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Chemical and biophysical insights into the propagation of prion strains

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Transmissible spongiform encephalopathies (TSEs) are lethal infectious neurodegenerative diseases. TSEs are caused by prions, infectious agents lacking informational nucleic acids, and possibly identical with higher-order aggregates of the cellular glycolipoprotein PrP^C. Prion strains are derived from TSE isolates that, even after inoculation into genetically identical hosts, cause disease with distinct patterns of protein aggregate deposition, incubation times, morphology of the characteristic brain damage, and cellular tropism. Most of these traits are relatively stable across serial passages. Here we review current techniques for studying prion strain differences *in vivo* and in cells, and discuss the strain phenomena in the general context of the knowledge gained from modeling prion fibril growth *in vitro* and in simple organisms. [DOI: 10.2976/1.2990786]

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Prion diseases or transmissible spongiform encephalopathies are neurodegenerative diseases that are, in most cases, infectious and invariably fatal. Similarly to other protein misfolding disease of the central nervous system, prion diseases manifest themselves through strong neurological signs, including rapidly progressive dementia, ataxia, and variable loss of brain function depending on the prion disease. Most reported human cases are sporadic in origin (sCJD), i.e., caused by unknown factors, less than 10% of the remaining cases are familial Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker syndrome, or fatal familial insomnia (Glatzel *et al.*, 2003; Collins *et al.*, 2004). Familial forms of prion diseases have all been linked to mutations in the gene encoding for the cellular prion protein (PrP^C), termed *Prnp* (Hsiao and Prusiner, 1990). Prions can also efficiently be transmitted from one indi-

vidual to another and even across animal species. Known cases involve transmission from human to human through ritual cannibalism (Kuru), through contaminated medical products and blood (iatrogenic CJD) and from cattle to humans, known as variant CJD (Gajdusek *et al.*, 1966; Collinge, 2001; Llewelyn *et al.*, 2004; Wroe *et al.*, 2006). Major neuropathological hallmarks of transmissible spongiform encephalopathies (TSEs) are extensive spongiosis, neuronal cell loss in the central nervous system, gliosis (DeArmond, 1993), and deposition of amyloid plaques or amorphous PrP aggregates (Bendheim *et al.*, 1984; DeArmond *et al.*, 1985).

PRION REPLICATION

The idea that a protein lacking any genetic material could operate as an infectious agent was first proposed by Griffith on the basis of experiments showing an unusually high level of re-

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sistance of the infectious agent (later termed the prion agent) to ultraviolet irradiation, high temperature, and other aggressive physical conditions, suggesting the absence of nucleic acid (Griffith, 1967). But it was the purification and the biochemical characterization of PrP^{Sc}, a protein form only found in scrapie infected animals, that led to the formulation of the “protein-only” hypothesis by Stanley Prusiner (Bolton *et al.*, 1982). Charles Weissmann and Bruce Chesebro then independently discovered that PrP^{Sc} was encoded by the *Prnp* gene, a gene also encoding for the normal cellular protein PrP^C, the physiological function of which remains unknown (Chesebro *et al.*, 1985; Oesch *et al.*, 1985). The protein-only hypothesis, the prevailing hypothesis on the nature of the infectious prion, proposes that the infectious disease causing agent consists essentially of PrP^{Sc}, an abnormally folded, protease resistant, β -sheet rich isoform of the normal cellular prion protein, denoted PrP^C (Prusiner, 1991). No known covalent modifications have been found to differentiate the two proteins PrP^C and PrP^{Sc} (Stahl *et al.*, 1993). It was therefore proposed that PrP^{Sc} is a posttranslational derivative of PrP^C having acquired a different three-dimensional conformational structure and that pathological PrP^{Sc} acts as a template to convert more PrP^C. The prion concept was further refined by Weissmann (1991) to denote an infectious protein that does not contain any coding nucleic acids and the infectivity of which propagates by recruitment and “autocatalytic” conformational conversion of cellular prion protein into disease-associated PrP^{Sc} (Aguzzi *et al.*, 2007). The current prevalent model is that it is not monomeric PrP^{Sc} that is responsible for the conversion of PrP^C, but rather oligomeric species bearing a certain range of stoichiometries (Silveira *et al.*, 2005). It is thought that PrP^{Sc} can form higher order aggregates that can act as a nucleation site for the growth of PrP^{Sc} fibrils. Here, the hypothesis is that the ends of these fibrils recruit PrP^C or PrP^{Sc} monomers and stabilize the PrP^{Sc} conformation through incorporation into the growing fibril (Aguzzi *et al.*, 2007).

