Predisposition of Prion Protein Homozygotes to Creutzfeldt–Jakob Disease Can Be Explained by a Nucleation-Dependent Polymerization Mechanism

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The transmissible spongiform encephalopathies (e.g., scrapie, Creutzfeldt–Jakob disease (CJD), and Gerstmann–Straüssler–Scheinker disease (GSS)), like Alzheimer's disease, are neurodegenerative diseases characterized by abnormal brain pathology and the deposition of extracellular protein aggregate, which can be in the form of amyloid.[1,2] These deposits consist predominately of a host-encoded protein, PrP.[3] Scrapie can be transmitted via an infectious particle (prion) that seems to consist only of an insoluble, protease-resistant form of PrP (PrP^{Sc}). PrP^{Sc} appears to be chemically,[4] but not conformationally,[5,6] identical to its cellular precursor (PrP^{C}). The prion converts host PrP^{C} into PrP^{Sc}.[7] One possible replication mechanism, which we favor,[1,8] assumes that PrP^{Sc} is an aggregate in which an alternative conformer of PrP is stabilized by intermolecular interactions. According to this mechanism, replication and infection involve the nucleation of polymerization.[8–10] A nonpathogenic polymorphism occurs at position 129 of PrP (valine or methionine). The homozygous genotype predisposes individuals to both sporadic[11] and iatrogenic CJD.[12] The chemical basis for this genetic effect can be investigated using peptide models of PrP. Peptides derived from the PrP 118–133 sequence, containing methionine (Met129, Figure 1) or valine (Val129) at position 129, form amyloid via a nucleation-dependent mechanism.[8] Preliminary studies suggest that homogeneous peptide amyloid (Met129 *or* Val129) is more stable than heterogeneous amyloid (Met129 *and* Val129).[8] In order to model possible mechanistic differences in prion formation between position 129 homozygotes and heterozygotes, we studied amyloid fibril formation from supersaturated peptide solutions, comparing homogeneous solutions (Met129 or Val129 to mimic homozygotes) to heterogeneous mixtures (1:1 Met129:Val129 to mimic heterozygotes), at the same total peptide concentration. We report herein kinetic and thermodynamic differences which suggest an explanation for the genetic predisposition.

Fibril assembly is characterized by slow nucleus formation, followed by rapid fibril growth (Figure 1).[9] A nucleus will form only if the concentration of the monomer is above the critical

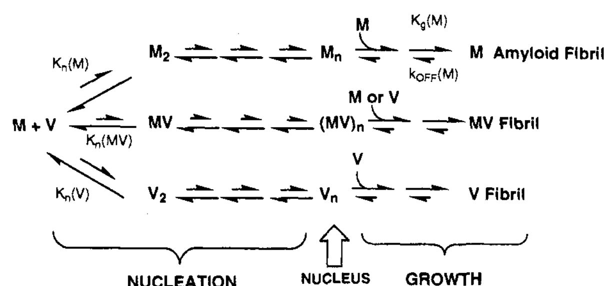

Figure 1. The sequence of the two peptides discussed herein is shown at the top. (A = Ala, G = Gly, L = Leu, M = Met, S = Ser, V = Val, Y = Tyr.) Below is a model for the formation of amyloid (M = peptide Met129 or protein PrP129M, V = peptide Val129 or PrP129V). The top and bottom pathways are available to homozygotes; all three pathways are available to heterozygotes, although the center pathway is disfavored. The slow step in polymerization is nucleus formation, due to a series of unfavorable association equilibria (K_n). The rate of nucleation is very sensitive to protein concentration (nth order) and nucleus size (n).

