

Characterization of Thermodynamic Diversity between Transmissible Spongiform Encephalopathy Agent Strains and Its Theoretical Implications*

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Some transmissible spongiform encephalopathy (TSE) (or “prion”) strains, notably those derived from bovine spongiform encephalopathy, are highly resistant to total inactivation by heat. When three TSE strains derived from sheep with scrapie were heated, little inactivation took place at low temperatures, but at higher temperatures, considerable inactivation occurred. The temperature at which substantial inactivation first occurred varied according to TSE strain, and it was calculated to be 70 °C for the 22C strain, 84 °C for ME7, and 97 °C for 22A by fitting the data to a model based on competition between a destructive and a protective reaction. However, PrP^{Sc} from mice infected with a range of TSE strains retained similar resistance to proteinase K digestion after heating to below or above these temperatures, showing that the properties of PrP^{Sc} responsible for proteinase resistance do not correlate with those conferring thermostability on the TSE agent. The simplest explanation of these data is that the causal agent contains a macromolecular component that is structurally independent of the host, that it varies covalently between TSE strains, and that it is protected by other macromolecular components. The model is in accord with the virino hypothesis, which proposes a host-independent informational molecule protected by the host protein PrP.

Transmissible spongiform encephalopathies (TSEs)¹ which include scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease, are a group of diseases that cause progressive neurological degeneration ending inevitably in death. The structure of the causal infectious agent is not known and is a matter of some controversy. The prion hypothesis proposes that the agent is comprised of a host glycoprotein, PrP, which alters its conformation into an infectious form (1). By contrast, the virino hypothesis proposes that the agent comprises an unidentified informational molecule (probably a nucleic acid) that is protected by the host protein PrP (2), and the virus hypothesis favors a structure sharing the essential elements of conventional viruses (3).

In mice, *Sinc* is the major gene controlling incubation periods

of different TSE strains (4, 5). It encodes PrP (6, 7), and it has been shown that PrP is an absolute requirement for the replication of TSE infectivity (8, 9). An altered form of PrP, designated PrP^{Sc}, is found in infected tissue. It is operationally differentiated from the normal form of the protein PrP^C by its relative resistance to protease digestion and its sedimentability after detergent treatment (10). PrP^{Sc} secondary structure has been shown to have a higher degree of β -sheet than PrP^C (11, 12). Correlations between amounts of TSE infectivity and amounts of PrP^{Sc} and their similar resistance to protease digestion are the main biochemical evidence supporting the role of PrP as a component of TSE agents (13, 14).

Despite a reputation for resistance to inactivation, heat does inactivate TSE infectivity (15), although a small, more resistant subpopulation of infectivity is sometimes recovered (16). Individual TSE strains vary in their thermostability (17). In one series of disinfection studies, autoclaving the 22A strain at 126 °C reduced the titer much less than for the 139A strain. Exposure to heat for short periods (5 min) markedly reduced titer for both strains (18). Longer exposures further reduced titers but at a much lower rate, giving biphasic inactivation curves with time. By contrast, exposure to SDS resulted in increasing losses of infectivity with time at monophasic rates characteristic of a first order reaction; these rates were significantly different for the two TSE strains studied. Also, whereas the 22A strain was more resistant to heat inactivation, 139A was more resistant to SDS at room temperature (18).

In a recent experiment, macerates of brains from both mouse *Sinc* genotypes (4) infected with five different TSE strains were investigated. Differences were found in their thermostability with respect to the strain of TSE agent when subjected to autoclaving with saturated steam at 126 °C for 30 min, but they were little affected by the host *Sinc* genotype (19), *i.e.* their PrP sequence. In a further experiment, three TSE strains were subjected to dry heat at 200 °C, but in this case, there was little difference with respect to TSE strain.²

In this study, we examine the effect of heating at different temperatures on three TSE strains and compare the effect on infectivity with that of a biochemical property of PrP^{Sc}. The data have practical value in allowing predictions of appropriate wet heat inactivation regimes (*i.e.* autoclaving). They also allow an examination of the thermodynamic properties of the molecule(s) involved in the structure of these infectious particles. These properties suggest a heteromeric structure involving two types of macromolecules in which one macromolecule, independent of the host, differs in its covalent structure between TSE strains.

