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Original Contribution

Prion protein does not redox-silence Cu^{2+}, but is a sacrificial quencher of hydroxyl radicals

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Abstract

Oxidative stress is believed to play a central role in the pathogenesis of prion diseases, a group of fatal neurodegenerative disorders associated with a conformational change in the prion protein (PrP^C). The precise physiological function of PrP^C remains uncertain; however, Cu^{2+} binds to PrP^C in vivo, suggesting a role for PrP^C in copper homeostasis. Here we examine the oxidative processes associated with PrP^C and Cu^{2+}. ^1H NMR was used to monitor chemical modifications of PrP fragments. Incubation of PrP fragments with ascorbate and $CuCl_2$ showed specific metal-catalyzed oxidation of histidine residues, $His^{96/111}$, and the methionine residues, $Met^{109/112}$. The octarepeat region protects $His^{96/111}$ and $Met^{109/112}$ from oxidation, suggesting that PrP(90–231) might be more prone to chemical modification. We show that $Cu^{2+/+}$ redox cycling is not 'silenced' by Cu^{2+} binding to PrP, as indicated by H_2O_2 production for full-length PrP. Surprisingly, although detection of Cu^+ indicates that the octarepeat region of PrP is capable of reducing Cu^{2+} even in the absence of ascorbate, H_2O_2 is not generated unless ascorbate is present. Full-length PrP and fragments cause a dramatic reduction in detectable hydroxyl radicals in an ascorbate/Cu^{2+}/O_2 system; however, levels of H_2O_2 production are unaffected. This suggests that PrP does not affect levels of hydroxyl radical production via Fentons cycling, but the radicals cause highly localized chemical modification of PrP^C.

Keywords: Oxidation; Reactive oxygen species; Hydrogen peroxide; Methionine sulfoxide; 2-Oxo-histidine

Introduction

Prion diseases, or transmissable spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative disorders. These diseases include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathies (BSE) in cattle, and scrapie in sheep. The pathology of these diseases is characterized by the deposition of a misfolded form of the prion protein (PrP) into insoluble aggregates. This abnormal isoform, PrP^{Sc}, is a protease resistant β-sheet-rich form of the normal prion protein (PrP^C). Oxidative stress is a key feature of the pathogenesis of prion diseases [1–5]. For example, levels of oxidative stress markers, namely malondialdehyde (MDA) and heme oxygenase-1 (HO-1) and additionally the generating rate of free radicals, are reported as being significantly increased in the brains of scrapie-infected mice [6–8]. It is also suggested that prion disease results from loss of antioxidant defense [9].

Prion diseases have been linked to metal-induced oxidative damage [10,11]. Oxidative chemical modification of PrP could be associated with PrP misfolding and aggregation in prion diseases. Indeed, metal-induced oxidation has been shown to provoke significant aggregation in the protein [11], which is a key feature linked to prion diseases. Reactive oxygen species (ROS) can mediate β-cleavage of the prion protein [12]. It has been shown that copper can catalyze the cleavage and dimerization of PrP [13], interestingly disulfide-bonded oligomers, created by a reduction–oxidation mechanism, exhibit

Abbreviations: BC, bathocuproine disulfonic acid; 3-CCA, 3-coumarin carboxylic acid; HO-1, heme oxygenase-1; MCO, metal-catalyzed oxidation; MDA, malondialdehyde; PrP, prion protein; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive species.

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seeding properties similar to PrP^{Sc} [14]. Oxidized methionine residues have been found at high levels in amyloid-β plaques of Alzheimers disease patients [15]. The fibrillogeneses of amyloid-β [16] and PrP(106–126) have been shown to be effected by the presence of Met^{ox} [17].

PrP^{C} contains two distinct structural domains [18–21]. The N-terminal region, residues 23–126, has little defined structure in the absence of Cu^{2+} ions [20] and has a high degree of main-chain flexibility [22]. In contrast, the C-terminal domain, residues 121–231, is largely α-helical [18]. The unstructured N-terminal domain contains an octapeptide sequence, PHGGGWGQ, which is repeated four times between residues 60 and 91. This octarepeat region binds up to four Cu^{2+} ions cooperatively [23–27]. At pH~6 it has been suggested that a single Cu^{2+} ion can bind multiple His residues, while at pH 7.4 coordination via single His residues dominates [27]. In addition, we have recently shown that two Cu^{2+} ions bind preferentially to a region outside of the octarepeats, between residues 90 and 126 where Cu^{2+} binding is centred at His^{96} and His^{111}, a region vital for prion propagation [28,29]. The affinity of Cu^{2+} for PrP is the subject of hot debate with reported dissociation constants varying between 10^{7} [30] and 10^{15} [31,32] for full-length PrP. There is more agreement for copper affinities of fragments of PrP with micromolar dissociation constants reported for the octarepeats [24] and nanomolar K_d for $His^{96/111}$ [28,29].