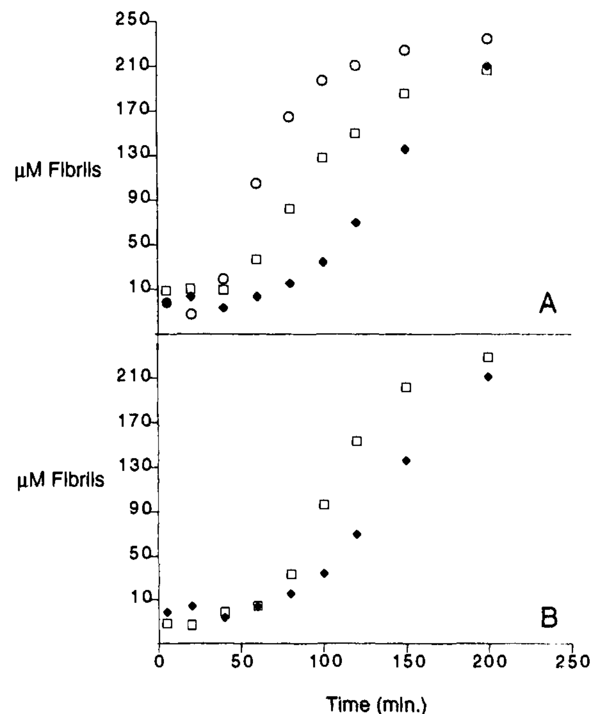

Figure 2. Amyloid fibril formation.[13] The "heterozygous" model (◆) forms fibrils more slowly. Each curve shows the amount of insoluble amyloid fibrils in suspension at a given time (total peptide − peptide in solution[13]). The curves shown are the average of at least three runs. (A) (○) 300 µM Met129; (□) 300 µM Val129; (◆) 150 µM Met129 plus 150 µM Val129. (B) (◆) 150 µM Met129 plus 150 µM Val129; (□) math addition of 150 µM Met129 and 150 µM Val129 (separate curves not shown). The nucleation times (defined herein as time at 20% of maximal fibril concentration) are as follows: Met129, 48 ± 11 min; Val 129, 76 ± 18 min; M/V129, 107 ± 14 min; math addition of 150 µM Met129 and 150 µM Val129, 88 ± 14 min. All errors reported herein are standard deviations.

concentration. Heterogeneous supersaturated solutions showed longer nucleation times (Figure 2A) than the homogeneous solutions, indicating that the heterogeneous nucleus ($(MV)_n$, Figure 1) is less stable than the homogeneous nuclei (M_n, V_n).[13] In the extreme case where heterogeneous association does not occur (that is, the center pathway in Figure 1 is not explored), the fibril nucleation time and growth rate from the heterogeneous solution at 300 µM would be identical to the sum of those two

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(10) An alternative mechanism holds that both forms of PrP are monomers and that prion replication involves catalysis of the conformational interconversion by a heterodimer (PrP^{C}–PrP^{Sc} to $(PrP^{Sc})_2$).[2]
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parameters for the separate homogeneous solutions at 150 μM. However, that was not the case (Figure 2B). The heterogeneous solution nucleated slightly more slowly, suggesting the formation of nonproductive heterogeneous oligomers, and grew more slowly, suggesting that Met129 inhibits growth of Val129 fibrils, and vice versa.

When Met129 fibrils were added to supersaturated homogeneous solutions of Met129 or Val129, seeding was observed, that is, the nucleation time was eliminated and a comparable growth rate was measured.[8] The latter observation indicates that seeding, unlike nucleus formation, is insensitive to the polymorphism. Seeding of the heterogeneous solution with Met129 fibrils was also successful. However, the observed seeded growth rate was slower than that of the homogeneous solutions, providing additional evidence for the proposal of mutual growth inhibition.

In order to compare the thermodynamic stability (K_g, Figure 1) of fibrils formed from homogeneous and heterogeneous solutions, solubilization (disaggregation) rates (k_{off}, Figure 1) were measured (Figure 3).[14] The fibrils from the heterogeneous solution dissolved at approximately twice the rate of the homogeneous fibrils and reached a final total peptide solubility which was greater than or equal to the sum of the solubilities of the two homogeneous solutions (Met129, 7.4 ± 0.4 μM; Val129, 4.4 ± 0.3 μM; and 1:1 M/V129, 15.4 ± 3.7 μM).[15,16] The solubilities suggest that the two peptides preferentially form separate homogeneous fibrils in the heterogeneous solution (i.e., the center path in Figure 1 is disfavored).

