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Major Myelin Proteolipid: The 4-α-Helix Topology

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Summary. Several conflicting models have been proposed for the membrane arrangement of the major myelin proteolipid (PLP). We have compared features of the sequence of PLP with those of other eukaryotic integral membrane proteins, with the view of identifying the most likely transmembrane topology. A new, simple model is suggested, which features four hydrophobic α-helices spanning the whole thickness of the lipid bilayer. Its orientation may be such that both the N- and C-termini face the cytosol. None of the biochemical, biophysical or immunological experiments hitherto reported provides incontrovertible evidence against the model. The effect or absence thereof of various PLP mutations is discussed in the frame of the proposed 4-helix topology.

Key Words PLP · DM20 · oligodendrocyte · myelin sheath · Pelizaeus-Merzbacher disease · dysmyelination · *jimpy* mutation · integral membrane protein · transmembrane topology · hydrophobic α-helix · hydrophobicity analysis · model building · prediction

Introduction

Proteolipid proteins (PLP, DM20) and myelin basic proteins (MBPs) are the two major protein families of myelin sheath (Lees & Brostoff, 1984). MBPs are peripheral membrane proteins associated with the cytoplasmic face of the oligodendrocyte plasma membrane (Roussel & Nussbaum, 1981). PLP and its splicing variant DM20 are integral (membrane-spanning) proteins, which appear to play an essential but poorly understood role in myelinization. Sequencing of complementary or genomic DNAs encoding the 276 amino acids of PLP shows high similarities (99–100% conservation) between cow (Lees et al., 1983; Stoffel et al., 1983), rat (Dautigny et al., 1985; Milner et al., 1985), man (Diehl et al., 1986), mouse (Macklin et al., 1987) and dog (Nadon, Duncan & Hudson, 1988) proteins.

Point mutations in PLP result in dysmyelinating diseases of various degrees of severity, such as some of the Pelizaeus-Merzbacher (P-M) diseases in man (Gencic et al., 1989; Hudson et al., 1989*b*; Trofatter et al., 1989), the *jimpy* mutations in mouse (Morello et al., 1986; Nave et al., 1986; Gencic & Hudson, 1990), the *md* mutation in rat (Boison & Stoffel, 1989) or the *shaking pup* mutation in dog (Nadon et al., 1988). Mutations may affect directly or indirectly the integration of PLP into the membrane during biosynthesis, its intracellular transport, its three-dimensional structure, its stability, or its interactions with other molecules in the cytoplasmic, lipid or extracellular phases. Formulating and testing hypotheses about the effect of a specific mutation requires a working model of the transmembrane topology of the protein, which would indicate which residues lie in which phase and which interactions they might conceivably establish. Three models have been hitherto proposed (Stoffel et al., 1983; Laursen, Samiulla & Lees, 1984; Stoffel, Hillen & Giersiefen, 1984; Hudson et al., 1989*a*) (*cf.* below, Fig. 4*a–c*). Despite numerous immunological, biochemical and biophysical studies, there is presently no agreement as to which of these models, if any, is correct.

During the past few years, understanding of the structure of integral membrane proteins has considerably progressed. Essential information has come from the determination of the structure of a complex of one peripheral and three integral proteins at atomic resolution [reviewed by Deisenhofer and Michel (1989) and Rees et al. (1989*b*)] and from the very rapid increase in the number of integral proteins whose sequences have been deduced from cDNA sequencing [reviewed by Popot and de Vitry (1990)]. Renaturation experiments have suggested that the folding of many integral proteins can be considered as a two-stage process: in the first stage, stable transmembrane α-helices establish themselves across the lipid bilayer; in the second stage, they pack without major rearrangement to yield the three-dimensional structure (Popot, Gerchman & Engelman, 1987;

Popot & Engelman, 1990). In this model, which is suggested to apply to most (but not all) integral membrane proteins, transmembrane α-helices are considered as autonomous folding domains similar to (but much smaller than) the folding domains evidenced in soluble proteins.

An essential assumption of the two-stage model is that transmembrane regions are made up of α-helices that would form stable transmembrane entities if inserted independently in a lipid bilayer. This implies almost necessarily that they be long enough to span the full thickness of the fatty acyl region of the bilayer, because of the high energy cost of burying in a hydrophobic medium polar helix ends and unsatisfied hydrogen bond donors and acceptors (*see* e.g. Henderson, 1977; Engelman & Steiz, 1981; Hol, 1985; Jacobs & White, 1989). There is presently no clear-cut counter-example to this rule. The anchoring C-terminal peptide of cytochrome b_5, reconstituted in its "tightly bound" form into lipid vesicles, may form a bent helix inserted part of the way into the bilayer. The evidence, however, is somewhat controversial (*see* Gogol, Engelman & Zaccaï, 1983; Takagaki et al., 1983; Gogol & Engelman, 1984; Rzepecki, Strittmatter & Herbette, 1986; Arinç, Rzepecki & Strittmatter, 1987) and not certain to reflect the native topology. To our knowledge, the two models initially proposed for PLP are unique in suggesting that a polytopic (multispanning) membrane protein features polypeptide segments looping back in the middle of the bilayer (Stoffel et al., 1983, 1984; Laursen et al., 1984; Fig. 4*a* and *b*).

A systematic examination of the hydrophobicity of putative transmembrane segments in proteins of known sequence leads to expectations about the probability that a sequence segment with a given hydrophobicity belongs to a transmembrane or an extramembrane region [*see* e.g. Klein, Kanehisa and Delisi (1985) and Popot and de Vitry (1990)]. The most recent model for PLP (Hudson et al., 1989*a*; Fig. 4*c*) is unexpected inasmuch as it assigns to the aqueous phase two sequence segments that are more hydrophobic than any of those yet encountered in extramembrane domains (*see below*).

In the present article, we discuss the relative merits of these proposals and of an alternative, more classical model for the transmembrane topology of PLP. The new model postulates that the transmembrane region of PLP is comprised of four hydrophobic α-helices spanning the whole thickness of the bilayer. Our approach is based on a comparison of features in the sequence of PLP with those encountered in other integral membrane proteins and a critical examination of experimental data.

