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Electrochemical and optical study of metallothionein interactions with prion proteins

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ABSTRACT

The prion protein (PrP^C) can be structurally shifted to its PrP^{Sc} isoform causing a wide range of neurodegenerative diseases, which are currently incurable. There is an evidence that metallothioneins (MTs), and especially MT-3, are associated with neurodegenerative diseases. PrP^C and MTs play pivotal roles in maintaining metal homeostasis; therefore, it is conceivable that each of them has its own significance in prion diseases. In this paper, we study the nature of interactions between PrP^C, MT, and copper ions, Cu(II), using the method of differential pulse voltammetry (DPV) coupled with adsorptive transfer stripping technique (AdTS). Electrochemical properties of PrP itself and its interactions with both the Cu(II) ions and MTs have been found. Based on the results obtained, we hypothesised the formation of the complex in molar ratio 2:1 (PrP^C:MT). Surface plasmon resonance imaging (SPRi) was used as a control reference assay to further confirm results obtained by the electrochemical approach, such as the specific interactions between PrP^C and MT-3.

1. Introduction

The prion protein (PrP^C) is a glycosylphosphatidylinositol-anchored host glycoprotein normally present in brain, where its amount exceeds those of other tissues by fifty times [1]. This protein, with a predominance of α-helix structures, can be converted to an abnormal protease-resistant isoform with the increased ratio of β-sheet structure called a prion (PrP^{Sc}) [2,3]. PrP^{Sc} isoform can cause a range of slow neurodegenerative disorders called transmissible spongiform encephalopathies (TSEs) [1]. While there is an established role for PrP^{Sc} in TSEs, the physiological role of PrP^C has still not been fully elucidated. Roles in neuroprotective signalling, lymphocyte activation, neurite growth, synaptogenesis, cellular signalling, cell viability, cellular response to oxidative stress and metal homeostasis have all been suggested [4–9].

The homeostasis of zinc and copper ions is tightly regulated and essential for brain physiology [10]. Proteomic studies have shown that PrP^C is one of the most important copper-chelating proteins in brain [11,12]. According to some authors, Cu(II) ions can stabilise the structure of PrP^C [13], but other studies indicated that copper ions can destabilise the native fold of PrP^C and can facilitate the conversion to PrP^{Sc} isoform [14]. According to several key papers, Cu(II) ions are selectively bound by prion proteins [15–17], as such complexes play a role in several processes including the pathological ones [18].

Other proteins, called metallothioneins (MTs), important in maintain of metal homeostasis, have been identified to fulfil multiple functions, including the involvement in zinc and copper homeostasis and protection against heavy metal toxicity and oxidative damage [19–22]. A brain-specific subtype of MT is called MT-3. This isoform binds Zn(II) through the array of 20 conserved cysteines into two metal–thiolate clusters. The latest studies show that MT-3 is able to bind Cu(II) when Cu homeostasis is disrupted and Cu(II) together with Zn(II) ions could be involved in the formation of amyloid plaques in neurodegenerative disorders as it was discussed by Adam et al. [23], whereas the results found in this field remain controversial. Moreover, the level of MTs has been found

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to be altered in some TSEs [24–26] and changes in normal homeostasis of essential transition metals such as Zn and Cu have been implicated as possible aetiology factors [27].

Minor changes in the structure of PrP^C and MTs due to the interaction with metal ions are difficult to observe. Methods of pulse voltammetry seem to be suitable for the detection of extremely small changes in a protein structure [28–30]. These electrochemical techniques are commonly used for protein determination and provide exceptional limits of detection at pM levels [31–33]. Differential pulse voltammetry (DPV) has been used for MT analysis several times [34–37], as well as for the analysis of PrP^C [4,38]. Moreover, PrP^C has been widely, electrochemically studied as, summarised in the following review [39]. In spite of the fact that considerable attention has been paid to the electrochemical behaviour of MT and PrP^C, prion-MT interactions have not been studied by DPV yet.

The first objective of our work was to characterise the electrochemical behaviour of PrP^C, and MT, under given conditions at a hanging mercury drop electrode (HMDE). The most important goal was an investigation of the interactions between the proteins. For these purposes, the fundamental electrochemical technique DPV was used in combination with AdTS. This approach allows analysing low sample volumes and analyte concentrations, which is extremely important in case of protein studies.

