The Anti-prion Activity of Congo Red

PUTATIVE MECHANISM*

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PrPSc, an abnormal conformational isoform of the normal prion protein, PrPc, is the only known component of the prion, a proteaceous agent that causes fatal neurodegenerative disorders in humans and other animals. The hallmark properties of PrPSc are its insolubility in non-denaturing detergents and its resistance to digestion by proteases. Anions such as Congo red (CR) have been shown to reduce the accumulation of PrPSc in a neuroblastoma cell line permanently infected with prions as well as to delay disease onset in rodents when administrated prophylactically. The mechanism by which such anti-prion agents operate is unknown. We show here that in vitro incubation with CR renders native PrPSc resistant to denaturation by boiling SDS. This resulted from PrPSc conformation, since neither the properties of PrPc nor those of predenatured PrPSc were changed by the addition of CR. CR-PrPSc could only be denatured by the addition of acidic 3 m guanidine thiocyanate. Since in vitro conversion experiments have suggested that partial denaturation may be required for PrPSc to serve as template in the PrPc → PrPSc conversion, we propose that CR inhibits prion propagation by overstabilizing the conformation of PrPSc molecules.

Prion diseases, such as Creutzfeldt-Jakob disease in humans or scrapie and bovine spongiform encephalopathies in animals, are fatal transmissible neurodegenerative diseases (1). The only component of the prion is PrPSc, an abnormal conformer of the host encoded PrPc. Both PrP isoforms share the same amino acid sequence, but, as opposed to PrPc, PrPSc is relatively resistant to digestion by proteases and insoluble in non-denaturing detergents (2). Unlike PrPc, which is mainly α-helical, PrPSc contains a considerable amount of β-sheet structures (3–5). Both isoforms are localized in cholesterol-rich membrane microdomains called rafts or caveolae-like domains (6–8). The cell biology of prion diseases as well as the metabolism of the prion protein isoforms, have been investigated in systems including prion diseases (26). In fact, its binding to protein aggregates is considered diagnostic of an amyloidogenic protein.

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1 The abbreviations used are: CR, Congo red; PK, proteinase K; Ab, antibody; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; GndSCN, guanidine thiocyanate; DLPC, detergent-lipid protein complexes.
genic process (27). Our results show that such an interaction of CR with the prion isoform exists and results in overstabilization of the PrPSc molecules, as can be seen by their increased resistance to denaturation. If so, CR inhibition of prion propagation may result from the inability of CR-PrPSc to convert into the partially denatured molecules required as template in the PrPSc → PrPSc reaction.

EXPERIMENTAL PROCEDURES

Chemicals and Tissue Culture Reagents—Congo red (98%) purchased from Aldrich was dissolved in distilled water and sterilized before use. Reagents for cell culture were from Biological Industries (Bat Haemek, Israel). G418 was from Calbiochem. α-t-Phosphatidylcholine was obtained from Avanti Biochemicals. Secondary antibodies were either from Promega (Madison, WI) or Jackson Immunoresearch (West Grove, PA). All other reagents were from Sigma.

Rabbit antiserum RO73 (10, 28) as well as monoclonal antibodies 3F4 (29, 30) and 13AS (31) were used for immunoblotting. Antibodies were used at a dilution of 1:3000 or 1:1000, respectively (of the serum or the ascitic fluid).

Cell Culture—ScN2a-c10 cells are scrapie-infected neuroblastoma cells that express the MMH2-Prp chimeric gene (30) driven by the commercial expression vector pCI-neo (Promega). The cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, glutamine, penicillin/streptomycin, and 1 mg/ml G418. ScN2a-c10 Cells Cultured with CR and Tunicamycin—Identically seeded and confluent ScN2a-c10 cells were cultured in 10-cm plates (about 10⁷ cells) and incubated for 16, 24, or 48 h in the presence of either 50 µg/ml CR, 1.5 µg/ml tunicamycin, or both. Cells were extracted in 1 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40. The samples were centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was concentrated by methanol precipitation and digested with 40 µg/ml proteinase K (PK) for 20 min at 37 °C. 3M acidic GndSCN was added to half of each sample before boiling in SDS sample buffer and immunoblotting with anti-PrP Ab 3F4.

