

# Unique Structural Features of Mule Deer Prion Protein Provide Insights into Chronic Wasting Disease

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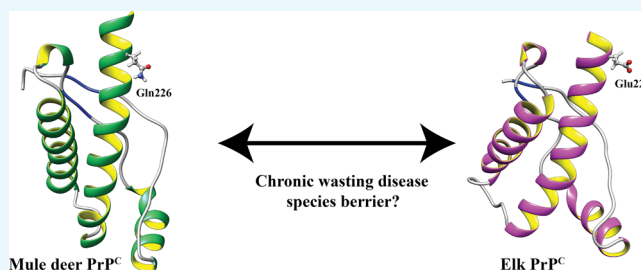
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## Supporting Information

**ABSTRACT:** Chronic wasting disease (CWD) is a highly infectious prion disease of cervids. Accumulation of prions, the disease-specific structural conformers of the cellular prion protein (PrP<sup>C</sup>), in the central nervous system, is the key pathological event of the disorder. The analysis of cervid PrP<sup>C</sup> sequences revealed the existence of polymorphism at position 226, in which deer PrP contains glutamine (Q), whereas elk PrP contains glutamate (E). The effects of this polymorphism on CWD are still unknown. We determined the high-resolution nuclear magnetic resonance structure of the mule deer prion protein that was compared to previously published PrP structures of elk and white-tailed deer. We found that the polymorphism Q226E could influence the long-range intramolecular interactions and packing of the  $\beta$ 2– $\alpha$ 2 loop and the C-terminus of the  $\alpha$ 3 helix of cervid PrP structures. This solvent-accessible epitope is believed to be involved in prion conversion. Additional differences were observed at the beginning of the well-defined C-terminus domain, in the  $\alpha$ 2– $\alpha$ 3 region, and in its interactions with the  $\alpha$ 1 helix. Here, we highlight the importance of the PrP structure in prion susceptibility and how single amino acid differences might influence the overall protein folding.



## INTRODUCTION

Chronic wasting disease (CWD) is an infectious prion disease of free-ranging cervids. It has been reported in both captive and wild cervid species, including elk (*Cervus canadensis*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), red deer (*Cervus elaphus*), reindeer (*Rangifer tarandus*), and moose (*Alces alces*).<sup>1–7</sup> The disease has now been reported in 26 states of the United States, three provinces of Canada, South Korea, Norway, Finland, and Sweden.<sup>7–12</sup>

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are rapid, progressive, and devastating neurodegenerative disorders, caused by misfolding events of the main  $\alpha$ -helical cellular prion protein (PrP<sup>C</sup>) to a  $\beta$ -sheet-enriched, partially protease-resistant, and infectious isoform (PrP<sup>Sc</sup> or prion).<sup>13,14</sup> Although there is a lack of high-resolution three-dimensional (3D) structural data for PrP<sup>Sc</sup> (mostly because of its insolubility and propensity to

aggregate),<sup>15</sup> the PrP<sup>C</sup> structure has been solved by solution-state nuclear magnetic resonance (NMR) and crystallographic methods. PrP<sup>C</sup> consists of a highly flexible N-terminal segment (residues 23–124) and a folded C-terminus domain (residues 125–231).<sup>15,16</sup> The C-terminus domain contains three  $\alpha$ -helices, spanning residues 144–154 ( $\alpha$ 1), 173–194 ( $\alpha$ 2), and 200–228 ( $\alpha$ 3), and two short antiparallel  $\beta$ -strands comprising residues 128–131 ( $\beta$ 1) and 161–164 ( $\beta$ 2). The PrP expressed by mammalian species exhibits a similar fold, with the local sequence and structure variations most prominently localized at the interface of the  $\beta$ 2– $\alpha$ 2 loop and in the C-terminus part of the  $\alpha$ 3 helix.<sup>17</sup>

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of NMR spectroscopy. A comparison to previously determined PrP structures from the white-tailed deer and Rocky Mountain elk<sup>36,37</sup> provides insights that may contribute to our understanding of how the single polymorphism Q226E between deer and elk can alter the structure and help to explain the substantial differences in biochemical properties, pathogenesis, and formation of different strains of CWD prions among cervids.<sup>38</sup> We hypothesized that the presence of polymorphism Q226E, as the most critical for CWD among the six identified differences in amino acid sequences, could influence the long-range intramolecular interactions including the packing of the  $\beta 2$ - $\alpha 2$  loop and the C-terminus of the  $\alpha 3$  helix. This solvent-accessible epitope has been studied greatly in view of its role in prion conversion.<sup>39,40</sup> Additionally, the changes from the neutral to negatively charged side chain at position 226 will influence the electrostatic surface potential in this region, which is of great relevance for the intermolecular interactions between PrP<sup>C</sup> and PrP<sup>Sc</sup> among cervids.

## RESULTS AND DISCUSSION

**Amino Acid Alignment and mdPrP Construct.** The amino acid sequences of PrPs from various cervid subspecies related to CWD are highly evolutionary-conserved. The alignment of amino acid sequences of mdPrP, white-tailed deer (wtdPrP), elk (ePrP), red deer PrP (reddPrP), American moose PrP (amPrP), Eurasian moose PrP (emPrP), and reindeer PrP (rdPrP) showed differences in the amino acid residues at positions 109, 123, 138, 176, 209, and 226 (Figure 1; numbering is based on the amino acid sequence of the mdPrP construct used herein for structure determination). A simple perusal of the differences shows that the three of them are positioned within the well-defined secondary structural elements. Truncated recombinant mdPrP from residues 94 to 233 with serine at position 138 and glutamine at position 226 was used for structural characterization and comparison with previously resolved wtdPrP and ePrP structures.

**Resonance Assignment and Structure Calculation.** The <sup>15</sup>N-heteronuclear single quantum coherence (HSQC) spectrum of <sup>13</sup>C, <sup>15</sup>N doubly labeled mdPrP presents a favorable dispersion of cross-peaks, indicating a high potential for in-depth structural determination (Figure 2). Standard two-dimensional and 3D NMR experiments were used for the assignment of backbone and side-chain resonances of mdPrP. In short, the sequence-specific assignment of the backbone <sup>1</sup>H, <sup>15</sup>N, <sup>13</sup>C <sub>$\alpha$</sub> , <sup>13</sup>C <sub>$\beta$</sub> , and <sup>13</sup>CO resonances for mdPrP was obtained using the <sup>15</sup>N-HSQC spectrum and triple-resonance HNCO, HN(CO)CA, HNCA, CBCA(CO)NH, and HNCACB experiments.<sup>41</sup> The <sup>1</sup>H and <sup>13</sup>C resonances of aliphatic and aromatic side chains were assigned using <sup>13</sup>C-HSQC in combination with HAHB(CO)NH, CC(CO)NH, (H)CCH- total correlation spectroscopy (TOCSY), and <sup>13</sup>C-edited nuclear Overhauser enhancement spectroscopy (NOESY)-HSQC experiments.<sup>42</sup> NOE contacts were determined in 3D <sup>15</sup>N and <sup>13</sup>C-edited NOESY-HSQC experiments. The overall completeness of chemical shift assignment was 99.1%. Noteworthy, the side-chain resonances including CH <sub>$\epsilon$</sub>  of His<sup>99</sup>, His<sup>114</sup>, and Tyr<sup>152</sup> and CH <sub>$\zeta$</sub>  of Phe<sup>178</sup> and Phe<sup>201</sup> could not be unambiguously assigned; however, this agrees very well with the final structure and properties of the studied protein. We have considered the use of residual dipolar couplings for structure improvement, especially interhelical orientations. However, the use of aligning media was showed to induce sample precipitation of PrPs.

