The crystal structure of an octapeptide repeat of the Prion protein in complex with a Fab fragment of the POM2 antibody

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ABSTRACT: Prion diseases are progressive, infectious neurodegenerative disorders caused primarily by the misfolding of the cellular prion protein (PrP^C) into an insoluble, protease-resistant, aggregated isoform termed PrP^{Sc}. In native conditions, PrP^C has a structured C-terminal domain and a highly flexible N-terminal domain. A part of this N-terminal domain consists of 4–5 repeats of an unusual glycine-rich, eight amino acids long peptide known as the octapeptide repeat (OR) domain. In this article, we successfully report the first crystal structure of an OR of PrP^C bound to the Fab fragment of the POM2 antibody. The structure was solved at a resolution of 2.3 Å by molecular replacement. Although several studies have previously predicted a β -turn-like structure of the unbound ORs, our structure shows an extended conformation of the OR when bound to a molecule of the POM2 Fab indicating that the bound Fab disrupts any putative native β turn conformation of the ORs. Encouraging results from several recent studies have shown that administering small molecule ligands or antibodies targeting the OR domain of PrP result in arresting the progress of peripheral prion infections both in *ex vivo* and in *in vivo* models. This makes the structural study of the interactions of POM2 Fab with the OR domain very important as it would help us to design smaller and tighter binding OR ligands.

Keywords: octapeptide repeats; POM2 Fab; complementarity determining regions; β -turns; backbone interactions; hydrophobic interactions

Additional Supporting Information may be found in the online version of this article.

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Introduction

Prion diseases including the Mad Cow disease in cattle, chronic wasting disease in cervids, and Creutzfeldt–Jakob disease (CJD) in humans are fatal, infectious neurodegenerative disorders caused by the self-catalytic misfolding and aggregation of the endogenous cellular Prion protein (PrP^C) into an infectious isoform, termed PrP^{Sc}. PrP^C is a Glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed predominantly in the brain but also found in stomach, kidneys, spleen, and blood.¹ The mature, full-length mouse PrP is 209 amino acids long with

a globular C-terminal domain (residues 125–231) comprising three α -helices and two short β -strands and an intrinsically disordered N-terminal domain (residues 23–124).^{2,3} Although not essential for pathogenicity, the N-terminal domain has been found to heavily influence higher-order aggregation of recombinant PrP *in vitro* as well as the rates of disease progression and the durations of disease incubation *in vivo*.^{4,5}

A major component of the N-terminal domain is the 4–5 tandem repeats of a glycine-rich octapeptide (PHGGG/SWGQ) known as the octapeptide repeat (OR) domain spanning residues 59–89. It is one of the most conserved domains of the Prion protein.⁶ The presence of one to nine additional copies of the OR is associated with familial forms of CJD^{7,8} whereas a lack of the OR domain in PrP^c slows the progress of the disease in mice.^{9,10} These studies indicate the possibility of a complex, modulatory role for the OR domain in the progression of Prion diseases. Several studies on potential biological binding partners of the OR domain showed that along with its flanking residues, it binds to a variety of ligands such as copper, zinc, sulfated aminoglycans, hemin, nucleic acids, stress inducible protein1, the laminin receptor, and the Low-density lipoprotein (LDL) receptor.¹¹

Many scientific groups have dedicated a better part of the last decade attempting to determine the structure of the OR domain both in its metal bound and in its metal-free forms.^{12–18} Millhauser and co-workers¹⁶ presented the first and so far the only crystal structure of the minimal binding fragment (HGGGW) of part of a single OR of PrP in complex with copper. In 2003, Nuclear Magnetic Resonance (NMR) studies on isolated peptide fragments (HGGGWGQP and its tandem repeats) of the OR domain at pH = 6.2¹⁷ suggested a β -turn-like conformation of the GWGQ portion of the octapeptide that was then found essential to the aggregation of PrP [PDB ID 1OEI (highlighted text) Supporting Information, Figure S1(A)]. In 2010, the solution NMR structure of the N-terminal domain fragment, PrP(23–106) in complex with a potentially therapeutic compound, pentosan polysulfate (PPS) was reported;¹⁹ PPS acts by promoting the aggregation of PrP^c and decreasing its availability for PrP^{sc} formation.²⁰ The NMR structure shows that the binding of PrP(23–106) to PPS involves a series of loops and turns in the OR domain that expose the tryptophan side chains to the solvent, thereby promoting PrP self-binding through possible aromatic interactions¹⁹ [PDB ID 2KKG (highlighted text) Supporting Information, Figure 1(B)].

Due to the absence of a well-defined tertiary structure, it has not been possible to study the native, unbound OR domain using the classical high-resolution technique of X-ray crystallography. However, when bound to specific ligands, the OR domain has been found to attain a certain level of order that would allow X-ray crystallographic

Figure 1. The asymmetric unit of the POM2 Fab-OR2 peptide complex crystal structure in the P2₁22₁ space group. The Fab molecules are shown in a cartoon representation, and the bound OR2 peptides have been displayed as sticks. In Mol1, the light chain, heavy chain, and the OR2 peptide are shown in orange, green, and magenta, respectively. In Mol2, the light chain, heavy chain, and the OR2 peptide are shown in blue, pink, and cyan, respectively (PDB ID 4J8R). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

studies.^{12,16} There have been several reports of antibodies with epitopes in the OR domain showing promising therapeutic efficacy by extending the survival of peripherally prion infected mice.^{10,21,22} These reports would clearly benefit from structural studies investigating the nature of the interactions of the OR domain with its various ligands at high resolution to enable the possibility of designing smaller and tighter binding ligands.

