Prion protein interconversions†

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The transmissible spongiform encephalopathies (TSEs), or prion diseases, remain mysterious neurodegenerative diseases that involve perturbations in prion protein (PrP) structure. This article summarizes our use of *in vitro* models to describe how PrP is converted to the disease-associated, protease-resistant form. These models reflect many important biological parameters of TSE diseases and have been used to identify inhibitors of the PrP conversion as lead compounds in the development of anti-TSE drugs.

Keywords: prion; transmissible spongiform encephalopathies; structure; conversion; Creutzfeldt–Jakob disease; bovine spongiform encephalopathy

1. INTRODUCTION

The transmissible neurodegenerative disease scrapie has been known as a problem in sheep for centuries. More recently, similar diseases, i.e. transmissible spongiform encephalopathies (TSEs) or prion diseases, have been identified in a wide variety of mammals ranging from rodents to humans. The first human TSE disease known to be transmissible was kuru in the Fore tribe of New Guinea. This cannibalistic tribe contracted kuru by eating family members who had died of the disease. Other notorious members of the TSE group of diseases include bovine spongiform encephalopathy (BSE) (mad cow disease), which appears to have caused variant Creutzfeldt–Jakob disease (vCJD) in humans who have consumed BSE-infected beef. In the USA, a TSE disease of deer and elk called chronic wasting disease (CWD) has become an increasing concern as it has appeared with high incidence in free-ranging herds in northern Colorado and Wyoming, as well as in game farms in other western US states (Williams *et al.* 2000).

A large body of evidence indicates that an important molecular event underlying most, if not all, TSE diseases is the conversion of the normal, protease-sensitive form of the host's prion protein (PrP^{sen} or PrP^C) to an abnormal, protease-resistant form (PrP^{res}) (for reviews, see Chesebro 1999; Horiuchi & Caughey 1999; Weissmann 1999). Unlike PrP^{sen}, PrP^{res} is insoluble in non-denaturing detergents and forms ordered aggregates, such as amyloid-like fibrils and amyloid plaques. PrP is a critical susceptibility factor for TSE diseases. For instance, the absence of PrP in a host (a PrP knockout mouse) makes the host resistant to TSE disease. Furthermore, the presence of PrP^{res} correlates with TSE infectivity. These and other considerations, such as the apparent lack of a TSE agent-specific nucleic-acid genome, have supported proposals that PrP^{res} alone may be the infectious agent, or prion, of TSEs. It appears that in human cases of

familial TSE disease associated with rare mutations in the PrP gene, there is spontaneous generation of PrP^{res} and infectivity; however, it is difficult to definitively exclude the role of an as yet unidentified virus in these cases. Unfortunately, despite intense effort, no one has succeeded in inducing the conversion of PrP^{sen} alone to an infectious agent, so the most basic prediction of the protein-only hypothesis for the TSE agent remains undemonstrated.

A critical challenge, then, is to understand how PrP^{res} is made in infected tissues. Experiments with scrapie-infected tissue culture cells showed that wild-type PrP^{sen} is converted to PrP^{res} after it is translated, glycosylated, linked to a glycosylphosphatidyl inositol (GPI) anchor and carried to the cell surface (Borchelt *et al.* 1990; Caughey & Raymond 1991). The conversion seems, therefore, to occur on the plasma membrane or in an endocytic vesicle (Caughey & Raymond 1991; Caughey *et al.* 1991; Borchelt *et al.* 1992). Familial TSE-associated mutant PrP molecules, on the other hand, can begin to show abnormal behaviour earlier in the biosynthetic pathway and this may reflect a tendency towards spontaneous rather than TSE-induced PrP^{res} formation by these mutants (Harris 1999).

2. SELF-PROPAGATING ACTIVITY OF PrP^{res}

A basic tenet of protein-only models of TSE infectious agents is that the abnormal, putatively infectious form of PrP is able to induce the conversion of normal PrP to the abnormal form in order to propagate itself. In an effort to obtain evidence as to whether PrP^{res} has such self-propagating activity, we incubated 35S-labelled PrP^{sen} with unlabelled PrP^{res} and digested with proteinase K (PK) to see if any of the PrP^{sen} was induced to convert to the protease-resistant state. 35S-labelled species exhibiting the characteristic partial PK-resistance of bona fide PrP^{res} were produced in the samples containing PrP^{res} but not in the absence of PrP^{res} or in the presence of denatured PrP^{res} (Kocisko *et al.* 1994). This cell-free conversion reaction provided direct evidence that PrP^{res} can induce its own formation.

