

Structure of myelin P2 protein from equine spinal cord

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Equine P2 protein has been isolated from horse spinal cord and its structure determined to 2.1 Å. Since equine myelin is a viable alternative to bovine tissue for large-scale preparations, characterization of the proteins from equine spinal cord myelin has been initiated. There is an unusually high amount of P2 protein in equine CNS myelin compared with other species. The structure was determined by molecular replacement and subsequently refined to an *R* value of 0.187 ($R_{\text{free}} = 0.233$). The structure contains a molecule of the detergent LDAO and HEPES buffer in the binding cavity and is otherwise analogous to other cellular retinol-binding proteins.

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1. Introduction

In our laboratory, equine tissue has replaced well characterized bovine tissue. Since most of our research involves spinal cord, there is an inherent risk of contracting variant Creutzfeldt–Jakob disease (the human form of bovine spongiform encephalopathy, BSE) if we were to continue using bovine tissue. As BSE presents problems in several countries, an alternate source of spinal cord is desirable: equine tissue provides the necessary amount for bulk experiments. Up until now very little if any work has been performed to characterize horse myelin. Although the myelin proteins are largely conserved across species, our initial investigations have shown that although similar to other well defined myelin proteins (*e.g.* bovine, human, rat, murine), equine myelin has some distinct differences. For example, our initial investigations into the major component of basic protein (Wood *et al.*, 2002) shows a higher molecular weight of 18766 compared with 18501 and 18635 for human and bovine, respectively, owing to the increased number of amino acids in the equine protein: 172 compared with 170 for human and 169 for bovine. While careful isolation of the MBP isoforms by CM52 cation-exchange chromatography shows the same panoply of charged isoforms, there was one unexpected result: the large amount of P2 protein present in equine CNS tissue.

Myelin P2 protein is normally found as a small (14.8 kDa) component of the peripheral nervous system (PNS), making up some 2–15% of the total PNS protein (Kadlubowski *et al.*, 1984). It is very probably localized to the cytoplasmic face of the Schwann cell membrane (Weize & Brostoff, 1985), where it associates with the membrane. Its exact function is unclear, although it is obvious from experimental evidence (Uyemura *et al.*, 1984) and its relation to other hydrophobic ligand-binding proteins that this function involves the binding of lipophiles, thereby suggesting a role in lipid transport and storage in myelinating Schwann cells (Uyemura *et al.*, 1984). (The paradox, however, is that most of the major lipids found

in myelin, *e.g.* cholesterol and phosphatidylcholine, are very much larger than the binding pocket in P2 and hence its role may be to shuttle the lipid components.)

In common with many myelin proteins, injection of purified P2 protein (Kadlubowski & Hughes, 1979) or neurotogenic peptides derived from it (Uyemura *et al.*, 1982) into experimental animals results in an experimental allergic neuritis (EAN). In the case of P2, an animal model for Guillain–Barré syndrome, a demyelinating disease of human PNS, is produced. Interestingly, in horses there is a condition known as neuritis of the cauda equina (NCE), which leads to paralysis of the tail, bladder and rectum. Although the aetiology of the condition is unknown, the paralysis of the trigeminal and facial nerves show similarities to Guillain–Barré syndrome in man and experimental allergic neuritis (EAN) in animals (Kadlubowski & Ingram, 1981; Greenwood *et al.*, 1973).

On the basis of both sequence and three-dimensional structure, myelin P2 protein is closely related to cellular lipophilic transport proteins such as cellular retinol-binding proteins (CRBP, CRBP II; Bashor *et al.*, 1973; Li *et al.*, 1986), heart fatty-acid-binding protein (Heuckeroth *et al.*, 1987), intestinal fatty-acid-binding protein (Sacchetti *et al.*, 1988) and the cellular retinoic acid-binding proteins (CRABP, CRABP II; Sundelin *et al.*, 1985; Bailey & Siu, 1988; Kitamoto *et al.*, 1988). The structures of several of these proteins are known, including that of bovine myelin P2 protein (Jones *et al.*, 1988; Cowan *et al.*, 1993), all of which show the same ten-stranded antiparallel β -barrel closed at one end by a helix–turn–helix motif in the large loop between strands 1 and 2 of the barrel. The other inter-strand loops are short. The serum retinol-binding protein family (serum RBP, β -lactoglobulin *etc.*) differs from the above, having an eight-stranded antiparallel β -barrel with N- and C-terminal extensions.