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¹ The abbreviations used are: TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy.

² D. M. Taylor, unpublished data.

EXPERIMENTAL PROCEDURES

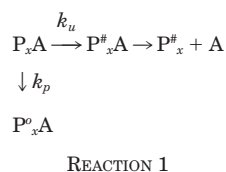
TSE Strains—The following scrapie strains were used: 22A, 22C, and ME7. 22A was serially passed from the SSBP/1 pool of serially passaged sheep scrapie into inbred VM (*Sinc^{o7}*) mice. 22C was serially passed from the same source, and ME7 was serially passed from a case of natural scrapie in a Suffolk sheep, both into C57BL (*Sinc^{o7}*) mice (20). The BSE-derived strains 301C and 301V were serially passed from a case of cattle BSE in C57BL or VM mice, respectively (21).

Heat Inactivation of TSE Infectivity Experiments—Brain homogenates (10%) in physiological saline were prepared using a Teflon glass homogenizer. Aliquots (0.3 ml) were placed in Teflon tubes and heated in a water bath ($\leq 95^\circ\text{C}$) or autoclave ($\geq 100^\circ\text{C}$) and heated at the set temperature for 30 min. The autoclave was depressurized after 30 min, and samples were removed as soon as possible after atmospheric pressure was reached. They were then allowed to cool gradually to room temperature and re-homogenized before injection.

Measurement of TSE Infectivity—10-fold serial dilutions with physiological saline were performed in the titration experiments, and a 10^{-2} dilution was used in the incubation period assay experiments. These were injected into groups of six mice intracerebrally (20 μl) or intraperitoneally (50 μl). Measurement of infectivity was performed as described before (4). Titers were calculated by the Kärber method (22).

PrP^{Sc} Resistance to Protease Digestion after Heating—Brain homogenates (10%) in phosphate-buffered saline were heated at 70, 90, or 100 $^\circ\text{C}$ in a hot block for 30 min. After cooling to room temperature, Sarkosyl (0.32%), Tris-HCl, pH 7.4, and proteinase K (100 or 400 μg) were added, and the samples were digested at 37 $^\circ\text{C}$ for 30 min. The digestions were halted with 1 mM phenylmethylsulphonyl fluoride. Samples were subjected to SDS-PAGE using the NuPage system and 12% gels (Invitrogen). They were immunoblotted using 6H4 antibody (Prionics) and visualized with SuperSignal7 West Dura Extended Duration Substrate (Pierce) in a Kodak 440 Imager Station. The amount of PrP^{Sc} was measured by determining the area under the curve generated by the PrP^{Sc} signal using the Kodak Digital Science 1D software.

Theoretical Approach—The inactivation of TSE agents by heat shows several distinctive properties: 1) After an initial rapid inactivation with time, the residual infectivity attains a plateau (18); 2) the degree of inactivation reached at the plateau depends only on this temperature³; 3) a consecutive inactivation at a similar temperature does not seem to increase the already attained degree of inactivation (16). Intuitively the heat inactivation data suggest biphasic curves with respect to temperature with little inactivation occurring below the inflection point but progressively greater inactivation with temperature above the inflection point. Such biphasic properties are consistent with the inactivation of heteromeric but not monomeric structures, involving at least two macromolecules. A reaction mechanism that fits the observed data⁴ is given by Reaction 1,



where P_xA is the infective agent consisting of x macromolecular species P and at least one other macromolecular species A, all with a well defined three-dimensional conformation and arranged in a specific three-dimensional structure. Thermal treatment leads to an unfolded state $\text{P}_x^\# \text{A}$ or to a protective state $\text{P}_x^\circ \text{A}$ in two competitive reactions. In the unfolded state, A can dissociate from P_x and both macromolecular species may denature separately into a disordered state in two irreversible reactions with differing reaction rates. In this disordered state, both macromolecular species have lost their initial chemical and biological properties.

