

Part I
Biochemical Studies

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The Role of Copper Ion and the Ubiquitin System in Neurodegenerative Disorders

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1.1 Introduction

Ubiquitin (Ub) plays a crucial role in intracellular protein degradation via the proteasome and the autophagy–lysosome pathways [1]. Failure to eliminate misfolded proteins can lead to the formation of toxic aggregates and cell death [2]. Insoluble protein aggregates enriched with Ub are a hallmark of most neurodegenerative disorders including Parkinson's, Alzheimer's, amyotrophic lateral sclerosis and prion diseases [3, 4]. All of these disorders have been linked to metal accumulation and disturbance of redox and metal homeostasis in the brain [5–7], and metal ions have been implicated in the aggregation of disease-related, amyloidogenic proteins [8–12]. The potential role of metal ions in the aggregation of Ub has recently been examined [13, 14]. Cu^{II} is different from Zn^{II} , Ni^{II} , Al^{III} , or Cd^{II} in that it binds to the N-terminal end of Ub, destabilizes the protein, and promotes its oligomerization into spherical particles. By mimicking the condition of low dielectric constant experienced near a membrane surface, the assembly of spherical oligomers of Ub yields a series of intermediate species leading to an extended nonfibrillar filament network. Aggregate disassembly is triggered by Cu^{II} chelation or reduction [14]. Intermediate annular and porelike structures, stabilized by the interaction of Cu^{II} -induced Ub oligomers with lipid bilayers, resemble toxic protofibrillar species produced by amyloidogenic proteins, which cause membrane permeabilization and disruption of metal homeostasis [15–19]. Susceptibility to aggregation of Ub represents a potential risk factor for disease onset or progression while cells attempt to tag and process toxic substrates. Cu^{II} binding and proximity to biological membranes appear to dramatically increase the aggregation propensity of Ub and other disease-related proteins, thus emphasizing the importance of preserving cellular compartmentalization and metal homeostasis for the correct functioning of protein degradation systems. Recent findings reinforce the vision of metal ions as key factors and promising therapeutic targets in protein conformational disorders [20, 21]. New strategies are being developed that will help to investigate their functional and pathogenic interactions *in vivo*.

1.2

Metal Ions in the Brain

The brain is a specialized organ that controls cognitive and motor functions. To carry out its functions the brain requires the highest concentrations of metal ions in the body and the highest per-weight consumption of body oxygen [22].

Metal ions in the brain fulfill catalytic and structural roles, which include the stabilization of biomolecules (e.g., Mg^{II} in nucleic acids, Zn^{II} in Zn-finger transcription factors) or dynamic processes (e.g., Na^I and K^I in ion channels, Ca^{II} in neuronal cell signaling) [23].

The dynamic partitioning of these metal ions is controlled by ion-specific channels that selectively allow passage of ions in and out of cells. In the brain, the uneven distribution of Na^I and K^I ions across a cell membrane creates a potential that enables transmission of nervous pulses. Ca^{II} is also a key modulator of molecular information transfer within and between cells during neurotransmission; most eukaryotic cells either export or store Ca^{II} within membrane-enclosed vesicles to maintain cytosolic-free Ca^{II} levels at 100–200 nM, roughly 10 000-fold less than in the extracellular space [23].

More recently, considerable attention has been directed to the role of transition metal ions in the brain [22, 24]. Zn, Fe, Cu, and related d-block metals are emerging as significant players in both neurophysiology and neuropathology, particularly with regard to aging and neurodegenerative diseases [25]. Relatively high concentrations of these d-block metals are present within the different cellular compartments, the values ranging from 100 to 1000 μM [22]. The metal concentrations in brain tissue are up to 10 000-fold higher than those in common neurotransmitters and neuropeptides. Not only do these metals serve as components of various proteins and enzymes essential for normal brain function, but, in the labile form, are also involved in specialized brain activities; therefore, if misregulation of their homeostasis occurs, toxicity, mediated also by oxidative stress in the case of Cu and Fe [26], could ensue. Oxidative stress has been identified in many neurodegenerative diseases, and is commonly associated with increased levels of at least one of these transition metal ions in specific brain regions [27].

Transporters for Cu, Zn, Fe, and Mn play an important part in the intracellular distribution of these metals [28], such that defects in their regulation, which could possibly occur with aging, may create an environment that could result in protein misfolding and aggregation, thereby accelerating degenerative conditions [2, 29, 30]. Notably, brain homeostasis of metals is intertwined with changes in one metal leading to changes in the levels of other metals [24]. This is well established for Cu and Fe, where decreased Cu bioavailability may result in altered Fe levels, and for Fe and Mn, where Fe deficiency leads to a significant increase in brain Mn.

The following discussion will be focused on basic aspects of brain Cu homeostasis. The widespread distribution and mobility of Cu required for normal brain function, along with the numerous correlations between Cu misregulation and a variety of neurodegenerative diseases, have prompted interest in studying its roles in neurophysiology and neuropathology [26, 31].

1.3 Brain Copper Homeostasis

Copper is the third-most abundant transition metal in the brain, after Fe and Zn, with average neuronal Cu concentrations of ~ 0.1 mM. This redox-active nutrient is distributed unevenly within brain tissue, as Cu levels in the gray matter are two- to threefold higher than those in the white matter. Cu is particularly abundant in the *locus coeruleus* (1.3 mM), the neural region responsible for physiological responses to stress and panic, as well as the *substantia nigra* (0.4 mM), the center for dopamine production in the brain [32]. The major oxidation states for Cu ions in biological systems are cuprous Cu^I and cupric Cu^{II}; the former is more common in the reducing intracellular environment, and the latter is dominant in the more oxidizing extracellular environment [33]. Levels of extracellular Cu^{II} vary from 10–25 μ M in blood serum, 0.5–2.5 μ M in cerebrospinal fluid (CSF), and 30 μ M in the synaptic cleft. Intracellular Cu levels within neurons can reach concentrations higher than 2–3 orders of magnitude [32]. Like Zn and Fe, brain Cu is partitioned into tightly bound and labile pools. Owing to its redox activity, Cu is an essential cofactor in numerous enzymes that handle the chemistry of oxygen or its metabolites, including cytochrome *c* oxidase (CcO), Cu, Zn superoxide dismutase (Cu,Zn-SOD1), ceruloplasmin (CP), dopamine β monoxygenase (D β M), peptidylglycine α -hydroxylating monoxygenase (PHM), and tyrosinase [31].

Because of its propensity to trigger aberrant redox chemistry and oxidative stress when unregulated, the brain maintains strict control over its Cu levels and distributions. An overview of homeostatic Cu pathways in the brain is given in Figure 1.1.

Many of the fundamental concepts for neuronal Cu homeostasis are derived from studies in yeast, but the brain provides a more complex system with its own unique and largely unexplored inorganic physiology. There is little “free” Cu in the yeast cytoplasm, which is due to the tight regulation of metallochaperones [35, 36]; however, many open questions remain concerning the homeostasis of organelle Cu stores, particularly in higher organisms with specialized tissues. Some data suggest that yeast and mammals possess pools of labile Cu in the mitochondrial matrix [37].

Uptake of Cu by the blood–brain barrier (BBB) is considered to occur through the P-type ATPase ATP7A, which can pump Cu into the brain [38]. Mutations in the related gene lead to Menkes disease, an inherited neurodegenerative disorder that is globally characterized by brain Cu deficiency. This phenotype is mirrored by Wilson disease, which involves mutations in the *ATP7B* gene responsible for excretion of excess Cu from the liver into the bile. Loss of ATP7B function leads to abnormal increase of Cu in the liver [39].