The physiological function of PrP^C is not well understood. PrP^C has been linked to neuronal signal transduction, lymphocyte function, copper-binding, as well as pro- and antiapoptotic functions (Aguzzi and Polymenidou, 2004). Mice devoid of PrP^C show only a mild phenotype, indicating that prion diseases are not caused by a loss-of-function (Büeler *et al.*, 1992). Indeed the most compelling evidence in favor of the protein only theory is that PrP-null mice are completely resistant to experimental transmission of prions, showing that PrP^C is crucial for prion replication (Büeler *et al.*, 1993). Furthermore, it was found that *de novo* prion infectivity can be generated, albeit inefficiently, by *in vitro* fibrillization or by sonicating a mixture of purified lipids, synthetic polyanions, and native PrP^C purified from normal hamster brain (Legname *et al.*, 2004; Deleault *et al.*, 2007).

The prion phenomenon exists also in non-mammalian eukaryotes such as yeast. Fungal prions are non-PrP re-

lated molecules, including HET-s, Ure2p, Sup35 proteins, that show prion properties in that they can adopt both non-amyloid and self-perpetuating amyloid structures. The conversion of these molecules has been shown to have important physiological functions. Conversion of Ure2p and Sup35 into their amyloid forms (URE3 and PSI+, respectively) regulates transcription and translation of specific yeast genes (Tanaka *et al.*, 2006; Immel *et al.*, 2007). HET-s in its amyloid form regulates heterokaryon incompatibility, a fungal self/nonself-recognition phenomenon that prevents different forms of parasitism (Wasmer *et al.*, 2008). So far there have been few examples of mammalian protein that are functionally, in a nonpathological way, regulated by interconversion between amyloid and nonamyloid forms. A remarkable example is the synthesis of melanine which has been shown to involve amyloid structures (Fowler *et al.*, 2007). In addition, it has been proposed that proteins involved in establishing long-term memory might do so by converting reversibly to and from a prion-like state (Si *et al.*, 2003; Shorter and Lindquist, 2005).

PRION STRAINS

In its most basic form, the seeding hypothesis is likely to represent an oversimplification, as it has been known for over 40 years that different prion strains can be isolated from the same species (Pattison and Millson, 1961). Prion strains are defined as infectious isolates that, when transmitted to identical hosts, exhibit distinct prion disease phenotypic traits. The main traits used to distinguish strains *in vivo* were historically based on the incubation time, which is the period it takes from the time point of an experimental inoculation to onset of clinical disease (Bessen and Marsh, 1992b). Other traits commonly used to differentiate strains include histopathological lesion profiling, i.e., the distribution and characteristics of PrP^{Sc} deposits and the degree of vacuolization in nine different specific brain regions, as well as clinical signs (Fraser and Dickinson, 1973; Bruce, 1993; Fraser, 1993; Sigurdson *et al.*, 2007). Phenotypic traits are mostly stable upon serial transmission (the continued passaging of infectious brain homogenate from a sick mouse into a healthy one), unless having been transmitted over a species barrier.

When prion isolates obtained from one species are transmitted experimentally into an animal of another species, often a characteristic delay in the onset of clinical disease is observed upon first passage, which is referred to as a species barrier. This incubation time is typically significantly reduced upon a second round of serial transmission into the same host-species, a phenomenon termed “adaptation” (Kimberlin *et al.*, 1987; Sigurdson *et al.*, 2007). The species barrier is thought to result from incompatibilities between the preferred host PrP structure in the fibrillary state and the specific conformation of PrP present in the inoculum. The species barrier can be unidirectional in that it inhibits the

transmission of the mouse-adapted scrapie strain 139A into rat, but not rat-adapted 139A into mice (Kimberlin *et al.*, 1987). Alternatively, it can be bidirectional, or in some instances it may not exist at all (Kimberlin *et al.*, 1987). Often new distinct strains can be observed upon transmission of prions across an interspecies barrier or into animals of the same species expressing different polymorphisms in the prion gene, a phenomenon sometimes referred to as a “strain mutation” (Bruce, 1993; Wadsworth *et al.*, 2004). New strains can arise upon cross-species barrier transmission even if the strains have been previously “cloned” (Kimberlin *et al.*, 1989). The latter procedure consists of selecting single prions by limiting endpoint dilution into mice, such as only one prion species should arise from an individual host (Bruce and Dickinson, 1987; Bruce, 1993). Therefore, it is not clear if one can indeed “clone” out a prion strain. Potentially, multiple strains exist in a cloned homogenate prior to transmission out of which one strain can become the dominant species depending on selection for the strain most congruent with the host PrP molecule. This interpretation is analogous to the quasispecies theory of RNA viruses (Biebricher and Eigen, 2006). Alternatively, new strains might be generated *de novo* due to strain “mutations” of unknown etiology (Weissmann, 1991). It is clear that in some patients suffering from CJD, multiple distinct types of CJD-associated PrP^{Sc} molecules coexist within the same patient, speaking in favor of the first hypothesis (Polymenidou *et al.*, 2005).

