Probing the Conformation of the Prion Protein within a Single Amyloid Fibril Using a Novel Immunoconformational Assay*

Vera Novitskaya1, Natallia Makarava1, Anne Bellon1, Olga V. Bocharova1, Igor B. Bronstein1, R. Anthony Williamson2, and Ilia V. Baskakov1

From the 1Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, Maryland 21201, 2Department of Biochemistry and Molecular Biology, University of Maryland, Baltimore, Maryland 21201, and the 6Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, the 6Institute for Animal Health, Compton Laboratories, Compton RG20 7NN, United Kingdom

The coexistence of multiple strains or subtypes of the disease-related isofrom of prion protein (PrP) in natural isolates, together with the observed conformational heterogeneity of PrP amyloid fibrils generated in vitro, indicates the importance of probing the conformation of single particles within heterogeneous samples. Using an array of PrP-specific antibodies, we report the development of a novel immunoconformational assay. Uniquely, application of this new technology allows the conformation of multimeric PrP within a single fibril or particle to be probed without pretreatment of the sample with proteinase K. Using amyloid fibrils prepared from full-length recombinant PrP, we demonstrated the utility of this assay to define (i) PrP regions that are surface-exposed or buried, (ii) the susceptibility of defined PrP regions to GdnHCl-induced denaturation, and (iii) the conformational heterogeneity of PrP fibrils as measured for either the entire fibrillar population or for individual fibrils. Specifically, PrP regions 159–174 and 224–230 were shown to be buried and were the most resistant to denaturation. The 132–156 segment of PrP was found to be cryptic under native conditions and solvent-exposed under partially denaturing conditions, whereas the region 95–105 was solvent-accessible regardless of the solvent conditions. Remarkably, a subfraction of fibrils showed immunoreactivity to PrPSc-specific antibodies designated as IgGs 89–112 and 136–158. The immunoreactivity of the conformational epitopes was reduced upon exposure to partially denaturing conditions. Unexpectedly, PrPSc-specific antibodies revealed conformational polymorphisms even within individual fibrils. Our studies provide valuable new insight into fibrillar substructure and offer a new tool for probing the conformation of single PrP fibrils.

Misfolding and aggregation of the prion protein (PrP) has been linked to several fatal neurodegenerative diseases, including Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker disease, and fatal familial insomnia (1). Prion maladies manifest themselves in sporadic, familial, or infectious forms (2). These diseases, including sporadic CJD, display substantial variations in clinical symptoms, in neuropathological profile, and in age at onset of disease (3, 4). This broad pathological and clinical heterogeneity is believed to be related, at least in part, to conformational variations in the disease-related isoforms of PrP (PrPSc).

Numerous studies have shown that different strains of transmissible spongiform encephalopathy are also linked to conformational differences within the PrPSc isoform (5–10). Coexistence of multiple types of PrPSc was recently shown in patients with sporadic CJD (11, 12) and in variant CJD (13).

The coincidence of multiple prion strains or subtypes in natural prion isolates demands the development of an assay that is able to assess the conformational heterogeneity in mixtures of abnormal PrP isoforms. Over the past decade, a variety of biochemical and immunological assays have been established that discriminate PrPSc from PrPc and distinguish different strains or subtypes of PrPSc.

These assays recognize different conformers of PrPSc by the extent of PK resistance, the size of the PK-resistant core, thermodynamic stability, or epitope presentation (7, 8, 14–18). All previously developed assays, however, assess bulk properties of PrP molecules averaged across the whole molecular population but not the conformation of individual fibrils or particles. When multiple strains or subtypes of PrPSc are present as a mixture, analysis of such samples is difficult and often produces conflicting results (3, 4, 12, 19).

Similar to the high heterogeneity of PrPSc subtypes generated in sporadic CJD, spontaneous polymerization of PrP in vitro under single growth conditions produces a range of amyloid fibrillar types (20). The specific conformational differences between fibrillar types have not yet been determined. Elucidation of the physical properties and conformational heterogeneity of individual synthetic fibrils is very important, considering that only a very small subfraction of fibrils produced in vitro appear to be infectious (21). Although the fibrillar form of recombinant PrP was shown to induce transmissible prion disease in animals (21, 22), our current knowledge about the specific structural features that underlie prion infectivity is very limited.

In recent studies, solid state NMR was used to determine the three-dimensional structure of fibrils produced from several amyloidogenic...
polypeptides, including Aβ(1–42) (23, 24), yeast prion Ure2p peptide 10–39 (25), and fungal prion HET-s peptide 218–289 (26). To date, the solid state NMR studies have been restricted to relatively short polypeptides that produce conformationally homogeneous well defined fibrils. The large size of full-length PrP molecules in combination with the highly heterogeneous nature of PrP fibrils limits the number of physical techniques that can be used for assessing the conformation of aggregated states of PrP. Antibodies have previously proved to be an informative tool for monitoring the structural transition from PrPSc to PrPSc and for probing the conformation of aggregated disease-specific isoforms of PrP (14, 15, 27–29). Using an array of specific antibodies recognizing different PrP epitopes and PrP conformations, we have developed a novel immunoconformational assay referred to as the dual color assay. In contrast to previously established techniques, the newly developed assay probes the conformation within a single fibril or particle of aggregated PrP. Using amyloid fibrils prepared from full-length recombinant PrP, we demonstrated the utility of this assay to define (i) the regions of PrP that are surface-exposed or buried in aggregated forms of the protein, (ii) the susceptibility of defined PrP regions to GdnHCl-induced denaturation, and (iii) the conformational heterogeneity of fibrils measured across either the whole fibrillar population or within individual fibrils.

