Biochimica et Biophysica Acta 1842 (2014) 2345–2356

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Methionine oxidation accelerates the aggregation and enhances the neurotoxicity of the D178N variant of the human prion protein

Boya Feng, Zonglin Wang, Ting Liu, Rui Jin, Shaobo Wang, Wei Wang, Gengfu Xiao *, Zheng Zhou *

State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, Hubei 430071, China
University of the Chinese Academy of Sciences, Beijing 100039, China

ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 27 August 2014
Accepted 24 September 2014
Available online 2 October 2014

Keywords:
D178N mutation
Fatal familial insomnia
Oxidative stress
Aggregates
Apoptosis

ABSTRACT

The D178N mutation of the prion protein (PrP) results in the hereditary prion disease fatal familial insomnia (FFI). Little is known regarding the effects of methionine oxidation on the pathogenesis of D178N-associated FFI. In the present study, we found that the D178N variant was more susceptible to oxidation than wild-type PrP, as indicated by reverse-phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) analysis. Circular dichroism (CD), differential scanning calorimetry (DSC), thioflavin T (ThT) binding assay studies demonstrated that methionine oxidation decreased the structural stability of the D178N variant, and the oxidized D178N variant exhibited a greater propensity to form β-sheet-rich oligomers and aggregates. Moreover, these aggregates of oxidized D178N PrP were more resistant to proteinase K (PK) digestion. Additionally, using fluorescence confocal microscopy, we detected a high degree of aggregation in D178N-transfected Neuro-2a (N2a) cells after treatment with hydrogen peroxide (H_2O_2). Furthermore, the oxidation and consequent aggregation of the D178N variant induced greater apoptosis of N2a cells, as monitored using flow cytometry. Collectively, these observations suggest that methionine oxidation accelerates the aggregation and enhances the neurotoxicity of the D178N variant, possibly providing direct evidence to link the pathogenesis of D178N-associated FFI with methionine oxidation.

1. Introduction

Prions are infectious pathogens that cause several invariably neurodegenerative diseases, including kuru, Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI), and Creutzfeldt–Jakob disease (CJD) in humans; bovine spongiform encephalopathy (BSE) in cattle; and scrapie in sheep [1,2]. The conversion of normal cellular prion protein (PrP^C) into a pathological infectious isoform (PrP^{Sc}) [3] lies at the heart of the disease pathogenesis. In contrast to the proteinase K (PK)-sensitive, α-helical monomeric PrP^C form, PrP^{Sc} is relatively resistant to proteinase K digestion and possesses a β-sheet-rich structure; moreover, PrP^C is soluble in detergent, whereas PrP^{Sc} is relatively insoluble in most detergents in the aggregated state [4,5].

Approximately 40 point mutations and polymorphisms in the human *PRNP* gene have been linked to inherited human prion diseases, including D178N-associated FFI. Fatal familial insomnia (FFI), which is the third most common hereditary prion disease worldwide, was first reported by Lugaresi [6] and is associated with a missense mutation of the prion protein (PrP) gene (*PRNP*) at codon 178 [7,8]. In a previous study, we identified several FFI pedigrees [9] in China. Numerous studies have reported that the combination of this D178N mutation in *PRNP* with a valine encoded at position 129 resulted in one of the subtypes of familial CJD (178CJD) [10,11]. However, the mechanism by which the D178N mutation induces FFI remains unclear.

Numerous studies have described an association between the pathogenicity of neurodegenerative disorders and oxidative stress [12–17]. Previous studies found the presence of methionine (Met)-oxidized PrP in PrP^{Sc} that was isolated from brain homogenates [18]. Canello et al. also detected that a large fraction of the Met residues in brain PrP^{Sc} were modified as methionine sulfoxides [19]. Recently, several studies using molecular simulation or experimental Met analogs have reported that Met oxidation may destabilize the structure of wild-type PrP and facilitate PrP^{Sc} formation [20,21]. Based on these studies, we inferred that methionine oxidation may play a special role in the pathogenicity of the disease-associated D178N variant. However, the relationship between methionine oxidation of the disease-associated D178N variant and its pathogenicity has thus far not been reported.