Procedures

Hydrophobicity analyses were performed using three different scales (Kyte & Doolittle, 1982; Eisenberg et al., 1984; Engelman, Steitz & Goldman, 1986). Hydrophobicity profiles were generated using either A. Goldman's FOAM program (Engelman et al., 1986) or a prediction program package purchased from A. Crofts (University of Illinois, Urbana). The algorithm of Klein et al. (1985) was used to determine the probability for a given segment to be transmembrane. Segment hydrophobicity was estimated using the GES scale (Engelman et al., 1986) as described by Popot and de Vitry (1990). Lateral amphipathy of putative transmembrane helices was examined using the program of Vogel, Wright and Jähnig (1985), adapted to use either the KD (Kyte & Doolittle, 1982) or the GES (Engelman et al., 1986) scale. Atomic models of the helices were built using the program POLYPEP written by Richard Lavery (Institut de Biologie Physico-Chimique), with conventional α-helix backbone angles (IUPAC-IUB Commission, 1970) and side chains arbitrarily set in the most frequent conformation observed in high-resolution crystallographic structures (Ponder & Richards, 1987). No attempt was made to remove steric clashes or otherwise optimize the structures. Programs were run on microcomputers or a Vax 750. Models were visualized on a Spectragraphics raster display using R. Lavery's program MACRASTER.

Sequence Analysis

Transmembrane Topology

As observed previously (Lees et al., 1983; Stoffel et al., 1983), the sequence of PLP contains four very hydrophobic stretches (Fig. 1), hereafter noted A–D. Using any of three usual hydrophobicity scales (Kyte & Doolittle, 1982; Eisenberg et al., 1984; Engelman et al., 1986), the four segments qualify as putative transmembrane α-helices.

It is well established that some soluble proteins or extramembrane domains of integral membrane proteins contain stretches of amino acids that are more hydrophobic than proven transmembrane segments of the same length, so that there is no clear-cut hydrophobicity threshold separating the two classes of segments (*see* e.g. Klein et al., 1985; Engelman et al., 1986; von Heijne, 1986; Popot & de Vitry, 1990). In order to estimate probabilities for a given segment to be transmembrane or not, Klein et al. (1985) have proposed an index based on the application of the KD scale (Kyte & Doolittle, 1982) to a database of soluble and integral proteins. Klein's index, calculated for segments A–D of the PLP sequence, gives all of them high probabilities of spanning the membrane (Table).

The average hydrophobicity of each segment on the GES scale (Engelman et al., 1986) is close to or higher than the average hydrophobicity of putative transmembrane segments in eukaryotic proteins [2.03 ± 0.36 kcal/mol (Popot & de Vitry, 1990);

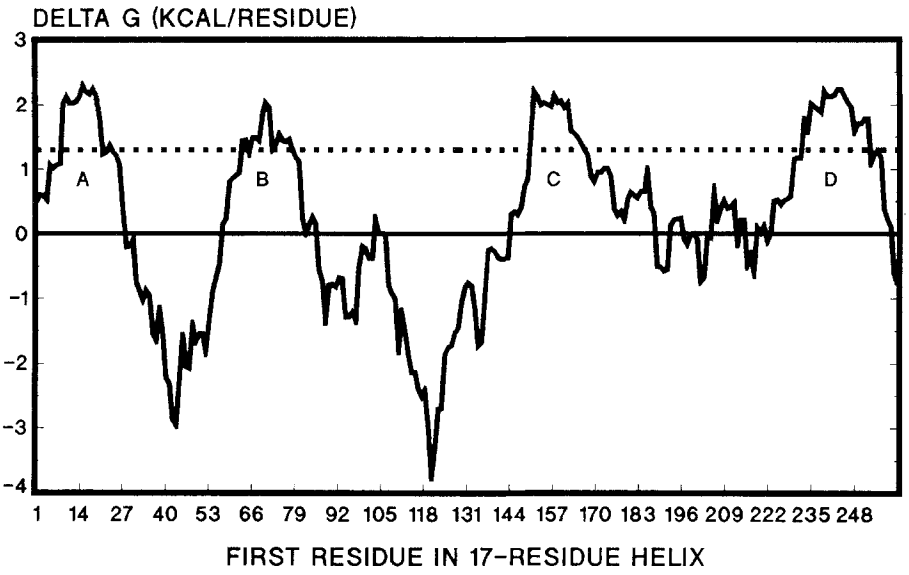

Fig. 1. Hydrophobicity profile of PLP. The free energy of transfer from lipids to water, calculated using the GES scale (Engelman et al., 1986), has been averaged over a 17-residue window. Four hydrophobic sequence segments are noted *A–D*. In other integral membrane proteins, sequence segments above the dotted line at 1.3 kcal/residue generally correspond to transmembrane α-helices (*see* Popot & de Vitry, 1990). The width of the peaks indicates that the hydrophobic stretches extend beyond 17 residues

Table]. Except for segment *B*, it also exceeds that of the most hydrophobic segment ever observed in a soluble protein (dogfish lactate dehydrogenase, 2.15 kcal/mol), itself highly atypical (Kyte & Doolittle, 1982), and that of the most hydrophobic segment yet found in the putative extramembrane regions of an integral membrane protein (2.05 kcal/mol; Popot & de Vitry, 1990). As a comparison, hydrophobicities of demonstrated transmembrane segments in the photosynthetic reaction center (Deisenhofer & Michel, 1989) and in bacteriorhodopsin (Henderson et al., 1990), measured on the same scale, range between 1.70 and 2.60 kcal/mol and between 0.94 and 2.02 kcal/mol, respectively (Popot & de Vitry, 1990). In the absence of experimental evidence to the contrary, it would seem most reasonable to assume that the four hydrophobic segments in PLP indeed span the membrane.

The thickness of the fatty acyl region of the bilayer in myelin is about 36 Å (*cf.* Kirschner, Brown & Singh, 1984). It takes an α-helix normal to the membrane plane ca. 24 residues to span this distance. While some of the hydrophobic segments are longer than this, none of them is long enough to form two transmembrane α-helices (Table). The most straightforward topological model for PLP therefore is one in which the polypeptide chain spans the membrane as four hydrophobic α-helices (Fig. 2). This 4-helix topology is consistent with the distribution of introns (Diehl et al., 1986; *cf.* Fig. 2), which, in integral proteins, are generally located in the loops between putative transmembrane helices (Jennings, 1989).

The putative extramembrane loops have distinct characteristics. The short A–B loop is hydrophilic, negatively charged, and rich in tyrosine residues. The B–C loop is long and basic; its second, most highly charged half is missing in the DM20 alternate transcript of PLP (Trifilieff et al., 1985, 1986). The long C–D loop is rather hydrophobic and carries an acylation site at position 198 (Stoffel et al., 1982). In both the Stoffel and Laursen models, this loop is postulated to be inserted half-way through the membrane (Fig. 4*a,b*). The possibility that the most hydrophobic segment in this loop (hydrophobicity 0.92 kcal/residue) forms a fifth transmembrane helix cannot be totally ruled out—such segments exist in bacteriorhodopsin or some receptors (*cf.* Popot & de Vitry, 1990)—but the probability is low (about 0.002 according to Klein's index). It has been suggested that the fatty acyl chain may play a role in myelin compaction by interacting with the opposite bilayer (Laursen et al., 1984).