MATERIALS AND METHODS

Protein Expression, Purification, and Conversion into Amyloid Fibril—Full-length mouse recombinant PrP encompassing residues 23–230 (rPrP) was expressed and purified as described earlier (30). The purified rPrP was confirmed by SDS-PAGE, analytical size exclusion chromatography, and electrospray mass spectrometry to be a single monomeric species with an intact disulfide bond. In vitro conversion of rPrP to amyloid fibrils was carried out under standard conditions (in 1 M GdnHCl, 3 M urea in 20 mM sodium acetate buffer, pH 5.0, at 37 °C) as previously described (30). At the end point of conversion (typically 24 h of incubation) the reaction was stopped, and rPrP fibrils were dialyzed induced denaturation, and (iii) the conformational heterogeneity of fibrils measured across either the whole fibrillar population or within individual fibrils.

Assessing Conformation within a Single PrP Fibril

FIGURE 1. A schematic representation of full-length rPrP 23–230. The known binding epitopes of Fabs D13, P, D18, R1, and Abs AH6 and AG4 are highlighted in light gray. When converted into fibrillar form, the PK-resistant cores consist of residues 138–230, 152–230, and 162–230, highlighted as dark gray boxes (31). Fabs 89–112 and 136–158 were generated by grafting mouse PrP sequences that correspond to amino acid residues 88–111 and 135–157, respectively, to recipient Ab (32).

primary antibodies was in TBS containing 0.25% Triton X-100, 5% normal horse serum, and 1% bovine serum albumin, and staining with the secondary antibodies was done in TBS containing 0.25% Triton X-100 and 2% bovine serum albumin. All antibodies were centrifuged for 10 min at 10,000 rpm prior to staining. 0.25% Triton X-100 was present throughout the whole staining procedure. Slides were mounted with antifade fluorescence mounting medium (DAKO, Denmark).

Immunofluorescence Imaging and Data Analysis—Fluorescence microscopy was carried out on an inverted microscope (Nikon Eclipse TE2000-U) with an illumination system X-Cite 120 (EXFO Photonics Solutions Inc.) connected through fiber optics using a 1.3 aperture Plan Fluor ×100 numerical aperture and ×60 objectives. Digital images were acquired using a cooled 12-bit CoolSnap HQ CCD camera (Photometrics). The exposure time for AG4 was 700 ms; for D13, D18, 89–112, and 136–158 it was 1400 ms; for P it was 800 ms; and for R1 and AH6 it was 1600 ms. The excitation irradiance was reduced twice when images were collected with a ×60 objective.

Images collected from the two channels were processed with WCIF Image software (National Institutes of Health). Manders’ Coefficients and Colocalization Threshold plugins were used to obtain two-dimensional fluorescence intensity scattering plots (2D-FIS). The colocalization threshold plugin was also used to calculate the values of linear regression slopes (LRS). S.D. of LRS values were calculated from the analysis of 3–5 images from the same experiment. Analysis of different subpopulations of fibrils observed after staining with Abs 89–112 or 136–158 was performed using the Colocalization Finder plugin, which allowed us to highlight and select the fibrils with a specified ratio of intensities measured in the “red” and “green” channels. Intensity profiles of single fibrils were built with the RGB profiler plugin.

RESULTS

Design of the Dual Color Immunoconformational Assay—Our assay consisted of double staining using different pairings of PrP-specific antibodies, where the reference Ab was specific to the epitope that is solvent-accessible, but the second Ab was specific to the epitope for which we wanted to assess the conformation (Fig. 2a). Because in PrPSc and in the fibrillar form of rPrP, the N-terminal region encompassing residues 23 to ~90 is proteinase K-sensitive and solvent-accessible (31), we used AG4 antibody specific to the epitope 37–59 as a reference Ab (Fig. 1). AG4 was used in pairs with one of the following Abs: D13 (epitope 95–105), D18 (epitope 132–156), R1 (epitope 224–230), 89–112, or 136–158 (Fig. 1, Table 1). IgGs 89–112 and 136–158 were previously shown to bind specifically to PrPSc but not to PrPSc (32). These Abs seem to recognize nonlinear epitopes, the locations of which are currently unknown (Fig. 1) (32). The secondary Ab to AG4 was labeled with Alexa-546 (red), and the secondary Ab to the remaining PrP specific Abs was labeled with Alexa-488 (green) (Fig. 2, a and b). When fluorescence microscopy was used for imaging, the double staining and subsequent
**Assessing Conformation within a Single PrP Fibril**

**TABLE 1**
The reactivity of the antibody to the amyloid fibrils as probed in the dual color assay

| Ab       | Epitope | Reference Ab | Immunoreactivity |
|----------|---------|--------------|------------------|
|          |         |              | No GdnHCl | 4 M GdnHCl |
| D13      | 95–105  | AG4          | +++       | +++       |
| D18      | 132–156 | AG4          | –         | ++        |
| AH6      | 159–174 | P            | –         | –/+       |
| R1       | 224–230 | AG4          | –         | –/+       |
| 89–112   | PrPSc-specific | AG4      | +++       | +/+       |
| 136–158  | PrPSc-specific | AG4      | +++       | +/+       |

*a The reactivity of IgG 89–112 is presented for the subpopulation of 89–112-positive fibrils.