Incubation of Syrian Hamster Brain Membranes with CR.—Normal and scrapie-infected Syrian hamster brains were kindly provided by Dr. S. B. Prusiner from the University of California, San Francisco. Membranes were prepared as follows. Brain (1 g) was homogenized in 10 ml of cold sucrose buffer (0.3 M sucrose, 10 mM Tris-HCl, pH 7.5) on ice. The homogenate was centrifuged at 2000 rpm for 15 min, and the supernatant was subsequently supplemented with 40 ml of sucrose buffer and centrifuged at 20,000 rpm for 30 min at 4 °C. The pellet was resuspended in 40 ml of cold 10 mM Tris-HCl, pH 7.5, and centrifuged again at 20,000 rpm for 30 min. The final pellet was resuspended to a concentration of 5 mg/ml in STES buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 2% Sarkosyl).

Normal and scrapie-infected hamster brain membranes were incubated with increasing concentrations of CR for 1 h at room temperature. Scrapie samples were digested with 40 µg/ml PK for 20 min at 37 °C, and all samples were boiled in SDS sample buffer before immunoblotting with mAb 3F4. The percentage of acrylamide for SDS-PAGE (10% for the scrapie samples or 12% for the normal samples) was determined to avoid comigration of the PrP band with the dye.

Incubation of Prion Rods with CR.—Syrian hamster prion rods (PrP-(27–30)) purified by the sucrose gradient method (32) were kindly provided by Dr. S. B. Prusiner. PrP-(27–30) was diluted to 1 mg/ml PK for 20 min at 37 °C, and all samples were boiled in SDS sample buffer before immunoblotting with mAb 3F4. Purified PrP-(27–30) was incubated with CR (as described under “Experimental Procedures”) and subsequently boiled in SDS sample buffer before immunoblotting.

RESULTS

CR Renders PrPSc Resistant to Denaturation by Boiling SDS—To investigate whether direct interaction of PrPSc with CR may result in inhibition of prion propagation, we tested the biochemical properties of the PrP proteins after incubation of membranes from normal or scrapie-infected hamster brains with CR. The hallmark property of PrPSc is its protease resistance. While PrPSc is highly sensitive to proteases, digestion of PrPSc with PK results in the protease-resistant core of PrPSc (34). To test whether incubation of PrPSc with CR will render this protein sensitive to PK, normal and scrapie brain samples were incubated with increasing concentrations of CR. Scrapie-infected hamster samples presumably contain both PrP isoforms, as opposed to normal brain membranes that contained only PrPSc (35). To differentiate the protease-sensitive PrPSc from protease-resistant PrPSc, the scrapie-infected brain samples (CR-treated and controls) were digested with PK (see “Experimental Procedures”) prior to PAGE analysis. All samples were subsequently boiled in SDS sample buffer and processed for immunoblotting. While the PrPSc pattern on the gel carrying only normal brain samples was not affected by the preincubation with CR (Fig. 1A), the presence of PrP-(27–30) on the immunoblot loaded with samples from scrapie-infected brains was reduced in a dose-dependent manner (Fig. 1B). These results suggested the intriguing possibility that direct incubation of the scrapie isoform with CR in vitro is enough to render PrPSc sensitive to digestion by proteases.

This attractive possibility, however, was discarded after the same experiments were repeated with purified PrP-(27–30). Purified PrP-(27–30) was incubated with CR (as described under “Experimental Procedures”) and subsequently boiled in SDS sample buffer before immunoblotting (Fig. 2A). As mem-

![FIG. 1. In vitro preincubation with CR differentiates PrPSc from PrPSc. Membranes from normal (A) and scrapie-infected (B) hamster brains (35) were digested to 5 mg/ml protein with STES buffer and incubated with increasing concentrations of CR for 1 h at room temperature. After the incubation, scrapie samples were digested with 40 µg/ml PK for 20 min at 37 °C. Samples were boiled in SDS sample buffer before immunoblotting with mAb 3F4 (29). The percentage of acrylamide for SDS page (10% for the scrapie samples or 12% for the normal samples) was determined to avoid comigration of the PrP band with the dye.](http://www.jbc.org/)

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CR renders purified PrP-(27–30) and membranal PrPSc resistant to denaturation by SDS boiling but not to denaturation by acidic guanidine. A, purified PrP-(27–30) diluted in STES buffer was incubated with increasing concentration of CR for 1 h at room temperature. Half the samples were then incubated for 2 min with 3 μM GdnSCN, pH 2–2.5 (lower part), and concentrated by methanol precipitation prior to PAGE analysis and immunoblotting. B, purified PrP-(27–30) was incubated as in A in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 200 μg/ml CR. After the incubation, all samples were digested with 20 μg/ml PK for 30 min at 37 °C. Prior to the PK digestion, samples 2 and 4 were boiled for 10 min in the presence of 1% SDS and then diluted 10-fold with STES buffer. Samples in the lower part were treated with 3 μM acidic GdnSCN and concentrated by methanol precipitation prior to SDS-PAGE. All samples were immunoblotted with anti-PrP mAb 3F4.

