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Journal

Biophysical Journal, 116(4)

ISSN

0006-3495

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Publication Date

2019-02-01

DOI

10.1016/j.bpj.2019.01.005

Peer reviewed

Molecular Features of the Zn^{2+} Binding Site in the Prion Protein Probed by ^{113}Cd NMR

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ABSTRACT The cellular prion protein (PrP^{C}) is a zinc-binding protein that contributes to the regulation of Zn^{2+} and other divalent species of the central nervous system. Zn^{2+} coordinates to the flexible, N-terminal repeat region of PrP^{C} and drives a tertiary contact between this repeat region and a well-defined cleft of the C-terminal domain. The tertiary structure promoted by Zn^{2+} is thought to regulate inherent PrP^{C} toxicity. Despite the emerging consensus regarding the interaction between Zn^{2+} and PrP^{C} , there is little direct spectroscopic confirmation of the metal ion's coordination details. Here, we address this conceptual gap by using Cd^{2+} as a surrogate for Zn^{2+} . NMR finds that Cd^{2+} binds exclusively to the His imidazole side chains of the repeat segment, with a dissociation constant of ~ 1.2 mM, and promotes an N-terminal-C-terminal *cis* interaction very similar to that observed with Zn^{2+} . Analysis of ^{113}Cd NMR spectra of PrP^{C} , along with relevant control proteins and peptides, suggests that coordination of Cd^{2+} in the full-length protein is consistent with a three- or four-His geometry. Examination of the mutation E199K in mouse PrP^{C} (E200K in humans), responsible for inherited Creutzfeldt-Jakob disease, finds that the mutation lowers metal ion affinity and weakens the *cis* interaction. These findings not only provide deeper insight into PrP^{C} metal ion coordination but they also suggest new perspectives on the role of familial mutations in prion disease.

INTRODUCTION

Transmissible spongiform encephalopathies, also known as prion diseases, are a class of fatal neurodegenerative diseases for which there is no cure or treatment (1). Examples of prion diseases are Kuru and Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, chronic wasting disease in cervids, and mad cow disease (2). Prion diseases originate from genetic, sporadic, or infectious routes and involve misfolding of the predominately helical cellular prion protein (PrP^{C}) to the β -sheet rich scrapie form (2). Similar to Alzheimer's disease, sporadic disease accounts for the majority of prion cases in humans.

PrP^{C} is expressed throughout the body but appears to be concentrated primarily at pre- and postsynaptic neuronal membranes (3–5). The precise physiological function of PrP^{C} is largely unknown; however, the protein's well-documented ability to coordinate Cu^{2+} and Zn^{2+} suggests a role in metal ion homeostasis (6–10). Mature human PrP^{C} is a 208-amino-acid protein with two N-linked glycans and a glycoposphatidylinositol moiety that anchors the protein to the extracellular membrane surface. The protein has

two distinct domains: the C-terminal domain (residues 126–230) composed of three α -helices, two short antiparallel β -strands, and a disulfide bond linking helices two and three and the N-terminal domain (residues 23–125), a flexible segment that coordinates both Cu^{2+} and Zn^{2+} in vivo (7,10–13).

Zn^{2+} is one of the most abundant trace metal in the brain with roles in diverse functions, including structural support in certain transcription factors, as catalytic elements in zinc metalloenzymes and as an abundant counterion in presynaptic glutamate vesicles (13,14). Glutamate release results in a synaptic (Zn^{2+}) spike, and recent findings suggest that Zn^{2+} binding to PrP^{C} stimulates Zn^{2+} transport back into neurons through α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, thereby restoring normal synaptic metal ion concentrations (14). Our lab demonstrated previously that Zn^{2+} binds to a PrP^{C} N-terminal segment composed of the sequence $(\text{PHGGGWGQ})_4$ (residues 60–91 in human PrP^{C}), termed the octarepeat (OR) domain, with a dissociation constant (K_d) of ~ 200 μM (Fig. 1) (10). This OR segment is essential for Zn^{2+} binding and subsequent transport of the ion through AMPA receptors.

Although once thought to be noninteracting, we recently demonstrated that PrP^{C} 's N-terminal and C-terminal domains have an important interdomain interaction driven

Submitted August 24, 2018, and accepted for publication January 4, 2019.

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Editor: Wendy Shaw.

<https://doi.org/10.1016/j.bpj.2019.01.005>

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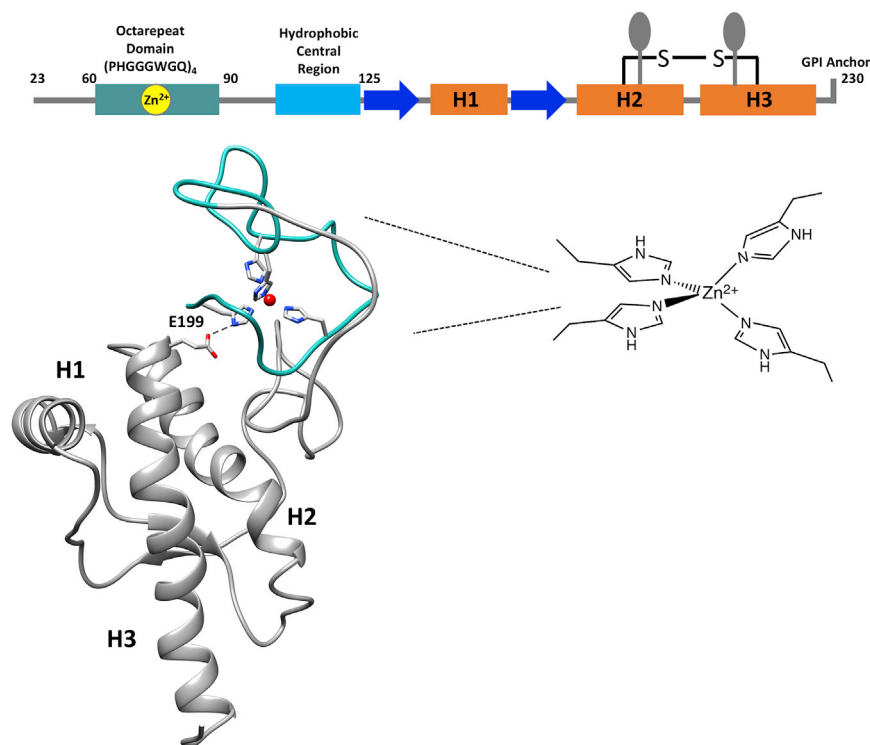


