Aggregation and Amyloid Fibril Formation Induced by Chemical Dimerization of Recombinant Prion Protein in Physiological-like Conditions*

Alireza Roostaei, Sébastien Côté, and Xavier Roucou

From the Department of Biochemistry, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

Prion diseases are caused by the conversion of a cellular protein (PrP\(^\text{C}\)) into a misfolded, aggregated isoform (PrP\(^\text{Res}\)). Misfolding of recombinant PrP\(^\text{C}\) in the absence of PrP\(^\text{Res}\) template, cellular factors, denaturing agents, or at neutral pH has not been achieved. A number of studies indicate that dimerization of PrP\(^\text{C}\) may be a key step in the aggregation process. In an effort to understand the molecular event that may activate misfolding of PrP\(^\text{C}\) in more relevant physiological conditions, we tested if enforced dimerization of PrP\(^\text{C}\) may induce a conformational change reminiscent of the conversion of PrP\(^\text{C}\) to PrP\(^\text{Res}\). We used a well described inducible dimerization strategy whereby a chimeric PrP\(^\text{C}\) composed of a modified FK506-binding protein (Fv) fused with PrP\(^\text{C}\) and termed Fv-PrP is incubated in the presence of a monomeric FK506 or dimerizing AP20187 ligand. Addition of AP20187 but not FK506 to recombinant Fv-PrP (rFv-PrP) in physiological-like conditions resulted in a rapid conformational change characterized by an increase in \(\beta\)-sheet structure and simultaneous aggregation of the protein. Aggregates were partially resistant to proteinase K and induced the conversion of soluble rFv-PrP in serial seeding experiments. As judged from thioflavin T binding and electron microscopy, aggregates converted to amyloid fibers. Aggregates were toxic to cultured cells, whereas soluble rFv-PrP and amyloid fibers were harmless. This study strongly supports the proposition that dimerization of PrP\(^\text{C}\) is a key pathological primary event in the conversion of PrP\(^\text{C}\) and may initiate the pathogenesis of prion diseases.

---

**Footnotes:**
* This work was supported by a grant from Canadian Institutes for Health Research (to X. R.).
1 To whom correspondence should be addressed: Dept. of Biochemistry, Faculty of Medicine, University of Sherbrooke, 3001 12th Ave. Nord, Sherbrooke, Quebec J1H 5N4, Canada. Tel.: 819-346-1110 (ext. 12248); Fax: 819-564-5340; E-mail: xavier.roucou@usherbrooke.ca.
2 The abbreviations used are: TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease in humans. In humans, these disorders can appear in three forms, sporadic, genetic, and iatrogenic. All forms involve modification of the normal prion protein (PrP\(^\text{C}\)), a secreted glycosylphosphatidylinositol-anchored sialoglycoprotein, into a disease-causing prion protein (PrP\(^\text{Res}\)) (1). Although the primary structures of PrP\(^\text{C}\) and PrP\(^\text{Res}\) have been found to be identical (2) the two isoforms have considerably different physiochemical properties. PrP\(^\text{C}\) is monomeric and degradable by proteinase K (PK), whereas PrP\(^\text{Res}\) exists as insoluble aggregates that show dramatically increased resistance to digestion by PK (1). Furthermore, spectroscopic data indicate that the two prion protein isoforms are characterized by different secondary structures; PrP\(^\text{C}\) is largely \(\alpha\)-helical, whereas PrP\(^\text{Res}\) is rich in \(\beta\)-sheet structure (3, 4). In designing strategies to prevent the propagation and the progression of TSEs, it is important to determine the molecular pathway leading to the aggregation of PrP\(^\text{C}\).

Substantial efforts have been devoted to reconstitute de novo misfolding of PrP\(^\text{C}\) and prion infectivity and/or toxicity. In the first approach, PrP\(^\text{Res}\) was amplified in PrP\(^\text{Res}\)-dependent conversion reaction experimental conditions (see Refs. 5 and 6 for reviews). Experiments included the use of ionic or non-ionic detergents, or repeated sonication steps, or shaking for a long period of time. In the second approach, recombinant PrP (rPrP) was mixed in a combination of low concentrations of guanidinium chloride (GdnHCl) (7, 8) or urea and NaCl (9–11) in acidic or neutral conditions. Recent investigations showed that acidic conditions alone can induce the conversion of rPrP (12). Addition of lipids to rPrP also induces a conformational change into a form reminiscent of PrP\(^\text{Res}\) (13). Furthermore, low concentrations of SDS induce a structural transition of PrP into a pathogenic conformation (14, 15). These approaches allowed purified PrP\(^\text{C}\) or rPrP to undergo a transition to an oligomeric \(\beta\)-sheet-rich structure with essential physiochemical properties similar to those of PrP\(^\text{Res}\) and, in some instances, to reconstitute infectivity (16, 17). Recently, thermal refolding and expression of PrP in form of a tandem repeat resulted in the formation of soluble oligomers with high \(\beta\)-sheet content (15, 18). In vitro PrP conversion techniques have already contributed significant insights into the molecular mechanisms of prion diseases. However, an important issue remains unresolved: physiological circumstances mimicking in vitro conditions used to induce misfolding of PrP\(^\text{C}\) are unlikely to occur in vivo. Thus, elucidating what molecular event may induce the aggregation of PrP\(^\text{C}\) in physiologically relevant conditions remains an important challenge.