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3. SPECIES SPECIFICITY OF PrP-res-INDUCED CONVERSION OF PrP-sen

Since then, many experiments have demonstrated a high degree of specificity in this reaction. For instance, substitution of PrP-res with another fibrillar amyloid protein, i.e. Alzheimer's beta amyloid, did not result in conversion (Kocisko *et al.* 1994). Even more impressive is the fact that the attempts to drive the reaction with PrP-res from a different host species can also result in little or no conversion (Kocisko *et al.* 1995; Bossers *et al.* 1997; Raymond *et al.* 1997; Horiuchi *et al.* 2000). The sequence specificity of this conversion reaction may provide the mechanism underlying the known requirement for certain PrP amino-acid sequence homologies between the inoculum and the host in interspecies TSE transmissions *in vivo*. Indeed, in reactions between PrP-sen and PrP-res molecules of different sequence, we have observed a widespread correlation between the efficiency of the cell-free reactions and known *in vivo* transmissibilities of TSE agents between the corresponding species (Raymond *et al.* 1997). For instance, BSE is known to be transmissible to cattle, mice, and certain genotypes of sheep, but not to hamsters or a BSE-resistant sheep genotype. In analogous cell-free conversion reactions, PrP^{BSE} induces the conversion of PrP-sen from cattle, mice and the BSE-susceptible genotypes of sheep more efficiently than the PrP-sen of hamsters or BSE-resistant sheep. This correlation has encouraged us to use the cell-free conversion reaction to gain clues to unknown interspecies transmissibilities such as that of BSE (Raymond *et al.* 1997) and CWD (Raymond *et al.* 2000) to humans.

4. OTHER CORRELATIONS BETWEEN CELL-FREE PrP CONVERSION REACTIONS AND TSE BIOLOGY

A number of additional findings have provided evidence that the reaction is related to the biology of TSE diseases. TSE strain-associated conformations of PrP-res have been identified (Bessen & Marsh 1994; Parchi *et al.* 1997; Somerville *et al.* 1997; B. Caughey *et al.* 1998; Rubenstein *et al.* 1998; Safar *et al.* 1998) and can be faithfully propagated in cell-free conversion reactions (Bessen *et al.* 1995). This observation provides a possible PrP-res conformation-based explanation for the propagation of TSE strains by a protein-only mechanism without variation of even the amino-acid sequence of the putative infectious protein. The reaction has been shown also to occur under conditions that are much more physiological than those described originally (DeBurman *et al.* 1997; Horiuchi *et al.* 1999, 2000) including intact brain slices (Bessen *et al.* 1997). Finally, a correlation between the cell-free conversion activity and scrapie infectivity has been observed in denaturation and centrifugation studies (Caughey *et al.* 1995, 1997).

5. MECHANISTIC INSIGHTS INTO THE PrP CONVERSION REACTION

A number of studies have provided evidence relating to the mechanism of the conversion reaction. The reaction is dependent upon the concentrations of both PrP-sen and PrP-res (Caughey *et al.* 1995). Reduction of the

single disulphide bond in either PrP-sen or PrP-res molecules (Herrmann & Caughey 1998) inhibits the conversion reaction. So far, we have been able to detect converting activity (and infectivity) associated with aggregates but not monomers of PrP-res (if the latter even exist) (Caughey *et al.* 1995, 1997). Complete disaggregation and denaturation of PrP-res results in a coincident loss of converting activity and scrapie infectivity (Kocisko *et al.* 1994; Caughey *et al.* 1997); however, partial reversible unfolding of PrP-res does not (Kocisko *et al.* 1996). The conversion reaction can be stimulated either by partially denaturing treatments or by the action of certain chaperone proteins, i.e. GroEL or hsp104 (DeBurman *et al.* 1997). In the course of conversion, the PrP-sen substrate binds to and remains associated with the PrP-res aggregate (Caughey *et al.* 1995; Bessen *et al.* 1997; DeBurman *et al.* 1997; Horiuchi *et al.* 1999, 2000). These observations are consistent with, but do not prove, a nucleated polymerization mechanism for the reaction.