STRAIN DISCRIMINATION

Strain-specific properties of prions could hypothetically be encoded by an ancillary genome consisting, e.g., of RNA species, or even of microRNA. Although anionic polymers including RNA species appear to facilitate prion conversion *in vitro*, no clear evidence in favor of the notion that nucleic acids determine the characteristics of prion strains has been forthcoming (Weissmann, 1991; Deleault *et al.*, 2003). Alternatively, PrP^{Sc} might possess several different disease-associated strain conformations, all of which can cause and transmit disease, with specific disease phenotypes being determined by specific conformations or aggregation number of PrP^{Sc} in the donor inoculum. Speaking in favor of this hypothesis, several different lines of circumstantial evidence seem to support that there are unique biochemical and biophysical characteristics of individual prion strains.

The most commonly used methods to biochemically describe prion strains are based on differences in electrophoretic mobility after proteinase K digestion and glycosylation patterns (the ratio between un-, mono-, and diglycosylated PrP) (Bessen and Marsh, 1992a; Collinge *et al.*, 1996; Parchi *et al.*, 1996; Khalili-Shirazi *et al.*, 2005). Sedimentation coefficients and the extent of PK resistance either in the absence (Bessen and Marsh, 1992a) or presence of different concentrations of chaotropic salt are strain de-

pendent, which suggest that different strains aggregate to a different extent and/or that they vary in their tertiary or quaternary fold and inherent stability to unfolding (Peretz *et al.*, 1997; Safar *et al.*, 1998). This notion is supported by the fact that certain antibody epitopes are buried within the globular protein domain of the prion protein to a different extent upon conversion. These buried epitopes can be exposed by chaotropic salts and the ratio of available to buried epitopes can be used to distinguish unique strains, the so-called conformation dependent immunoassay (Safar *et al.*, 1998).

The different lesion profiles seen in the brains of mice infected with different prion strain isolates suggest that stability and conformation of PrP^{Sc} are not the only differences between strains (Tremblay *et al.*, 2004). It appears that there is a disparity between the cell tropism of various strains. Indeed it can be shown that prion strains upon intraperitoneal inoculation replicate in the spleen to a variable extent prior to entry into the CNS (Aguzzi and Sigurdson, 2004). Recently, the phenomenon of cellular prion tropism was clearly illustrated by the work of Weissmann’s group who could show that a panel of cell lines would replicate prion strains to differing degrees. All of the cell lines were fully capable of supporting prion replication by the mouse-adapted scrapie strain 22L, but only one cell line, the CAD5 cell line, would support the replication of mouse-adapted BSE strain 301C. And whereas CAD5 cells would replicate all strains, R33 cells would only replicate the 22L strain and not 301C, RML, and Me7 (Mahal *et al.*, 2007). This study clearly illustrates that at present we lack the full knowledge of what a prion strain is. The differential cell tropism of the various prion strains clearly implies the requirement for cell-specific cofactors, be it chaperones, specific uptake receptors, RNA species, a particular lipid environment, a specific post-translationally modified PrP molecule, or a particular prion replicating subcellular environment. Currently, there is no consensus on the nature of this cofactor, but much work in different groups is currently focused on identifying the type of cofactors implicated in prion growth in living systems.

STRAINS AND SMALL MOLECULE DYES

Most detection methods for distinguishing individual prion strains rely on biochemical techniques applied to homogenates of prion-containing samples or transmission studies, neglecting the aspect of spatial distribution of prions. The presence of histologically visible β -sheet rich protein aggregates, called amyloid deposits, can be visualized by small amyloidotropic dyes, such as derivatives of Congo red, and thioflavins (Nilsson, 2004). These dyes bind with various degrees of selectivity to protein aggregates that display extensive cross β -pleated sheet conformations with a high degree of structural symmetry. Hence, the presence of mature amyloid deposits is detected by these dyes as enhanced fluorescence (thioflavins) or apple-green birefringence under cross

polarized light (Congo red). Congo red, an aromatic sulfonated azo dye, was introduced more than 80 years ago and its gold-green birefringence under polarized light has been the gold standard for amyloid detection ever since (Bennhold, 1922; Divry, 1927). However, these dyes are not suitable for recognizing “prefibrillary” species (which are often, by way of a circular argument, defined as non-congophilic amyloids). Also, amyloid deposits of diverse morphological origin, such as prion strains, cannot be separated.