In this paper, we describe the purification and structure determination of P2 protein from horse central nervous system (CNS) tissue to high resolution. We routinely extract P2 from our detergent-solubilized fractions of MBP and PLP and, as an added experiment, decided to keep the solubilizing detergent LDAO (lauryl dimethylamine-*N*-oxide) present at its CMC (0.1%) throughout the purification and crystallization protocol, almost treating it like a membrane protein in many respects (although it is not), just to see if the copious lipid present would be incorporated into the active site or liganded to the exterior surface of the protein. P2 can also be easily extracted from equine CNS tissue by simply using buffer and 200 mM NaCl and gently agitating by homogenizing or gentle sonication. Visual inspection of the remaining solution by SDS–PAGE indicates that P2 extraction is almost complete. Since there is a relatively large amount of P2 in equine myelin (between 5 and 10% of the total myelin proteins), this initial pre-solubilization is a useful clean-up step prior to removal of MBP and PLP proteins.

2. Materials and methods

All materials used in the purification were of reagent grade or higher. Lauryl dimethylamine-*N*-oxide (LDAO) was from

Fluka, Dorset, England. Cation-exchange chromatography used Whatman CM52 cellulose and Superdex 75 (Amersham Bioscience, UK) was used for gel filtration.

2.1. Purification of P2 protein

Myelin was purified from horse spinal cord by the method of Norton & Poduslo (1973). P2 protein was extracted from myelin using a low concentration of the detergent LDAO and purified by chromatography on CM52 cellulose. Briefly, myelin was suspended at ~ 2 mg ml⁻¹ protein in 10 mM PIPES pH 6.8, 5 mM EDTA, 200 mM KCl, 0.1% (*v/v*) LDAO by gentle stirring. The suspension was sonicated for 2 min (bath sonicator) and then stirred gently for 20 min. Following centrifugation (16 000 rev min⁻¹, 20 min, 277 K) to separate the extract, the residue was re-extracted a further three times. The soluble extracts were pooled and dialysed overnight against 10 mM Tricine pH 8.9, 1 mM EDTA, 0.1% (*v/v*) LDAO and centrifuged [18 000 rev min⁻¹, 30 min, 277 K] to remove precipitated lipid and contaminating cyclic nucleotide 3'-phosphatase (CNPase). The clarified extract was concentrated (Amicon Ultrafiltration, YM10 membrane from Millipore UK Ltd) and applied onto a 1 \times 20 cm column of CM 52 cellulose equilibrated with 10 mM PIPES pH 6.8, 1 mM EDTA, 0.1% LDAO. After washing, the bound proteins, including P2, were eluted with a linear 0–0.5 M gradient of 1 M NaCl in equilibration buffer. Fractions containing P2, as judged by SDS–PAGE, were pooled, concentrated to ~ 1 ml and applied onto a Superdex 75 (1.6 \times 50 cm column) in the above equilibration buffer to remove minor contaminants. The S-75 eluate was concentrated, typically to 5 mg ml⁻¹ protein, and stored at 253 K.

Pure P2 protein was subject to electrospray mass spectrometry following inline HPLC to remove buffer components. From the deconvoluted ion series, the major sample component gave a weight of 14 761 Da.

