The role of metals in protein conformational disorders - The case of prion protein and Aβ-peptide

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Abstract. Protein conformational disorders are members of a vast class of pathologies in which endogenous proteins or peptides undergo a misfolding process by switching from the physiological soluble configuration to a pathological fibrillar insoluble state. An important, but not yet fully elucidated, role in the process appears to be played by transition metal ions, mainly copper and zinc. X-ray absorption spectroscopy is one of the most suitable techniques for the structural characterization of biological molecules in complex with metal. Owing to its chemical selectivity and sensitivity to the local atomic geometry around the absorber, it can be successfully used to study the environment of metal ions in complex with proteins and peptides in physiological conditions. In this paper we present X-ray absorption spectroscopy studies of the metal ions coordination modes in systems where metals are complexed with specific amyloidogenic proteins and peptides. In particular, we show results concerning the Amyloid β peptide, that is involved in Alzheimer’s disease, and the Prion protein, that is responsible for the Transmissible Spongiform Encephalopathy. Our findings suggest that the copper and zinc ions may play a crucial role in the aggregation and fibril formation process of these two biomolecules. Elucidating this kind of interaction could be a key preliminary step before any viable therapy can be conceived or designed.

1. Introduction

It is a well established notion that the function of a protein is strictly related to its structure. Protein folding is a very complicated process that not always ends with the ‘right’ three-dimensional configuration. Being protein function strictly related to its folding, it is straightforwardly understood how errors in the folding can be at the basis of pathologies. Indeed, protein conformational disorders are pathologies caused by the transition of endogenous proteins and peptides from the physiological globular configuration to a misfolded harmful fibrillar state. Regardless of the nature of the proteins by which they are formed, fibrils have common histochemical properties and ultrastructural morphology.

Among this class of pathologies, many neurodegenerative diseases have a major impact on human health, in particular, Transmissible Spongiform Encephalopathies (TSEs), Alzheimer’s (AD) and Parkinson’s (PD) diseases. The molecular basis of these diseases is not yet completely understood. An important, not yet fully elucidated, role appears to be played by transitions metal ions (mainly Cu²⁺ and Zn²⁺) that have been observed to be present in fairly large amount in the aggregates. The presence
of these metal ions in amyloid fibrils evokes the possibility that they might trigger, prevent or promote amyloid aggregates formation [1, 2].

In this general framework, it appears to be of the utmost importance to understand and clarify whether and how metal ions cross-interact with aggregating proteins and peptides. Here, we present the analysis, at a structural level, of the mechanism by which Cu$^{2+}$ and Zn$^{2+}$ ions appear to compete in the binding to both the prion protein, which is involved in TSEs, and to the A$\beta$ peptides, which is involved in AD.

1.1. Transmittable Spongiform Encephalopathies (TSEs) and Prion protein (PrP)
TSEs are fatal infectious neurodegenerative diseases that affect several animal species. Examples of TSEs include mad cow disease in cattle, scrapie in goats and sheep, and Kuru and Creutzfeldt–Jakob diseases in humans. All TSEs are characterized by spongiform degeneration (formation of fluid-filled holes in the brain tissue) and astrocytic gliosis (proliferation and branching of glial) of the central nervous system that unavoidably lead to death. There is increasing evidence that in all TSEs the occurrence of an abnormal form of a protein called Prion protein (contraction of “proteinaceous infectious protein” [3]) is of crucial importance. The PrP is a membrane protein composed by about 260 amino acids anchored to the membrane surface via a glycosyl-phosphatidyl inositol group. It exists in two alternative conformers: the cellular native conformer, PrP$^C$, rich in $\alpha$-helix, and the aberrant pathogenic conformer, PrP$^{Sc}$ (or scrapie PrP) endowed with aberrant self-replicating properties, comparatively rich in $\beta$-sheet [4]. Although the physiological role of PrP$^C$ is not known, the ability of PrP to bind both Cu$^{2+}$ and Zn$^{2+}$ ions in vivo has led to the suspicion that this protein may be involved in metal homeostasis in the brain [5-8]. The observation that Cu and Zn homeostasis is severely altered in the brain tissues of infected organisms compared with normal tissues led to the hypothesis of an involvement of these two metals in the illness genesis.

From a structural point of view, the N-terminal region of PrP, up to residue 120, is unstructured and highly flexible. A characteristic feature of this region is the so-called octarepeat domain composed by a number of repeats (up to six, depending on the species) of the eight-residue sequence PHGGGWGQ. It has been shown that this region hosts the binding sites with the highest affinity to metals [6, 9] (see Figure 1a).