As noted in §3, it seems clear that the conversion process involves precise interactions between PrP-sen and PrP-res. Accordingly we have been interested in defining the sites of interaction between the two types of PrP in order to understand the conversion mechanism and the basis for species barriers in TSE transmissions. Analyses of sequence homology requirements between mouse and hamster PrP-sen and PrP-res molecules, and chimeras thereof, indicated that efficient PrP-res formation in cell culture and *in vivo* (Scott *et al.* 1992; Priola *et al.* 1994; Priola & Chesebro 1995) depended on homology in the central third of the PrP molecule. Indeed, differences in as little as one residue at position 138 can dramatically diminish PrP-res formation in scrapie-infected neuroblastoma cells (Priola & Chesebro 1995). The importance of intermolecular interactions in this portion of the PrP sequence was further indicated by studies showing that synthetic peptides corresponding to this general region of the molecule, especially residues 119–136, profoundly inhibit PrP-res formation (Chabry *et al.* 1998, 1999).

Kinetic analyses of the conversion reaction indicate that the initial binding and conversion steps can be separable (DeBurman *et al.* 1997; Horiuchi *et al.* 1999, 2000). To analyse the binding and conversion reactions as they occur under physiological conditions, we developed cell-free conversion reactions in which either KCl or NaCl was substituted for guanidine hydrochloride or urea (Horiuchi *et al.* 1999, 2000). Under similar conditions, we found that when PrP-res was incubated with cell lysates or tissue culture supernatants containing complex mixtures of protein, only PrP-sen (a GPI-negative construct) was observed to bind and co-sediment with PrP-res. Thus the binding between PrP-sen and PrP-res is highly selective.

In another approach to mapping the sites of interaction between PrP-sen and PrP-res, we tested the ability of antibodies to different PrP epitopes to block binding and conversion (Horiuchi *et al.* 1999). Only the binding of an antiserum to C-terminal residues 218–232 of PrP-sen blocked its initial binding to PrP-res and, thereby, its subsequent conversion. However, removal of the epitope sequence itself from PrP-sen did not inhibit binding and conversion. We interpret these results to mean that the

initial binding occurs not via residues 218–232, but via a surface on PrP-sen that is sterically hindered or conformationally altered by the binding of the antibody to those residues. Three-dimensional nuclear magnetic resonance structures of PrP-sen (Riek *et al.* 1997; Donne *et al.* 1997) indicate that two surfaces would probably be blocked by this antibody but not by the other antibodies tested or by the presence of N-linked glycans at residues 181 and 197. These surfaces are formed by the loop of residues at about 165–174 and helical residues at about 206–223 and, on the other face of the molecule, by the extended chain formed by residues at about 119–141. The importance of the latter region has also been implicated by the peptide inhibition, cell culture, and *in vivo* studies described above. Further cell-free studies with mouse and hamster PrP molecules that differ at residues in this region indicate that, although conversion reactions between the heterologous PrP molecules is inefficient, binding still occurs relatively efficiently (Horiuchi *et al.* 2000). Thus, initial binding between PrP-sen and PrP-res of these species seems to occur somewhat promiscuously via one of the above-mentioned sites near in space to the C-terminus. However, the subsequent conversion to protease resistance requires further interactions and/or conformational changes that depend on sequence homologies at the polymorphic residue 138.