In order to address these limitations, luminescent conjugated polymers (LCPs) were recently developed as a novel class of amyloidotropic dyes (Herland *et al.*, 2005; Nilsson *et al.*, 2005; 2006). These dyes contain a swiveling thiophene backbone and the optical processes, e.g., the fluorescence from the dye, are highly sensitive to the geometry of the thiophene backbone. Upon interaction with protein aggregates of distinct morphologies, the rotational freedom of the LCP backbone is restricted in specific ways, disrupting the conjugation of the π electron systems within the polythiophene chains. This generates optical fingerprints that are often unique to given protein conformations. Instead of simply measuring the total amount of aggregated protein, heterogeneous populations of specific protein aggregates can be differentiated by LCP staining. This phenomenon was recently observed in a transgenic mouse model with AD pathology, where a striking heterogeneity in the characteristic plaques composed of the beta-amyloid peptide ($A\beta$) was identified with the LCPs (Nilsson *et al.*, 2007). LCP staining of brain tissue slices revealed different subpopulations of plaques, seen as plaques with different colors. The spectral features of LCPs enabled an indirect mapping of the plaque architecture, as the different colors of the LCPs are associated with different conformations of the thiophene backbone. Further evidence for the idea that the prion strain phenomenon is encoded in the structure of the prion aggregates is provided from the analysis of brain sections stained by LCPs from mice infected with distinct prion strains (Sigurdson *et al.*, 2007). The LCPs not only bound specifically to the prion deposits, even those which were negative for other amyloidotropic dyes (Congo red and ThT), but also different prion strains could be separated due to individual staining patterns of LCPs with distinct ionic sidechains. Furthermore, the anionic LCP, PTAA, emits light of different wavelengths when bound to distinct protein deposits associated with a specific prion strain [Figs. 1(a)–1(c)]. As the emission profiles of LCPs are associated with geometrical changes of the LCP backbone, ratios of the intensity of the emitted light at certain wavelengths can be used as an indicator of the geometry of the LCP chains (Berggren, 1999; Nilsson *et al.*, 2002). For example, nonplanar and separated LCPs chains emit light around 530–540 nm, whereas a planarization of the thiophene backbone will shift the emission maximum (Emax) towards

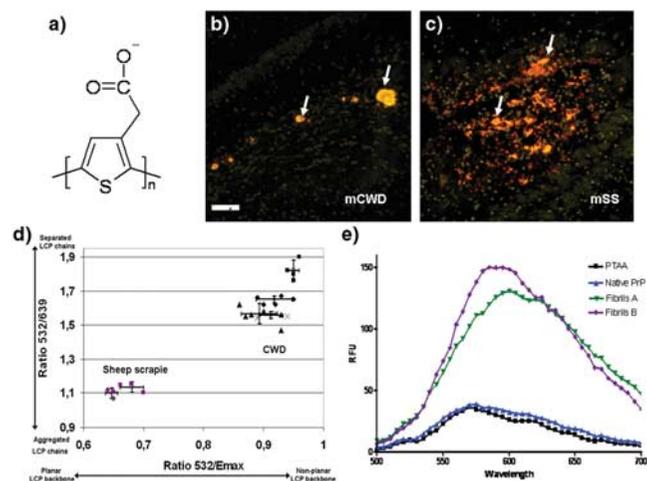


Figure 1. Prion strain discrimination by LCPs. (a) Chemical structure of the anionic LCP, PTAA. (b)–(c) Fluorescence images showing PTAA bound to PrP aggregates from mCWD (b) and mSS (c). Typical PTAA stained aggregates are seen in yellow-red and are indicated with white arrows. Scale bars represent 50 μm . (d) Correlation diagram of the ratios of emitted intensities, R532/639 and R532/Emax, of the intensity of the emitted light from PTAA bound to PrP plaques in individual cases of mCWD and mSS. Each symbol represents the mean value from 3–5 different plaques within a single mouse. For mCWD or mSS, data was collected from four or two different passages, respectively. (e) Emission spectra of PTAA bound to native or fibrillar recombinant mouse PrP. RFU, relative fluorescence units.

longer wavelengths. A planar backbone might also give rise to an aggregation of LCP chains, seen as an increase of the intrinsic emission around 640 nm. When plotting the ratio 532/Emax and the ratio 532/639 nm in a correlation diagram, prion aggregates associated with distinct prion strains, mouse-adapted chronic wasting disease (mCWD), mouse-adapted sheep scrapie (mSS), and mouse-adapted BSE were easily distinguished from each other, illustrating the usefulness of spectral properties of LCPs for the classification of protein deposits [Fig. 1(d)]. These conformation dependent spectral characteristics can only be afforded by LCPs and provide the opportunity to acquire optical fingerprints for protein aggregates which correlates with distinct prion strains.