Aguzzi and coworkers developed a unique panel of 19 monoclonal antibodies (mAbs) in *PrP*^{0/0}-knockout mice, named POM1 through POM19; these antibodies have epitopes spanning the entire sequence of the mature prion protein.²³ Of these antibodies, the POM2 antibody recognizes an epitope in the OR domain. As the crystallization of IgG molecules is a challenging process due to the presence of glycosylation sites, we chose to use the Fab fragments of POM2 IgG for our crystallization studies. In this article, we show for the first time the crystal structure of a tandem OR of PrP^c comprising 16 residues (OR2) in complex with the POM2 antibody Fab fragment. The structure shows a unique extended conformation of one of the ORs revealing how the POM2 Fab binding disrupts the reported β -turn conformation of the ORs.^{17,19}

Results

Overall structure of the OR2-POM2 Fab complex

The complex of the POM2 Fab bound to the OR2 peptide was successfully co-crystallized in the space

Table I. Summary of the Data Collection and Refinement Statistics

Data collection and refinement statistics			
Wavelength (Å)	0.979		
Mathew's coefficient (Å ³ Da ⁻¹)	2.57		
Space group	P2 ₁ 22 ₁		
Unit-cell parameters			
a (Å)	65.41		
b (Å)	71.57		
c (Å)	207.59		
Resolution range ^a (Å)	39.6–2.3 (2.31–2.30)		
Total no. of reflections	319,135 (51,348)		
Unique reflections	44,080 (6956)		
R _{meas} ^b (%)	16.1 (186.5)		
Average I/σ(I)	10.82 (1.16)		
CC _{1/2} ^c (%)	99.7 (35.0)		
No. of pairs of reflections	44,047 (6924)		
Completeness (%)	99.8 (99.4)		
Multiplicity	7.24 (7.38)		
Refinement statistics			
R _{work} ^d (%)	24.7		
R _{free} ^d (%)	25.7		
Number of atoms			
Total	6791		
Protein	6626		
Water	165		
Mean B-factor (Å ²)		Mol1	Mol2
Overall	56.2		
Solvent	54.0		
POM2 Fab		55.8	56.3
OR2		73.8	63.5
RMSD bonds (Å)	0.03		
RMSD angles (°)	1.9		
Ramachandran plot			
Favoured (%)	97.23		
Allowed (%)	1.85		
Outliers (%)	0.92		
PDB ID	4J8R		

^a Values in the parentheses are for the highest resolution shell.

^b $R_{meas} = \sum_{hkl} (\sqrt{n(n-1)}) \sum_{j=1}^n |I_{hkl,j} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_j I_{hkl,j}$ is the redundancy independent indicator of data quality.

^c CC_{1/2} = percentage of correlation between intensities from random half-datasets.

^d R_{work} and $R_{free} = \sum_{hkl} |F_{obs}| - |F_{calc}| / \sum_{hkl} |F_{obs}|$ for reflections in the working and test (5% of the data) sets.

group P2₁22₁. The asymmetric unit is composed of two molecules of the POM2 Fab-OR2 complex—Mol1 and Mol2 (Fig. 1). As suggested previously,²⁴ the high-resolution cut-off of 2.3 Å was determined based on the CC_{1/2} factor of the diffraction data instead of being based on the average I/σ(I). The final model was refined to a crystallographic R_{work} factor of 24.7% and R_{free} of 25.8% with 876 protein residues and 165 water molecules. The difference electron density map ($|F_o| - |F_c|$) clearly shows an OR2 peptide bound to each of the POM2 Fab molecules in the asymmetric unit. The data processing and refinement statistics are summarized in Table I. A MOLPROBITY validation report shows that only

eight residues (0.92%) are in the disallowed region of the Ramachandran plot. All the residues of the POM2 Fab are numbered according to the standard Kabat and Wu numbering system;^{25,26} this numbering system describes the precise delineation of the complementarity determining regions (CDRs) of both light and heavy chains of antibodies in general. Throughout this article, the residue numbers of the OR2 peptide, POM2 Fab light, and heavy chains will be preceded by the letters P, L, and H, respectively.

Structure of the bound POM2 Fab

The global folding of the POM2 Fab molecule is characteristic of that of an immunoglobulin molecule. Each Fab molecule contains a light chain (L) and a heavy chain (H) with each domain containing two disulphide bonds. Each of the L and H chains contain a constant and variable domain and the variable domains in turn, contain three CDRs—loops L1, L2, L3 in the L chain and loops H1, H2, and H3 in the H chain [see Fig. 2(A)]; in POM2 Fab, all the six CDRs have well-defined electron densities in the $2|F_o| - |F_c|$ map. The elbow angle of POM2 Fab which is an indicator of the relative disposition of its variable domain with respect to its constant domain was calculated to be 161.6° for Mol1 and 166.3° for Mol2 and is within the expected range for IgGs (<http://proteinmodel.org/AS2TS/RBOW/index.html>).

