Surface charge of polyoxometalates modulates polymerization of the scrapie prion protein

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Prions are composed solely of an alternatively folded isoform of the prion protein (PrP), designated PrPSc. N-terminally truncated PrPSc, denoted PrP 27–30, retains infectivity and polymerizes into rods with the ultrastructural and tinctorial properties of amyloid. We report here that some polyoxometalates (POMs) favor polymerization of PrP 27–30 into prion rods, whereas other POMs promote assembly of the protein into 2D crystals. Antibodies reactivating with epitopes in denatured PrP 27–30 also bound to 2D crystals treated with 3 M urea. These same antibodies did not bind to either native PrPSc or untreated 2D crystals. By using small, spherical POMs with Keggin-type structures, the central heteroatom was found to determine whether prion rods or 2D crystals were preferentially formed. An example of a Keggin-type POM with a phosphorous heteroatom is the phosphotungstate anion (PTA). Both PTA and a Keggin-type POM with a silicon heteroatom have low charge densities and favor formation of prion rods. In contrast, POMs with boron or hydrogen heteroatoms exhibiting higher negative charges encouraged 2D crystal formation. The 2D crystals of PrP 27–30 produced by selective precipitation by using POMs were larger and more well ordered than those obtained by protocols employing sucrose gradient centrifugation. Our findings argue that the negative charge of Keggin-type POMs determines the quaternary structure adopted by PrP.

Results

2D Crystals Composed of PrP 27–30. In fractions purified by sucrose gradient centrifugation, we initially found numerous rods composed of PrP 27–30 (5). Subsequently, we identified 2D crystals in these same fractions (Fig. 14) (11, 12). Because these fractions contained primarily one protein by silver staining, we concluded that both the rods and 2D crystals were composed of PrP 27–30 (Fig. 1D). Using Keggin-type POMs, we found larger and more ordered 2D crystals (Fig. 1 B and C) than previously observed in the sucrose gradient preparations. As shown by silver staining and Western blot analysis, the crystals appear to be composed of PrP 27–30 (Fig. 1E). The silver-stained bands from the PTA precipitate are primarily N-terminally truncated glycoforms of PrPSc (Fig. 1E, lane 3). When the ammonium salt of the POM HTA (NH3)8[H2W12O40] was used to precipitate PrP 27–30, the proteolysis catalyzed by proteinase K, which digested the precursor PrPSc (15, 16). During the development of an improved immunnoassay, we found that salts of the phosphotungstate anions (PTA; Na2H[PW12O40]) selectively precipitate PrPSc (17).

We report here that PTA precipitation can be used in place of sucrose gradient centrifugation for the preparation of 2D crystals of PrP 27–30 and that a series of compounds with structures similar to PTA (18) controls the quaternary structure of PrP 27–30. By using small, spherical POMs with Keggin-type structures (19), the central heteroatom was found to determine whether prion rods or 2D crystals were preferentially formed. Both PTA and a Keggin-type POM with a silicon heteroatom have low charge densities and favor formation of prion rods. In contrast, POMs with boron or hydrogen heteroatoms exhibiting higher negative charges encouraged 2D crystal formation. The 2D crystals of PrP 27–30 produced by selective precipitation by using POMs were larger and more well ordered than those obtained by protocols employing sucrose gradient centrifugation. Our findings argue that the negative charge of Keggin-type POMs determines the quaternary structure adopted by PrP. Large and highly organized crystals are essential for high-resolution analyses by cryo low-dose electron crystallography. Whether POMs can be used to create well-ordered 3D crystals amenable to atomic structure determinations is unknown.
Fig. 1. Two-dimensional crystals of PrP 27-30 prepared by different purification protocols: traditional sucrose-gradient procedure (10) (A), precipitation with PTA (B), and precipitation with HTA (C). The 2D crystals from the sucrose-gradient procedure tended to be smaller and contained more defects than those obtained by POM precipitation. For all 3 preparations, the lattice parameters were essentially identical (a and b ~ 6.9 nm; γ ~ 120°) and as previously determined (11, 12). (Scale bar: A, 100 nm; applies to all micrographs.) (D) and (E) SDS/PAGE analysis of a sucrose gradient–derived sample (D) and of samples from POM precipitations (E). After SDS/PAGE, gels were silver-stained or immunoblotted by using anti-PrP polyclonal antiserum W5517, as labeled. Lanes 1, molecular mass standards, in kilodaltons (kDa), that can be visualized in silver stains and Western blots (Chemicon). PrP 27-30 samples purified by the sucrose-gradient protocol (lanes 2), precipitated with PTA (lanes 3) or with HTA (lanes 4). Although the bands of PrP 27-30 from sucrose gradient purification are the dominant protein bands, a small contaminating peptide of 16 kDa can also be seen (D). In comparison, samples precipitated by the POMs (E) consist mostly of PrP 27-30 with a few contaminating peptides at ~16 kDa. PrP 27-30 samples purified by sucrose gradient (A and D) were derived from Syrian hamster Sc237 prions; samples purified by PTA and HTA (B, C, and E) were derived from mouse RML prions.