Transmembrane Orientation

PLP is synthesized without a cleavable N-terminal signal sequence (Milner et al., 1985). It is therefore uncertain *a priori* whether its N-terminus is exposed to the extracellular medium or to the cytosol. Acylation, the only known post-translational modification of PLP with the removal of the initiating methionine, gives no clue about the orientation of the protein in the membrane, since both cytosolic and extracellular sites of ester-linked acylation are known (Towler et al., 1988).

Hartmann, Rapoport and Lodish (1989) have

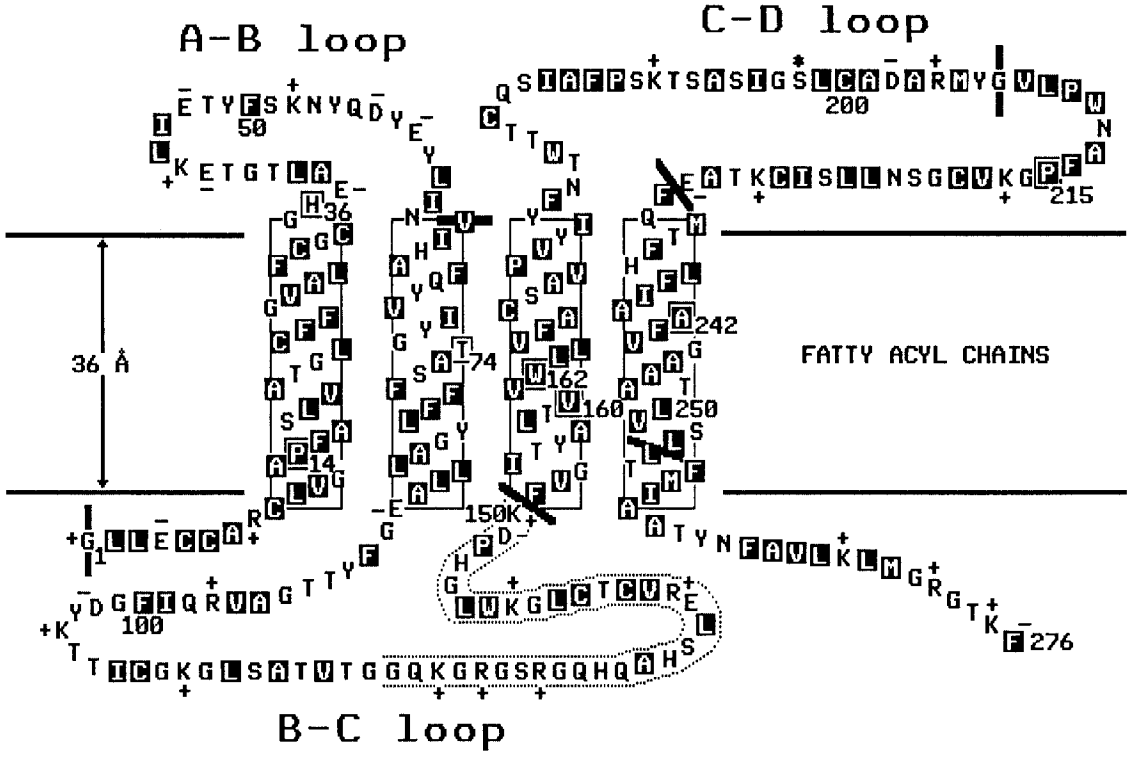

Fig. 2. A schematic representation of the 4-helix model of PLP. Hydrophobic residues [free energy of transfer from lipids to water > 1.2 kcal/residue on the GES scale (Engelman et al., 1986)] are shown on black background. Residues whose substitution is discussed in the text are boxed in and numbered. The positions of introns in the human PLP gene (Diehl et al., 1986) are marked by black bars. Dotted lines surround the region that is deleted in DM20 as a consequence of alternative splicing (Trifilieff et al., 1986). A star indicates acylation at position 198 (Stoffel et al.,1982). Helices are given the approximate length needed to span the hydrophobic core of the membrane, but could actually continue into the polar head region

analyzed the orientation of the first transmembrane helix in eukaryotic integral proteins without cleavable signal sequence as a function of the distribution of charges in the extramembrane regions immediately upstream and downstream of the helix. These regions were taken to include 15 residues, starting with the first charged residue on each side of the helix. Histidine was counted as half a positive charge. A striking correlation was observed between the balance of charges and the orientation; namely: among 91 proteins examined, all of the 37 proteins with an excess of negative charges on the C-terminal side of the first helix had their N-terminus located in the cytosol; 51 out of the 54 proteins with either an excess of negative charges on the N-terminal side or a perfect balance of charges had their N-terminal in the extracellular medium; three proteins were oriented with their N-terminal in the cytosol despite it being less positive or more negative than the C-terminus side.

Applying the rules of Hartmann et al. (1989) to PLP gives a balance of charges (C–N) of −2.5, negative on the C-terminal side of segment *A* (*cf.* Fig. 2). This suggests that this putative helix is oriented so that its N-terminus faces the cytosol. In the following discussion, the 4-helix model oriented according to this rule and that oriented in the opposite way will be referred to as the N-in and N-out models, respectively (Fig. 4*d,e*). Topology and orientation of PLP according to the 4-helix N-in model are similar to those proposed for synaptophysin (Leube et al., 1987) and liver gap junction connexin (Milks et al., 1988), two membrane proteins of comparable length.

Helix Orientation with Respect to the Lipids

Transmembrane helices in the photoreaction centers and in bacteriorhodopsin tend to be oriented so that their most hydrophobic faces are turned toward the

Table. Estimates of the position in sequence and hydrophobicity of PLP's four putative transmembrane α-helices

	Putative transmembrane segments			
	A	B	C	D
Position in sequence of the most hydrophobic 17-residue segments[a]:				
KD scale (maximal extension)	18–34 (9–36)	70–86 (70–87)	157–173 (150–179)	238–254 (234–262)
GES scale (extension to 25 residues)	15–31 (10–34)	70–86 (63–87)	151–167 (151–174)	242–258 (234–258)
Probability of spanning the bilayer[b]	99.7%	98.9%	99.8%	99.9%
Average hydrophobicity (kcal/residue)[c]	2.29	1.98	2.18	2.22