2.2. Crystallization, data collection, structure solution and refinement

Purified P2 protein formed crystals in several of the Hampton Research A screen solutions. Crystals grown using sitting-drop vapour diffusion in optimized condition No. 41 (0.1 M HEPES pH 5.6, 20% PEG 4000, 15% 2-propanol) diffracted well. Data were collected to better than 2.1 Å at stations 9.6 and 14.1 at SRS, Daresbury on a crystal form with space group $P3_22_1$ and unit-cell parameters $a = b = 45.9$, $c = 125.8$ Å, $\gamma = 120^\circ$ at 100 K using 10% glycerol as cryoprotectant. Data were reduced using *DENZO/SCALEPACK* (Otwinowski & Minor, 1997); subsequent data processing used the *CCP4* suite. Interestingly, we frequently obtained drops in which two crystal forms had grown simultaneously: the form used in this analysis, shaped like thick prisms, and also fine long needle-shaped crystals. Synchrotron data were collected from several of these needle-shaped crystals, but they only diffracted to about 4.5 Å in monoclinic space group *C2*.

The structure was solved by molecular replacement using *AMoRe* (Navaza, 1994). P2 bovine myelin protein stripped of

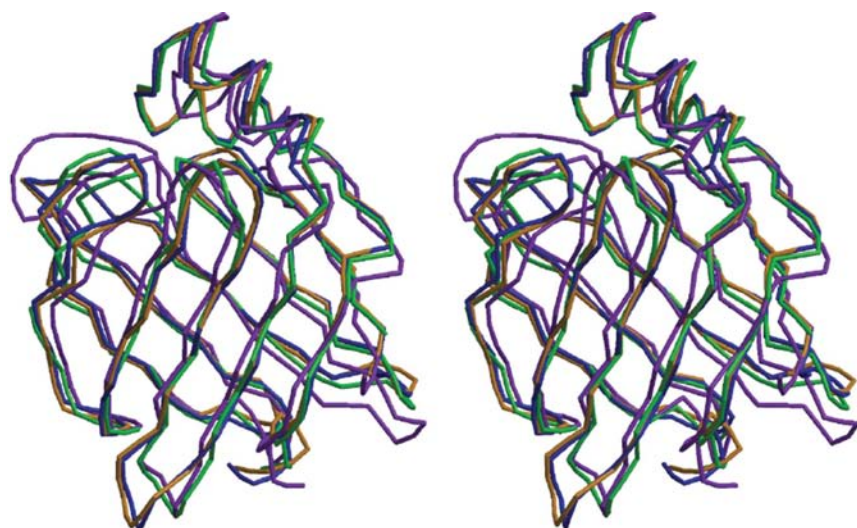


Figure 1
Stereo overlay of equine (dark blue), rat (green), bovine (orange) and human (purple) P2 structures.

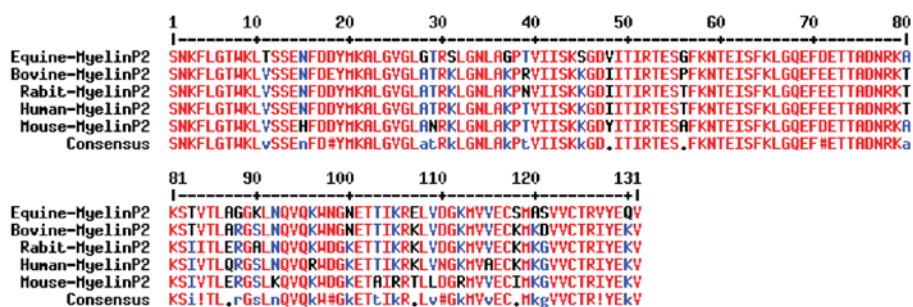


Figure 2
Sequence alignment of equine, bovine, rabbit, human and mouse myelin P2 proteins.