The high-resolution structure of mdPrP was calculated using 545 intraresidual, 618 sequential, 482 medium-range, and 559 long-range distance restraints complemented with 168 backbone torsion angle restraints (Table 1). The calculated

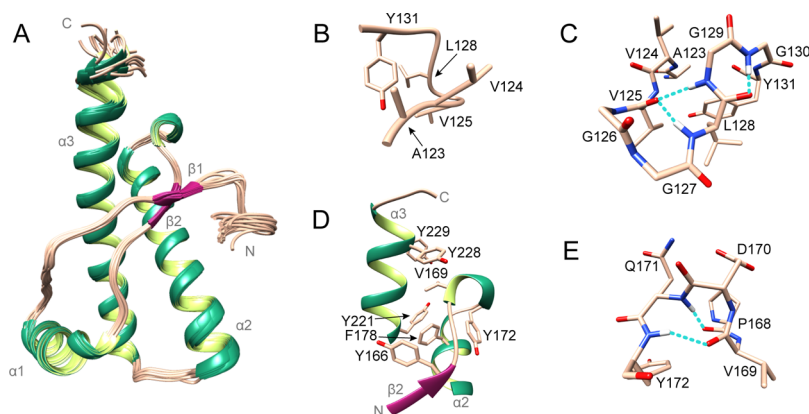
**Table 1. NMR Restraints and Structural Statistics for an Ensemble of 20 Lowest Energy Structures of mdPrP**

NOE upper distance limits <sup>a</sup>	
total	2204
intraresidue ( $ i - j  = 0$ )	545
sequential ( $ i - j  = 1$ )	618
medium-range ( $1 <  i - j  < 5$ )	482
long-range ( $ i - j  \geq 5$ )	559
torsion angle restraints <sup>a</sup>	
backbone ( $\varphi/\psi$ )	168
rmsd to the mean coordinates (Å)	
ordered backbone atoms (123–231)	0.42 ± 0.08
ordered heavy atoms (123–231)	0.78 ± 0.09
Ramachandran plot (123–231) <sup>b</sup>	
residues in most favored regions (%)	95.0
residues in additional allowed regions (%)	5.0
structure Z scores <sup>b</sup>	
first generation packing quality	0.763 ± 0.513
second generation packing quality	5.102 ± 1.464
Ramachandran plot appearance	−0.453 ± 0.282
chi-1/chi-2 rotamer normality	−3.717 ± 0.483
backbone conformation	−0.541 ± 0.234
rms Z scores <sup>b</sup>	
bond lengths	1.143 ± 0.003
bond angles	0.465 ± 0.011
omega angle restraints	0.481 ± 0.032
side-chain planarity	0.358 ± 0.029
improper dihedral distribution	0.571 ± 0.017
inside/outside distribution	1.030 ± 0.011

<sup>a</sup>None of the 20 structures exhibits distance violations over 0.2 Å and torsion angle violation over 5°. <sup>b</sup>ensemble of structures was analyzed by PROCHECK-NMR<sup>43</sup> and WhatIF programs incorporated in ICING structure evaluation package<sup>44</sup> and PSVS.<sup>45</sup>

structure of mdPrP (PDB ID: 6FNV) is composed of two distinct domains. The highly disordered N-terminal domain consists of residues from Gly<sup>94</sup> to Gly<sup>122</sup>, whereas the well-defined C-terminus domain is composed of residues from Ala<sup>123</sup> to Ala<sup>233</sup> and exhibits a backbone root-mean-square deviation (rmsd) of 0.42 Å (Figure 3A and Table 1).

The C-terminus domain of mdPrP is characterized by a compact set of three  $\alpha$ -helices and a short antiparallel  $\beta$ -sheet. The  $\alpha 1$  helix is composed of residues from Asp<sup>147</sup> to Asn<sup>156</sup> and is followed by the  $3_{10}$ -helix turn from Met<sup>157</sup> to Arg<sup>159</sup>. The geometry of  $\alpha 1$  helix is classified as kinked according to the HELANAL web server.<sup>46,47</sup> The  $\alpha 2$  and  $\alpha 3$  helices are longer than the  $\alpha 1$  helix and are composed of residues from Gln<sup>175</sup> to Lys<sup>197</sup> and from Glu<sup>203</sup> to Gln<sup>230</sup>, respectively. The geometries of  $\alpha 2$  and  $\alpha 3$  helices are linear and of curved type, respectively. The helices  $\alpha 2$  and  $\alpha 3$  form a twisted V-shaped skeleton that serves as a platform for anchoring the  $\alpha 1$  helix and  $\beta$ -sheet. The antiparallel  $\beta$ -sheet is formed at the beginning of the C-terminus domain and consists of two  $\beta$  strands,  $\beta 1$  and  $\beta 2$ , that are composed of residues from Met<sup>132</sup> to Leu<sup>133</sup> and from Tyr<sup>165</sup> to Tyr<sup>166</sup>, respectively. The structure of mdPrP is



**Figure 3.** Structure of mdPrP. (A) Ensemble of 20 lowest energy structures of mdPrP (residues from Ala<sup>123</sup> to Ala<sup>233</sup>).  $\alpha$ -Helices and  $3_{10}$ -helix are colored green,  $\beta$ -sheets are colored magenta, and loops are colored champagne pink. (B) Well-defined region between residues Ala<sup>123</sup> and Tyr<sup>131</sup>. (C) Residues from Ala<sup>123</sup> to Tyr<sup>131</sup> involved in the formation of  $\alpha$ -helical turn (Val<sup>125</sup>–Leu<sup>128</sup>) and  $\gamma$ -turn (Leu<sup>128</sup>–Gly<sup>130</sup>). (D) Hydrophobic pocket in the proximity of the  $\beta 2$ – $\alpha 2$  loop and the C-terminus of the  $\alpha 3$  helix. (E)  $3_{10}$ -Helix from residues Pro<sup>168</sup> to Tyr<sup>172</sup> inside the  $\beta 2$ – $\alpha 2$  loop. Residues are presented as sticks in champagne pink and the hydrogen bonds in panels (C,E) are shown as dashed lines in cyan.