FIGURE 1 Sequence and structural model of mouse PrP^C-Zn²⁺ complex. (top) Linear sequence diagram of PrP^C(23–230) (mouse sequence) with numbering indicating important N-terminal segments, along with secondary structure and posttranslational modifications of the folded C-terminal domain. Coloring shows the octarepeat (OR) domain (light green) that coordinates Zn²⁺, β sheets (blue), helices (orange), CR segment (light blue), glycans (ovals), disulfide bond linking helices two and three, and glycosphosphatidylinositol anchor at residue 230. (bottom) Three-dimensional ribbon model of PrP^C with Zn²⁺ (red sphere) coordinated to the OR domain His residues and docked against a C-terminal cleft formed by helices 1–3 (Spevacek et al. (15)). Also shown is residue E199 (mouse sequence) with its carboxylate group forming a hydrogen bond with the remote NH of a coordinating His residue.

by the addition of physiological metal ions (6,7,15). Evidence for this *cis* interaction came from detailed double electron-electron resonance (DEER) EPR and ^1H - ^{15}N heteronuclear single-quantum coherence (HSQC) NMR. Specifically, Spevacek et al. (15) demonstrated that the addition of Zn²⁺ drives the N-terminal OR to make a direct contact with a negatively charged patch on C-terminal surface composed of residues from helices two and three as well as the N-terminal end of the β 1- α 1 loop extending to the beginning of helix 1. Interestingly, a significant number of pathological mutations responsible for inherited prion disease reside on this surface, and those tested systematically weakened the observed *cis* interaction (15). Therefore, it was hypothesized that PrP^C's *cis* interaction plays a role in regulating the prion protein (PrP), with a decrease in this *cis* interaction promoting prion-mediated toxicity.

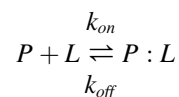
In addition to Zn²⁺, Cu²⁺ also drives a *cis* interaction, as shown by Evans et al. (6). This was demonstrated using paramagnetic relaxation enhancement NMR and DEER EPR. Paramagnetic relaxation enhancement broadening of PrP^C ^1H - ^{15}N HSQC NMR crosspeaks with 1.0 equivalent of Cu²⁺ localized the *cis* interaction to the same C-terminal PrP^C surface identified in the Zn²⁺ studies. The specific location of the Cu²⁺ ion was further refined using DEER-EPR-derived distance restraints, along with trilateration calculation (6). These structural data suggest that physiologically relevant metal ions are essential for stabilizing higher order structure in PrP^C. Recent monoclonal antibody and

electrophysiology experiments underscore the importance of this newly discovered *cis* interaction (16). These experiments show consistently that the PrP^C C-terminal domain regulates the otherwise toxic N-terminal executive domain. Using mutagenesis and select PrP^C constructs, we have provided strong evidence that this regulatory function requires the Zn²⁺/Cu²⁺-promoted *cis* interaction identified by our magnetic resonance experiments (6,7,15–17).

Cu²⁺ is a paramagnetic species, which enables EPR experiments for assessing details of the metal ion's coordination environment. Using EPR with 1.0 equivalent of Cu²⁺, we showed that the copper ion is bound to the four imidazole side chains of the OR histidines (9). This coordination environment is preserved in both the OR domain alone when expressed as a polypeptide as well as in the full-length protein. This information was essential in developing a structural model of Cu²⁺-occupied PrP^C.

Unlike Cu²⁺, there is no convenient magnetic resonance method for probing the coordination environment of Zn²⁺. However, given the essential role of Zn²⁺ in PrP^C physiology, as evidenced by the ion's ability to trigger divalent ion transport through the AMPA receptor (14), it is very important to evaluate the precise details of how this physiological ion coordinates within PrP^C. We have shown previously with indirect methods such as mass spectrometry mapping applied to OR peptides that, much like Cu²⁺, Zn²⁺ binds to the OR through imidazole coordination (10). However, this has yet to be demonstrated in the full-length PrP. As such, it is unclear whether Zn²⁺ remains

Analysis leading to the conclusion of fast exchange in the ¹¹³Cd spectra utilized the following relations:



$$K_d = \frac{k_{off}}{k_{on}}$$

$$k_{ex} = [P]k_{on} + k_{off}$$

$$\Delta\omega = 2\pi\Delta\delta\nu_o,$$

where $\Delta\delta$ is the difference in ¹¹³Cd chemical shift between free Cd²⁺ and the fully bound metal ion, and ν_o is the spectrometer frequency. k_{on} is estimated to be diffusion controlled and $\sim 10^9 \text{ M}^{-1}\text{s}^{-1}$. With a protein concentration of 300 μM , $k_{ex} \gg \Delta\omega$ thereby satisfying fast exchange consistent with a single ¹¹³Cd NMR line.

ITC

ITC experiments were conducted using a MicroCal VPITC calorimeter. Zinc chloride titrations were performed by adding 2.0 mM zinc chloride into each PrP^C construct (18–45 μM) at pH 7.4 in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS). Cadmium chloride titrations were performed by titrating 20 mM cadmium chloride into each PrP^C construct (45–60 μM) at pH 6.0 in 10 mM MES. Each construct was dialyzed overnight before running the experiments in either 50 mM MOPS pH 7.4 (Zn²⁺ titrations) or 10 mM MES pH 6.0 (Cd²⁺ titrations). Metal ions used in the titrations were prepared by diluting a 1.0 M stock in water to either 2.0 mM for zinc chloride or 20 mM for cadmium chloride using the MOPS or MES dialysis buffer, respectively. Because of the large heats of dilution with cadmium chloride, the integrated ITC data were background corrected. Data analysis was performed using the Origin calorimetry software package. Experiments were replicated two to three times for each construct, and the reported error is the difference between the highest and lowest K_d measured for each set of measurements.

