In Vitro Amplification of Protease-Resistant Prion Protein Requires Free Sulfhydryl Groups†

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ABSTRACT: Prions, the infectious agents of transmissible spongiform encephalopathies, are composed primarily of a misfolded protein designated PrP^{Sc}. Prion-infected neurons generate PrP^{Sc} from a host glycoprotein designated PrP^{C} through a process of induced conformational change, but the molecular mechanism by which PrP^{C} undergoes conformational change into PrP^{Sc} remains unknown. We employed an *in vitro* PrP^{Sc} amplification technique adapted from protein misfolding cyclic amplification (PMCA) to investigate the mechanism of prion-induced protein conformational change. Using this technique, PrP^{Sc} from diluted scrapie-infected brain homogenate can be amplified >10-fold without sonication when mixed with normal brain homogenate under nondenaturing conditions. PrP^{Sc} amplification *in vitro* exhibits species and strain specificity, depends on both time and temperature, only requires membrane-bound components, and does not require divalent cations. *In vitro* amplification of Syrian hamster Sc237 PrP^{Sc} displays an optimum pH of ∼7, whereas amplification of CD-1 mouse RML PrP^{Sc} is optimized at pH ∼6. The thiolate-specific alkylating agent *N*-ethylmaleimide (NEM) as well as the reversible thiol-specific blockers *p*-hydroxymercuribenzoic acid (PHMB) and mersalyl acid inhibited PrP^{Sc} amplification *in vitro*, indicating that the conformational change from PrP^{C} to PrP^{Sc} requires a thiol-containing factor. Our data provide the first evidence that a reactive chemical group plays an essential role in the conformational change from PrP^{C} to PrP^{Sc}.

Transmissible spongiform encephalopathies such as kuru, variant Creutzfeldt Jakob disease (vCJD),[1] bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD), and scrapie are fatal, infectious, neurodegenerative diseases. These diseases appear to be caused by unconventional infectious agents termed prions (*1*). Much evidence supports the claims that prions lack nucleic acids, consist primarily of a misfolded protein designated PrP^{Sc}, and replicate by inducing a host-encoded glycoprotein designated PrP^{C} to undergo a conformational change to form new PrP^{Sc} molecules in a self-propagating process (*2*).

Whereas PrP^{C} is composed of 42% α-helix and 3% *β*-sheet, PrP^{Sc} contains 30% α-helix and 43% *β*-sheet (*3*, *4*). These differences in secondary structure make PrP^{Sc} substantially more detergent-insoluble and protease-resistant than PrP^{C}, allowing the two isoforms to be differentiated biochemically. Purified preparations of PrP^{C} and PrP^{Sc} predominantly contain proteins that have a C-terminal glycophosphatidylinositol (GPI) anchor (*5*), two N-linked oligosaccharides attached to residues N181 and N197, and a single intramolecular disulfide bridge between residues C179 and C214 (*6*).

Currently, the central mechanisms by which infectious PrP^{Sc} proteins induce host PrP^{C} molecules to undergo conformational change and create new PrP^{Sc} molecules remain undetermined. It is unknown whether any cellular factors other than PrP^{Sc} and PrP^{C} are required to produce new prions. The structural and chemical dynamics of the conversion process are also obscure. For instance, it has not been determined whether the intramolecular disulfide bridge in PrP^{C} remains intact during the conformational change to PrP^{Sc}. In the past, a factor that precluded the biochemical investigation of prion propagation has been the lack of an *in vitro* PrP^{Sc} amplification assay. A radiolabel conversion technique using purified PrP molecules recapitulates many features associated with prion transmission *in vivo* (*7*−*10*), but this method requires a 50-fold molar ratio of PrP^{Sc} to PrP^{C} to drive the formation of protease-resistant PrP. More recently, Saborio and Soto developed a method termed protein misfolding cyclic amplification (PMCA) that efficiently amplified PrP^{Sc} in hamster brain homogenates through repeated cycles of direct sonication in the presence of the anionic detergent sodium dodecyl sulfate (SDS) (*11*).

To investigate the mechanism of prion propagation biochemically, we modified the PMCA protocol. We reasoned that sonication and/or the use of SDS might denature cellular protein factors and alter the normal biochemical reactions

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[1] Abbreviations: vCJD, variant Creutzfeldt Jakob disease; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; GPI, glycophosphatidylinositol; PrP, prion protein; PrP^{C}, cellular isoform of the prion protein; PrP^{Sc}, scrapie isoform of the prion protein; RML, Rocky Mountain Laboratory; NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuribenzoic acid; PMCA, protein misfolding cyclic amplification; AEMTS, 2-(aminoethyl)methane thiosulfonate; SPM, synaptic plasma membrane.

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that we intended to study. We therefore omitted these two processes form the modified protocol, and used this nondenaturing amplification procedure to characterize and discover several fundamental aspects of PrP^{Sc} formation *in vitro*, including the requirement for a thiol-containing factor.

EXPERIMENTAL PROCEDURES

Chemical Reagents. *N*-Ethylmaleimide (NEM), PHMB, mersalyl acid, and homocysteine were purchased from Sigma. All reagents were of the highest grade available.

Preparation of Brain Homogenates. Whole brains, including cerebellum and brainstem, were dissected from normal, specific-pathogen-free 3-week-old female golden Syrian hamsters or 5-week-old CD-1 mice purchased from Charles River Laboratories. Brains were weighed and homogenized in 10 volumes (w/v) of ice-cold phosphate-buffered saline without calcium or magnesium (PBS) plus Complete protease inhibitors (Roche) using a Wheaton glass-glass Potter homogenizer. Crude homogenates were centrifuged for 30 s at 200*g*, and aliquots of the supernatant were frozen at −70 °C for subsequent experiments.

Frozen scrapie-infected hamster or mouse brains were homogenized in 10 volumes (w/v) of ice-cold phosphate-buffered saline without calcium or magnesium (PBS) containing 1% Triton X-100 using a Wheaton glass-glass Potter homogenizer. Crude homogenates were centrifuged for 30 s at 200*g*, and aliquots of the supernatant were frozen at −70 °C for subsequent experiments.

Frozen $Prnp^{0/0}$ mouse brains (kindly provided by S. B. Prusiner, San Francisco, CA) were prepared as described above for normal and scrapie-infected brains, for use as control homogenates.

