (Palmiter and Findley, 1995). Its expression is regulated by Zn, and loss of function of mouse *Znt1* is embryonic lethal. ZnT-2 is found in the membrane of an acidic endosomal/lysosomal compartment and may play a role in zinc sequestration (Palmiter et al., 1996a). The mRNA of ZnT-3 is expressed particularly in brain and testis, although the protein is not found in testis. ZnT-3 is most abundant in the neurons of the hippocampus and the cerebral cortex, and is localized to synaptic vesicles of glutaminergic neurons which sequester Zn, suggestive that ZnT-3 transports zinc into this compartment (Palmiter et al., 1996b). Loss of mouse *Znt-3* prevents Zn accumulation in these synaptic vesicles.

It is possible that in humans Zn can also be transported across membranes by other proteins, like the SLC11 gene family of metal ion transporters which are energized by H^+ electrochemical gradients (for a review see McKenzie and Hediger, 2004). This family has two members, DMT1 and NRAMP1. DMT1 is involved in the transport of dietary iron and perhaps zinc across the brush border membrane of the intestine and the transport of iron out of the endosomal compartment in the transferrin-cell cycle described above. NRAMP1

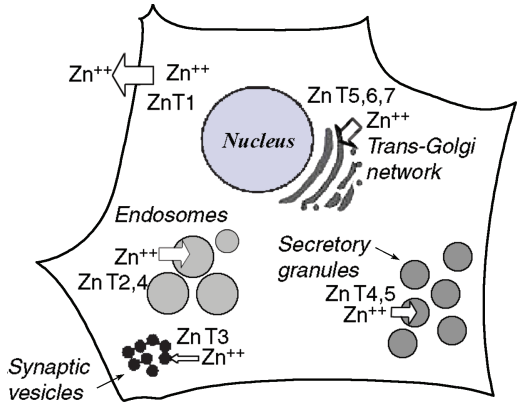


Figure 1.10: Cellular localization of SLC30 class of Zn efflux transporters. ZnT1 is targeted to the plasma membrane where it facilitates Zn efflux from cells: all the other members facilitate Zn transport into various intracellular compartments. From Palmiter and Huang (2004). Reproduced with kind permission of Springer Science and Business Media.

(natural resistance-associated macrophage protein-1) confers resistance to microbial infection, and functions at the phagolysosomal membrane of macrophages and neutrophils, either by extruding essential metal ions (including Mn^{2+} and possibly Fe^{2+}) from the phagolysosome, or alternatively, by concentrating metal ions like Fe^{2+} within the phagolysosome, resulting in the generation of toxic free radicals.

Zn metabolism itself seems to be regulated essentially through Zn-dependent control of transcription, translation and intracellular trafficking of transporters as well as its storage, like copper, in metallothionein. It is likely that Zn homeostasis in humans requires the coordinated activity of members of several Zn transporter families, particularly those involved in Zn ingress and egress from cells and organelles.

1.5 METAL ION TRANSPORT AND STORAGE – OTHER METALS

While specific transporters in animal cells for iron, copper and zinc have been described, there are other important metal ions which must also be accumulated, like manganese, a key component of mitochondrial superoxide dismutase, cobalt, with its important role as a cofactor in cobalamine (vitamin B12) enzymes, and molybdenum, present for example in xanthine oxidase. It is likely that some of the systems mentioned previously for Zn and Fe transport are also used by other divalent transition metal ions, like Mn and Co. This is most notably the case for the SLC11 and for the SLC39 gene families, which are, by definition, divalent metal ion transporters. Of the SLC11 family, DMT1, is widely expressed in human tissues, including brain, and accepts a broad range of metal ions, including Mn^{2+} and Co^{2+}, and NRAMP1 (see above), which is clearly involved in transporting Mn out of the phagolysosome in activated macrophages. Among the members of the SLC39 family, the Atx2 protein seems to transport Mn out of the Golgi apparatus in yeast (Lin and Culotta, 1996). The role of these families of proteins in the transport of Mn and Co in human cells is still not clear, while for Mo we have virtually no information.

1.6 METALS AND THEIR HOMEOSTASIS (WITH PARTICULAR REFERENCE TO IRON AND COPPER)

Cellular iron homeostasis is regulated at the level of translation of pre-existing mRNAs into protein by the ribosomal protein synthesizing machinery. These systems have been characterized both *in vitro* in cell lines as well as *in vivo* in tissues after iron loading and depletion. However, iron homeostasis in neurons, astrocytes and microglia has received little attention.