[a] Limits given correspond successively to the most hydrophobic 17-residue segments according to the KD scale (Kyte & Doolittle, 1982), their maximal extension according to Klein et al. (1985), the most hydrophobic 17-residue segments according to the GES scale (Engelman et al., 1986) and their extension to 25 residues.
[b] Probability that a given segment spans the bilayer according to Klein et al. (1985).
[c] Average hydrophobicity of the most hydrophobic 17-residue segments calculated using the GES scale (Engelman et al., 1986; *cf.* Popot & de Vitry, 1990).

outside of the molecule or complex [*see* reviews by Rees et al. (1989*b*) and Popot & Engelman (1990)]. The lateral "amphipathy" of hydrophobic helices (differential hydrophobicity of opposite faces; *cf.* Eisenberg et al., 1984) can be used as a tentative indication of their orientation with respect to the inside and outside of PLP transmembrane region (Rees, DeAntonio & Eisenberg, 1989*a*). A very hydrophobic face on helix IV of the nicotinic acetylcholine receptor subunits was suggested on this basis to face the lipids (Popot & Changeux, 1984) and was later found to be labeled by a photoreactive lipid analog (Giraudat et al., 1985). Helices C and D of PLP both exhibit a strongly hydrophobic face (shaded in Fig. 3), suggestive of an exposure to lipids. Helix B is more polar but somewhat amphipathic. Helix A contains few hydrogen-bonding side chains. Its amphipathy is not pronounced, but it is interesting to note that Ser_{17}, Thr_{21}, Cys_{24} and Cys_{32} are grouped on the same helix face.

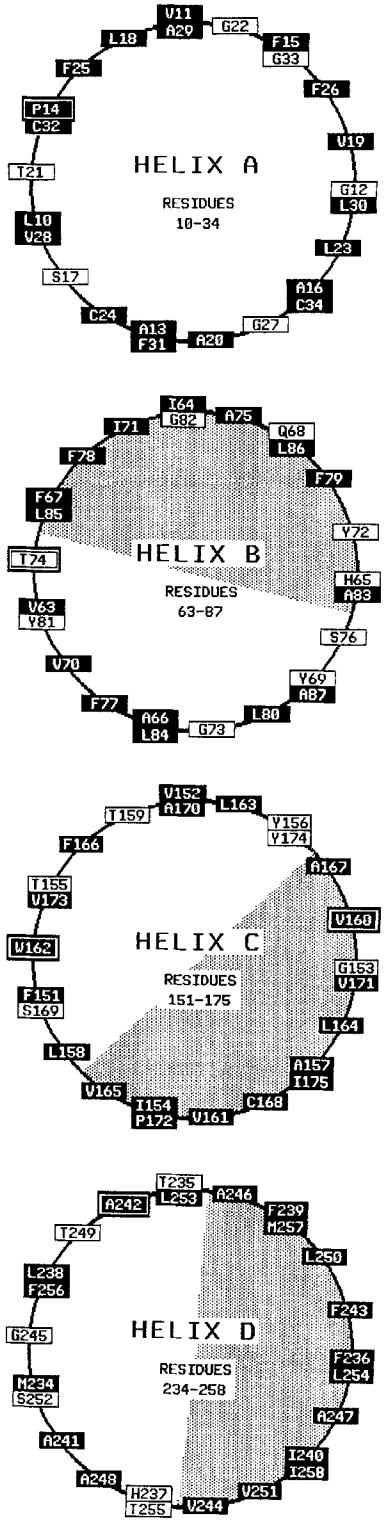

Fig. 3. Wheel projections of helices A–D. Helices are seen from the N-terminus. The most hydrophobic face of helices *B–D* according to Jähnig's algorithm (Vogel et al., 1985) is shaded. Hydrophobic residues [free energy of transfer from lipids to water >1.2 kcal/residue on the GES scale (Engelman et al., 1986)] are shown on black background. Residues whose substitution is discussed in the text are boxed in