The extracellular trafficking of brain Cu differs from that in the rest of the body. CSF, the extracellular medium of the brain and central nervous system, possesses a distinct Cu homeostasis from blood plasma, which carries Cu to organs in the rest of the body. Cp, a multicopper oxidase that is essential for Fe metabolism, is

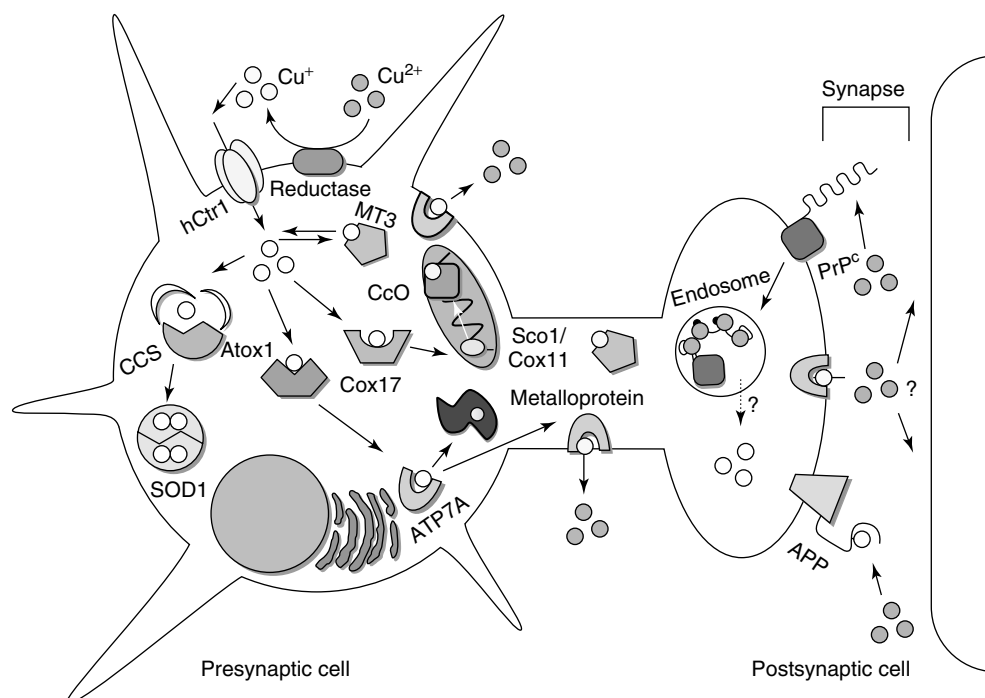


Figure 1.1 A schematic model of neuronal copper homeostasis. Reprinted with permission from [34]. Copyright 2008 American Chemical Society.

the major carrier of Cu^{II} in the plasma, but houses less than 1% of Cu in CSF [32]. The primary protein or small-molecule ligands for Cu in CSF remain unidentified. Uptake of Cu into brain cells requires reduction of Cu^{II} to Cu^{I} . Steap proteins may fulfill this role like the yeast ferric and cupric reductases Fre1 and Fre2 [40]. Following reduction, Cu^{I} ions can be transported into cells through a variety of trafficking pathways [41, 42].

A major class of proteins involved in cellular Cu uptake is the copper transport (Ctr) protein family. Ctr1 is a representative member that is ubiquitously expressed. It resides predominantly in the plasma membrane and is essential for the survival of mammalian embryos and for Cu import into neurons and astrocytes [43]. Elevated Cu stimulates rapid endocytosis and degradation of Ctr [44]. Ctr1 contains three transmembrane helices, an N-terminal extracellular domain, and a C-terminal cytosolic domain. Electron crystallography revealed that Ctr1 is trimeric and possesses the type of radial symmetry associated with the structure of certain ion channels [45]. A region of low protein density at the center of the trimer is consistent with the existence of a Cu permeable pore. Mutagenesis studies have established that a methionine(Met)-rich motif in the N-terminal domain and a Met-rich motif at the extracellular end of the second transmembrane helix of Ctr1 play a pivotal

role in the mechanism of Cu uptake [46, 47]. The mechanisms of Cu translocation across cellular membranes, however, remain largely unknown.

In addition to Ctr1, prion protein (PrP) and amyloid precursor protein (APP) are two other abundant Cu-binding proteins, found *specifically* at brain cell surfaces, implicated in Cu uptake/efflux [48, 49]. In particular, PrP is localized in synaptic membranes of presynaptic neurons. Mammalian PrP contains at least four octapeptide repeats in the N-terminal region that can bind Cu^{II}. Millimolar concentrations of Cu^{II} induce endocytosis of PrP, suggesting that PrP may act as a buffer for Cu in the synaptic cleft, maintaining presynaptic Cu concentrations while preventing Cu^{II}-related toxicity in the extracellular space [49, 50].

Upon its entry into brain cells, Cu^I can be funneled to its ultimate intracellular destinations through the use of Cu chaperone proteins or buffering by metallothioneins (MTs), such as MT1 (ubiquitously expressed) and MT3 (expressed in the brain) [51]. The metallochaperones function not only as intracellular Cu delivery proteins but also as protective agents against toxicity resulting from unbound and unregulated Cu ions [35, 36].

Three human Cu chaperones have been characterized so far: Atox1, CCS, and Cox17. Atox1 loads Cu^I into the Menkes and Wilson P-type ATPases, ATP7A and ATP7B, which mediate Cu delivery to the secretory pathway from the trans-Golgi network (TGN) to the plasma membrane [41]. Both Atox1 and ATP7A/B contain CXXC sequence motifs that are essential for Cu^I binding and exchange of Cu^I between the two partner proteins [52]. The combination of available structural and biochemical data suggests a docking model that involves Cu^I transfer through two- and three-coordinate intermediates [53–56].

ATP7A and ATP7B play multiple roles in neurons from the delivery of Cu to cuproenzymes involved in neurotransmitter synthesis and metabolism, such as D β M, to the removal of excess Cu via secretion or vesicular sequestration [39]. To carry out this function, ATP7A undergoes Cu-stimulated translocation from the Golgi to the plasma membrane [57]. Metabolic studies also revealed that translocation of ATP7A after *N*-methyl *D*-aspartate (NMDA) receptor activation is associated with rapid release of Cu from hippocampal neurons [58, 59], a finding that suggests a role for Cu in the modulation of synaptic activity [60].

The copper chaperone for superoxide dismutase (CCS) inserts Cu into SOD [61]. Cu,Zn-SOD1 is a ubiquitous component of the cellular antioxidant system, which catalyzes disproportionation of the superoxide anion to oxygen and hydrogen peroxide [62]. Active Cu,Zn-SOD1 is a dimer; each subunit binds one Cu and one Zn ion, and contains an *intramolecular* disulfide bond [63]. On the contrary, in the immature, apo form of SOD1, cysteines are in the reduced state and the protein is a monomer [64, 65]. CCS docks with and transfers the Cu ion to the latter form of SOD [66], and also catalyzes disulfide bond formation [67, 68]. CCS is made of three domains: the N-terminal domain I has a fold similar to Atox1 and contains a conserved CXXC motif, domain II has a fold similar to SOD1 and participates in target recognition, domain III is constituted by ~30 residues and contains a CXC Cu-binding motif [69]. While domain III is essential for CCS activity *in vivo*, the requirement for domain I is only apparent under Cu-limiting conditions [69].

A third Cu chaperone, Cox17, is involved in Cu delivery to CcO in mitochondria [70, 71]. CcO is a 13-subunit complex embedded in the mitochondrial inner membrane and a key component of the respiratory chain that reduces oxygen to water [72]. Two Cu ions form a dicopper cluster, designated Cu_A, in a CcO subunit, while a third Cu ion, designated Cu_B, forms a dinuclear site with heme *a* in another CcO subunit [72]. Cox17 acts as Cu^I donor for Sco1 and Cox11 [73]. Sco1, in turn, transfers Cu to Cu_A [74], while Cox11 is involved in Cu_B assembly [75]. Cox17 contains two CX₉C motifs implicated in two *intramolecular* disulfide bonds [76] and a conserved CC motif essential for Cu^I binding [77]. Fully reduced Cox17 binds up to four Cu^I ions in a polycopper cluster and undergoes oligomerization [76, 78].