Iron Responsive Elements (IREs) are RNA-hairpin structures which are found in the 3′-untranslated region of transferrin receptor mRNA, and in the 5′-untranslated region of ferritin subunits (**Figure 1.11**). The regulation of the translation of these mRNAs involves cytosolic IRE-binding proteins, called Iron Responsive Proteins (IRP), which in conditions of iron deficency bind to the IREs, permitting synthesis of transferrin receptor, and preventing ferritin synthesis. In iron overload, the IRPs do not bind to the mRNAs, and as a consequence, ferritin is synthesized and transferrin receptor mRNA is degraded (Crichton, 1991). This ensures that when iron is in short supply, transferrin receptors are expressed allowing the cell to take up iron from circulating transferrin, while blocking synthesis of the un-required storage protein. When the cellular levels of iron are adequate, the potentially toxic iron excedent in the LIP can be stored in ferritin, while in the absence of IRP protection, the transferrin receptor mRNA is subjected to nuclease digestion, and destroyed.

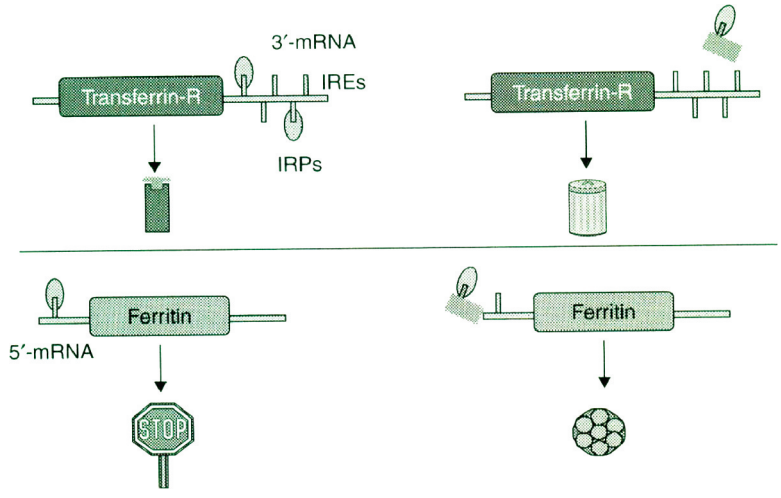


Figure 1.11: Production of transferrin receptor and of ferritin are regulated at the level of mRNA. Iron Responsive Elements (IREs) in mRNA of the transferrin receptor (TfR) are localized in the 3′mRNA region, and in mRNA of ferritin in the 5′mRNA promotor region. The system is designed to allow the cells to procure iron from the plasma, by expressing more transferrin receptors, if they need iron for production of proteins, and to protect cells against potentially toxic iron by expressing ferritin molecules, able to hide Fe(III) within its core. The system is regulated by Iron Responsive Protein 1 (IRP). In iron deficiency IRP inhibits RNase attack of TfR mRNA, and inhibits production of ferritin while sitting on an IRE in the 5′mRNA region. If the LIP contains abundant amounts of iron, modified IRP has no affinity for IRE, resulting in destruction of TfR mRNA. At the same time free IRE on ferritin mRNA allows sufficient expression of ferritin. From Crichton, 2001.

There are two closely related Iron Responsive Proteins, designated IRP-1 and IRP-2 in many mammalian cell types, which act as iron sensors by existing in two different conformations. When iron is in short supply, both IRPs can bind with high affinity to the IREs. Both IRP-1 and IRP-2 are induced in iron-deficient cells but lose their IRE-binding capacity after iron administration (**Figure 1.12**). Both IRP-1 and IRP-2 have been identified in perinatal rat brain (Siddappa et al., 2002). Immunolocalization studies show a quite distinct distribution of the two IRPs in mouse brain (Liebold et al., 2001), which may be indicative of distinct roles for IRP-1 and IRP-2 in the regulation of iron homeostasis. It is suggested that IRP1 may provide a maintenance function whereas IRP-2 could participate in modulating central autonomic network functions, including cardiopulmonary, gustatory and fine motor control. Targeted deletion of IRP-2 in mice leads to misregulation of iron metabolism in the intestinal mucosa and the CNS, which leads to neurodegenerative disease associated with a movement disorder in adulthood, characterized by ataxia, bradykinesia and tremor (La Vaute et al., 2001). Iron accumulates in the cytosol of neurons and oligodendrocytes in distinctive brain regions. Abnormal accumulations of ferritin colocalize with iron accumulations in populations of neurons that degenerate, and iron-loaded oligodendrocytes

