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The kinetics of proteinase K digestion of linear prion polymers

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Transmissible spongiform encephalopathies such as scrapie are caused by a protein-only infectious agent, known as a prion. It is not clear how a protein can be capable of replicating itself, and the mechanism remains controversial. One influential model hypothesizes that prions are nucleated, macroscopically linear polymers. We investigated the theoretical kinetics of this model and derived predictions which could be used to test the model. In the model, the polymerization and depolymerization rates are independent of polymer size. This leads to an exponential size distribution at equilibrium. In agreement with a prediction stemming from this size distribution, the average size of PrP-res polymers was proportional to the square root of the concentration of PrP-res in a published study of *in vitro* conversion. Prion digestion by proteinase K (PK) is predicted to be biphasic. The second phase of digestion should be virtually independent of the PK concentration and should depend on the initial size distribution of prion polymers. For initially equilibrated polymers with an exponential size distribution, phase two digestion is exponential at a predicted rate. This rate varies in a defined way with the concentration used for equilibration and with other parameters which affect the average polymer size.

Keywords: prion disease; replication mechanism; nucleated polymerization; proteinase K; mathematical model

1. INTRODUCTION

The 'protein-only' or prion theory of transmissible spongiform encephalopathies such as scrapie and Creutzfeldt–Jakob disease (Griffith 1967; Prusiner 1991) has not been directly proved, but an abundance of circumstantial evidence has won it widespread support (Aguzzi & Weissmann 1997; Caughey & Chesebro 1997; Prusiner 1997; Chesebro 1998). According to this protein-only theory, an infectious form of the PrP protein (PrP^{Sc}) converts the normal form (PrP^{C}) into PrP^{Sc}. PrP^{C} is sensitive to digestion by proteinase K (PK), while PrP^{Sc} is partially resistant and therefore they are referred to as PrP-sen and PrP-res, respectively. The larger protein PrP-res 33–35 is reduced to a smaller protein PrP 27–30 upon PK digestion (Meyer *et al*. 1986). After this initial reduction in size, PrP-res resists further PK digestion, but can eventually be digested by prolonged exposure (McKinley *et al*. 1983).

Self-replication of a protein agent is a novel concept and no consensus has been reached on the mechanism. Three mechanisms have been proposed. According to the heterodimer mechanism (Cohen *et al*. 1994; Eigen 1996), a single PrP-res molecule catalyses the conformational change of a single PrP-sen molecule into PrP-res. According to the cooperative autocatalysis hypothesis (Eigen 1996; Laurent 1997), a mixed aggregate of PrP-res and PrP-sen converts to an aggregate of PrP-res via allosteric interactions. According to the nucleated polymerization hypothesis (Jarrett & Lansbury 1993; Eigen 1996; Harper & Lansbury 1997; Nowak *et al*. 1998; Masel *et al*. 1999) (figure 1), PrP-res is a polymeric form of PrP, while PrP-sen is monomeric. Polymerization is very slow below a critical size. Above this size, the polymer is stabilized and further polymerization is comparatively rapid. The slow nucleation process can be circumvented by adding an infectious 'seed'.

The heterodimer mechanism seems implausible on kinetic grounds (Eigen 1996). Cooperative autocatalysis (Eigen 1996) is a very general formulation of a polymerization mechanism. Common formulations of nucleated polymerization (Harper & Lansbury 1997; Masel *et al*. 1999) can be thought of as a limiting case of cooperative autocatalysis in which the polymers are linear at a macroscopic level, polymers have no theoretical maximum size and conformational change is rapid. Macroscopic linearity of polymers is an essential feature of these proposed models and experimental evidence is needed to prove or disprove this linearity. In support of linearity, PrP-containing, scrapie-associated fibrils or prion rods appear as unbranched linear polymers under electron microscopy (Jeffrey *et al*. 1995).

