pH-dependent Prion Protein Conformation in Classical Creutzfeldt-Jakob Disease*

Received for publication, August 14, 2001, and in revised form, August 27, 2001

Published, JBC Papers in Press, September 19, 2001. DO1 10.1074/jbc.C100458200

Gianluigi Zanusso‡, Alessia Farinazzo§, Michele Fiorini‡, Matteo Gelati‡, Annalisa Castagna§, Pier Giorgio Righetti§, Nicola Rizzato‡, and Salvatore Monaco‡

From the ‡Department of Neurological and Visual Sciences, University of Verona, Piazzale L. A. Scuro, 10, 37134 Verona, Italy and the §Department of Agricultural and Industrial Biotechnologies, University of Verona, Strada Le Grazie 15, 37134 Verona, Italy

In transmissible spongiform encephalopathies, the cellular prion protein (PrP C) undergoes a conformational change from a prevailing α-helical structure to a β-sheet-rich, protease-resistant isofrom, termed PrP Sc. PrP C has two characteristics: a high affinity for Cu 2+ and a strong pH-dependent conformation. Lines of evidence indicate that PrP Sc conformation is dependent on copper and that acidic conditions facilitate the conversion of PrP C → PrP Sc. In each species, PrP Sc exists in multiple conformations, which are associated with different prion strains. In sporadic Creutzfeldt-Jakob disease (sCJD), different biochemical types of PrP Sc have been identified according to the size of the protease-resistant fragments, patterns of glycosylation, and the metal-ion occupancy. Based on the site of cleavage produced by proteinase K, we investigated the conformational stability of PrP Sc under acidic, neutral, and basic conditions in 42 sCJD subjects. Our study shows that only one type of sCJD PrP Sc, associated with the classical form, shows a pH-dependent conformation, whereas two other biochemical PrP Sc types, detected in distinct sCJD phenotypes, are unaffected by pH variations. This novel approach demonstrates the presence of three types of PrP Sc in sCJD.

The mammalian prion diseases are a group of fatal neurodegenerative disorders that exhibit sporadic, inherited or infectious presentation (1). These disorders are characterized by the accumulation in affected brains of a misfolded pathogenic isoform of host-encoded cellular prion protein (PrP C), which is insoluble in non-denaturing detergents and partially resistant to proteolysis (2). PrP Sc and PrP C differ in their secondary and tertiary structures, but not in the amino acid sequence (3), as an effect of increased β-sheet content and decreased α-helices in the pathological protein (4, 5).

PrP Sc is a glycosylphosphatidylinositol-anchored glycoprotein (6) with two potential N-linked glycosylation sites, encoded by a single gene located on human chromosome 20 (7). In its mature form the human protein spans residues 23–231 of the polypeptide predicted from cDNA sequence and exists under diglycosylated, monoglycosylated, and unglycosylated species. PrP Sc is composed of an N-terminal unstructured domain, which contains four copies of an octapeptide sequence (PHGGGGWQ) between residues 60 and 91, and a C-terminal region with two short β-sheet structures of four residues each and three α-helical regions (5). Previous studies have identified the octarepeat sequence as a Cu 2+ binding motif and have assessed that, in the presence of copper, each octapeptide forms an α-helix and acts as an α-helical formation promoter on the region comprising residues 84–103 (8). Copper binding stoichiometry is pH-dependent: four octapeptides chelate four Cu 2+ at pH 7.4, but bind only two Cu 2+ at pH 6. Under acidic conditions, Cu 2+ is bound by two histidine residues of different octapeptides, whereas the His-triglycine segment of a single octapeptide represents the binding site at pH 7.4 (9). The effect of pH on PrP C conformation is not, however, limited to its influence on Cu 2+. At low pH, recombinant forms of human 231 (10) and murine 121–231 (11) PrP C undergo conformational changes, with an increase in β-sheet content and loss of α-helical structures.

Within a single mammalian species, PrP Sc exists under multiple conformations that are associated with distinct disease phenotypes. Upon experimental transmission, prion strains are distinguished according to differences in incubation time, regional distribution of cerebral lesions, and the pattern of proteinase K (PK) 1-induced proteolytic cleavage of PrP Sc (12). Biochemical evidence for the notion that PK cleaves the N-terminal region of PrP Sc at different sites in different strains has been obtained so far both in animal (13) and human (14–16) prion diseases. In addition, based on the occupancy of the N-glycosylation sites or the ratio of the three PK-resistant PrP Sc glycoforms (diglycosylated, monoglycosylated, and unglycosylated), further biochemical types of PrP Sc have been classified (14). Decoding the biochemical signature of individual prion strains may allow for a more precise molecular classification and may reveal environmental risk factors into human disorders with unknown etiology, such as sporadic Creutzfeldt-Jakob disease (sCJD).