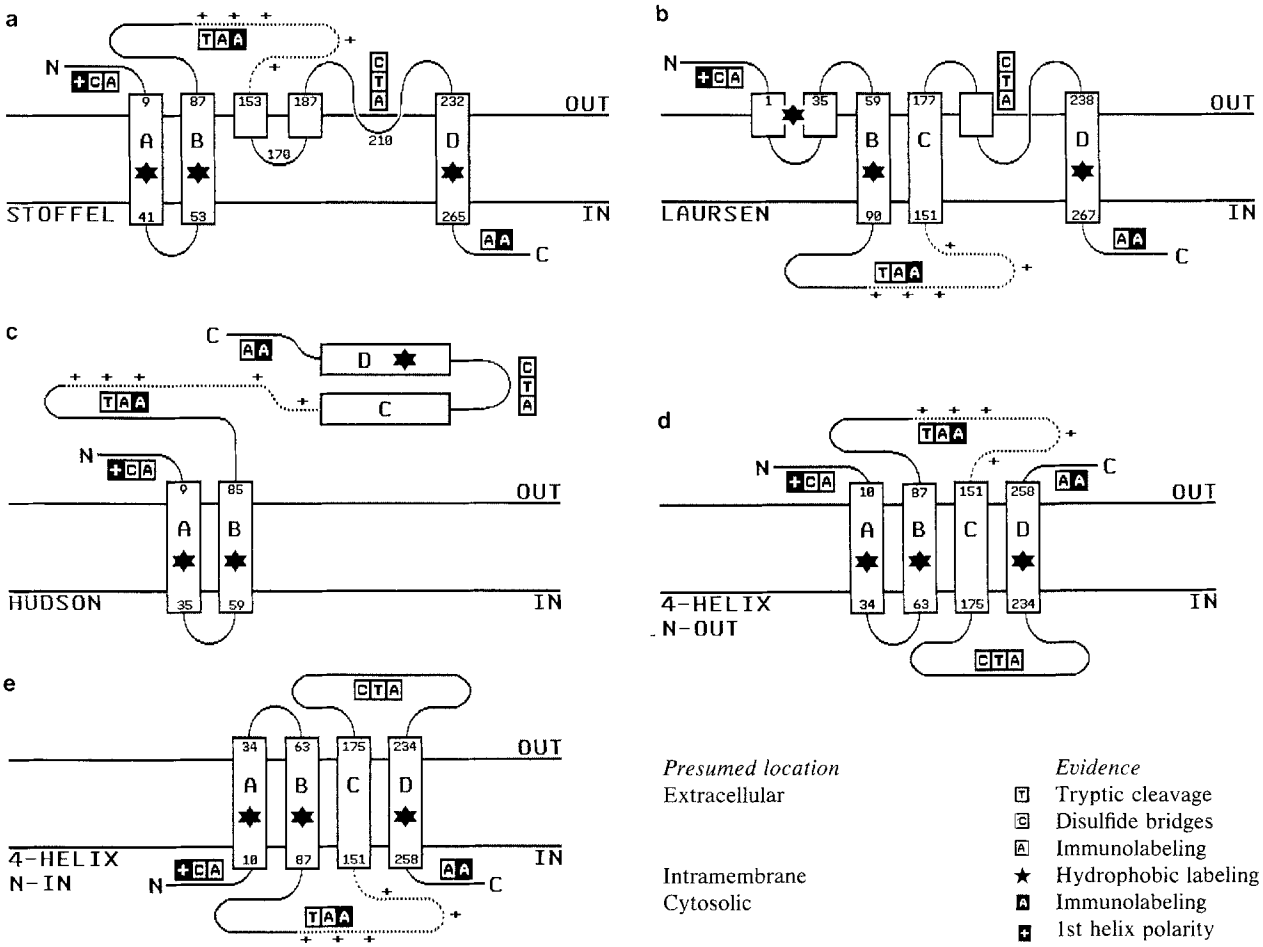

Fig. 4. A schematic representation of five models for PLP transmembrane topology. Models *a–c* summarize the proposals of Stoffel et al. (1983, 1984), Laursen et al. (1984) and Hudson et al. (1989*a*), respectively, and models *d* and *e* the 4-helix model discussed in the present paper in its two possible orientations. + + + +, basic region (residues 120–136). ....., region of loop B–C that is lacking in the DM20 variant of PLP (Trifilieff et al., 1986). ★, segments labeled by a lipophilic reagent (Kahan & Moscarello, 1985). Regions presumed to face either aqueous phase are indicated by symbols on white (extracellular) or black (cytosol) background. T, regions accessible to trypsin in osmotically swollen myelin (Stoffel et al., 1984). C, regions yielding peptides containing half-cystines (Jollès et al., 1979; Stoffel et al., 1983; Lees et al., 1983); unpublished data quoted by Stoffel et al. (1984) (*see* text) have not been taken into account. A, A, immunolabeling: regions reported to bind antibodies in oligodendrocyte cultures (Hudson et al., 1989*a*), A inside-out myelin vesicles (Lin et al., 1987) (A) or permeabilized cells (Konola et al., 1990) (A). +, cytosolic orientation of the N-terminus predicted (Hartmann et al., 1989) from the distribution of charges on either side of helix A

Biochemical, Biophysical and Immunological Data

The two 4-helix models (N-in and N-out) suggested by this simple-minded examination of the sequence differ from earlier suggestions (schematized in Fig. 4*a–c*) as regards the number and arrangement of transmembrane segments and the assignment of extramembrane regions to either of the two aqueous phases. In the following, we examine the degree of agreement of each of the five models with available biochemical, biophysical and immunological data. Particular attention is paid to those experiments whose results could permit ruling out one or the other of the two 4-helix models.

Labeling with a Hydrophobic Reagent

Kahan and Moscarello (1985) labeled PLP with radioactive 3-(trifluoromethyl)-3-(*m*[^{125}I]iodophenyl) diazirine (TID), a lipophilic photoactivatable label. They found evidence for TID binding between residues 1–52, 53–97, and 205–268, i.e., regions encompassing segments A, B and D. Peptides encompassing segment C were not recovered, but a comparison of the specific activities of whole PLP with that of recovered peptides suggested that additional labeled peptides had been lost. The extent of labeling was not affected by the presence of scavenging thiol in the buffer and was similar in peptide

203–268 (helix D) to that on the other sites. These observations are more easily reconciled with the Stoffel, Laursen and 4-helix models than with the Hudson model, that places segments C and D in the extracellular medium (Fig. 4*c*).

X-Ray Diffraction

X-ray diffraction data overall are more easily reconciled with the Hudson model than with any other (Inouye & Kirschner, 1989). The evidence is indirect, however, and its interpretation relies on a number of assumptions. Conclusions should probably be regarded as indicative rather than compelling.

Inouye and Kirschner (1988*a*,*b*, 1989) have measured X-ray diffraction patterns from mouse optic nerves equilibrated with low ionic strength buffer at various pH. The myelin period remained relatively constant between pH 2.5 and 5; between pH 5 and 8 the diffraction patterns became indistinct; a swollen state with a larger period was observed at pH 8–9 (Inouye & Kirschner, 1988*a*). The behavior of myelin between pH 5 and 8 was interpreted as resulting from physical association between apposed external membrane surfaces (bridging molecules) that would be overcome by electrostatic repulsion only at pH 8 and above (Inouye & Kirschner, 1988*b*).

The effects of pH changes on swelling give indications about the distribution of protonable groups on both faces of the membrane. If the effect of physical association is neglected, the Hudson model and, to a lesser extent, the Stoffel model (both of which place the positively charged B–C loop in the extracellular phase; *cf.* Fig. 4) predict that swelling should start at higher pH than the Laursen model does and therefore fit the data better (Inouye & Kirschner, 1988*b*, 1989). The same reasoning would favor the N-out 4-helix model, which predicts a distribution of charges virtually identical to the Stoffel model, over the N-in model, which is more similar to the Laursen model. The analysis, however, necessitates numerous assumptions (such as a homogeneous planar distribution of surface charges), neglects contributions from proteins other than MBP and PLP [which represent close to 30% of the myelin proteins; *cf.* Inouye and Kirschner (1988*b*)] and requires a scaling factor of uncertain origin in order to match the charge density calculated from the membrane composition to that required by the theoretical treatment of the swelling data. Furthermore, it assumes that the physical association postulated to account for the anomalous behavior of myelin between pH 5 and 8 does not play any role on the onset of swelling, which is the major criterion used to discriminate among models.

Electron densities in the extracellular and cytoplasmic phases calculated from X-ray data are similar (Kirschner et al., 1984). Given the cytoplasmic location of MBP, this suggests that other proteins are more concentrated in the extracellular phase. Models built up of lipids, MBP and PLP alone do not account well for this observation. However, the Hudson model, which places more material out of the membrane and into the external medium, predicts more similar electron densities than any of the other models would do (Inouye & Kirschner, 1989). The analysis again neglects contributions from proteins other than MBP and PLP.

Altogether, X-ray data on native myelin tend to favor the Hudson model. It is, however, uncertain how strong a refutation of the other models these estimates actually provide. It should be noted that X-ray diffraction patterns of samples reconstituted from myelin lipids and PLP, free from other proteins, indicated that most of the PLP mass actually spanned the bilayer (Kirschner et al., 1988), as proposed by all but the Hudson model. This apparent contradiction could be due to the reconstituted protein not having adopted its native conformation (Kirschner et al., 1988), but it is also conceivable that this simpler system eliminates sources of ambiguity.