However, as the difference in elbow angles of the two Fab molecules in the asymmetric unit is considerable, we calculated the root-mean-square-deviations (RMSD) of the variable and constant domains of the two Fab molecules separately; the RMSD values are 0.37 and 0.54 Å² for the variable and constant domains, respectively. Thus, the structures of the two POM2 Fab molecules in the asymmetric unit are quite similar.

Out of the 18 standard canonical conformations described for the CDR loops, POM2 Fab CDR loops L1, L2, L3, H1, and H2 fall into canonical classes κ1,1,1,1, and 3, respectively.²⁷ The POM2 Fab CDR H3 loop [Fig. 2(B)] exhibits a commonly observed β-bulged torso at Asp H101 stabilized by an Arg H94–Asp H101 salt bridge and a hydrogen bond between the backbone carbonyl oxygen of Met (H100E) and the Nε1 of a highly conserved Trp H103.^{28–30} Interestingly, of all six CDRs of the POM2 Fab, only the CDR H3 loop makes hydrogen bonded interactions with the OR2 peptide, whereas the CDRs in the light chain make interactions with the OR2 peptide via van der Waals and aromatic contact interactions.

The POM2 Fab CDR H3 loop has the AAAP-TYYAM sequence; it contains two adjacent Tyr residues flanked by small, hydrophobic residues that function to keep the loop conformationally flexible to facilitate antigen recognition. Several studies have shown that the large, polar, aromatic Tyr residues play dominant roles in antigen-antibody

Figure 2. The CDR loops of the POM2 Fab molecule. (A) The light (orange) and heavy (green) variable domains of Mol1 with the CDR loops highlighted in blue. The bound OR2 peptide is displayed as sticks in magenta. (B) The intramolecular contacts within the CDR H3 loop and its neighboring residues in Mol1. Only the residues in the CDR H3 loop are colored in blue, whereas its neighboring residues are colored in green. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

contacts.^{31–33} In the POM2 Fab, residue Tyr H100B forms a hydrogen bond with Asp H58 of the CDR H2 loop and critical interactions with the OR2 peptide, whereas Tyr H100C forms an important hydrogen bond with the backbone carbonyl oxygen of Ala H97 that keeps the CDR H3 loop conformationally stable [Fig. 2(B)].

The structure of the OR2 peptide

The 16 residue OR2 peptide is bound to POM2 Fab in a cleft spanning the interface between the

variable domains of the L and H chains of the POM2 Fab. In the $2|F_o| - |F_c|$ electron density map, the OR2 peptide in Mol1 could be traced up to Pro P9 [Fig. 3(A)] whereas in Mol2, the peptide could only be traced up to Gln P8 [Fig. 3(B)]. Neither of the OR2 peptide chains shows sufficient electron density to unambiguously model the second OR of OR2. The OR2 peptide is bound to the POM2 Fab in an extended conformation with a distance of 16.19 and 14.13 Å between the Pro P1 to Gln P8 residues in Mol1 and Mol2, respectively. The shape

Figure 3. The final $2|F_o| - |F_c|$ difference electron density map contoured at 1σ for the OR2 peptides at 2.3 Å resolution represented as chicken wire in (A) Mol1 and (B) Mol2. The OR2 peptides are shown as sticks and the POM2 Fab molecules are displayed as cartoon along with a surface representation (in light grey). The color scheme is similar to the one in Figure 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4. The intermolecular contacts between the OR2 peptide and the POM2 Fab molecule in (A) Mol1 and (B) Mol2. The color scheme is similar to the one in Figure 1. The water molecule is shown as a sphere (light blue). The contacts between the OR2 peptide and the Fab molecule are coloured in black, whereas those within the POM2 Fab molecule are colored in green and pink in (A) and (B), respectively (PDB ID 4J8R). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

complementarity scores calculated by the Sc program³⁴ of the CCP4 suite for the POM2 Fab H chain and OR2 peptide surfaces are 0.72 and 0.75 for Mol1 and Mol2, respectively whereas the Sc scores for L chain of POM2 Fab and the OR2 peptide are 0.52 and 0.71 for Mol1 and Mol2, respectively. A score of 1 indicates perfect shape complementarity and 0 indicates uncorrelated topography. Except in the case of the L chain of Mol1, the Sc scores indicate good surface complementarity between the POM2 Fab molecule and the OR2 peptide.

The overall RMSD between the C_{α} atoms of the OR2 peptide in Mol1 and Mol2 is 1.28 Å. The percentage buried surface areas of the OR2 peptide on binding to the POM2 Fab are 46.5% (575 Å²) and 59.8% (653.2 Å²) for Mol1 and Mol2, respectively.³⁵ The overall average B-factors of the OR2 peptide chains is 73.8 and 63.5 Å² in Mol1 and Mol2, respectively. The residues of the OR2 peptide that are critical to the complex formation with POM2 Fab have been determined by the nature and number of contacts formed by each peptide residue with the Fab molecule [Fig. 4(A,B)]. In Mol1 and Mol2, the side chain of Pro P1 dips into a binding pocket formed by the three CDR loops of the H chain of the POM2 Fab. The amide nitrogen of Pro P1 residue forms a hydrogen bond with a water molecule situated in this pocket which in turn hydrogen bonds with the Glu H35 residue of the CDR H1 of the POM2 Fab.