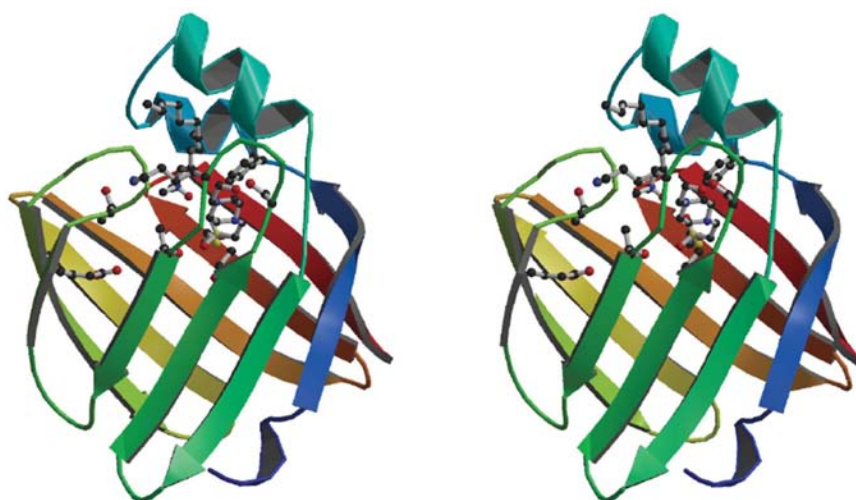


Figure 3
View of binding pocket with HEPES and LDAO.

water molecules (PDB code 1pmp) was used as the initial search molecule. Before refinement, 5% of the observed reflections were flagged for calculation of the free R factor, R_{free} . The structure was initially refined using *REFMAC* (Murshudov *et al.*, 1997) and the P2 structure was manually

built into the resulting electron-density map using *QUANTA*. As no sequence is available for equine P2, the assignment of amino acids was based on sequence alignment of four other myelin P2 sequences (Fig. 2). Side chains were added when electron density permitted. Several iterative rounds of model building and refinement allowed over 95% of the protein model to be built. Subsequent rounds of model building and refinement (again using *REFMAC*) involved TLS and restrained refinement (Winn *et al.*, 2001). The final model contains 1016 protein atoms, 102 water molecules, a HEPES molecule and a detergent LDAO molecule. The final R factor is 0.187 and R_{free} is 0.233. Table 1 outlines the refinement parameters. (space group $P3_22_1$ was confirmed over the symorphic $P3_12_1$ by initial trial refinements in each space group).

3. Results and discussion

The overall structure is homologous to other P2 and CRBP structures determined to date, comprising a single domain consisting of a ten-stranded up-and-down β -barrel with two orthogonal β -sheets. The top sheet consists of strands 1–4 and the bottom sheet of strands 5–10; strand 1 is shared by both sheets, with a classic β -bulge (Chan *et al.*, 1993) seen at residue 11. The centre of the barrel is closed at one end by two short α -helices. Residues Met119 and Trp97 and a salt bridge between OD1 and OD2 on Asp76 and NE and NH2 on Arg78 on neighbouring loops also close the opening. The entrance at the other end of the barrel is restricted by a number of conserved hydrophobic residues pointing into the barrel. These include Ser1, Lys44, Ile49, Leu66 and Leu91. Access to the interior of the barrel is gained by a small orifice formed between the second helix and the second hairpin turn involving Thr29, Leu32, Phe57 and Lys58. The opening is controlled by the conformation of

Phe57 (Cowan *et al.*, 1993). Fig. 1 illustrates the similarity in secondary-structure topology by overlaying the current equine structure (dark blue) with three other CRBP structures (rat, green, PDB code 1crb; bovine, orange, 1pmp; human, purple, 1cbq). The equine, rat and bovine structures have a

Table 1
Refinement parameters.

Space group	$P3_22_1$
Unit-cell parameters (Å, °)	$a = 45.846, b = 45.846, c = 125.778,$ $\alpha = 90.0, \beta = 90.0, \gamma = 120.0$
Resolution limits	41.89–2.10
No. of unique reflections	8255
Completeness (%) (high-resolution shell)	91.51 (67.93)
$R_{\text{work}}/R_{\text{free}}$ (%)	18.6/23.3
R.m.s. bond lengths (Å)	0.018
R.m.s. bond angles (°)	1.831
No. of protein residues	131
No. of water molecules	102
No. of hetero molecules	LDAO, HEPES
Average B factor (Å ²)	43.22
Percentage of residues in Ramachandran plot regions	
Most favoured	92.4
Additional allowed	5.1
Generously allowed	1.7

near-identical match, with the human structure showing slight deviations in the loop between β -strands 5 and 6 (at the open end of the barrel) and the small loop between strands 8 and 9.