In acquired TSEs, PrP\(^\text{Res}\) physically interacts with PrP\(^\text{C}\) and may force PrP\(^\text{C}\) molecules to dimerize/oligomerize and aggregate (19–22). Thus, we reasoned that dimerization may be a key
Dimerization-induced Prion Protein Aggregation

step toward aggregation of PrP in vivo. In favor of this hypothesis, several observations indicate that PrP can form dimers that could be intermediate states in PrP formation (23). Recently, we used a well described chemical dimerization strategy to test this proposition. In this technology, a synthetic homodimerizing ligand termed AP20187 binds to and cross-links two FK506 binding domains (Fv). A fusion protein between PrPC and Fv was engineered, and addition of AP20187to cultured cells expressing Fv-PrP resulted in the formation of extracellular deposits that are insoluble in non-ionic detergents and partially resistant to PK (24). These findings demonstrated that homologous interactions between PrP molecules constitute a minimal intrinsic property of the protein, or require other cellular factors. In the present study, we addressed this issue by inducing dimerization of purified recombinant Fv-PrP (rFv-PrP) in physiological-like conditions. We show that addition of AP20187 but not control monomeric FK506 to rFv-PrP results in spontaneous misfolding and aggregation as judged by an increase in turbidity, by differential centrifugation, and by electron microscopy. CD experiments confirmed that AP20187 induces a structural conversion characterized by a decrease in α-helices and a concomitant increase in β-sheets. Furthermore, rFv-PrP aggregates induced the conversion of soluble rFv-PrP in serial seeding experiments. These results demonstrate that dimerization is a sufficient molecular event responsible for initiating misfolding and aggregation of PrP in vitro and support the hypothesis that dimerization of PrP is a possible initial step in the pathogenesis of prion diseases.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Recombinant Proteins—Recombinant human Fv-PrP (rFv-PrP) was amplified from pCEP4-β-FvPrP (24). PCR primers were forward 5'-ggaattccatagagaagccgcaagctgag-3' and reverse 5'-ccggaattccatagaggaagagcgccagctgag-3'. Recombinant Fv-PrP and rFv-PrP were generated by overlap extension PCR. For rFv-PrP, Fv was amplified from pC4-Fv1E (vector kindly provided by ARIAD Pharmaceuticals) using PCR primers forward 5'-actacatacataggctttgctggattgtgctgagct-3' and reverse 5'-ggcgcggcgtttcctctcgagctgctgctg-3'. Fv-PrP was amplified from pCEP4-β-FvPrP (24), using PCR primers forward 5'-ccggaattccatagagaagccgcaagctgag-3' and reverse 5'-ccggaattccatagagaagccgcaagctgag-3'. rFv-PrP was generated by using the products of these two PCR as primers for the overlap extension. For rFv-PrP, Fv was amplified from pC4-Fv1E using PCR primers forward 5'-actacatacataggctttgctggattgtgctgagct-3' and reverse 5'-ggcgcggcgtttcctctcgagctgctgctg-3'. Fv-PrP was amplified from pCEP4-β-FvPrP (24), using PCR primers forward 5'-ccggaattccatagagaagccgcaagctgag-3' and reverse 5'-ccggaattccatagagaagccgcaagctgag-3'. The PCR product was inserted in the natural SmaI site of mouse Prnp at bp 116. Mouse Fv-PrP was amplified using PCR primers forward 5'-gggatccatagagaagccgcaagctgag-3' and reverse 5'-ccggaattccatagagaagccgcaagctgag-3'. Recombinant human PrP was amplified from pCEP4-β-PrP using PCR primers forward 5'-attactagagagagactgctgctgctg-3' and reverse 5'-ggcgcggcgtttcctctcgagctgctgctg-3'. The PCR products were inserted between the NdeI and EcoRI sites in pET28a(+), creating NHEI-His2 tag constructs. PCR amplification fidelity was confirmed by sequencing. Constructs were overexpressed in BL-21 (DE3) pLys S cells, and inclusion bodies were purified (25). Purification of recombinant proteins from the inclusion bodies was performed using the artificial chaperone-assisted method (26). Inclusion bodies were resuspended in buffer D containing 6 M GdnHCl, 100 mM Na2HPO4, 10 mM Tris-HCl, 10 mM reduced glutathione, 1 mM Tris(2-carboxyethyl)phosphine, pH 8.0. Soluble protein fraction obtained after harvesting (15,600 × g, 45 min) was added to 20 ml of nickel-nitritolriatriac acid resin followed by incubation for 3 h at 4 °C. Buffer D1 was gradually substituted by buffer D2 (4 mM GdnHCl, 100 mM Na2HPO4, 10 mM Tris-HCl, 5 mM reduced glutathione, 0.5 mM Tris(2-carboxyethyl)phosphine, pH 8.0). GdnHCl was then replaced by 14 mM cetrimonium bromide in buffer D2, and samples were incubated at 4 °C for 2 h. Buffer D2 was exchanged by buffer R (100 mM NaH2PO4, 10 mM Tris-HCl, 0.5 mM Tris(2-carboxyethyl)phosphine, 5 mM β-cyclodextrin, pH 8.0) followed by overnight incubation at 4 °C. After a washing step with buffer R devoid of β-cyclodextrin (Buffer W), the protein was eluted with the same buffer containing 500 mM imidazole and 100 mM GdnHCl (Buffer E). The eluted fractions were concentrated (27) in buffer W. All buffers contained 30 mM imidazole, unless indicated otherwise.