The Pro P1 amide also forms a hydrogen bond with the backbone carbonyl oxygen of the Thr H100A residue. Pro P1 is further anchored into this pocket by a hydrogen bond between the backbone amide of His P2 to the carbonyl oxygen of Pro H100. Also seen in both Mol1 and Mol2 are the backbone hydrogen bonding interactions between the amide of Tyr H100B to the carbonyl oxygen of Gly P3 and the amide of Ser P5 to the backbone carbonyl oxygen of Tyr H100B that contribute to anchoring the peptide to the CDR H3 loop of POM2 Fab. The residue Gly P4 does not contribute to any hydrogen bonding interactions with the POM2 Fab. The side chain of Ser P5 is also seen participating in hydrogen bonding interactions with the side chain O γ 1 of Thr H100A.

There are also some differences observed in the hydrogen bonding of the OR2 peptides in Mol1 and Mol2. In Mol2, the Trp P6 residue is better positioned than in Mol1 in the binding cleft of the POM2 Fab facilitating the hydrogen bonding from N ϵ 1 in Trp P6 to the backbone carbonyl oxygen of Ala H100D. In addition, the side chain Gln P8 O ϵ 1 in Mol2 forms a hydrogen bond with backbone amide of Ala H97. A summary of the various hydrogen bonding interactions of the two chains of OR2 peptides with their respective POM2 Fab molecules is presented in Table II.

An analysis of the hydrophobic interactions between the Fab molecule and the OR2 peptide

Table II. Summary of the Intermolecular Interactions between the POM2 Fab and the OR2 Peptide in Mol 1 and Mol 2

OR2	HOH	POM2 Fab	Distance (Å)	
			Mol1	Mol2
Pro P1 N	HOH196	Glu H35 δ2	3.89	
	HOH196		2.87	
Pro P1 N	HOH 6	Glu H35 δ2		3.59
	HOH 6			3.11
Pro P1 N		Thr H100A O	3.78	3.41
His P2 N ε1		Pro H100 O	2.89	2.74
Gly P3 O		Tyr H100B N	2.76	2.75
Ser P5 N		Tyr H 100B O	3.49	3.27
Ser P5 O _γ		Thr H100A O _γ 1	3.21	3.81
Trp P6 Nε1		Ala H100D O		2.88
Gln P8 O ε1		Ala H97 N		3.44

using LIGPLOT³⁶ reveals that there are 31 and 33 hydrophobic contacts in Mol1 and Mol2, respectively, between the OR2 peptide and POM2 Fab

(Supporting Information, Fig. S2). In both of the peptide chains in the asymmetric unit, the two residues Pro P1 and Trp P6 make the greatest contributions to the formation of the peptide-antibody complex. Pro P1 makes 41.9 and 45.4% of the hydrophobic contacts in Mol1 and Mol2, respectively, and interacts with the residues Val H50, Asn H58, Pro H100, and Tyr H100B [Fig. 5(A)], whereas Trp P6 makes 51.6 and 45.4% of the hydrophobic contacts in Mol1 and Mol2, respectively, and is well-placed in a hydrophobic binding pocket to make a T-shaped (or edge-on) geometry favored³⁷ aromatic interactions with Tyr H100C, Tyr L90, and Trp L48 residues [Fig. 5(B)]. In Mol1, Trp P6 contributes more toward the hydrophobic binding, whereas in Mol2, both Pro P1 and Trp P6 make equal contributions. In addition, an analysis of the electrostatic potential surface map of the POM2 Fab-OR2 complex indicates partial electrostatic surface complementarity [Fig. 6(A,B)]. The electrostatic potential was

Figure 5. Stereo representation of the hydrophobic binding pockets around the Pro P1 and the Trp P6 residues of the OR2 peptides with the Mol1 and Mol2 molecules superimposed. (A) The hydrophobic binding pocket around the Pro P1 residue in Mol1 and Mol2; (B) The hydrophobic binding pocket around the Trp P6 residue in Mol1 and Mol2. All the residues involved in hydrophobic binding (calculated using LIGPLOT) are shown as sticks. In addition, the residues of POM2 Fab are shown with a mesh around a sphere (grey) representation. The color scheme is similar to the one shown in Figure 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 6. An electrostatic potential-based surface representation of the POM2 Fab and the OR2 peptide. The electrostatic potential was calculated using the programs PDB2PQR³⁸ and APBS.³⁹ The color range extends from $-5kT/e$ (red) to $+5kT/e$ (blue). (A) The OR2 peptide (stick representation) bound to the POM2 Fab in Mol1 (surface representation) (B) The electrostatic potential surface representation of the OR2 peptide removed from the binding site by a 180° rotation about the vertical axis shown. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

calculated using the programs PDB2PQR³⁸ and APBS.³⁹ The first four residues of the OR2 peptide form a basic patch that complements an acidic patch on the POM2 Fab binding cleft.