In Vitro PrP^{Sc} Amplification Technique. We employed a modification of the protein misfolding cyclic amplification (PMCA) technique of Saborio and Soto (*11*). Typically, 50 μL of normal brain homogenate was mixed with 50 μL of scrapie-infected brain homogenate diluted 1:50 into PBS with 1% Triton. Thus, the final concentration of normal brain homogenate was 5% (w/v), and that of scrapie brain homogenate was 0.1% (w/v). The normal and scrapie brain homogenate mixture was incubated for 16 h at 37 °C with continuous shaking. Following incubation, 50 μg/mL proteinase K (Roche) was added to each sample, and the samples were incubated at 37 °C for 1 h. Proteinase K digestion was terminated by addition of an equal volume of SDS loading buffer (containing 1.4 M β-mercaptoethanol unless indicated otherwise) and boiling for 10 min.

Electrophoresis and Immunoblotting. SDS−polyacrylamide gel electrophoresis (PAGE) was performed on 1.5 mm, 12% acrylamide gels with an acrylamide:bisacrylamide ratio of 29:1. Following electrophoresis, proteins were blotted onto charged PVDF membranes (Millipore) using a Hoeffer TE 50X transfer apparatus set at 1.5 A for 2 h. Membranes were pretreated with 0.2 N NaOH for 10 min, rinsed for 3 × 5 min with TBST [10 mM Tris (pH 7.2), 150 mM NaCl, and 0.1% Tween 20], and blocked with 5% Carnation nonfat milk powder in TBST for 1 h at room temperature. Blocked membranes were incubated with 3F4 mAb (Signet) diluted 1:5000 in TBST or D13 humanized Fab (kindly provided by S. B. Prusiner) diluted 1:3000 in TBST overnight at 4 °C. Following incubation with the primary antibody, membranes were washed for 3 × 10 min in TBST, incubated with either a horseradish peroxidase (HRP)-labeled anti-mouse IgG secondary antibody conjugate (Amersham) diluted 1:5000 in TBST or a HRP-labeled anti-human Fab2 conjugate (Pierce) diluted 1:30000 in TBST for 1 h at 4 °C, and washed again for 3 × 10 min in TBST. After chemiluminescent development with the ECL reagent (Amersham) for 1−15 min, blots were sealed in plastic covers and exposed to Super RX film (Fujifilm). Films were processed automatically in a Kodak M35A X-OMAT film processor. Unless otherwise indicated, blots were developed using a 3F4 primary antibody, and lines indicating apparent molecular masses based on migration of prestained protein standards (Bio-Rad) are 36.2, 29.0, and 21.4 kDa. For each experiment, densitometric measurement of film signals was performed through the analysis of multiple film exposures to ensure that comparisons were made within the linear range of the film. Signals within the linear range were quantitated using the magic wand and histogram functions in Adobe Photoshop and calibrated against standard dilutions of scrapie brain homogenate.

RESULTS

Characterization of Nondenaturing PrP^{Sc} Amplification in Vitro

To characterize the process of conformational change from PrP^{C} to PrP^{Sc} biochemically, it was necessary first to determine that PrP^{Sc} could be amplified *in vitro* without sonication or SDS. Our results show that a mixture of 5% normal brain homogenate and 0.1% scrapie brain homogenate in PBS with 1% Triton after 16 h produced a 6-fold increase in the level of proteinase K-resistant PrP^{Sc} compared to that of input PrP^{Sc} (Figure 1A, compare lane 3 to lane 4). These results resemble those reported by Saborio and Soto when PMCA was performed in the absence of sonication (*11*). PrP^{Sc} could be amplified from either hamster Sc237 scrapie brain homogenate or mouse RML scrapie brain homogenate by mixing with normal brain homogenate from the corresponding species (Figure 1A, top and bottom panels). The glycoform distributions and apparent molecular masses of the PrP^{Sc} molecules formed by *in vitro* amplification match those of the corresponding input PrP^{Sc} molecules for both species. To ensure that unconverted PrP^{C} molecules were completely digested under our experimental conditions, we included as controls normal brain homogenate samples that were not mixed with diluted scrapie brain. These samples contained no protease-resistant PrP bands, indicating that protease digestion of PrP^{C} is complete under the conditions that were used (Figure 1A, lane 2). Comparisons between newly formed PrP^{Sc} bands and input PrP^{C} bands (Figure 1A, compare lanes 1 and 4) suggest that 5−10% of input PrP^{C} molecules are converted into PrP^{Sc} molecules *in vitro* during the 16 h incubation period.

To determine the optimal pH for nondenaturing *in vitro* PrP^{Sc} amplification, we incubated mixtures of normal and diluted scrapie brain homogenates for 16 h at 37 °C at various pH values between 5 and 10. Following incubation, all samples were neutralized and subjected to proteinase K digestion. Our results indicate that PrP^{Sc} amplification efficiency varied as a function of incubation pH; amplification was optimal at pH ∼7 for hamster Sc237 scrapie and at