RESULTS

Cadmium induces a *cis* interaction between PrP^C N-terminal and C-terminal domains

Previous ¹H-¹⁵N HSQC NMR experiments performed on uniformly ¹⁵N-labeled PrP^C with one to three equivalents of Zn²⁺ identified a collection of C-terminal residues that exhibited both linewidth broadening and/or changes in chemical shift of select crosspeaks. The affected residues, when mapped to the three-dimensional structure of the C-terminal domain of PrP^C, identified a shallow cavity localized primarily to a surface patch composed of adjacent sides of helices 2 and 3 ($\alpha 2$ and $\alpha 3$), along with the residues corresponding to the $\beta 1$ - $\alpha 1$ loop. When combined with EPR DEER experiments and suitable controls, these NMR data demonstrated that the Zn²⁺-OR segment docks to a well-defined, negatively charged patch on the PrP^C C-terminal domain (Fig. 1) (15). Divalent Zn is diamagnetic; we

therefore attributed changes in the line shapes and chemical shifts of the affected C-terminal residues to intermediate exchange molecular dynamics at the interface between the interacting N-terminal and C-terminal domains. To confirm the use of Cd²⁺ as a viable Zn²⁺ surrogate, we performed parallel ¹H-¹⁵N HSQC NMR experiments at 25°C on ¹⁵N-labeled wild-type PrP^C(23–230) (300 μM) in the presence of 1.0 mM Cd²⁺ (added as CdCl₂) at pH 6.0 and pH 7.0.

Fig. 2 A shows a segment of the 800-MHz ¹H-¹⁵N HSQC NMR spectrum. As with Zn²⁺, we find that select crosspeaks exhibit changes in linewidth and chemical shift (15). To evaluate these spectral changes, values for intensity ratio (I/I_o) were calculated for all assigned C-terminal resonances. These ratios are obtained by dividing the crosspeak intensity in the presence of Cd²⁺ by the intensity of the crosspeak before addition of Cd²⁺. The average and SD of the I/I_o values were calculated, and standard Z-score analysis (6) was performed to determine a threshold for residues broadened more than 1.0 SD from the mean by the presence of Cd²⁺. In addition, crosspeaks exhibiting changes in chemical shift greater than 0.1 ppm (measured by $[(\Delta\delta^1\text{H})^2 + (\Delta\delta^{15}\text{N}/9)^2]^{1/2}$) were deemed to be significantly affected by the addition of Cd²⁺. The results are summarized in Fig. 2 B, which shows I/I_o versus residue position for the C-terminal domain. Affected residue crosspeaks exceeding statistical significance are noted by dark blue (I/I_o exceeding one SD the mean), medium blue (I/I_o greater than 0.5 SD with a chemical shift change greater than 0.1 ppm), and in light blue (0.1 ppm or greater change in chemical shift) (6,35). When plotted onto surface (Fig. 2 C) or ribbon (Fig. 2 D) diagrams, the data identify a patch of affected residues similar to those observed by the addition of Zn²⁺. Specifically, the Cd²⁺ caused either broadening, shifting or broadening and shifting of proximal residues on $\alpha 2$, $\alpha 3$, and the $\beta 1$ - $\alpha 1$ loop. The 27 affected residues are all proximal to each other and, of these, 12 are equivalent to those affected by Zn²⁺ (Fig. S1). Spectra acquired at pH 6.0 and 7.0 gave similar results; however, for experiments that follow, we standardized on the lower pH value because it was found to give narrower ¹¹³Cd line shapes (see below, Fig. 5). Noting that Zn²⁺ does not bind to PrP^C(90–230), these results show that like Zn²⁺, Cd²⁺ drives a well-defined *cis* interaction between the PrP^C Cd²⁺-occupied N-terminal domain and the shallow cavity of the C-terminal domain.

Cd²⁺ coordinates to imidazole groups of OR histidines

We previously used chemical mapping to show that Zn²⁺ coordinates specifically to His imidazole residues in the PrP^C OR segment with a K_d of $\sim 200 \mu\text{M}$ (10). To examine Cd²⁺ coordination details and measure its binding affinity, we applied ¹H-¹⁵N HSQC with pulse sequence modifications to highlight ²J_{NH} scalar couplings (30). With this pulse