As demonstrated herein, a conservative sequence polymorphism (Met *vs* Val) can have a dramatic effect on nucleation-dependent amyloid formation by peptide mixtures, by influencing nucleation time and critical concentration. It is a consequence of the exponential dependence of nucleation time on concentration that small changes in association energetics (K_n, Figure 1), for example, between a heterogeneous pair as opposed to a homogeneous pair, can translate into large changes in nucleation time.[9] The model system discussed herein demonstrates that peptide mixtures nucleate and grow more slowly and are more soluble than pure peptides. By analogy, the PrP critical concentration for nucleus formation would be higher in a heterozygote than in a homozygote. Therefore, the *in vivo* PrP concentration would be more likely to be below the critical concentration and heterozygotes would be protected against amyloid formation (Figure 3).

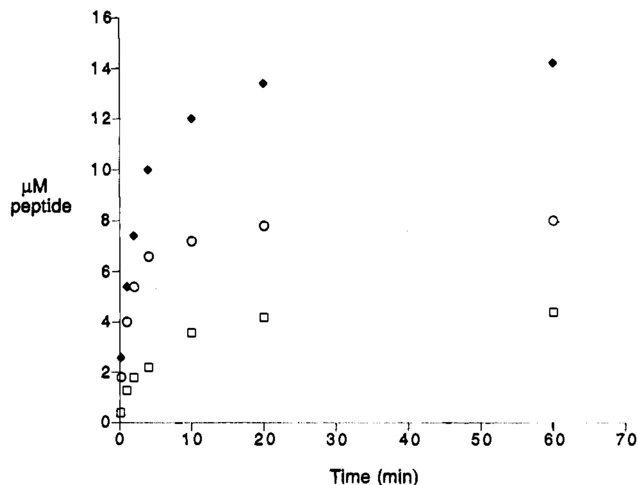

Figure 3. Amyloid fibril dissolution. The "heterozygous" model (◆) reaches a greater solubility. All fibrils were formed from a 300 μM solution of peptide.[14,16] Each curve shown is the average of three runs: (○) Met129 fibrils; (□) Val129 fibrils; (◆) 1:1 Met129:Val129 fibrils.

A situation similar to this proposed scenario is known to exist in sickle-cell anemia.[17,18] Sickle-cell hemoglobin heterozygotes are protected from severe symptoms, apparently by the coproduction of normal hemoglobin. Mixtures of normal adult or fetal hemoglobin (soluble under physiological conditions) with the sickle-cell form show increased *in vitro* solubility and fibril nucleation time compared to pure sickle-cell hemoglobin. One therapeutic strategy involves the induction of fetal hemoglobin in order to produce the same effect *in vivo*. Similar strategies may be utilized against the prion diseases and other amyloidoses, such as Alzheimer's disease.

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(13) We have refined our kinetic aggregation assay by measuring the amount of soluble ^{3}H-labeled peptide (peptides synthesized as reported in ref 8, ^{3}H incorporated by acetylation with [^{3}H]Ac_2O; initial peptide concentration determined by absorbance at 276 nm; (ϵ = 1700 as determined by amino acid analysis) in a stirred supersaturated solution (100 mM NaCl, 50 mM phosphate, pH = 7.4)) over time. Supersaturated solutions were made by dissolving films of peptide formed from hexafluoro-2-propanol in deionized water and filtering through 0.22 μm filters (Millipore). We were not able to measure differences in rates between homogeneous and heterogeneous solutions using a kinetic assay based on turbidity.[8]

(14) Amyloid fibril disaggregation was measured by following the dissolution of peptide fibrils. Fibrils were formed by adding DMSO solutions to buffer (final concentration = 300 μM), these suspensions were stirred for 14 days, and fibrils were harvested by centrifugation. The fibrils were taken up in 3 mL of fresh buffer (final fibril concentration = 30 μM, time = 0), and after a given time, aliquots were filtered through 0.22 μm filters (Millipore) and soluble peptide was measured by scintillation counting.

(15) The final solubility from the kinetic aggregation assay was determined to be higher than that measured in the disaggregation assay: 36 ± 6 μM for Met129, 23 ± 4 μM for Val129, 44 ± 4.6 μM for M/V129. This solubility showed a dependence on the initial concentration of the supersaturated solution, which may account for the difference shown; 150 μM solutions had approximately half the soluble peptide of 300 μM solutions after fibril formation. These discrepancies may involve fibril–fibril interactions.

(16) The solubility was measured for the disaggregation of fibrils which were allowed 2 weeks to form. Fibrils which were formed by stirring for 1 day were soluble at approximately 2–3 times the level of the "aged" fibrils.

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