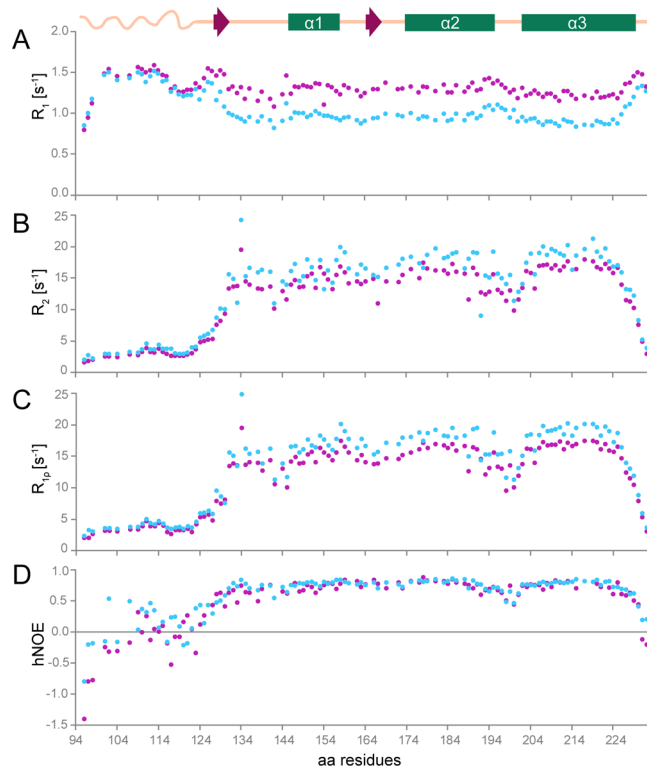
stabilized by a disulfide bond between Cys<sup>182</sup> and Cys<sup>217</sup> that is located in the middle of  $\alpha 2$  and  $\alpha 3$  helices.

**Unique Structural Features of the mdPrP Protein.** We observed the structuring of the region at the beginning of the C-terminus domain of mdPrP. This region consists of nine residues from Ala<sup>123</sup> to Tyr<sup>131</sup> and adopts a well-defined structure with the backbone rmsd of 0.22 Å (Figure 3B). It is characterized by an  $\alpha$ -helical turn and a  $\gamma$ -turn that are stabilized by three hydrogen bonds (Figure 3C). The carbonyl group of Val<sup>125</sup> is involved in a bifurcated hydrogen bond with the amide protons of Leu<sup>128</sup> and Gly<sup>129</sup> in the  $\alpha$ -helical turn. In addition, a hydrogen bond is formed between the carbonyl group of Leu<sup>128</sup> and the amide proton of Gly<sup>130</sup> in the  $\gamma$ -turn. An hydrophobic pocket in this region is formed by Val<sup>125</sup>, Leu<sup>128</sup>, and Tyr<sup>131</sup> (Figure 3B,C).

Additionally, the  $\beta 2$ – $\alpha 2$  loop of the mdPrP structure is well defined with one turn of  $3_{10}$ -helix from the residues Val<sup>169</sup> to Gln<sup>171</sup> (Figure 3D) held together by the Gln<sup>171</sup>H<sup>N</sup>–Pro<sup>168</sup>O and Tyr<sup>172</sup>H<sup>N</sup>–Val<sup>169</sup>O hydrogen bonds (Figure 3E). The  $\beta 2$ – $\alpha 2$  loop is further stabilized by hydrophobic and aromatic interactions with the nearby amino acid residues at the C-terminus of the  $\alpha 3$  helix. The hydrophobic pocket defined by the  $\beta 2$ – $\alpha 2$  loop and the C-terminus of the  $\alpha 3$  helix is composed of residues Tyr<sup>166</sup>, Val<sup>169</sup>, Tyr<sup>172</sup>, Phe<sup>178</sup>, Tyr<sup>221</sup>, and Tyr<sup>228</sup> (Figure 3D).

The above structuring of the region before the well-defined C-terminus domain and the  $\beta 2$ – $\alpha 2$  loop is supported further with the study of backbone dynamics. We analyzed 118 resolved amide resonances of mdPrP on a fast (picoseconds to nanoseconds) timescale with the use of <sup>15</sup>N relaxation time measurements at two magnetic fields (14.1 and 18.8 T). The resulting <sup>15</sup>N longitudinal ( $R_1$ ), transverse ( $R_2$ ), and rotating frame ( $R_{1\rho}$ ) relaxation rates combined with <sup>1</sup>H–<sup>15</sup>N heteronuclear NOE (hNOE) conform to the flexible N-terminal tail and a well-structured C-terminus domain of the mdPrP structure (Figure 4).

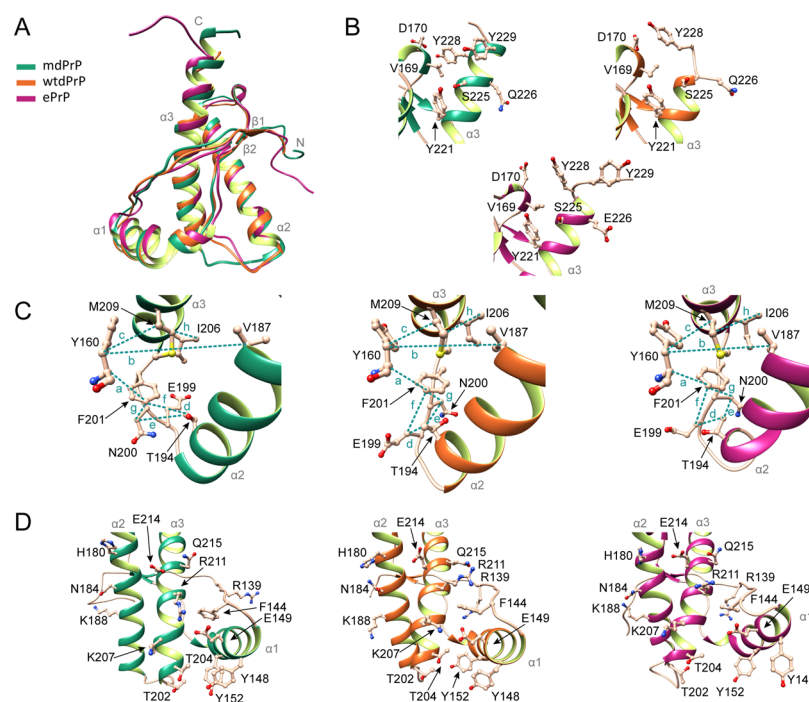
Furthermore, <sup>15</sup>N relaxation data indicate structuring for residues from Ala<sup>123</sup> to Tyr<sup>131</sup> at the beginning of the C-terminus domain and for the  $\beta 2$ – $\alpha 2$  loop. On the other hand, <sup>15</sup>N relaxation data show an increased mobility for residues from Lys<sup>197</sup> to Phe<sup>201</sup> that connect  $\alpha 2$  and  $\alpha 3$  helices and for residues from Tyr<sup>229</sup> to Ala<sup>233</sup> at the C-terminus of the mdPrP structure. However, the relative lower values of  $R_2$  and  $R_{1\rho}$