*b The reactivity of IgG 136–158 is presented for the subpopulation of 136–158-positive fibrils.

---

**FIGURE 2. Schematic representation of a dual color assay.**

- **a**: schematic representation of an amyloid fibril illustrating that the N-terminal region referred to as N (residues 23 to ~90) is solvent-exposed, whereas the C-terminal region (residues ~90–230) is assembled into a fibrillar structure. The epitopes to AG4 (residues 37–50) were used as the reference epitope, the immunoreactivity of which was monitored in the red channel. The epitopes within the region ~90–230 are partially or fully hidden; their immunoreactivities were monitored in the green channel. Shown are examples of fluorescence microscopy images of amyloid fibrils stained with AG4/D13 (b) or with AG4/89–112 (c). The upper panels show images collected in the red channel (AG4); middle panels show images collected in the green channels (D13 or 89–112); and lower panels show the merged images. Scale bars, 15 μm.

---

merge of images collected in the red and green channels provided information regarding the solvent accessibility of the epitope of interest within individual fibrils (Fig. 2, b and c). A predominantly red color in the merged images indicated that the epitope of interest was largely buried; predominantly green color meant that the epitope of interest was solvent-accessible; whereas different tints of yellow and orange reflect slight differences in the accessibility of epitopes to the solvent (Fig. 2, b and c, bottom panels).

In the Fibrillar Form, the Epitope 95–105 Was Solvent-accessible, whereas the Epitope 132–156 Was Buried—Double staining of amyloid fibrils with AG/D13 revealed that under native conditions the epitope 95–105 was solvent-accessible (Fig. 3a). To analyze the fluorescence intensities in more detail, the microscopy images were transformed into 2D-FIS plots, where red fluorescence intensities are plotted on the horizontal axis, and the green intensities are plotted on the vertical axis (see insets to Fig. 3). As evident from the 2D-FIS plots, the amyloid fibrils consisted of a single population, which was relatively homogeneous with respect to the accessibility of the D13 epitope. From the 2D-FIS plot, the LRS can be calculated, a parameter that we used to measure the intrinsic accessibility of a particular epitope to a solvent. The LRS value of 1.307 ± 0.073 indicates that the D13 epitope is solvent-exposed under native conditions (Fig. 3d).

The reactivity of D18 to PrP amyloid fibrils was substantially lower than that of D13, as reflected by the predominantly red color in microscopy images and by the low value of the LRS (0.349 ± 0.037) calculated from the 2D-FIS plot (Fig. 3e). Taken together, these data show that the epitope 132–156 was predominantly buried under the native conditions, whereas the epitope 95–105 was solvent-accessible (Table 1).

To determine whether partial denaturation alters the accessibility of 95–105 and 132–156 epitopes, the fibrils were exposed to 4 M GdnHCl for 1 h prior to the dual color assay. We previously showed that the C1/2 value for the GdnHCl-induced denaturation of amyloid fibrils produced in vitro was 4.2 M (31); therefore, at 4 M GdnHCl, the amyloid fibrils were expected to preserve most of the cross-β-sheet structure; however, some of the fibrillar regions could undergo local unfolding. Upon partial denaturation, the color distribution in samples stained with D18 shifted toward the “green” sector, as was evident from significant shift of the LRS value from 0.349 ± 0.037 to 0.845 ± 0.139 and from the broadening of the fluorescence intensity distribution in the 2D-FIS plot (Fig. 3d). These changes indicated that the D18 epitope that was cryptic under the native conditions became partially exposed to the solvent upon partial denaturation. In contrast to the D18 epitope, the accessibility of the noncryptic D13 epitope remained the same (LRS value unchanged) regardless of whether amyloid fibrils were preincubated with GdnHCl (Fig. 3, compare a and c, Table 1).

Highly Denaturing Conditions Were Required to Expose Epitopes 159–174 and 224–230—Our former studies revealed that in the fibrillar form, the PK-resistant cores are constructed from the C-terminal regions that encompass residues 152–230 and 162–230 (31). These C-terminal regions are believed to compose the most thermodynamically stable cross-β-sheet structures of fibrils. To test the accessibility of epitopes within the C-terminal region, we used IgG AH6 and Fab R1, which bind to the residues 159–174 and 224–230, respectively (Fig. 1). Here, to avoid unwanted cross-reactivity with the secondary Ab to IgG, IgG AG4 (red channel) was used as a reference in a pair with Fab R1 (green channel), and Fab P (epitope 95–105, red channel) was paired with IgG AH6 (green channel) (Table 1).

When stained with either R1 or AH6, the amyloid fibrils showed no detectable immunoreactivity in the green channels in the absence of GdnHCl, suggesting that both epitopes were completely buried in the fibrillar interior (Fig. 4, a and b, left panels). After incubation with 4 M GdnHCl, a subfraction of fibrils displayed a dotted pattern of fluorescence in the green channels when stained with either R1 or AH6 (Fig. 4, a and b, middle panels). Such dotted patterns could be due to the local exposure of epitopes to R1 or AH6 occurring at the fibrillar edges, at the sites of occasional fibrillar bending or fragmentation, or at the junction of several fibrils. The dotted pattern observed with R1 or AH6 was in sharp contrast to the relatively smooth and uniform pattern of fluorescence observed in fibrils that were pretreated with 4 M GdnHCl and stained with D18 (Fig. 4c).