Discussion

As the OR domain of PrP^c is intrinsically disordered in the native solution, the popular approach to study its structural characteristics has been to truncate the OR domain to variants of its fundamental binding unit¹⁵ and then stabilize them by ligand binding. We have adopted a similar approach and have successfully crystallized a tandem OR of PrP^c in complex with the POM2 Fab molecule. The crystal structure of the POM2 Fab-OR2 peptide shows for the first time an extended conformation adopted by a single OR of PrP^c when in complex with a binding partner. The structure shows that the monoclonal antibody fragment, POM2 Fab has a continuous epitope in the OR domain comprising residues PHGGSW, thus confirming previous epitope mapping experiments.²³ The OR2 peptide binds to the POM2 Fab by embedding the Pro P1 residue in a hydrophobic binding pocket followed by firm anchoring of the HGGS residues to the CDR H3 loop by backbone hydrogen bonding interactions. In addition, there is good surface complementarity as well as ample hydrophobic and aromatic interactions between the POM2 Fab and the OR2 peptide surfaces. Our structure highlights the importance of the Pro and the Trp residues of the ORs in being essential for the hydrophobic interactions between the peptide and Fab molecule. In addition, the aromatic

residues Tyr H100B and Tyr H100C in the CDR H3 loop of POM2 Fab play critical roles in the OR binding.^{33,40} The presence of all the above interactions explains the tight binding (K_d of $20 \times 10^{-9}M$) observed between a monovalent POM2 scFv and a single OR using surface plasmon resonance.²³

Considerable advances have been made in the field of Prion disease therapeutics as OR domain-binding polyanionic ligands such as heparin/heparan sulphate,⁴¹ PPS^{20,42} have been reported as potential antiprion compounds based on numerous *ex vivo* and *in vivo* experiments. PPS specifically has shown remarkable results in prolonging the incubation periods for PrP^{sc} formation when administered intracerebrally in prion-infected animal models and has now progressed to human clinical trials showing encouraging results by extending the survival of some patients.⁴³ However, the capacity of PPS to cross the blood-brain-barrier (BBB) has not been very encouraging. Despite the availability of numerous functional studies on these polyanionic compounds, there is a definite lack of molecular level, structural studies of their interactions with the ORs. Currently, the NMR structure of PPS bound to the OR domain is the only high-resolution structure available in the literature for a potentially therapeutic compound binding to the OR domain of PrP^c.¹⁹ The main highlight of the NMR model of the PPS-OR domain complex is the presence of the loop-turn structure of the OR domain resulting in the solvent exposure of the Tryptophan residues potentially enabling PrP self-binding through aromatic interactions.

The past decade has also seen considerable advances made in the field of prion immunotherapy using mAbs with epitopes in specific regions of the PrP^c.²² In 2003, seminal work by White *et al.*,⁴⁴ has shown the efficacy of passive immunization in protecting wild-type mice from peripheral prion inoculations. Considerable work has been reported on the mAbs with epitopes in the OR domain of PrP^c (anti-OR antibody) as they showed very encouraging results with their neuroprotective action in the face of peripheral prion challenge.^{10,21,45} As the Fab fragments of these mAbs failed to cross the BBB, they were found ineffective against intracerebral infection. Anti-OR antibodies—mAb110, SAF34—were found to inhibit PrP^{sc} formation in prion-infected neuroblastoma cells. It was suggested that mAb 110 acts by promoting cell surface retention of PrP^c thereby preventing PrP^{sc} accumulation inside the cells and SAF34 acts by preventing the formation of the molecular complexes between PrP^c and PrP^{sc}.²¹ In a completely different study,¹⁰ spinal cord lesions and other morphological abnormalities observed in the peripheral nervous system of rats caused by increased PrP^c levels induced by cobalamin deficiency were treated successfully with anti-OR antibodies. These functional studies greatly increase the importance of the structural study of an anti-OR antibody fragment such as the POM2 Fab and its potential in the development of antibody-based therapy for prion diseases and the recently discovered cobalamin-deficiency induced pathologies.

Conclusions

Our structure of the OR2 peptide complexed with the POM2 Fab is the first reported crystal structure of a tandem OR, PHGGSWGQPHGGSWGQ that shows one complete OR in complex with a potentially therapeutic antibody. Hydrophobic as well as backbone interactions between the residues of the OR and the POM2 Fab make this a high affinity interaction. Previously, two studies^{17,19} have highlighted the importance of the β -turns and the exposure of the Trp residues of the ORs to the aggregation of the PrP molecules. The POM2 Fab, however, binds to the ORs by completely disrupting their β -turns and by completely burying the Trp as well as the Pro residues from solvent exposure. It would be interesting to see if the rest of the potentially therapeutic anti-OR antibodies bind the ORs in a similar fashion that result in their disrupting the aggregation of PrP. The results would then highlight the importance of β -turns, the Trp residues as well as the Pro residues to the aggregation of PrP molecules. It is noteworthy to mention that the PPS-PrP(23–106) complex was made at pH=5.0, whereas the OR2-POM2 Fab complex was formed at pH= 7.0 and its crystals were obtained at pH=7.5.

Finally, the POM2 Fab-OR domain peptide system has the potential to be a very useful framework for carrying out further aggregation and structural studies on the OR domain and its numerous ligands. With more encouraging reports on the therapeutic uses of anti-OR antibodies, our structure could prove to be important in designing smaller, tighter binding, and BBB permeable recombinant anti-OR antibody fragments.