silver-stained band at the top of the polyacrylamide gel diminished, suggesting improved purification (Fig. 1E, lane 4). The band at ~16 kDa was found with all 3 purification protocols; extracts of this band from the gel produced no interpretable signal by mass spectrometry analysis. This observation suggests that the ~16-kDa band may consist of several different polypeptides.

Still concerned that the crystals might be composed of a non-PrPSc minor contaminant, we undertook a quantitative immunostaining EM study. Previously, we found that the 3F4 mAbs bound to the 2D crystals after exposure to 2 M urea (12). Because quantification of the findings with 3F4 mAbs produced results of limited statistical significance, we selected 2 other mAbs, D13 and F4–31, that bind to epitopes that are exposed in denatured PrPSc but buried in native PrPSc. All 3 antibodies, D13, F4–31 and 3F4 failed to bind native 2D crystals (Fig. 2A, C, and E) but recognized PrPSc after exposure to 3 M urea (Fig. 2B, D, and F). By using 3 M urea, all 3 α-PrP mAbs showed statistically significant differences between binding to native and denatured crystals (Fig. 2H and Table 1). The P value for labeling density of native versus denatured 2D crystals with the 3F4 mAb was <0.005, and the P values for D13 Fab and F4–31 mAb were <0.001. These findings argue that the 2D lattices are formed of native conformers of PrP 27-30 with buried epitopes, which become exposed after treatment with 3 M urea.

POM-Mediated Crystallization of PrP 27-30. In an effort to improve the 2D crystallization procedure, we tested the effects of ionic and nonionic detergents, proteases, buffers, chelators, lipids, and other additives [supporting information (SI) Table S1]. More than 300 grids were stained and inspected for the presence of 2D crystals; ~30% of the samples contained 2D crystals (Fig. S1A). Neither 2D crystals nor prion rods were found in fractions prepared from the brains of un inoculated mice (Fig. S1C).

Several of the additives increased the yield of 2D crystals (Table S1), but none improved their quality. For example, we observed that higher concentrations of proteinase K (PK) increased the yield, even though the N-terminal truncation of PrPSc to form PrP 27-30 was accomplished at lower concentrations of PK as judged by Western blot analysis. These findings raised the possibility that protein or peptide contaminants adversely affect 2D crystallization. Impurities may hinder 2D crystallization by binding to the crystal lattice, preventing the lattice from extending. A comparison of silver-stained gels and Western blots revealed that PrP 27-30 was only a minor component at this stage of the preparation. Considering the amount of impurities, it is surprising that PrP 27-30 crystallized at all. The importance of protein purity and homogeneity for 2D crystallization is well recognized (20, 21).

Because we had found that PTA, [PW12O40]3−, selectively
precipitates PrPSc and PrP 27-30 (17), we asked whether PTA might be used to isolate 2D crystals. Using PTA, we found both prion rods and 2D crystals (Fig. S1 d and e). The crystals were generally of higher quality than the best 2D crystals obtained by any of the other protocols previously used. Preparations from the brains of uninoculated control animals consistently failed to show any 2D crystals or prion rods (Fig. S1f), supporting the argument that the 2D crystals are composed of PrP 27-30. Image processing revealed the amount of structural detail that can be resolved from these 2D crystals (Fig. S2).

Within the framework of the PTA precipitation method, relatively minor changes in the purification procedure had major effects on 2D crystallization. For example, when we added the detergent Sarkosyl after the initial centrifugation used for clarification (see Methods), the amount of contaminating lipids was reduced, which, in turn, led to a noticeable increase in the yield of 2D crystals. Doubling the concentration of the brain homogenate from 5% to 10% (wt/vol) in the PTA precipitation step also increased the yield of 2D crystals. In both cases, the protein:l lipid and the protein:detergent ratios were modified. The relative proportions of protein, lipid, and detergent are well-known factors influencing the 2D crystallization of membrane proteins (20, 21).