Fig. 2. CR renders purified PrP-(27–30) and membranal PrPSc resistant to denaturation by SDS boiling but not to denaturation by acidic guanidine. A, purified PrP-(27–30) diluted in STES buffer was incubated with increasing concentration of CR for 1 h at room temperature. Half the samples were then incubated for 2 min with 3 μM GdnSCN, pH 2–2.5 (lower part), and concentrated by methanol precipitation prior to PAGE analysis and immunoblotting. B, purified PrP-(27–30) was incubated as in A in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 200 μg/ml CR. After the incubation, all samples were digested with 20 μg/ml PK for 30 min at 37 °C. Prior to the PK digestion, samples 2 and 4 were boiled for 10 min in the presence of 1% SDS and then diluted 10-fold with STES buffer. Samples in the lower part were treated with 3 μM acidic GdnSCN and concentrated by methanol precipitation prior to SDS-PAGE. All samples were immunoblotted with anti-PrP mAb 3F4.

Fig. 3. CR does not affect the properties of predenatured PrPSc. A, purified PrP-(27–30) was incubated in the presence or absence of 3 M GdnSCN followed by methanol precipitation. The precipitated samples were treated with and without CR as described in Fig. 2B (500 μg/ml CR) and subsequently immunoblotted with mAb 3F4. B, PrP-(27–30) was boiled in the presence of SDS for 5 min and subsequently diluted before incubation in the presence of 0, 100, or 500 μg/ml CR. All samples were immunoblotted with mAb 3F4. C, PrP-(27–30) was incubated in the presence and absence of 200 μg/ml CR and applied to SDS-PAGE after boiling in SDS sample buffer.

SDS-boiled PrP-(27–30) untreated with CR (Fig. 2B). CR-treated PrP-(27–30) was boiled in 1% SDS for 10 min and subsequently digested with 20 μg/ml PK at 37 °C for 30 min after a 10-fold dilution of the SDS. As opposed to untreated PrP-(27–30), which was converted into protease-sensitive form by boiling SDS; CR-treated PrP-(27–30) was resistant to PK digestion even at these conditions, reinforcing the conclusion that CR renders PrP-(27–30) resistant to denaturation by established procedures such as boiling in the presence of SDS (Fig. 2B, +). That CR does not inhibit the activity of PK can be inferred from Fig. 1, since PrPSc was completely digested to PrP-(27–30) in the presence of high CR concentrations. As in Fig. 2A, the presence of the scrapie protein on the gel could be detected only after acidic GdnSCN was added to the sample buffer (Fig. 2B, –). Interestingly, acidic GdnSCN revealed some PK-resistant PrP-(27–30) also in the SDS-boiled control samples, suggesting that PrP-(27–30) rods comprise a core of very stable PrP-(27–30) even before the addition of CR.

CR Does Not Interfere with the Dispersion of PrP-(27–30) into DLPC—In addition to protease resistance, another hallmark property of the scrapie prion protein is its insolubility in non-denaturing detergents (Fig. 4A). The insolubility of PrPSc has been utilized in the protocol developed for its purification as PrP-(27–30) rods (37). Interestingly, as opposed to its behavior in many detergents, the insoluble rods could be dispersed into DLPC (33). When PrP-(27–30) in prion rods was sonicated in the presence of phosphatidylcholine and 2% cholate and subsequently centrifuged at high speed, most of the PrP remained in the supernatant, as opposed to the samples without lipids, which are pelleted at these conditions (Fig. 4B). Although it is difficult to conclude whether PrP-(27–30) is completely solubilized to monomers by this procedure, it is obviously more dispersed than in rods and, as a result of this, more infectious (38).

To investigate whether CR competes with lipids for binding sites on PrPSc, we tested whether CR-PrP-(27–30) could also be incorporated into DLPC. As can be seen in Fig. 4B, when CR-treated PrP-(27–30) was dispersed into DLPC by the same protocol used for control PrP, most of the CR-treated protein remained in the supernatant. As in the previous experiments, this could be visualized only after the addition of acidic GdnSCN to the samples prior to their analysis by PAGE (Fig. 4B).