Materials and Methods

Preparation of POM2 Fab and the OR2 peptide

The POM2 IgG hybridoma cell line was generated as described previously²³ and cultured in the Roswell Park Memorial Institute media 1640(GIBCO) supplemented with 5% fetal bovine serum. The IgG-rich hybridoma culture supernatant was purified by affinity chromatography using a Protein G sepharose (PIERCE) column. Pure POM2 IgG was eluted from the column with 0.1M Glycine pH 2.8. Papain (SIGMA) 0.25%(w/v) was used to digest POM2 IgG into Fab fragments as previously described.⁴⁶ The digestion was allowed to take place for 5 h in a water bath at 37°C in freshly prepared 50mM Tris, 150mM NaCl pH 8.0, 20mM EDTA, 20mM cysteine. The digestion was stopped by adding 30mM Iodoacetamide. The POM2 Fab molecules were separated from their cleaved F_c fragments and undigested POM2 IgG molecules using a Protein A sepharose (PIERCE) column followed by further purification on a Hiload 16/60 Sephadex 75 (Amersham Biosciences) gel filtration column in 50mM Tris, 25 mM NaCl pH 7.0 buffer. The pure POM2 Fab obtained was concentrated to 20mg/mL using centrifugal filter units (Millipore).

The peptide OR2 with the sequence PHGGSWGQPHGGSWGQ was synthesized by and purchased from the Institute for Biomolecular Design at the University of Alberta, Canada. There were no protective groups added to either end of the peptide. The peptide was found to be soluble in water.

Crystallization

POM2 Fab was mixed with a twofold molar excess of the OR2 peptide and incubated at room temperature for 30 min. The complex resulted in a clear solution with no precipitation visible to the naked eye. The resulting solution was concentrated to 20mg/mL with a 30 kDa cut-off centrifugal filter units to remove any unbound OR2 peptide. Pure POM2 Fab-OR2 peptide complex was stored in 50mM Tris, 25mM NaCl, 1mM NaN₃ pH 7.0 buffer at 4°C. Commercial crystal screening solutions from the crystal screen and index screen (Hampton Research) were used to explore a variety of crystallization conditions by the sitting drop vapour diffusion method in the 96-well Intelli-Plates (Hampton Research) set up using a crystallization robot (Hydra 96 Plus One,

Robbins Scientific). A crystallization hit was obtained with 0.2M CaCl₂·2H₂O, 0.1M HEPES pH 7.5, 28% PEG1000 as the reservoir solution at room temperature. The sitting drop was prepared by mixing 1 μL of the protein solution with 1 μL of the reservoir solution. Diffraction quality crystals were obtained after 14 days. The crystals were flash-cooled in liquid N₂ after adding 20% glycerol as the cryoprotectant.

Data collection and structure determination

A full data set was collected to 2.3 Å resolution at the beamline 9-2 in the Stanford Synchrotron Radiation Lightsource (SSRL)⁴⁷ from one crystal. The data had an overall completeness of 99.80%. The AUTOXDS script developed at SSRL was used for data processing, scaling, and integration.⁴⁸ The data collection statistics are presented in Table I. The crystals were indexed in the orthorhombic space group P2₁2₂1 with two 1:1 POM2 Fab-OR2 peptide complexes in the asymmetric unit. The structure of Fab POM2-OR2 was solved by molecular replacement using the program PHASER⁴⁹ in the CCP4 suite⁵⁰ with POM1 Fab⁵¹ protein as the search model (PDB ID 4DGI).

Structure refinement

The PHENIX suite was used to perform crystallographic refinement⁵² on the molecular model of the POM2 Fab-OR2 peptide complex. 5% of the reflections from the data collected were randomly selected and set aside to calculate the R_{free} factor for monitoring the progress of refinement. In the initial rounds of refinement, translation/libration/screw and the individual atomic displacement parameter refinements along with the Non-crystallographic symmetry (NCS) restraints were applied to the model. The electron density of the OR2 peptide was clearly visible in the $|F_o| - |F_c|$ difference map calculated after the first round of refinement. In the final rounds of refinement, coordinates' refinement was performed by applying stereochemical restraints on the molecular model. The positions of water molecules were initially identified using PHENIX and their positions were subsequently confirmed by manually checking for positive peaks in both the $2|F_o| - |F_c|$ and the $|F_o| - |F_c|$ electron density maps. Water molecules with B-factors greater than 60 Å² were removed. The program, *Coot* was used for manual model building.⁵³ Structure validation was performed with the program MOLPROBITY.⁵⁴ Potential hydrogen bonds and van der Waals contacts were analyzed using the program LIGPLOT.³⁶ PyMOL (<http://www.pymol.org>) was used to calculate the RMSDs and to create all the figures. The coordinates and the structure factors for the model have been deposited in the PDB with an accession ID of 4J8R.

