Biophysical Chemistry 156 (2011) 140–145

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Salt bridges in prion proteins are necessary for high-affinity binding to the monoclonal antibody T2

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ARTICLE INFO

Article history:
Received 22 January 2011
Received in revised form 31 March 2011
Accepted 31 March 2011
Available online 8 April 2011

Keywords:
Prion protein
Salt bridges
Mutation
Conformation
Interaction
Antibody

ABSTRACT

We studied the role of the 2 salt bridges (Asp143–Arg147 and Asp146–Arg150) in helix 1 of mouse prion protein (PrP) on the formation of the complex between PrP and the monoclonal antibody T2. We introduced 6 charge-changing mutations to the amino acid residues associated with the salt bridges. Analysis of the circular dichroism spectra of the mutant PrPs showed that the salt bridge mutations did not change the secondary structures. We analyzed the kinetics of the association and dissociation of the PrPs with the T2 antibody. The results showed that the association kinetics were not significantly different among the variants except Arg150Lys, while the dissociation rate of the neutralized-charge variants was 2 orders of magnitude higher than that of the wild type. These results indicate that salt bridges make the interaction of PrP with T2 tighter by slowing down dissociation.

1. Introduction

Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Sheinker disease in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep and goats are lethal neurodegenerative illnesses [1,2]. All of these are prion diseases, and the main pathogenic factor involved in their development is considered to be an abnormal prion protein (PrP^{Sc}) generated by posttranslational modification of the host-encoded cellular prion protein PrP^{C} [3]. Although the mechanism of conversion from PrP^{C} to PrP^{Sc} has not been elucidated in detail, the conversion is known to be initiated by the contact of PrP^{C} with exogenous PrP^{Sc}. Diagnosis of prion diseases has thus far been carried out by immunohistochemical examination [4,5] and western blotting [6] to detect accumulated PrP^{Sc}. Various antibodies have been prepared and used for diagnosis and epidemiologic surveillance of prion protein diseases. Some anti-PrP^{C} antibodies can inhibit the conversion of PrP^{C} to PrP^{Sc} and the accumulation of PrP^{Sc} by interfering with the association of PrP^{C} with PrP^{Sc} [7–11]. Investigation of the mechanisms by which the antibodies recognize the prion proteins will be helpful for elucidating the mechanism underlying the conversion of PrP^{C} to PrP^{Sc}.

The anti-PrP monoclonal antibody T2 was developed from PrP-knockout mice immunized with recombinant mouse PrP120–230. This antibody has been used to detect BSE in Japan because it shows strong cross-reactivity with human and bovine PrPs [6]. Additionally, it was reported that the T2 antibody inhibits PrP^{Sc} accumulation in cultured cells [12]. Recently, we reported that this antibody recognized both a disulfide bond and salt bridges in PrP [13]. Nuclear magnetic resonance (NMR) analysis has revealed that the recombinant mouse PrP fragment PrP120–230 consists of 2 short β-strands (strand 1, 126–129 and strand 2, 159–162) and 3 α-helices (helix 1, 142–152; helix 2, 177–191; and helix 3, 198–215) [14]. The chemical bonds, a disulfide bond (Cys178–Cys213) and 2 salt bridges (Asp143–Arg147 and Asp146–Arg150), are important in maintaining the tertiary structure of PrP. For example, the disulfide bond connects helix 2 to helix 3. The conversion of PrP^{C} to PrP^{Sc} was found to be inhibited by reduction and alkylation treatment of the disulfide bond in a cell-free conversion system [15]. The salt bridges contribute to the stability of the helix more than the typical salt bridges found in most proteins. Helix 1 consists mainly of hydrophilic residues (DWEDRYYRENM) without hydrophobic contacts with other protein

Abbreviations: BSE, bovine spongiform encephalopathy; CD, circular dichroism; k_a, association rate constant; k_d, dissociation rate constant; K_D, dissociation constant; PAGE, polyacrylamide gel electrophoresis; PrP, prion protein; PrP^{C}, cellular prion protein; PrP^{Sc}, abnormal prion protein; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