Despite its ability to increase the yield of 2D crystals, PTA failed to stain previously formed 2D crystals. Other heavy metal stains, such as uranyl acetate or ammonium molydate, were unable to stain previously formed 2D crystals. Other heavy metal stains, such as uranyl acetate or ammonium molydate, were unsuccessful to visualize 2D crystals of PrP 27-30 (11, 12). In contrast, prion rods were decorated with PTA (17). Apparently, the PTA binding site is not available in the 2D crystals and may participate in forming crystallographic contacts. The removal of PTA via dialysis further improved the yield and quality of the 2D crystals, reduced the number of prion rods. Presumably, dialysis caused the partial disassembly of the PTA/prion rod complexes, thereby increasing the amount of nonfibrillar, crystallization-competent PrP 27-30. Alternatively, PTA-bound PrP 27-30 may not be able to form 2D crystals on account of interference by PTA.

Precipitation of PrP 27-30 by Using Other Polyoxometalates. Given the improvements in 2D crystallization that were observed by employing PTA, we decided to explore other parameters related to the nature of the POM and their effect on PrP 27-30 (Table 2). We tested AsTA (Na4[As4W40O140]), TTA (Na4[As2W4O40]), and 4 Keggin-type POMs (Fig. 3A) for their ability to precipitate PrP 27-30 and analyzed the resulting pellets by quantitative electron microscopy and a conformation-dependent immunoassay (CD1) (Fig. 3B). In addition to PTA, the Keggin-type POMs evaluated were SiTA (SiW12O40), BTA (K5[BW12O40]), and HTA (NH4)6[H2W12O40]). The yield of fibrils was quantified by taking advantage of the inherent electron density and the contrast of the POM-PrP 27-30 complexes (17). Fibrils were seen in all samples, but the quantities varied depending on which species of POM was used. Initially, we expected that, for the different POMs, the fibril content would correlate directly with the yield of precipitated PrP 27-30 as determined by CD1 (17). However, the correlation between the fibril and PrP 27-30 contents was poor, with a coefficient of determination \( r^2 \) of 0.20 (Fig. 3B).

On closer examination, two of the POMs (BTA and HTA) produced substantial numbers of aggregates other than fibrils, e.g., 2D crystals. The other POMs produced predominantly fibrillar aggregates and relatively few other structures. The propensity to form nonfibrillar structures explained the poor correlation between the fibril and the PrP 27-30 contents (Fig. 3B). When the data for BTA and HTA were excluded, the correlation between the fibril and PrP 27-30 contents was excellent, with an \( r^2 \) of 0.91 (Fig. 3B, solid line).

Impact of POM Charge on 2D Crystallization. We next examined whether the charge of the POM might modify polymeric forms of PrP 27-30. Keggin-type POMs have diameters of \( \sim 1 \) nm and...
and the number of crystals for the series of Keggin-type POMs, we quantified the total area covered by the 2D crystals of prion rods.

Structured POMs show a reduced propensity to induce formation of prion rods. Those used here, PTA, SiTA, and BTA dissociate into lacunary complexes by loss of a single $[\text{WO}_4]^{2-}$ unit. An earlier investigation of the solution properties of these POMs suggested that, under conditions similar to those used here, PTA, SiTA, and BTA dissociate into lacunary complexes by loss of a single $[\text{WO}_4]^{2-}$ unit (18, 24). This dissociation should increase the negative charge of the resulting lacunary structures to $[\text{PW}_{11}\text{O}_{39}]^{8-}$, $[\text{SiW}_{11}\text{O}_{39}]^{8-}$, and $[\text{HBW}_{11}\text{O}_{39}]^{8-}$, respectively (compare the supporting information of ref. 18), thereby abolishing any meaningful correlation between the charge density and quaternary structure of PrP 27–30. It is conceivable, however, that the solution structure of the POMs does not represent the form that actually binds to prions. Additional investigations are needed to clarify what POM species is responsible for binding to PrP 27-30 and how the quaternary structure is affected by it.

Discussion

In the studies reported here, we present 5 different lines of evidence that the 2D crystals found in our preparations are composed largely of PrP 27-30. First, the 2D crystals were only found in fractions prepared from the brains of mice and hamsters infected with prions. No crystalline structures were found in fractions prepared from the brains of mice or hamsters infected with prions. No crystalline structures were found in fractions prepared from the brains of mice or hamsters infected with prions. No crystalline structures were found in fractions prepared from the brains of mice or hamsters infected with prions.
control fractions prepared from the brains of uninfected rodents.