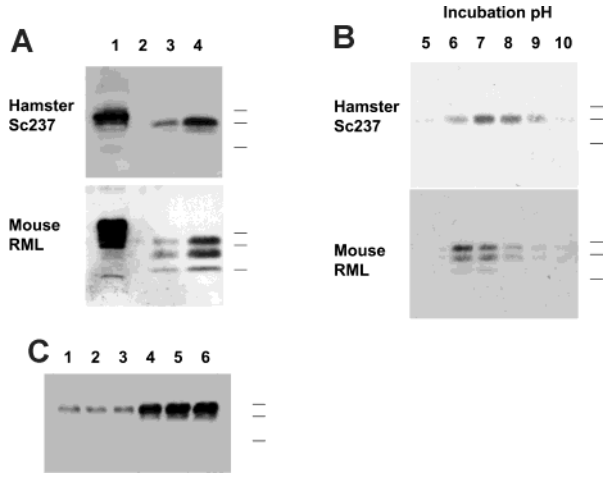

FIGURE 1: Nondenaturing amplification of PrP^{Sc} *in vitro*. (A) *In vitro* amplification of hamster and mouse PrP^{Sc}. (1) Normal brain homogenate not treated with proteinase K. (2) Normal brain homogenate and diluted $Prnp^{0/0}$ brain homogenate digested with proteinase K. (3) $Prnp^{0/0}$ brain homogenate and diluted scrapie brain homogenate digested with proteinase K. (4) Normal brain homogenate and diluted scrapie brain homogenate digested with proteinase K. The top blot contained samples of hamster brain homogenate mixed with Sc237 scrapie brain homogenate and was developed with primary antibody 3F4. The bottom blot contained samples of mouse brain homogenate mixed with RML scrapie brain homogenate and was developed with primary Fab D13. (B) Determination of pH optima for PrP^{Sc} amplification *in vitro*. Normal hamster or mouse brain homogenates prepared in H_2O with Complete Protease Inhibitors were adjusted to the indicated pH values with 20 mM sodium acetate (pH 5), sodium phosphate (pH 6−8), or Tris (pH 9−10) buffer prior to mixture with Sc237 or RML scrapie brain homogenate diluted 1:50 into the corresponding 20 mM buffer solutions with 1% Triton. Following amplification, all samples were neutralized by addition of 0.1 M HEPES (pH 7.0) and digested with proteinase K. The top blot contained samples of hamster brain homogenate mixed with Sc237 scrapie brain homogenate and was developed with primary antibody 3F4. The bottom blot contained samples of mouse brain homogenate mixed with RML scrapie brain homogenate and was developed with primary Fab D13. (C) Effect of divalent cation chelation on PrP^{Sc} amplification *in vitro*. Diluted Sc237 scrapie brain homogenate was mixed with (1) $Prnp^{0/0}$ mouse brain homogenate and 2.5 mM EGTA [added from a 0.2 M EGTA stock (pH 8.5)], (2) $Prnp^{0/0}$ mouse brain homogenate and 2.5 mM EDTA [added from a 0.2 M EDTA stock (pH 8.0)], (3) $Prnp^{0/0}$ mouse brain homogenate, (4) normal hamster brain homogenate and 2.5 mM EGTA, (5) normal hamster brain homogenate and 2.5 mM EDTA, or (6) normal hamster brain homogenate. All samples were treated with proteinase K.

pH ∼6 for mouse RML scrapie (Figure 1B). In contrast, pretreating normal and scrapie brain homogenates with mildly acidic or alkaline buffers before mixing at neutral pH did not create significant differences in PrP^{Sc} amplification (data not shown). The results suggest that pH directly influences either the binding of PrP^{Sc} to PrP^{C} or the conformational change process. All subsequent experiments were conducted in PBS buffer (pH 7.3) except where indicated.

We also investigated whether additional chelation of divalent cations might influence PrP^{Sc} amplification *in vitro*. Supplementation with the calcium-specific chelator, EGTA, or the nonspecific divalent cation chelator, EDTA, failed to change the level of PrP^{Sc} amplification appreciably (Figure 1C). Complete removal of chelators, achieved by preparing normal brain homogenates with protease inhibitor cocktail lacking EDTA, also did not alter the PrP^{Sc} amplification efficiency (data not shown). All subsequent amplification reactions were conducted with 0.5 mM EDTA, routinely added as part of the protease inhibitor cocktail used to prepare normal brain homogenates.

To characterize the kinetics of PrP^{Sc} amplification *in vitro*, we incubated mixtures of normal brain homogenate and diluted scrapie brain homogenate at different temperatures for varying times up to 2 days. The results show that PrP^{Sc} can be amplified at 25 and 37 °C, but not at 4 °C (Figure 2A). After incubation at 37 °C for 48 h, PrP^{Sc} was amplified 15.4-fold compared to input PrP^{Sc}. The PrP^{Sc} amplification rate changes over time; at 37 °C, the initial doubling time of ∼2.5 h increases to ∼24 h after 2 days [Figure 2B (●)].

Specificity of PrP^{Sc} Amplification in Vitro

We performed a series of experiments to demonstrate that our method of PrP^{Sc} amplification recapitulates several specific properties of prion propagation *in vivo*.

Species Specificity. Transmission barriers limit the efficient propagation of infectious prions between different animal species (*12*, *13*). The molecular basis for species-specific transmission barriers appears to be differences in the amino acid sequences of PrP molecules between the inoculum and host (*14*, *15*). A barrier to transmission of scrapie between mice and hamsters has been particularly well documented (*16*). We examined whether a similar species-specific barrier would be observed during PrP^{Sc} amplification *in vitro*. Normal Syrian hamster brain homogenate was incubated with either Sc237 hamster scrapie brain homogenate (Figure 3A, lane 1) or RML mouse scrapie brain homogenate (Figure 3A, lane 5). Following amplification and proteinase K digestion, hamster PrP^{Sc} was detected specifically by monoclonal antibody (mAb) 3F4, which recognizes an epitope present in hamster PrP but not mouse PrP. The results show that RML mouse scrapie brain homogenate is unable to convert hamster PrP^{C} to PrP^{Sc} *in vitro* (Figure 3A, bottom panel, lane 5). Thus, the well-documented prion transmission barrier between mice and hamsters *in vivo* is reproduced as a species-specific barrier to PrPSc amplification in our *in vitro* assay. To investigate whether the efficiency of PrP^{Sc} amplification *in vitro* correlates with species susceptibility to prion infection *in vivo*, we compared Sc237 PrP^{Sc} amplification in three different hamster species whose PrP amino acid sequences are mismatched at six positions. It has been previously established that Syrian, Armenian, and Chinese hamsters have different scrapie incubation times when inoculated intracerebrally with Syrian hamster-derived Sc237 prions. Whereas Syrian hamsters had a scrapie incubation time of ∼69 days, Armenian hamsters had an incubation time of ∼174 days, and Chinese hamsters had an incubation time ∼344 days (*17*). We prepared brain homogenates from each hamster species and separately mixed these homogenates with Sc237 scrapie brain homogenate. Our results demonstrate an inverse correlation between PrP^{Sc} amplification and scrapie incubation time (Figure 3B). Thus, the rank order of Sc237 PrP^{Sc} amplification efficiency was as follows: Syrian > Armenian > Chinese. Thus, the degree of PrP^{Sc} amplification *in vitro* correlates with the degree of species susceptibility to the infectious prion *in vivo*.