1.4 Brain Copper and Neurodegenerative Disorders

Disruption of Cu homeostasis is implicated in a number of neurodegenerative diseases, including Alzheimer's disease (AD), prion diseases, Parkinson's disease (PD), familial amyotrophic lateral sclerosis (ALS), Menkes disease, and Wilson disease [5]. In all these disorders, the deleterious effects of Cu stem from its dual abilities to bind ligands and trigger uncontrolled redox chemistry. A dominant risk factor associated with most neurodegenerative diseases is increasing age. A positive correlation with chronic occupational exposure to Cu and other metal ions in industrialized countries has also been recognized [79, 80].

Several studies have reported a rise in the levels of brain Cu from youth to adulthood [32]. However, biologically *available* Cu levels drop markedly with advanced age and in AD brain [20, 81]. The connection between Cu and AD pathology is due mainly to its reactions with APP and its β -amyloid cleavage product ($A\beta$), that result in imbalance of extracellular and intracellular brain Cu pools [20]. Aberrant binding of Cu^{II} to APP triggers its reduction to Cu^I with concomitant disulfide bond formation; this intermediate can then participate in reactive oxygen species (ROS) production [82]. Extracellular $A\beta$ deposits from AD brains (amyloid plaques) are rich in Cu, in addition to Zn and Fe [83]. The MT3, released in the synaptic cleft by neighboring astrocytes, has the potential to ameliorate this adverse interaction, but is downregulated in AD [84]. Moreover, the β -secretase β -site of amyloid precursor protein cleaving enzyme (BACE1), involved in APP cleavage, possesses a Cu^I-binding site in its C-terminal cytosolic domain through which it interacts with domain I of CCS, indicating that intracellular Cu levels can have an impact on $A\beta$ generation [85]. Altered brain Cu distribution in AD, with abnormal accumulation of Cu in amyloid plaques and Cu deficiency in neighboring cells, is accompanied by a loss of Cu-dependent enzymes (e.g., CcO, Cu,Zn-SOD1, CP). Therefore, administration of Cu chelators such as clioquinol, that can reverse $A\beta$ aggregation and redistribute brain Cu pools acting as ionophores, can have dual beneficial effects [20]. It is also found that Cu-bound clioquinol and other Cu complexes can exhibit proteasome-inhibitory abilities [86, 87].

Prion diseases are also linked to brain Cu misregulation, where opposing Cu^{II} and Mn^{II} levels may influence the conversion of PrP into the toxic, protease-resistant form, PrP^{Sc} [88, 89]. PrP may act as a Cu-chelating agent, when extracellular Cu reaches high concentration peaks (15–300 μM) such as during synaptic transmission and depolarization [8]. Another hypothesis is that the binding of Cu to PrP could act directly to detoxify ROS, performing SOD-like activity [90]. In one proposal for prion toxicity, excess free Cu further exacerbates the disease by promoting oxidative stress [91].

Onset of PD is accompanied by death of dopaminergic neurons and intracellular accumulation of Lewy bodies [92], which are protein aggregates containing α -synuclein (α -syn), an abundant protein in the brain whose function is still unclear [93]. Monomeric α -syn, which has no persistent structure in aqueous solution, is known to bind anionic lipids [94] with a resulting increase in α -helix structure [95, 96]. Factors including oxidative stress and presence of various metal ions promote its fibrillation [97, 98]. Cu^{II} also promotes the self-oligomerization of α -syn [10] and its oxidation and aggregation in the presence of H₂O₂ [99]. Although α -syn is widely expressed in the brain, inclusions of α -syn are commonly localized in the *substantia nigra*, *locus coeruleus*, and cerebral cortex, which are the regions where Cu is abundant [32]. In PD brain, increased levels of Cu are found in the CSF [100].

Familial ALS is an inherited neurodegenerative disorder stemming from mutations in Cu,Zn-SOD [101, 102]. Three main hypotheses exist regarding the molecular mechanisms of this disease: (i) the *loss-of-function* mechanism, which results in toxic accumulation of superoxide by lack of SOD1 protection, (ii) the *gain-of-function* mechanism, in which SOD1 exhibits enhanced peroxidase activity by aberrant redox chemistry, and (iii) the aggregation mechanism, where SOD1 aggregates are induced by decreased availability of Cu and Zn ions and are stabilized by *intermolecular* disulfide bonds [103, 104]. The role of Cu homeostasis in this disease remains uncertain, however molecular machineries controlling redox homeostasis in mitochondria [68] appear to be essential for *intramolecular* disulfide bond formation and correct metal incorporation into SOD1, two processes involving the CCS metallochaperone [105].

1.5 The Role of Ubiquitin in Protein Degradation

The unique morphology of neurons (with specialized zones for presynaptic neurotransmitter release and postsynaptic receptor activation) and the plasticity of synapses (which is tightly coupled to changes in the synaptic proteome) impose special challenges on the cellular machinery for both protein synthesis and degradation [106]. Protein degradation has important roles in both neuronal development and long-term synaptic plasticity. Moreover, many neurodegenerative diseases are associated with abnormal protein aggregates, implicating degradative dysfunction [4, 107].

Major proteases in eukaryotic cells are confined to specialized protein complexes (proteasomes) and organelles (lysosomes) to prevent nonspecific proteolysis. The ubiquitin-proteasome system (UPS) is responsible for degrading most intracellular, soluble proteins, but it can also degrade transmembrane proteins if they are extracted from the membrane into the cytosol (Figure 1.2). The lysosome degrades most membrane and endocytosed proteins, but it can also digest cytosolic proteins through autophagy (Figure 1.3) [1]. In both cases ubiquitin (Ub) plays a crucial role.

Ub is a small protein of 76 aminoacids folded in a compact globular structure in which a mixed parallel/antiparallel β -sheet packs against an α -helix generating a hydrophobic core [108]. Not found in bacteria, this protein is ubiquitous in eukaryotes and has highly conserved sequences, the human and the yeast proteins differing by only three residues. The remarkable degree of sequence conservation underscores its important physiological role [109].

Ubiquitination is a posttranslational modification that forms an isopeptide bond between a lysine residue on the protein and the C-terminus of Ub. The ubiquitination requires four different classes of enzymes: E1–E4 [110, 111]. First, Ub is covalently conjugated to E1 (Ub-activating enzyme) in an ATP-dependent reaction, and then it is transferred to E2 (Ub-conjugating enzyme). E3 (Ub-protein ligase) then transfers Ub from E2 to the substrate protein and is largely responsible for target recognition through physical interactions with the substrate. After the first Ub has been attached (monoubiquitination), E3 can also elongate the Ub chain by creating Ub–Ub isopeptide bonds. Finally, E4 enzymes (chain elongation factors) are a subclass of E3-like enzymes that only catalyze chain extension (Figure 1.2) [110, 111].

Ub has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), all of which are available and indeed used *in vivo* for chain extension. The significance of complex ubiquitination patterns is only partially understood: K48 chains lead to degradation of the substrate by the 26S proteasome, whereas monoubiquitination and K63 chains are known to activate cell signals in several pathways including tolerance of DNA damage, inflammatory response, ribosomal protein synthesis, endocytosis, and protein trafficking [111].

The 26S proteasome is a large multisubunit complex of ~ 2 MDa, localized in the cytosol and nucleus, and composed of a 20S proteolytic core and one or two 19S regulatory caps. After substrate–proteasome association, deubiquitinating enzymes (DUBs) and ATP-dependent unfoldase activities help the substrate to enter the proteolytic lumen of the 20S core by regenerating monomeric Ub [110, 111]. Notably, the cleavage of the isopeptide bond between the substrate and the most proximal Ub of the polyUb chain requires the metalloprotease activity of a 19S proteasome subunit, which contains a JAMM (JAB1 (Jun activation domain-binding protein-1)/MPN (Mpr1 Pad1 N-terminal domain)/Mov34 metalloenzyme) domain with a coordinated Zn^{II} ion [112, 113].