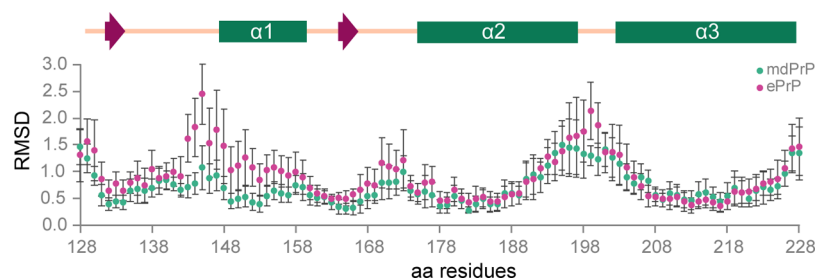
**Figure 4.** <sup>15</sup>N amide backbone relaxation rates and hNOE of mdPrP. (A) <sup>15</sup>N longitudinal ( $R_1 = 1/T_1$ ), (B) transverse ( $R_2 = 1/T_2$ ), (C) spin–lattice relaxation rates in the rotation frame ( $R_{1\rho} = 1/T_{1\rho}$ ), and (D) hNOE at 298 K at a magnetic field of 14.1 (magenta) and 18.8 T (blue). A schematic presentation of the secondary structure elements of mdPrP is at the top of the figure. For clarity, error bars are not shown here as they are within the size of the data points in the above graphics but are presented in the Supporting Information in Figure S1.

relaxation rates for residues around Ile<sup>142</sup> and Gln<sup>189</sup> are not indicative of a well-defined secondary structure and suggest more complex dynamics coupled to their intricate tertiary interaction. Few amino acid residues could not be analyzed because of the cross-peak overlap (for details, see Methods).

**Comparison of Structures of mdPrP and Other Cervids.** Cervid prion proteins exhibit a well-conserved



**Figure 5.** Comparison of mdPrP, wtdPrP, and ePrP structures. (A) Superposition of well-defined C-terminus domains from amino acids Ala<sup>123</sup>–Ala<sup>233</sup> of mdPrP (green), wtdPrP (orange), and ePrP (magenta). The selected residues are presented as ball-and-stick and colored in champagne pink with marked heteroatoms. (B) Structural diversity at the end of the  $\alpha_3$  helix and the  $\beta_2$ – $\alpha_2$  loop. (C) Spatial orientation of residues in the proximity of the  $\alpha_2$ – $\alpha_3$  loop with marked distances. Selected distances among residues are indicated with dashed lines and small letters (see Table 2 for distance information). (D) Structural differences in orientations at the  $\alpha_1$  helix with respect to the  $\alpha_2$ – $\alpha_3$  V-shaped skeleton.



**Figure 6.** Local rmsd values for backbone atoms per residue (from 128 to 228) of mdPrP (green) and ePrP (magenta) with respect to the wtdPrP structure that was determined by X-ray. Standard deviations are reported for the ensemble of 20 lowest energy structures of mdPrP and ePrP.

amino acid sequence, which may suggest similarity of their 3D structures. We compared our mdPrP structure with the structures of previously determined PrPs from white-tailed deer and Rocky mountain elk and observed several differences. For easier comparison of cervids' PrP structures, we unified the residue numbering based on the mdPrP amino acid sequence. Here, we have to mention that the wtdPrP structure was determined in complex with an antibody fragment POM1 at pH 6.8; however, the PDB entry for wtdPrP includes residues from 128 to 228 (PDB ID: 4YXH).<sup>36</sup> The ePrP structure consists of residues from 124 to 234 (PDB ID: 1XYW) and was determined at pH 4.5.<sup>37</sup> We used pH of 5.5 for structure determination because the lower pH prevents aggregation and enables longevity of the prion protein samples that is necessary for structure determination by NMR spectroscopy. Additionally, it has been suggested that misfolding of PrPs in prion disease occurs in endosomes that exhibit a low pH (pH  $\approx$  5).<sup>48</sup> In general, the fold of mdPrP is grossly similar to wtdPrP and ePrP structures (Figure 5A), even though the structures were determined under different sample conditions.

We compared the chemical shifts ( $\delta$ ) of amide proton ( $H^N$ ),  $C\alpha$ , and  $C\beta$  atoms of amino acids from 124 to 233 between the mdPrP and ePrP structures determined by NMR spectroscopy (Figure S2). The chemical shifts of  $H^N$ ,  $C\alpha$ , and  $C\beta$  atoms of mdPrP and ePrP showed good agreement along the sequence. Slight, if not negligible, differences have been observed for  $\delta(H^N)$  and  $\delta(C\alpha)$  for the amino acid residues in the  $\alpha_2$ – $\alpha_3$  loop, which could indicate different long-range interactions among amino acids in this region. The calculated rmsd for the protein backbone of the three compared structures (residues 128–228) is 1.2 Å. The local backbone rmsd values per residue are in good agreement with the observed differences among the examined structures (Table S1, Figures 5 and 6). The main differences in backbone rmsd values between the mdPrP and ePrP structures have been detected at the N-terminal of the  $\alpha_1$  helix and at the  $\alpha_2$ – $\alpha_3$  loop (Figure 6).

However, despite a very high level of numerical similarity, structural differences are observed at the beginning of the C-terminus domains,  $\beta_2$ – $\alpha_2$  loops and their interactions with  $\alpha_3$

helices, at the beginning of  $\alpha 1$  helices and their interactions with  $\alpha 2$  and  $\alpha 3$  helices, and  $\alpha 2$ – $\alpha 3$  loops (Figures 5 and S3).

Residues from Val<sup>124</sup> to Gly<sup>130</sup> in ePrP exhibit an extended backbone conformation. Similarly, the residues from Leu<sup>128</sup> to Gly<sup>130</sup> in the wtdPrP structure have no observed structuring. Both regions of ePrP and wtdPrP structures exhibit no hydrogen bonds in contrast to mdPrP, where we observed three hydrogen bonds (Figures 3C, 5A and S3). No hydrophobic pocket was observed in this region of ePrP or wtdPrP structures, whereas mdPrP exhibits a well-defined hydrophobic pocket composed by the residues Val<sup>125</sup>, Leu<sup>128</sup>, Tyr<sup>131</sup>, and Ile<sup>185</sup>. The backbone structures of the three proteins exhibit similar conformations after the residue Gly<sup>130</sup>. Interestingly, within the examined structures, Tyr<sup>131</sup> exhibits a diverse side-chain orientation and distinct stacking with Tyr<sup>166</sup> and Ile<sup>185</sup>. The relative position of Tyr<sup>131</sup> side chain and its interactions with the residues in proximity might have an impact on the formation of the  $\alpha$ -helical turn and  $\gamma$ -turn in mdPrP in contrast to wtdPrP and ePrP. Solvent accessibility analysis with the GETAREA program<sup>49,50</sup> showed that the Tyr<sup>131</sup> residue is protected from exchange with solvents in all three structures (Figure S4). However, the distance between Tyr<sup>131</sup> and Ile<sup>185</sup> in the  $\alpha 2$  helix is 2 times longer in mdPrP and ePrP structures in comparison to the wtdPrP structure (Table S2). In contrast, the distance between Leu<sup>128</sup> and Ile<sup>185</sup> is shorter in the mdPrP structure with respect to the distances in ePrP and wtdPrP structures (Table S2). These observations indicate differences in interactions between the  $\beta 1$ – $\alpha 1$ – $\beta 2$  and  $\alpha 2$ – $\alpha 3$  subdomains of the compared structures. It was previously proposed that different side-chain orientations of Tyr<sup>131</sup> play an important role in the interactions between these subdomains and furthermore could also affect the flexibility of the  $\beta 2$ – $\alpha 2$  loop region.<sup>36,55</sup> In this way, the region from the residues Ala<sup>123</sup> to Tyr<sup>131</sup> could additionally stabilize the mdPrP structure through its interactions with the antiparallel  $\beta$ -sheet and  $\alpha 2$  helix and in this way prevent PrP<sup>C</sup>-to-PrP<sup>Sc</sup> conversion.