After exposure to a more severe denaturing environment (6 M GdnHCl), the number of spots detectable in green channels and the...
fluorescence intensity of the spots increased in fibrils stained with either R1 or AH6 (Fig. 4, a and b, right panels, as indicated by the green arrows). These reactive spots were often seen at the fibrillar edges or at sites of fibrillar junctions or overlaps. The appearance of large amorphous spots was indicative of extensive denaturation and loss of fibrillar shape. Notable differences in patterns of staining observed between D18 and either R1 or AH6 suggest strikingly different roles that the regions 132–156 and 159–230 play in fibrillar structure. The epitope to D18 became solvent-accessible under conditions where fibrils still maintained fibrillar shape, whereas full denaturation and loss of fibrillar shape seem to be required for the R1 and AH6 epitopes to be solvent-accessible.

**PrPSc-specific Fabs Distinguished Two Subpopulations in Fibrils**—To probe the extent to which the amyloid fibrils produced in vitro are similar to PrPSc, we used IgGs 89–112 and 136–158. Both IgGs 89–112 and 136–158 were previously shown to bind specifically to PrPSc but not to PrPSc (32). These motif-grafted Abs were generated by replacing the complementarity-determining region 3 in heavy chains of recipient Ab with mouse PrP sequences that correspond to amino acid residues 88–111 and 135–157, respectively (32).

Staining of fibrils with IgG 89–112 revealed that two major subpopulations of fibrils were produced under single growth conditions (Fig. 5a, left panel). The coexistence of two subpopulations was evident directly from visual analysis of the microscopy images and also from the 2D-FIS plots (Fig. 5a, left panel, inset). We noticed that fibrils that lacked immunoreactivity with IgG 89–112 were typically aggregated in clusters and were large in size, whereas the fibrils that showed binding of 89–112 were much smaller in size and nonaggregated (Figs. 6a and 2c, bottom).
Assessing Conformation within a Single PrP Fibril

The IgG 89–112-positive and 89–112-negative subpopulations showed substantially different LRS values: 2.04 ± 0.43 and 2.27 ± 0.06, respectively (Fig. 6, b and c). In addition to the IgG 89–112-positive and 89–112-negative fibrils, we noticed that a minor subtraction of fibrils displayed heterogeneity within individual fibrils (Fig. 6d). This type of fibrils showed segments of variable colors observed along the fibrillar axis within individual fibrils; the conformational heterogeneity was also reflected by an intermediate LRS value of 1.24 ± 0.08.

Upon exposure to 4 M GdnHCl, the color distribution of the IgG 89–112-positive fibrils shifted toward the red sector in the 2D-FIS plot (Fig. 5a, compare left and right panels). This change suggests that the epitope recognized by IgG 89–112 is sensitive to GdnHCl and loses its PrPSc-like conformational properties under partially denaturing conditions. This GdnHCl-induced decrease in the reactivity of fibrils to IgG 89–112 contrasted with the increase in binding of D18 observed under the same experimental conditions. Such opposite effects are not surprising, considering that PrPSc-specific conformation is required for binding of 89–112, whereas binding of D18 depends solely on the solvent accessibility of linear epitope.

In a manner similar to IgG 89–112, IgG 136–158 distinguished two subpopulations of fibrils, where smaller fibrils showed binding of 136–158, but the larger and aggregated fibrils lacked immunoreactivity with this Ab (Fig. 5a, right). In contrast to the strong negative effect of GdnHCl on reactivity of IgG 89–112, the reactivity of fibrils to IgG 136–158 dropped only slightly following the exposure to the denaturant. 4 M GdnHCl induced only a minor shift of the color distribution toward the red sector for the IgG 136–158-positive fibrils (Fig. 5b, compare left and right panels). Such differences in responses between the IgGs 89–112 and 136–158 suggest that the epitope bound by IgG 136–158 is more stable to GdnHCl and capable of maintaining its PrPSc-like conformation despite exposure to partially denaturing conditions. Taken together, the data presented here suggest that only a subfraction of fibrils generated in vitro resemble PrPSc with respect to the conformations of epitopes bound by IgGs 89–112 and 136–158.

To determine whether the PrPSc-specific conformation was peculiar to amyloid fibrils, we were interested in testing the immunoreactivity of small soluble β-sheet-rich oligomeric particles referred to as β-oligomers to IgGs 89–112 and 136–158 (30) (Fig. 5, c and d). As judged from the 2D-FIS plot, the reactivity of the β-oligomers to IgG 89–112 was similar to that seen for the 89–112-negative fibrils (Fig. 5, compare c and the left plot in a). The reactivity of the β-oligomers to IgG 136–158 was somewhat higher than that of the β-oligomers to IgG 89–112 (Fig. 5, compare d and c) but was not as high as reactivity of fibrils with IgG 136–158 (Fig. 5, compare d and the left plot in b). Taken together, these data illustrate that there are conformational differences between the amyloid fibrils and the β-oligomers with respect to PrPSc-specific epitopes. These results also demonstrate the ability of the dual color assay to distinguish between distinct isoforms of PrP or conformationally distinct subpopulations of the same isoform that might be present as a mixture in one sample.

