Gerstmann-Sträussler-Scheinker Disease Amyloid Protein Polymerizes According to the “Dock-and-Lock” Model*

Marco Gobbi,† Laura Colombo,‡ Michel A. Morbin,§ Giuida Mazzoleni,† Elena Accardo,§ Marco Vanoni,∥ Elena Del Favero,∥ Laura Cantú,∥ Daniel A. Kirschner**∥∥∥, Claudia Manzoni,∥ Marten Beeg,∥ Paolo Ceci,∥ Paolo Ubezio,∥ Gianluigi Forloni,∥ Fabrizio Tagliavini,∥ and Mario Salmina

From the †Istituto di Ricerche Farmacologiche “Mario Negri,” 20100 Milano, Italy, the ‡Istituto Nazionale Neurologico “Carlo Besta,” 20100 Milano, Italy, the ¶Dipartimento di Biotecnologie e Bioscienze, Università di Milano Bicocca, 20100 Milano, Italy, the †Dipartimento di Chimica Biochimica e Biotecnologie per la Medicina, Università degli Studi di Milano, 20100 Milano, Italy, and the **Biological Department, Boston College, Chestnut Hill, Massachusetts 02467

Prion protein (PrP) amyloid formation is a central feature of genetic and acquired prion diseases such as Gerstmann-Sträussler-Scheinker disease (GSS) and variant Creutzfeldt-Jakob disease. The major component of GSS amyloid is a PrP fragment spanning residues 82–146, which when synthesized as a peptide, readily forms fibrils featuring GSS amyloid. The present study employed surface plasmon resonance (SPR) to characterize the binding events underlying PrP82–146 oligomerization at the first stages of fibrillization, according to evidence suggesting a pathogenic role of prefibrillar oligomers rather than mature amyloid fibrils. We followed in real time the binding reactions occurring during short term (seconds) addition of PrP82–146 small oligomers (1–5 mers, flowing species) onto soluble prefibrillar PrP82–146 aggregates immobilized on the sensor surface. SPR data confirmed very efficient aggregation/elongation, consistent with the hypothesis of nucleation-dependent polymerization process. Much lower binding was observed when PrP82–146 flowed onto the scrambled sequence of PrP82–146 or polymerization process. Much lower binding was observed when PrP82–146 flowed onto the scrambled sequence of PrP82–146 or onto prefibrillar Aβ42 aggregates. As previously found with Aβ40, SPR data could be adequately fitted by equations modeling the “dock-and-lock” mechanism, in which the “locking” step is due to sequential conformational changes, each increasing the affinity of the monomer for the fibril until a condition of irreversible binding is reached. However, these conformational changes (i.e. the locking steps) appear to be faster and easier with PrP82–146 than with Aβ40. Such differences suggest that PrP82–146 has a greater propensity to polymerize and greater stability of the aggregates.

Prion diseases are transmissible neurodegenerative disorders characterized by the transition of the prion protein (PrP) from an α-helix-rich soluble isoform (PrP0) into disease-specific insoluble species (PrPSc) with a prevalent β-sheet structure (1, 2). A major feature of PrPSc is partial resistance to proteinase K digestion, with the protease-resistant core assembling into insoluble fibrillar structures with the properties of amyloid (3). Amyloid formation is a major feature of variant Creutzfeldt-Jakob disease and Gerstmann-Sträussler-Scheinker disease (GSS). GSS is a prion disease caused by germ line mutations in the PRNP gene, causing deposition of amyloid whose major component is a PrP fragment of ~7 kDa, spanning residues 81–82 to 144–153 (4–6), which is very similar in patients with different mutations (i.e. A117V, F198S, and Q217R). This fragment is an integral part of the minimal sequence that sustains prion replication (7, 8), so it might play a central role in the conformational transition of PrP0 into PrPSc and PrPSc propagation. When synthesized as a peptide, PrP82–146 adopts a secondary structure primarily composed of β-sheet and turn and readily forms fibrils with the staining and ultrastructural features of GSS amyloid (9).

It is generally thought that the polymerization of amyloid peptides is a stepwise process, with early events leading to formation of small oligomers, followed by assembly into soluble protofibrils first and insoluble fibrils later (10). This process has been examined in detail for amyloid β (Aβ) of Alzheimer’s disease using a variety of methods to unravel the different steps of fibril formation and growth. A “dock-and-lock” model has been proposed for fibrillogenesis (11, 12) where Aβ binding to growing fibrils (locking) is followed by a conformational rearrangement of the bound monomer (locking), which increases its affinity for the fibril.