Second, two different purification protocols used for enriching PrP 27-30 and prion infectivity produced fractions containing both prion rods and 2D crystals (Fig. 1). One protocol used sucrose gradient centrifugation and the other used POM precipitation. Third, purified fractions containing rods and crystals had predominantly one protein, PrP 27-30, as demonstrated by silver staining; the protein was identified by immunostaining on Western blots (Fig. 1). Fourth, the diameter of the 2D crystal unit cells (Fig. 1) is similar to the diameter of negatively stained prion rod protofilaments. Fifth, α-PrP mAbs immunostained the 2D crystals (Fig. 2) (12). Taken together, these 5 lines of evidence argue that the 2D crystals found in purified fractions enriched for prion infectivity are composed largely, if not entirely, of PrP 27-30.

Because the number of 2D crystals is relatively small compared with the plethora of rods even under conditions that favor 2D crystal formation (Fig. 4), we were unable to apply techniques like optical spectroscopy to measure directly the β-sheet content of PrP 27-30 in the crystals. However, several lines of investigation argue that the PrP 27-30 molecules in the 2D crystals possess a conformation similar to that found in the rods (3–5). First, the epitopes in PrP 27-30 that are inaccessible to antibody binding when the protein is polymerized into prion rods are also buried in the 2D crystals (13, 14). These cryptic epitopes in both the rods and crystals were exposed by denaturants such as 3 M urea (Fig. 2 and Table 1) (12). Second, PrP 27-30 in prion rods and 2D crystals showed similar levels of resistance to digestion by proteasome K, in contrast to PrPSc, which is readily degraded by proteases. Third, PrP 27-30 in both the 2D crystals and the rods binds to POMs (Fig. 4), whereas PrPSc does not form precipitable complexes with POMs (17, 18, 25). Our results argue that the prion rods and 2D crystals represent alternative quaternary structures of PrP 27-30, but it seems likely that variations in the tertiary structure of PrP 27-30 are responsible for these two distinct polymeric forms.

The binding of a panel of mAbs directed to an array of epitopes in PrP 27-30 provides a sensitive tool for comparing the structure of PrP 27-30 in rods and 2D crystals. The R1 and R2 Fabs directed at the C terminus of PrP 27-30 reacted with native rods and crystals (12). As reported here, the 3F4 and F4-31 mAbs as well as the D13 Fab did not react with PrP 27-30 in native rods and crystals but did bind to the protein after exposure to 3 M urea. The concurrent behavior of PrP 27-30 immunoreactivity in the rods and crystals contend that the conformation of the protein is likely to be similar in these two macromolecular complexes. Our findings with PrP 27-30 contrast with studies on fibrillar and crystalline forms of insulin. In crystals, native insulin forms complexes. Our findings with PrP 27-30 provide direct evidence that the prion rods and 2D crystals represent alternative quaternary structures of PrP 27-30, but it seems likely that variations in the tertiary structure of PrP 27-30 are responsible for these two distinct polymeric forms.

The reciprocal relationship between prion rods and 2D crystals shown in Fig. 4 requires qualification: purified samples were used for counting the number of 2D crystals and crude fractions were used for determining the number of rods (see also SI Methods). To obtain reproducible results, the samples for rod counts were taken immediately after the fibrillation reaction and before centrifugation. This step prevented the prion rods from aggregating and allowed us to distinguish individual fibrils and small clusters of fibrils at relatively low magnifications (1,700 to 5,000×). In contrast, the 2D crystals could only be identified at higher magnifications (>25,000×), which made it necessary to use concentrated samples. Currently, we have no reliable means to calculate the precise number of PrP 27-30 molecules per length of prion rod because key aggregation parameters are unknown. Although we know the lattice parameters of the 2D crystals, the thickness of the crystals is unknown. Once the packing arrangement of the PrP 27-30 molecules in the prion rods is understood and the thickness of the 2D crystals can be determined, it will be possible to calculate more accurate comparisons.