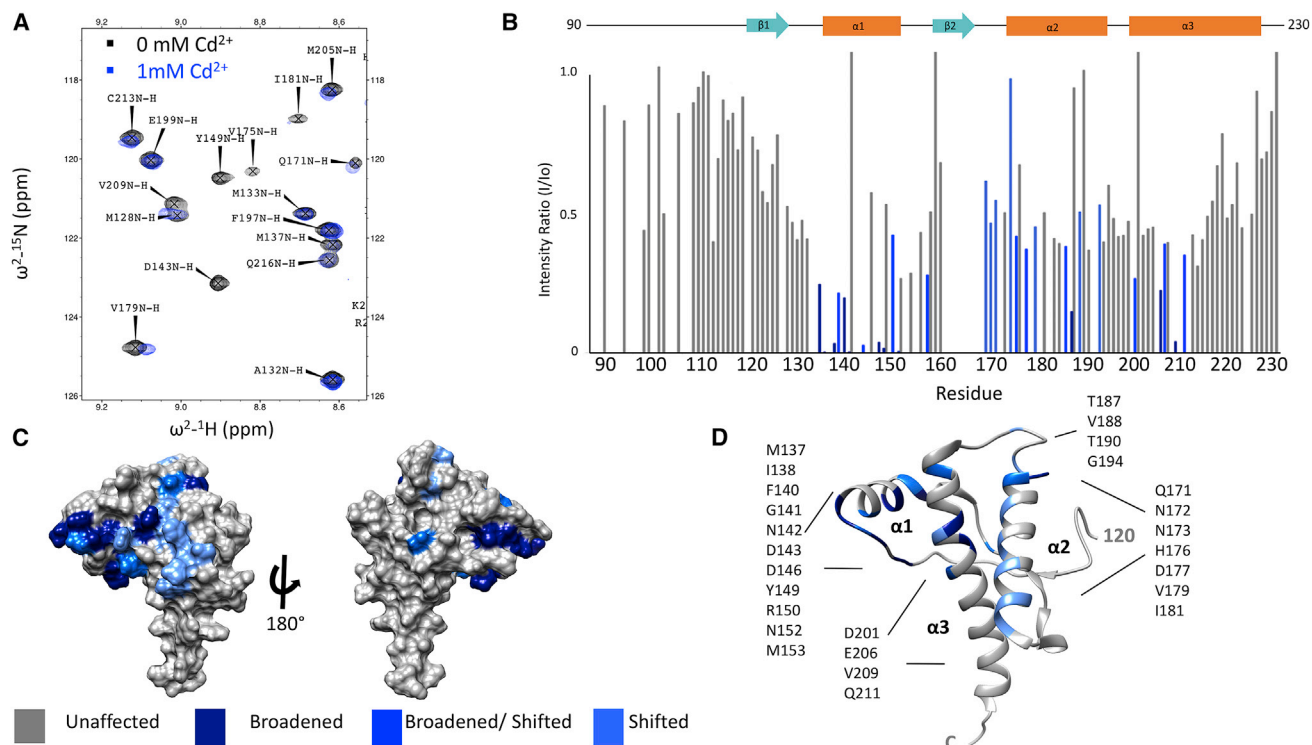


FIGURE 2 Cd^{2+} promotes an interdomain interaction wild-type PrP at pH 6.0. (A) A selected region of the ^1H - ^{15}N HSQC of wild-type PrP in the absence of metal (black) and in the presence of 1.0 mM of Cd^{2+} (blue). (B) A bar graph of I/I_0 for residues 90–23 of PrP^C in the presence of Cd^{2+} . (C and D) Surface and ribbon plots, respectively, of C-terminal residues affected by the presence of Cd^{2+} (coordinates from Protein Data Bank (PDB): 1XYX). Affected residues are noted specifically on the ribbon diagram.

sequence, His side chains give characteristic patterns depending on the protonation state of the imidazole ring. Spectra of full-length PrP^C and PrP (23–125), both in the absence of Cd^{2+} , are compared in Fig. 3. PrP^C contains 9 His residues, 6 of which are in the flexible region 23–125. Examination of Fig. 3 shows that H ϵ 1 crosspeaks with ^1H chemical shifts above 8.1 ppm are readily assigned to this N-terminal segment. In addition, prominence of the specific crosspeak patterns of N δ 1-H ϵ 1, N ϵ 2-H ϵ 1, and N ϵ 2-H δ 2 for each imidazole is consistent with protonation of the ϵ 2 nitrogen of the imidazole ring. Three separate patterns are observed for PrP (23–125) with approximate volume ratios of 4:1:1. We therefore assigned the four OR His residues to more intense set crosspeaks.

Next, we added increasing concentrations of Cd^{2+} to a solution of 300 μM PrP (23–125) and observed a progressive chemical shift changes of the N ϵ 2-H ϵ 1 and N ϵ 2-H δ 2 crosspeaks, assigned to the OR His residues (Fig. 4). The peaks exhibited only slight broadening with increasing $[\text{Cd}^{2+}]$, consistent with fast exchange. These data suggest that Cd^{2+} coordinates preferentially to the ϵ 2 nitrogen, perhaps displacing the exchangeable proton. Plotting the N ϵ 2 ^{15}N chemical shifts derived from the N ϵ 2-H ϵ 1 and N ϵ 2-H δ 2 crosspeaks gave saturable binding curves, which we fit independently to a standard model for equilibrium fast exchange (35). Both curves gave similar results with an approximate

K_d of 1.2 mM (Table 1). We also attempted titration with full-length PrP^C; however, the relevant $^2\text{J}_{\text{NH}}$ crosspeaks exhibited significant broadening, likely because of intermediate exchange, and were therefore not amenable to fast-exchange binding analysis. Taken together, these NMR

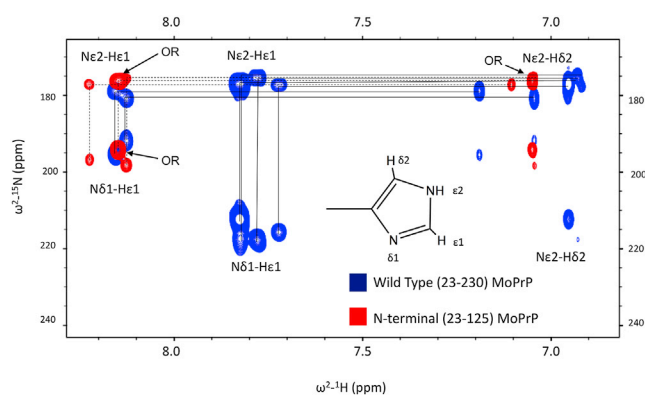


FIGURE 3 $^2\text{J}_{\text{NH}}$ -HSQC of ^{15}N -labeled N-terminal PrP and full-length PrP^C. ^1H - ^{15}N -HSQC spectra with pulse sequence modified to highlight $^2\text{J}_{\text{NH}}$ couplings showing the imidazole region of the spectrum for mouse PrP(23–125) (red, correlated crosspeaks connected by dashed lines) and full-length PrP (blue, peaks connected by solid lines). The pattern of connections from N δ 1-H ϵ 1 \rightarrow N ϵ 2-H ϵ 1 \rightarrow N ϵ 2-H δ 2 is characteristic of the protonation at N ϵ 2. Crosspeaks from the octarepeat (OR) His residues are labeled “OR.”