Moreover, PrP-(27–30) in DLPC was still susceptible to CR, since acidic GdnSCN was required to visualize on the immunoblot PrP-(27–30) to which CR was applied after the disper-
CR Inhibited New PrPSc Synthesis and Old PrPSc Degradation—The best cell culture model developed to date for prion infection is the ScN2a system. These scrapie-infected mouse neuroblastoma cells produce PrPSc as well as infectivity (11, 40). When ScN2a-c10 cells were cultured in the presence of CR, new PrPSc was synthesized and subsequently accumulated (41). We tested the fate of PrPSc in these cells to resolve whether in previous experiments some of the “old” scrapie protein may not have been accounted for because of the insufficient denaturation that results from the addition of CR. ScN2a-c10 cells, a ScN2a clone expressing a chimeric mouse/hamster PrP (47), were incubated with and without CR and in the presence or absence of tunicamycin, a glycosylation inhibitor, for 18, 30, or 48 h. Cell lysates were then digested with 20 μg/ml PK for 30 min at 37 °C. In the presence of tunicamycin, new PrP is synthesized as an unglycosylated protease-resistant protein (42), while fully glycosylated PrPSc, higher in Mr, present prior to the addition of tunicamycin is degraded very slowly. Such an experiment is shown in Fig. 6. In the absence of CR, fully glycosylated “old” PrP (upper band of the three that comprise the PrP immunoblott profile) was partially degraded after 48 h in tunicamycin, while the lower band signal (representing unglycosylated PrP) was considerably enhanced as compared with the signal obtained before the addition of tunicamycin. When tunicamycin was added to the cells incubated in the presence of CR, no new protease-resistant PrP was synthesized, as seen by the fact that there was no increase in the signal of the low molecular weight band. In effect, no difference at all could be appreciated in the banding pattern of PK-resistant PrP in the presence or absence of tunicamycin. These results suggest that no new PrPSc was made by ScN2a-c10 cells in the presence of CR, although the concentration of the old PrPSc remained high. As with the previous experiments performed on membranes or purified PrP-(27–30), the total concentration of PrPSc in the CR-treated cells could be visualized only when acidic GdnSCN was added to the denaturing sample buffer.

**DISCUSSION**

Prions appear to propagate by refolding mature PrPSc, a membrane glycoprotein of unknown function, into an isofrom of aberrant conformation denominated PrPot. This refolded protein is the only known component of the prion. Thus, the two isoforms of PrP serve, at the same time, as substrate and template in the conversion process that results in the accumulation of more prions. During the conversion, PrPSc somehow confers to PrPot its own and distinct structural conformation, which is extremely stable and comprises large β-sheet segments (5). Although the very nature of the conversion process is unknown, *in vitro* studies have suggested that partial denaturation of PrPSc may be a crucial requirement for this molecule to act as a template in the further conformational conversion of PrPSc (43). In addition, theoretical considerations also suggested the involvement of a partially denatured molten globule in the formation of PrPSc (44). The requirement of the chaperon HSP104 for the propagation of the prion like Ψ of yeast also suggested that template “breathing” may feature in the propagation of conformers (45). Therefore, it is possible that reagents that stabilize PrPSc so that it cannot be partially denatured will inhibit prion propagation.
The conversion of PrPC. This is probably why it has been very metabolically stable PrP will be less infectious, since it cannot

Conversely, it is possible that highly aggregated as well as than the same sample in the more aggregated form (33, 46).

Indeed, PrP-(27–30) in DLPC, a more dispersed form of PrP, 

Whether the anti-prion effect of CR is caused by stabilizing the scrapie conformation only or also by blocking the binding of PrPSc molecules to conversion sites, the fact that direct interaction of CR with PrPSc inhibits new PrPSc synthesis reinforces the notion that PrPSc is probably the crucial if not the only 

What molecules in the cell, in addition to PrPSc, participate in the conversion reaction? Several molecules have been shown to either bind to PrP or be altered in scrapie-infected cells or brains (49–52). Experiments with transgenic mice have postulated the existence of a species-specific factor, denominated Protein X, which is yet to be identified (15). The presence of glycosaminoglycan molecules such as heparan sulfate in every amyloid was proposed as evidence of their possible role in the scrapification of PrP (25). However, the fact that PrPSc, although at a low efficiency, can be converted in vitro to a PrPSc- like molecule (43), suggests that such molecules may be important mostly for the kinetics of the reaction or for the pathogenesis of the prion disease. As proposed above, it is the very nature of PrPSc conformation that probably hinders the finding of such molecules.