Although it was shown that the emission profile of LCPs could be used to characterize protein deposits, further evidence was necessary to enable relating the geometrical alterations of the LCPs to a structural variance of the protein deposits associated with the distinct prion strains. By taking recombinant mouse prion protein (mPrP) and converting it into two different types of amyloid fibrils by using varying conditions for fibrillation, Sigurdson *et al.* were able to show that the emission profile of PTAA could be used to distinguish the two fibril preparations [Fig. 1(e)] (Sigurdson *et al.*, 2007). As these two preparations of fibrils were chemically identical, having the same protein (mPrP) and being dialyzed

against the same buffer, the spectral differences seen for PTAA were most likely due to structural differences between the fibrils. Hence, LCPs provide indirect structural insights into the morphology of individual prion aggregates and can be used as a complementary technique to conventional staining protocols for the characterization of protein deposits associated with individual prion strains. However, further studies of complexes between *in vitro* produced prion aggregates with defined conformations and LCPs with distinct ionic sidechain functionalities or different chain lengths will likely be necessary in order to understand the origin of the correlation of the spectroscopic readout from the LCP and the molecular structure of the prion aggregate. Although the achievement of obtaining certain spectroscopic LCP signatures from protein aggregates associated with distinct prion strains are beneficial compared to conventional amyloidotropic dyes, correlating this spectroscopic signature to a specific form of the aggregated protein is still necessary in order to gain novel insight into the pathological process of the disease. Nevertheless, the LCPs can be useful for comparison of heterogeneous prion aggregates in well-defined experimental systems and offers a novel tool to study prion strain adaptation and competition between distinct prion strains.

ELEMENTARY STEPS OF PRION PROPAGATION

The physical basis for the propagation of prions constitutes a key element in refining our fundamental understanding of their biological activity. It is increasingly apparent that many aspects of prion behavior such as the species barrier for their transmission and the existence and stable propagation of multiple prion strains can be rationalized based on a finite set of kinetic and structural conditions on the elementary steps which underlie prion growth. In agreement with this idea, the fact that the strain phenomena is ubiquitous in prion biology, being found for mammalian prions (Safar *et al.*, 1998; Jones and Surewicz, 2005; Sigurdson *et al.*, 2007) as well as for naturally occurring and artificial fungal prions (Glover *et al.*, 1997; Chien and Weissman, 2001; DePace and Weissman, 2002), strongly suggests that it arises from common physical determinants rather than from specific sequence dependent effects—an intriguing manifestation of universality within a complex biological setting. The aim of this section is to give a brief overview of recent progress towards understanding the physical principles which govern prion growth and to discuss some of the implications of such theories for the biology of these systems.

The realization that certain types of natural epigenetic information transfer processes in fungal species are mediated by prion forms of endogenous proteins has provided an experimentally tractable framework for the detailed study of mechanisms associated with prion propagation. In many cases the current level of biophysical information available for prions in fungi is more detailed than for mammalian pri-

ons. Strong analogies and similarities, some discussed below, are, however, emerging and many of the findings for yeast prions have parallels in mammalian prion biology and in some cases even in the wider class of noninfectious self-propagating β -sheet rich amyloid fibrils.

The process by which proteins convert from their normal soluble state into the prion form according to the protein only mechanism (Prusiner, 1982; King and Diaz-Avalos, 2004; Tanaka *et al.*, 2004) can conceptually be separated into two steps (Collins *et al.*, 2004), namely: (1) the conformational rearrangement using the end of an existing prion aggregate as a template to misfold the protein monomer and enable its incorporation into the growing fibril; and (2) the multiplication of prions through fragmentation of existing structures (Fig. 2). This mechanism has been experimentally verified for yeast prions (Collins *et al.*, 2004; King and Diaz-Avalos, 2004; Tanaka *et al.*, 2004; Brachmann *et al.*, 2005). There are indications that similar processes govern the growth of mammalian prions (Legname *et al.*, 2004; Castilla *et al.*, 2005; Legname *et al.*, 2005; Weber, 2006) and indeed also of non-prion related amyloid fibrils (Dobson, 2003). In addition, for many amyloid systems spontaneous nucleation (Xue *et al.*, 2008), i.e., the creation of a propagating entity from soluble protein, is an important contribution to the overall polymerization reaction. For prion growth, however, this spontaneous nucleation process is, in general, very slow, a fact which underlies the capability of prion mediated inheritance in fungi to function as a bistable switch (Shorter and Lindquist, 2005; Wickner *et al.*, 2007). The slow rate of nucleation (or the efficient clearance of seeds) is also implied by the low fre-