Kinetically, linearity means that polymerization and depolymerization only occur at the polymer ends, i.e. at only one or two sites per polymer, irrespective of polymer size. It seems unlikely that the presence or absence of additional residues at one end of a linear polymer could influence the polymerization and depolymerization rates at the opposite end. The polymerization and depolymerization rates of a linear polymer should therefore be independent

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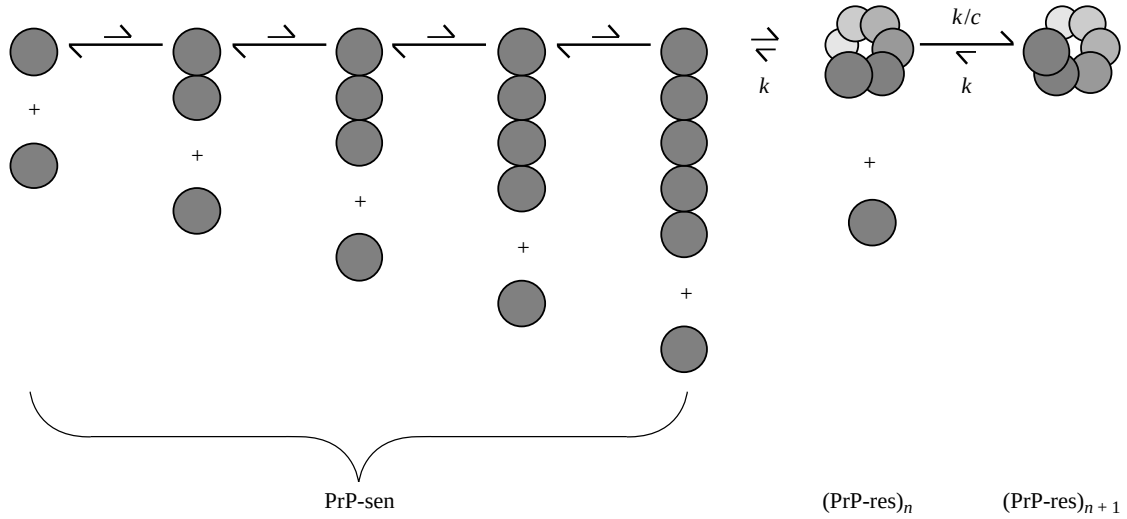

Figure 1. A schematic representation of the nucleated polymerization mechanism. The formation of aggregates below a critical nucleation size n, shown here as 6, requires sequential, kinetically unfavourable steps. Once a polymer of size n exists, the presence of additional stabilizing bonds within the polymer means that further polymerization is favoured and comparatively rapid. A solution of monomers above a critical concentration c is supersaturated and fails to polymerize because of the kinetic barrier to the creation of a nucleus. However, polymerization will occur if a pre-existing aggregate or 'infectious seed' is added to the solution. Previous work has shown that the critical concentration is equal to the ratio of the depolymerization and polymerization rates (Oosawa & Kasai 1971).

of polymer size. This is not true for a polymer extending in more than one dimension, when the available surface varies with polymer size. This difference can potentially be used to test whether prion polymers are linear. In this paper, we derive the specific kinetics of PK digestion of linear polymers. This allows us to make testable predictions for evaluating the hypothesis of nucleated linear polymers.

2. EQUILIBRIUM STATE OF PRIONS

To understand PK digestion, we first consider the state of prions before the addition of PK. This state is analogous to polymers of the cytoskeletal protein actin, whose equilibrium state has been extensively studied (Oosawa & Kasai 1971; Oosawa & Asakura 1975; Timasheff 1981).

According to most formulations of the nucleated polymerization theory, monomers are the normal form of PrP and polymers are the pathogenic form. In fact, a preparation of pathogenic PrP should be a mixture of monomers and polymers at equilibrium. The terms PrP^{Sc} and PrP-res are often used interchangeably. Here we define them more precisely, letting PrP-res or P be the polymer and PrP-sen or M be the monomer. PrP^{Sc} is a mixture of the two and PrP^{C} is a preparation containing only monomer. A distinct monomeric form of PrP has been observed that is only slightly less resistant to PK than aggregated PrP (Jackson *et al*. 1999). This form has only been seen at low pH and very low salt concentration and following the reduction of a disulphide bond. If this form is an intermediate in polymerization and depolymerization, then it is highly unstable under the conditions normally used for PK digestion and can be ignored for the kinetics analysed in this paper.

Let the number concentration of polymers of length i be y_i, the total number concentration of polymers be y, the concentration of PrP participating in polymers be z and the minimum nucleation size be n. Polymerization occurs at rate k/c and depolymerization occurs at rate k. The ratio c between these two rates is equivalent to the critical concentration (Oosawa & Kasai 1971). Polymers P of lengths i and $i+1$ interconvert according to

$$\mathrm{P}_i + \mathrm{M} \underset{k}{\overset{k/c}{\rightleftharpoons}} \mathrm{P}_{i+1}, \tag{1}$$

for $i \geqslant n$.