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References

1. Zomosa-Signoret V, Arnaud JD, Fontes P, Alvarez-Martinez MT, Liautard JP (2008) Physiological role of the cellular prion protein. *Vet Res* 39:9.
2. Riek R, Hornemann S, Wider G, Glockshuber R, Wuthrich K (1997) NMR characterization of the full-length recombinant murine prion protein, mPrP(23–231). *FEBS Lett* 413:282–288.
3. Knaus KJ, Morillas M, Swietnicki W, Malone M, Surewicz WK, Yee VC (2001) Crystal structure of the human prion protein reveals a mechanism for oligomerization. *Nat Struct Mol Biol* 8:770–774.
4. Frankenfield KN, Powers ET, Kelly JW (2005) Influence of the N-terminal domain on the aggregation properties of the prion protein. *Protein Sci* 14:2154–2166.
5. Yam AY, Gao CM, Wang X, Wu P, Peretz D (2010) The octarepeat region of the prion protein is conformationally altered in prp^{sup}/sc^{sup}. *PLoS ONE* 5:e9316.
6. Wopfner F, Weidenhofer G, Schneider R, von Brunn A, Gilch S, Schwarz TF, Werner T, Schatzl HM (1999) Analysis of 27 mammalian and 9 avian prps reveals high conservation of flexible regions of the prion protein. *J Mol Biol* 289:1163–1178.
7. Campbell TA, Palmer MS, Will RG, Gibb WR, Luthert PJ, Collinge J (1996) A prion disease with a novel 96-base pair insertional mutation in the prion protein gene. *Neurology* 46:761–766.
8. Pietrini V, Puoti G, Limido L, Rossi G, Di Fede G, Giaccone G, Mangieri M, Tedeschi F, Bondavalli A, Mancina D, Bugiani O, Tagliavini F (2003) Creutzfeldt-jakob disease with a novel extra-repeat insertional mutation in the prnp gene. *Neurology* 61:1288–1291.
9. Flechsig E, Shmerling D, Hegyi I, Raeber AJ, Fischer M, Cozzio A, von Mering C, Aguzzi A, Weissmann C (2000) Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knock-out mice. *Neuron* 27:399–408.
10. Scalabrino G, Mutti E, Veber D, Rodriguez Menendez V, Novembrino C, Calligaro A, Tredici G (2011) The octapeptide repeat prp(c) region and cobalamin-deficient polyneuropathy of the rat. *Muscle Nerve* 44:957–967.
11. Caughey B, Baron GS (2006) Prions and their partners in crime. *Nature* 443:803–810.
12. Stockel J, Safar J, Wallace AC, Cohen FE, Prusiner SB (1998) Prion protein selectively binds copper(II) ions. *Biochemistry* 37:7185–7193 PMID: 9585530.
13. Viles JH, Cohen FE, Prusiner SB, Goodin DB, Wright PE, Dyson HJ (1999) Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc Natl Acad Sci USA* 96:2042–2047.
14. Miura T, Hori-i A, Mototani H, Takeuchi H (1999) Raman spectroscopic study on the copper(II) binding mode of prion octapeptide and its pH dependence. *Biochemistry* 38:11560–11569 PMID: 10471308.