Electron Paramagnetic Resonance (EPR) experiments, performed on hamster PrP, have shown that the copper coordination environment depends critically on the [Cu$^{2+}$]:[octarepeat] concentration ratio. At low Cu$^{2+}$ concentration, the four octarepeat His imidazole side chains bind simultaneously to a single Cu$^{2+}$ [10]. At intermediate Cu$^{2+}$ concentration, the octarepeat takes up two copper equivalents, each coordinated by two His side chains. At higher copper concentration, the octarepeat domain saturates at 4 copper equivalents [11]. It has been also demonstrated [5, 11] that Zn$^{2+}$ is able to perturb the Cu$^{2+}$ coordination mode to the tetra-octarepeat region. In particular, it has been suggested that, even if Zn$^{2+}$ is not able to completely remove Cu$^{2+}$, by increasing its concentration the way Cu$^{2+}$ is bound to the tetra-octarepeat can be progressively changed. The effect of Zn$^{2+}$ on Cu$^{2+}$ binding is stronger at moderate Cu$^{2+}$ concentration and progressively disappears while Cu$^{2+}$ concentration is increased.

1.2. Alzheimer disease (AD) and Amyloid $\beta$ peptide (A$\beta$)
AD is a type of dementia that causes problems with memory, thinking and behavior. Symptoms usually develop slowly and get worse over time, becoming severe enough to interfere with daily tasks. The brain of people affected by AD shows the presence of two microscopic kind of aggregates: the senile (or amyloid) plaques, which are found between neurons, and the neurofibrillary tangles, which are found inside neurons. Amyloid plaques are formed by the aggregation of A$\beta$. A$\beta$ is originated by the proteolytic cleavage of a membrane protein called Amyloid Precursor Protein (APP) that is encoded in chromosome 21. Under physiological conditions, various secretases are known to cut the APP. If $\alpha$ and $\gamma$ secretases are involved in the cutting, the result is a non-pathological, harmless A$\beta$-peptide form. However, if $\beta$ and $\gamma$ secretases are involved, the result is a pathological, harmful A$\beta$-
peptide form. The formation of amyloid plaques from the peptides is a complex and not yet fully elucidated process. At the basis of this aggregation process there is a conformational change of the peptide that leads to the formation of an extended β-sheet like structure, capable of grouping together other peptides to form antiparallel β-sheet with intermolecular binding. It has been observed that plaques contain large amounts of transition metal ions (Cu²⁺ and Zn²⁺ are the most abundant). The physiological role possibly played by metal ions has not been yet fully understood. It has been shown [12], however, that the nature of the metal binding can influence the protein folding/misfolding and aggregation process, thus possibly affecting the pathology progression.

In Aβ the highest affinity metal binding sites are located in the first sixteen amino acids of the N-terminal fragment [13, 14] (see Figure 1b). Most of the studies in literature state that copper has an intra-peptide coordination involving the imidazole groups of three histidines (His₆, His₁₃, His₁₄). The nature of other amino acids possibly involved in Cu²⁺ coordination is still undefined. In many EPR works [15-17] it has been shown that Cu²⁺ coordination is heterogeneous at physiological pH. Namely two binding ways coexist and are termed Component I and Component II depending on the number of histidine residues bound to the metal (two His in Component I and one His in Component II).

The Zn²⁺ binding mode is more controversial and a few different structures in which Zn²⁺ is coordinated to different numbers of His residues have been proposed [18, 19]. Among them, one of the most accredited is an inter-peptide coordination mode in which Zn²⁺ is bound to four histidine residues [13, 20]. The simultaneous presence of both metal ions, that is the normal situation in vivo, is still poorly investigated. A multi techniques study of Damante et al. [21] suggested that the addition of Zn²⁺ does not promote Cu²⁺ ions release, but it significantly modifies the Cu²⁺ mode of coordination in Aβ. EPR studies of Silva et al. [15] have recently shown that after the addition of Zn²⁺ to the already formed Cu-complexes, Zn²⁺ is able to replace only Cu²⁺ coordinated in Component I, while Component II bound Cu²⁺ remain unmodified.

![Figure 1](image_url) Primary structure of the region of PrP involved in metal binding (panel a) and of Aβ peptide (panel b). Regions with the highest binding probability are highlighted.

2. XAS – X-ray absorption spectroscopy

Owing to its chemical selectivity and sensitivity to local atomic arrangement around the absorber, X-ray absorption spectroscopy (XAS) can be successfully used for structural studies on biological materials, in particular when one is interested in characterizing the structural environment of metal ions (that are the absorbers in the systems studied in this paper) complexed with proteins and peptides in physiological conditions. X-ray photons used for XAS experiment are usually produced by synchrotrons, which provide intense and tunable X-ray beams. The X-ray beam is then focused on the samples and the structural information about the atomic environment around the absorber are obtained through the study of the absorption coefficient as a function of the photon energy. The absorption spectrum (XAS), whose oscillation originate from the interference between the outgoing and the backscattered electron waves, contains detailed information about the geometrical distribution of the scatterers around the absorber. The XAS spectrum is normally divided in two regions, the XANES (X-ray Absorption Near Edge Structure) and the EXAFS (Extended X-ray Absorption Fine Structure) (see Figure 2). XANES, where the kinetic energy of the ionized electron is low, is dominated by multiple scattering events, while the EXAFS region, that conventionally starts about 100 eV after the edge (i.e. the ionization energy of the 1s electron of the absorbing atom) is dominated by single scattering
events. Compared to the EXAFS region, XANES are richer in geometrical information that, however, are much more difficult to extract.