So at equilibrium,

$$y_{i+1} = y_i \frac{[\mathrm{M}]}{c} \text{ and } y_i = y_n \lambda^{i-n} \text{ where } \lambda = \frac{[\mathrm{M}]}{c}, \tag{2}$$

for $i \geqslant n$.

This equation shows that the ratio y_{i+1}/y_i is constant and equal to λ for $i \geqslant n$. This constant ratio means that the distribution of sizes is exponential, i.e. if abundance y_i is plotted against size i, an exponential curve is obtained. In theory, an exponential size distribution is achieved so slowly that it may not apply (Oosawa 1970). In practice, the distribution is a good description of actin polymers (Kawamura & Maruyama 1969).

For polymers below the minimum nucleation size n, a similar argument applies: the ratio y_i/y_{i+1} is a different constant for $i < n-1$, which is also proportional to the monomer concentration. The threshold ratio y_{n-1}/y_n gives a third value proportional to [M]. This yields

$$y_n \propto [\mathrm{M}]^n \propto \lambda^n. \tag{3}$$

So from equation (2), the total number of polymers is

$$y = \sum_n^\infty y_i = \frac{y_n}{1-\lambda} \propto \frac{\lambda^n}{1-\lambda}. \tag{4}$$

When the mean polymer length $s \gg n$, then $y \gg y_n$ and λ is close to 1, i.e. the monomer concentration [M] is just under the critical concentration c. As the number of polymers y increases, λ asymptotically increases to

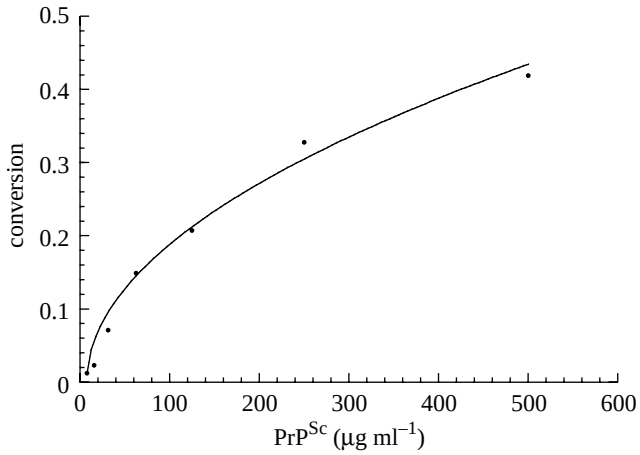

Figure 2. The data points are taken from Caughey *et al.* (1995) and indicate the proportion of labelled PrP-sen that can be converted into PrP-res by a given concentration of added PrP^{Sc}. These data points fit the parabola predicted by equation (5).

approach 1. λ is so close to 1 that, for most purposes, the monomer concentration [M] in a mixture of monomers and polymers can be approximated by c.

An exponential size distribution (equation (2)) implies that the number of polymers y is proportional to the square root of the number of subunits participating in polymers $\sqrt{z}$ (see equation (A1)). This is equivalent to saying that the mean length $s = z/y$ is proportional to $\sqrt{z}$.

PrP^{Sc} which has been pre-treated with 3 M guanidine hydrochloride (GdnHCl) can convert labelled PrP-sen into PrP-res (Kocisko *et al.* 1994). Conversion can be quantified as the amount of PK-resistant label. The extent of conversion is proportional to [PrP-sen], which represents the number of monomers (Caughey *et al.* 1995) and we also expect it to be proportional to the number of polymers y. The number of protein subunits participating in polymers z before the addition of labelled monomers is equal to the total PrP^{Sc} less the monomer concentration. The monomer concentration can be approximated by the critical concentration at 3 M GdnHCl, denoted $c_{3\,\mathrm{M\,GdnHCl}}$, so we expect

$$\text{conversion} \propto y \propto \sqrt{[\text{added } PrP^{Sc}] - c_{3\,\mathrm{MGdnHCl}}}. \qquad (5)$$

The data from a conversion study (Caughey *et al.* 1995) were replotted in figure 2 and an excellent fit to this relationship was found. This provides indirect evidence that the size distribution is exponential. An exponential size distribution in turn supports the chemical equation (equation (1)) for linear polymers. The critical concentration at 3 M GdnHCl can be calculated from the intercept of the curve as $20.8\,\mu g\,ml^{-1}$.