Abbreviations: PrP, prion protein; PrP^C, the normal cellular PrP; FFI, fatal familial insomnia; huPrP, human prion protein; HPLC, high performance liquid chromatography; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; LC, liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; CD, circular dichroism; DSC, differential scanning calorimetry; SDD-AGE, semi-denaturing detergent agarose gel electrophoresis; ThT, thioflavin T; ANS, 8-Anilino-1-naphthalen-sulfonic acid; PK, proteinase K; N2a, neuro-2a

* Corresponding authors at: State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, Hubei 430071, China. Tel./fax: +86 27 87198352.
E-mail addresses: xiaogf@wh.iov.cn (G. Xiao), Zhouzheng@wh.iov.cn (Z. Zhou).

http://dx.doi.org/10.1016/j.bbadis.2014.09.012
0925-4439/

As a common oxidant, H_2O_2 is routinely used to study Met oxidation in numerous proteins [21–23]. The methionine oxidation of PrP by H_2O_2 has previously been characterized using mass spectrometry [20,22,24]. Here, we investigated the potential effects of methionine oxidation on the aggregation of the FFI-associated D178N variant. Using several biophysical methods, we found that the D178N variant was more susceptible to oxidation than wild-type PrP and exhibited a greater propensity to form β-sheet-rich oligomers and aggregates. Oxidative treatment of N2a cells transfected with the D178N variant resulted in a large number of aggregates that exhibited increased proteinase K resistance and, subsequently, induced greater cell apoptosis.

2. Materials and methods

2.1. Plasmid construction and protein purification

The plasmid construction of recombinant human PrP23-231 (huPrP) was performed as described previously [25]. The D178N huPrP variant was constructed by site-directed mutagenesis using a Quick Change kit and the appropriate primers. Recombinant huPrP and D178N huPrP variant were expressed in *Escherichia coli* BL21 (DE3). The inclusion bodies were purified and refolded on a Ni-NTA-agarose column by decreasing Gdn-HCl gradient concentrations as described [26]. The purified protein was dialyzed overnight against 25 mM sodium phosphate buffer at pH 6.0. The concentrations of proteins were determined using the molar extinction coefficient (at 280 nm) of 57,995 M^{-1} cm^{-1}. The full-length human PrP1-253 gene was inserted into a eukaryotic expression vector pcDNA3.1 (+) (Invitrogen, USA) using EcoRI/XhoI sites. PrP1-22 (signal peptide) and PrP23-253 were separately fused in the pDsRed-monomer-N1 vector (Clontech, USA) using the restriction sites EcoRI/XhoI and NotI, respectively, to generate the sp-RFP-PrP for the expression of RFP fused PrP in cells. The plasmids D178N huPrP1-253 and D178N sp-RFP-PrP variant were also constructed by site-directed mutagenesis using a Quick Change kit and the appropriate primers.

2.2. Oxidation of PrP and reverse-phase HPLC analysis

Wild-type and D178N PrP were oxidized by incubation with hydrogen peroxide (H_2O_2). The concentration of PrP in the samples was 35 μM, and H_2O_2 was added to a final concentration of 50 mM. The reaction mixture was incubated at 37 °C for the indicated times. Samples were rapidly dialyzed against 25 mM sodium phosphate buffer at pH 6.0 to eliminate unreacted H_2O_2.

Reverse-phase HPLC was performed using an Agilent 1200 series HPLC system equipped with a C8 column (3.9 × 150 mm, 5 μm, Waters, USA). Samples of PrP oxidation reactions taken at various times were centrifuged at 15,000 rpm for 5 min and were subsequently loaded and eluted using a gradient beginning with solvent A (0.1% trifluoroacetic acid in ddH_2O) and then including an increasing percentage of solvent B (0.1% trifluoroacetic acid in acetonitrile). The gradient used was as follows: 0–10% solvent B in 5 min, 10–30% solvent B in 20 min, 30–40% solvent B in 5 min, and, finally, a return to 0% solvent B in 5 min.

2.3. Mass spectrometry

2.3.1. MALDI-TOF mass spectrometry

Oxidized and unoxidized D178N and wild-type PrP samples were dialyzed overnight against 25 mM sodium phosphate buffer at pH 6.0 and then desalted using C4 ZipTips (Millipore, MA) according to the manufacturer's protocol. Saturated sinapinic acid was used as the matrix for MALDI-TOF analysis and was prepared in an aqueous solution containing 45% acetonitrile and 0.1% trifluoroacetic acid. The sample was mixed with the matrix on a ground steel target plate and allowed to air-dry at room temperature prior to MALDI-TOF analysis. MALDI-TOF mass spectra were acquired using an UltraFlex I MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany).