The reaction rate constants k_u and k_p are characteristic for the overall inactivation reaction, *i.e.* they are both small in comparison with the reaction rate constants of the consecutive reactions of dissociation and denaturation. The infectivity is then given by the sum of the concentrations of P_xA and $\text{P}_x^\circ \text{A}$, as shown in Eq. 1,

$$c_{\text{infect}} = [\text{P}_x\text{A}] + [\text{P}_x^\circ \text{A}] \quad (\text{Eq. 1})$$

and the time dependence of both concentrations is given by Eq. 2.

$$d[\text{P}_x\text{A}]/dt = -(k_u + k_p) \cdot [\text{P}_x\text{A}] \text{ and } d[\text{P}_x^\circ \text{A}]/dt = +k_p \cdot [\text{P}_x\text{A}] \quad (\text{Eq. 2})$$

After solving these equations with the initial infectivity c_0 at time $t = 0$, the infectivity as a function of time shows a rapid decrease with time and attains a plateau due to the increase of the concentration of protected agents $[\text{P}_x^\circ \text{A}]$. The infectivity as a function of temperature for infinite time $t \rightarrow \infty$ is then given by Eq. 3,

$$c_{\text{infect}} = c_0 \{ k_{ou}/k_{op} \cdot \exp[-(E_{Au} - E_{Ap})/RT] + 1 \} \quad (\text{Eq. 3})$$

where c_{infect} is the concentration of infectivity (after heat treatment at temperature T), c_0 is the initial concentration of infectivity (before heat treatment), k_{ou}/k_{op} is the ratio of the reaction rate constant of the unfolding reaction, and the protective reaction, $E_{Au} - E_{Ap}$, is the difference between the activation energies of the unfolding reaction and the protective reaction; R is the gas constant, and T is the absolute temperature.

Eq. 3 shows a constancy of infectivity with time at low temperatures and an inflection toward a hyperbolic decrease when the logarithm of infectivity as a function of temperature is plotted. The intersection between the initial constant straight line and the hyperbola is called the inflection point (in $^\circ\text{C}$), and the slope of the hyperbola at the intersection is called slope (in K^{-1}) in the following. From both values, k_{ou}/k_{op} and $E_{Au} - E_{Ap}$ can be deduced.

The incubation time as a function of infectivity can be approached in the range of infectivities greater than the infective dose at which 50% of the injected mice become infected by Eq. 4,⁵

$$\log_{10}(t/t_{50}) = -A \cdot \tanh [B \cdot \log_{10}(\text{ID}/\text{ID}_{50})] \quad (\text{Eq. 4})$$

where $\log_{10}(t/t_{50})$ is the logarithm of the ratio between the incubation time at a given infective dose and the incubation time at the infective dose where 50% of the mice become infected, $\log_{10}(\text{ID}/\text{ID}_{50})$ is the logarithm of the ratio between the applied dose and the dose where 50% of the mice get infected, and A and B are constants that are assumed to be independent of strain and treatment. The strain- and treatment-specific constant in Eq. 4 is t_{50} .

RESULTS

Effect of Heat on 22A and 22C (Measured by Titration)—Brain homogenates from mice infected with two TSE strains, 22C and 22A, were heated at a range of temperatures at 10^{-1} dilution and then titrated. There was little loss of infectivity below 60 $^\circ\text{C}$ for the 22C samples or below 90 $^\circ\text{C}$ for the 22A samples, but at higher temperatures, there was a rapid reduction in infectivity (Fig. 1). Initially the data were fitted empirically using the computer program GraphPad Prism to biphasic straight lines ($R^2 = 0.98$ for 22C and 0.97 for 22A) with inflection points equivalent to 75 $^\circ\text{C}$ for 22C and 98 $^\circ\text{C}$ for 22A. The slopes of the straight lines before ($p = 0.63$) or after ($p = 0.32$) the inflection points were not significantly different. The inflection points were significantly different ($p = 0.001$). Four samples of brain homogenate at 10^{-2} dilution from mice infected with 22A strain were also heated at 105, 110, 115, and 120 $^\circ\text{C}$. They gave similar values to those heated at 10^{-1} dilution but were not included in the calculations. The data were also fitted to Eq. 3 from which inflection points equivalent to 70 $^\circ\text{C}$ for 22C and 97 $^\circ\text{C}$ for 22A were derived. The inflection points, slopes, ratios of rate constants, and differences of activation energies for 22A and 22C calculated from Eq. 3 are summarized in Table I. These data suggest that the inactivation reaction includes an unfolding step typical for a protein denaturation reaction (23) with an activation energy of >200 kJ/mole, whereas the protective reaction may have a rather low activation energy.