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doi:10.1016/j.bpc.2011.03.011

regions. Without forming hydrophobic contacts with the protein core for stabilization, helix 1 is stabilized by the 2 internal salt bridges [16]. Molecular dynamics simulations have shown that a double mutant (mouse PrP: D146A and R150A) with alterations that affect the second salt bridge has increased conformational fluctuations [17]. In addition, it has been shown that the introduction of mutations to the salt bridges inhibits the conversion of PrP^{C} to PrP^{Sc} in a cell-free conversion system [18]. Our previous experiments have revealed that 4 mutations (C178M, C213M, D146N, and R150H) reduced PrP reactivity with the T2 antibody compared to wild-type PrP; in particular, the salt bridge mutants D146N and R150H were found to have almost completely lost reactivity to the T2 antibody [13]. Most specific antibodies, which inhibit the conversion and accumulation of PrP^{Sc}, interact with the helix 1 region of PrP^{C} [7–11], which is the case for the T2 antibody [13]. Therefore, characterizing the mechanisms by which the T2 antibody recognizes the salt bridges will provide an insight into the PrP^{C}-to-PrP^{Sc} conversion mechanism. We introduced mutations into the 4 residues that make up the 2 salt bridges (Asp143–Arg147 and Asp146–Arg150) and produced 6 mutants. In 4 of the 6 mutants, the aspartic acid and arginine were replaced with asparagine and histidine, respectively, to significantly reduce the charge (neutralized-charge variants: D143N, D146N, R147H, and R150H), while in the other 2 mutants, the aspartic acid and arginine were replaced with glutamate and lysine, respectively, (like-charge variants: D146E and R150K). We evaluated the secondary structure of the 6 mutants via circular dichroism (CD) studies. In addition, we analyzed the kinetics of the association and dissociation of the PrP fragment and its variants with the T2 antibody to examine the specific binding mechanisms. We found that the salt bridges in helix 1 affect the dissociation kinetics and dominate the affinity of the PrP fragment to the T2 antibody.

2. Material and methods

2.1. Antibody

The anti-PrP monoclonal antibody T2, which was used in all the experiments, was provided by Dr. T. Yokoyama of the National Institute of Animal Health (NIAH, Japan).

2.2. Expression of recombinant PrPs

The amino acid sequence used was based on that of mouse PrP. DNA for the wild-type fragment and PrP120–230 salt bridge mutants was synthesized by GenScript (NJ, USA), digested with *Eco*RI and *Xho*I restriction enzymes, and inserted into the pET22b(+) vector for periplasmic protein expression. After the nucleotide sequences were confirmed, the resulting expression plasmids were transformed in *Escherichia coli* BL21 (DE3) (Novagen, CA, USA). The transformants were cultured at 37 °C in 250 mL Luria–Bertani (LB) medium containing 50 μg/mL of ampicillin until an A_{595} of 0.4 was reached. The cells were then incubated at 25 °C for an additional 6 h in the presence of 0.1 mM isopropyl-β-D(−)-thiogalactopyranoside and pelleted by centrifugation.