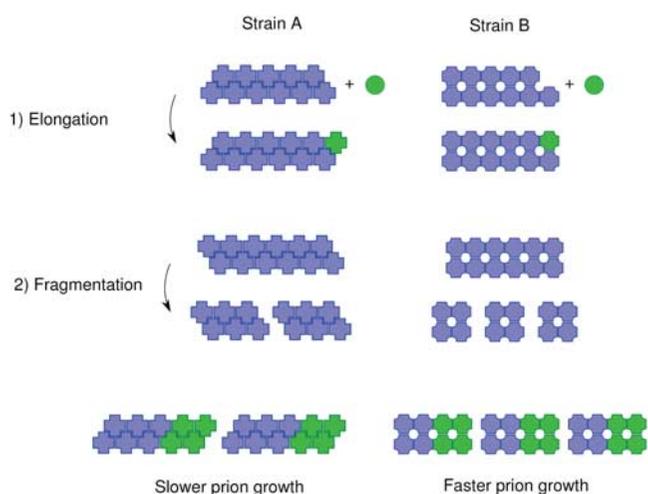


Figure 2. Fibril replication model. Nucleated polymerization model as a basis for understanding prion growth. Monomers (circle) are incorporated into growing fibrils in the elongated step (1) and multiplication of active ends occurs through fragmentation (2). The strain-dependent frangibility of aggregates leads to different propagation rates: a mechanically robust strain A has a stable core and fragments slowly, whereas a more frangible strain B multiplies faster resulting in more efficient incorporation of cellular protein (green) into the aggregates.

quency of onset of sporadic versus transmitted mammalian prion conditions (Aguzzi *et al.*, 2007). Recent studies linking expression levels of proteins in a cell with their intrinsic aggregation propensity (Tartaglia *et al.*, 2007) suggest that the natural stability of proteins in their native soluble state is, in general, guaranteed through kinetic, not thermodynamic, barriers. This kinetic stability of soluble cellular prion proteins is then perturbed by the presence of preformed aggregates which accelerate the conversion to the prion isoform. Within this framework, the ability of prions to proliferate in a given environment is governed by nucleated polymerization theories (see Fig. 2) defined through a set of rate constants (Masel *et al.*, 1999; Pöschel *et al.*, 2003; Hall and Edskes, 2004; Carulla *et al.*, 2005) for the different processes. In this picture, growth—expressed by the elongation and division rates—competes against the removal of prions through cellular clearance mechanisms.

PHYSICAL AND STRUCTURAL DETERMINANTS OF PRION STRAINS

The apparent simplicity in the elementary steps of prion growth raises the question of how they can account for the biological complexity encountered in prion biology and, in particular, how they can generate the prion strain phenomena. First hints at the answer came from the study of yeast prions. It is increasingly apparent that the mechanical fragility (the propensity to break) of formed prion fibrils plays a key role in this context (Tanaka *et al.*, 2006). Yeast prions have been shown to possess inherent fragilities which differ substantially between strains but are relatively homogeneous within one class of fibrils belonging to a given strain (Tanaka *et al.*, 2006; Immel *et al.*, 2007). For instance, some of the stronger yeast prion strains have been found to be composed of amyloid fibrils possessing the largest propensity to fragment, thereby leading to a more aggressive tendency to multiply *in vivo*. It is interesting to note that in terms of their mechanical rigidity, amyloid fibrils can be very heterogeneous (Knowles *et al.*, 2007) implying substantial potential for intrinsic variability in their breakage rates. Although prion propagation in mammalian tissues may inherently be a more convoluted process, recent observations hint that a similar kinetic description which has been validated for fungi could be important for the propagation of mammalian prion strains. Indeed, shorter fibrils such as those which would result from breakage of frangible structures are more infectious than longer fibrils (Silveira *et al.*, 2005), a conclusion which is consistent with the fact that a larger number of free ends in a short fibril population leads to a more rapid conversion of soluble cellular prion protein into misfolded fibrillar form, thereby overwhelming and overcoming cellular clearance mechanisms. In addition, the chemical stability and resistance to disaggregation by *ex vivo* amyloid material is highly strain dependent (Safar *et al.*, 1998), indicating that differences in the robustness of the aggregates could charac-

terize the differential propagation of strains. In agreement with this idea, an inverse correlation has recently been proposed between the stability of the prion aggregate and their incubation times *in vivo* (Legname *et al.*, 2006), a finding which is closely analogous to the corresponding results obtained for yeast prions (Toyama *et al.*, 2007; Tanaka *et al.*, 2004). It is frequently found that for *in vitro* growth assays of many amyloid fibril systems agitation significantly enhances the overall conversion rate of proteins into fibrillar form (Sluzky *et al.*, 1991; DePace *et al.*, 1998; Serio *et al.*, 2000; Ohhashi *et al.*, 2005; Atarashi *et al.*, 2007; Kim *et al.*, 2007), an indication that modulations of fibril breakage are essential factors determining the rate of amyloid formation in general. In living systems, the rate constants for the multiplication of prions are clearly influenced not only by the intrinsic strength of the aggregates but also by other cellular components; molecular chaperones in yeast, for instance, have been identified as vital actors in this context (DeBurman *et al.*, 1997; Shorter and Lindquist, 2004).