Ub is also involved in the lysosomal degradation pathway. Lysosomes are organelles that contain acid hydrolases that break down biomolecules. The hydrolases in the lumen of lysosomes (pH 4–5) and late endosomes (pH 5–6) are highly active in acidic environments but lose their activities in the cytosol (pH ~ 7.2) [114]. Many types of signals can regulate endocytosis and sorting of lysosomes, including

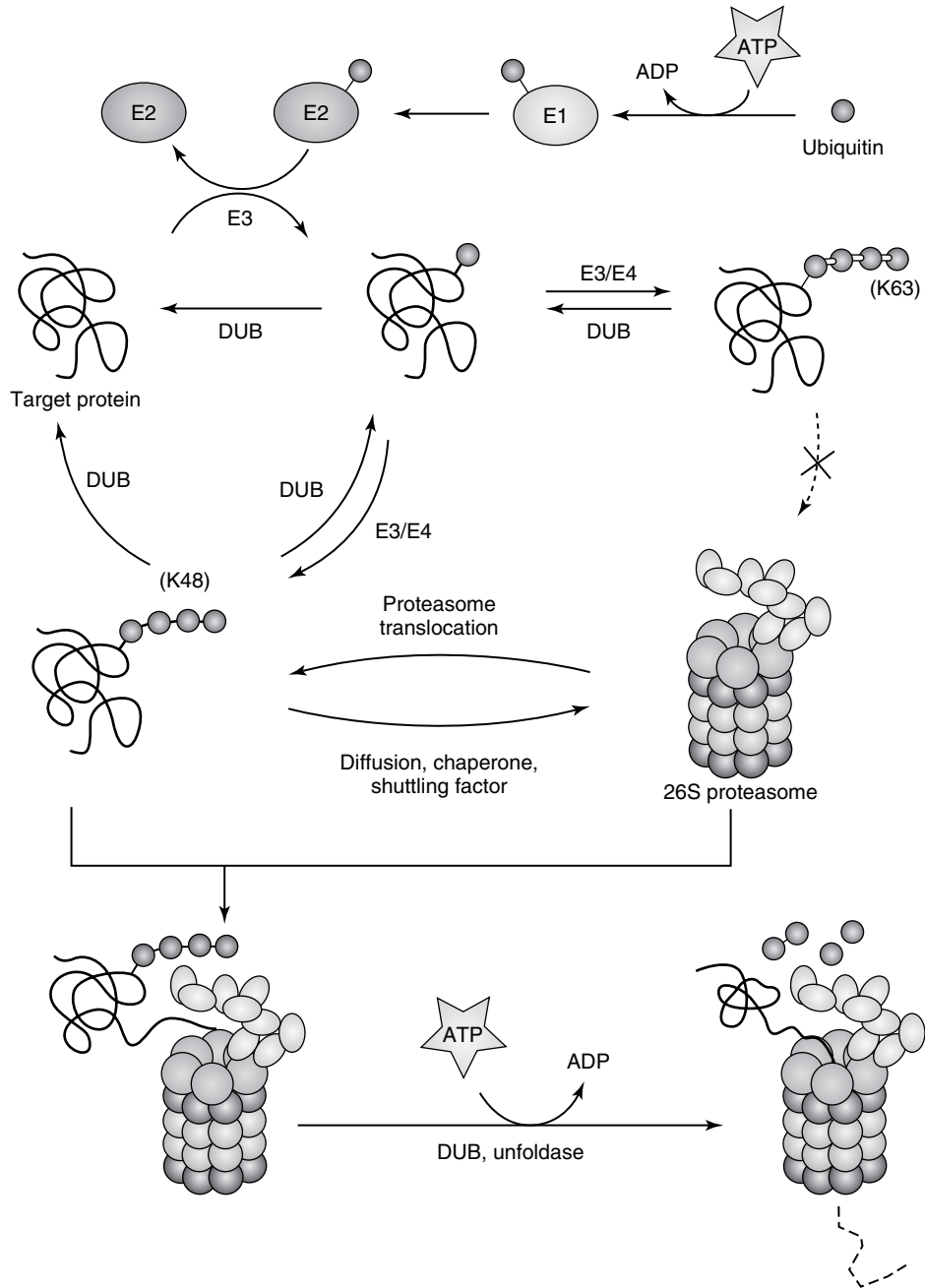


Figure 1.2 The ubiquitin-proteasome system. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Neuroscience* [1], copyright 2008.

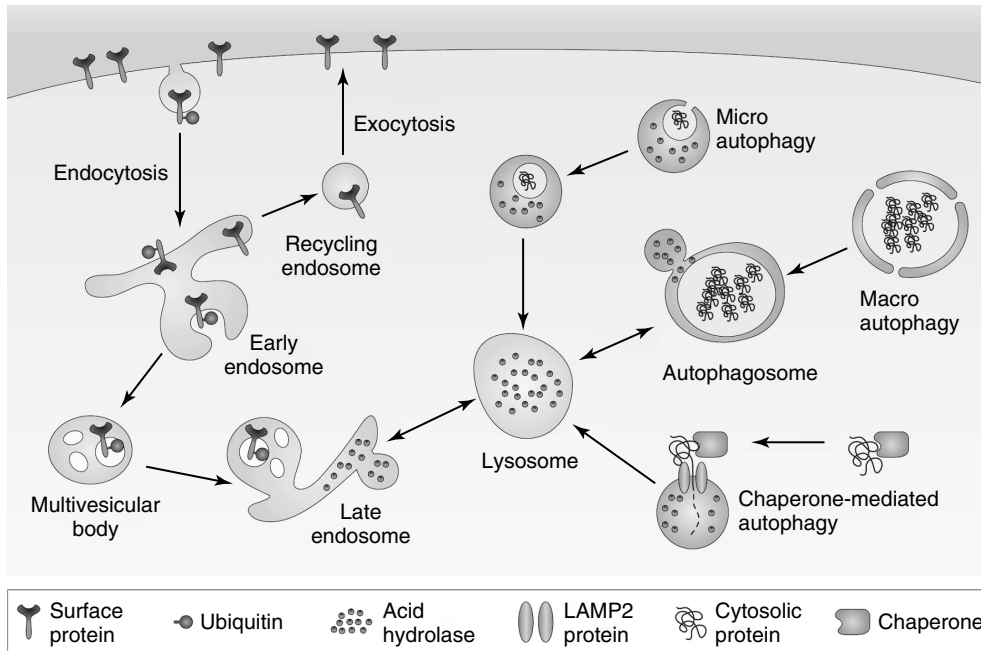


Figure 1.3 The autophagy-lysosome pathway. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Neuroscience* [1], copyright 2008.

monoubiquitination and K63-polyubiquitination [115, 116]. Intracellular proteins can enter lysosomes through several autophagic mechanisms. In macroautophagy, large amounts of cytosolic materials or even organelles are surrounded by a double-membrane structure (autophagosome) that fuses with lysosomes. In microautophagy, a small amount of the cytoplasm is internalized through lysosomal invagination (Figure 1.3) [117, 118].

Different classes of misfolded proteins partition between two separate intracellular compartments, one next to the nucleus (*juxtannuclear*) and the other near vacuoles (*perivacuolar*). Soluble ubiquitinated proteins go to the juxtannuclear compartment, whereas insoluble proteins accumulate in the perivacuolar compartment [119]. In addition to the spatial segregation, the fate of the proteins in these two compartments is divergent. Proteins in the juxtannuclear compartment are in close proximity to the 26S proteasome, whereas the perivacuolar compartment is targeted by proteins implicated in autophagy. Misfolded proteins may interact inefficiently with the normal quality control machinery and as a consequence are shunted to the perivacuolar pathways, as observed for PrP [119]. Enhancing the ubiquitination of a PrP enhances its targeting to the juxtannuclear compartment. Likewise, blocking the ubiquitination of proteins that normally go to the juxtannuclear compartment leads them to the perivacuolar compartment.

1.6

Failure of the Ubiquitin System in Neurodegenerative Disorders

Protein misfolding and the subsequent assembly of protein molecules into aggregates of various morphologies represent common mechanisms that link a number of important human disorders, known as *conformational diseases*. These disorders include numerous neurodegenerative diseases and many systemic, localized, and familial amyloidoses [29]. A hallmark of these diseases is the accumulation of insoluble protein aggregates, termed *amyloid*, within affected cells. Amyloid fibrils have a characteristic cross- β structure with a ribbon-like β -sheet that extends over the length of the fibril and is comprised of β -strands that run approximately perpendicular to the long axis of the fibril. Backbone H-bonds that link the β -strands are nearly parallel to the fibril axis [30].