Strain Specificity. A single animal species can host several different prion strains that produce distinct clinical and

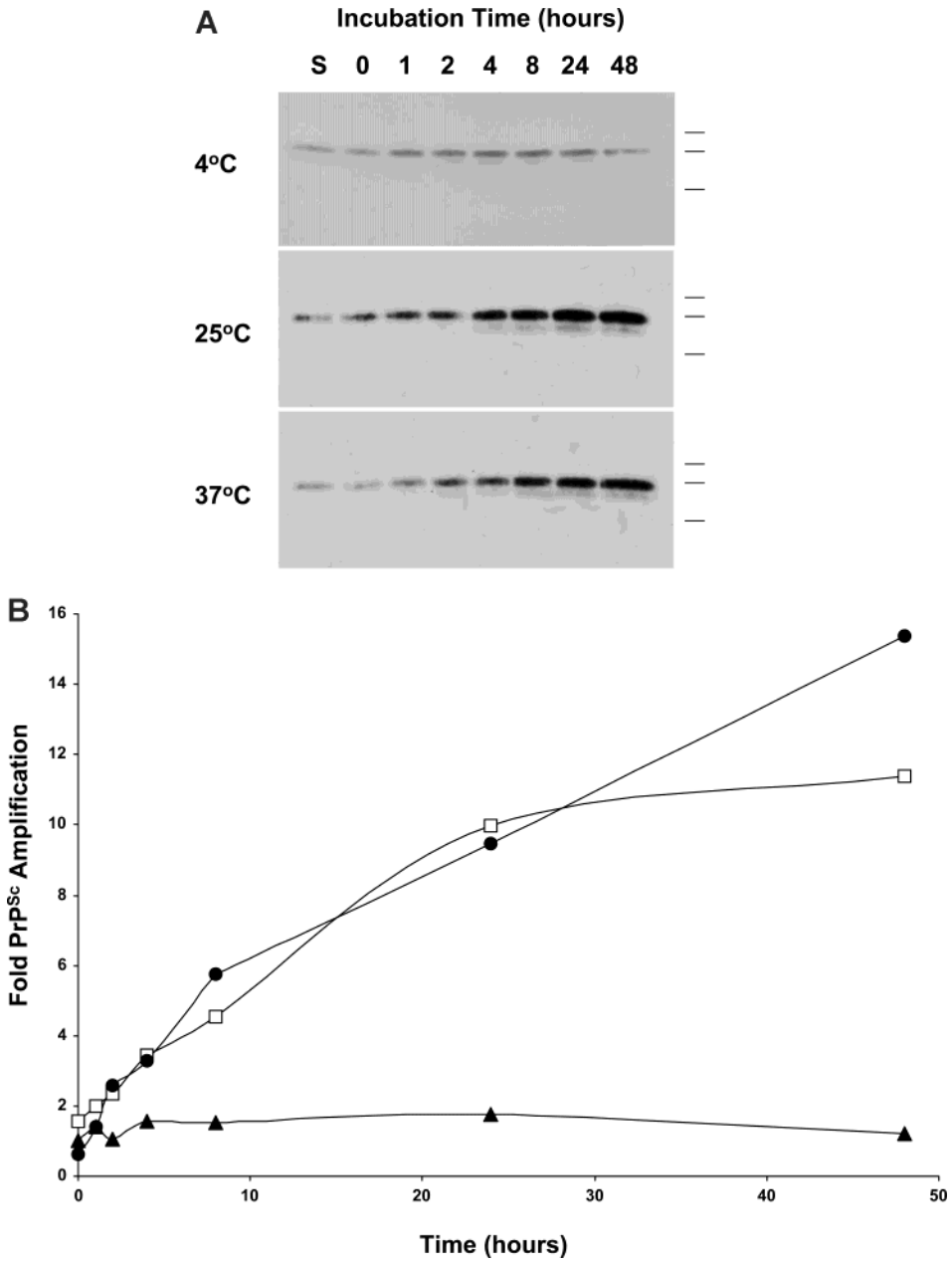

FIGURE 2: Effect of time and temperature on PrP^{Sc} amplification *in vitro*. (A) Normal hamster brain homogenate was mixed with diluted Sc237 scrapie brain homogenate and incubated at the indicated temperatures and time intervals. S represents the control sample containing diluted Sc237 brain homogenate alone. All samples were treated with proteinase K. (B) Graphic representation of densitometric measurements of blots presented in panel A: (▲) 4, (□) 25, and (●) 37 °C. Densitometric measurements that are shown represent the mean values from five individual scans. The values obtained at 25 and 37 °C are statistically different from the values obtained at 4 °C for time points after 8 h using the Student's T-test ($p < 0.001$).

pathological phenotypes (*18*, *19*). The ability of each strain to produce a unique disease phenotype is preserved upon serial passage. In some cases, PrP^{Sc} molecules from different strains can be distinguished biochemically. An example of a strain containing altered PrP^{Sc} molecules is drowsy, which was originally isolated from prion-infected mink and subsequently introduced into hamsters (*20*−*22*). Drowsy scrapie prions contain PrP^{Sc} molecules that migrate ∼2 kDa faster than other hamster scrapie strains, such as Sc237 and 139H, following proteinase K digestion. We used drowsy prions to determine whether PrP^{Sc} molecules amplified *in vitro* maintain strain-specific biochemical characteristics. Our results show that drowsy-amplified PrP^{Sc} molecules migrated ∼2 kDa faster than Sc237- or 139H-amplified PrP^{Sc} molecules when analyzed by SDS−polyacrylamide electrophoresis (Figure 3C). The ∼2 kDa difference in PrP^{Sc} migration patterns between drowsy-amplified samples and Sc237- and 139H-amplified samples was also observed after removal of N-linked oligosaccharides with PNGase F, indicating that the strain-specific difference in migration patterns was not caused by differences in PrP^{Sc} glycoform distribution (Figure 3C). These results indicate that the strain specificity of prion propagation *in vivo* is reproduced *in vitro* by our nondenaturing PrP^{Sc} amplification technique.