Effect of Heat on ME7 and 22C (Measured by Incubation Period)—Brain homogenates from ME7- and 22C-infected mice were heated and then assayed for TSE infectivity at 10^{-2} dilution using an incubation period assay (Fig. 3). The data

³ U. Havekost, S. Hamilton, R. A. Somerville, and R. C. Oberthür, unpublished results.

⁴ U. Havekost, S. Hamilton, R. A. Somerville, and R. C. Oberthür, in preparation.

⁵ R. C. Oberthür, unpublished results.

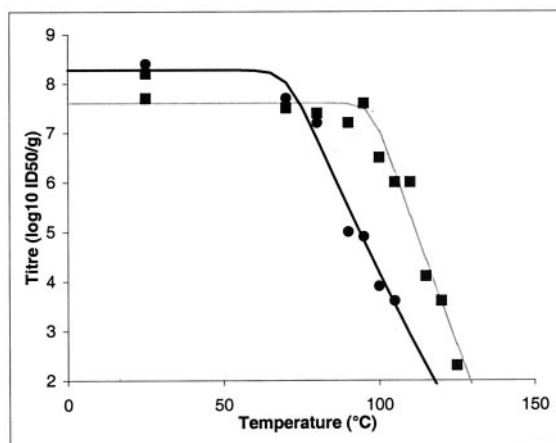


FIG. 1. Heat inactivation of 22A and 22C. Homogenates from brains infected with two TSE strains, 22A (■) and 22C (●), were heated for 30 min at a range of temperatures and titrated using the intracerebral route. The graph shows the titers, *i.e.* number of ID₅₀ per gram of tissue (log₁₀) with respect to temperature. The curves were predicted by calculation according to Eq. 3.

TABLE I

Heat inactivation values of three scrapie strains calculated from Eq. 1

TSE strain	Inflection point	Slope	log ₁₀ (k _{0in} /k _{0pr})	E _{Ain} -E _{Apr}	r.m.s.d. ^a
	°C	K ⁻¹		kJ/mol	Log units
Data from titration assays					
22A	97	0.19	71	510	0.39
22C	70	0.15	52	340	0.30
Data from incubation period assays					
ME7	82	0.25	88	600	19

^a r.m.s.d., root mean square deviation.

again show biphasic responses to heat, and when fitted to biphasic straight lines, they gave inflection points at 87 °C for ME7 by the intracerebral route or 87 °C by the intraperitoneal route and are not significantly different ($p > 0.95$). The slopes of the straight lines before and after the inflection point are not significantly different ($p = 0.78$ and 0.33 , respectively). The inflection point for 22C is at 79°, significantly different from ME7-intracerebral and ME7-intraperitoneal data ($p < 0.0001$) and close to the empirically estimated value of 75 °C obtained by the titration of heated 22C in the experiment above (Fig. 1). After the inflection point, the 22C-intracerebral slope is significantly different from ME7-intracerebral ($p < 0.0001$) but not significant from ME7-intraperitoneal ($p = 0.07$).

Previously, it has been shown that intracerebral titrations of boiled ME7 have a longer incubation period for the same dose of infectivity (24) than control samples, an effect not found after intraperitoneal injection. This effect might have compromised the use of incubation period assays in this experiment (Fig. 3) but did not do so. Analysis of the dose response curves (*i.e.* the relationship between incubation period and dose of infectivity) from the data generated for Fig. 1 suggests that the 22C-intracerebral assays are little affected by heat (data not shown). ME7-intraperitoneal assays are not altered by boiling (24), and the incubation periods from this experiment fall within the normal range for control intraperitoneal assays of ME7 and are probably not affected. However, the intracerebral assays of ME7 probably were affected at the higher temperatures, as indicated by the greater slope of the second part of the biphasic curve (ME7-intracerebral = -12.55 , ME7-intraperitoneal = -10.5 , 22C-intracerebral = -7.5) and as indicated from the calculated t_{50} value from Eq. 4 discussed below. In