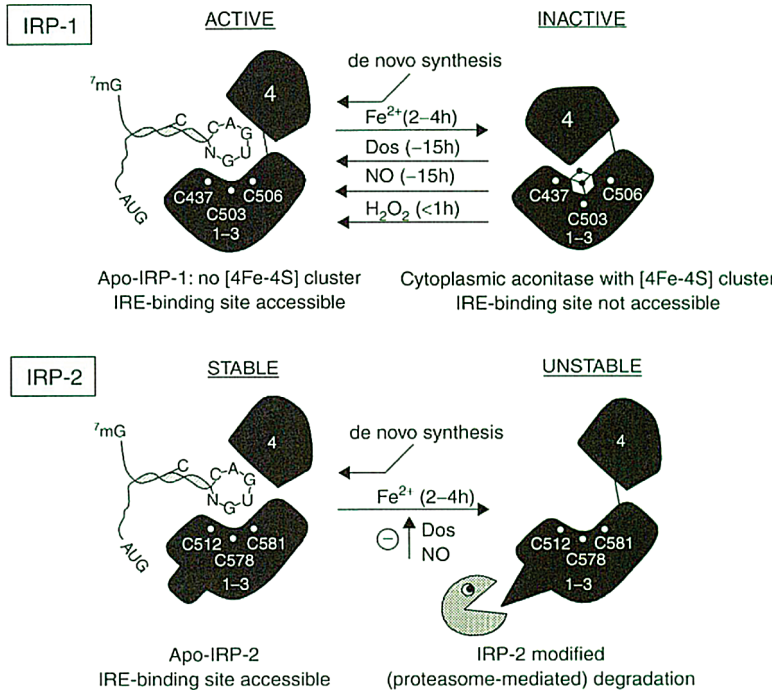


Figure 1.12: Regulation of IRP-1 and IRP-2. The two IRPs are shown as homologous four domain proteins that bind to IREs (*Left*) In iron-replete cells, IRP-1 assembles a cubane Fe-S cluster that is liganded via cysteines 437, 503 and 506. Similar cysteines are conserved in IRP-2 (Cys 512, 578 and 581), but it is unresolved whether they also coordinate an Fe-S cluster. (*Right*) In iron-replete cells, IRP-2 is targeted for destruction via a specific region (shaded in black), whereas IRP-1, with a 4Fe-4S cluster, is stable and active as a cytoplasmic aconitase. Multiple signals induce IRE-binding by IRP-1 with distinct kinetics. Whether or not NO and H_2O_2 induce IRP-1 by apoprotein formation remains to be addressed directly. From Hentze and Kühn, Copyright (1996) National Academy of Sciences, USA.

accumulate ubiquitin-positive inclusions. Mice which are homozygous for targeted deletion of IRP-2 and in addition heterozygous for targeted deletion of IRP-1 develop a much more severe form of neurodegeneration with extensive axonopathy in white matter tracts associated with marked increases in ferritin and ferric iron, and eventually vacuolization, notably in the substantia nigra (Smith et al., 2004). Axonal degeneration is apparent before evidence for abnormalities or loss of neuronal cell bodies can be detected. At a later stage, neuronal cell bodies degenerate in the substantia nigra and other vulnerable areas, microglia are activated and vacuoles appear. Gait and motor impairment are seen when axonopathy is pronounced but loss of neuronal cell bodies is minimal. A high resolution magnetic resonance imaging (MRI) study has been carried out on mice which are homozygous for targeted deletion of IRP-2 (Grabill et al., 2003) and a distinctive MRI pattern was found, reflecting both ferritin accumulation in areas known to accumulate ferritin iron in this animal model, but also increased water content in several areas of the brain, including the substantia nigra and superior colliculus. Subsequent histological examination showed the presence of relatively small, previously unappreciated vacuoles in these brain regions. If this high resolution technique can be applied to human subjects, it could represent a very significant advance in both the diagnosis and therapeutic monitoring of human neurological diseases. Iron homeostasis in neurons, astrocytes and microglia has received little attention. Whether these cells behave like parenchymal or RE cells is unknown. Studies of an *in vitro* iron-loaded immortalized microglial cell line, showed similar results to that of the inflammatory processes identified in the iron-loaded macrophage, i.e. a reduction in iNOS (Ward and Ledeque, unpublished results).