In earlier large scale studies, conflicting results have been obtained on the types of PrP Sc in sCJD. Parchi et al. (15, 17) have proposed a molecular classification of sCJD based on the analysis of PrP codon 129, encoding either methionine (M) or valine (V), and the physicochemical properties of two types of the PK-resistant PrP Sc, as resolved after an SDS 13% polyacrylamide gels electrophoresis (SDS-PAGE) separation: type 1, with a core fragment (the PK-resistant PrP Sc polypeptide backbone after removal of sugar chains) of 21 kDa, and type 2, migrating 2 kDa faster. On the contrary, Wadsworth et al. (18) were able to characterize three PrP Sc types after separation on 16% Tris-glycine gels, with type 1 found in subjects of the MM

* The abbreviations used are: PK, proteinase K; sCJD, sporadic Creutzfeldt-Jakob disease; PNGase F, N-glycosidase F; PAGE, polyacrylamide gel electrophoresis; FL, full-length; T, truncated.
genotype, type 2 in all genotypes, and type 3 only in MV or VV genotypes. These three types of PrPSc are further distinguished based on their metal-ion occupancy. Type 1 and type 2 PrPSc upon metal-ion chelation undergo conformational changes that expose an apparently common PK cleavage site, whereas type 3 is unaffected by metal chelators (18). Given the well-known influence of pH on PrPSc conformation and the lack of reports addressing the influence of pH on the conformational stability of PrPSc, we undertook a biochemical study on sCJD PrPSc in an attempt to assess pH-dependent strain-specific conformations.

EXPERIMENTAL PROCEDURES

Brain Tissues—Forty-two subjects with neuropathologically confirmed diagnosis of sCJD were investigated in the present study. In all patients genomic DNA was extracted from frozen brain tissues, and the prion protein gene was sequenced as described previously (19).

Homogenization and Fractionation—Five samples of ~10 mg each were obtained from the frontal cortex, occipital cortex, striatum, and cerebellum of each subject investigated. Each sample was dissolved in 9 volumes of lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris) at pH values 4, 6, 7.4, and 8, respectively, and clarified by centrifugation at 1,000,000 x g for 10 min. The supernatant was stored at −80 °C until use, whereas the pellet was discarded. Detergent-insoluble fractions were obtained by two cycles of centrifugation at 100,000 x g for 90 min, as reported previously (20).

Proteinase K and N-Glycosidase F Treatments—The protease resistance was assayed by incubating sample aliquots, containing 40 μg of total protein, with 100 μg/ml of PK (Roche Molecular Biochemicals) for 1 h at 37 °C. The digestion was blocked by the addition of phenylmethylsulfonyl fluoride to 3 mM. Samples were deglycosylated with PNGase F according to the manufacturer’s instruction (Roche Molecular Biochemicals).

Immunoblots—Proteins were denatured by boiling in SDS sample buffer, separated in 13% SDS-PAGE gels, and then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) for 2 h at 60 V. Membranes were blocked with 1% nonfat dry milk in TBS (TBST with 0.05% Tween 20) and probed with 3F4 antibody (Dako, Denmark), recognizing human PrP residues 109–112, and 582 (21), directed to N-terminal residues 23–40. After washing the membranes were incubated with a peroxidase-conjugated anti-mouse IgG, developed using a chemiluminescent substrate (ECL, Amersham), and visualized using Biomax MR films (Eastman Kodak Co.).

RESULTS

We first used conventional Western blot methods, with lysis buffer at pH 7.4, to analyze protease-resistant PrPSc in all cases. Based upon the banding pattern of the three PK-resistant PrPSc species and the apparent molecular mass of the core fragment, two types of PrPSc were observed. In 24 subjects (MM and VV genotypes), PK-cleavage products of the three PrPSc glycoforms migrated at ~30, 27, and 21 kDa (Fig. 1, lane 1), all of them shifting to a 21-kDa zone upon deglycosylation (Fig. 1, lane 3), whereas in 18 subjects (MM, MV, VV genotypes) PK-resistant PrPSc species were resolved at ~28, 26, and 19 kDa (Fig. 1, lane 2), with enzymatic deglycosylation yielding a band at ~19 kDa (Fig. 1, lane 4). When we investigated the physicochemical properties of these two types of PrPSc at pH values other than 7.4, three patterns could be distinguished, based on the size of PK cleavage products.