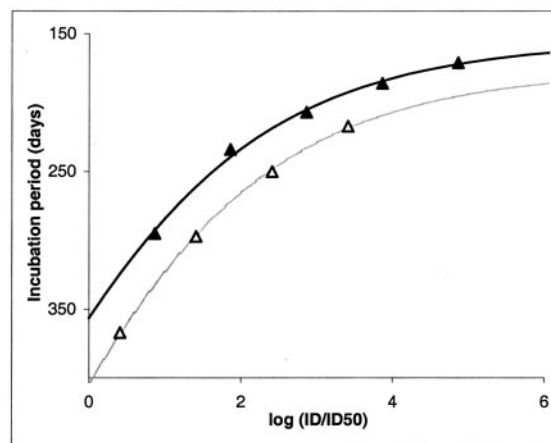


FIG. 2. Dose-response curves of unheated and heated and heated ME7. Incubation period of ME7 as a function of the injected dose divided by the dose at which 50% of the injected animals get infected (ID₅₀). Lower curve, heat-treated samples (dry heat at 160 °C) (△) with $t_{50} = 405$ days. Upper curve, native sample with $t_{50} = 356$ days (▲). Mean incubation period data are from Taylor *et al.* (25). The curves were calculated according to Eq. 4.

practice there was little effect of route on the values calculated for the ME7 inflection point.

These data can also be interpreted by a combination of Eqs. 3 and 4. Eq. 4 is used to calculate the incubation time as a function of the logarithm of the number of ID₅₀. To test Eq. 4, data from Taylor *et al.* 1996 (25) in which ME7 was heated to 160 °C dry heat and an unheated control were analyzed. An excellent agreement is obtained between the experimental data and Eq. 4 with the constants $A = 0.36$ and $B = 0.28$. The parameter t_{50} distinguishes between the heat-treated and the unheated sample and has values of $t_{50} = 356$ days for the control and $t_{50} = 405$ days for the heat-treated sample (Fig. 2).

These values ($A = 0.36$ and $B = 0.28$) are used for evaluation of the ME7 data in Fig. 3 with t_{50} as an additional variable. The initial dose is obtained from the upper curve in Fig. 2 and the average incubation time at 25 °C. It is calculated to be $\log_{10}(\text{ID}_{\text{initial}}/\text{ID}_{50}) = 4.9$. The incubation time t_{50} at ID₅₀ of 380 days for the intracerebral route is slightly higher than the corresponding value for unheated ME7 material as obtained in Fig. 2 but not as high as for the 160 °C-treated material. The calculated inflection point for ME7 combining the intracerebral and the intraperitoneal data is at 82 °C (Fig. 3). There are insufficient data to calculate the inflection point of the 22C data independently of the data in Fig. 1.

The quality of the different fits of Eqs. 3 and 4 to the experimental points can be judged from the root mean square deviation of the experimental points with respect to the calculated curves given in Table I. The deviations are within the limits of experimental errors, which are in the range of 0.3–0.5 log units for the titers and in the range of 18 days for the incubation times with a tendency of increasing standard deviations for the incubation time with decreasing infective dose.

Protease Resistance of Heated PrP^{Sc}—Brain homogenates from mice clinically affected with two different TSE strains were heated at 70, 90, and 100 °C, values below and above the calculated inflection points for 22C and 22A. After cooling, they were suspended in Sarkosyl and subjected to digestion with proteinase K (400 µg/ml). There was little degradation of PrP^{Sc} after heating to 70° (Fig. 4). Indeed the amount of PrP^{Sc} detected was greater; possibly solubilization by SDS was assisted by the prior heating. Some PrP^{Sc} was degraded in some models at 90 °C, and at 100 °C, most had lower amounts of detectable PrP^{Sc}. Similar results (not shown) were obtained from samples

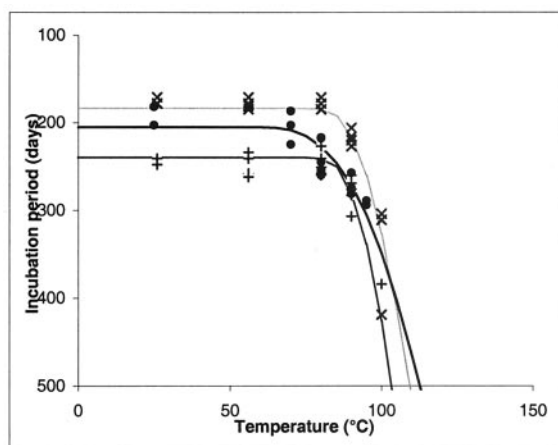


FIG. 3. **Heat inactivation of 22C and ME7.** Homogenates from brains from two TSE strains, 22C-intracerebral (●), and ME7-intracerebral (X) and ME7-intraperitoneal (+), were heated at a range of temperatures. Incubation periods were measured at 10^{-2} dilution after intracerebral or intraperitoneal inoculation. The graph shows the incubation periods of individual mice with respect to temperature. The curves are calculated according to Eqs. 3 and 4 with $t_{50} = 360$ days for the intracerebral assay and $t_{50} = 469$ days for the intraperitoneal assay of ME7 and 431 days for the intracerebral assay of 22C.