Pharmacological Specificity. To validate further the specificity of PrP^{Sc} amplification *in vitro*, we tested the abilities of several anti-prion compounds to inhibit PrP^{Sc} amplification *in vitro*. Previous work has shown that conversion of purified PrP^{C} to a protease-resistant state is blocked by Congo Red, but not by E64 or quinacrine (*23*, *24*). Those

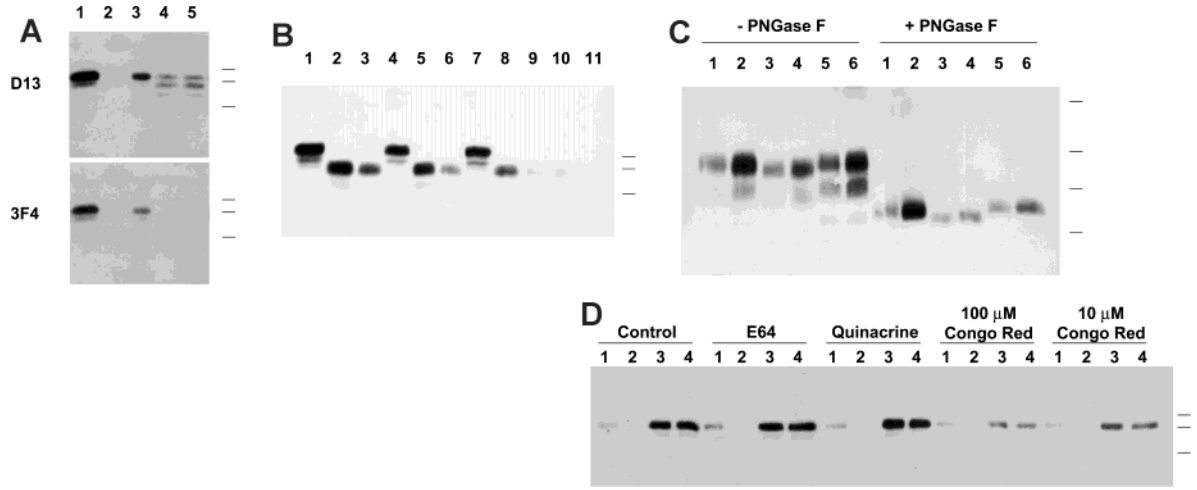

FIGURE 3: Specificity of PrP^{Sc} amplification *in vitro*. (A) Species barrier to PrP^{Sc} amplification *in vitro*. (1) Normal hamster brain homogenate mixed with diluted hamster Sc237 scrapie brain homogenate. (2) Normal hamster brain homogenate mixed with diluted $Prnp^{0/0}$ mouse brain homogenate. (3) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 scrapie brain homogenate. (4) $Prnp^{0/0}$ brain homogenate mixed with diluted mouse RML scrapie brain homogenate. (5) Normal hamster brain homogenate mixed with diluted mouse RML scrapie brain homogenate. Amplification reactions were carried out at pH 7, and all samples were treated with proteinase K. The PVDF membrane was developed first in D13, stripped, and then probed with primary antibody 3F4. (B) Species susceptibility to Sc237 PrP^{Sc} amplification. (1) Normal Syrian hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:50. (2) Normal Syrian hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:20. (3) Normal Syrian hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:50. (4) Normal Armenian hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:50. (5) Normal Armenian hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:20. (6) Normal Armenian hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:50. (7) Normal Chinese hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:50. (8) Normal Chinese hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:20. (9) Normal Chinese hamster brain homogenate mixed with Syrian hamster Sc237 scrapie brain homogenate diluted 1:50. (10) $Prnp^{0/0}$ brain homogenate mixed with Sc237 scrapie brain homogenate diluted 1:20. (11) $Prnp^{0/0}$ brain homogenate mixed with Sc237 scrapie brain homogenate diluted 1:50. Samples 1, 4, and 7 were undigested, and all other samples were treated with proteinase K. This blot was developed with primary Fab D13. (C) Strain-specific PrP^{Sc} amplification *in vitro.* Diluted hamster scrapie brain homogenates were mixed with either $Prnp^{0/0}$ brain homogenates (odd lanes) or normal hamster brain homogenate (even lanes) and amplified *in vitro*. Lane assignments of strains: (1 and 2) Sc237, (3 and 4) drowsy, and (5 and 6) 139H. All samples were treated with proteinase K. Following proteinase K digestion, half of each sample (50 μL) was removed, denatured, and treated with 1 unit of PNGase F (New England Biolabs) according to the manufacturer's protocol. Apparent molecular masses based on migration of prestained protein standards are 50, 37, 25, and 15 kDa. (D) Effect of anti-prion compounds on PrP^{Sc} amplification *in vitro*. (1) Diluted Sc237 scrapie brain homogenate. (2) Normal hamster brain homogenate. (3 and 4, duplicate samples) A mixture of the two homogenates incubated with 100 μM E64, 100 μM quinacrine sulfate, or Congo Red as indicated. All samples were digested with proteinase K.

results suggest that Congo Red interacts directly with PrP^{Sc} molecules whereas E64 and quinacrine may block PrP^{Sc} accumulation though indirect cellular mechanisms. We obtained similar results when we added these anti-prion compounds to PrP^{Sc} amplification reaction mixtures. Congo Red inhibited PrP^{Sc} amplification *in vitro* in a dose-dependent manner, whereas E64 and quinacrine did not block PrP^{Sc} amplification (Figure 3D).

Thiol Blockade Inhibits PrP^{Sc} Amplification in Vitro

To test the free sulfhydryl requirement of PrP^{Sc} amplification *in vitro*, we incubated reaction mixtures with 5 mM *N*-ethylmaleimide (NEM) at pH 6.0. At this pH value, NEM specifically forms covalent bonds with free thiolate groups, and does not reduce disulfide bonds (*25*). We found that 5 mM NEM completely inhibits PrP^{Sc} amplification (Figure 4A, top panel, lane 8). The IC_{50} for NEM-mediated inhibition of PrP^{Sc} amplification is ~1 mM (data not shown). Similar results were obtained with the mercurial compounds *p*-hydroxymercuribenzoic acid (PHMB) and mersalyl acid (Figure 4A, middle and bottom panels, lane 8). PHMB and mersalyl acid are highly specific, noncovalent inhibitors of free sulfhydryl groups and also do not reduce disulfide bonds (*25*). As a control to show that the thiol blocking agents are not inhibiting PrP^{Sc} amplification by reducing disulfide bonds, we found that two reducing agents, dithiothreitol (DTT) and β-mercaptoethanol (βME), inhibited PrP^{Sc} amplification with IC_{50} values of >3 mM (Figure 4B). Since DTT and β-ME have 100000-fold greater affinity for disulfide bonds than NEM, PHMB, and mersalyl acid (*25*), the inhibition of PrP^{Sc} amplification by thiol blocking agents cannot be explained by disulfide bond reduction in our system.

To demonstrate that NEM inhibits PrP^{Sc} amplification by covalently modifying a free thiolate group, we quenched the reactive maleimide group of 5 mM NEM with an equivalent concentration of homocysteine, a thiol-containing amino acid. As expected, quenched NEM did not inhibit PrP^{Sc} amplification (Figure 4C, lane 3).