6. INHIBITION OF PrP-res FORMATION AND PROSPECTS FOR TSE THERAPEUTICS

One of the major approaches to TSE therapy is to inhibit PrP-res formation. Many inhibitors have been identified and some have been shown to prolong the lives of scrapie-infected animals if administered near the time of infection (Priola *et al.* 1999). However, none has proven to be effective for treating TSE disease in clinical settings after the appearance of symptoms. Recently discovered new lead compounds include the tetrapyrroles (porphyrins and phthalocyanines) (W. S. Caughey *et al.* 1998; Priola *et al.* 1999, 2000), sirius red and other sulphonated dyes related to Congo red (Demaimay *et al.* 2001), lysosomotropic amines, and cysteine protease inhibitors (Doh-ura *et al.* 2000). Members of each of these classes of compounds were first found to block PrP-res formation in scrapie-infected neuroblastoma cells. Certain tetrapyrroles and sulphonated dyes were also found to inhibit the cell-free conversion reaction (W. S. Caughey *et al.* 1998). Most recently, Priola *et al.* (2000) demonstrated that the tetrapyrroles can dramatically improve the life span of scrapie-infected rodents. It is hoped that with improved understanding of the mechanism of PrP-res formation, inhibitors with clinical applicability can be obtained.

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Discussion

M. B. Pepys (*Department of Medicine, Royal Free and University College Medical School, London, UK*). Are you suggesting that we should all be getting some gene therapy with a nice innocent PrP to stop us going down with BSE or vCJD in 10, 20 or 30 years' time?

B. Caughey. If you can figure out how to do it, it might work.

M. B. Pepys. That was a facetious question, but it had a serious content. Do you think that there are therapeutic opportunities in blocking the binding, and thereby the conversion?

B. Caughey. People are attempting to develop such strategies. If you could figure out how to get the appropriate PrP^{sen} expressed in the right place at the right time, it could conceivably work.

J. W. Kelly (*Department of Chemistry, Scripps Research Institute, La Jolla, CA, USA*). The peptide work is interesting to me from the perspective of trying to sort out, using the cell culture experiments, where the conversion is taking place. For example, my anticipation would be that the peptide would not get into the cell, so that if it inhibits the conversion, this is likely to be occurring outside. Can you comment on what you know about this?

B. Caughey. I did not get a chance to show you at the end that one of our major thrusts is to identify inhibitors of this process. We have identified a great many compounds that are profound inhibitors, in addition to the peptides. They can all be added to the medium of the tissue culture cells and block the conversion reaction. I showed you on the first slide of the cell biology that we think the conversion is occurring either on the plasma membrane or in some endocytic vesicle. Even in endocytic vesicles, the external medium is being internalized, so molecules, even if they cannot cross membranes, should be able to reach either the endocytic or cell surface site where we think this conversion is occurring, at least in the scrapie-infected cell.

J. W. Kelly. The quantification is interesting, since I would expect the inhibitor concentration in the cells to be low.

B. Caughey. In the endosome I would expect the concentration to be the same as in the medium because the medium is being engulfed. We have reason to think that these inhibitors bind to PrP; we know that these peptides inhibit the conversion reaction by binding to PrP^{sen} rather than binding to PrP^{res}. If these inhibitors can bind to PrP, they may be concentrated.

R. J. Ellis (*Department of Biological Sciences, University of Warwick, UK*). I should like to ask any of the prion

speakers whether they have an estimate as to the number of different strains that can replicate in the same mouse or type of mouse, because that would give us some idea of how many different conformations could be generated, each of them infectious, from one primary structure.

B. Caughey. There has been a debate about how many prion strains there might be that can replicate inside a given inbred strain of mouse. Some people argue that it is only several, but others have argued it might be as many as 20. I do not know how to arbitrate on this point.

J. Collinge (*Medical Research Council Prion Unit, Imperial College School of Medicine, London, UK*). This point remains controversial. I do not think that there is clear evidence that there could be as many as 20 strains of sheep scrapie that can replicate in the same mouse strain, but there is clear evidence of seven or eight propagating in the same mice. That is the sort of number we see with distinct human strains in the same genotype of transgenic mice.

M. F. Perutz (*Medical Research Council Unit of Molecular Biology, Cambridge, UK*). I should like to ask about the chemical basis of species specificity. Is it simply a function of the number of amino-acid substitutions between two species or are there certain key residues which determine whether the prion of a particular species is infectious to humans or not?

B. Caughey. There are definitely key residues that are much more important than others. In the mouse and hamster models Sue Priola has shown that there is a particular residue, 138, which, if it differs between the mouse and hamster, can completely shut down the conversion reaction. So there are definitely key residues that are important in determining the species barrier.