IRP-1 is expressed in all vertebrate tissues, whereas IRP-2 is less abundant than IRP-1 in most cells, but is highly expressed in intestine and brain. IRP-1 is a monomeric 889 amino acid protein with an amino acid sequence which shows extensive homology to mitochondrial and bacterial aconitases. These 4Fe-4S cluster enzymes convert citrate to isocitrate, and IRP-1 has three of the cysteine residues (Cys 437, 503 and 506) which anchor the iron-sulphur cluster into the mitochondrial and bacterial enzymes (Hirling et al., 1994). Their mutagenesis to Ser converts IRP-1 to a form which constitutively binds to IREs. When iron supply to cells is increased, the IRE binding capacity of IRP-1 is lost, by incorporation of the 4Fe-4S cluster (**Figure 1.12**).

Human IRP-2 shares 57% homology with human IRP-1, but is slightly larger than IRP-1, due to the presence of a 73-residue inclusion. IRP-2 has no aconitase activity, although three cysteine residues similar to those that coordinate the iron-sulphur cluster in IRP-1 are conserved (Cys 512, 578 and 581). IRP-2 binds to IREs like IRP-1, however, in iron-replete cells, IRP-2 is degraded (**Figure 1.12**). This degradation is mediated through the inclusion domain (Iwai et al., 1995), and is prevented by inhibitors which block proteosome formation, but not by inhibitors of lysosomal proteases or calpain (Iwai et al., 1995; Guo et al., 1995). It seems likely that the proteosome (and also ubiquitinylation) is involved in IRP-2 degradation. IRP-2 is sensitive to iron status and can compensate for loss of IRP-1 by increasing its binding activity in animals. Therefore IRP-2 dominates post-transcriptional regulation of iron metabolism.

Since this mode of protein degradation will be relevant for later Chapters on the formation of aggregated protein inclusion bodies in many neurodegenerative diseases, we present here a brief summary of the operation of this important protein degradation mechanism. Eukaryotic cells have two systems for protein degradation, lysosomal mechanisms and ATP-dependent cytosol localized mechanisms. The lysosomal compartment fuses with autophagic vesicles, consisting of membrane-enclosed bits of cytosol, and the

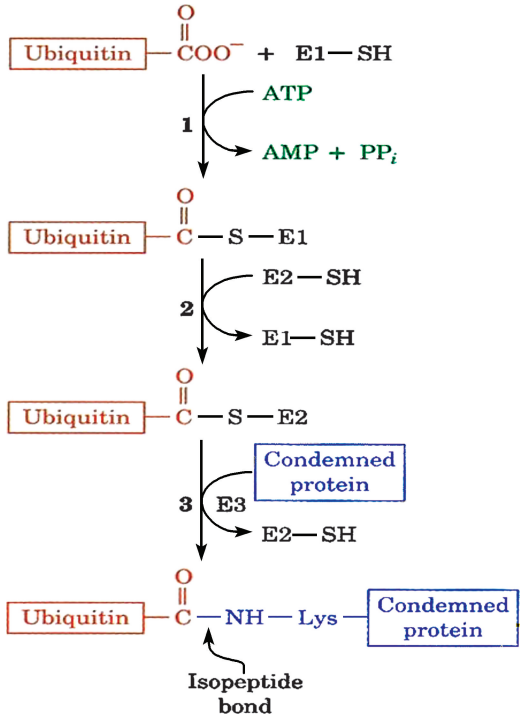


Figure 1.13: The reactions involved in the attachment of ubiquitin to a protein. In the first part the carboxyl group of ubiquitin is coupled to E_1 by a thioether linkage in a reaction driven by ATP hydrolysis. The activated ubiquitin is subsequently transferred to a sulphydryl group of E_2 and then in a reaction catalysed by E_3 to the ε-amino group of a Lys residue on a condemned protein, thereby flagging the protein for proteolytic degradation by the 26S proteosome. From Voet and Voet, 1995.

contents are degraded to their basic building blocks which are recycled in a non-selective manner.