The discovery reported here that PTA favors rod assembly and HTA favors crystal formation should provide tools that can be used to answer several fundamental questions about the prion polymerization pathways. Can prion rods be converted into crystals by exposure to HTA? Can crystals be transformed into rods by using PTA? Can conditions be identified in which all PrP 27-30 polymers are crystals? Given the formation of lacunary structures, what are the species of PTA and HTA that actually bind to PrP 27-30? We do not know whether it will be possible to convert crystals into soluble, oligomeric complexes composed of native PrPSc. Attempts to solubilize native PrPSc from rods have been disappointing (10, 35, 36) although a small fraction of low-molecular-weight prions can be recovered after size fractionation (37).

Earlier experimental and computational analyses of the 2D crystals argue for a trimer of parallel left-handed, β-helices as the fundamental unit of PrPSc structure (12, 38). This model is consistent with ionization radiation experiments, which identified the target size of the proteinaceous part of the smallest infectious unit as being 55 ± 9 kDa (39, 40). For comparison, the molecular mass of trimers of the polypeptide chains of PrPSc and PrP 27-30 are 69 and 48 kDa, respectively.

The use of POMs to manipulate the polymeric forms of PrP 27-30 is particularly intriguing because it may be possible to create conditions where PrPSc polymerizes into 3D crystals that are suitable for high-resolution X-ray structure determination. The mechanism by which HTA stimulates 2D crystal formation is unknown. One factor may be the apparent ability of HTA precipitation to reduce the impurities in purified fractions compared with PTA (Fig. 1E). It is possible that, as the rods are elongated, they trap more impurities than crystals do as they form. The ability to fine-tune the molecular properties of the POM particles, as demonstrated by changes in the valency of the central heteroatom that resulted in increased charge densities, improves their versatility as tools to study the structure of oligomeric and polymeric protein assemblies. The use of HTA or...
a POM with an even higher surface-charge density might be ideal for encouraging large 3D crystal formation.

**Methods**

Immunoeassays, preparation of 2D crystals and POM stock solutions, image processing, fibril and 2D crystal content determinations, and statistical analyses are described in the *SI Methods*.

**POM Precipitation of RML and Sc237 Prions.** PrPs 27-30 was prepared from the brains of scrapie-sick, wild-type FVB mice infected with RML prions or Syrian hamsters infected with Sc237 prions. The PTA precipitation protocols were based on published procedures (17). Ten percent brain homogenates (BH) were prepared in Ca\(^{++}\)/Mg\(^{++}\)-free PBS. Homogenates were centrifuged at 500 × g for 5 min in a tabletop centrifuge. The resulting supernatant was diluted to a final 5% (w/vol) by using PBS containing 4% (w/vol) Sarkosyl. The diluted samples were digested with 25 μg/mL protease K (PK) for 60 min at 37 °C with constant agitation. After incubation, protease inhibitors (0.5 mM PMSF, aprotinin, and leupeptin, 2 μg/mL each) were added and the samples were mixed with the POM stock solutions. After either 1 or 16 h of incubation at 37 °C on a rocking platform, the samples were centrifuged at 14,000 × g for 30 min at room temperature (RT). The resulting pellets were resuspended in PBS containing 2% Sarkosyl and the precipitation was repeated with the same concentration of POM. The final pellet was resuspended in PBS with 0.2% Sarkosyl containing protease inhibitors (see above).

For the quantification of the 2D crystal contents, 20% BH was used instead of the usual 10% and the centrifuged supernatant was adjusted to 10% BH equivalent before the addition of the POM stock solutions. This variation in the protocol ensured a more reliable production of 2D crystals.

**Electron Microscopy.** Negative staining was performed on Formvar-carbon-coated, 200-mesh copper grids (Ted Pella, Inc.) that were glow-discharged before staining. Five-microliter samples were adsorbed for 30 s, briefly washed with 0.1 M and 0.01 M ammonium acetate buffer, pH 7.4, and stained with 2 drops (0.5 μL each) of freshly filtered 2% (w/vol) uranyl acetate solution. Positively stained samples relied on the contrast provided by the POMs used to precipitate PrP 27-30. Samples were loaded onto the grids, adsorbed, and washed with ammonium acetate buffers as described above, but without any additional staining steps. After drying, the samples were viewed with a FEI Tecnai F20 electron microscope (FEI Company) at an acceleration voltage of 80 kV. Electron micrographs were recorded on a Gatan UltraScan 4000 CCD camera.

**ImmuGold Labeling of the 2D Crystals.** ImmunoGold labeling was performed essentially as described in refs. 12 and 42. To reduce the background intensity, denatured forms of PrP were removed by briefly incubating the grid with 50 μg/mL PK for 15 min at RT. To enhance the labeling intensity, antibodies were incubated for 2 h.

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