Dual Color Assay for Assessing PrP Conformation within Single Fibrils—Whereas the current assays measuring PrP conformation yield values averaged across a whole population of PrP particles, the dual color assay, combined with microscopy imaging, probes conformation within a single amyloid fibril or a single particle. When D13 or D18 was used for analysis, the fibrils generated under single growth conditions displayed a relatively homogeneous conformation as measured across the whole population as well as within individual fibrils (Fig. 3). PrP conformation within single fibrils was assessed by comparison of the fluorescence intensities collected along the fibrillar axis in red and green channels. When stained with D13 or D18, the shape of the profile of the fluorescence intensities observed in a green channel generally mimicked the profile of the intensities monitored for the reference Ab AG4 in a red channel (Fig. 7, a and b). After normalizing by intensities in the red channel, the profile of fluorescence intensities in the green channel did not oscillate (data not shown), suggesting that the D13 and D18 epitopes maintained a relatively uniform conformation along fibrillar axis.

In contrast to D13 and D18, staining with PrPSc-specific IgGs 89–112 and 136–158 revealed the heterogeneity across the fibrillar population (Fig. 5a and b) and within a subpopulation of individual fibrils (Fig. 7, c and d). These fibrillar subpopulations stained with 89–112 or 136–158 showed a dotted pattern of fluorescence in a green channel. Detailed analysis of fluorescence intensities monitored along the fibrillar axis revealed that the profiles of intensities recoded in green and red channels did not follow each other and sometimes oscillated in contraphase (Fig. 7, c and d). These results suggest that the conformations recognized by PrPSc-specific IgGs were not presented in a uniform manner along the fibrillar axis.

DISCUSSION

In the present study, we have described a novel dual color immunosay to probe PrP conformation of fibrillar and nonfibrillar aggregates.

FIGURE 4. A dual color assay of amyloid fibrils stained with R1 and AH6. Fluorescence microscopy images of fibrils stained with AG4/R1 (a) or with P/AH6 (b). The fibrils were kept under native conditions (left panels) or exposed to 4 M GdnHCl (middle panels) or to 6 M GdnHCl prior to staining (right panels). Secondary Abs to AG4 and Fab P were labeled with Alexa-546 (red), and secondary Abs to Fab R1 and IgG AH6 were labeled with Alexa-488 (green). Spots with high immunoreactivity to R1 or to AH6 are indicated by green arrows. Scale bars, 10 μm. c, magnified images of fibrils pretreated with 4 M GdnHCl and stained with AG4/R1 (left panels), with P/AH6 (middle panels), or with Ag4/D18 (right panels). The upper panels show images taken in the green channel, and lower panels show merges of images taken in green and red channels.
FIGURE 5. A dual color assay of amyloid fibrils and the β-oligomer stained with PrPSc-specific IgGs. Shown are fluorescence microscopy images of fibrils stained with AG4/89–112 (a) or with AG4/136–158 (b); the fibrils were kept under native conditions (left panels) or exposed to 4 M GdnHCl prior to staining (right panels). Fluorescence microscopy images of the β-oligomers were stained with AG4/89–112 (c) or with AG4/136–158 (d). Secondary Ab to AG4 was labeled with Alexa-546 (red), and secondary Ab to IgGs 89–112 and 136–158 was labeled with Alexa-488 (green). 2D-FIS plots are shown in the insets, where red fluorescence intensity is given on the horizontal axis, and green fluorescence intensity is given on the vertical axis. Scale bars, 20 μm.
Assessing Conformation within a Single PrP Fibril

FIGURE 6. PrPSc-specific IgG revealed conformational heterogeneity in population of amyloid fibrils. Shown is a fluorescence microscopy image of fibrils stained with AG4/99–112 (a) and the same field of view showing the fibrils selected by the Colocalization Finder plugin (WCIF Image J software; see “Materials and Methods”) as 89–112-positive fibrils (b), 89–112-negative fibrils (c), or fibrils with conformational polymorphism (d). Scale bars, 15 μm.
The dual color assay provides information regarding (i) the regions of PrP that are surface-exposed or buried, (ii) the effect of GdnHCl-induced denaturation upon those regions, and (iii) the conformational heterogeneity of fibrils, which could be measured across the whole population of fibrils or within individual fibrils.

Using this dual color assay, we analyzed the conformation of fibrils generated in vitro from the full-length rPrP (Table 1). We found that the epitopes to AH6 and R1 (residues 159–174 and 225–230, respectively) were the most resistant to the GdnHCl-induced denaturation; these epitopes displayed immunoreactivity only upon treatment with high concentrations of GdnHCl. This result is consistent with our previous studies, in which the C-terminal fragments of 152–230 and 162–230 residues were shown to be the most proteolytically resistant regions in fibrillar form (31). Staining with AH6 and R1 displayed a dotted pattern, suggesting that partial denaturation resulted in the exposure of the AH6 and R1 epitopes at the fibrillar edges or at sites of occasional fibrillar bending or fragmentation. These data along with our previously published results (31) suggest that the epitopes to AH6 and R1 compose the most thermodynamically stable cross-β-core of the fibrils.

The epitope bound by D18 (residues 132–156) was found to be cryptic under the native conditions; however, it became partially exposed to the solvent after treatment at the partially denaturing conditions (4 M GdnHCl). When fluorescence intensities were monitored along the fibrillar axis of individual fibrils, staining with D18 displayed relatively smooth fluorescence profiles regardless of whether the fibrils were exposed to GdnHCl. These results illustrate that (i) unfolding and solvent exposure of the D18 epitopes did not require global unfolding of the fibrillar structure, and (ii) the D18 epitope was presented in a relatively uniform manner or relatively homogeneous conformation along the fibrillar axis. We have shown previously that the region encompassing residues 138–152 was partially resistant to PK digestion in the fibrillar form; specifically, it remained intact only at low concentrations of PK (31). It is reasonable to suggest that the epitope to D18 is localized close to the fibrillar periphery and/or on an interface between filaments and therefore is not directly involved in the cross-β-core.