There are two significant mutations in equine P2 when compared with other myelin P2 sequences (Fig. 2): Arg88 and Lys120 are both mutated to Ala. This would deplete the overall positive charge on the molecule by 2 when compared with other myelin P2s. A lack of density in these regions suggested other assignments to be inappropriate. In several cellular retinol-binding proteins (CRBPs), Arg88 is replaced with a glycine. Since there is no sequence information for equine P2, the side-chain assignments were made solely from electron-density modelling, taking cognisance of sequence information from other species. The electron density for the structure was very good and these different side-chain residues were only confirmed after careful refinement. Other minor mutations were Val for Ile48 and Ala for Thr80 and Ser90. Fig. 2 shows the sequence alignment of equine, bovine, rabbit, human and mouse myelin P2, illustrating the high degree of conservation between species. Electrospray mass spectrometry gave a mass of 14761, while the calculated mass from the sequence gives 14288. However, when the contribution from the HEPES and LDAO molecules that are found in the cavity is added in, this brings the weight to 14756 Da, which gives further confidence to the assigned residues.

3.1. Binding site and ligands

Two molecules have been found in the binding site: the detergent LDAO and HEPES buffer, both sequestered from the crystallization conditions (Fig. 3). The HEPES buffer molecule is at the hydrophilic end of the compartment. Each of the O atoms on the sulfonyl head group makes contact with different moieties: O(1) to water 61, O(2) to Cys117 and O(3) to the oxygen at the head of the detergent, LDAO. Interestingly, the hydroxyl group of the buffer points inwardly rather than in an extended conformation to allow favourable contacts with Ser55 OG and the carbonyl O atom of Lys58. With the close proximity of the adjacent hydrophobic region of LDAO this O–H rotation makes sense. There are nine water molecules that appear to form a halo-type motif around

the HEPES group. The positioning of a number of water molecules (usually between five and nine) at this end of the barrel seems to be a common feature of P2 and CRBP structures.

The tail of the detergent makes favourable hydrophobic interactions with the residues present in the opening at the second hairpin turn, while the head group makes interactions with HEPES O(3) and a number of water molecules in the ‘halo’. There is also a hydrophobic interaction (3.78 Å) between C(8) of HEPES and C(4) of LDAO. The above arrangement is not dissimilar to that found in the insect fatty-acid-binding protein isolated from *Manduca sexta* (Benning *et al.*, 1992), where a sulfate ion derived from crystallization in ammonium sulfate is also seen at the hydrophilic end of the barrel along with the fatty acid, palmitic acid. Initially, we tried to model a retinol molecule into this site since the aromatic ring was clearly defined in the initial density. However, there was no connective density from this proposed head group to the unsaturated chain (of the subsequent LDAO).

4. Conclusion

Structurally, equine P2 is practically identical to other P2 and CRBP structures, even though the equine myelin was derived from CNS rather than PNS tissue. Careful examination of the residual density from difference maps showed no evidence of lipid or other molecules associated with the exterior of the protein despite careful extraction, purification and crystallization under detergent conditions. This is in contrast to the lipid-bound form of bovine P2 crystallized by Sedzik *et al.* (2003). SDS–PAGE clearly shows lipid being carried forward in our purification steps and probably into crystallization. It may be that the second form of poorly diffracting rod-shaped crystals does have associated lipid and hence the heterogeneity of the system prevents the growth of better diffracting crystals. The presence of HEPES and LDAO molecules in the binding pocket is not surprising since these molecules, derived from purification and crystallization procedures, fit the requirements of the binding pocket: a charged entity at the hydrophilic end and a hydrophobic tail pointing towards the opening at the second hairpin turn.

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