The  $\beta 2$ – $\alpha 2$  loop of mdPrP comprising residues 168–178 is well-defined, composed of  $3_{10}$ -helix, and stabilized with aromatic and hydrophobic interactions with the nearby residues. The backbone orientations of  $\beta 2$ – $\alpha 2$  loops of the three examined structures are similar, whereas small differences are notable in the side-chain orientations of Val<sup>169</sup> and Asp<sup>170</sup> (Figure 5B). Moreover, different orientations of glutamine and asparagine side chains in the  $\beta 2$ – $\alpha 2$  loops (residues Gln<sup>171</sup>, Asn<sup>173</sup>, Asn<sup>174</sup>, and Asn<sup>176</sup>) are observed and might be related to the long-range interactions and orientations of the aromatic moiety of Tyr<sup>131</sup> in the three compared structures (Figure S3). The structural features of  $\beta 2$ – $\alpha 2$  loop have been extensively discussed in the literature.<sup>22,56–60</sup> The presence of Asn/Gln residues in the  $\beta 2$ – $\alpha 2$  loop can be a strong determinant for prion conversion that overrides the differences in the sequence and has influence on the appearance of prions according to the zipper model between the cervid and human PrP.<sup>22</sup> Moreover, insertion of additional Gln residues into the  $\beta 2$ – $\alpha 2$  loop of mouse PrP promotes prion protein conversion,<sup>61–63</sup> whereas several substitutions (at positions 169, 171, 173, and 177; residues numbering based on the mdPrP sequence) in the  $\beta 2$ – $\alpha 2$  loop of PrP<sup>C</sup> are believed to prevent the spontaneous prion formation by influencing the structural stability of the  $\beta 2$ – $\alpha 2$  loop.<sup>56–59,64</sup>

Additionally, the structures and interactions of side chains in the  $\beta 2$ – $\alpha 2$  loops are influenced by the orientations of side chains in the C-terminus of the  $\alpha 3$  helix including the residue

at position 226. The  $\alpha 3$  helix of wtdPrP is shorter and ends with Ser<sup>225</sup>, possibly because of the shorter amino acid sequence<sup>36</sup> with respect to the mdPrP structure. The C-terminus of ePrP protein is unstructured after Tyr<sup>228</sup> (Figure 5B). Tyr<sup>228</sup> and Tyr<sup>229</sup> have different side-chain orientations in mdPrP in comparison to the ePrP structure, as a result of their distinct relative orientation, that lead to hydrophobic interactions with the residues Val<sup>169</sup>, Asp<sup>170</sup>, and Ser<sup>225</sup> and additionally stabilize the end of the C-terminus part in mdPrP (Figure 5B and Table S2). Tyr<sup>228</sup> of the wtdPrP structure is involved in stacking interactions with Asp<sup>170</sup> in the  $\beta 2$ – $\alpha 2$  loop. However, the interactions between the residues at the end of the  $\alpha 3$  helix and Gln<sup>226</sup> are not observed in wtdPrP, resulting in higher solvent accessibility of Ser<sup>225</sup> and Gln<sup>226</sup> in comparison to mdPrP and ePrP (Figures 5B and S3). Importantly, beside the polymorphism Q226E, mule deer exhibits serine-to-asparagine polymorphism at position 138, which is processed as a pseudogene,<sup>69,70</sup> and serine-to-phenylalanine polymorphism at codon 225.<sup>30</sup> Allele Phe<sup>225</sup> in mule deer could contribute to CWD resistance in view of the reported prolonged incubation period with respect to the Ser<sup>225</sup> mule deer homozygote.<sup>30</sup> Interestingly, it has been shown that polymorphisms at residues 225 and 226 affect the interactions between the  $\beta 2$ – $\alpha 2$  loop and  $\alpha 3$  helix and therefore prion propagation within deer and elk.<sup>40,71</sup> Our results showed that Ser<sup>225</sup> is protected from solvents as it is involved in the interaction with Tyr<sup>228</sup> in mdPrP. Additionally, Tyr<sup>228</sup> in mdPrP is protected from solvents by the stacking interaction with Val<sup>169</sup>. These data contribute to the understanding at the molecular level and are in agreement with the structural and molecular dynamics studies of inter- and intraspecies PrP transmission related to cervids that pointed out a critical role of residues 225 and 226 in PrP<sup>C</sup>-to-PrP<sup>Sc</sup> conversion and strain propagation.<sup>40</sup>

In the three structures,  $\alpha 2$  and  $\alpha 3$  helices form a V-shaped skeleton that slightly differs in the spatial orientation of the helices. The interhelical angle between the  $\alpha 2$  and  $\alpha 3$  helices of mdPrP is 44.5°, whereas its value in wtdPrP and ePrP is 49.8 and 52.2°, respectively. The hydrophobic and aromatic residues of  $\alpha 2$  and  $\alpha 3$  helices have preserved architectures that are stabilized by a disulfide bond in the three structures. However, significant differences are observed for the side-chain orientations of His<sup>190</sup> and Thr<sup>194</sup> in the  $\alpha 2$  helix and Ile<sup>206</sup>, Met<sup>209</sup>, and Glu<sup>210</sup> in the  $\alpha 3$  helix (Figures 5C and S1). These residues are spatially close to the loop that connects  $\alpha 2$  and  $\alpha 3$  helices. Surprisingly, the  $\alpha 2$ – $\alpha 3$  loop of mdPrP exhibits a unique backbone conformation with different orientations of the side chains of Glu<sup>199</sup>, Asn<sup>200</sup>, and Phe<sup>201</sup> with respect to wtdPrP and ePrP. However, the hydrophobic interactions of Phe<sup>201</sup> and Tyr<sup>160</sup> are preserved in the three structures. Major differences are observed for distances Tyr<sup>160</sup>C <sub>$\alpha$</sub> –Val<sup>201</sup>C <sub>$\beta$</sub>  and Glu<sup>199</sup>C <sub>$\beta$</sub> –Phe<sup>201</sup>C <sub>$\zeta$</sub>  that are up to 2 Å longer in mdPrP with respect to wtdPrP and ePrP (Table 2). The opposite is observed for distances Thr<sup>194</sup>C <sub>$\gamma 2$</sub> –Asn<sup>200</sup>C <sub>$\beta$</sub> , Asn<sup>200</sup>C <sub>$\beta$</sub> –Phe<sup>201</sup>C <sub>$\zeta$</sub> , and Ile<sup>206</sup>C <sub>$\gamma 2$</sub> –Met<sup>209</sup>C <sub>$\gamma$</sub>  that are shorter in mdPrP with respect to the other two cervid structures (Figure 5C and Table 2). The residue Glu<sup>199</sup> is more exposed to the solvents, whereas residue Asn<sup>200</sup> is less solvent-exposed in mdPrP in comparison to wtdPrP and ePrP (Figure 7). Different side-chain orientations in the  $\alpha 2$ – $\alpha 3$  loop could influence the interactions of residues in helices that are spatially close to this region. In early events of oligomerization, it is believed that the  $\alpha 1$  helix moves away from the  $\alpha 2$ – $\alpha 3$  V-shaped skeleton. This