Other investigators previously demonstrated that PrP^{C} is enriched in synaptic plasma membrane (SPM) fractions (*26*). To determine whether thiol blockade would inhibit PrP^{Sc} amplification in enriched membrane fractions, we prepared SPM from Syrian hamster brains using a variation of the protocol described previously (*26*), modified to avoid hypotonic lysis and protease digestion. Our results show that NEM, PHMB, and mersalyl acid all block PrP^{Sc} amplification in our SPM preparation (Figure 4D). Thus, the free sulfhydryl group required for PrP^{Sc} amplification must reside on either PrP^{C}, PrP^{Sc}, or another molecule present in our SPM

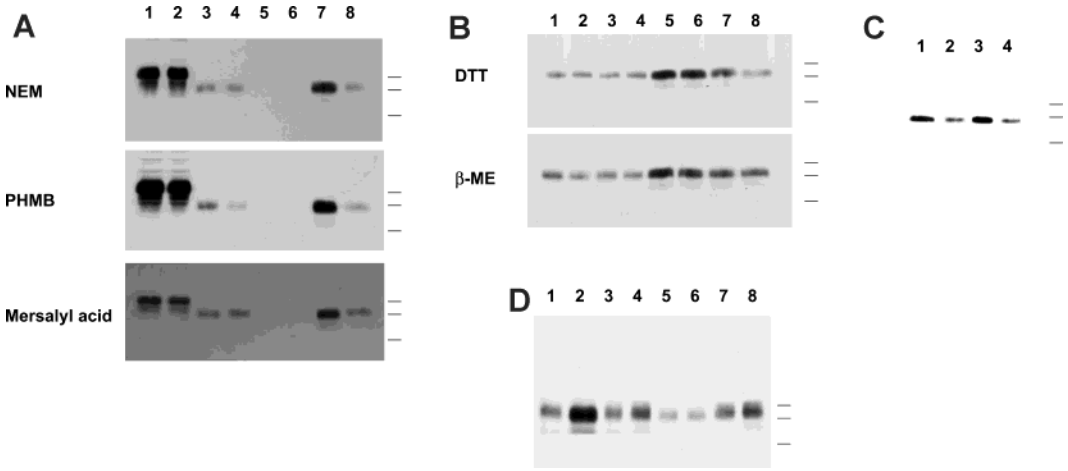

FIGURE 4: Effect of thiol blocking compounds on PrP^{Sc} amplification *in vitro*. (A) Effect of thiol blocking compounds on PrP^{Sc} amplification in crude membranes. Normal brain homogenates were centrifuged at 20800*g* for 10 min at 4 °C in a 5417C centrifuge (Eppendorf), and pellet fractions were resuspended in original volumes of buffer. For experiments with NEM, the pellet fractions were resuspended in PBS titrated pH 6.0 with Complete Protease Inhibitors, and for experiments involving PHMB and mersalyl acid, the pellet fractions were resuspended in PBS titrated to pH 8.0 with Complete Protease Inhibitors. Amplification reactions were performed at 37 °C for 16 h as described in Experimental Procedures. (1) Normal hamster brain homogenate not subjected to proteinase K digestion. (2) Normal hamster brain homogenate and 5 mM thiol-reactive compound not subjected to proteinase K digestion. (3) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate. (4) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 scrapie brain homogenate and 5 mM thiol-reactive compound. (5) Normal hamster brain homogenate mixed with diluted $Prnp^{0/0}$ brain homogenate. (6) Normal hamster brain homogenate mixed with diluted $Prnp^{0/0}$ brain homogenate and 5 mM thiol-reactive compound. (7) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate. (8) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate and 5 mM thiol-reactive compound. In lanes 3−8, samples were subjected to limited proteinase K digestion as described in Experimental Procedures. (B) Effect of reducing agents on PrP^{Sc} amplification *in vitro*. Amplification reactions were performed at 37 °C as described in Experimental Procedures with addition of dithiothreitol (DTT) or β-mercaptoethanol (β-ME). (1) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate. (2) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate and 1 mM reducing agent. (3) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate and 3 mM reducing agent. (4) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate and 10 mM reducing agent. (5) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate. (6) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate and 1 mM reducing agent. (7) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate and 3 mM reducing agent. (8) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate and 10 mM reducing agent. All samples were subjected to limited proteinase K digestion as described in Experimental Procedures. (C) Quenching of NEM by homocysteine. Normal brain homogenates were centrifuged at 20800*g* for 10 min at 4 °C in a 5417C centrifuge (Eppendorf), and pellet fractions were resuspended in original volumes of PBS titrated to pH 6.5 with Complete Protease Inhibitors. Amplification reactions were performed at 37 °C for 16 h as described in Experimental Procedures. (1) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate. (2) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate. (3) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate and 5 mM NEM and 5 mM homocysteine. (4) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate and 5 mM NEM. All samples were subjected to limited proteinase K digestion as described in Experimental Procedures. (D) Effect of thiol blocking compounds on isolated synaptic plasma membranes (SPM). Modified SPM fractions were prepared from Syrian hamster brains as described previously, except that Complete Protease Inhibitors were included in all buffers and the crude synaptasomal pellet was resuspended in 5 mM Tris (pH 7.5) and centrifuged immediately without incubation (*26*). Even-numbered lanes contained samples in which 150 μg of SPM protein was mixed with diluted Sc237 scrapie brain homogenate, and odd-numbered lanes contained samples with diluted Sc237 scrapie brain homogenate alone as input controls: (1 and 2) no compound added, (3 and 4) 5 mM NEM, (5 and 6) 5 mM PHMB, and (7 and 8) 5 mM mersalyl acid. All samples were amplified and subjected to limited proteinase K digestion as described in Experimental Procedures.

preparation. Furthermore, these results suggest that the essential free sulfhydryl-containing compound is unlikely to be a small, soluble molecule such as glutathione because such small molecules should have been removed by the fractionation procedure.