Numerous recent reports suggest that the toxicity of amyloidogenic proteins lies not in the insoluble fibrils that accumulate but rather in the soluble oligomeric intermediates [16]. Early oligomers have been identified as the primary toxic species for a number of amyloid diseases and shown to be cytotoxic to cells in culture and in tissue [15, 120]. These soluble oligomers include spherical particles and curvilinear structures, called *protofibrils*, that appear to represent strings of the spherical particles [30]. An antibody specifically recognizes soluble oligomers formed by several types of amyloidogenic proteins and peptides, which indicates that they have a common structure and may share a common pathogenic mechanism [17]. Such a mechanism appears to be related to the ability of these oligomers to interact with cell membranes and to form annular protofibrils that cause membrane permeabilization [19, 121, 122]. Disruption of intracellular Ca^{II} homeostasis and ROS production are among the earliest biochemical modifications following the interaction of protofibrillar assemblies with membranes [123]. In the case of extracellular aggregates, cell damage may be accomplished via a nonspecific pathway (pores) or through oligomer interaction with specific cell surface receptors, such as glutamatergic receptors involved in Ca^{II} influx [124].

Most, if not all, protein inclusions associated with neurodegenerative diseases are enriched with Ub [4]. Lewy bodies in PD, amyloid plaques and neurofibrillary tangles in AD, skeinlike inclusions and Lewy body-like inclusions in ALS, polyglutamine inclusions in Huntington's disease (HD): all these inclusions show Ub immunoreactivity (Figure 1.4). Ub immunoreactivity is also present in vesicles in some areas of degeneration in AD [3, 125].

The frequent detection of proteasomes and lysosomes around Ub-enriched aggregates in *postmortem* brains implies that proteins within these inclusions are marked for degradation but not efficiently removed. Hence, it has been proposed that neurodegeneration might be linked to degradative dysfunction by several mechanisms, and protein aggregates might arise, at least in part, from impaired proteasomal and/or autophagic removal of the damaged proteins [4, 107].

One prevalent idea is that misfolded proteins resist degradation by, but not engagement with, the proteasome, resulting in proteasome inhibition. In this model, soluble misfolded proteins would be tagged with Ub and partly enter the

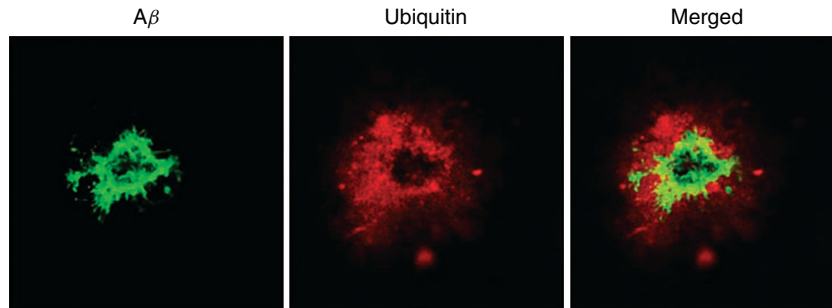


Figure 1.4 Ubiquitin-positive amyloid plaques from AD brain revealed by immunofluorescence. Reprinted by permission from Wiley-Blackwell: *Journal of Cellular and Molecular Medicine*, copyright 2008 [126].

proteasome, but because of their abnormal structures, would resist full entry and cause steric occlusion [127]. In particular, β -sheet structures, which have been implicated in aberrant conformations in several diseases, seem to be difficult for the proteasome to process when they occur at the site at which misfolding initiates. Moreover, overall proteasome activity in brain tissue decreases with age, and further loss is observed in degenerative conditions such as AD and PD [128]. Importantly, impairment of the UPS is an early response to *soluble* protein aggregates and not a consequence of inclusion formation [129].

Alternatively, the impairment of degradation might be responsible for the etiology of disease, and aggregate formation might be a secondary phenomenon [4]. The link between the Ub system and neurodegeneration has been strengthened by the identification of disease-causing mutations in genes coding for Ub [130] and several ubiquitination enzymes related to AD and PD [131].

The most compelling evidence for the involvement of the UPS in AD pathogenesis comes from a transcriptional misreading, which causes the deletion of two nucleotides in the mRNA coding for Ub [130]. The resulting *frameshift* mutant form of Ub, called UBB^{+1} , has a 19-aminoacid extension at the C-terminus and cannot bind to target proteins. However, it can be ubiquitinated by wild-type Ub. The polyubiquitinated UBB^{+1} cannot be degraded by the proteasome and probably inhibits its activity. Furthermore, it cannot be deubiquitinated [132].

One of the more common types of familial PD is caused by mutations in the gene that encodes the E3 Ub-ligase parkin [133]. Parkin is reported to possess monoubiquitination and K63-polyubiquitination activities [134]. In addition to parkin, an increasing number of E3 genes are now linked to neurodegenerative disorders [1]. Mutations in parkin cause early disease onset, with the loss of dopaminergic neurons in the *substantia nigra* in the general absence of Lewy bodies. On the other hand, the genetic association of UCHL1 (ubiquitin C-terminal hydrolase L1), a DUB, with familial and sporadic PD is controversial, but UCHL1 is found in Lewy bodies [131].

UCHL1 is a neuron-specific DUB and one of the most abundant proteins in the brain, which helps to maintain monomeric Ub levels. It possesses DUB activity in its monomeric form and ligase activity in its dimeric form. A mutation linked to familial PD promotes its dimerization and the K63-polyubiquitination of α -syn [135].

The cellular concentrations of the two forms of Ub, free Ub and polyUb chains, are closely interconnected and may change because of various cellular events; for instance heat stress induces an increase in polyUb chains at the expenses of free Ub [136]. The finding that modest reduction in the level of free Ub in brain is linked to synaptic dysfunction and neuronal degeneration associated with loss-of-function mutations in DUBs, suggests that adequate neuronal Ub supply should be maintained. Decreased Ub availability in neurons of mice is sufficient to cause neuronal dysfunction and death [137]. On the other hand, increased Ub concentrations in CSF of patients affected by neurodegenerative diseases and amyloidosis may have a neuroprotective effect [138].

The presence of Ub within inclusion bodies was noted in a large variety of neurodegenerative diseases [3, 4, 107, 125], suggesting that disruption of Ub homeostasis may be a common factor in the pathogenesis of these disorders [137].

1.7 Interaction of Ubiquitin with Metal Ions

1.7.1 Thermal Stability of Ubiquitin

Ub has been widely used as model for stability, folding, and structural studies, the protein remaining soluble and folded over a wide pH range and at high temperatures [139]. The effect of various cations on the thermal stability of Ub was assessed by differential scanning calorimetry (DSC) experiments carried out on Ub solutions of different metals (Cu^{II} , Zn^{II} , Ni^{II} , Al^{III} , and Cd^{II}). Cu^{II} , in contrast with other cations, has a specific negative effect on the thermal stability of Ub, both in terms of unfolding temperature (T_m) and enthalpy (ΔH) [13]. Far-UV circular dichroism (CD) spectra indicated very little overall change in Ub secondary structure upon Cu^{II} binding, however thermal denaturation curves revealed a decrease of T_m from 100 °C for native Ub to 90 °C for the Cu^{II} -Ub system.