In 15 subjects (MM and VV) with the 21-kDa PrPSc, hereafter designated as group 1, the cleavage products of the three PrPSc bands, corresponding to the di-, mono-, and unglycosylated PK-resistant PrPSc, as obtained at pH 4 and 6, had a molecular mass value ~1.5 kDa higher than digestion fragments generated at pH 7.4; conversely, when the samples were solubilized in lysis buffer at pH 8, the three PK-resistant PrPSc glycoforms showed a faster migration, corresponding to an additional loss of ~1 kDa as compared with species generated at pH 7.4 (Fig. 2, A and B). The possibility of a direct pH influence on the electrophoretic mobility of PrPSc was ruled by exposing PK-treated and untreated samples to low and high pH before electrophoretic separation. Subjects of group 1 had a distinct clinical course with early dementia, myoclonus, and periodic sharp-wave electroencephalographic activity, overlapping the typical myoclonic sCJD variant, or cortical visual disturbances, consistent with the Heidenhain variant. These two sCJD variants represent clinical phenotypes of the classical form (17).

In marked contrast, in 9 subjects, MM genotypes and termed group 2, with the PK-resistant fragment of 21 kDa, the migration of digestion products was insensitive to variations in pH (Fig. 2, C and D). In these cases the three glycoforms migrated to zones of ~30, 27, and 21 kDa either at low or high pH. At variance with group 1, subjects of this group had an ataxic-dementia sCJD variant of longer duration. Our findings suggest that the physicochemical properties of PrPSc in group 1 and group 2, although apparently similar when examined at pH 7.4, may be further distinguished upon conformational changes induced by acidic and basic pH values.

In sCJD subjects (MM, MV, and VV genotypes, group 3) with
resistant PrPSc types, disease phenotype and codon 129 genotype, but not from group 1 (lane 2), there are significant amounts of truncated PrPSc fragments.

The results obtained in each single subject were highly reproducible in at least three repetitions for a given sample and were detected in all investigated brain regions.

To confirm that changes in migration similarly affected glycosylated and unglycosylated PrPSc species, we examined PK digestion products after removal of sugar chains. As expected, changes in pH affected only the migration of the PrPSc core fragment of group 1 subjects (Fig. 2G), whereas the size of the PrPSc core fragment of group 2 (Fig. 2H) and 3 (Fig. 2I) subjects was unchanged.

To gain insight into whether naturally occurring species of the PrPSc types detected in the three groups differed in their polypeptide backbone, detergent-insoluble PrPSc-enriched fractions were prepared from the frontal cortex of each case and analyzed by immunoblot. In group 1 subjects, the insoluble PrPSc had an intact N-terminal region, as suggested by comparable signal intensities with 3F4 and 5B2 antibodies (Fig. 3, lanes 1 and 2). Conversely, the remaining cases of groups 2 and 3 were all characterized by prevailing amounts of truncated PrPSc fragments, as suggested by a more intense signal with 3F4 (Fig. 3, lanes 4 and 6) as compared with anti-N antibody (Fig. 3, lanes 3 and 5), reflecting the presence of additional bands which lack the N-terminal region. These results suggest that the PrPSc is mostly present under full-length isoforms in group 1, whereas it is mostly recovered under N-terminally truncated fragments in groups 2 and 3. Accordingly, we designated the three types of PrPSc as type-1FL (full-length), type-2T (truncated) and type-3T.

In summary, the PrPSc bands of group 1, named type-1FL, naturally occur as full-length species, change in conformation over the pH range 4–8, and migrate to a 21-kDa zone after PK treatment at pH 7.4 and enzymatic deglycosylation. Conversely, type-2T of group 2 and type-3T detected in group 3 mostly exist as N-terminally truncated fragments, show no response to pH, and generate PK-resistant PrPSc core fragments of 21 and 19 kDa, respectively.

Based on physicochemical properties of our three PrPSc types under the same experimental conditions of Parchi et al. (15, 17) (see all lanes at pH 7.4 of Fig. 2, A–I), it is evident that both type-1FL and type-2T correspond to their type 1, whereas the type-3T matches their type 2.

Regarding the classification of Wadsworth et al. (18) no appropriate biochemical relation can be defined due to different experimental conditions.

Although caution is dictated, our present results point to differences in the polypeptide backbone of the naturally occurring PrPSc, which contains the N terminus in group 1, whereas is mostly N-terminally truncated in the other two groups. This suggests that the effect of pH on the different types of PrPSc is strictly dependent on the presence of the N-terminal portion of the protein. Based on the present observations, it can also be suggested that, at variance with the N-terminal region, the conformation of the C-terminal portion of PrPSc is apparently insensitive to changes in pH. This property further differentiates PrPSc from PrPc (10).