Due to the high level of protein conservation across several species [33] it is believed that PrP^{C} has an important, but as yet undefined, biochemical role. PrP is found at high levels on the surface of stem cells; furthermore, PrP-null bone marrow exhibited impaired self-renewal [34]. This observation suggests that PrP could protect stem cells when under stress. Expression of PrP^{C} has been linked to enhanced binding of copper to the outer plasma membrane as well as an increase in antioxidant enzyme activity [35], and PrP knockout mice are found to be more sensitive to oxidative stress [36]. Furthermore, an artificially tethered N-terminus of PrP compromises the cellular response to oxidative stress [37]. Prion protein-deficient neurons are also more sensitive to oxidative stress [38,39] and the toxic effects of copper [40]. In vivo and in vitro studies have shown the ability of PrP^{C} to bind Cu^{2+} which suggests a role for PrP^{C} in copper homeostasis [41] or to copper-dependent enzymatic functions [42]. Elevated levels of copper induce endocytosis of the protein [43,44]; however, the degree of PrP expression does not influence cuproenzyme activity [45].

The ability of PrP to bind Cu^{2+} ions and the oxidative damage associated with prion diseases suggests that $Cu^{2+/+}$ redox cycling may be the cause of ROS generation. In the presence of suitable reducing agents, for example ascorbate, redox active metal ions, in particular $Cu^{2+/+}$ (and $Fe^{3+/2+}$), are capable of catalyzing the activation of oxygen to various reactive oxygen species (ROS), which readily react with biomolecules, compromising cell membranes, and protein structure and function. Much of the damage resulting from oxidative stress has been attributed to the highly reactive hydroxyl radical. Fentons and the Haber Weiss reactions involve the reduction of copper (or iron) with the subsequent reoxidation of the metal ion coupled with the production of highly reactive and damaging $^{\bullet}OH$ radicals and other ROS [46–49].

The aim of this study is to investigate the redox cycling of Cu^{2+} when incubated with PrP^{C}. This was done by examining the effects that full-length recombinant PrP, as well as specific prion protein fragments and constructs, have on the production of ROS, under physiologically relevant levels of ascorbate and Cu^{2+} ions. In addition, we have also monitored the nature and susceptibility of PrP to metal-catalyzed oxidation. PrP copper chelation has been suggested to effectively redox-silence copper [50]. In contrast, others suggest that PrP can directly reduce Cu^{2+} [51,52]. To date both antioxidant [9,50,53–56] and prooxidant [52,57] roles have been proposed for PrP^{C}. In addition, chemical modification of PrP through Cu^{2+} and ascorbate has been reported, although the nature of this modification is disputed with some groups showing only methionine oxidation [58], while Requena et al. report histidine oxidation [11]. Here we aim to address some of these contradicting observations. ^{1}H NMR has been used to directly monitor metal-catalyzed oxidative modification of PrP over time in solution, on a per residue basis. In addition a comprehensive range of assays for detecting various ROS has been used to understand the Cu^{2+}-PrP redox system, and furthermore, Cu^{2+} reduction is directly monitored. We believe, that by using a wide range of approaches a more complete understanding of the Cu^{2+}-PrP redox system has been obtained.

Experimental procedures

Protein expression and purification

Recombinant mouse protein was expressed in *Escherichia coli.* PrP(23–231) and PrP(23–231Δ51–90) (the coding region of the full-length mouse PrP-(23–231) with a deleted octarepeat region) was cloned into a pET-23 vector to produce a tag-free protein as previously described [28,56]. Both PrP constructs were purified with a copper-charged metal affinity column made from chelating Sepharose (Amersham Biosciences). The protein was eluted from the column using imidazole (300 mM). The purity of the protein was detected by polyacrylamide electrophoresis and Coomassie staining. The protein was refolded by successive rounds of dilution in distilled water, concentrated using a vivaspin concentrator (Vivascience), and dialyzed with a 12,000–14,000 molecular weight cutoff dialysis tube at 4°C against water overnight to remove residual urea. To inhibit bacterial growth sodium azide was added, 0.005% (w/v).

Peptide syntheses and purification

Peptides representing various fragments and constructs of the prion protein were synthesized by employing solid-phase F-moc chemistry (ABC, Imperial College, London). After removal from the resin and deprotection, the samples were purified using reverse-phase HPLC and characterized using mass spectrometry and ^{1}H NMR.