Elucidating the diverse mechanisms by which the known anti-prion agents operate may help in understanding the biology of prion diseases and may also be a crucial step for the preparation of reagents that can be used in vivo experiments and clinical trials. Some of them will eventually also be useful for the treatment of other amyloid disorders.

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Fig. 6. CR presence inhibits new PrPSc synthesis in Scn2a-c10 cells. Scn2a-c10 cells were cultured in the absence or presence of 50 μg/ml CR and in the absence or presence of 1.5 μg/ml tunicamycin for 16, 24, and 48 h. After extraction in 1% Nonidet P-40 lysis buffer, cell samples were digested with 40 μg/ml PK for 20 min at 37 °C. Half of each sample was denatured with 3 μl acidic GndSCN before boiling in SDS sample buffer and immunoblotting with the anti-PrP Ab 3F4.

Our results show that CR can act directly on PrPSc, even in vitro, and that its effect can be evaluated in a simple assay. Since reagents such as CR inhibit further propagation of prions, they must interfere with one or more steps in the conversion process. While other anti-prion agents may disrupt the PrPSc conformation and render this protein protease-sensitive, as expected from an anti-prion agent, CR seems to reinforce the template PrPSc structure and, as a result of this, inhibit its denaturation. In view of this, we suggest that CR may hinder prion propagation and PrPSc formation by stabilizing the template PrPSc so that the partial denaturation needed for the conversion of additional PrPSc molecules is prevented.

The effect of CR on PrPSc is not a function of the PrP sequence or its glycosylation profile but rather of its conformation. We conclude this from the fact that neither PrPSc (Fig. 1) nor predenatured PrPSc (Fig. 3) changes its basic properties when incubated in the presence of CR. It has been shown in the past that CR does not inhibit the synthesis of PrPSc (24).

CR association abolished the affinity of PrP-(27–30) to its antibodies. This affinity, very poor in purified PrP rods, was significantly enhanced only after PrP-(27–30) dispersion into DLPC. In addition, the comparison of the lines in Fig. 6 with and without guanidine denaturation suggests that inhibition of synthesis of new PrPSc by CR occurs even in the presence of the PrPSc molecules that were present in the cells before the addition of CR.

Our results are consistent with yet another plausible conclusion. Since in the presence of CR, PrPSc seems to be “invisible” to either antibodies or metabolic cell components, it may also be invisible to components of the PrPSc → PrPSc conversion site. Whether this effect is due to direct inhibition of binding by CR or is also a result of the increased stability conferred to PrPSc by CR remains to be established.

Scrapie incubation time may be determined not only by the concentration of PrPSc in the inoculum, but also by the number of PrPSc molecules available for binding to conversion sites. Indeed, PrP-(27–30) in DLPC, a more dispersed form of PrP, has been shown to be more infectious, by a factor of 10 or 100, than the same sample in the more aggregated form (33, 46).

Conversely, it is possible that high aggregation as well as metabolically stable PrP will be less infectious, since it cannot interact efficiently with the crucial cell components needed for the conversion of PrPSc. This is probably why it has been very difficult to correlate PrPSc concentrations with infectivity titer (47). As stated above, Scn2a cells did not produce new PrPSc in the presence of CR, although acidic guanidinium revealed the presence of large concentrations of very stable PrPSc.

The results presented here suggest an interesting feature of transmissible amyloid diseases. While it is necessary for the aberrant isofrom of the amyloidogenic protein to be stable enough to metabolic degradation, so that it can be present long enough in the cell to transform many normal molecules, an overstabilized aberrant conformation will inhibit the partial denaturation required for template formation. Interestingly, the amyloid aggregates from a nontransmissible brain neurodegenerative disorder, Alzheimer’s disease, cannot be denatured by SDS boiling but by much harsher conditions such as 6 M urea (48).

Whether the anti-prion effect of CR is caused by stabilizing the scrapie conformation only or also by blocking the binding of PrPSc molecules to conversion sites, the fact that direct interaction of CR with PrPSc inhibits new PrPSc synthesis reinforces the notion that PrPSc is probably the crucial if not the only prion component.
Anti-prion Activity of Congo Red

3489

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