![Schematic view of XAS oscillations due to the photoelectron scattering from neighbour atoms. XANES and EXAFS regions are highlighted.](image)

**Figure 2** Schematic view of XAS oscillations due to the photoelectron scattering from neighbour atoms. XANES and EXAFS regions are highlighted.

### 3. Results and Discussion

Metal homeostasis is necessary to maintain essential transition metal ions at the required cellular concentration to both prevent their excess and their lack. There are evidences that a breakdown in metal trafficking regulation has a significant impact in the development of age-related neurodegenerative diseases [22]. PrP proteins and Aβ peptides are both present in brain. They display different affinity for specific metal ion binding and may possibly interact one with the other [23, 24]. Taken together, these three observations lead to the hypothesis that the two peptides may play complementary roles in the Cu²⁺ and Zn²⁺ ions homeostasis.

In the past years, many experimental techniques have been employed to characterize the structure of metal binding site in amyloid peptides, among which EPR [11, 15, 16, 21, 25], Nuclear Magnetic Resonance (NMR) [18, 19, 26], Circular Dichroism (CD) [25-27] and XAS [4-5, 12, 13, 28-31] are the most prominent ones. Using XAS, we investigated the way Cu²⁺ and Zn²⁺ reciprocally affect the way they bind to both PrP and Aβ peptide. Structural information was obtained both from the XANES and the EXAFS regions of the spectrum.

#### 3.1. Prion Protein (PrP)

Systematic XAS experiments were performed on samples containing the tetra-octarepeat (4R₈) region of the PrP in the presence of Cu²⁺ and Zn²⁺. In order to explore the possibility that the way the two metal ions compete and interfere in protein binding may also depend on the order they are added to the peptide containing solution, we prepared solutions where the Cu²⁺ (Zn²⁺) ions were added after few hours of Zn²⁺ (Cu²⁺) incubation in PrP solutions [5, 31].

The first result obtained through the EXAFS analysis is that both Cu²⁺ and Zn²⁺ bind to the 4R₈ region via histidine residues when separately added, thus they compete for the same binding site. The second result is that Zn²⁺ binding mode changes upon Cu²⁺ addition to accommodate the copper ion. On the contrary, when Zn²⁺ is dissolved in a solution pre-incubated with Cu²⁺, it does not modify the way Cu²⁺ binds the protein. The XAS experimental outcome is pictorially schematized in Figure 3.
Figure 3 Schematic illustration of the suggested effect of adding Cu\(^{2+}\) and Zn\(^{2+}\) to the 4R\(_{8}\) solution in different order. Only the bonds between metals and His residues are drawn as short blue segments. In the first line, we depict the effect of adding Cu\(^{2+}\) to already formed 4R\(_{8}/Zn^{2+}\) complexes. In the second one, we show the effect of adding Zn\(^{2+}\) to already formed 4R\(_{8}/Cu^{2+}\) complexes. In the different panels, the number of orange (Cu\(^{2+}\)) and black (Zn\(^{2+}\)) dots per 4R\(_{8}\) is indicative only and does not represent the real proportion among tetra-octarepeats, Cu\(^{2+}\) and Zn\(^{2+}\).

3.2. A\(_{\beta}\)-peptide

XAS has been also used to study the metal binding mode to the A\(_{\beta}\) peptide, showing that it depends on the nature and concentration of the complexed metal ion [32]. On one hand, XAS data are always consistent with an intra-peptide (involving a single copy of the A\(_{\beta}\) peptide) Cu\(^{2+}\) mode of binding involving three His residues coordinated to the ion. On the other hand, XAS data are consistent with either an intra- or an inter-peptide Zn\(^{2+}\) mode of binding, depending on solution conditions [12, 30].

As in the case of PrP, an obvious extension of this work is to study the way the two metals influence reciprocally their mode of binding to the A\(_{\beta}\) peptide when they are simultaneously present. This study has already been performed using EPR that, however, cannot give information on the Zinc ion [15]. It is also worth studying the effect of the order in which metals are added to the peptide solution [33].

3.3. Conclusions

The general idea that emerges from the studies presented here is that both PrP and A\(_{\beta}\) peptide may be involved in metal trafficking and homeostasis. The role played by metals in the pathological misfolding and aggregation processes is not yet clarified, but there are convergent evidences that Zn\(^{2+}\) play an important role at least in the first phases of aggregation. This role is possibly modulated by Cu\(^{2+}\) presence.

We are confident that, given the fact that the resolution of experimental techniques is constantly getting higher and higher and the parallel computing platforms is allowing to perform simulations of
larger and larger (more detailed) model systems, we will collect enough information to at least clarifying the structural relevant steps of the misfolding-aggregation process involving metal ions.

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