**Table 2. Distances between C Atoms of Selected Amino Acid Residues in Proximity of the  $\alpha 2$ – $\alpha 3$  Loop in mdPrP, wtdPrP, and ePrP Structures<sup>a</sup>**

mark <sup>b</sup>	distance	mdPrP (Å)	wtdPrP (Å)	ePrP (Å)
A	Tyr <sup>160</sup> C <sub><math>\alpha</math></sub> –Phe <sup>201</sup> C <sub><math>\zeta</math></sub>	6.5 ± 0.3	5.3	5.5 ± 0.4
B	Tyr <sup>160</sup> C <sub><math>\beta</math></sub> –Val <sup>187</sup> C <sub><math>\beta</math></sub>	11.5 ± 0.4	9.1	8.9 ± 0.3
C	Tyr <sup>160</sup> C <sub><math>\beta</math></sub> –Met <sup>209</sup> C <sub><math>\beta</math></sub>	5.2 ± 0.1	4.7	5.1 ± 0.3
D	Thr <sup>194</sup> C <sub><math>\gamma 2</math></sub> –Glu <sup>199</sup> C <sub><math>\beta</math></sub>	4.8 ± 0.4	5.7	4.2 ± 0.6
E	Thr <sup>194</sup> C <sub><math>\gamma 2</math></sub> –Asn <sup>200</sup> C <sub><math>\beta</math></sub>	5.7 ± 0.5	8.3	6.4 ± 1.3
F	Glu <sup>199</sup> C <sub><math>\beta</math></sub> –Phe <sup>201</sup> C <sub><math>\zeta</math></sub>	7.3 ± 0.5	6.8	6.5 ± 0.7
G	Asn <sup>200</sup> C <sub><math>\beta</math></sub> –Phe <sup>201</sup> C <sub><math>\zeta</math></sub>	4.0 ± 0.2	7.5	7.5 ± 0.4
H	Ile <sup>206</sup> C <sub><math>\gamma 2</math></sub> –Met <sup>209</sup> C <sub><math>\gamma</math></sub>	4.8 ± 0.1	6.5	6.8 ± 0.2

<sup>a</sup>Reported distances are average values obtained from the coordinates of the structural ensemble for mdPrP (PDB id 6FNV) and ePrP (PDB id 1XYW) that were determined by NMR and for wtdPrP (PDB id 4YXH) determined by X-ray crystallography. Standard deviations are reported for the ensemble of 20 lowest energy structures for mdPrP and ePrP. <sup>b</sup>Letters specify the position of distances in Figure 5C.

is responsible for the increased local structural dynamics that is reflected in greater exposure of the amide hydrogen atoms in the  $\alpha 1$  helix of mouse PrP.<sup>65–68</sup> Importantly, the stabilization of interactions or covalent linkage by a disulfide bond between the subdomains  $\beta 1$ – $\alpha 1$ – $\beta 2$  and  $\alpha 2$ – $\alpha 3$  is supposed to prevent the oligomerization.<sup>65</sup>

Structure variations were also observed in the orientations of the three helices and at the end of the  $\alpha 1$  helix and its interactions with the  $\alpha 2$ – $\alpha 3$  loop. The aromatic interactions between Tyr<sup>148</sup> and Tyr<sup>152</sup> differ among the three structures (Figure 5D). Furthermore, 2 times shorter distances of Tyr<sup>148</sup> with Thr<sup>202</sup> and Thr<sup>204</sup> are observed in mdPrP and ePrP structures compared to wtdPrP (Table S2). A similar trend in distances was observed between Tyr<sup>152</sup> and Thr<sup>202</sup>. In contrast, the distances between Tyr<sup>152</sup> and Thr<sup>204</sup> are similar for all the three structures. Thr<sup>202</sup> and Thr<sup>204</sup> are more solvent-exposed in the ePrP structure with respect to mdPrP and wtdPrP structures (Figure 7). We observed a closer anchoring of the  $\alpha 1$  helix to  $\alpha 2$  and  $\alpha 3$  helices in the mdPrP structure with respect to ePrP and wtdPrP.

**Effect of Polymorphism Q226E on Electrostatic Surface Potential.** MdPrP, wtdPrP, and ePrP are known for their polymorphisms at positions 138 and 226. S138N and Q226E polymorphisms have a major impact on the electrostatic surface potential of the examined structures (Figure 8).

Our results show that variations in the electrostatic surface potential among the three proteins are mostly clustered at the  $\beta 1$ – $\alpha 1$  loop, at the beginning of the  $\alpha 2$  helix, at the V-shaped skeleton where the antiparallel  $\beta$  sheet is in proximity to the  $\alpha 2$

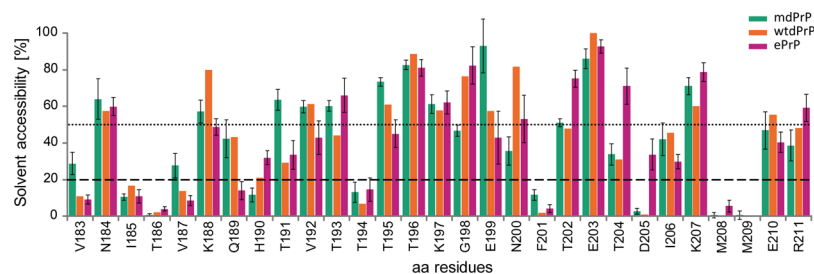
and  $\alpha 3$  helices, and at the C-terminus of the  $\alpha 3$  helix (Figure 8).

In contrast to the wtdPrP and ePrP structures, a large contiguous area of positive electrostatic potential is observed on the surface of the mdPrP structure. The region around residue 138 is positively charged in mdPrP, whereas the corresponding region in wtdPrP and ePrP proteins is neutral. Additional variations of positive charge in the mdPrP and wtdPrP structures to a predominantly neutral state in the ePrP structure are observed in the middle of  $\alpha 2$  and  $\alpha 3$  helices. Polymorphism Q226E is reflected in the charge of preferentially positive surface areas in the  $\beta 2$ – $\alpha 2$  loop and the C-terminus of the  $\alpha 3$ -helix in mdPrP and wtdPrP in comparison to the negative electrostatic potential in ePrP. However, the C-terminus of wtdPrP is negatively charged to mdPrP and ePrP. Residues 225 and 226 are located in a distal region of the  $\alpha 3$  helix that participates in interactions with the  $\beta 2$ – $\alpha 2$  loop to form a solvent-accessible contiguous epitope.<sup>72</sup> Our data suggest that different distributions of electrostatic potential between mdPrP and ePrP proteins may facilitate intramolecular interactions between two allelic variants in deer subspecies in case of S225F and Q226E polymorphisms and influence the early stages of prion conversion and neuropathology of CWD among cervids.