Assessment of in Vitro Aggregation—Recombinant proteins (0.2 mg/ml) were incubated in the presence or absence of increasing concentrations of AP20187 or FK506 at 37 °C in 0.5 ml of 25 mM sodium phosphate buffer, pH 7.4. After 30 min, the apparent optical density, which is proportional to turbidity, was measured at 340 nm using a Beckman model DU 70, multisample UV-visible spectrometer. After recordings, samples including AP20187 were transferred to fresh tubes and spun at 15,600 × g for 15 min, and the absorbance of supernatant was recorded at 340 nm.

Aggregation was also assessed by differential centrifugation. Recombinant proteins (0.2 mg/ml) were incubated in the absence or in the presence of increasing concentrations of AP20187 in 0.5 ml of a buffer containing 10 mM Tris-HCl and 100 mM Na2HPO4, pH 7.4. After 30 min at 37 °C, samples were centrifuged at 15,600 × g for 15 min. Supernatants containing soluble proteins were collected in fresh tubes, and proteins were precipitated with trichloroacetic acid, and pellets were solubilized in 30 μl of SDS loading buffer (0.5% SDS (w/v), 1.25% β-mercaptoethanol (v/v), 4% glycerol (v/v), 0.01% bromophenol blue (w/v), 15 mM Tris-HCl, pH 6.8). Pellets containing aggregated proteins were directly solubilized in 30 μl of SDS loading buffer. A 10-μl aliquot of each sample was analyzed in 12% SDS-PAGE and proteins were stained with Coomassie Blue.
CD Spectroscopy—CD measurements were carried out as previously described (28) using a protein concentration of 6 μM in a 25 mm sodium phosphate buffer at pH 7.4 (PBS) and at 37 °C. The secondary structure changes of rFv-PrP were evaluated by monitoring the ellipticity in the far-UV region (190–250 nm). In some experiments, CD data were collected at various incubation time intervals after addition of AP20187. Three consecutive scans were accumulated, and the average spectra were stored. Protein thermal denaturation curves were determined by monitoring the CD values at 222 nm, with increasing temperature from 10 °C to 90 °C. The temperature dependence of the mean residue ellipticity (θ) at 222 nm was fitted using an in-house non-linear least-squares fitting program (29). To obtain the thermodynamic parameters (T°, ΔH° (T°), ΔS° (T°), and ΔG° (T°)) describing the stability of rFv-PrP structural species, thermal denaturation curves of rFv-PrP were simulated assuming a two-state unfolding mechanism (27–29). These experiments were performed in triplicates.

Data Analysis—The temperature dependence of mean residue ellipticity (θ) at 222 nm obtained from thermal denaturation profiles were fitted using an in-house non-linear least-squares fitting program assuming a two-state unfolding reaction as in Equation 1,

\[
\theta(T) = (1 - P_u(T)) \cdot \theta_u(T) + P_u(T) \cdot \theta_N(T) + \theta_{N0}(T)
\]

where θ_N(T) and θ_u(T) are the temperature dependence of the mean residue ellipticity of the macroscopic folded and unfolded states, respectively. They were both assumed to be linear, i.e. \( \theta_N(T) = \theta_N(0) - d\theta_N(T)/dT \cdot T \) and \( \theta_u(T) = \theta_u(0) - d\theta_u(T)/dT \cdot T \), where \( \theta_N(0) \) and \( \theta_u(0) \) are the mean residue ellipticities at 0 °C for the folded and unfolded states, respectively; correspondingly \( d\theta_N(T)/dT \) and \( d\theta_u(T)/dT \) are the constant slopes of \( \theta_N(T) \) and \( \theta_u(T) \). \( P_u(T) \), the population of the unfolded state, is given by Equation 2,

\[
P_u(T) = \frac{\exp(-\Delta G_u(T)/RT)}{1 + \exp(-\Delta G(T)/RT)}
\]

where the temperature dependence of the free energy of unfolding (\( \Delta G_u(T) \)) is described by Equation 3,

\[
\Delta G_u(T) = \Delta H_u^0 \left(1 - \frac{T}{T^*}\right) + \Delta C_{p,u}(T - T^*) - T \ln \left(\frac{T}{T^*}\right)
\]

where \( T^* \) is the melting temperature, \( \Delta H_u^0 \) is the apparent enthalpy of unfolding at \( T^* \) and \( \Delta C_{p,u} \) is the temperature-independent (30) heat capacity of unfolding. In the present study, \( \Delta C_{p,u} \) was considered constant and equal to 1 kcal/mol·K (1 kcal = 4.184 kJ).

Thioflavin Assay—The kinetics of amyloid fibril formation was monitored by a benzoiazole dye thioflavin (ThT) binding assay (31). Briefly, 5 μM AP20187 was added to 50 μM rFv-PrP in a physiological buffer (PBS buffer) (32), containing 10 mM K2HPO4, 1 mM KCl, 50 mM CH3COOH, 5 mM KH2PO4, 5 mM Na2HPO4, pH 7.4, followed by incubation at 37 °C. Aliquots of 30 μl were withdrawn and diluted to a final concentration of 5 μM rFv-PrP in PBS buffer (pH 8) containing 10 μM ThT. ThT fluorescence emission at 482 nm (450 nm excitation) was measured on an Hitachi F-2500 spectrofluorometer. For seeding experiments, freshly prepared seeded amyloid fibrils obtained after 48 h of incubation in the presence of AP20187 were sonicated on ice to disrupt large aggregates (33), and 2% (w/w) fibrillar material was added to 50 μM rFv-PrP.

PK Sensitivity—Analysis of PK resistance was performed by incubating 10 μg/ml of each protein for 15 min in the presence of various concentrations of PK at 37 °C in PB buffer. The samples were then precipitated with four volumes of methanol, resuspended in the loading buffer, and analyzed by Western blotting with 3F4 (Chemicon) or EP1802Y (Abcam) antibodies.