3. PROTEINASE K DIGESTION

The resistant portions of linear PrP-res polymers can potentially be digested in two ways. First, they might be directly digested by PK, albeit very slowly. Second, monomers may dissociate off the ends of the polymers, convert back to PrP-sen and then be digested. In this section we assume that the second digestion method is the faster of the two and, thus, dominates the kinetics of PK digestion. This allows us to make predictions about the effects of PK which could be used to test this assumption. If this assumption turns out to be true, then PK may be used as a tool to investigate the original testable hypothesis of polymer linearity.

When PK is introduced into the system, we should observe biphasic kinetics, as illustrated in figure 3. In the first phase, the concentration of PrP-sen drops to negligible levels, since PrP-sen is rapidly digested by PK (Buschmann *et al.* 1998). At the end of this phase, the concentration of polymers z is equivalent to the total protein concentration. The sensitive portion of PrP-res is also rapidly digested. The total protein concentration falls rapidly, while the number of polymers y is constant. The PK concentration determines the rate of this phase of the digestion.

In the second phase, all monomer is digested and the chemical equilibrium in equation (1) shifts to the left. PrP-sen starts dissociating from the ends of PrP-res at rate k and is immediately digested. The digestion rate of the second phase is slower than that of the first phase and is virtually independent of the PK concentration. For an exponential size distribution, we show that digestion of both y and z is exponential at rate $k/(s - n + 1)$ (equation (A2)), i.e. the average polymer length stays constant during digestion. This is counter-intuitive, since every polymer is progressively being digested. This result holds for the limit in which the number of polymers is infinite and arbitrarily long polymers can be found. In reality, there will always be a finite number of polymers and a maximum polymer length. We simulated second phase digestion with a maximum polymer length, as described in Appendix A. In these simulations, the average length is very close to constant until digestion has progressed a long way.

4. TESTABLE PREDICTIONS

The number of polymers y should correspond approximately to the number of LD_{50} units. The conversion assay can give a more precise estimate of y, but may not be feasible in all circumstances. For the purposes of this section, z is simply the total concentration of prion protein present during digestion.

The time-courses of y and z can be carefully monitored during PK digestion of purified prions to see whether biphasic digestion can be observed, i.e. whether rapid decay in z with no decay in y is followed by slow decay of both y and z. The biphasic kinetics of PK digestion also mean that a longer digestion with less enzyme should digest more PrP-res than a short digestion with more enzyme. This can have practical application in improving yields of digested PrP-res.

If these biphasic kinetics are ruled out, then PK does not operate in the manner postulated here. As an alternative to PK, dialysis of PrP^{Sc} in tubing that is permeable to monomer but not to polymer should generate the kinetics described in this paper.

The first phase of digestion is probably too rapid to quantify accurately, but the second phase could be measured and compared to the predicted behaviour. If y and z do not decay exponentially at almost equal rates in the second

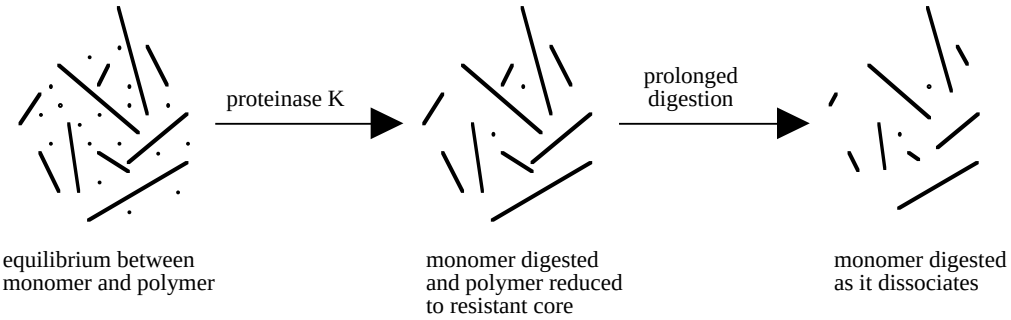

Figure 3. The two phases of linear polymer digestion by PK. In the first phase, free monomer and the sensitive portions of polymers are rapidly digested. Remaining polymer is resistant to digestion. In the second phase, the monomer–polymer equilibrium is disrupted, favouring dissociation. Subunits are digested as they dissociate, causing each polymer to shrink.

phase, then the size distribution is not exponential. Anything that reduces the average length of PrP-res polymers, such as sonication, should reduce their resistance to PK digestion. We have shown in figure 2 that the mean length s is proportional to $\sqrt{z}$ for PrP^{Sc} pre-treated with GdnHCl. If digestion is exponential, then the digestion rates can be compared to those predicted by the expression $k/(s-n+1)$. If they disagree, then the size distribution is not exponential.