Testing the Disulfide Shuffling Hypothesis

Welker et al. (*27*) hypothesized that new PrP^{Sc} molecules might be formed through a process termed disulfide shuffling. A unique feature of this proposed mechanism is that PrP^{Sc} is hypothesized to contain a terminal free thiol group that is required for propagation (Figure 5, mechanism 4). We tested the disulfide shuffling hypothesis by pretreating normal or scrapie-infected brain homogenates with NEM prior to PrP^{Sc} amplification. Our results indicate that pretreatment of the normal brain homogenate with NEM (Figure 6A, lanes 3 and 4) abolishes PrP^{Sc} amplification *in vitro*, whereas pretreatment of the scrapie-infected brain homogenate with NEM did not have a significant effect on PrP^{Sc} amplification (data not shown).

As another test of the disulfide shuffling hypothesis, we investigated whether PrP^{Sc} formed by *in vitro* amplification contains intermolecular disulfide bonds. To do this, we prepared amplified and control PrP^{Sc} samples in a nonreducing loading buffer and looked for high-molecular mass multimers by SDS−PAGE. The data show that no multimers could be detected in amplified PrP^{Sc} samples prepared under nonreducing conditions (Figure 6B, lane 3). However, it is important to caution that this result alone cannot rule out the possibility that intramolecular disulfide bonds are destroyed by the process of disulfide shuffling (*27*) because we denatured our samples by boiling them in 1% SDS prior to electrophoresis.

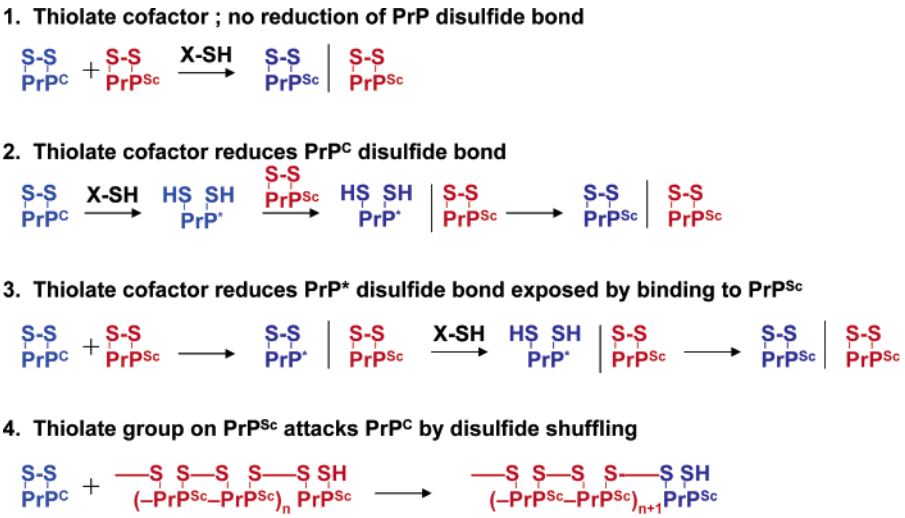

FIGURE 5: Hypothetical models for the location and mechanistic role of the free thiol group required for the conformational change from PrP^C to PrP^{Sc}.

DISCUSSION

In this paper, we describe and use a nondenaturing procedure refined from PMCA to characterize the amplification of PrP^{Sc} *in vitro*. The stoichiometric ratio of scrapie to normal brain homogenate that is added to each reaction mixture is 1:50. Acquisition of protease resistance by PrP^C is dependent on both time and temperature, and PrP^{Sc} amplification reaches levels of >10-fold at 25 °C over 2 days. Like prion propagation *in vivo*, nondenaturing PrP^{Sc} amplification *in vitro* exhibits species and strain specificity.

Using this procedure as a tool to investigate the mechanism of prion conversion, we demonstrated that blockade of free sulfhydryl groups inhibits PrP^{Sc} amplification *in vitro*. Several lines of evidence support our conclusion that thiol blockade functionally inhibits the conversion of PrP^C to PrP^{Sc} *in vitro*. (1) Three different selective thiol blockers inhibit PrP^{Sc} amplification under nondenaturing conditions (Figure 4A). (2) These selective thiol blockers also inhibit PrP^{Sc} amplification in a purified membrane preparation (Figure 4D). (3) Prequenching NEM with an equimolar concentration of homocysteine prevents inhibition (Figure 4C). (4) Pretreatment of normal brain homogenate with NEM inhibits subsequent PrP^{Sc} amplification (Figure 6A). The discovery that thiol blockers inhibit PrP^{Sc} amplification represents the first reported evidence that a reactive group is functionally required for PrP conformational change, and provides insight into the catalytic chemistry of that process. The amino acid sequence of PrP contains two cysteine groups at positions 179 and 214. Preparations of PrP^C and PrP^{Sc} isolated from hamster brain predominantly contain molecules with intramolecular disulfide bridges.

Currently, it is unknown whether the disulfide bond of PrP^C must be broken during the process of conformational change to PrP^{Sc}. Denaturation of purified preparations of PrP^{Sc} aggregates by guanidinium chloride releases PrP monomers with an intramolecular disulfide bond (*6*). To explain this observation, some investigators have suggested that PrP conformational change occurs without reduction of the disulfide bond (*28*), and others have hypothesized that PrP^{Sc} contains intermolecular disulfide bonds, which undergo disulfide shuffling during denaturation (*27*, *29*, *30*). Reduction of recombinant PrP polypeptides causes those molecules to shift conformation from predominantly α-helical to predominantly *β*-sheet (*31*−*33*). Thus, it is also possible that reduction and re-formation of the intramolecular disulfide bond occur during the conformational change from PrP^C to PrP^{Sc}.

We believe that at least four different hypothetical mechanisms could explain the thiol requirement for PrP conformational change (Figure 5). In mechanism 1, PrP intramolecular disulfide bond breakage does not occur when PrP^C is converted into PrP^{Sc}, but a cofactor "X" with an active site sulfhydryl group catalyzes the conformational change. In mechanism 2, reduction of the PrP intramolecular disulfide bond is needed before PrP^C can unfold into an intermediate conformation capable of interacting with PrP^{Sc}. In mechanism 3, binding of PrP^C to PrP^{Sc} causes partial unfolding of PrP^C, exposing the intramolecular disulfide bond to reduction. Reduction of the exposed disulfide bond then allows bound, partially unfolded PrP^C to complete the conformational change into PrP^{Sc}. In mechanism 4, the disulfide shuffling hypothesis described by Welker (*27*) proposes that native PrP^{Sc} is a polymer in which PrP monomers are covalently joined by intermolecular disulfide bonds. New PrP^C molecules are incorporated into the elongating PrP^{Sc} polymer when a terminal free sulfhydryl group on PrP^{Sc} attacks the PrP^C disulfide bond.