This model has been confirmed by studies with surface plasmon resonance (SPR) biosensors (13) that served to follow in real time the binding reactions between Aβ fibrils, immobilized on a sensor chip, and Aβ monomers continuously flowing onto the chip at a constant concentration (14–16). This technology enables us to study association and dissociation rates on a very short time scale (seconds), thus approaching the single events underlying fibril elongation.

Understanding the early phases of Aβ misfolding and aggregation is particularly important for the development of prevention strategies, because oligomers rather than mature amyloid fibrils may feature in nerve cell dysfunction and degeneration in animal models of disease. Similarly, critical oligomeric states have been described in the pathway of PrP fibril formation (17–19), including species with β-turn-rich conformation and neurotoxic properties (19). However, information is limited on the binding events underlying PrP oligomerization, particularly at the first stages of fibrillization. We therefore used SPR to obtain kinetic data regarding these early binding events, after short term flow of low order oligomers onto soluble, prefibrillar PrP82–146 aggregates immobilized on the SPR sensor chips. Our data indicate that the PrP82–146 polymerization reaction is well explained by the dock-and-lock model, the “locking” step actually being due two sequential isomeriza-
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The dissociation steps, each increasing the affinity of the monomer for the fibril until a condition of irreversible assembly is reached. These studies highlight similarities and differences between Aβ and PrP82–146.

**EXPERIMENTAL PROCEDURES**

**Peptides**—PrP82–146 and PrP82–146 with a totally scrambled sequence (PrP82–146scr) were synthesized and purified as previously described (9, 20). β-Amyloid 1–42 (Aβ42) was purchased from U.S. Peptide Inc. (Pomona, CA).

**Peptide Immobilization on Sensor Surface**—The peptide solutions used for immobilization on the BIACORE sensor chip were prepared to facilitate the formation of high order aggregates. Thus, 200 μM PrP82–146 were incubated for 18–24 h at room temperature in 2× Dulbecco’s phosphate-buffered saline (PBS; pH 7.4; Invitrogen). In these conditions PrP82–146 readily formed visible aggregates. Just before the immobilization procedure, the solutions were rapidly centrifuged (800 rpm for 30 s); the supernatants (containing “prefibrillar aggregates”; see “Results”) were diluted four times with deionized water and used for the immobilization procedure. The peptides were covalently linked to the sensor chips with carboxymethylated dextran surface (CM4 and CM5; Biacore Inc., Uppsala, Sweden) using amine-immobilizing chemistry (21).

After surface activation, the peptide solutions were injected for 2–10 min (flow rate, 5 μl/min), and the remaining activated groups of the dextran surface were blocked with a 15-min injection of 1× ethanolamine, pH 8.0 (21). A reference cell was always prepared in parallel by the same immobilization procedure (activation/deactivation steps) without the addition of peptides. In some experiments the scrambled PrP82–146 peptide (9) was immobilized using the same procedure described for wild-type PrP82–146. As a further control in one experiment, we immobilized Aβ42 using a peptide solution containing prefibrillar aggregates morphologically similar to those used for PrP82–146 immobilization. To this aim, a 220 μM Aβ42 solution was incubated for 1 h in 2× PBS, diluted four times with deionized water, and centrifuged for 30 s at 800 rpm. The supernatant was diluted five times with acetate buffer, pH 4.0, and used for the immobilization procedure.

**SPR Analysis of PrP82–146 Extension and Dissociation**—The flowing PrP82–146 solutions were prepared to minimize aggregation reactions and favor the presence of low order oligomers. The peptide was dissolved in ice-cold, sterile deionized water at a final concentration of 1–2 mM, kept on ice, and used within 8 h. Just before injection, the peptide solution was diluted to 3–40 μM using BIACORE running buffer (HBS, 10 mM Hepes, 150 mM NaCl, 3.0 mM EDTA, and 0.005% surfactant P20, pH 7.4). In one experiment only (see Fig. 6) we injected the PrP82–146 solution containing higher order assemblies, prepared by diluting the supernatant used for the immobilization procedure (see above) 10-fold with HBS.