Labelled PrP-res formed by the conversion assay should be found on the ends of polymers and should be digested much faster than the total PrP-res. If not and if PK operates as it is postulated to, this disproves the hypothesis of linear polymers and possibly of any large polymers at all.

Assuming end vulnerability to digestion, more labelled PrP should be recoverable from a conversion assay by sedimentation than by PK digestion. This has been observed (DebBurman *et al*. 1997). The conversion assay is normally initiated by a change in solvent conditions, such as dilution of GdnHCl. If this decreases the ratio c between the depolymerization and polymerization rates, then the chemical equilibrium in equation (1) shifts to the right and unlabelled monomer present in the initial PrP^{Sc} preparation will polymerize. This should shield labelled PrP-res from digestion. The extent of the protection will be reflected in the ratio of labelled PrP recovered by sedimentation to labelled PrP recovered by PK digestion. This can explain the difference in this ratio between two conversion experiments, the first using PrP^{Sc} pre-treated with GdnHCl and the second using untreated PrP^{Sc} and the chaperone GroEL added at the same time as labelled PrP-sen (DebBurman *et al*. 1997). Treatment with GdnHCl or GroEL probably raises c. In the first case, c falls at the start of conversion, leading to protection, whereas in the second case c rises at the start of conversion.

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APPENDIX A

At equilibrium, $y_i = y_n \lambda^{i-n}$, where $\lambda = [M]/c$ is a little less than 1 (equation (2)). The total number of polymers is given by equation (4) as

$$y = \sum_n^\infty y_i = \frac{y_n}{1-\lambda},$$

and the total number of polymer subunits is

$$z = \sum_n^\infty i y_i = \frac{y_n(n+\lambda-n\lambda)}{(1-\lambda)^2}.$$

The ratio

$$\frac{\sqrt{z}}{y} = \sqrt{\frac{n(1-\lambda)+\lambda}{y_n}} \approx \sqrt{\frac{\lambda}{y_n}}.$$

Since y_n varies with λ^n (equation (3)), we have

$$\frac{\sqrt{z}}{y} \propto \lambda^{(1-n)/2}.$$

λ does not vary very much, so this ratio is nearly constant when n is small, i.e.

$$y \propto \sqrt{z}. \tag{A1}$$

We can now find an explicit solution for digestion over time. The mean length is

$$s = \frac{z}{y} = \frac{n+\lambda-n\lambda}{1-\lambda},$$

so

$$\lambda = 1 - \frac{1}{s-n+1}.$$

Substituting into equation (2), we obtain

$$y_i(0) = y_n(0)\left(1 - \frac{1}{s-n+1}\right)^{i-n},$$

for $i \geqslant n$ and when the first digestion phase is complete we have $y_i(t) = 0$ for $i < n$. PK digestion implies the set of differential equations

$$\frac{\mathrm{d}y_i}{\mathrm{d}t} = k(y_{i+1} - y_i)$$

for $i \geqslant n$.

We can solve this explicitly to get

$$y_i(t) = y_i(0)\mathrm{e}^{-kt/(s-n+1)} \text{ and } z_i(t) = z_i(0)\mathrm{e}^{-kt/(s-n+1)} \tag{A2}$$

for $i \geqslant n$.

Here, we have shown that y and z decay exponentially at the same rate, i.e. the average size remains constant during digestion. This solution assumes the existence of arbitrarily long polymers. In reality, there will be a maximum polymer length m. Define three new series: $w_i = y_i/y$ with $w_i = 0$ for $i<n$ and $i>m$, $b_i = \sum_{j=n+1}^{m} w_j$ and $c_i = \sum_{j=1}^{m} b_j$. The number of dissociation events undergone by a single polymer in a given time is given by a Poisson distribution. This gives

$$y_i(t) = \mathrm{e}^{-kt} \sum_{j=i}^{m} \frac{(kt)^{j-i}}{(j-i)!} y_j,$$

and we can derive the formulae

$$y(t) = \sum_{i=n}^{m} y_i(t) = y(0)\mathrm{e}^{-kt} \sum_{i=0}^{m} \frac{(kt)^i}{i!} b_i,$$

and

$$z(t) = \sum_{i=n}^{m} i y_i(t) = y(0)\mathrm{e}^{-kt} \sum_{i=0}^{m} \frac{(kt)^i}{i!} (nb_i + c_{i+1}),$$

which can be used to run simulations with a finite maximum polymer length.

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