Electron Microscopy of rFv-PrP Samples—50 μM rFv-PrP was incubated with 5 μM AP20187 for 2 or 48 h at 37 °C. A 10-μl aliquot of protein preparation was applied to Formvar- and carbon-coated grids. The excess fluid was drained with filter paper, and the sample was stained for 1 min with 2% uranyl acetate. The grid was air-dried and examined in a Hitachi H-2500 electron microscope at 80 kV at a magnification of 15–75000.

Dimerization-induced Seeding Reactions—In a total reaction volume of 100 μl, 50 μM rFv-PrP was aggregated by addition of 5 μM AP20187 in a buffer, including 130 mM NaCl, 50 mM Na2HPO4, pH 7.4, at 37 °C. From the reactions aliquots were withdrawn after 1 h of incubation and used in seeding experiments after dilution 20 times in the same buffer containing 10 μM rFv-PrP in the absence of AP20187. After dilution, samples were subjected to protein misfolding amplification cycles with shaking/incubation periods (34). Each cycle included 2-min shaking and 30-min incubation at 37 °C. PK digestion of the samples was performed using 2 μg/ml PK. Proteins were precipitated with four volumes of cold methanol and analyzed by Western blotting.

The following modifications were introduced for rFv-MoPrP. Aliquots were withdrawn after 5 h of incubation with AP20187, and shaking and incubation times were 5 and 60 min, respectively. For the experiment in Fig. 10 (see below), rFv-PrP was aggregated with 0.1% SDS and periodic sonication as previously described (35).

In Vitro Toxicity Experiments—For toxicity monitoring, N2a cells were seeded at a density of 0.5 × 10⁴ cells per well in a 6-well plate. After 1 day in culture, cells were incubated in different conditions for 72 h. To keep steady culture medium concentrations, the proteins were diluted in 2× culture medium, and the proper volumes of water and vehicle solution were added to get a final 1× concentration. rFv-PrP aggregates were obtained by incubating freshly prepared 1 mM rFv-PrP with 100 μM AP20187 for 30 min. AP20187-treated rFv-PrP was directly added to cells at a final concentration of 10 μM. Staurosporine was used at a concentration of 100 nM to induce apoptosis in control experiments (36). Control aggregates of stereoidogenic acute regulatory protein (StAR) protein (27) were obtained with 2 M urea at pH 5.0 and added to cells in the same concentration as rFv-PrP.

RESULTS

Dimerization-induced Aggregation of rFv-PrP—To test whether dimerization of PrPC induces a conformational change, rFv-PrP was expressed and purified as described under "Experimental
Dimerization-induced Prion Protein Aggregation

FIGURE 1. Expression and purification of rFv-PrP and detection of rFv-PrP aggregates by light scattering and Coomassie Blue staining. A, rFv-PrP was produced in Escherichia coli cells, BL21(DE3)pLys S as described under “Experimental Procedures.” A, 2 μg of rFv-PrP was subjected to the SDS-PAGE analysis (left panel). Immunoblotting assay was performed using 3F4 antibody (right panel). B, light scattering; rFv-PrP (0.2 mg/ml, final volume 0.5 ml) was incubated in the presence of increasing concentrations of AP20187 (●) or FK506 (○) ligands at 37 °C. Optical density resulting from light scattering of rFv-PrP aggregates or soluble proteins were measured at 340 nm by UV-visible spectroscopy. Samples, including AP20187, were centrifuged, and the absorbance of the supernatant is represented (▲). Values are expressed as the mean ± S.E. of optical density at 340 nm from three independent experiments (p < 0.5). C, Coomassie Blue staining: 50 μM rFv-PrP was incubated in the presence of the indicated concentrations of AP20187 or FK506 at 37 °C for 30 min. After centrifugation, the supernatants were separated and precipitated by trichloroacetic acid. Aggregated rFv-PrP pellets and precipitated supernatant were solubilized in SDS loading buffer, and a 10-μl aliquot from each sample was subjected to a 12% SDS-PAGE analysis.

Procedures.” SDS-PAGE analysis showed that the 38-kDa rFv-PrP protein was the predominant polypeptide in the purified fraction (Fig. 1A, left panel). The identity of the protein was also confirmed by immunoblotting analysis, using a monospecific antibody (Fig. 1A, right panel).

Recombinant Fv-PrP was incubated in the presence of increasing concentration of the dimerizing ligand AP20187, and the turbidity associated with the formation of aggregated proteins was measured at 340 nm. A large increase in absorbance was observed upon addition of AP20187, and maximal aggregation was achieved at 1.4 μM AP20187 (Fig. 1B, filled circles). Furthermore, all absorbance was lost after centrifugation of the samples in a table top centrifuge set at 15,600 × g (Fig. 1B, empty circles). Addition of FK506, a monovalent ligand unable to induce the dimerization of Fv domains did not induce any change in absorbance, indicating that dimerization is essential for the aggregation of rFv-PrP (Fig. 1B, filled triangles). Aggregation of rFv-PrP was confirmed by SDS-PAGE and Coomassie Blue staining (Fig. 1C). In the absence of AP20187, rFv-PrP was detected in the supernatant after centrifugation at 15,600 × g. Addition of increasing concentrations of AP20187 induced the transfer of the protein from supernatant to pellet, while FK506 did not modify the distribution of rFv-PrP (Fig. 1C).