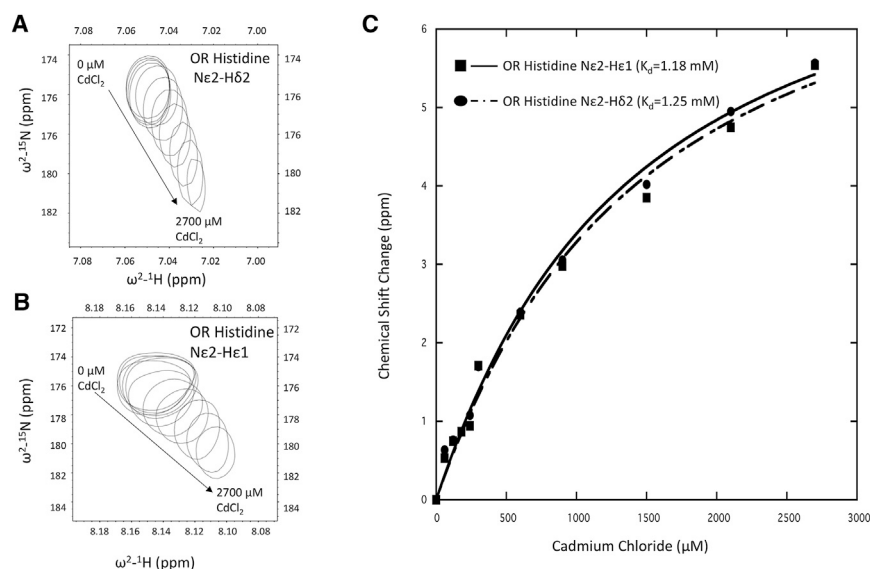


FIGURE 4 Determination of K_d from $^2J_{NH}$ HSQC chemical shifts versus Cd^{2+} . (A) $Ne2-H\delta2$ and (B) $Ne2-He1$ $^2J_{NH}$ HSQC crosspeaks of N^{15} -labeled PrP (23–125) shift as a function of added cadmium chloride. (C) A plot of ^{15}N chemical shift change versus $CdCl_2$. Data were fitted to the K_d expression derived for fast chemical exchange (Materials and Methods) to determine binding constant for Cd^{2+} to the OR domain (Table 1). Resulting K_d values derived from the $Ne2-H\delta2$ and $Ne2-He1$ crosspeaks are ~ 1.25 and 1.18 mM, respectively.

experiments demonstrate that Cd^{2+} , like Zn^{2+} , binds to the OR His residues but with a significant reduction in affinity.

¹¹³Cd NMR of wild-type PrP^C and relevant mutants

With the goal of assessing the Cd^{2+} coordination environment, we carried out direct NMR measurements on ^{113}Cd combined with PrP^C and various relevant constructs. With an 11.7 T magnetic field (500 MHz for 1H), the ^{113}Cd resonance frequency is 110.9 MHz (29). All spectra were referenced to the chemical shift of 0.10 M Cd (ClO₄)₂ (aq), which places the chemical shift of aquo ^{113}Cd at 0 ppm (29). Given the low gyromagnetic ratio of ^{113}Cd , it was essential to adjust sample conditions to give the best possible spectra. Consequently, we prepared peptides and proteins to concentrations of 300 μM , which is near the PrP^C solubility limit, along with 1.0 mM $^{113}CdCl_2$. Under these conditions, a spectrometer fitted with a cryoprobe was capable of acquiring resolvable spectra in approximately 12 h.

Resulting spectra are shown in Fig. 5. As shown in the insert, nitrogen coordination leads to deshielding of the ^{113}Cd center, with concomitant resonances of higher chemical shift values. Oxygen coordination produces resonance signals of lower chemical shifts (23). The differences between nitrogen and oxygen coordination are reflected in the comparison of imidazole (175 ppm) and EDTA (108 ppm).

TABLE 1 K_d for OR Histidine ϵ Nitrogen

	K_d (μM)	Error (μM)
OR histidine $Ne2-He1$	1187	± 200
OR histidine $Ne2-H\delta2$	1253	± 245

The top represents OR histidine $Ne2-He1$, and the bottom represents OR histidine $Ne2-H\delta2$.

All peptide and protein samples gave only a single resonance line consistent with fast exchange between the coordinated species and free Cd^{2+} in buffered solution, as seen with the $^1H-^{15}N$ HSQC above (Fig. 2). A peptide containing the isolated 4-Octa peptide, OR segment (KKRPKP-PrP (56–90)-NH₂) produces a single line with a ^{113}Cd chemical shift close to that of imidazole, consistent with our findings above that Cd^{2+} coordinates primarily through His side chains. Full-length PrP^C is further deshielded relative to the isolated OR by ~ 5 ppm. Consequently, either the *cis* interaction described above further stabilizes OR- Cd^{2+} coordination or the Cd^{2+} coordination shell is additionally enhanced by C-terminal His residues. To test for this latter case, we prepared PrP^C(H139Y, H176Y), which replaces two C-terminal His residues at the Cd^{2+} -OR docking interface, specifically, H139 on the $\beta1-\alpha1$ loop and H176 on $\alpha2$. The resulting ^{113}Cd spectrum is equivalent to that of wild-type PrP^C, suggesting that either Cd^{2+} remains fully confined by coordination to the OR His residues or that one or two of the C-terminal His residues replace OR His residues thereby maintaining a four-His coordination environment.

To directly test the involvement of Cd^{2+} binding to the flexible N-terminal PrP^C domain, we prepared a mutant in which all OR His residues, His95, and His110 were mutated to Ala. This His-to-Ala mutant shows a strong shift toward a more shielded ^{113}Cd signal of ~ 35 ppm, close to that of $CdCl_2$ in aq solution. We therefore conclude that, indeed, the N-terminal segment provides the primary coordination environment for Cd^{2+} with a three- or four-His coordination shell, as previously found for Zn^{2+} .

The fast-exchange conditions observed for ^{113}Cd provide an opportunity to estimate the chemical shift of the OR- $^{113}Cd^{2+}$ species. Under fast exchange, the observed signal is a weighted average of the free and fully PrP^C-bound ^{113}Cd chemical shifts. Using a $K_d = 1.2$ mM determined