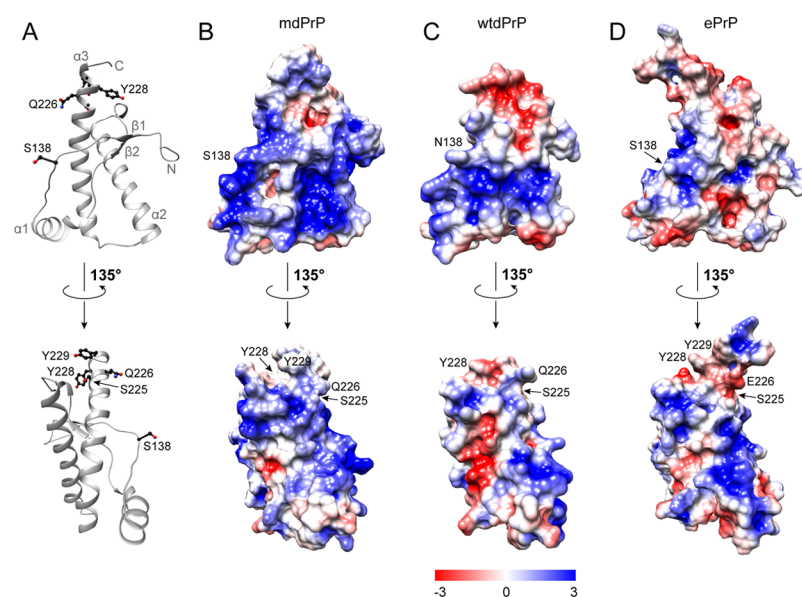
## CONCLUSIONS

The spread of CWD in North America and the most recent cases of CWD-infected cervid subspecies in Europe have raised concerns for public health and perceived risks for possible CWD transmission to humans through the consumption of CWD-infected venison.<sup>8,51–53</sup> The possible spontaneous spread of the disease among cervid subspecies in Eurasia represents a global threat.<sup>54</sup> Structural studies on mdPrP at the molecular level are important for understanding the still unknown reasons for the appearance of the detected and confirmed cases of CWD in captive mule deer<sup>3</sup> and the progressive spread and identification of the disease in other cervids.

Previous findings suggest that the primary structural differences at residue 226 identify biologically distinct prion strains on the basis of different disease progressions in deer and elk<sup>33</sup> and have a role in dictating the selection of different CWD prion strains in gene-targeted mice.<sup>35</sup> These findings suggest that the observed differences are related to an altered structure of PrP<sup>C</sup> caused by the Q226E polymorphism, highlighting the importance of amino acid sequence variations affecting the local changes of 3D structures, whereas the globular fold remains similar. A detailed comparative structural



**Figure 7.** Solvent accessibility of selected residues that belong to the  $\alpha 2$  and  $\alpha 3$  helices. Hatched and dotted lines at 20 and 50% indicate the limits of amino acid residue accessibility to solvents (>50%) or burial in solvent-inaccessible regions (<20%). Standard deviations are reported for the ensemble of 20 lowest energy structures of mdPrP and ePrP that have been determined by NMR.



**Figure 8.** Electrostatic surface potential of the three cervid PrPs. (A) Ribbon presentation of the mdPrP backbone orientation used in panels (B–D). Residues Ser<sup>138</sup>, Ser<sup>225</sup>, Gln<sup>226</sup>, Tyr<sup>228</sup>, and Tyr<sup>229</sup> are presented as ball-and-stick and colored black. Electrostatic surface potentials of (B) mdPrP, (C) wtdPrP, and (D) ePrP. Regions of positive and negative charges are depicted from blue to red according to the presented charge legend. Orientation of structures is preserved in all panels. The lower set of structures is rotated by 135°.

analysis of the examined PrP of cervids could provide insights into pathogenesis suggesting that the structures of deer and elk prion proteins may determine prion strain mutation in these cervids.

Our comparative analysis uncovered the structural determinants of mdPrP that are manifested in diverse structural rearrangements and distinct electrostatic surface potentials with respect to the wtdPrP and ePrP structures. The region at the beginning of the C-terminus domain could protect the  $\beta$ -sheet from solvents, force the closer packing of  $\beta 1$ – $\alpha 1$ – $\beta 2$  to  $\alpha 2$ – $\alpha 3$  subdomains and raise the structural stability of mdPrP. These structural features could have a major effect on the prion conversion. In our previous studies, we have found that amino acid substitution at position 226 has dramatic effects on CWD prion replication, pathogenesis, and biochemical properties.<sup>73</sup> Tg(DeerPrP) has a longer incubation time compared with Tg(ElkPrP) mice after inoculation with CWD prions. On the other hand, Tg(DeerPrP) mice were susceptible to SSBP/1,<sup>74</sup> whereas Tg(ElkPrP) mice were completely resistant.<sup>71</sup> In addition, Q226 CWD prions display more resistance to guanidine denaturation than the E226 CWD prions.<sup>35</sup> Our current findings suggest that the long-range interactions in the mdPrP protein might stabilize the overall structure, thus impacting the PrP<sup>C</sup>-to-PrP<sup>Sc</sup> conversion. The distribution of electrostatic surface potential on the mdPrP protein may lead to different intermolecular interactions between PrP<sup>C</sup> and PrP<sup>Sc</sup> and in this way may represent a step toward understanding the underlining mechanism of CWD prion transmission.

## METHODS

### Plasmid Construction for NMR Sample Preparation.

The recombinant dePrP (94–233) was obtained using the QuikChange kit (Stratagene) utilizing primers 5'-CAGAGA-GAATCCCAGGCTTATTACCAAAGA-3' and 5'-TCTTTGGTAATAAGCCTGGGATTCTCTCTG-3' and ePrP(94–234) as templates. The DNA product was then

inserted into pProExHTa (Invitrogen), containing the cleavage site between the His<sub>6</sub> tag and the protein fragment. The cloned DNA sequences were verified by sequencing.

