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Fibrillation of hen egg white lysozyme triggers reduction of copper(II)

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ABSTRACT

Copper is known to exert diverse effects on the self-association of proteins and has been found in amyloid deposits that are involved in neurodegenerative disease processes. The effects of the metal ion on the protein during fibrillation were investigated by fluorescence, circular dichroism spectroscopy and fluorescence microscopy. We report for the first time, the complete reduction of Cu(II) → Cu(I) *in vitro* during fibrillation of hen egg white lysozyme at pH 7. This was confirmed by the lack of any signal for Cu(II) in electron paramagnetic resonance spectroscopy and quantification of Cu(I) was achieved by a bathocuproine disulfonate based assay.

1. Introduction

Copper(II) plays a central role in pathological disorders where toxic fibrillar deposits have been found to be enriched with Cu(II) [1,2]. Recent studies demonstrate remarkable effects of Cu(II) which can either potentially promote or inhibit amyloid formation of numerous neurodegenerative disease related/unrelated proteins such as Aβ-peptides, α-synuclein, prion protein, ubiquitin, human serum albumin, human peptide of amylin, β-2-microglobulin and β-lactoglobulin A [3–13]. In addition, the amyloid precursor protein (APP), $A\beta_{1-40}$ peptide, α-synuclein and prion (PrP) are capable of reducing Cu(II) to Cu(I) [14–17]. Reduction of Cu(II) to Cu(I) results in the generation of reactive oxygen species and leads to oxidative stress or protein damage which in turn is linked to neurological disorders [15,18]. It has been observed that for the $A\beta_{1-40}$ peptide both the monomer and fibrillar forms act as potent reductants of Cu(II) [15]. $A\beta_{1-16}$ peptide monomer shows a higher affinity for Cu(I) compared to Cu(II) [19]. In case of α-synuclein, reduction of Cu(II) to Cu(I) occurs in the absence of oxygen whereas reoxidation during aggregation takes place in the presence of oxygen [16]. It has been illustrated that binding of Cu(I) to α-synuclein involves specific Met residues [20]. In case of the human prion protein, the N-terminal tandem repeat region was found to reduce Cu(II) to Cu(I) where Trp was found to be the key amino acid residue [17]. In addition to this His, Cys and Trp were also found to play a pivotal role in Cu(II) reduction for $A\beta_{1-40}$, APP, PrP respectively [21]. In case of the recombinant ovine prion protein, apart from reduction of Cu(II) → Cu(I), fragmentation of the protein occurred that was believed to facilitate amyloid formation [22].

Hen egg white lysozyme (HEWL) is a globular protein with 129 amino acid residues that comprise two different domains cross-linked by means of four disulfide bonds [23]. It is known to undergo amyloid formation under variable conditions such as change in pH, elevated temperature, presence of denaturants and *via* chemical modifications [24–27]. Human lysozyme, a homologue of HEWL has been found to be associated with hereditary systemic amyloidosis due to a point mutation in the lysozyme gene [28]. Human lysozyme forms fibrillar species *in vitro* similar to those obtained from individuals suffering from disease [29]. In addition to the structural homology, fibrils formed from both human lysozyme and HEWL were found to be extremely similar [25,29]. Moreover the observed membrane activity of HEWL fibrils and their ability to induce apoptosis in neuroblastoma cells has proven that HEWL can act as a suitable model *in vitro* [30,31]. The binding of Cu(II) to HEWL has been studied by both enzymatic and spectroscopic techniques including electron paramagnetic resonance (EPR) along with structural studies [32,33]. Studies indicate the involvement of Asp 52, in the vicinity of Trp 108 at site A (of the hexasaccharide binding region comprised of subsites A–F). In case of site B, the Cu(II) ion was found to form intermolecular bridges by being bound in an equivalent fashion to two symmetry related molecules [32,33].

We have previously shown that there is enhanced fibrillation of human serum albumin (HSA) at physiological pH (7.4) in the presence of a stoichiometric amount of Cu(II) [10]. In this study we have monitored the effect of Cu(II) on HEWL fibrillation at pH 7 and characterized the fibrillar species *via* ThT fluorescence, circular dichroism (CD) spectroscopy, fluorescence microscopy and transmission electron microscopy (TEM). Recently salen–manganese complexes have been found to exhibit potent inhibitory and

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disintegrating effects against HEWL fibrillation at acidic pH [34], but there is no prior report of the effect of Cu(II) on HEWL fibrillation. We report here the complete reduction of Cu(II) → Cu(I) during fibrillogenesis under aerobic conditions. This is unlike that found in case of α-synuclein where the reduction occurred in the absence of oxygen [16]. The most interesting finding in this work has been the observed reduction of Cu(II) to Cu(I) during the fibrillation process of HEWL that was previously unknown. The reduction was confirmed by EPR spectroscopy and information about the coordination environment obtained from Raman spectroscopy. Structural insights into the protein during fibrillation and the effect of the metal ion were investigated by fluorescence and CD measurements. Quantification of Cu(I) was performed through the bathocuproine disulfonate (BCS) based assay. This study not only provides further guidelines into the effects of metal ions on the fibrillation process of the protein, but also the effect of the protein environment on the metal ion itself.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL) and thioflavin T (ThT) were purchased from Sigma Chemical Co. (St. Louis, USA) and used as received. Copper sulphate pentahydrate ($CuSO_4{\cdot}5H_2O$) and all other chemicals were obtained from SRL (India).