Recently, other investigators reported that *in vitro* conversion of purified, radiolabeled PrP^C to a protease-resistant state was not inhibited by 50 mM NEM or 70 mM 2-(aminoethyl)-methane thiosulfonate (AEMTS) (*34*). Those results argue against the disulfide shuffling hypothesis (Figure 5, mechanism 4). Our findings that pretreatment of normal brain homogenate with NEM inhibits PrP^{Sc} amplification (Figure 6A) and that disulfide-bonded multimers could not be detected in amplified PrP^{Sc} samples (Figure 6B) provide additional evidence that disulfide shuffling is an unlikely mechanism of PrP^{Sc} formation. Because PrP^C molecules do not contain free thiol groups (*6*), the remaining possible mechanisms of PrP^{Sc} formation are consistent with the existence of a novel thiol-containing cofactor required for PrP conversion (Figure 5, mechanisms 1−3). The eventual identification of this novel cofactor by biochemical techniques will be of interest.

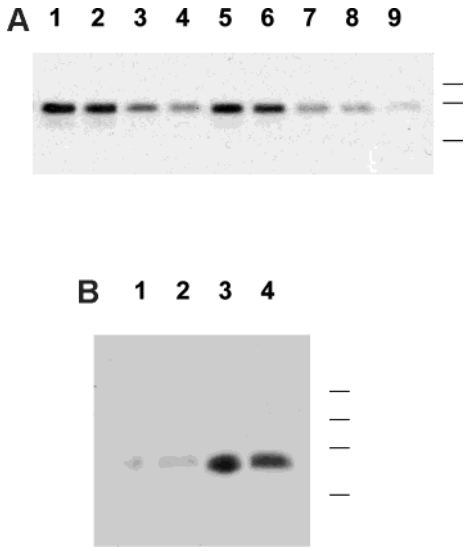

FIGURE 6: Experiments testing the disulfide shuffling hypothesis. (A) Effect of NEM pretreatment on PrP^{Sc} amplification *in vitro*. All samples were amplified for 16 h at 37 °C and subjected to limited proteinase K digestion as described in Experimental Procedures. (1−4) Normal Syrian hamster brain homogenates in PBS (pH 7.0) were pretreated for 3 h at 25 °C with (1 and 2) vehicle control or (3 and 4) 15 mM NEM and centrifuged at 20800*g* for 10 min at 4 °C, and pellet fractions were resuspended in original volumes of PBS and mixed with an equal volume of Sc237 scrapie brain homogenate diluted into PBS and 1% Triton. (5−8) As inhibition controls, normal Syrian hamster brain homogenates were incubated with diluted Sc237 scrapie brain homogenate in PBS with 0.5% Triton X-100 and (5 and 6) vehicle control or (7 and 8) 7.5 mM NEM for 3 h at 25 °C and then centrifuged at 20800*g* for 10 min at 4 °C. Pellet fractions were then resuspended in original volumes of PBS and 0.5% Triton X-100, and amplification was continued overnight. In even-numbered lanes, the normal brain homogenate was centrifuged at 20800*g* for 10 min at 4 °C and the pellet was resuspended in the appropriate buffer at the beginning of the experiment. (9) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate. (B) Effect of reducing conditions on PrP^{Sc} mobility in SDS−PAGE. All samples were amplified for 16 h at 37 °C and subjected to limited proteinase K digestion as described in Experimental Procedures. (1) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate, with electrophoresis samples boiled in SDS loading buffer without reducing agents. (2) $Prnp^{0/0}$ brain homogenate mixed with diluted Sc237 brain homogenate, with electrophoresis samples boiled in SDS loading buffer with β-mercaptoethanol. (3) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate, with electrophoresis samples boiled in SDS loading buffer without reducing agents. (4) Normal hamster brain homogenate mixed with diluted Sc237 scrapie brain homogenate, with electrophoresis samples boiled in SDS loading buffer with β-mercaptoethanol. Apparent molecular masses based on the migration of prestained protein standards are 75, 50, 37, and 25 kDa.

We found that pH values between 6 and 8 were optimal for PrP^{Sc} amplification, depending on the animal species that was analyzed (Figure 1B). This determination suggests that the pathogenic conformational change from PrP^{C} to PrP^{Sc} would occur most efficiently in cellular locations with nearly neutral pH environments, such as on the extracellular surface of the plasma membrane (*35*) or within the cytoplasm (*36*). It is unlikely that the pH optimum of PrP^{Sc} amplification reflects titration of the required thiolate anion because the pK_a for thiol groups in aqueous solutions is 8.3.

Our results also indicated that divalent cation chelation did not affect PrP^{Sc} amplification *in vitro* (Figure 1C). This finding argues that divalent ions are not absolutely necessary for generation of PrP^{Sc} from PrP^{C}, and therefore do not participate directly in the conversion process *in vitro*. However, our observations do not rule out an important role for divalent cations in determining PrP^{Sc} conformation. Other investigators have found that, in some experiments, copper assists refolding of denatured PrP^{Sc} into infectious prions (*37*). Also, chelation of divalent cations alters strain-specific biochemical characteristics of PrP^{Sc} molecules in CJD-infected brains (*38*). Thus, binding of metal ions such as copper and manganese to PrP^{Sc} may influence its conformation and generate different prion strains.

In summary, by modifying the PMCA procedure originally developed by Saborio and Soto (*11*), we have demonstrated that PrP^{Sc} can be specifically amplified *in vitro* under nondenaturing conditions, which more closely resemble the native state. Using this technique, we have characterized several fundamental aspects of the PrP^{Sc} formation process *in vitro*: (1) retention of species, strain, and pharmacological specificity, (2) pH optimum between 6 and 8, (3) time and temperature dependence, (4) lack of dependence on divalent cations, and (5) requirement for a free thiol-containing factor.

The requirement of a free thiol group in the pathogenic conformational change from PrP^{C} to PrP^{Sc} is a particularly significant discovery. It is, to our knowledge, the first evidence that a reactive chemical group is necessary for PrP^{Sc} formation. Reactive chemical groups represent novel and potentially attractive therapeutic targets in prion disease, and deciphering the mechanism by which the essential sulfhydryl group participates in PrP conformational change will be of considerable interest.

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