1.7.2 Spectroscopic Characterization of Cu^{II} Binding

Electrospray ionization mass spectrometry (ESI-MS) indicated binding of two Cu^{II} ions to the protein with \sim fourfold different affinities. The first Cu^{II} site has an affinity constant of $\sim 10^7 \text{ M}^{-1}$, as determined from spectrophotometric measurements, and the EPR (electron paramagnetic resonance) parameters ($g_{\parallel} = 2.30$ and $A_{\parallel} = 159 \times 10^{-4} \text{ cm}^{-1}$) are consistent with a tetragonal N_1O_3 (type II)

Cu^{II} site [13]. The unpaired electron of a type II Cu^{II} site is characterized by relatively long electronic relaxation times (10^{-8} – 10^{-9} seconds); therefore, its coupling with the nuclear spins has a dramatic effect on the nuclear relaxation and consequently severely affects the NMR linewidths [140]. For most residues in the proximity of the metal center that completely escaped signal identification in ¹H-detected heteronuclear single quantum coherence (HSQC) spectra, ¹³C resonances could be recovered using tailored NMR experiments based on ¹³C direct detection [141, 142]. These latter experiments are intrinsically less affected by paramagnetism-enhanced relaxation than conventional experiments. As the paramagnetic dipolar contributions to nuclear relaxation depend on the square of the gyromagnetic ratio of the observed nucleus, going from ¹H ($\gamma_{\text{H}} = 2.67 \times 10^8$) to ¹³C ($\gamma_{\text{C}} = 6.73 \times 10^7$) detection produces a decrease in relaxation rate by a factor of ~ 16 [140, 143].

The “protonless” approach was originally applied to a Cu^{II}-binding protein involved in Cu trafficking and homeostasis in bacteria [144, 145], and subsequently extended to human Cu,Zn-SOD1 [146, 147]. In the case of Ub, conventional and ¹³C-detected experiments allowed the identification of resonances for most amino acid residues with the only exception of those directly coordinated to the Cu ion: the first Cu^{II} site is located at the N-terminus of the protein and involves the Met1 nitrogen and three oxygen donor ligands (residues 16–18) in a tetragonal arrangement; the second Cu^{II}-anchoring site involves the imidazole nitrogen of His68 [13] (Figure 1.5).

Cu^{II} has excited electronic states of relatively high energy, which produce small orbital contributions to the electronic spin moment [140, 143]. As a consequence, the magnetic susceptibility anisotropy of Cu^{II} is generally small. Nonetheless some nuclei of Ub experienced small but measurable pseudocontact shifts (PCSs) arising from dipolar coupling with the unpaired electron [13]. The size and the sign of PCS allowed to define the orientation of the magnetic susceptibility tensor χ , whose main axis is nearly orthogonal to the tetragonal plane formed by the Cu^{II} donor atoms. Such a Cu^{II} coordination environment was also supported by the presence

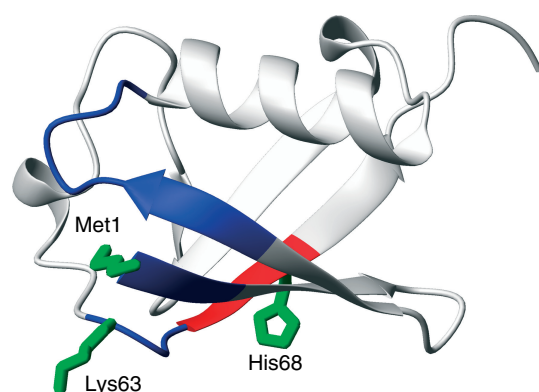


Figure 1.5 The two Cu^{II}-binding sites of ubiquitin determined by paramagnetic NMR [13].

of a weak absorption band at 680 nm in the visible spectrum, assignable to the $d-d$ electronic transition [13].

All NMR spectral changes induced by Cu^{II} on Ub are abolished upon addition of stoichiometric amounts of ascorbic acid and reduction of Cu^{II} to Cu^{I} [13, 148]. Therefore, under “normal” conditions, in which intracellular Cu is mainly Cu^{I} , Ub does not bind Cu. On the other hand, addition of the tridentate ligand iminodiacetic acid (IDA) mobilizes Cu^{II} from the first to the second affinity site, in which three coordination positions of Cu^{II} are firmly taken by the IDA ligand, and the protein only provides the fourth donor atom coincident with an easily accessible nitrogen of the His68 imidazole ring [13, 149] (Figure 1.5). Cu reduction or chelation can suppress the Cu^{II} -induced protein destabilization.

1.7.3

Possible Implications for the Polyubiquitination Process

Since residues close to the N-terminus are involved in the formation of a β -strand, Cu^{II} binding may destabilize the protein starting from its N-terminal region. Potential oxygen donor ligands for the first site are Met1 and Val17 carbonyl groups, and Glu16 and Glu18 carboxylate groups [13]. In native Ub, the first residue, Met1, is involved in two key H-bonds [108]: one occurring between the amino-terminal group and the CO of Val17 and the other between the side chain sulfur atom of Met1 and the amide NH of Lys63 (Figure 1.5). Therefore, Cu^{II} binding to Ub might hamper the proteins' turnover and other *in vivo* signaling events regulated by Lys63-polyubiquitination [111] such as the autophagic clearance of protein inclusions [150].

In the polyubiquitination process, Lys63 of the acceptor Ub attacks Gly76 (the C-terminal residue) of the donor Ub forming a Lys63–Gly76 isopeptide bond. The reaction is catalyzed by the Ub-conjugating enzyme Ubc13 and a Ub-ligase. The structural elucidation of Ub covalently bound to Ubc13 has revealed the molecular determinants of Lys63-polyubiquitination [151]. In this structure, several H-bonds help positioning the Lys63 of the acceptor Ub in the active site of Ubc13 near the C-terminus of the donor Ub [151]. On this basis, binding of Cu^{II} to the acceptor protein could compromise the correct positioning of Lys63 by affecting the electrostatic interactions at the interface between the conjugating enzyme and the acceptor Ub.

1.7.4

Cu^{II} -Induced Self-Oligomerization of Ub

Since conditions that destabilize the native state of a protein render the macromolecular system more prone to aggregation, the interaction of Ub with Cu^{II} was hypothesized to be a factor promoting Ub aggregation. Furthermore, Cu^{II} ions were found to target the most aggregation-prone regions of Ub (the N-terminus and His68), as predicted by an algorithm based on the propensity of two residues to be found paired in neighboring strands within a β -sheet [152].

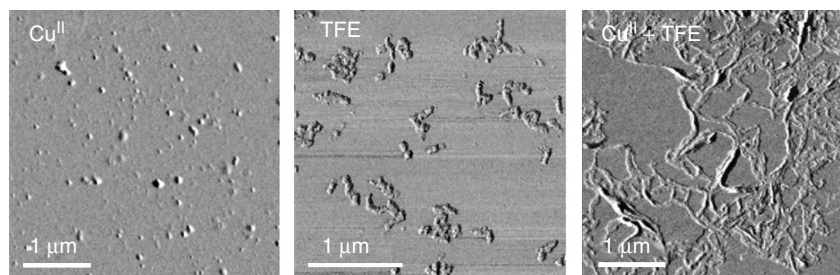


Figure 1.6 AFM images of ubiquitin aggregate morphologies upon long-term incubation with Cu^{II} and/or TFE [14].

These predictions were corroborated by an *in vitro* study of Ub aggregation [14]. It was observed that micromolar concentrations of Cu^{II} ions were sufficient to induce self-oligomerization of Ub. Ub oligomers appear as spherical particles ranging from 5 to 25 nm (Figure 1.6), that can progress from dimers to large Ub conglomerates resistant to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cu^{II} reduction by ascorbic acid or Cu^{II} chelation by ethylenediaminetetraacetic acid (EDTA) or IDA, can trigger disruption of Ub oligomers. On the other hand, when the Cu^{II} -stabilized oligomers were added to a low-polarity medium, Ub aggregation increased dramatically.

1.7.5

Cooperativity between Cu^{II} -Binding and Solvent Polarity

An aqueous solution with a moderate amount (20%, v/v) of 2,2,2-trifluoroethanol (TFE) was used to mimic the local decrease of dielectric constant in the proximity of a membrane surface [153]. In the absence of Cu^{II} , 20% TFE does not affect Ub secondary structure; the protein forms short beaded chains composed of SDS-sensitive spherical subunits of 6–8 nm (Figure 1.6). In contrast, in the mixture containing both Cu^{II} and TFE, the Ub structure shows an increase of β -sheet content, as determined by far-UV CD and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy [14]. Atomic force microscopy (AFM) indicates that the aggregation process proceeds through distinct steps characterized by clustering of spherical particles in annular species, formation of trigonal branched structures growing radially from the annular species, and interconnection of these branched structures in filament networks (Figure 1.6). Large aggregates can be disrupted by Cu^{II} reduction yielding a homogeneous population of spherical particles similar to those formed in TFE alone. The Cu^{II} -stabilized spherical oligomers of Ub form annular and porelike structures, in liposomes and planar phospholipid bilayers respectively [14] (Figure 1.7). The membrane-penetrating structures are not observed after pretreatment with EDTA, which was shown to destroy the Ub oligomers formed by incubation with Cu^{II} . The negativity to tests for amyloid confirmed that the aggregation process of Ub does not lead to fibril formation [14].