As an alternative explanation, both Cu2⁺ binding at the N-terminal region could be simultaneously responsible for changes observed in type-1FL PrPSc; it is of interest to note that the 19-kDa core fragment, treatment at different pH values did not affect the molecular masses of PrPSc species generated by PK digestion (Fig. 2, E and F).

The results of the present study show that the conformation of the PrPSc associated with the classical form of sCJD, encompassing the myoclonic and Heidenhain variant, is dependent on pH and clearly distinguishable from the pathological isoforms detected in two other sCJD phenotypes. Interestingly, this distinctive physicochemical property clearly differentiates two PrPSc types, namely type-1FL and type-2T, found in classical sCJD and in the ataxic-dementing variant, respectively, which, upon conventional Western blot analyses, share the migration of the three glycoforms and the size of the PrPSc core fragment. Although having in common the codon 129 genotype, subjects with type-2T PrPSc show different clinical presentation and disease duration significantly longer than patients with type-1FL PrPSc (data not shown), thus supporting the biochemical detection of two PrPSc strains.

Recent sequencing studies have shown that the major PK cleavage site of the 21-kDa isoform associated with sCJD occurs at Gly-82 (22), although different secondary cleavages are observed depending on the genotype. Accordingly, in MM and MV genotypes a secondary cleavage is observed at Gly-78, whereas in VV subjects additional N-terminal species, starting at Gly-86, are detected (22). Interestingly, all these cleavage sites occur in the region of the third and fourth octapeptide, a region that under normal and pathological conditions may bind Cu²⁺. On the contrary, N-terminal species of the 19-kDa isoform start at Gly-97, in a Cu²⁺-free post-octapeptide domain.

Two physicochemical properties of the PrPSc associated with classical sCJD are: the acquisition of its conformation in the presence of copper (18) and a pH-dependent conformational stability (present study). Upon metal-ion chelation (in a lysis buffer at pH 7.4 and with Cu²⁺-chelators titrated to pH 8) (18), PK digestion products have molecular masses lower than PK-resistant PrPSc species obtained in the absence of metal chelators, thus suggesting that removal of Cu²⁺ induces a loss of β-sheet conformation in the octapeptide region. Our results, showing an increase in molecular masses of PK-resistant species at low pH and a decrease at pH 8, speak in favor of an increase in β-sheet conformation under acidic conditions and a loss of β-sheets under basic conditions. Therefore, the expected loss of two Cu²⁺ ions per PrPSc molecule at low pH is not in keeping with the present findings, unless, as demonstrated at pH 6 for the normal octapeptide motif (9), residual Cu²⁺ ions are shared between two histidine residues of different octapeptide chains. However, the presumed effect of pH on Cu²⁺ binding does not fully explain results obtained at pH 4 and 8. Although it remains to be determined whether either the stoichiometry or the type of Cu²⁺ binding are maintained after the conversion of PrPc into PrPSc, our result may suggest a direct effect of pH on PrPSc conformation.

Why Is PrPSc conformation pH-sensitive only in one type of the sCJD-associated proteins?

Although caution is dictated, our present results point to differences in the polypeptide backbone of the naturally occurring PrPSc, which contains the N terminus in group 1, whereas is mostly N-terminally truncated in the other two groups. This suggests that the effect of pH on the different types of PrPSc is strictly dependent on the presence of the N-terminal portion of the protein. Based on the present observations, it can also be suggested that, at variance with the N-terminal region, the conformation of the C-terminal portion of PrPSc is apparently insensitive to changes in pH. This property further differentiates PrPSc from PrPc (10).
the pattern of progressive fragmentation in going from pH 4 to pH 8 (see G) follows the titration curve of the imidazole ring in His. Assuming a $pK_a = 6.5,$ the latter would be fully protonated at pH 4 and extensively protonated ($70\%$) at pH 6.0, but would only be $11\%$ protonated at 7.4 and would bear only $5\%$ positive charge at pH 8.0. The extent of His protonation, together with possible differences in the polypeptide backbone, might be responsible for the conformational transitions occurring in the type-1 PrPSc reported here.

Taken together, our data show evidence of three PrPSc types in our unselected consecutive series of sCJD patients and provide a novel reproducible method to differentiate PrPSc types in prion diseases.

REFERENCES

pH-dependent Prion Protein Conformation in Classical Creutzfeldt-Jakob Disease
Gianluigi Zanusso, Alessia Farinazzo, Michele Fiorini, Matteo Gelati, Annalisa Castagna,
Pier Giorgio Righetti, Nicola Rizzuto and Salvatore Monaco

doi: 10.1074/jbc.C100458200 originally published online September 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.C100458200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 22 references, 9 of which can be accessed free at http://www.jbc.org/content/276/44/40377.full.html#ref-list-1