The epitope bound by D13 was found to be immunoreactive regardless of whether the fibrils were pretreated with GdnHCl, indicating that residues 95–105 are solvent-exposed in the fibrils. The solvent-exposed conformation of the D13 epitope correlated well with its high PK sensitivity (31). However, in most PrPSc strains, but not in the PrP fibrils used in these studies, the region corresponding to the D13 epitope is PK-resistant, suggesting that there are notable conformational differences between PrPSc generated in vivo and fibrils produced in vitro. Contradicting data exist with respect to whether the D13 epitope is buried or exposed in native PrPSc (14, 29). Peretz et al. (14) reported that this epitope has low immunoreactivity in ELISA against purified hamster Sc237 prions, whereas Khalili-Shirazi et al. (29) showed that IgGs to the epitopes 93–105 and 97–105 efficiently immunoprecipitated human variant CJD and mouse RML prions, arguing that the residues 93–105 are exposed, at least in part, in native PrPSc found in some prion diseases but not others. The differences observed in solvent accessibility of the residues 93–105 in different studies could be either due to conformational differences between strains of hamster, mouse, and human prions or due to different procedures for the preparation of PrPSc. In the studies by Peretz et al. (14), hamster Sc237 was purified from brain homogenate via high speed ultracentrifugation, a procedure that is known to cause aggregation of PrPSc, whereas Khalili-Shirazi et al. (29) precipitated PrPSc directly from brain homogenates, a procedure where undesired aggregation of PrPSc is minimized. To avoid aggregation of the amyloid fibrils in the current study, we undertook precautions designed to prevent formation of fibrillar clumps.

In contrast to D13 and D18, which showed a uniform presentation of the epitopes 95–105 and 132–156, respectively, on the surface of fibrils, PrPSc–specific motif-grafted Abs revealed conformational heterogeneity in fibrils produced under single growth conditions. IgGs 89–112 and 136–158 distinguished two major subpopulations of fibrils, which differ from each other by immunoreactivities to each of these PrPSc–specific Abs. Because the epitopes for IgGs 89–112 and 136–158 are currently unknown, we cannot assign the differences between these two subpopulations to specific regions of the PrP molecule. Of note, and in agreement with the results presented here, previous studies showed that only a subfraction of PrPSc was immunoreactive to IgG 89–112 or 136–158 (32). To the extent that PrPSc–specific Abs capture the peculiar physical properties of PrPSc, our studies illustrate that a substantial fraction of the fibrils generated in vitro displayed a conformation similar to that of PrPSc, at least for specific surface-exposed epitopes. In dual color imag-
Assessing Conformation within a Single PrP Fibril

ing. IgGs 89–112 and 136–158 were diluted to a final concentration of 1 nM, which was 8.5-fold lower than that of the reference Ab AG4 used for the same experiments, indicating that the 89–112- and 136–158-reactive fibrillar subpopulations had high affinity for the motif-grafted IgGs.

It remains unclear whether or not the two subpopulations of fibrils that had different reactivities to PrPSc-specific Abs emerged via two independent folding pathways or simply represent different stages of polymerization. Conformational polymorphism is not restricted to PrP fibrils and was previously observed for amyloid fibrils produced from other amyloidogenic polypeptides, including Aβ (23, 24). Conformational heterogeneity observed within individual PrP fibrils is consistent with the hypothesis that PrPSc-specific Fabs recognize specific epitopes on the surface of low order fibrils and that these epitopes are involved in the formation of an interface between filaments upon their lateral association into higher order fibrils and/or formation of fibrillar aggregates. Our previous studies using atomic force microscopy revealed a highly hierarchical mechanism of assembly, which proceeds through several steps of lateral association of lower order into higher order fibrils (20).

The assay described in the current study proved to be extremely illuminating for probing the conformation of fibrils across an entire population as well as the conformation of individual fibrils. To present the newly developed assay in perspective, we need to briefly review the assays that were used previously for assessing conformations of multimeric PrP isoforms. In order to develop an assay capable of determining the amounts of PrPSc in complex mixtures, Peretz and co-workers identified PrP regions that are buried in PrPSc but solvent-accessible in PrPC (14). Toward this goal, immunoreactivity of PrPSc and PrPC was measured under native and fully denatured conditions (3 M guanidine thiocyanate) using a panel of Fabs raised to different linear and conformational epitopes within PrP polypeptide. In a manner similar to the current study, the epitopes that were buried showed substantial increase in immunoreactivity upon the addition of denaturant, whereas the reactivity of the epitopes that were solvent-accessible did not change with denaturation (14). This approach identified those regions of PrP that were largely cryptic in native PrPSc but solvent-accessible in PrPSc, an observation that was further exploited to design the immunoconformational assay known as CDI (7, 33). CDI was not only able to quantify the concentration of PrPSc in a mixture with PrPSc but also reveal conformational differences between PrPSc strains (7, 33). Specifically, prion strains were found to differ from each other by the extent to which the immunoreactivity of the epitopes that were buried under native conditions increased upon partial denaturation (7, 33). Using an alternative strategy, Cashman and co-workers (15) raised an antibody to the PrP epitope that is buried in the native PrPSc isoform but became solvent-exposed upon conversion of PrPSc to PrPSc. Under the native conditions, this antibody recognized PrPSc but not PrPSc, as was shown by immunoprecipitation.