Accessibility to Proteases

Stoffel et al. (1984) subjected PLP to tryptic degradation after hypoosmotic shock of myelin. They reported cleavage at positions 97 and 127 (in the basic loop between B and C) and 191 (loop C–D). This observation formed the basis for placing these two loops in the extracellular space by breaking helix C into two membrane-penetrating segments (Fig. 4*a*). An extracellular exposure of both the B–C and the C–D loops is incompatible with all but the Stoffel and Hudson models (Fig. 4).

Exclusion of trypsin from the cytoplasmic face of the membrane was deduced from the fact that MBP was not digested (Stoffel et al., 1984). The SDS-PAGE experiment shown, however, suggests a diminution of the intensity of the MBP band. As the B–C loop of PLP is rich in basic residues, it is likely to be particularly prone to tryptic attack. Disruption of part of the myelin as a consequence of the hypoosmotic shock—not an unlikely event—may have been sufficient to give trypsin access to the cytoplasmic side of some of the PLP molecules.

Disulfide Bridges

Given the reducing environment of the cytosol, disulfide bridges are a very strong indication that the

sequence regions involved face the outside medium. Sequencing of purified peptides has suggested the presence of cystyl residues close to the N-terminus (Jollès et al., 1979; Lees et al., 1983) and in the C–D loop (Lees et al.,1983; Stoffel et al., 1983). Stoffel et al. (1984) mention unpublished data showing that disulfide bridges associate the N-terminal region to the B–C loop and the B–C loop to the C–D loop. Had these data been obtained under conditions where disulfide rearrangement or formation was prevented (Creighton, 1989), they would constitute definitive evidence against all but the Stoffel and Hudson models (Fig. 4). However, such was not the case in those experiments that have been described in detail, which were not designed for topological purposes (Jollès et al., 1979; Lees et al., 1983; Stoffel et al., 1983). Spontaneous formation of disulfide bridges occurs upon exposure of cysteyl residues to air, as has been characterized in detail for erythrocyte anion exchange protein (Rao, 1979). A number of free sulfhydryles do disappear during PLP purification, presumably due to oxidation (Lees, Leston & Marfey, 1969; Nicot et al., 1973; Cockle et al., 1980). It is therefore probable that some of the disulfide bridges found in the purified peptides do not exist in vivo.

Immunolabeling

Immunochemical evidence regarding the topology of PLP is contradictory.

Trifilieff et al. (1986) and Konola, Tyler and Lees (1990) report that primary cultures of oligodendrocytes can be labeled by antibodies against either the B–C loop or the C-terminus only after the cells have been permeabilized, suggesting that these two regions are exposed to the cytosol. Lin, Bartlett and Lees (1987), using inside-out myelin vesicles and antipeptide sera in ELISA tests, concluded that the C-terminus faces the cytosol and the C–D loop the extracellular medium. These preliminary observations would be consistent with either the Laursen or the 4-helix N-in models (Fig. 4).

Conflicting results have been obtained by Hudson et al. (1989*a*). Antipeptide immunoglobulins applied to nonpermeabilized primary brain cultures enriched for oligodendrocytes were found to detect the 10 C-terminal residues, the 8 N-terminal residues, and regions of the B–C and C–D loops. An antibody against part of the A–B loop reacted only after treatment of the cells with Triton X-100, but this in an unspecific manner, apparently labeling proteins other than PLP. Proteolytic treatment of the cells reduced staining and complement-dependent lysis by variable extents. These results form the basis for the Hudson model shown in Fig. 4*c*.

Some difficulties of these experiments should be noted. For one thing, PLP was revealed at the surface of the cells only after a very long time (>20 days), when some cell death already became apparent. This observation is consistent with the earlier work of Trifilieff et al. (1986), who detected no antibody binding to the B–C loop of PLP in intact oligodendrocytes at day 17, but did not examine older cultures. By contrast, anti C-terminus antibodies bind to permeabilized cells after 6–9 days (Hudson et al., 1989*a*; Espinosa de los Monteros, Roussel & Nussbaum, 1986). Additional sources of concern are the known tendency of oligodendrocytes to bind immunoglobulins nonspecifically (Lubetzki et al., 1986) and the fact that labeling also occurred at the surface of astrocyte cells, which do not express PLP (Hartman et al., 1982). Given these difficulties, it may be deemed uncertain whether each antipeptide antibody actually labeled only the specific sequence segment it was raised against, on PLP molecules normally integrated in the plasma membrane of viable cells.

The general difficulty of using antibodies for mapping the topology of membrane proteins has been illustrated by recent studies of the nicotinic acetylcholine receptor. Apparently clear-cut localization of anti C-terminal monoclonal antibodies by electron microscopy were subsequently invalidated by biochemical approaches [*see* McCrea, Popot and Engelman (1987, 1988) and DiPaola, Czakhòwski and Karlin (1989), and references therein]. The reason for erratic immunolabeling has not been reported. It may be that monoclonal antibodies that have proven specific at the nanomolar concentrations used in immunoblotting experiments cross-react with extraneous epitopes when they are used at micromolar concentrations in labeling experiments and the peptide they are supposed to bind to is either inaccessible or not recognizable (*see also* Maelicke et al., 1989). The risk of cross-reaction is probably even higher when polyclonal antibodies are used, as has been the case for PLP. In any case, given these sources of concern, topological conclusions based on immunolabeling alone should probably be regarded as tentative.

Overall Balance of Experimental Data

As should be apparent from the above discussion, there is presently a dearth of very conclusive experimental data on which to base a choice between models. For each of the extramembrane regions—ex-

cepting the C–D loop that hitherto has consistently been found outside of the cell—whatever evidence there is is contradictory. The degree of agreement or disagreement of the five models with biochemical and immunological data is schematized in Fig. 4. It should be remembered, however, that most of this evidence is open to criticism on one ground or another.

The Hudson model is particularly difficult to evaluate, because it predicts that most of PLP lies in the extracellular phase. Methods of topological investigations hitherto employed generally aimed at identifying outwardly exposed sequence regions. Inwardly facing regions were deduced only on the basis of negative evidence, and any loophole in the procedures would lead to the incorrect assignment to the extracellular phase of regions that actually face the cytosol. We have mentioned that, in our view, immunolabeling data should be considered with caution. We note, however, that the only experiments giving some positive identification of inward facing segments (immunolabeling on permeabilized cells or inverted membranes), while preliminary, do not support the Hudson model, inasmuch as they place the C-terminus of PLP in the cytosol. Furthermore, the Hudson model is strongly at odds both with reasonable expectations regarding the transmembrane location of segments C and D and with actual lipophilic labeling experiments. One prediction unique to this model, which does not seem to have been tested yet, is that tryptic cleavage of the B–C loop should release the C-terminal half of PLP from the membrane.