2.3.2. Electrospray ionization mass spectrometry (ESI-MS)

During oxidation, aliquots of D178N variant and wild-type PrP samples were removed and analyzed in 15% SDS-PAGE gels using SDS loading buffer containing 4.5 M urea; the proteins were detected using GelCode Blue Stain Reagent (Thermo). After tryptic digestion, the peptide mixture was desalted using C18 ZipTips (Millipore, MA) according to the manufacturer's protocol. The extent of Methionine oxidation of oxidized D178N PrP samples was confirmed by high-resolution mass analysis using nano-ESI-based LC–MS/MS. All nano-ESI-based LC–MS/MS experiment were performed on a TripleTOF 5600+ System (AB SCIEX, Foster City, CA) coupled with a splitless Ultra 1D Plus (Eksigent, Dublin, CA) system. The desalted peptides were dissolved in 0.1% formic acid/2% acetonitrile/98% H_2O, loaded into a C18 trap column (5 μm, 5 × 0.3 mm, Agilent Technologies, Inc.) at a flow rate of 5 μL/min, and subsequently eluted from the trap column over the C18 analytic column 6, 9, 10 (75 μm × 150 mm, 3 μm particle size, 100 Å pore size, Eksigent) at a flow rate of 300 nL/min in a 100 min gradient. The mobile phase consisted of two components: component A was 3% DMSO/97% H_2O with 0.1% formic acid, and component B was 3% DMSO/97% acetonitrile with 0.1% formic acid. The IDA (information dependent acquisition) mode was used to acquire MS/MS. Survey scans were acquired in 250 ms and 40 product ion scans were collected in 50 ms/per scan. The precursor ion range was set from m/z 200 to m/z 1500, and the product ion range was set from m/z 100 to m/z 1500. Tandem mass spectra were extracted by Peakview version 2.0.

2.3.3. Analysis of MS data for protein identification

Analysis of the raw MS spectra generated by LC–MS/MS analyses were performed with the ProteinPilot 4.5 software program (AB SCIEX, USA) using the Paragon algorithm. The data analysis parameters were as follows: Sample type: Identification; Cys Alkylation: Iodoacetamide; Digestion: Trypsin; Instrument: TripleTOF 5600; Special Factors: Urea denaturation; ID Focus: Biological modifications, Amino acid substitution; Search Effort: Thorough ID; Detected Protein Threshold [Unused ProtScore (Conf)]: 1.3 (95.0%); Database: Uni-prot.

2.4. Secondary structure and thermal stability

2.4.1. CD spectroscopy

Circular dichroism spectra were obtained using a Chirascan CD spectrometer (Applied Photophysics, United Kingdom) using a 1-mm pathlength cuvette at 25 °C. Far-UV CD spectra were recorded from 195 to 250 nm. A protein concentration of 10 μM was used. Each spectrum represents an average of three scans that were corrected by subtracting a buffer blank. The experimental results were expressed in units of mean residue molar ellipticity (deg × cm^2 × d^{-1}) using the relationship $[\theta] = \theta_{obs} \times MRW/(10 \times l \times c)$, where MRW is the mean residue molecular weight, θ_{obs} is the observed ellipticity in degrees, c is the concentration in g/ml, and l is the path length in centimeters.

Equilibrium thermal-unfolding CD analysis of protein stability was performed at a protein concentration of 10 μM. The CD signal was monitored at 222 nm in the temperature range of 25–90 °C using a heating rate of 1 °C/min.

2.4.2. Differential scanning calorimetry (DSC)

The thermal stabilities of unoxidized and oxidized PrP samples were further monitored using a capillary cell Nano DSC (TA Instruments). All experiments used 45 μM protein in 25 mM sodium phosphate buffer at pH 6.0, and the samples were degassed prior to use. A heating rate of 1 °C/min was used, and the scanning was performed from 25 to 100 °C under nitrogen pressure. A baseline was obtained by scanning the buffer using an identical heating rate, which was subtracted from the experimental runs. The T_m value was obtained from curve fitting