2. Materials and methods

2.1. Chemicals

PrP^C-recombinant bovine PrP^C [highly purified protein (rec bovPrP)], amino acid sequence corresponds to mature bovine PrP^C (amino acids 25–242), expressed in an *Escherichia coli* K12 strain [12] with molecular weight (MW) 23,686 Da, was purchased from Prionics AG (Zurich, Switzerland). MT (MW 6500 Da) was purchased from Ikzus (Alessandria, Italy). Both Cu(II) and Zn(II) salts ($Cu(NO_3)_2{\cdot}3H_2O$ and $Zn(NO_3)_2{\cdot}6H_2O$), bovine serum albumin (BSA), mercaptoundecanoic acid (MUA), ethanolamine (EA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), 11-mercaptoundecanol (MU) and 6-mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless noted otherwise. Some chemicals were further used for SPRi-Biochips™ (Horiba Scientific, France) bio-functionalisation and surface saturation. Working standard solutions were prepared daily by the dilution of the stock solutions. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot, Weilheim, Germany). The pH-electrode (SenTix H, pH 0–14/3 M KCl) was regularly calibrated by a set of WTW buffers (Weilheim, Germany). High-purity deionised water (Milli-Q Millipore 18.2 MΩ/cm, Bedford, MA, USA) was used throughout the study.

2.2. Electrochemical measurements

Electrochemical measurements were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA Stand 663 (Metrohm, Herisau, Switzerland), using a standard cell with three electrodes. The working electrode was HMDE with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl/3M KCl electrode and the auxiliary electrode was a glassy carbon electrode (GCE, 2 mm diameter, Metrohm, Herisau, Switzerland). For smoothing and baseline correction, the software GPES 4.4 (Metrohm) supplied by EcoChemie was employed.

2.2.1. Adsorptive transfer stripping technique (AdTS)

The principle of the AdTS was based on the strong adsorbing of the studied analyte on the electrode surface at an open electrode circuit. The excess of analyte was rinsed from the surface of the working electrode in sodium phosphate and/or acetate buffer. The adsorbed analyte was finally detected in the presence of supporting electrolyte. Volume of test sample was 5 μL for all measurements. The optimal time of adsorption was determined to be 120 s.

2.2.2. Differential pulse voltammetry (DPV)

PrP^C and MT samples were analysed using AdTS combined with DPV in sodium phosphate buffer (0.2 M, pH 7.0). DPV parameters were as follows: start potential 0.0 V, end potential −1.5 V, scan rate 47.25 mV/s, modulation time 57 ms, time interval 200 ms, equilibration time 5 s, step potential of 9.45 mV/s, and modulation amplitude of 25.05 mV.

Cu(II) and its interactions were measured in acetate buffer (0.2 M, pH 5.0). DPV parameters: start potential −0.3 V, end potential +0.1 V, deposition potential −0.3 V, deposition time 0 s, scan rate 47.25 mV/s, modulation time 57 ms, time interval 200 ms, equilibration time 10 s, step potential of 9.45 mV/s, and modulation amplitude of 25.05 mV.

All experiments were carried out at 24 °C. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s.

2.3. Surface plasmon resonance imaging (SPRi)

PrP^C and MT interaction (without metals in solution) was further monitored and proved by SPRi with SPRi Lab+ (Horiba Scientific; Edison, NJ, USA). SPRi technology is increasingly exploited for clinical analysis and diagnostics applications [40], and it was extensively described in [41,42] while the experimental instrumental asset together with the micro-welled poly (dimethylsiloxane) (PDMS) mask (Sylgard 184 Silicone Elastomer Kit, Dow Corning, UK) preparation for multi-arrayed biomolecules immobilisation was reported in [43]. To achieve final MT immobilisation, first 1 mM MUA in Milli-Q water (0.8 μL) was spotted in the micro-welled PDMS mask on the gold chip surface overnight. After activation of the carboxylic group with NHS/EDAC solution, MT was covalently immobilised at five different surface concentrations (15.9; 31.8; 63.5; 127.0; and 254.0 ng/mm^2, respectively) through the respective deposition of 0.8 μL MT (15.7; 31.3; 62.5; 125.0; and 250.0 μg/mL, respectively). BSA was also immobilised on the activated sensing surface at the level of 100 ng/mm^2 as reference control (deposition of 0.8 μL at 100 μg/mL). Immobilisations were performed in acetate buffer (0.2 M, pH 5.0). Subsequently 250 μg/L PrP^C target solution in 200 mM PBS buffer pH 7.4 (100 μL) were injecting under a constant flow of 6 μL/min, and the interaction with immobilised MT was monitored for 30 min.

2.4. Descriptive statistics

Data were processed using Microsoft Excel®, version 2010 (Washington, USA). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise.

3. Results and discussion

Protein-protein interactions are key ones in numerous cellular biochemical pathways, as these depend on various factors including outer chemical moieties and/or ions exchanging. Looking deeply at the Cu(II) and Zn(II) ions homeostasis in the brain, MT-3 plays an important role as the transporter of those ions, which are also structural components of PrP^C. There should be active interactions between these proteins; to our knowledge, *in vitro* tests to confirm