Changes in the Secondary Structure of rFv-PrP—The dramatic propensity of rFv-PrP to undergo dimerization-induced aggregation prompted us to examine whether AP20187 induces a structural change characterized by modifications in the content of α-helices and β-sheets. Fig. 3A shows the far UV CD spectra for rFv-PrP in PBS, pH 7.4, at a protein concentration of 6 μM. In the absence of AP20187, the spectrum of rFv-PrP displayed a double minimum at 222 and 208 nm, characteristic of α-helical structure. Remarkably, upon the addition of AP20187, we observed a concentration-dependent decrease in negative ellipticity, comitant with a change in the shape of the spectrum to one characteristic of β-sheet structure. The continuous helical destabilization can be monitored by an isodichroic point near 203 nm (37), which was recorded after an increasing concentration of dimerizing ligand (Fig. 2A, arrow). The percentages of protein secondary structure from CD spectra using the K2d program confirmed the structural changes and indicated similar proportions of α-helix, β-sheet, and disordered structures after treatment with 3 μM AP20187 (Fig. 2B). Importantly, the values obtained were consistent with values obtained for brain-derived PrPRes and after in vitro conversion of PrPRes by protein misfolding cyclic amplification (Table 1 and Ref. 17). The spectra and values (Fig. 2 and Table 1) show a still relatively high content of α-helical structure after AP20187 treatment, as expected for disease-associated misfolded prion protein (38). A low content or lack of α-helical structure is considered a drawback for many in vitro PrP refolding/misfolding assays in which the misfolded PrPRes form is almost entirely organized in an
aggregated β sheet structure (38). The addition of FK506 did not change the secondary structure of rFv-PrP (not shown).

**Dimerization Increases the Conformational Stability of rFv-PrP**—To gain more insights into the mechanism of PrP dimerization and its aggregation, the stability of rFv-PrP in the monomeric and aggregated states was studied by temperature-induced denaturation monitored by CD spectroscopy. In this context, changes in heat-induced conformational stability of rFv-PrP were determined at 222 nm ([θ]222) over a range of 10–90 °C different times after addition of AP20187. The unfolding profiles of rFv-PrP were associated with a single and cooperative transition (Fig. 3A). Using an in-house nonlinear least-squares fitting program, temperature-denaturation curves ([θ]222 (T)) were fitted to Equation 1, to obtain Tm, ΔH°u (Tm), ΔS°u (Tm), and ΔG°u (Tm). All of the thermodynamic parameters were measured from the equations described under “Experimental Procedures.” Thermal denaturation of rFv-PrP initially revealed a midpoint of thermal transition (Tm) of 70 °C (Fig. 3A, filled circles), in accordance with values published in the literature for rPrP (39, 40). The addition of AP20187 resulted in a slight decrease of the Tm value to 68.6 °C (Fig. 3A, empty circles). This can be assumed as the initial step of the binding where AP20187 docks into the Fv domain, which in turn increases the dynamics of binding site and a global destabilinity in rFv-PrP structure. However, the stability of rFv-PrP was restored in a time-dependent manner with an increase in Tm after 1 h (Fig. 3A, filled triangles). Maximum stability was observed 24 h following chemical dimerization of rFv-PrP with a Tm value of 79.8 °C (Fig. 3A, empty triangles; Table 2). A comparison of stability curves revealed that dimerization of rFv-PrP favored spontaneous transformation of the protein into more stable conformations (Fig. 3B). As depicted in Table 2, at 37 °C the free energy of unfolding of the aggregated states reached a maximum value [ΔG°u (37 °C) = 7.1 ± 0.2 kcal.mol⁻¹] when compared with that of the native soluble rFv-PrP [ΔG°u (37 °C) = 4.9 ± 0.1 kcal.mol⁻¹], with a ΔΔG°u (37 °C) = 2.2 ± 0.1 kcal.mol⁻¹. To analyze the nature of this favorable structural transformation, variation in enthalpy and entropy of unfolding were determined as a function of temperature (Fig. 3B). A comparison of ΔH°u (37 °C) of rFv-PrP unfolding during the aggregation indicated that less favorable (enthalpic) interactions are broken during the structural transformation (Table 2). However, the variation in entropy ΔS°u (37 °C) of unfolding of rFv-PrP caused by the dimerization-induced aggregation was calculated to be 12.2 ± 0.3 kcal.mol⁻¹ after 24 h. That is, the unfolding of the aggregated rFv-PrP is accompanied with a reduction of favorable (less positive) entropy. This behavior could be the result of the exposure of more buried hydrophobic surfaces upon chemical dimerization and structural transformation of the protein.

**Dimerization-induced Amyloid Formation of rFv-PrP and Self-seeded Conversion**—To further characterize the nature of dimerization-induced rFv-PrP aggregates, we used the ThT assay. ThT specifically binds to amyloid fibrils (31). The structure of rFv-PrP was very stable and no ThT binding occurred for over 60 h of incubation at 37 °C (Fig. 4A, empty squares). The addition of AP20187, but not monomeric FK506 to rFv-PrP resulted in a time-dependent increase in ThT binding (Fig. 4B), while the addition of AP20187 to rPrP did not show any ThT binding (Fig. 4C)
Dimerization-induced Prion Protein Aggregation

**FIGURE 4.** rFv-PrP fibrilization monitored by ThT-binding fluorescence assay. A, 50 μM rFv-PrP was incubated in the absence (■), or in the presence of 5 μM AP20187 (▲) or 5 μM FK506 (▲) in the standard physiological buffer. In the seeded reactions, 2% w/w of preformed seeds were incubated with rFv-PrP (▲) or rFv-PrP/AP20187 (●). The formation of amyloid fibrils was also induced in physiological buffer containing 100 mM GdnHCl and 1 M urea (△). All reactions were performed at pH 7.4 at a temperature of 37 °C. B and C, ThT fluorescence of rFv-PrP observed in a fluorescence microscope after excitation with UV light. rFv-PrP was incubated for 48 h in the presence of 3 μM AP20187 (B) or 3 μM FK506 (C).