Taken together, the observations discussed above suggest that differences in the kinetics of the elementary steps of prion growth underlie the differential proliferation of prion strains. In order to be successfully propagated, these differences have to stem from well-defined molecular level changes in the structures of the insoluble prion aggregates (Aguzzi, 2004). Concrete indications that this is indeed the case have recently accumulated. Elegant studies using hydrogen/deuterium exchange probed by solution NMR (Toyama *et al.*, 2007), fluorescence spectroscopy (Krishnan and Lindquist, 2005), and x-ray crystallography (Sawaya *et al.*, 2007; Nelson *et al.*, 2005) have recently highlighted widespread structural polymorphism and identified some of the characteristics differentiating prion strains. Emerging as an important factor here is the size of the stabilizing cross- β amyloid core which appears to define the physical properties of the resulting structures such as their propensity to fragment, with small core sizes leading to enhanced fragility. Furthermore, covalent crosslinking of the precursor proteins at well-defined locations to favor small or alternatively large core sizes in the seed material is sufficient to result in the production of a population of fibrils of the corresponding strains (Krishnan and Lindquist, 2005). Indications of self-propagating strain dependent conformational changes have similarly been identified for three mammalian PrP amyloid fibril systems (Jones and Surewicz, 2005). These fibrils, when growing in cross-seeding experiments, adopt the structural features determined by the seed prion fibrils, even in cases where this does not coincide with the preferred morphology of the monomer when polymerized *de novo*. This type of seed determined polymorphism is increasingly appearing as a generic feature of self-propagating amyloid structures more generally. For instance A β (1–42) fibrils of the type associated with Alzheimer's disease have been found to exist in two structurally and morphologically dis-

tinct conformations which accurately propagate the structure-encoded information to subsequent generations of fibrils (Petkova *et al.*, 2005). Interestingly, recent experiments in transgenic mice created to develop β -amyloid plaques further suggest that the analogies between prions and $A\beta$ aggregates could be broader than was initially suspected. When these mice were injected with brain homogenate containing $A\beta$ amyloid, a seeding effect was observed, causing an accelerated deposition of $A\beta$ plaques. In addition, certain strain patterns, partially encoded by the injected seeds, appeared to be observable suggesting that there are still unexplored similarities between Alzheimer's and prion diseases (Meyer-Luehmann *et al.*, 2006). Measurements by atomic force microscopy of three nondisease related amyloid systems has yielded indications that several, typically of the order of 5–10, energetically close but structurally different packings for polypeptide chains can exist within different fibrils, and that subsequent monomer addition onto these templates preserves the packing type (Knowles *et al.*, 2006). These numbers are interestingly of a similar order of magnitude to the numbers of prion strains identified *ex vivo* based on their biochemical characteristics (Safar *et al.*, 1998).

The intrinsic susceptibility towards polymorphic aggregation is also increasingly appearing as a determining factor in the species barrier phenomenon, which prevents certain prion aggregates, but not others, from proliferating in a host organism producing cellular prion protein with a different sequence to that of the initial infectious seed material. For instance, amyloid fibrils formed from a fragment of the mammalian prion protein have been shown to have the fastest growth rates *in vitro* in cross-seeding experiments when the preferred fibrillar structures of the growth protein and seed protein are similar (Jones and Surewicz, 2005). In cases where significant differences in fibril core structure were identified by infrared spectroscopy and atomic force microscopy, the cross-seeding capacity was abolished. The primary sequence of the prion protein appears therefore only to intervene in the species barrier through the preferred fibril structure it confers. This preference for a given conformation of a protein in the fibrillar state seems furthermore to stem predominantly from specific, in many cases short, portions of the polypeptide sequence (Santoso *et al.*, 2000; Tessier and Lindquist, 2007). Yeast prion proteins which possess two such “recognition elements” corresponding to two different strains have consequently been shown to possess the ability to fibrillize efficiently in the presence of seeds from either strain (Tessier and Lindquist, 2007) whereas proteins with only one such element can only grow into fibrils when seeded with aggregates from the corresponding strain.