**Prion Protein Expression and Purification.** A freshly transformed overnight culture of *E. coli* BL21 (DE3) cells (Stratagene) was added at 37 °C to 2 L of the minimal medium plus ampicillin (100 mg/mL). For isotope labeling, 4 g/L [<sup>13</sup>C<sub>6</sub>] glucose and 1 g/L [<sup>15</sup>N] ammonium chloride were added. At 0.8 OD<sub>600</sub>, expression was induced with isopropyl  $\beta$ -D-galactopyranoside to a final concentration of 0.8 mM. Cells were grown in a Biostat B plus 2 L vessel (Sartorius) and harvested 18 h after inoculation. The bacterial paste was resuspended in 25 mM Tris-HCl, 0.8% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0, and lysed by a Panda homogenizer. A crude extract was loaded onto a 5 mL HisTrap column (GE Healthcare) equilibrated in a binding buffer [2 M GndHCl, 500 mM NaCl, 20 mM Tris-HCl, and 20 mM imidazole (pH 8.0)] and eluted with 500 mM imidazole. The purified protein was lyophilized and dissolved in 8 M GndHCl. The protein was diluted to a final concentration of 0.5 mg/mL in a tobacco etch virus (TEV) reaction buffer (50 mM Tris base, 1 mM ethylenediaminetetraacetic acid, and 5 mM dithiothreitol), and TEV protease was added to the final concentration of 75  $\mu$ g/mL. The reaction was incubated at 22 °C overnight. The cleaved sample was loaded onto a 5 mL HisTrap column (GE Healthcare) equilibrated with a binding buffer [500 mM NaCl and 20 mM Tris-HCl (pH 8.0)], and the flow-through containing only the cleaved protein was collected. The purified protein was lyophilized and redissolved in 8 M GndHCl. Refolding was performed by dialysis against a refolding buffer [20 mM sodium acetate and 0.005% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (pH 5.5)] using a Spectra/Por membrane (molecular weight, 3000). The purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, western blot, and electrospray mass spectrometry. The purification and expression of TEV protease were obtained as described earlier.<sup>75</sup>



**NMR Spectroscopy and Structure Calculation.** All NMR experiments used for structure determination were performed on a  $^{13}\text{C}$ ,  $^{15}\text{N}$  isotopically labeled mdPrP sample on a Varian VNMRs 800 MHz spectrometer equipped with a triple  $^1\text{H}/^{13}\text{C}/^{15}\text{N}$  resonance cryogenic probe head operating at 25 K with inverse detection. The sample temperature was calibrated using the methanol- $d_3$  standard sample to ensure consistent sample temperature. The sample temperature for all experiments was 298 K. The sample contained 0.48 mM of mdPrP in a 20 mM sodium phosphate buffer, pH 5.5. NMR experiments for NH and HC detection were performed in 90%/10%  $\text{H}_2\text{O}/\text{D}_2\text{O}$  and in a 100% deuterated buffer, respectively. The sequence-specific assignment of the backbone  $^1\text{H}$ ,  $^{15}\text{N}$ ,  $^{13}\text{C}_\alpha$ ,  $^{13}\text{C}_\beta$ , and  $^{13}\text{C}$  resonances for mdPrP was obtained using the  $^{15}\text{N}$ -HSQC spectrum and triple-resonance NMR experiments HNC(O), HN(CO)CA, HNCA, CBCA-(CO)NH, and HNCACB.<sup>41</sup> The  $^1\text{H}$  and  $^{13}\text{C}$  resonances of aliphatic and aromatic side chains were assigned using  $^{13}\text{C}$ -HSQC in combination with HAHB(CO)NH, CC(CO)NH, (H)CCH-TOCSY, and  $^{13}\text{C}$ -edited NOESY-HSQC experiments.<sup>42</sup> NOE contacts were determined in 3D  $^{15}\text{N}$  and  $^{13}\text{C}$ -edited NOESY-HSQC experiments. Structure modeling of mdPrP was performed using the program CYANA 3.1.<sup>76</sup> Structure refinement using the explicit solvent model was performed by the YASARA program.<sup>77</sup> An ensemble of 20 lowest energy structures of mdPrP was validated by the web server software ICING<sup>44</sup> and PSVS.<sup>45</sup>

Backbone amide relaxation measurements including  $^{15}\text{N}$  longitudinal ( $R_1$ ), transverse ( $R_2$ ), rotating frame ( $R_{1\rho}$ ) relaxation rates, and  $\{^1\text{H}\}$ - $^{15}\text{N}$  heteronuclear NOE were obtained at two different magnetic fields (14.1 and 18.8 T) at 298 K.<sup>78</sup> Residues Gln<sup>95</sup>, Thr<sup>98</sup>, Ser<sup>100</sup>, Asn<sup>103</sup>, Ser<sup>106</sup>, Met<sup>137</sup>, His<sup>143</sup>, Tyr<sup>165</sup>, Asp<sup>170</sup>, Gln<sup>171</sup>, Asn<sup>174</sup>, Asn<sup>176</sup>, His<sup>180</sup>, Cys<sup>182</sup>, Val<sup>187</sup>, Met<sup>216</sup>, and Ile<sup>218</sup> could not be analyzed because of the cross-peak overlap.

All recorded spectra were processed with NMRPipe software<sup>79</sup> and analyzed with CARA<sup>80</sup> and SPARKY software.<sup>81</sup> The prediction of backbone dihedral angles was made by the TALOS+ program.<sup>82</sup> Alignment was prepared using ClustalO.<sup>83</sup> An analysis was performed by the web server GETAREA.<sup>49,50</sup> The potentials were calculated at an experimental pH of 5.5 of mdPrP using PDB2PQR server,<sup>84</sup> APBS,<sup>85</sup> and PROPKA.<sup>86,87</sup>

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02824.

$^{15}\text{N}$  amide backbone relaxation rates and hNOE of mdPrP; chemical shifts of  $\text{H}^{\text{N}}$ ,  $\text{C}_\alpha$ , and  $\text{C}_\beta$  atoms of mdPrP and ePrP structures; structural diversity of mdPrP, wtdPrP, and ePrP; and solvent accessibility of the selected amino acid residues of mdPrP, wtdPrP, and ePrP (PDF)

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U.S. and G.S. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

The atomic coordinates and structure factors of mdPrP (UniProtKB—P47852, PRIO\_ODOHE) have been deposited in the Protein Data Bank (PDB ID: 6FNV) and Biological Magnetic Resonance Bank (BMRB ID: 34236).

## ■ ABBREVIATIONS

$\delta$ , chemical shift; amPrP, America moose prion protein; CWD, chronic wasting disease; ePrP, elk prion protein; emPrP, Eurasian moose prion protein; HSQC, heteronuclear single quantum coherence; hNOE,  $\{^1\text{H}\}$ - $^{15}\text{N}$  heteronuclear nuclear Overhauser enhancement; mdPrP, mule deer prion protein; PrP, prion protein; PrP<sup>C</sup>, cellular isoform of prion protein; PrP<sup>Sc</sup>, prion-protease-resistant and infectious isoform;  $R_1$ , longitudinal relaxation rate ( $1/T_1$ );  $R_2$ , transverse relaxation rate ( $1/T_2$ );  $R_{1\rho}$ , relaxation rate in rotating frame ( $1/T_{1\rho}$ ); reddPrP, red deer prion protein; rdPrP, reindeer prion protein; TOCSY, total correlation spectroscopy; TSE, transmissible spongiform encephalopathy; wtdPrP, white-tailed deer prion protein

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