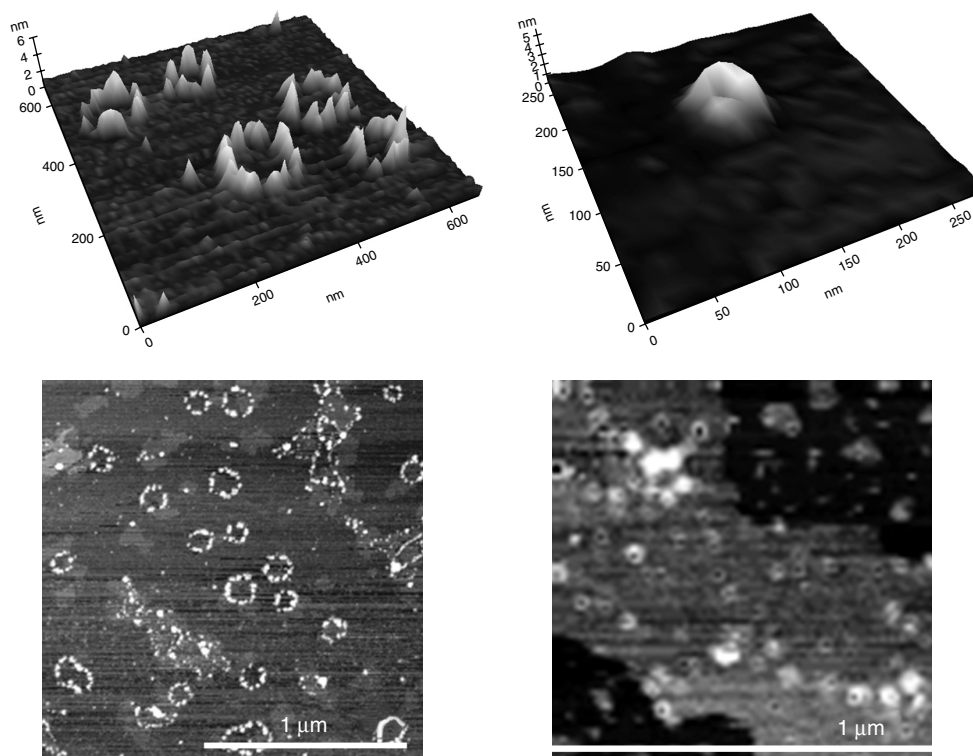


Figure 1.7 AFM images of annular and porelike structures formed by Cu^{II}-stabilized ubiquitin oligomers in phospholipid bilayers [14].

Kinetic stabilization of α -syn protofibrils, under conditions inhibiting their conversion to mature fibrils, was shown to enhance the harmful effects of aggregation and to accelerate disease progression [15, 154–156]. It was also shown that natural lipids destabilize and rapidly revert inert A β amyloid fibrils to soluble neurotoxic protofibrils [157]. Changes in the A β peptide structure promoted by metal ions appear to modify the effect of A β on lipid membranes. In particular, addition of Cu^{II} induces a greater peptide association with lipids and membrane insertion, and also results in an increased β -sheet content for the peptide [158].

1.7.6

Comparison with Other Metal Ions

Cu^{II} appears to play a unique role in Ub destabilization and aggregation. Other metal ions (Zn^{II}, Ni^{II}, Al^{III}, and Cd^{II}) do not affect Ub stability, even at very high metal : protein ratios [13]. The different behavior of various metal ions could be ascribed to differences in their coordination properties. The N-terminus of Ub is the preferred Cu^{II}-binding site in pure water as well as in water with 20%

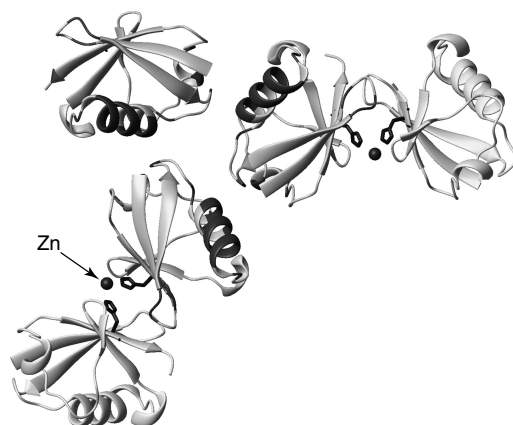


Figure 1.8 Packing of ubiquitin molecules in orthorhombic Zn^{II} -ubiquitin crystals [159].

TFE, as determined by NMR spectroscopy [14]. Despite the affinity of Cu^{II} for the imidazole nitrogen of histidine, the lack of a preorganized set of donor atoms near His68 renders the binding of Cu^{II} to this site less effective than its binding to the N-terminus, where the presence of carboxylate groups (Glu16 and Glu18) helps to accommodate the metal ion in a preorganized anchoring site. In contrast, His68 is the favorite binding site for Zn^{II} , as deduced from the available X-ray structure of the Zn^{II} -Ub adduct [159] (Figure 1.8). The Zn^{II} ion completes its tetrahedral coordination by binding two water molecules and His68 from an adjacent Ub molecule. In the solid state, the adjacent Ub molecule plays a role similar to the IDA ligand in solution experiments, that is, to provide additional donor atoms to the metal ion. All trials to crystallize Ub in the presence of Cu^{II} were unsuccessful, even when using very small amounts of metal [159]. This could be a consequence of Cu^{II} destabilizing the protein structure and hampering crystallization.

The analysis of molecular packing shows that in orthorhombic crystals of the Zn^{II} -Ub adduct, the N-terminus of one Ub molecule packs against helix $\alpha 1$ of another molecule. Each of the two Ub molecules, in turn, establishes complementary contacts with a third molecule, thus giving rise to a symmetric trimer stabilized by a series of intermolecular electrostatic interactions (H-bonds and salt bridges) (Figure 1.8). Helix $\alpha 1$ of native Ub contains a large number of unshielded backbone H-bonds, called *dehydrons* [160], which are generally correlated with membrane association [161] and aggregation propensity of a protein [162]. Mapping on the Zn-Ub structure reveals that dehydrons are clustered in the core of the trimer [159] (shown in black in Figure 1.8). Therefore, missing H-bond protection in the isolated Ub molecules is partially offset by crystal packing. Similarly, protein desolvation associated with a decrease in dielectric constant near a membrane surface is expected to foster intermolecular electrostatic interactions at the trimer interface. From this analysis it is also inferred that Cu^{II} binding at the N-terminus may enhance the aggregation propensity of these surface regions of Ub, thus shifting the equilibrium toward the formation of oligomeric species with bridging metal ions.

1.8 Biological Implications

1.8.1 The Redox State of Cellular Copper

Cu^{II} reduction to Cu^I offsets Ub destabilization and aggregation [14]. As described above, Cu is generally believed to be transported in the cells by the plasma membrane permease Ctr1 in the +1 oxidation state; it is yet unproven, however, that Cu^I is the only permeant species [163]. There is unequivocal evidence that Ctr1 is not the only protein capable of mediating Cu entry into mammalian cells, and it is quite possible that Cu^{II} rather than Cu^I is transported by Ctr1-independent mechanisms, based on divalent metal ion inhibition [164, 165]. Consistent with the reducing environment of the cytosol, X-ray absorption spectroscopy indicates the presence of low-coordinate monovalent Cu^I in this compartment [33, 166, 167]; however, Cu^{II} appears to be abundant inside both normal and PD neurons of *substantia nigra* [168]. That Cu^{II} could gain access to special intracellular districts of normal or diseased cells is also supported by the recent identification of the Fre6 vacuolar cupric reductase [169].