4A, filled triangles and filled squares, respectively). The kinetic curves for the assembly of amyloid structures were characterized by a lag phase of 4 h that was completely eliminated after addition of a small amount (2% wt/wt) of the preformed rFv-PrP amyloid to monomeric rFv-PrP (Fig. 4A, compare filled triangles with empty and filled circles). These results indicate that the conformational conversion of rFv-PrP is a self-seeded reaction that requires dimerization-induced oligomeric nuclei. In a positive control experiment, 0.1 mM GdnHCl and 1 M Urea induced in physiological buffer containing 100 mM GdnHCl and 1 M urea (△). All reactions were performed at pH 7.4 at a temperature of 37 °C. B and C, ThT fluorescence of rFv-PrP observed in a fluorescence microscope after excitation with UV light. rFv-PrP was incubated for 48 h in the presence of 3 μM AP20187 (B) or 3 μM FK506 (C).

**FIGURE 5.** Morphology of rFv-PrP amyloid fibrils. A–D, electron micrographs of negatively stained rFv-PrP taken after 2 h (A) or 48 h (B and C) of incubation in physiological buffer at 37 °C in the presence of AP20187, or 48 h in the presence of FK506 (D). Note the presence of amyloid fibers after 48 h (B and C). C, higher magnification of section indicated by a black box in B. The bar represents 100 nm.

digestion. In these experiments, aggregates and amyloids were produced after incubation of rFv-PrP with 3 μM AP20187 for 1 h and 48 h, respectively. Untreated (not shown) or FK506-treated rFv-PrP was fully degraded in a 15-min incubation at 37 °C in the presence of as little as 0.5 μg/ml PK (Fig. 6A, left panel). In contrast, rFv-PrP incubated with AP20187 was slightly resistant to PK, and a number of fragments persisted at 2 μg/ml of PK (Fig. 6A, middle panel). A concentration of 8 μg/ml was required to completely proteolyze amyloid fibers (Fig. 6A, right panel). These results were not specific to the human protein, because the mouse protein (rFv-MoPrP) also acquired some resistance to PK treatment after incubation with AP20187 (Fig. 6B).

The C-terminal Structured Domain Is Responsible for Dimerization-induced Aggregation of PrP—To test which domain of PrP is involved in the aggregation of the protein after dimerization, we engineered two recombinant proteins, rFv-PrP23–124, representing the N-terminal unstructured domain between residues 23 and 124, and rFv-PrP124–231, representing the folded C-terminal domain between residues 124 and 231. Similar to rFv-PrP, these two truncated molecules were also fused to Fv. rFv-PrP23–124 was slightly insoluble, because some protein was recovered in the pellet in the absence of any ligand after centrifugation at 15,600 × g (Fig. 7A). Addition of AP20187 or FK506 did not result in a significant increase of the protein in the pellet fraction, indicating that dimerization of rFv-PrP23–124 does not induce its aggregation. In contrast, addition of AP20187 but not FK506 to rFv-PrP124–231 triggered the transfer of the protein from the soluble to the insoluble fraction (Fig. 7A). In accordance to these results, aggregates formed

Dimerization-induced Aggregates and Amyloids Are Slightly Resistant to PK Digestion—Next we sought to investigate the extent to which AP20187-treated rFv-PrP is resistant to PK
after dimerization of rFv-PrP124–231 were slightly resistant to PK treatment (Fig. 7B). Incubation of rFv-PrP23–124 with AP20187 did not modify the resistance of the protein to PK treatment. Interestingly, rFv-PrP124–231 was more sensitive to PK than rFv-PrP (compare Figs. 6A and 7B), suggesting that the N-terminal domain of PrP is important to acquire full resistance to PK. All together, these results show that the C-terminal domain of PrP is essential for dimerization-mediated aggregation of PrP.

Recombinant Fv-PrP Conversion by Shaking—PrP<sub>res</sub>-seeded conversion of rPrP is an efficient method to demonstrate the presence and propagation of PrP<sub>res</sub> (34, 35). Because dimerization-induced rFv-PrP aggregates have seeding activity in amyloid formation assays (Fig. 4A), we tested if they also display seeding activity in misfolding cyclic amplification assays using the quaking-induced conversion method recently described (34). rFv-PrP aggregates were diluted 20 times into a buffer containing soluble rFv-PrP, and the reaction mixture submitted to quaking-induced conversion. The generation of aggregates was determined by Western blotting after PK digestion at a concentration of 2 μg/ml PK (Fig. 9A). This concentration of PK allowed the detection of aggregated rFv-PrP in the first shaking cycle (Fig. 9A). A concentration of 10 μg/ml of PK was otherwise required to completely digest rPrP aggregates seeded by rFv-PrP aggregates (Fig. 9B), and soluble rPrP was completely degraded with 2 μg/ml PK (Fig. 9C). The conversion of rPrP with aggregated Fv-PrP was observed through at least six serial reactions (Fig. 9A). In a reverse experiment, aggregated rPrP was also able to convert soluble rFv-PrP in shaking reactions (Fig. 9D).