The remaining four models are contradicted to variable extents by some biochemical, biophysical or immunological data, most of which are suggestive but not compelling. Taking experimental data at their face value, the Stoffel model would fare best, being contradicted only by some immunolabeling data, themselves controversial. The remark already made about the Hudson model largely applies to the Stoffel one, however, namely that most conceivable sources of ambiguity in current data tend to favor it because it places little material in the cytosol. The Laursen model is the most difficult to distinguish experimentally from the 4-helix N-in model, as it differs only in the transmembrane arrangement of helix A, the position of the N-terminus and the partial insertion of loop C–D into the lipid bilayer. The two 4-helix models are thermodynamically the most sound and should probably be given serious consideration. Of those, the N-in model is the most appealing, as it does not contradict the rule of Hartmann et al. (1989) for the orientation of the first helix and conflicts less with experimental data.

Genetic Data

In this section, we briefly discuss the putative effect of several mutations on the structure of PLP, according to the 4-helix model(s). Stress is placed on those mutations whose interpretation is model dependent.

Mutations in Helices A and B

Three mutations leading to dysfunction involve proline residues within or close to putative transmembrane segments A and B. The rat *md* mutation results from the replacement of a threonine with a proline at position 74, in the middle of helix B (Boison & Stoffel, 1989). As noted by Boison and Stoffel (1989), this is a major change that would prevent the helix from adopting a regular conformation (*see* e.g. Piela, Némethy & Scheraga, 1987); however, it is not likely to prevent its insertion. The general topology of the mutant protein should remain that of wild type PLP, but packing of the transmembrane region would be affected.

The *shaking pup* mutation (Nadon et al., 1988) substitutes a proline to a histidine at position 36, and a mutation responsible for a P-M disease in man (Trofatter et al., 1989) substitutes a leucine to a proline at position 14. These two mutations occur at either extremities of helix A and it is not certain from mere inspection of the sequence which of the affected residues actually belongs to the putative helix.

Conservative Substitutions in Helices C and D

Sequence comparisons have shown that, in integral as in soluble proteins, most variability occurs at the surface (*cf.* Rees et al., 1989*a*). Amino acid substitutions in PLP transmembrane segments should stand a greater chance of being tolerated if they occur on helix faces exposed to lipids, e.g. the most hydrophobic faces of helices C and D (Fig. 3). The opposite prediction would be made by the Hudson model, which places these two segments in the extracellular medium where they would be expected to bury their most hydrophobic residues away from the solvent.

There are unfortunately no known PLP sequences from nonmammals, and the variability between known sequences is too low to permit a stringent test of these predictions. The dog PLP sequence

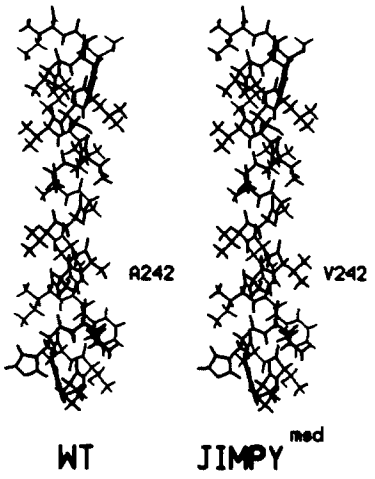

Fig. 5. A skeleton representation of helix D showing the effect of substituting Ala_{242} (wild type) by a valine (*jimpy*^{msd} mouse). Side chains are given arbitrarily the conformation most frequently observed in crystallographic structures (Ponder & Richards, 1987)

(Nadon et al., 1988) presents a substitution of an isoleucine for a valine at position 160, which does not affect function. Val_{160} is located at the edge of the most hydrophobic face of helix C (Fig. 3) and may therefore face the lipids. Addition of one methyl group, however, is a very small perturbation wherever the residue lies and therefore not very informative.

The PLP gene of *jimpy*^{msd} mouse mutant features the substitution of a valine for an alanine at position 242, which is very likely responsible for oligodendrocyte death (Gencic & Hudson, 1990). This is a remarkable effect for a very conservative substitution (addition of two methyl groups; *see* Fig. 5), and an indication that residue 242 is probably buried in a tightly packed protein/protein interface. A similar situation has been described for T4 lysozyme, in which replacement by a valine of alanine 98, which lies at a helix-helix contact, strongly destabilizes the protein (Alber, 1989). Ala_{242} is located on the least hydrophobic face of helix D (close, however, to the limit of the most hydrophobic face; *cf.* Fig. 3).

The inocuity of the Val_{160} substitution and the deleterious effect of the *jimpy*^{msd} mutation therefore are consistent with the orientations of helices C and D that could be expected if they are indeed transmembrane. Nevertheless, they constitute only very circumstancial evidence.

The Pelizaeus-Merzbacher Mutation at Position 162 (Helix C)

The replacement of a tryptophan by an arginine at position 162, in the middle of segment C, causes another form of P-M disease (Hudson et al., 1989*b*). Tryptophans are usually well conserved and the mutation is likely to cause a major disruption whether segment C is transmembrane or not. Hudson et al. (1989*b*) proposed that it would perturb interactions between hydrophobic helices in the extracellular medium.

In the frame of the 4-helix model, two possible effects of the mutation can be envisaged. The hydrophobicity of helix C is so high that it may be able to drag even as hydrophilic a residue as arginine into the membrane (calculated hydrophobicity of the modified segment on the GES scale 1.35 kcal/residue). Helix assembly would likely be strongly perturbed, however, both for steric reasons and because the guanidinium group of the arginine would seek to associate with hydrogen-bonding groups at the expense of normal bonds.

An alternative, perhaps more likely effect of the substitution would be to displace the third transmembrane segment down the sequence, leaving the arginine residue access to the aqueous phase. The resulting transmembrane segment ought to be stable by itself (hydrophobicity $\geq$ 1.44 kcal/residue), but it would lack the proper surfaces of interaction with the other helices, which should result in misfolding. The shortening of the C–D loop and corresponding lengthening of the B–C loop would likely prevent correct folding and interactions as well.