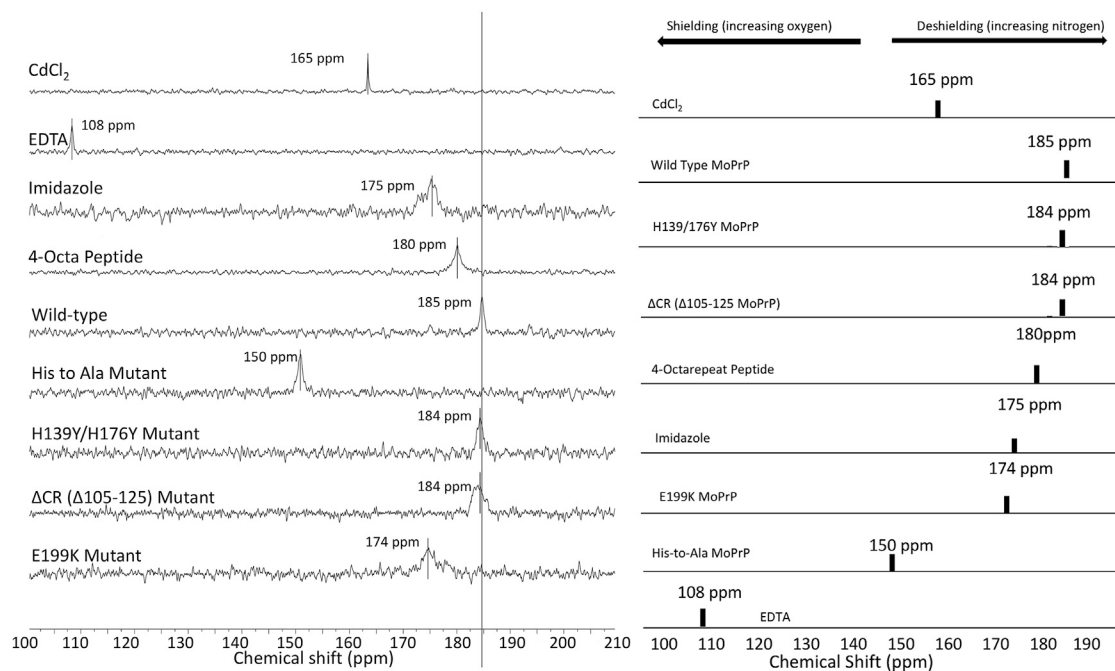


FIGURE 5 ^{113}Cd NMR spectra of controls, octarepeat (OR) segment, full-length PrP^C, and relevant mutants. (left) ^{113}Cd NMR spectra with chemical shifts referenced to 0.1 M $\text{Cd}(\text{ClO}_4)_2$ (aq). Spectra for the OR peptide (4-Octa peptide) and all proteins were acquired with 300 μM peptide/protein and 1.0 mM $^{113}\text{Cd}^{2+}$. The vertical line is drawn as a reference against wild-type protein. (right) A stick diagram of the ^{113}Cd NMR spectra ordered by chemical shift from high to low (with reference CdCl_2 at the top). As noted at the top, nitrogen coordination decreases chemical shielding, leading to higher chemical shift values, whereas oxygen coordination shifts in the opposite direction.

for the isolated 4-Octa OR segment (peptide concentration = 300 μM and $[\text{Cd}^{2+}] = 1.0 \text{ Mm}$), we calculate that the concentration of the bound OR- Cd^{2+} species is 126 μM or 12.6% of the total $^{113}\text{Cd}^{2+}$ in solution. Given the free $^{113}\text{Cd}^{2+}$ signal of 165 ppm (from CdCl_2) and exchanged average signal of 180 ppm, the fully bound species is determined to be approximately 300 ppm. This value aligns well with published values of 310–320 ppm for superoxide dismutase, which coordinates through a histidine-rich environment (36–38).

Next, we evaluated two important mutants that link directly to prion disease. ΔCR PrP^C is a designed deletion mutant in which residues 105–125, corresponding to the central region, are eliminated. Loss of this 21-residue segment between the OR and globular C-terminal domain drives severe cerebellar degeneration and neonatal lethality in laboratory mice (39). Electrophysiological experiments performed on cells transfected with the ΔCR PrP^C gene show spontaneous cationic currents associated with the early stages of prion disease (40,41). In cell culture, these currents are inhibited by the addition of Cu^{2+} , which binds with high affinity to the OR (16). Similar to Zn^{2+} , Cu^{2+} drives a *cis* interaction, and previous work from our lab showed a loss of this copper-promoted interaction in ΔCR PrP^C. Fig. 5 shows that ΔCR PrP^C gives a ^{113}Cd spectrum approximately equivalent to that of wild-type PrP^C. Consequently, there is no loss of Cd^{2+} coordination in ΔCR PrP^C; however, we cannot rule out a potential loss of the metal-ion-promoted *cis* interaction.

Finally, we examined murine PrP^C(E199K), a mutation that corresponds to familial E200K in humans (42,43). Families that carry this fully penetrant E200K mutation develop midlife CJD (44). Spevacek et al. demonstrated that the E199K mutation exhibits a weakened *cis* interaction, relative to wild-type, upon the addition of Zn^{2+} (15). They postulated that because E199 is located on the N-terminal end of helix 3 contributing to a large concentration of negatively charged C-terminal residues, the mutation to a Lys confers toxicity by reducing this localized negative charge thereby weakening electrostatic contributions to the *cis* interaction (15). The ^{113}Cd spectrum of PrP^C(E199K) shows a significant shift relative to that obtained from wild-type PrP^C, with a chemical shift reflecting a partial loss of nitrogen coordination. Complementing the findings of Spevacek et al. (15), these data suggest partial release of the metal ion.

We next performed ITC measurements on both wild-type PrP^C and the E199K mutant to test whether the difference in ^{113}Cd chemical shift arises from a change in metal ion affinity. K_d values were determined for Cd^{2+} and Zn^{2+} binding to both proteins, as well as Zn^{2+} binding to the N-terminal peptide segment (PrP(23–125)). K_d values are reported in Table 2 (ITC curves are in Fig. S2). Wild-type PrP^C gives a K_d of $\sim 3.0 \text{ mM}$, in reasonable agreement with 1.2 mM obtained from NMR using the fast exchange approximation (above). Comparing wild-type and mutant PrP^C, we find that there is a very slight increase in K_d for Cd^{2+} binding to PrP^C(E199K) relative to wild-type PrP^C; however, the

TABLE 2 K_d for Zn^{2+} and Cd^{2+} Determined by ITC

	Zn^{2+} K_d (μM) ^a	Cd^{2+} K_d (mM) ^a
Wild-type PrP ^C	16.9 ± 1.2	3.0 ± 1.8
PrP ^C (E199K)	33.6 ± 0.3	3.3 ± 0.8
N-terminal PrP(23–125)	38.0 ± 0.1	N/A

N/A, not acquired.