PrP 82–146 solutions were injected at a rate of 3–30 μl/min for 2–8 min, as indicated. At the end of the injection, the sensor surface was washed, and the dissociation phase data were collected for 15–60 min. The assays were done at 25 °C. The solutions were run simultaneously over two to four flow cells. The sensogram (time course of the SPR signal, expressed in resonance units (RU)) observed in the cell immobilizing the peptide was corrected by subtracting the response observed in the empty cell (reference cell) and usually normalized to a baseline of 0 RU.

The dissociation curves are classically analyzed using exponential equations with the simplest situation, i.e., from a simple bimolecular or Langmuir interaction, modeled by a single exponential decay. Here, double and triple exponential equations were also used to fit the data to see whether more complicated models were significantly better than the simpler ones, by the F-test (extra sum-of-squares test; GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA). Finally, kinetic rate constants were obtained by the global fitting of both association and dissociation phases according to the three-step model (16) described by the following equations,

\[
d[B]/dt = -k_1[A][B] + k_{-1}[AB],
\]

(Eq. 1)

\[
d[AB]/dt = k_1[A][B] - (k_{-1} + k_2)[AB] + k_{-2}[A*B],
\]

(Eq. 2)

\[
d[A*B]/dt = k_2[AB] - (k_{-2} + k_3)[A*B] + k_3[A**B],
\]

(Eq. 3)

\[
d[A**B]/dt = k_3[A*B] - k_{-3}[A**B],
\]

(Eq. 4)

where \(k_1, k_2, k_3, k_{-1}, k_{-2}, \) and \(k_{-3} \) are the values of the rate constants of the forward and reverse reactions, \([A]_0\) is the concentration of injected PrP82–146 peptide, \([B]_0\) is the amount (RU) of unbound immobilized PrP82–146 fibrils, \([AB]_t\) is the amount of bound peptide (RU), \([A*B]\), and \([A**B]\) represent subsequent conformational changes (see “Results”), and the dependence on time is explicitly indicated. Less complex models were also considered, by setting \(k_2 = 0\) (two-step model) or \(k_{-3} = 0\) (simple bimolecular interaction).

With any given set of values of the rate constants, and of \([A]_0\), the equations were solved numerically using a Runge-Kutta second order approximation in a spreadsheet of Microsoft Excel, and the simulated response (SR) of the SPR measurement was calculated (SR = C[AB] + [A*B] + [A**B]), where C is a suitable instrumental constant).

SR was compared with the measured response (R), and the values of the rate constants were optimized minimizing the sum of square errors (2)(SR – R)². For this purpose, we used the Solver function associated to each spreadsheet of Microsoft Excel, which performs a constrained nonlinear fitting based on the generalized reduced gradient algorithm.

**Nature of the Oligomers in the Peptide Solutions Used for SPR Studies**—The ultrastructure of soluble peptide aggregates used for immobilization on sensor surface for SPR analysis was determined by electron microscopic analysis. Five μl of PrP82–146 solutions were applied to 200-mesh Formvar carbon-coated nickel grids for 6 min, gently removed using Whatman No. 1 paper, air-dried, and negative stained with uranyl acetate for 5 min. The samples were observed with an electron microscope (EM109; Zeiss, Oberkochen, Germany) operated at 80 kV at a standard magnification, calibrated with an appropriate grid. The mean diameter of peptide aggregates in the supernatant was measured on computer captured images using a computer-assisted image analyzer (Nikon Eclipse E-800) (9, 20). The presence, amount, size, and morphology of materials detected by electron microscopy were semiquantitatively evaluated by three independent operators.

To verify the nature of the species formed by PrP82–146 in flowing solution, we performed an immunogold labeling using the monoclonal antibody 3F4 that recognized an epitope corresponding to residues 108–111 of human and hamster PrP proteins (22). Five μl of PrP82–146 flowing solutions were applied to 200-mesh Formvar carbon-coated nickel grids for 6 min, gently removed, and fixed with 5 μl of PBS containing 4% paraformaldehyde and 0.05% glutaraldehyde. Five min later the fixing buffer was replaced with 5 μl of incubation buffer (PBS with 2% bovine serum albumin) containing 5% horse serum and further incubated for 20 min. The grids were then incubated for 2 h with 3F4 anti-
bipyridyl)-ruthenium (II) and 1 mM sodium phosphate, pH 7.4, and the mixture was irradiated using a 150-watt short arc Xenon lamp positioned 15 cm from the reaction tube. Unless otherwise stated, the irradiation time was 10 s, and the irradiation was performed on the flowing solution (25, 26). PrP82–146 monomers-to-trimers are presumably in a dynamic equilibrium, as indicated by preliminary data showing that the ultrastructural examination of the solution used for PrP82–146 immobilization on the sensor chip disclosed that most of the materials aggregated in a loose meshwork of little chains formed by small round-shaped particles having a diameter of 19 ± 7 nm (range 8–52 nm; Fig. 1c). Moreover electron microscopy analysis disclosed the presence of a few straight, short filaments with a diameter of ~5.5 nm, whereas no fibrils were detected.