2.2. Preparation of fibrillar solutions

HEWL was dissolved in double distilled water and the concentration determined using a molar extinction coefficient of 37,646 M^{-1} cm^{-1} at 280 nm [35]. Fibrils were prepared in the presence of 80% ethanol (*v*/*v*) and 20 mM phosphate buffer of pH 7 containing 20 mM NaCl keeping protein and metal ion ratio of 1:1 (150 μM) after heating at ∼60 °C for 6 h followed by incubation at room temperature. To maintain the desired concentration in the final working solution 20 mM phosphate buffer of pH 7 was used in each study.

2.3. Thioflavin T fluorescence

The change in ThT intensity was measured after heating at ∼60 °C for 6 h. After the samples attained room temperature, ThT was added and the mixture incubated for 5 min and scanned in a Jobin Yvon Fluoromax 4 spectrofluorimeter. In each case, samples were diluted with 20 mM phosphate buffer (pH 7) keeping the protein and dye concentration at 2 μM and 5 μM respectively. Excitation and emission maxima were at 450 and 482 nm respectively. Slit width and integration time were fixed at 5 nm and 0.3 s. Each spectrum was corrected with respect to the corresponding blank.

2.4. Circular dichroism

Far-UV circular dichroism spectra were acquired using a JASCO-810 spectrophotometer keeping a protein concentration of 20 μM in the scanning range of 190–240 nm (25 °C) at a scan rate of 50 nm/s. A quartz cuvette of 0.1 cm path length was used. Protein secondary structure content was estimated using DICHROWEB, an online server [36].

2.5. Higher order oligomer formation: SDS–PAGE

Electrophoresis was carried out under reducing conditions. Fibrillar solutions (50 μM) were mixed with sample buffer (2×) containing SDS (4 g), bromophenol blue (1%, 4 ml), β-mercaptoethanol (10 ml), glycerol (20 ml) and Tris–HCl [pH 6.8, 1 M, 12.5 ml] (the volume was adjusted to 100 ml with water for the sample buffer). Samples were boiled for 2 min and then applied to a 15% resolving gel and electrophoresis conducted in a Mini Dual vertical electrophoresis unit (Tarson). Gels were stained with Coomassie brilliant blue (SRL, India). Destaining was done using a mixture of $CH_3COOH/MeOH/H_2O$ (37.5 ml, 25 ml, 430 ml) with moderate agitation.

2.6. Fluorescence microscopy

10 μl of ThT was mixed with 5 μl of each sample, placed on a glass slide and covered with a cover slip. Images were observed under a Leica DM 2500M microscope equipped with a fluorescence attachment. For ThT excitation and emission filter cube no 2 (Leica I3 11513878, BZ: 01) was used. The images were captured with a Leica DFC 310 FX camera attached with the microscope. All observations were performed at 10×/0.25 (N PLAN EPI).

2.7. Transmission electron microscopy

HEWL fibrillar solutions were diluted to a concentration of 100 μM and placed on TEM grids. Samples were negatively stained with an aqueous solution of uranyl acetate [1% (*w*/*v*)], air dried and scanned in a TECNAI G^2 20S-TWIN transmission electron microscope operating at an accelerating voltage of 80 kV.

2.8. Electron paramagnetic resonance spectroscopy

Fibrillar samples of [HEWL–Cu(II)] were taken in a standard quartz tube and frozen at 77 K. The X-band EPR spectra were recorded on a JEOL-JES-FA 200, 9.13 GHz spectrometer.

2.9. Raman spectroscopy

Fibrillar samples (previously formed HEWL fibrils both in the absence and presence of Cu(II) at pH 7 after heating at ∼60 °C for 6 h followed by incubation at room temperature for 10 days) were subjected to Raman scattering keeping a 180° scattering geometry using a micro-Raman spectrometer with a 488 nm argon ion laser. The spectrometer is equipped with an optical microscope (Model BX 41, Olympus, Japan), single monochromator (Model TRIAX550, JY, Horiba, France), an edge filter, and a Peltier cooled CCD (1024 × 256 pixel) detector. The laser power on the samples was 5 mW. The data acquisition time for each Raman spectrum was 300 s. The laser irradiation under the given experimental conditions does not affect the protein conformation [37]. For Raman measurements, five drops of each sample were set to dry on a thoroughly cleaned Al foil at room temperature for 10 h. Raman scattered light was collected from at least six different spots of each drop using a 50× objective lens.

2.10. Equilibrium dialysis and copper reduction assay

Dialysis of the fibrillar solution of HEWL in the presence of Cu(II) at pH 7 was carried out using dialysis tubing with a 12.4 kDa cutoff (25 mm × 16 mm). Prior to dialysis, the cellulose membrane was washed initially with 50% (*v*/*v*) ethanol, followed by 0.025 (M) $NaHCO_3$ for 30 min using a magnetic stirrer and then finally with distilled water for another 30 min. Fibrillar solutions were added to respective dialysis bags and placed in 250 ml beakers filled with 60 ml of 20 mM phosphate buffer of pH 7 and stirred for 24 h. The dialysates were collected and fibrillar solutions redialyzed in the same manner. The total amount of dialysates was then concentrated and used for Cu(I) detection using bathocuproine disulfonate (BCS), a known Cu(I) chelator. The $[Cu(BCS)_2]^{3-}$ complex has an absorption maxima at 483 nm (ε_{483} = 13,300 M^{-1} cm^{-1}) [38]. For