Finally, it is interesting to speculate that general mechanisms given by the statistical physics of heterogeneous chain-like molecules lie behind the widespread polymorphism of proteins in prion states, a feature which is generally absent or less pronounced for native states where a given se-

quence encodes a unique three-dimensional conformation. The energy landscape governing according to the “new view” of protein folding (Frauenfelder *et al.*, 1991; Dobson *et al.*, 1998) the reliable acquisition of native states of proteins with given amino-acid sequences has been optimized by evolutionary pressures to be smooth and free of frustration in order to avoid the situation where competing interactions could trap the protein into local energy minima corresponding to partially folded nonfunctional states (Dobson *et al.*, 1998; Dobson, 2003). However, prions and amyloid structures more generally by their very nature circumvent the normal folding funnel (Dobson, 2003), and are therefore likely to experience a rougher energy landscape characteristic of frustrated systems where interactions which cannot be simultaneously satisfied lead to multiple distinct energy minima. The connectivity imposed by the polypeptide backbone together with the requirement for a stable cross- β core hinders the independent search for an optimal chemical environment for the individual amino acids, for instance through their differential positioning in the fibril core or alternatively in solvent exposed parts outside of it. This type of intrinsic frustration then implies that several distinct arrangements which favor a certain subset of globally incompatible interactions are possible, reflecting for instance in the observed strain-dependent differences in the parts of the sequence incorporated into the fibril core (Toyama *et al.*, 2007). In this sense the strain phenomenon can be seen to follow naturally from the physical features of the protein folding landscape when sampled outside of the range normally used by nature.

CONCLUSION

Although many similarities to other neurodegenerative protein misfolding diseases such as Alzheimer's, Huntington's, and Parkinson's disease have been described (DeArmond, 1993; Aguzzi and Haass, 2003), prion diseases were thought to be unique in that they are transmissible and likely show protein structure encoded strain properties. However, as an acceleration of disease onset and certain strain properties appear to be encoded in experimentally transmitted $A\beta$ seeds, it would appear that there are still unexplored similarities between Alzheimer's and prion diseases to be discovered (Meyer-Luehmann *et al.*, 2006). Despite our increased understanding of the epidemiology and general biology of prion diseases many key questions are still unanswered (Table I). As new and more advanced techniques for studying prion biology are being developed continuously, our hope and belief is that some of these questions will be addressed within the foreseeable future. New tools such as LCPs, solid state nuclear magnetic resonance, advanced cell culture models, including the cell panel assay or the prion organotypic slice culture assay (POSCA; prion replication in *ex vivo* slices of living brain tissue) (Mahal *et al.*, 2007; Falsig *et al.*, 2008) can hopefully help us address some of these important

Table I. Some topics in need of research in the prion field.

Open questions in prion biology	Current hypotheses and state of the art
What constitutes the infectious prion particle?	Experimental evidence indicates that the primordial infectious prion particle has a size compatible with 14–28 monomers of PrP monomers (Silveira <i>et al.</i> , 2005). It is unclear whether this material consists exclusively of PrP, or is associated with proteins or other constituents.
What is the structure of mammalian prions/what is the structural determinants of prion strains?	The fine structure of HET-s yeast prions was recently solved by solid-state nuclear magnetic resonance (Wasmer <i>et al.</i> , 2008). This is the only available structure of infectious prions. Only hypothetical models are currently available for mammalian prions.
What is the molecular mechanism underlying the replication kinetics of mammalian prions?	As models now exist for the growth of amyloid and prion fibrils <i>in vitro</i> and in yeast (Tanaka <i>et al.</i> , 2006) it will be interesting to see them applied to more complex systems such as mammalian cells or tissue.
What determines the formation of the initial seed/what is the etiology of sporadic CJD?	This and the previous question may potentially have the same answer. One hypothesis is that PrP ^{Sc} is produced continuously, and that the failure to degrade PrP ^{Sc} leads to seed formation. Other ideas posit that sCJD is caused by somatic mutations in the <i>Prnp</i> gene encoding for PrP ^C (similar to transformation of tumor cells), or that the formation of PrP ^{Sc} is a rare stochastic event.
What is the mechanism of prion-induced neurotoxicity?	This is an underdeveloped subject in prion biology due to the lack of appropriate experimental models. Cell lines that do replicate prions do not show any effect on cell viability. Maybe aggregation of PrP ^{Sc} on the cell surface leads to the propagation of some neurotoxic signal (Solforosi <i>et al.</i> , 2004).

issues, including the structure of amyloids, the molecular basis underlying the prion strain phenomena and the cause of neurodegeneration.

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