from ME7-, 301C-, and 301V-infected brains that were digested with 100 or 400 $\mu\text{g/ml}$ proteinase K. In all cases, including that of 22C, PrP^{Sc} survived digestion at a temperature at which the infectivity of 22C was reduced by >99.9% (Fig. 4).

DISCUSSION

The heat inactivation properties of some TSE strains lie near but within the extremes of stability of biological molecules. Of greater importance for understanding the structure of the causal agent may be their diversity of properties, which can be usefully compared with the chemistry of other biological molecules and infectious agents. As Rohwer (15, 26) pointed out, much infectivity can be destroyed by relatively mild treatments that also destroy many viruses, and it is only a small proportion of infectivity that survives, possibly through some protective mechanism. With respect to heating of hydrated TSE strains, 22C is heat-stable only up to 70 °C, a relatively low temperature compared with some other microorganisms (27). However, 22A is much more stable (up to 97 °C), and BSE-derived strains are more stable still (19, 28),⁶ although detailed heating curves are not available for comparison. The data are consistent with the heat denaturation of a component of the agent, which differs in structure between strains.

The data do not fit a model in which the infectious agent is simply destroyed or dissociated by heat. To develop a mathematical model that described the data adequately, it was necessary to propose an infectious particle composed of at least two different macromolecules (heteromeric model): one molecule acting as the carrier of information (the informational molecule) and the other protecting this informational molecule. Heat treatment leads either to a rearrangement of these macromolecules, resulting in a more effective protection of the informational molecule, or to a partial unfolding of the tertiary structure of at least one component, resulting in the dissociation of the informational molecule and the protective molecule where both components are sensitive to irreversible degradation but at different rates. This model is mathematically expressed in Eq. 3. The informational molecule must irreversibly degrade especially rapidly, whereas the protective components degrade more slowly and reconstitute their structure after

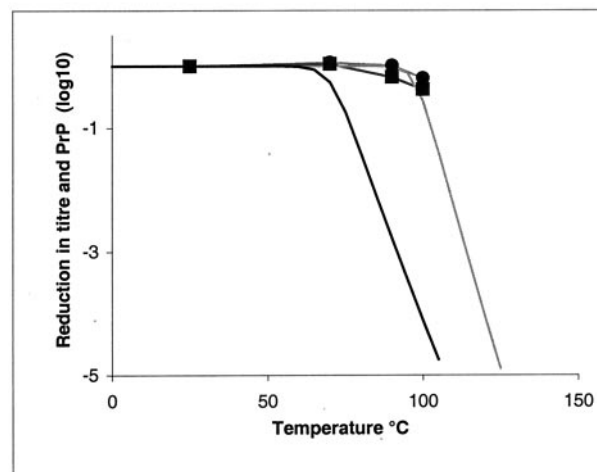
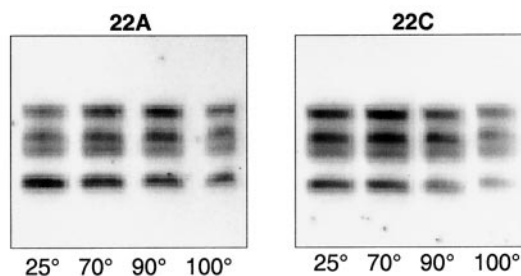


FIG. 4. **Comparison of the effect of heat on TSE infectivity.** Homogenates from brains infected with two TSE strains, 22A (■) and 22C (●), were heated for 30 min at a range of temperatures, and after cooling, were subjected to digestion with 400 μg of proteinase K. The two immunoblots show PrP^{Sc} from 22A- or 22C-infected brain after heating at the indicated temperatures and subsequent digestion with proteinase K. The graph plots the log of the change in the amount of PrP against the log of temperature and is compared with the calculated curve of reduction in log titer after heating, recalculated from Fig. 1, of 22A (light line) and 22C (dark line).

cooling. This model leads to the testable prediction that more of the protected form will be created by heating at temperatures just above the inflection point. Similarly more of the protected form will be created if samples are heated slowly to their target temperature than if the temperature rise is rapid. Furthermore it may be possible to promote the destruction or sequestration of components of the agent when in the disassociated state.