In the current study, we present a new strategy where the solvent accessibility of specific regions was assessed by comparing the immunoreactivity of one Ab specific to the epitopes of interest with that of a reference Ab. Our assay does not require digestion with PK and could be used for probing the conformation of PK-sensitive and PK-resistant states of the disease-specific PrP isoforms in the absence of PK treatment. Another advantage of our approach was the ability to combine dual color staining with the fluorescence microscopy that provides a tool for assessing the conformation within individual fibrils or particles. We demonstrated that, in this format, the dual color assay was able to distinguish conformationally different fibrils present in mixtures. A majority of the previously developed techniques are based on ELISA, immunoprecipitation, or Western blots, which often require PK digestion and have a limited capacity to discriminate conformationally distinct forms that are present in complex mixtures. Natural isolates of TSE or sporadic CJD were shown to exist as a mixture of different strains or subtypes that co-exist in a single brain (11, 12). It is noteworthy that successful transmission of prions across a species barrier was shown to result in bifurcation of prions, leading to generation of multiple strains within a single isolate (34). Because existing assays measure macroscopic properties that are averaged across a whole molecular population, they do not discriminate clearly different subtypes of PrPSc and sometimes provide confusing results with respect to current CJD classifications (3, 4, 12).

The approach presented here could provide valuable new information about the surface-exposed epitopes in disease-specific PrP isoforms and potential changes in these epitopes occurring at different stages in prion disease. Subtle differences of these surface-exposed epitope could be important factors in identifying infectious versus toxic states of PrPSc, or PK-resistant versus PK-sensitive PrPSc isoforms. To date, there are no precisely defined biochemical markers that directly associate with prion infectivity. Numerous recent studies indicate that the correlation between PK resistance and prion infectivity that appeared to be well established in older studies is, in fact, not absolute (35–38). It is also well known that the acquisition of β-sheet-rich structure by PrP molecules is not exclusively associated with the generation of prion infectivity either (39, 40). It would be interesting to determine in future studies whether the infectious subpopulation of abnormally folded PrP states has a unique or distinctive pattern of surface-exposed epitopes.

Acknowledgments—We thank J. M. Manser and the Transmissible Spongiform Encephalopathies Resource Center (Institute for Animal Health, United Kingdom) for providing anti-PrP monoclonal antibody and Pamela Wright for editing the manuscript.