Small round shaped material, often organized to form little chains, was present in the solution of Aβ42 immobilized on sensor chip as an internal control (Fig. 1d). For Aβ42, however, the globular units appeared smaller (8 ± 2 nm; range, 3–15 nm).

When examined by quasi-elastic laser light scattering, the PrP82–146 “flowing solution” contained species with an average hydrodynamic radius ($R_H$, by cumulant analysis) of 250 nm. Multiexponential analysis of the correlation function decay (Fig. 2A) showed that most of these species (>99% by number) had average $R_H = 12$ nm, corresponding to small oligomers. Much larger species (average $R_H = 1700$ nm) were found in the PrP82–146 solution used for immobilization on the sensor surfaces.

SDS-PAGE on both the PrP82–146 solutions, without any irradiation, showed monomers to trimers (Fig. 2B, lanes 1, 2, 5, and 6). Dimers and trimers are therefore very stable, being resistant to a procedure involving boiling in SDS. For comparison, in the same experimental conditions Aβ40 was detected as monomers only, as previously described (25, 26). PrP82–146 monomers-to-trimers are presumably in a dynamic equilibrium, as indicated by preliminary data showing that isolation of the monomers promptly results in the formation of dimers and trimers.
good reproducibility between the responses obtained when repeating peptide injections over the same flow cell (see for example the replicates in Fig. 3, A and B). Furthermore, the cumulative amount of peptide remaining pseudo-irreversibly bound after consecutive cycles of injections could greatly exceed (up to 30 times) the amount of peptide that had been initially immobilized on the chip, strongly suggesting an aggregation/elongation process.

The association phase of specific binding (i.e. corrected for the signal measured in the empty cell; Fig. 3D) was essentially linear, although in some cases we noted some slight curvature. Under our experimental conditions (up to 8 min of injection), however, the reaction never reached equilibrium. The association rates, calculated between 30 and 90 s, were linearly correlated with the concentration of injected peptide (Fig. 3). Similarly, the association rates measured on injecting 10 μM PrP82–146 were also linearly correlated with the fibril surface concentration (not shown).

PrP82–146 dissociation (see a representative sensorgram in Fig. 5) appeared to be multibasichic because the single exponential fitting was significantly worse than the double exponential fitting, which was significantly worse than the triple exponential fitting. According to the triple exponential fitting shown in the inset of Fig. 5, 9 ± 5% (n = 8) of the bound PrP82–146 dissociates with a very short half-life (0.05 ± 0.02 min), 9 ± 5% dissociates with a longer half-life (1.1 ± 0.9 min), and 12 ± 5% dissociates with an even longer half-life (6 ± 5 min). The remaining fraction appears to be irreversibly bound.

Global fits of the association and dissociation phases were also done, using equations corresponding to different models. At least a three-step model similar to that previously described for Aβ40 (16) was necessary to fit the data adequately. This model assumes that once bound to the fibril, the peptide undergoes two conformational changes, each of them described by first order rate constants, leading to a more stable complex.

The association phase constant ($K_\text{a}$) of PrP82–146 is lower than that of Aβ40, but this is compensated by a lower dissociation rate constant ($K_\text{d}$). Thus, apparent $K_{\text{eq}}$ values calculated for the initial binding step of Aβ40 and PrP82–146 (123 and 105 μM, respectively) are very similar. Once docked PrP82–146 undergoes dissociation or the first isomeriza-
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FIGURE 5. Time course of PrP82–146 dissociation. Representative BIACORE sensorgram obtained by injecting 20 μM PrP82–146 (small oligomers) on the flow cell coated with PrP82–146 prefibrillar aggregates (10,000 RU). PrP82–146 dissolved in HB5 was injected for 2 min at a flow rate of 30 μL/min, and the dissociation phase was followed for 60 min. The sensorgram is corrected for the signal measured in the empty cell to show the “specific” binding (see Fig. 3). The response was adjusted to 0 just before the injection. The inset highlights the dissociation phase and also shows the fitting of the data points by a triple exponential function, which was significantly better than the double and single exponential fittings (sum-of-squares, 2.8, 11, and 98, respectively, using the three equations). The goodness of the fitting by the triple exponential equation is also highlighted by the small residuals.