Aggregates but Not fibrils Are Toxic to Cultured Cells—The toxicity of dimerization-induced aggregates was tested in N2a cells by the internucleosomal DNA fragmentation assay. Aggregates and amyloid fibers were produced as described.

FIGURE 6. Differential resistance of the rFv-PrP species to PK digestion. A, rFv-PrP (2 μg) was incubated in PB buffer at 37 °C in the presence of FK506 for 48 h, or in the presence of AP20187 for 1 h or 48 h. Proteins were then subjected to PK digestion at various concentrations of PK ranging from 50 ng to 3.2 μg in a total volume of 200 μl. Western blot analysis was performed using 3F4 antibody. The positions of rFv-PrP and PK-resistant (PK-res.) fragments are indicated. B, rFv-MoPrP (2 μg) was incubated in PB at 37 °C in the presence of FK506 or AP20187 for 48 h. Proteins were then subjected to PK digestion at various concentrations of PK ranging from 30 ng to 3.2 μg in a total volume of 200 μl. Western blot analysis was performed using EP1802Y antibody.

FIGURE 7. Aggregation of truncated mutants of rFv-PrP. A, Coomassie Blue staining: dimerization of 50 μM rFv-PrP<sub>23–124</sub> or rFv-PrP<sub>124–231</sub> was induced using the indicated concentrations of AP20187 at 37 °C for 30 min. After centrifugation at 15,600 × g for 15 min supernatants were separated and precipitated by trichloroacetic acid. Pellets and precipitated supernatant were solubilized in SDS loading buffer, and a 10-μl aliquot from each sample was subjected to a 12% SDS-PAGE analysis. B, differential resistance of rFv-PrP<sub>23–124</sub> and rFv-PrP<sub>124–231</sub> to PK digestion. Recombinant proteins (2 μg) were incubated in PB buffer at 37 °C in the presence of FK506 or AP20187 for 48 h. Proteins were then subjected to PK digestion at various concentrations of PK ranging from 50 to 250 ng in a total volume of 200 μl. Western blot analysis was performed using 3F4 (rFv-PrP<sub>23–124</sub>) or EP1802Y (rFv-PrP<sub>124–231</sub>) antibodies.
Dimerization-induced Prion Protein Aggregation

**FIGURE 8. Quaking reactions seeded with rFv-PrP or rFv-MoPrP aggregates.** A, rFv-PrP was incubated in the presence of 5 μM AP20187 or FK506 for 1 h. Samples were diluted 20 times into freshly prepared soluble rFv-PrP and submitted to quaking reactions as described under "Experimental Procedures." Dilutions and quaking reactions were repeated five times. Proteins were then subjected to 2 μg/ml PK digestion and Western blot analysis using 3F4 antibody. The positions of rFv-PrP and PK-resistant (PK-res.) fragments are indicated. The concentration of AP20187 and FK506 remaining in each reaction is also indicated. B, rFv-MoPrP was incubated for 5 h in the presence of 5 μM AP20187. Samples were submitted to five cycles of quaking reactions as described in A. Proteins were then subjected to 2 μg/ml PK digestion and Western blot analysis using EP1802Y antibody.

above. Exposure of cells to 10 μM rFv-PrP aggregates for 72 h resulted in clear DNA fragmentation, similar to cells exposed to the apoptosis inducer staurosporine (Fig. 10). In contrast, amyloid fibers were completely innocuous, demonstrating that only non-amyloid PrP aggregates are toxic species. FK506-treated rFv-PrP was also not toxic to N2a cells. Aggregates from the steroidogenic acute regulatory protein (StAR) were innocuous, indicating that toxicity is specifically associated with PrP aggregates.

**DISCUSSION**

Misfolding of PrpC is the central pathogenic event in TSEs, yet what triggers de novo conformational change and aggregation of the protein in physiological conditions (e.g. no sonication, no chaotropic or denaturing agent, neutral pH) and in the absence of PrPRes remains unknown. The most important finding of this study is the recognition of dimerization as a minimal and sufficient mechanism leading to spontaneous aggregation of PrP in physiological conditions. Furthermore, dimerization-induced aggregation is an intrinsic property of PrP, because it occurs in vitro using the purified recombinant protein.

Several lines of evidence indicate that dimerization-induced misfolding of PrpC may be relevant to the aggregation of the protein in prion diseases. First, our findings are consistent with previous reports based on theoretical and experimental data suggesting that dimerization has an important role in conformational change of PrpC (reviewed in Ref. 23). We have directly tested and confirmed this hypothesis by using a conditional dimerization strategy. Most importantly, aggregates obtained after dimerization of rFv-PrP display seeded activity in amyloid fibrils formation and quaking conversion assays. Second, the proposition that dimerization may be a key event in PrP aggregation implies that PrP dimers should exist in both physiological and pathological conditions. Indeed, the presence of PrP dimers has been detected in non-infected hamster and mouse brains (42) and in murine neuroblastoma cell line expressing hamster PrP (43). Dimers were also detected in Creutzfeldt-Jakob infectious material disaggregated in gentle conditions (44) and in infected hamster brains (43). Third, monomeric rPrP is able to spontaneously dimerize during its crystallization process (45). The structure of the dimer suggested that dimerization may constitute an important step in the conversion PrpC → PrPRes. Finally, an in vitro study on the mechanism of PrP assembly into amyloid demonstrated that the precursor exists in a monomer-dimer equilibrium (46). The contact sites in the dimer were identified to residues 90–106 and 152–156.