REFERENCES

1. Prusiner, S. B. (2001) N. Engl. J. Med. 344, 1516–1526
2. Prusiner, S. B. (1997) Science 278, 243–251
3. Hill, A. F., Joiner, S., Wadsworth, J. D. F., Sidle, K. C. L., Bell, J. E., Budla, H., Ironside, J. W., and Collinge, J. (2003) Brain 126, 1333–1346
4. Parchi, P., Giese, A., Capellari, S., Brown, P., Schulz-Schaeffer, W., Windl, O., Zerr, I., Budla, H., Kopp, N., Piccardo, P., Poser, S., Rojiani, A., Streicherenberger, N., Julien, J., Vital, C., Ghetti, R., Gambetti, P., and Kretzschmar, H. (1999) Ann. Neurol. 46, 224–231
5. Bessen, R. A., and Marsh, R. F. (1994) J. Virol. 68, 7859–7868
6. Telling, G. C., Parchi, P., DeArmond, S. J., cortelli, P., Montagna, P., Gabizon, R., Mastrianni, J., Lugaresi, E., Gambetti, P., and Prusiner, S. B. (1996) Science 274, 2079–2082
7. Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E., and Prusiner, S. B. (1998) Nat. Med. 4, 1157–1165
8. Peretz, D., Scott, M., Groth, D., Williamson, A., Burton, D., Cohen, F. E., and Prusiner, S. B. (2001) Protein Sci. 10, 854–863
9. Cashen, R., Raymond, G. J., and Bessen, R. A. (1998) J. Biol. Chem. 273, 32230–32235
10. Thorning, A., Spassov, S., Friedrich, M., Naumann, D., and Beekes, M. (2004) J. Biol. Chem. 279, 33854
11. Polymenidou, M., Stoeck, K., Glatzel, M., Vey, M., Bellon, A., and Aguzzi, A. (2005) Lancet Neurol. 4, 805–814
12. Schoch, G., Seeger, H., Bogosladysky, J., Trolay, M., Janzer, R. C., Aguzzi, A., and Glatzel, M. (2006) PLoS Med. 3, 236–244
13. Yull, H. M., Ritchie, D. L., Langeveld, J. P., van Zijldeveer, F. G., Bruce, M. E., Ironside, J. W., and Head, M. W. (2006) Am. J. Pathol. 168, 151–157
14. Peretz, D., Williamson, R. A., Matsunaga, Y., Serban, H., Pinilla, C., Bastidas, R. B., Rozenshtein, R., James, T. L., Houghten, R. A., Cohen, F. E., Prusiner, S. B., and Burton, D. R. (1997) J. Mol. Biol. 273, 614–622
15. Paramithiotis, E., Pinard, M., Lawton, T., LeBoissiere, S., Leiters, V. L., Zou, W., Estey, L. A., Lamontagne, J., Lehto, M. T., Kondejewski, L. H., Francoeur, G. P., Papadopoulos, M., Haghhighat, A., Spatz, S. J., Head, M., Will, R., Ironside, J. O’Rouke, K., Tonelli, Q., Ledebur, H. C., Chakrabarty, A., and Cashen, N. R. (2003) Nat. Med. 9, 893–899
16. Bessen, R. A., and Marsh, R. F. (1992) *J. Virol.* **66**, 2096–2101
17. Kuczius, T., and Groschup, M. H. (1999) *Mol. Med.* **5**, 406–418
18. Bellon, A., Seyfert-Brandt, W., Lang, W., Baron, H., Groner, A., and Vey, M. (2003) *J. Gen. Virol.* **84**, 1921–1925
19. Notari, S., Capellari, S., Giese, A., Westner, I., Baruzzi, A., Ghetti, B., Gambetti, P., Kretzschmar, H. A., and Parchi, P. (2004) *J. Biol. Chem.* **279**, 16797–16804
20. Anderson, M., Bocharova, O. V., Makarava, N., Breydo, L., Salnikov, V. V., and Baskakov, I. V. (February 20, 2006) *J. Mol. Biol.* **364**, 645–659
21. Legname, G., Baskakov, I. V., Nguyen, H.-O. B., Riesner, D., Cohen, F. E., DeArmond, S. J., and Prusiner, S. B. (2004) *Science* **305**, 673–676
22. Legname, G., Nguyen, H.-O. B., Baskakov, I. V., Cohen, F. E., DeArmond, S. J., and Prusiner, S. B. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 17342–17347
23. Chan, J. C. C., Oyler, N. A., Yau, W.-M., and Tycko, R. (2005) *Biochemistry* **44**, 10669–10680
24. Ritter, C., Maddalone, M. L., Siermer, A. B., Luhrs, T., Ernst, M., Meier, B. H., Saupé, S. J., and Riek, R. (2005) *Nature* **435**, 844 – 848
25. Peretz, D., Williams, R. A., Kaneko, K., Vergara, J., Leclerc, E., Schmitt-Ulms, G., Mehlhorn, I. R., Legname, G., Wormald, M. R., Rudd, P. M., Dwek, R. A., Burton, D. R., and Prusiner, S. B. (2001) *Nature* **412**, 739 – 743
26. Leclerc, E., Peretz, D., Ball, H., Sakurai, H., Legname, G., Serban, A., Prusiner, S. B., Burton, D. R., and Williamson, R. A. (2001) *EMBO J.* **20**, 1547–1554
27. Khalili-Shirazi, A., Summers, L., Linehan, J., Mallinson, G., Anstre, D., Hawke, S., Jackson, G. S., and Collinge, J. (2005) *J. Gen. Virol.* **86**, 2635–2644
28. Bocharova, O. V., Breydo, L., Parfenov, A. S., Salnikov, V. V., and Baskakov, I. V. (2005) *J. Mol. Biol.* **346**, 645–659
29. Bocharova, O. V., Breydo, L., Salnikov, V. V., Gill, A. C., and Baskakov, I. V. (2005) *Protein Sci.* **14**, 1222–1232
30. Moroncini, G., Kan, N., Solfiori, L., Abalos, G., Telling, G. C., Head, M., Ironside, J., Brockes, J. P., Burton, D. R., and Williamson, R. A. (2004) *Proc. Acad. Natl. Sci. U. S. A.* **101**, 10404–10409
31. Safar, J. G., Scott, M., Monaghan, J., Deering, C., Didorenko, S., Vergara, J., Ball, H., Legname, G., Leclerc, E., Solfiori, L., Serban, H., Growth, D., Burton, D. R., Prusiner, S. B., and Williamson, R. A. (2002) *Nat. Biotechnol.* **20**, 1147–1150
32. Korth, C., Kaneko, K., Groth, D., Heye, N., Telling, G., Mastrianni, J., Parchi, P., Gambetti, P., Will, R., Ironside, J., Heinrich, C., Tremblay, P., DeArmond, S. J., and Prusiner, S. B. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 4784–4789
33. Lasmézas, C. L., Deslys, J.-P., Robain, O., Jaegly, A., Beringue, V., Peyrin, J.-M., Fourmier, J.-G., Hazout, J.-J., Rossier, J., and Dormont, D. (1997) *Science* **275**, 402–405
34. Tremblay, P., Ball, H. L., Kaneko, K., Groth, D., Hegde, R. S., Cohen, F. E., DeArmond, S., Prusiner, S. B., and Safar, J. (2004) *J. Virol.* **78**, 2088–2099
35. Manuelidis, L., Fritsch, W., and Xi, Y.-G. (1997) *Science* **277**, 94–98
36. Barron, R. M., Thomson, V., Jameison, E., Melton, D. W., Ironside, J., Will, R., and Manson, J. C. (2001) *EMBO J.* **20**, 5070–5078
37. Baskakov, I. V., Legname, G., Baldwin, M. A., Prusiner, S. B., and Cohen, F. E. (2002) *J. Biol. Chem.* **277**, 21140–21148
38. May, B. C. H., Govaerts, C., Prusiner, S. B., and Cohen, F. E. (2004) *Trends Biochem. Sci.* **29**, 162–165

Assessing Conformation within a Single PrP Fibril

JUNE 2, 2006 • VOLUME 281 • NUMBER 22

JOURNAL OF BIOLOGICAL CHEMISTRY 15545