TABLE 1
Kinetic rate constants for PrP82–146 aggregation

| Kinetic rate constants for PrP82–146 aggregation |
|-----------------------------------------------|
| Kinetic rate constants for PrP82–146 aggregation were obtained by fitting sensorgrams according to the three-step model previously used for Aβ40. This involves a first “docking” step between the flowing small oligomers and the immobilized aggregates, with k1 and k-1, as the corresponding association and dissociation constants. This binding step is then followed by two conformational changes of the bound peptide (locking steps, characterized by k2 and k-2 rates) leading to a more stable complex. As for Aβ40, this model was required to adequately fit the experimental data obtained with PrP82–146. Seventeen sensorgrams from four experimental sessions were analyzed, and the calculated constants are shown in the table as the means ± S.E. (n = 17).

|        | PrP82–146 | Aβ40* |
|--------|-----------|-------|
| k1 (s⁻¹) | 3.9 ± 0.6 × 10⁻² | 6.6 ± 1.5 × 10⁻¹ |
| k-1 (s⁻¹) | 4.0 ± 1.0 × 10⁻² | 8.1 ± 1.4 × 10⁻¹ |
| k2 (s⁻¹) | 2.3 ± 0.3 × 10⁻³ | 6.4 ± 0.6 × 10⁻³ |
| k-2 (s⁻¹) | 2.3 ± 1.0 × 10⁻³ | 4.4 ± 0.4 × 10⁻³ |
| k3 (s⁻¹) | 4.4 ± 1.0 × 10⁻³ | 4.6 ± 0.2 × 10⁻³ |
| k-3 (s⁻¹) | 7.2 ± 3.3 × 10⁻⁵ | 4.3 ± 0.3 × 10⁻⁴ |

* The data for Aβ40 are from Cannon et al. (16) and are shown here for comparison.

tion with similar rates (compare K₁ with K₃), whereas dissociation is favored with Aβ40. Once isomerized first, the reaction proceeds toward further isomerization (compare K₃ with K₋₃) with low values of K₋₃ and, mainly, K₋₂, indicating more stable states.

The absolute amount of PrP82–146 remaining pseudo irreversibly bound to (incorporated into) the immobilized peptide structures was proportional to the concentration of both the injected and the immobilized peptide. In all cases this amounted to 68.1 ± 2.7% of the maximal PrP82–146 binding measured at the end of the 2-min injection (mean ± S.E. n = 11). The proportion was significantly higher (77.7 ± 2.7%, n = 6, p < 0.05, Student’s t test) when the association time was increased to 8 min.

FIGURE 6. PrP82–146 prefibrillar aggregates do not bind to similar high order structures immobilized on the chip. A shows the sensorgrams obtained by injecting a PrP82–146 solution prepared by diluting 10-fold with HB5 the same supernatant used for the immobilization procedure to have a theoretical peptide concentration of 20 μM. The peptide was injected for 2 min at a flow rate of 30 μL/min, on the peptide-coated cell (Fc2, ~450 RU) and on the empty cell (Fc1). B shows the sensorogram calculated as the difference between the signal in Fc2 and the signal in Fc1 (lower sensorgram) comparing it with the corresponding sensorgram obtained by injecting 20 μM of small PrP82–146 oligomers. The plots were created by adjusting the response (RU) to 0 just before the injection.

The sensorgrams were analyzed, and the calculated constants are shown in the table as the means ± S.E. (n = 17).

**DISCUSSION**

SPR technology has been previously used in several investigations focusing on PrP and PrP-related proteins. Thus, for example, SPR
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allowed the study of the interaction of recombinant PrP with lipid membranes (27), protein kinase CK2 (28), oligomeric actin-interacting protein2 (29), polyanions (30), and quinoline derivatives (31) or the interaction of the N-terminal fragment PrP23–110 with different plasminogen activators (32, 33). SPR data also concurred to exclude a direct interaction between the prion domain of Ure2p (Ure2p1–80) with the C-terminal (81–354) functional nitrogen regulation domain of this yeast protein (34). Most of these studies, however, provided only a qualitative analysis of SPR data, whereas the binding constants were not determined because of the complex association and dissociation kinetics, not fitting the classic 1:1 interaction model. Complex binding kinetics were always found when PrP was used as the flowing analyte, suggesting oligomerization of PrP on the sensor chip (27, 28, 30, 32).