Reticulocytes, which do not contain lysosomes, nonetheless degrade abnormal proteins. This observation led to the discovery of the ATP-dependent ubiquitin system for protein degradation, which also involves the 26S proteosome (**Figure 1.13**). Proteins that have been marked out for degradation are identified by covalent coupling to **ubiquitin**, a 76-residue protein which is both ubiquitous and abundant in eukarotes. Ubiquitin is very highly conserved–identical in human, fish, frogs and fruit fly (*Drosophila*) and with only 3 residues different between human and baker's yeast. The attachment of the ubiquitin tag to the condemned protein involves three steps, as described in **Figure 1.13.** In the first step, ubiquitin is bound by its carboxyl terminus to **ubiquitin-activating enzyme**, $\boldsymbol{E_1}$ in an ATP-dependent process. Most organisms, including humans, have only one $\boldsymbol{E_1}$. Ubiquitin is then transferred to the thiol group of one of a number of **ubiquitin-conjugating enzymes**, $\boldsymbol{E_2}$s, of which there are over 20 in mammals. Then, the **ubiquitin-protein ligase** ($\boldsymbol{E_3}$) transfers the ubiquitin from E_2 to the amino group of a Lys residue of its target protein, forming an isopeptide bond. Mammalian cells contain a large number of $\boldsymbol{E_3}$s, each mediating the ubiquitination of a specific set of proteins. Ubiquinated proteins are subsequently proteolytically degraded by the large (2000kD) multisubunit protein complex known as the 26S

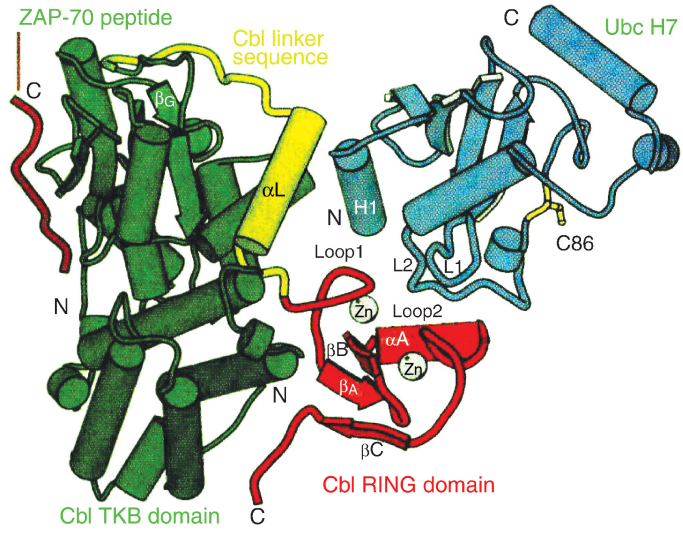


Figure 1.14: The structure of the ternary complex, consisting of an E_2 ubiquitin-conjugating enzyme, an E_3 RING finger ubiquitin ligase and the target protein's ubiquitination domain. From Voet and Voet, 2004.

proteosome. In this process, the ubiquitin molecules are not degraded, but are returned to the cell for reutilization.

An important family of ***E₃*** ubiquitin-protein ligases are members of the so-called **RING finger** (RING for *R*eally *I*nteresting *N*ew *G*ene) protein family. They all contain a RING finger, a 40- to 60-residue motif which binds two Zn^{2+} ions in a characteristic consensus sequence (like the zinc-finger motif in certain DNA-binding proteins). The RING finger interacts with both the ubiquitin-conjugating E_2 and with the domain of the condemned protein containing the Lys residue to be ubiquinated (**Figure 1.14**). A number of post-transcriptional modifications in condemned proteins have been described, including phosphorylation, proline hydroxylation and glycosylation as recognition signals for E_3. We might point out here that **parkin**, the product of a gene linked to Parkinson's disease, is a RING finger ***E₃*** ubiquitin-protein ligase (Shimura et al., 2000). Recently (Yamanaka et al., 2003) the RING finger protein **HOIL-1** has been shown to act as an E_3 ligase for oxidized IRP-2, suggesting that oxidation is the specific recognition signal for ubiquination. In iron-rich cells oxidation of IRP-2 is generated by haem and by oxygen, which might indicate that iron sensing by IRP-2 depends on haem synthesis and availability.

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