In neurodegenerative diseases the intravesicular material undergoes ubiquitination [3, 170]. It has been also shown that a portion of α -syn is present in the lumen of intracellular vesicles and can be secreted to the extracellular space, thus possibly contributing to disease propagation [171]. Intravesicular α -syn is more prone to aggregation than its cytosolic counterpart [171] and this could be explained by the microenvironment of the vesicular lumen and increased metal ion concentrations. Preliminary findings indicate that α -syn and Ub, the two main components of Lewy bodies, form hybrid oligomers in the presence of Cu^{II} (Arnesano *et al.*, unpublished observations). Intravesicular aggregates and Lewy bodies can then be propagated from diseased tissues to the cytosol of healthy neurons through prion-like transmission [172].

Importantly, it has been reported that extracellular aggregates including AD amyloids, can be internalized by mammalian cells, gain access to the cytosol, and colocalize with Ub and UPS components [173]. The mechanism of internalization is not clear; however, the recent discovery that PrP mediates A β -induced synaptic dysfunction [174], has led to the hypothesis that internalization of PrP may allow A β oligomers to reach intracellular compartments and interfere with proteasomal degradation [175] (Figure 1.9).

Altogether, these findings strongly indicate that Ub may come in contact, in the cytosol, with extracellular aggregates enriched with oxidized Cu^{II}. Elemental mapping of amyloid deposits of AD brain revealed “hot spots” of accumulated metal ions, particularly Cu and Zn [83, 176]. Moreover, an increment of Cu density was observed in brain tissues that were positively stained for Ub [177], and AD amyloid plaques can be dissolved by Cu^{II} chelators [178].

Therefore, the interaction of Ub with Cu^{II} could be a pathological event taking place inside intracellular organelles or in the cytosolic compartment of cells which

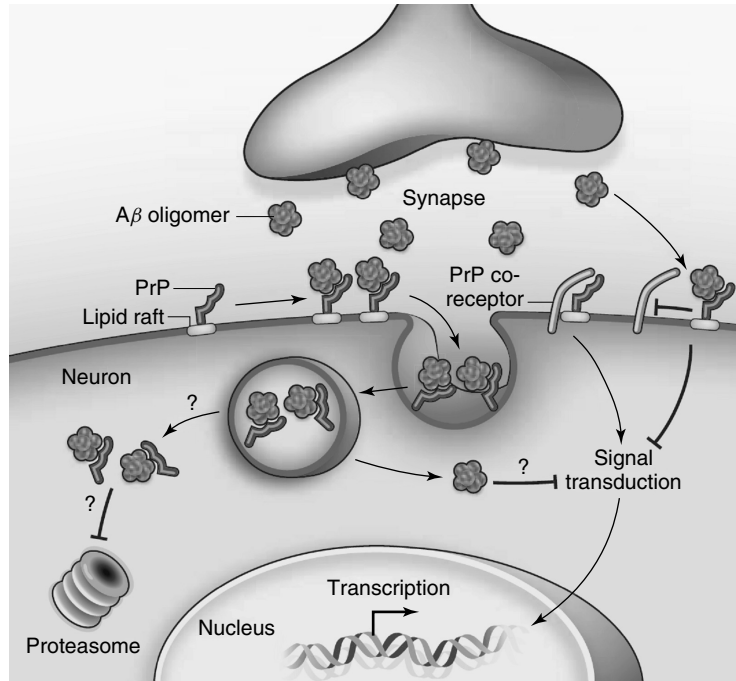


Figure 1.9 Prion and A β -induced synaptic toxicity. Reprinted by permission from Macmillan Publishers Ltd: *Nature* [175], copyright 2009.

attempt to tag and process toxic aggregates. Oxidative stress, membrane breaching, abnormal metal ion homeostasis, and metal miscompartmentalization can foster this process [6, 179].

1.8.2

Ubiquitin and Phospholipids

Factors that favor protein and metal desolvation (e.g., lower dielectric constant near a membrane surface or in the proximity of inclusion bodies) [180] may significantly increase the Cu^{II} binding affinity and aggregation propensity of Ub. As an example, the Cu^{II} binding affinity of α -syn was shown to increase by 1 order of magnitude in the presence of lipids [181]. Lipids and vesicle membranes were found in Lewy bodies on autopsy [182], and lipid-bound soluble cytosolic oligomers were increased in brain extracts from patients with PD or dementia, with Lewy bodies [183].

The lipid composition of different subcellular compartment membranes can modulate protein folding and aggregation rates. Intracellular accumulation of A β was seen predominantly in multivesicular bodies and lysosomes and A β fibrillogenesis was found to be accelerated in the presence of endosomal and

lysosomal membranes. Furthermore, A β oligomerization was accelerated through the interaction with ganglioside clusters in lipid rafts [184].

Many physiologically relevant functions of Ub are carried out in the proximity of membrane surfaces. The ubiquitination process regulates endocytosis of membrane proteins, multivesicular body formation, and Golgi and endoplasmic reticulum functions [115]. A membrane-bound form of Ub, phosphatidyl-Ub, has been found in baculovirus particles [185] and in the virions of several other enveloped viruses [186]. The release of Ub from phosphatidyl-Ub, rather than the free cellular pool of Ub, facilitates protein ubiquitination events at membranes [187].

In-cell NMR spectroscopy allows one to determine how protein structures are influenced by their intracellular environment [188]. The application of this technique to the study of Ub in human cells has revealed an increase of protein dynamics and a decrease of folding stability *in vivo* as compared to those *in vitro*, as assessed by hydrogen exchange measurements [189]. This somewhat unexpected behavior has been attributed to nonspecific interactions of Ub with intracellular macromolecules, the cytoskeleton, and inner membranes [189], which may decrease the folding stability of Ub in cells. Ub exhibits large structural heterogeneity both in the free form and after binding to different partners [190]. Thus, binding events in cells might cause interconversion between different conformations of Ub, which may also destabilize folding and increase hydrogen exchanges rates. Alternatively, some of the substrates may preferentially bind to less folded states of Ub [190].

1.9 Conclusions and Perspectives

We found that Ub, the protein responsible for the cellular clearance of toxic aggregates linked to neurodegenerative disorders, is able to coordinate Cu^{II} and form *in vitro* assemblies similar to those it is supposed to break up [14]. The new findings underscore the importance of preserving cellular compartmentalization and Cu homeostasis for the correct functioning of protein degradation systems. Ub stability and function might be otherwise compromised [13].

The inorganic chemistry of the brain is inherently rich and remains an open frontier. The results linking Cu trafficking to neurodegenerative disorders are engaging, but much work remains to be done to fully elucidate the molecular roles of bound and labile metal ions, their physiological redox state and speciation, and their contributions to basic aspects of signaling within and between brain cells.

Considerable attention is presently focused on the early stages of protein misfolding and aggregation (i.e., protein destabilization and oligomerization), with the aim of elucidating the molecular and biochemical basis of conformational and neurodegenerative disorders. Biophysical studies have been increasingly influential owing to their ability to provide a mechanistic rationale to better explain the effects of disease-causing mutations, oxidative stress, and the exacerbating role of Cu and other metal ions.

Of the features discussed above, NMR characterization of paramagnetic Cu^{II} proteins represents a challenge, however NMR experiments based on ¹³C direct detection open new possibilities for solution and solid-state studies of disease-related Cu^{II}-binding proteins and their oligomeric assemblies [140, 143].

The obvious shortcoming of such studies is that they require experimental conditions that have little resemblance to the human brain. Moreover, the pathological processes are difficult to study *in vitro*, because it is difficult to reconstitute protein aggregation in an artificial environment. However, the advancements of *in-cell* NMR techniques [188], coupled to a variety of molecular and elemental imaging techniques including fluorescence and X-ray detection methods [34, 191], will allow detailed investigations at the atomic and microscopic level of globular, amyloid-forming, or intrinsically unstructured proteins in neuronal cells, and will help to characterize functional and pathogenic interactions of metal ions *in vivo*.

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