2.3. Purification of recombinant PrPs from periplasmic extracts

The pelleted cells that expressed the wild-type fragment and His-tagged PrP120–230 salt bridge mutants were suspended in resuspension buffer (30 mM Tris–HCl, 20% sucrose). EDTA was added at a final concentration of 1.0 mM, and the suspension was rotated at 4 °C for 10 min. The cell lysates were centrifuged at 8000×*g* at 4 °C for 10 min. The pellets were collected, resuspended in 5 mM $MgSO_4$, rotated at 4 °C for 10 min, and centrifuged at 8000×*g* at 4 °C for 20 min. The periplasmic supernatants were collected and diluted with binding buffer (20 mM phosphate, 0.5 M NaCl, and 20 mM imidazole [pH 7.6]). The diluents were filtered through a 0.22-μm filter and loaded onto a 1-mL HisTrap-FF column (GE Healthcare, MA, USA) that was equilibrated with binding buffer. The His-tagged fragments were eluted with a linear gradient of 20–500 mM imidazole in binding buffer. The purified PrP fragments were dialyzed against phosphate-buffered saline (PBS) (pH 7.4) and stored at −30 °C. The purified PrP fragments were dissolved in sample buffer (2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, 62.5 mM Tris–HCl [pH 6.8]) and boiled at 95 °C for 5 min. The samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a denaturing running buffer (containing 0.1% SDS). The gel was stained with coomassie brilliant blue (Bio-safe Coomassie; Bio-Rad Laboratories, CA, USA) or transferred by electrophoresis to polyvinylidene difluoride (PVDF) membranes using a transfer buffer (without SDS). The blots were blocked in PBST (PBS with 0.05% Tween 20) containing 5% nonfat dry milk. After they were washed with PBST, the blots were incubated with the T2 antibody for 2 h and washed with PBST. Subsequently, they were incubated with anti-mouse IgG antibody conjugated to horseradish peroxidase for 1 h, washed with PBST, and developed using a chemiluminescent HRP substrate kit (Immobilon Western; Millipore, MA, USA), and the chemiluminescence signal was detected using Chemi-Stage CC16mini (KURABO, Osaka, Japan).

2.4. CD spectroscopy

CD spectroscopy was performed using a J820 CD spectropolarimeter (JASCO, Tokyo, Japan) in a 0.1-cm cuvette for far-UV CD spectra. Spectra were measured at protein concentrations of 0.1–0.25 mg/mL in PBS (pH 7.4) at 25 °C. The spectra were scanned from 200 nm to 250 nm 4 times with a 0.2-nm scanning interval and were corrected for the buffer.

2.5. Kinetic analysis by surface plasmon resonance

Kinetic analysis was performed using a Biacore J system (GE Healthcare, MA, USA). The T2 antibody was amine coupled to the CM5 sensor chip as instructed by the manufacturer (NHS/EDC coupling kit, GE Healthcare). Wild-type PrP120–230 and its salt bridge mutants were dissolved in HBS-EP buffer (0.01 M HEPES [pH 7.4], 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20) and injected as the analyte solution. All measurements were performed at a flow rate of 30 μL/min at 25 °C, and the interaction surface was regenerated with glycine-HCl (pH 1.5). Data were evaluated using the Biaevaluation software (version 4.1; GE Healthcare).

3. Results

3.1. Affinity of PrP120–230 salt bridge mutants to the T2 antibody as determined by western blot analysis

The T2 antibody recognizes 2 contiguous regions of the PrP120–230 fragment located far apart from each other along the primary structure, indicating that the antibody recognizes the tertiary structure of PrP120–230 [13]. Helix 1 of mouse PrP is located in 1 of these recognition regions, which has 2 salt bridges (Asp143–Arg147 and Asp146–Arg150) [14]. In our previous study, we reported that the mutations introduced into Asp146 and Arg150 decrease the reactivity of the resultant protein with the T2 antibody [13]. To systematically analyze the influence of these 2 salt bridges on the recognition of the PrP120–230 fragment by the T2 antibody, we introduced mutations into the 2 salt bridge residues (Asp143–Arg147 and Asp146–Arg150) to change their charges to be neutral or significantly reduced (Fig. 1(a)). The amino acids of the first salt bridge, Asp143 and Arg147, were replaced with residues with a neutral charge (Asp to Asn or Arg to His), and the amino acids of the second salt bridge, Asp146 and Arg150, were replaced with neutrally (Asp to Asn or Arg to His) or similarly charged residues (Asp to Glu or Arg to Lys). The periplasmic extract of the recombinant proteins was