^aNote different units for Zn^{2+} (μM) and Cd^{2+} (mM).

difference is within experimental error. By contrast, the K_d difference for Zn^{2+} shows a factor of two increase for PrP^C(E199K) compared to wild-type PrP^C, thus reflecting a lower affinity for the mutant. In addition, K_d for PrP^C(E199K) is approximately equivalent to that obtained from N-terminal PrP (23–125). Together, these data demonstrate that the C-terminal domain in wild-type PrP^C enhances the OR domain's affinity for Zn^{2+} and that this enhancement is lost in PrP^C(E199K).

To further explore the consequence of the E199K mutation, we performed ^{15}N - ^1H HSQC NMR in the presence of 1.0 mM CdCl_2 . Fig. 6 shows that the E199K mutation significantly weakens the observed *cis* interaction relative to wild-type, similar to our previous observations with Zn^{2+} . The majority of the remaining broadened residues are localized to helix 1; line shape broadening of crosspeaks from residues at the respective N-termini of helices 2 and 3 is much less pronounced. Collectively, with the ITC

measurements above, these data suggest that residues E199 in the wild-type protein promotes the *cis* interaction and enhances metal ion binding affinity.

DISCUSSION

PrP^C is a Zn^{2+} binding protein, and emerging evidence suggests that uptake of this essential metal ion of the central nervous system drives an intramolecular *cis* interaction that is critical for regulating PrP^C function and arresting inherent toxicity. Previous to this current study, we used chemical mapping performed on an OR peptide to suggest that Zn^{2+} coordinates exclusively to OR His side-chain imidazole groups (10). Here, we used Cd^{2+} as a Zn^{2+} surrogate to carefully investigate the metal ion coordination features and the resulting *cis* interaction. As with Zn^{2+} , ^1H - ^{15}N HSQC NMR experiments show that the binding of Cd^{2+} to the N-terminal OR segment of PrP^C leads to broadening of select C-terminal residues located to a shallow, negatively charged cleft formed primarily by three α -helices. Next, we used a modified ^1H - ^{15}N HSQC NMR sequence that selects for $^2J_{\text{NH}}$ scalar couplings to assess the Cd^{2+} -PrP^C K_d , in turn establishing optimal conditions for ^{113}Cd NMR studies (23,30,31). Finally, ^{113}Cd NMR studies allowed us to compare Cd^{2+} complexation features among the isolated OR, the full-length PrP^C protein, as well as important mutants.

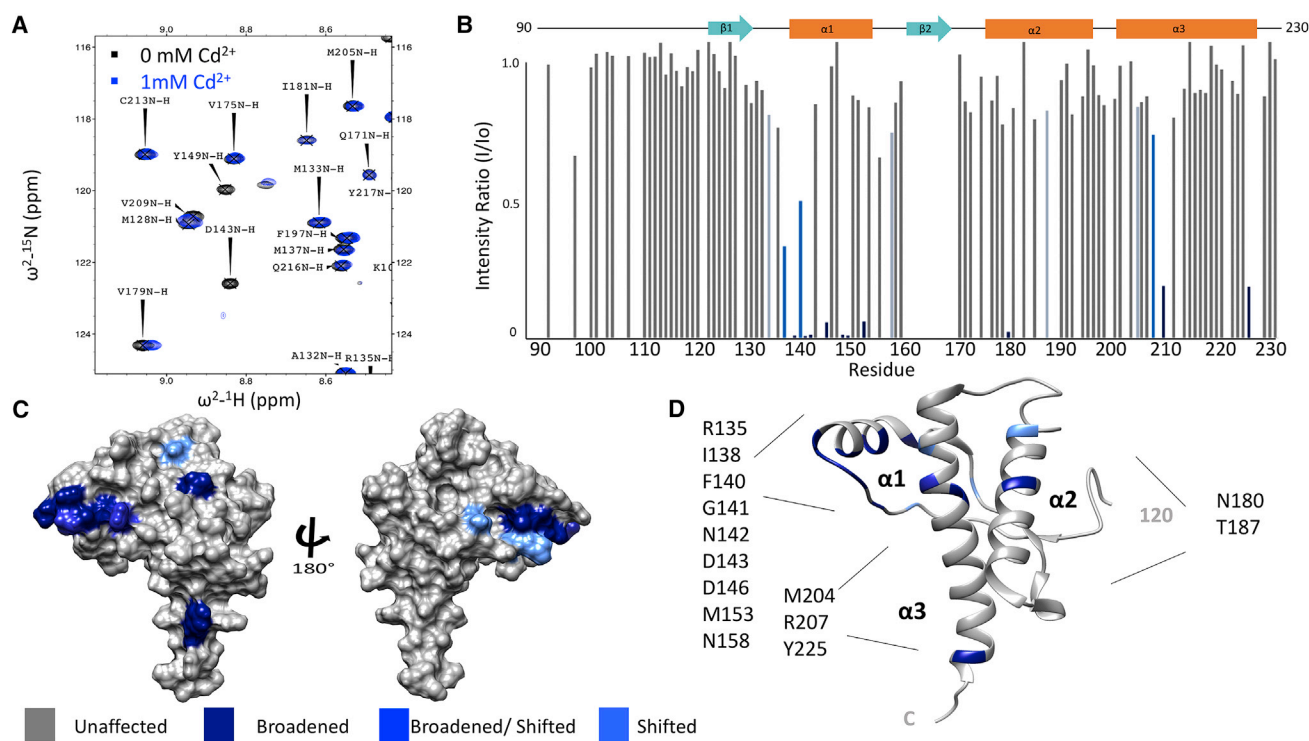


FIGURE 6 Cd^{2+} -promoted interdomain interaction PrP^C(E199K) is significantly weakened relative to the wild-type. The PrP^C mutation E200K (E199K in mouse) correlates with the human prion CJD. (A) A selected region of the ^1H - ^{15}N HSQC of wild-type PrP in the absence of metal (black) and in the presence of 1.0 mM of Cd^{2+} (blue). (B) A bar graph of I/I_0 for residues 90–23 of PrP^C in the presence of Cd^{2+} . (C and D) Surface and ribbon plots, respectively, of C-terminal residues affected by the presence of Cd^{2+} (coordinates from PDB: 1XYX). Affected residues are noted specifically on the ribbon diagram.