The *jimpy* Mutation (C–D Loop, Helix D, C-Terminus)

The *jimpy* mutation causes erroneous splicing of PLP mRNA, resulting in the deletion of exon V (second half of the C–D loop) and a frame shift responsible for an abnormal, proline- and cysteine-rich C-terminal sequence beyond Tyr_{206} (Morello et al., 1986; Nave et al., 1986; Hudson et al., 1987; Macklin et al.,1987; Nave, Bloom & Milner, 1987; Ikenaka et al., 1988):

Y_{206}PNDLPPVYCCICGGCSYTGFPAHLHDCCHLQLCRP

The aberrant C-terminal sequence contains a moderately hydrophobic segment (1.36 kcal/residue, underlined above), which may be able to span the membrane as a distorted α-helix (*cf.* Fig. 6). In the frame of the 4-helix N-in model, the overall topology of the protein would not change, but the

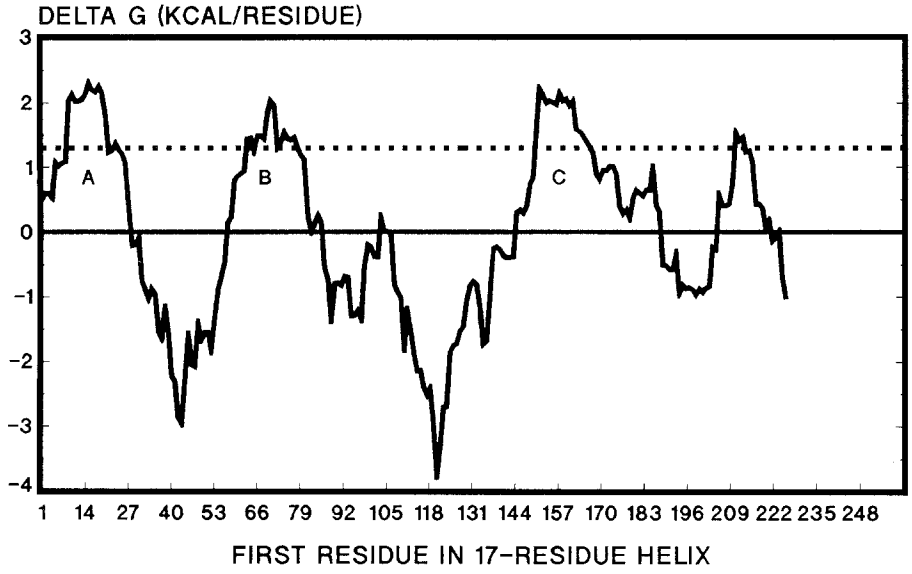

Fig. 6. Hydrophobicity profile of PLP from the *jimpy* mouse. Calculations were as in Fig. 1

structure of the C–D loop, C-terminus and transmembrane region would be strongly affected. Alternatively, the C-terminal hydrophobic segment may slip through the bilayer during biosynthesis, carrying into the lumen of the ER the whole C-terminus, where, in addition to other perturbations, its numerous cysteine residues would be likely to oxidize and cause aggregation (Nave et al., 1986). Intracellular transport of *jimpy* PLP from the ER to the Golgi apparatus is blocked (Roussel et al., 1987). Aggregation, retention into the ER and degradation are classical consequences of misfolding and in some cases have been shown to involve aberrant interchain disulfide crosslinks (Hurtley & Helenius, 1989).

The C–D Loop

It has been proposed that, in addition to their role in the formation of the myelin sheath, PLP and its alternative splicing form DM20 represent membrane-bound signals involved in regulating oligodendrocyte proliferation (Hudson et al., 1987). According to the 4-helix N-in model, the only extended region of PLP to face the extracellular medium is the loop between helices C and D. Perturbation of this loop should result from the recently described substitution of a serine to a proline at position 215, which causes a form of P-M disease (Gencic et al., 1989).

Conclusion

Only four integral membrane proteins are presently known in any structural detail (Deisenhofer & Michel, 1989; Rees et al., 1989*b*; Henderson et al., 1990). To which extent they can be taken as prototypes in interpreting the sequence of other proteins is currently a matter of debate. It is clearly important to our understanding of membrane proteins to explore the limits of variability of transmembrane arrangements. While exceptions to the hydrophobic α-helix bundle scheme do exist (bacterial porins, possibly voltage-gated channels), our analysis suggests that there is to date no compelling demonstration that the structure of PLP presents such original features as helices looping back half-way through the lipid bilayer or highly hydrophobic extramembrane segments. A "classical" topology, in which four hydrophobic α-helices fully span the bilayer, cannot at that point be rejected on the basis of incontrovertible experimental data and permits reasonable interpretations of the effect—or absence thereof—of various amino acid substitutions. It should probably be given consideration in the interpretation of future biochemical or genetic evidence.

The two 4-helix models permit a number of testable predictions. They exclude, for example, the existence of disulfide bridges linking the N-terminus to either the A–B or the C–D loops. The 4-helix N-in model is the only model to predict that the N-terminus of PLP faces the cytosol. The N-in orientation virtually excludes any disulfide bridges involving either the N-terminal region or the B–C loop, given the reducing environment of the cytosol. Bridges should be restricted to the A–B and C–D loops (and, possibly, to transmembrane segments A and C). Both the Laursen and 4-helix N-in models suggest that lactoperoxidase-catalyzed iodination from the outside of intact myelinated fibers (Poduslo

& Braun, 1975) should label primarily the tyrosine-rich A–B loop.

Understanding of the biosynthesis, folding and interactions of PLP, and elucidation of the mechanism of PLP-linked dysmyelinating diseases, would greatly benefit from experiments aimed at identifying the correct transmembrane topology.

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Note Added in Proof

After this paper was submitted for publication, O.A. Bizzozero, L.K. Good and J.C. Evans reported that Cys_{108} of bovine PLP is palmitoylated (*Biochem. Biophys. Res. Commun.* **170:**375–382, 1990). These authors noted a striking similarity between the sequence surrounding Cys_{108} (TTICGK) and that surrounding the pair of palmitoylated cysteyl residues in rhodopsin (TTICCGK in human rhodopsin); they suggested that palmitoylation of PLP may play a role in the transduction of intracellular signals. Palmitoylation of the homologous cysteine in human β_2-adrenergic receptor appears to control coupling to the G_s protein (B.F. O'Dowd et al., *J. Biol. Chem.* **264:**7564–7569, 1989). Recently, S.M. Strittmatter et al., reported that the N-terminal region of the intracellular growth cone protein GAP-43 regulates GTP-γ-S binding to G_0 and noted that this N-terminus (MLCCMRR in man), which is palmitoylated in membrane-bound forms of GAP-43, presents sequence similarities with the palmitoylated region of the B_2-adrenergic receptor and other G-protein linked receptors (*Nature* **344:**836–841, 1990). We note that a marked similarity exists between some of these sequences, particularly that of the β_1-adrenergic receptor (GLLCCAR), and the N-terminus of PLP (GLLECCAR). If the 4-helix N-in topology proposed in the present paper is correct, PLP and DM20 therefore would expose to the cytosol two sequence segments similar to segments shown in other proteins to be involved in G-protein regulation.