M. F. Perutz. What is the amino acid?

B. Caughey. Residue 138 is methionine in the hamster. If you put methionine into the mouse PrP it becomes incompatible with the mouse PrP-res.

M. F. Perutz. What about humans, between sheep scrapie and bovine?

J. Collinge. Differences in the primary structure are important, but it is clearly not the whole story. As far as BSE is concerned it seems to be hardly any of the story. For instance, if you take isolates of classical CJD, where one is dealing with the normal human PrP, this transmits rather easily to mice expressing the same PrP. If you take vCJD, which we now know is the BSE strain, the PrP that you extract from the brain of these individuals has the same primary structure as that from classical CJD, but this does not transmit efficiently to mice expressing human PrP. It does transmit efficiently to wild-type mice, where there are many amino-acid changes. Another example is Prusiner's recent result showing the ease of transmission of vCJD to mice expressing the bovine PrP. So with BSE it seems to be breaking the rules, in that the primary structure of the PrP in the host seems to be of little consequence. I think we have to talk about transmission barriers rather than species barriers, the latter being something of a misnomer. We have to think of transmission barriers as having multiple components, or certainly two components, one of which is about the mismatch

between the primary structure of the PrP in the inoculum and the host, and the other is related to the strain of the agent. If strain is indeed to do with the tertiary conformation of PrP scrapie this makes sense, because the primary structure is presumably having some effect on the tertiary structure. In the case of PrP there is not simply one tertiary structure dictated by that primary structure; there are several. With the BSE strain this heterogeneity of conformation overpowers the effect of the amino-acid differences. It seems the strain-specific effect is the dominant effect determining the species barrier rather than the differences in primary structure.

M. F. Perutz. Does glycosylation play a part?

J. Collinge. Yes, that is something that makes BSE stand out. I have evidence that BSE has this remarkable glycoform pattern, which is maintained in all sorts of intermediate hosts, not just in mice. You can pass it through sheep, various sorts of exotic gazelle, monkeys and humans, and it retains its particular glycosylation profile. So it looks as though glycosylation is part of what is going on, determining the ease of interactions between PrP scrapie in the inoculum and those in the host. So the primary structure is just the first approximation to this; we are seeing examples now where it is not only not a good first approximation, it does not seem to be relevant at all.

B. Caughey. In the interspecies conversion reactions we study *in vitro*, we find little or no influence of glycosylation. You can get this profound species specificity with unglycosylated forms of PrP, so the primary structure seems to be most important. That does not exclude the possibility that glycosylation could modulate events under certain circumstances.

M. Pepys. What is the stoichiometry in your *in vitro* conversion system, and how does that relate to the putative stoichiometry of these conversions happening *in vivo*?

B. Caughey. We usually have a 50-fold excess of PrP-res relative to the radioactively labelled PrP substrate. We cannot put in a small amount of seed and nucleate the polymerization of huge stoichiometric excesses of soluble protein continuously. We have tried hard to achieve this but so far without success. This failure may be because it is difficult to convert the protein to a β -sheet amyloid form, and we do not have the conditions optimized for this. Alternatively some other factor may be missing.

J. Masel (*Department of Zoology, University of Oxford, UK*). This heterologous or mutant PrP strategy seems to be very effective in controlling amyloidosis in your experiments. Do you think that the body uses that kind of strategy under normal circumstances as one of the ways it might have evolved to prevent any arbitrary chain from forming amyloid and causing disease?

B. Caughey. One possible example of that might be the apparently greater resistance that human heterozygotes at codon 129 have towards various types of CJD. So heterozygosity may have a protective effect. Whether there is an evolutionary selection for this is unknown.

J. Collinge. I wonder whether you could summarize the attempts you have made to demonstrate whether there is any infectivity being generated *de novo* in your experiments?

B. Caughey. This is an important point. We have attempted this, but it is a difficult proposition to demonstrate whether we have generated infectivity in our

conversion reactions, because of the stoichiometry. We, like you, have attempted to change the tropism of the agent we put into the reaction mixture to drive it, by doing heterologous conversions. In agreement with your published work, we have no evidence for changes in tropism in these sorts of experiments. Nor do we have evidence for infectivity resulting from the production of protease-resistant PrP in our conversion reactions.