Although assayed in mice, the determination of thermostability properties is not dependent on pathogenic interactions between the recipient mouse and the inoculum and so differs from the phenotypic properties of incubation period, pathology, and PrP^{Sc} properties that are normally measured for strain typing. By contrast, thermostability and other inactivation properties are presumably intrinsic to the structural components of the infectious agent. They could be used for strain typing purposes, too.

Biological Extremes, Thermophilic Enzymes as Models of Variable Thermostability—Comparison of TSE agent strain stabilities with macromolecules from mesophilic and thermophilic bacteria shows that protein structure and function can evolve to be stable above 100 °C. They provide a model of how macromolecular structures are stabilized at high temperatures and in what ways differences in thermostability may be accounted for (29). Heat denatures hydrated proteins by breaking non-covalent bonds, but their structures can be stabilized by a series of amino acid replacements that increase the energy required for denaturation. This has been demonstrated by the progressive selection at increasing temperatures of an enzyme from a mesophilic bacterium (30) where changes lead to small

⁶ D. M. Taylor *et al.*, in preparation.

incremental stabilization steps in secondary structural elements. There are no specific rules about amino acid substitutions, although there is a trend to the use of amino acid residues that stabilize the secondary and tertiary structure (30, 31). Although wet heat tends to hydrolyze peptide bonds at temperatures above 100 °C and individual residues can also be covalently modified, the effects can be inhibited by local structural features (29). Enzyme activity can survive temperatures up to 130 °C for 10 min (29). High thermostability tends to correlate with stability to other types of denaturation and to degradation. Denaturants such as heat and high pH tend to work cooperatively (29). Nucleic acids can be stabilized (32) through base substitution and high salt concentrations (33) to give increased cooperativity and higher functional stability (34). Differences in thermodynamic parameters such as the enthalpy and the entropy of the unfolding of enzymes with different thermostabilities are similar to the differences in thermodynamic parameters found between ME7, 22C, and 22A as given in Table I and are consistent with the more thermostable strain being stabilized by a small number of additional or stronger non-covalent bonds.

Kinetics of TSE Inactivations—The reactions associated with monophasic inactivation of TSE infectivity, ionizing radiation (35), and treatment with diethylpyrocarbonate (36) presumably directly destroy a critical component of the agent. By contrast, the protein denaturants SDS and guanidine inactivate biphasically with respect to concentration (18, 37–39). Only when the required concentration has been reached does inactivation occur significantly, presumably when denaturation of a protein component of the infectious particle occurs. Similarly heat inactivates biphasically with respect to temperature: significant inactivation only occurs once a certain temperature has been reached. Heat also inactivates biphasically with respect to time: after initial rapid inactivation, there is little further effect. These inactivation curves (15, 18, 40) are similar to the biphasic curves obtained when oligomeric enzymes are denatured (29, 41) but not to monomeric enzymes, which are denatured monophasically. In contrast, SDS at room temperature denatured TSEs monophasically with time and was more effective against the more thermostable TSE strain (15, 18). Overall, the differing kinetics suggest that the infectious agent offers a complex target to inactivation processes. Different mechanisms operate and/or different parts of the agent's structure are being denatured or otherwise damaged. In particular, biphasic inactivation curves suggest that heteromeric structures are involved in the inactivation process, whereas monophasic curves suggest that one component of the structure is damaged.