Given the remarkable chemical shift sensitivity of ^{113}Cd , the agreement between the isolated OR and the full-length protein provides strong evidence that the OR alone is responsible for direct Cd^{2+} coordination (23). Consistent with this proposal, elimination of N-terminal His residues leads to a significant change in ^{113}Cd chemical shift. Moreover, analysis of the fast-exchange signal finds a calculated chemical shift of the Cd^{2+} -PrP^C species to be consistent with coordination by three- or four-His nitrogen atom configuration (35,45). Together, these findings with Cd^{2+} as a surrogate support our previous proposal that PrP^C takes up Zn^{2+} with three- or four-His coordination in the repeat domain.

After our chemical mapping studies (10), several extended x-ray absorption fine-structure (EXAFS) and related x-ray studies investigated Zn^{2+} coordination to PrP^C (46,47). Stellato et al. probed the competition between Zn^{2+} and Cu^{2+} coordination and showed that at low Cu^{2+} occupancy, Zn^{2+} partially displaces His side chains from the copper centers (48). In a separate study, this group found that Zn^{2+} does not fully coordinate to all OR His residues but instead may facilitate OR peptide clustering (47). Given that this work was performed exclusively on OR peptides, it is not clear whether this type of metal ion-assisted cross-linking applies to full-length PrP^C. Pushie et al. (46) combined density functional theory and EXAFS to carefully examine Zn^{2+} occupation of the OR. Interestingly, their calculations found little energy difference between Zn^{2+} coordinated to three imidazole groups and a single water versus Zn^{2+} coordinated to four imidazoles. This finding was supported by analysis of the EXAFS data, which were well fit with a model of only three imidazole groups (46). Our findings here suggest three- or four-His coordination as determined by extrapolation from the fast-exchange signal and comparison of the estimated chemical shift to that of known His-rich Cd^{2+} complexes. As such, our result, although an approximation, is consistent with the findings of Pushie et al. (46).

A surprising finding of our study is the significant change in chemical shift associated with the E199K mutation. In humans, the parallel E200K mutation potently confers familial CJD (44,49). Residue 200 is located near the N-terminal end of $\alpha 3$, so it is therefore reasonable to hypothesize that this mutation destabilizes the protein thereby promoting aggregation (6). However, biophysical studies with NMR and circular dichroism find that PrP^C(E200K) maintains the same fold and stability as the wild-type protein (15,50,51). Residue E200 contributes to a highly conserved, negatively charged electrostatic patch on the PrP^C C-terminal domain (15). In our previous investigations with PrP^C- Zn^{2+} binding, we noted that all C-terminal disease promoting mutations involving acidic or basic residues results in a reduction of the patch's negative charge character. Using ^1H - ^{15}N HSQC NMR, we directly tested the influence of the Glu \rightarrow Lys mutation and found a significant loss of the Zn^{2+} -promoted *cis* interaction, especially in the vicinity of

$\alpha 3$ (15). This finding led to the proposal of a new paradigm for understanding the E200K and related mutations in which alteration of the protein's electrostatics results in a weakening of the regulatory *cis* interaction. Our findings here take this concept further by suggesting a loss of metal ion affinity. Testing this directly with ITC indeed shows that the affinity for Zn^{2+} is reduced by the Glu \rightarrow Lys mutation, consistent with the change in ^{113}Cd chemical shift found for PrP^C(E199K) possibly from a loss of nitrogen coordination.

Integrating the observations above suggests a new scheme for understanding the role of residue E200 in stabilizing the PrP^C *cis* interaction. The ^{113}Cd NMR spectra comparing wild-type PrP^C with the OR peptide reflects little change in the metal ion coordination environment. However, mutation to Lys causes a loss of the observed PrP^C *cis* interaction promoted by either Zn^{2+} or Cd^{2+} . These findings may be explained by proposing that E200 contributes to the second coordination sphere of the metal ion. Previous work by Spevacek et al. (15) used molecular dynamics simulations with distance restraints from DEER EPR to localize the OR- Zn^{2+} domain relative to the interfacial binding surface of the C-terminal domain. Inspection of the coordinates from these simulations finds that the side-chain carboxylate of E200 is within hydrogen bonding distance of an imidazole ring NH opposite to the nitrogen that coordinates Zn^{2+} , as shown in Fig. 1. In this paradigm, the mutation E200K does not destabilize the C-terminal domain; instead mutation of the acidic glutamate results in the loss of a Glu-His hydrogen bond that is essential for regulating the otherwise toxic PrP^C N-terminal executive domain.

In summary, ^{113}Cd NMR has provided new and important insights into metal ion coordination in the PrP. Our studies continue to support the concept of an interdomain *cis* interaction promoted by coordination of Zn^{2+} to the OR; mutations that weaken this interaction correlate with inherited prion disease. This concept may prove useful in the continued study of PrP^C function and treatment of prion diseases.

SUPPORTING MATERIAL

Two figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(19\)30021-9](http://www.biophysj.org/biophysj/supplemental/S0006-3495(19)30021-9).

AUTHOR CONTRIBUTIONS

K.A.M. and G.L.M. designed the experiments. K.A.M., G.P.R., R.B.L., and H.-W.L. performed the experiments. K.A.M. and G.L.M. wrote the manuscript. K.A.M., G.P.R., and G.L.M. edited the manuscript.

ACKNOWLEDGMENTS

The authors extend their sincere gratitude to professor Ian M. Armitage, University of Minnesota, for helpful advice on the use of ^{113}Cd as an

NMR probe of metal ion coordination centers and to Kevin Schilling, University of California, Santa Cruz, who generously contributed protein for ¹¹³Cd NMR studies. We further thank Dr. Mark Swanson, PhD, San Francisco State University NMR Facility Manager, for help with acquisition of ¹¹³Cd spectra.

This work was funded by National Institutes of Health instrumentation grant S10OD018455, which supported acquisition of the University of California, Santa Cruz 800 MHz NMR spectrometer, and National Institutes of Health research grant R01GM065790 (awarded to G.L.M.). The San Francisco State University NMR instrument was supported by National Science Foundation Major Research Instrumentation grant DBI1625721.

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