15. Aronoff-Spencer E, Burns CS, Avdievich NI, Gerfen GJ, Peisach J, Antholine WE, Ball HL, Cohen FE, Prusiner SB, Millhauser GL (2000) Identification of the Cu²⁺ binding sites in the N-terminal domain of the prion protein by EPR and CD spectroscopy. *Biochemistry* 39:13760–13771.
16. Burns CS, Aronoff-Spencer E, Dunham CM, Lario P, Avdievich NI, Antholine WE, Olmstead MM, Vrielink A, Gerfen GJ, Peisach J, Scott WG, Millhauser GL (2002) Molecular features of the copper binding sites in the octarepeat domain of the prion protein. *Biochemistry* 41:3991–4001 pMID: 11900542.
17. Zahn R (2003) The octapeptide repeats in mammalian prion protein constitute a pH-dependent folding and aggregation site. *J Mol Biol* 334:477–488.
18. Chattopadhyay M, Walter ED, Newell DJ, Jackson PJ, Aronoff-Spencer E, Peisach J, Gerfen GJ, Bennett B, Antholine WE, Millhauser GL (2005) The octarepeat domain of the prion protein binds Cu(II) with three distinct coordination modes at pH 7.4. *J Am Chem Soc* 127:12647–12656.
19. Taubner LM, Bienkiewicz EA, Copié V, Caughey B (2010) Structure of the flexible amino-terminal domain of prion protein bound to a sulfated glycan. *J Mol Biol* 395:475–490.
20. Kamatari YO, Hayano Y, Yamaguchi K, Hosokawa-Muto J, Kuwata K (2013) Characterizing anti-prion compounds based on their binding properties to prion proteins: implications as medical chaperones. *Protein Sci* 22:22–34.
21. Perrier V, Solassol J, Crozet C, Frobert Y, Mourton-Gilles C, Grassi J, Lehmann S (2004) Anti-prp antibodies block prpsc replication in prion-infected cell cultures by accelerating prpc degradation. *J Neurochem* 89:454–463.
22. Muller-Schiffmann A, Korth C (2008) Vaccine approaches to prevent and treat prion infection : progress and challenges. *BioDrugs* 22:45–52.
23. Polymenidou M, Moos R, Scott M, Sigurdson C, Shi Y, Yajima B, Hafner-Bratkovič I, Jerala R, Hornemann S, Wuthrich K, Bellon A, Vey M, Garen G, James MNG, Kav N, Aguzzi A (2008) The POM monoclonals: a comprehensive set of antibodies to non-overlapping prion protein epitopes. *PLoS ONE* 3:e3872.
24. Karplus PA, Diederichs K (2012) Linking crystallographic model and data quality. *Science* 336:1030–1033.
25. Abhinandan K, Martin AC (2008) Analysis and improvements to kabat and structurally correct numbering of antibody variable domains. *Mol Immunol* 45:3832–3839.
26. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of proteins of immunological interest, NIH 5 edition (1991). NIH Publication No. 91-3242, US Department of Health and Human Services, Public Health Service, National Institute of Health.
27. Al-Lazikani B, Lesk AM, Chothia C (1997) Standard conformations for the canonical structures of immunoglobulins. *J Mol Biol* 273:927–948.
28. Morea V, Tramontano A, Rustici M, Chothia C, Lesk AM (1998) Conformations of the third hypervariable region in the vh domain of immunoglobulins. *J Mol Biol* 275:269–294.
29. Richardson JS, Getzoff ED, Richardson DC (1978) The beta bulge: a common small unit of nonrepetitive protein structure. *Proc Natl Acad Sci USA* 75:2574–2578.
30. Stanfield RL, Zemla A, Wilson IA, Rupp B (2006) Antibody elbow angles are influenced by their light chain class. *J Mol Biol* 357:1566–1574.
31. Mian IS, Bradwell AR, Olson AJ (1991) Structure, function and properties of antibody binding sites. *J Mol Biol* 217:133–151.
32. Koide S, Sidhu SS (2009) The importance of being tyrosine: lessons in molecular recognition from minimalist synthetic binding proteins. *ACS Chem Biol* 4:325–334 pMID: 19298050.
33. Ramaraj T, Angel T, Dratz EA, Jesaitis AJ, Mumey B (2012) Antigen–antibody interface properties: composition, residue interactions, and features of 53 non-redundant structures. *Biochim Biophys Acta* 1824:520–532.
34. Lawrence MC, Colman PM (1993) Shape complementarity at protein/protein interfaces. *J Mol Biol* 234:946–950.
35. Hubbard S, Thornton J (1993) “naccess”. Department of Biochemistry and Molecular Biology, University College London. Computer Program.
36. Wallace AC, Laskowski RA, Thornton JM (1995) Ligplot: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng* 8:127–134.
37. Hunter CA, Sanders JKM (1990) The nature of .pi-.pi. interactions. *J Am Chem Soc* 112:5525–5534.
38. Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, Klebe G, Baker NA (2007) Pdb2pqr: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. *Nucleic Acids Res* 35:W522–W525.
39. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci* 98:10037–10041.
40. Fellouse FA, Barthelemy PA, Kelley RF, Sidhu SS (2006) Tyrosine plays a dominant functional role in the paratope of a synthetic antibody derived from a four amino acid code. *J Mol Biol* 357:100–114.
41. Warner RG, Hundt C, Weiss S, Turnbull JE (2002) Identification of the heparan sulfate binding sites in the cellular prion protein. *J Biol Chem* 277:18421–18430.
42. Dealler S, Rainov NG (2003) Pentosan polysulfate as a prophylactic and therapeutic agent against prion disease. *IDrugs* 6:470–478.
43. Tsuboi Y, Doh-ura K, Yamada T (2009) Continuous intraventricular infusion of pentosan polysulfate: clinical trial against prion diseases. *Neuropathology* 29:632–636.
44. White AR, Enever P, Tayebi M, Mushens R, Linehan J, Brandner S, Anstee D, Collinge J, Hawke S (2003) Monoclonal antibodies inhibit prion replication and delay the development of prion disease. *Nature* 422: 80–83.
45. Kim CL, Karino A, Ishiguro N, Shinagawa M, Sato M, Horiuchi M (2004) Cell-surface retention of prpc by anti-prp antibody prevents protease-resistant prp formation. *J Gen Virol* 85:3473–3482.
46. Andrew SM, Titus JA (2001) Fragmentation of immunoglobulin G. *Curr Protoc Immunol*. John Wiley & Sons, Ltd. Book Chapter 2: Unit 2.8.
47. Soltis SM, Cohen AE, Deacon A, Eriksson T, Gonzalez A, McPhillips S, Chui H, Dunten P, Hollenbeck M, Mathews I, Miller M, Moorhead P, Phizackerley RP, Smith C, Song J, van dem Bedem H, Ellis P, Kuhn P, McPhillips T, Sauter N, Sharp K, Tsyba I, Wolf G (2008) New paradigm for macromolecular crystallography experiments at ssrl: automated crystal screening

- and remote data collection. *Acta Crystallogr D Biol Crystallogr* 64:1210–1221.
48. Gonzalez A, Tsai Y autoxds. (2010). http://smb.slac.stanford.edu/facilities/software/xds/#autoxds_script.
 49. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ (2007) Phaser crystallographic software. *J Appl Crystallogr* 40:658–674.
 50. Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AGW, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A, Wilson KS (2011) Overview of the ccp4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* 67:235–242.
 51. Baral PK, Wieland B, Swayampakula M, Polymenidou M, Rahman MH, Kav NNV, Aguzzi A, James MNG (2012) Structural studies on the folded domain of the human prion protein bound to the fab fragment of the antibody pom1. *Acta Crystallogr D Biol Crystallogr* 68:1501–1512.
 52. Afonine PV, Grosse-Kunstleve RW, Echols N, Headd JJ, Moriarty NW, Mustyakimov M, Terwilliger TC, Urzhumtsev A, Zwart PH, Adams PD (2012) Towards automated crystallographic structure refinement with *phenix.refine*. *Acta Crystallogr D Biol Crystallogr* 68:352–367.
 53. Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of *Coot*. *Acta Crystallogr D Biol Crystallogr* 66:486–501.
 54. Chen VB, Arendall III WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC (2010) *MolProbity*: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* 66:12–21.