Recent reports, however, suggested that SPR technology has the potential to provide quantitative kinetic data for the fibril elongation reactions too, as shown by studies carried out using Aβ (14–16). In the present study, we therefore used SPR to characterize the reactions underlying the oligomerization of the fibrillogenic prion fragment PrP82–146, i.e. the major component of GSS amyloid (9). In particular, our aim was to determine the kinetic constants of the early binding events, as observed after short term addition of low order oligomers (1–5-mers) to soluble pre fibrillar PrP82–146 aggregates immobilized on the sensor surface.

The polymerization states of the PrP82–146 actually involved in the binding reactions were confirmed by quasi-elastic laser light scattering, electron microscopy, and photo-induced cross-linking of unmodified proteins studies. SDS-PAGE data obtained without previous irradiation suggested high stability of dimers and trimers, which might well, together with monomers, be the constituent molecular “units” underlying PrP82–146 aggregation.

The SPR binding data suggest a very efficient aggregation/elongation process, in which the binding of the flowing small oligomers to the pre fibrillar aggregate is followed by isomerization of the bound peptide leading to pseudo-irreversible binding (see below) with the generation of new binding sites. Thus, 1) a significant amount (more than 60%) of the peptide bound at the end of each injection does not dissociate; 2) the peptide remaining irreversibly bound did not change the binding capacity nor the kinetic constants, of the subsequent injections cycles; and 3) consecutive binding cycles could increase the peptide mass on the chip up to 30-fold the originally immobilized mass, with no apparent saturation. These findings confirm that the binding partners of the flowing PrP82–146 small oligomers are high order oligomers further supporting the idea of nucleation-dependent polymerization (35), in which the formation of the nucleus (such as the pre fibrillar aggregates) is the rate-limiting step in fibril formation. Much lower PrP82–146 binding was found either on immobilized PrP82–146sc, previously characterized as a nonaggregating peptide (9), and on immobilized Aβ42 pre fibrillar aggregates. These data support the concept that the PrP82–146 elongation/aggregation requires sequence-specific binding interactions. Finally, our SPR data also showed that pre fibrillar PrP82–146 aggregates bind together very poorly.

The dissociation phase after PrP82–146 injection was multiphasic, as previously described with Aβ40 (14–16), and for both peptides global sensorgrams could be adequately fitted only by equations modeling the dock-and-lock mechanism, in which the locking step is due to at least two sequential isomerization steps, each increasing the affinity of the monomer for the pre fibrillar aggregate until a condition of irreversible binding is attained. As for Aβ40 (14), the dock-and-lock model was confirmed by the observation that the fraction of PrP82–146 remaining irreversibly bound to the immobilized aggregates is proportional to the association time (i.e. the time available to the bound monomer to undergo isomerization process). Some differences emerged, however, between Aβ40 and PrP82–146. Thus, Aβ40 dissociation showed fast exponential decay followed by slow, but continuous, linear decay (15, 16), suggesting that the rapidly dissociating peptide is docked but not yet locked into the mature fibril conformation, whereas the locked material (i.e. stably incorporated, possibly via isomerization steps) dissociated at a much slower rate. The PrP82–146 dissociation curve, however, follows a multieponential-decay kinetics with ~70% of the total PrP82–146 bound, which apparently does not dissociate. Comparison of the kinetic constants obtained by globally fitting the sensorgrams according to the three-step model (16) showed that: 1) the initial binding step between small PrP82–146 oligomers and PrP82–146 aggregates has an apparent Kd value (105 M) similar to that found with Aβ40 (123 μM), although kinetic analysis indicates that this is due to slower association and slower dissociation, and 2) the conformational changes (i.e. the locking steps) appear to be favored with PrP82–146 more than with Aβ40. These differences suggest higher propensity to polymerize and greater stability of the PrP82–146 aggregates. Interestingly, it has been reported (32) that flowing PrP23–110 binds to immobilized PrP23–110 in a manner suggesting the formation of multimers on the chip surface, although the visual inspection of the corresponding sensorgrams indicate that the binding kinetics are very different from those of PrP82–146.

As demonstrated here, SPR measurements can be particularly useful for kinetic analysis of PrP polymerization. We expect that the application of this approach to the problem of PrP strains and species barriers will provide structural molecular insight into the role of docking and locking in the transmission of prion disease.

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