What may trigger the dimerization of PrpC in prion diseases? In TSEs acquired by infection, PrPRes interacts with and could force the dimerization/oligomerization of PrpC molecules (22, 47, 48). In TSEs non-acquired by infection, dimerization of PrpC may be triggered either by an unknown ligand (49), or by the concentration of the protein in a restricted environment.

Our thermodynamic data indicate that the population of rFv-PrP dimers is more stable than that of the soluble monomers. A progressive increase in the entropy of reaction (Table 2) is a consequence of the disruption of a hierarchy of different kinds of intermolecular interactions (hydrogen bonds, electrostatic, dispersion forces, and disulfide bridges) at the monomer-monomer interface. This evolution of internal dynamics in association with strong hydrophobic forces helps the system to cross the energy barriers according to the energy landscape model (50). This can result in the formation of a stable "seed," which serves as a template for other soluble monomers. A global structural transformation in population of soluble rFv-PrP monomers shifts the equilibrium toward the massive rFv-PrP aggregation and subsequent amyloid fibril formation.

It is noteworthy that dimerization-induced aggregates form ThT-positive structures with typical amyloid morphology after 48 h of incubation. Previous reports describing the
production of amyloid fibers from rPrP are all associated with the use of denaturants, elevated temperatures, or a combination of phospholipids bicelles and elevated temperature (11, 51, 52). Our observation indicates that dimerization-induced aggregates are competent to form amyloids and demonstrate that it is possible to achieve the generation of PrP amyloid fibers in vitro in physiological-like conditions.

Another interesting finding in this study is that rFv-PrP aggregates but not amyloids are cytotoxic. This result confirms previous evidence that small aggregates rather than amyloids are the toxic species in prion diseases and in other neurodegenerative diseases (3, 15, 53) and underscores the relevance of dimerization in prion diseases.

From our results, it is tempting to speculate that dimerization of PrPC in vivo would be a noxious event. This hypothesis is comforted by the observation that enforced dimerization of PrPC using cross-linking antibodies results in neuronal toxicity in mice (54). In that study, the presence of PrP aggregates was not investigated. However, in contrast to chemical dimerization, it is likely that cross-linking antibody-mediated dimerization may interfere with further conformational changes and aggregation.

The present study establishes dimerization of PrPC as a potential toxic mechanism in TSEs. Future therapeutic strategies may be aimed at preventing the contact between PrPC monomers.

**Acknowledgments**—We thank Victor M. Rivera (ARIAD Pharmaceuticals, Cambridge, MA) for providing the AP20187 chemical dimerizer. We also thank the people from the electron microscopy facility of the Faculty of Medicine at the University of Sherbrooke.

**REFERENCES**

1. Prusiner, S. B. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 13363–13383
2. Stahl, N., Baldwin, M. A., Teplow, D. B., Hood, L., Gibson, B. W., Burlingame, A. L., and Prusiner, S. B. (1993) Biochemistry 32, 1991–2002
3. Caughey, B. W., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, W. S. (1991) Biochemistry 30, 7672–7680
4. Pan, K. M., Baldwin, M., Nguyen, J., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E., et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10962–10966
5. Supattapone, S. (2004) J. Mol. Med. 82, 348–356
6. Baskakov, I. V. (2007) FEBS J. 274, 576–587
7. Swietnicki, W., Morillas, M., Chen, S. G., Gambetti, P., and Surewicz, W. K. (2000) Biochemistry 39, 424–431
8. Jackson, G. S., Hosszu, L. L., Power, A., Hill, A. F., Kenney, J., Saibil, H., Craven, C. J., Wahlto, J. P., Clarke, A. R., and Collinge, J. (1999) Science...
Dimerization-induced Prion Protein Aggregation

283, 1935–1937
9. Morillas, M., Vanik, D. L., and Surewicz, W. K. (2001) Biochemistry 40, 6982–6987
10. Baskakov, I. V., Legname, G., Prusiner, S. B., and Cohen, F. E. (2001) J. Biol. Chem. 276, 19687–19690
11. Bocharova, O. V., Breydo, L., Parfenov, A. S., Salnikov, V. V., and Baskakov, I. V. (2001) J. Mol. Biol. 346, 645–659
12. Cobb, N. J., Apetri, A. C., and Surewicz, W. K. (2008) J. Biol. Chem. 283, 34704–34711
13. Wang, F., Yang, F., Hu, Y., Wang, X., Wang, X., Jin, C., and Ma, J. (2007) Biochemistry 46, 7045–7053
14. Legname, G., Baskakov, I. V., Nguyen, H. O., Riesner, D., DeArmond, S. J., and Prusiner, S. B. (2004) Science 305, 673–676
15. Castilla, J., Saá, P., Hetz, C., and Soto, C. (2005) Cell 121, 195–206
16. Rezaei, H., Eghiaian, F., Perez, J., Doulet, B., Choquet, Y., Haertle, T., and Grosclaude, I. (2005) J. Biol. Chem. 347, 665–679
17. Prusiner, S. B., Scott, M., Foster, D., Pan, K. M., Groth, D., Mirenda, C., Parfenov, A. S., Salnikov, V. V., and Oldstone, M. B., Skyes, B. D., Hodges, R. S., and Kay, C. M. (1995) J. Biocomm. 7045–7053
18. Knaus, K. J., Morillas, M., Surewicz, W. K., and Yee, V. C. (2001) Trends Biochem. Sci. 26, 673–686
19. Horiiuchi, M., and Caughey, B. (1999) EMBO J. 18, 3193–3203
20. Dimerization-induced Prion Protein Aggregation

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 284 • NUMBER 45 • NOVEMBER 6, 2009

30916