PrP^{Sc} Resistance to Proteinase K Digestion—The residual PrP^{Sc}, which resists digestion by proteinase K after heating, does not follow the decrease of infectivity as monitored by bioassay (Fig. 4), *e.g.* only 10–30% of PrP^{Sc} from 22C-infected brain is digested by proteinase K digestion after heating to a temperature of 100 °C, which destroys >99.9% of infectivity. This result shows that the heat-sensitive component of the infectious agent is not associated with the structure of PrP^{Sc} responsible for proteinase K resistance. These data suggest that it is inappropriate to use PrP^{Sc} as a surrogate for TSE infectivity in inactivation experiments. In other respects, too, the association between PrP^{Sc} and TSE infectivity can be altered. For example, treatment with certain detergents (42, 43), detergent at high pH (44, 45), or detergent in the presence of dimethyl sulfoxide (46, 47) can separate some PrP that is normally associated with the infectious fraction from TSE infectivity. These results raise questions about the association between all of the PrP molecules in the PrP^{Sc} fraction and the

infectious agent. It remains a possibility that a subfraction of the PrP population in infected tissue is a component of the structure of the infectious agent.

Models of the Agent and Diversity of Thermostability—The prion hypothesis proposes that conformational change of the host glycoprotein PrP^C makes PrP infectious and that differences in conformation determine TSE strain properties, *i.e.* they encode the information required to specify TSE agent strain properties. Differences in the host PrP sequence have also been proposed to contribute to determining TSE strain properties. Consequently, a strain-specific conformation of PrP^{Sc} should transfer this conformation (*i.e.* secondary or higher structure) to a host PrP with different sequence (primary structure). If the prion hypothesis is correct, then it has to account for differences in TSE strain thermostability and their survival at high, near denaturing temperatures. It has been predicted that conformational change should be achievable *in vitro*, *e.g.* by using infectious PrP to “seed” conversion in the test tube. PrP^{Sc} can indeed seed conversion of PrP to an abnormal form (48), but there is no evidence that the converted form is infectious (49). Similarly if there are strain-specific conformations as is proposed (50, 51), then it is entirely feasible that partially denaturing physical or chemical treatments could alter the conformation to a more thermodynamically stable form, which should then have a different strain phenotype. Again there is no evidence of this occurring, although it is a prediction that can be specifically tested. Perhaps TSE strain-specific conformations are as resilient, or even more so, to denaturation than the element of the structure that is disrupted when infectivity is destroyed, although this seems unlikely.

The phenotypic properties of TSE agents include relative incubation period differences, distribution and severity of pathological lesions, both vacuolation and PrP deposition, degree of PrP^{Sc} glycosylation, and its apparent size after proteinase digestion. Properties that are intrinsic to the infectious agent include its ability to mutate (52), differences in thermostability, and differences in resistance to inactivation by SDS (18). All these properties must be encoded in the genome of the agent, independently of the host. They demonstrate that the genome of the agent, independently of the host, is information-rich and is therefore more likely to comprise the biologically orthodox, *i.e.* a nucleic acid, which is known to be capable of encoding large quantities of biological information, rather than the heterodox proposal of TSE strain-specific conformational change to host protein (PrP) independent of its own primary structure and triggered by an infecting seed. Even if the latter proposal is biologically plausible, no mechanisms have been proposed for how so much biological information could be encoded by conformational change, can be replicated faithfully within and between species (21, 53), and can interact in TSE strain-specific fashion with host pathways to control phenotypic properties. Hypotheses that propose an independent informational molecule account for these data better and more simply. They include the virino hypothesis, which proposes that PrP and an agent-specific informational molecule (most likely to be a nucleic acid) comprise the infective agent, and the virus hypothesis, although this latter hypothesis would need to involve PrP, possibly as a host-coded binding site.

TSE heat inactivation properties are consistent with the inactivation properties of thermostable proteins and nucleic acids and with a heteromeric structure. The data fit with a model in which the infectious agent comprises at least two components that interact in a multimer. To account for differences in TSE thermostabilities, one (biochemically unidentified) molecule in the structure varies between TSE strains and is the component responsible for differences in thermostability. It is not host-encoded and differs in primary sequence between strains. When

heated, it loses secondary structure, and/or its interaction with other molecules is disrupted. One other molecule is most likely to be PrP, present as dimers or higher multimers (54), but need not be so. It is predicted that differences in the primary sequence of the informational molecule of the TSE agent (since PrP is host-encoded and remains constant) are responsible for differences in thermostability. Wet heat inactivation either directly disrupts the interaction between the two molecules or does so indirectly through the disruption of the secondary structure of the variable sequence molecule.

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