Biol. Chem. Hoppe-Seyler
Vol. 374, pp. 75–83, January 1993

A Novel Mutation in the Proteolipid Protein Gene Leading to Pelizaeus-Merzbacher Disease

Bernd Otterbach[a], Wilhelm Stoffel[a] and Vincent Ramaekers[b]

[a] Institut für Biochemie, Medizinische Fakultät, Universität Köln
[b] Kinderhospital, Medizinische Fakultät, RWTH Aachen

(Received 26 October 1992)

Summary: Point mutations of the gene of human proteolipid protein (PLP) have been recognized as the molecular basis of one form of leukodystrophy, the X-chromosome-linked Pelizaeus-Merzbacher disease (PMD). We report the molecular analysis of four PMD patients in three unrelated families and describe a point mutation (G→A transition) in exon V which leads to the substitution of Gly^{216} by a serine residue in a highly conserved extracytosolic domain and a *Mae* I RFLP. Molecular modelling with energy minimization indicates that this seamingly minor alteration of the amino-acid sequence induces a considerable conformational change and tight packing of the polypeptide chain apparently not compatible with the regular PLP function in oligodendrocytes.

This mutation has been detected and characterized by PCR amplification of genomic DNA using intron and exon primers and the complete sequence analysis of the seven exons and a 300 bp promotor region of the PLP gene of two affected brothers. The sequence analysis of a PCR fragment representing exon V amplified from genomic DNA of different kindreds of the pedigree revealed the mother as the only carrier indicating that the mutation has occurred de novo in the mother's germline.

PLP gene (including the 8.8 kb intron I) rearrangements have been excluded by Southern blot hybridization and overlapping PCR amplification of genomic DNA.

Eine neuartige Mutation im Gen des Proteolipidproteins führt zur Pelizaeus-Merzbacher-Krankheit (PMD)

Zusammenfassung: Punktmutationen des menschlichen Proteolipidprotein-Gens (PLP) sind die molekulare Grundlage einer bestimmten Leukodystrophie-Form, der X-chromosomalen Pelizaeus-Merzbacher-Krankheit (PMD). Wir berichten über die molekulare Analyse von vier PMD-Patienten in drei nicht miteinander verwandten Familien und beschreiben eine Punktmutation (G→A-Transition) im Exon

---

Enzymes:
Alkaline phosphatase, orthophosphoric-monoester phosphohydrolase (alkaline optimum) (EC 3.1.3.1) also named alkaline phosphomonoesterase;
Proteinase K, microbial serine proteinases (EC 3.4.21.14);
Pancreatic ribonuclease, RNAse A (EC 3.1.27.5);
Polydeoxyribonucleotide synthase (ATP), T_4 DNA ligase (EC 6.5.1.1);
Polynucleotide 5′-hydroxyl-kinase, ATP:5′-dephosphopolynucleotide 5′-phosphotransferase (EC 2.7.1.78), also named T_4 polynucleotide kinase;
DNA-directed DNA polymerase, deoxyribonucleoside-triphosphate:DNA deoxynucleotidyltransferase (DNA-directed) (EC 2.7.7.7), also named Taq polymerase;
Type II site-specific deoxyribonuclease (EC 3.1.21.4), also named type II restriction enzyme.

Abbreviations:
CNS, central nervous system; PCR, polymerase chain reaction; PLP, proteolipid protein; PMD, Pelizaeus-Merzbacher disease; RFLP, restriction fragment length polymorphism.

V, die zum Austausch von Gly^{216} durch Serin in einer sehr stark konservierten extrazytosolischen Domäne sowie zu einem *Mae* I-RFLP führt. Das Ergebnis einer molekularen Simulation durch Energieminimierung weist darauf hin, daß diese scheinbar unbedeutende Änderung der Aminosäuresequenz eine beträchtliche Änderung der Konformation, nämlich eine dichte Zusammenlagerung der Polypeptidkette, hervorruft, die mit der normalen PLP-Funktion in Oligodendrozyten nicht vereinbar ist.

Die Mutation wurde entdeckt und charakterisiert durch PCR-Amplifikation der genomischen DNA mit Hilfe von Intron- und Exon-Primern und der kompletten Sequenzanalyse der sieben Exons sowie einer 300-bp-Promotorregion des PLP-Gens zweier erkrankter Brüder. Die Sequenzanalyse des durch PCR der genomischen DNA amplifizierten Exons V der verschiedenen Mitglieder des Stammbaums zeigte, daß die Mutter die einzige Trägerin ist, so daß die Mutation de novo in der Keimbahn der Mutter eingetreten ist.

PLP-Gen-Umlagerungen (auch innerhalb des 8.8 kb langen Introns I) wurden durch Southern-Blot-Hybridisierung und überlappende PCR-Amplifikation der genomischen DNA ausgeschlossen.

Key terms: proteolipid protein, point mutation, dysmyelinosis of PMD type.

Dysmyelinoses or leukodystrophies are autosomal recessive or X-chromosomal recessive inherited diseases showing an impaired myelination. They are primarily restricted to the oligodendroglial cells. X-chromosomal recessive diseases with vertical inheritance are known in animals (jimpy mouse[1], md-rat[2], shaking pup[3,4], msd-mouse[5], rumpshaker mouse[6,7]) and in humans as sudanophilic leukodystrophy or Pelizaeus-Merzbacher disease (PMD), a rare neurodegenerative disease in which oligodendrocytes and white matter are affected. The clinical criteria (nystagmus, tremor, seizures, pyramidal and cerebellar signs, mental retardation) and neuropathological findings (lack of myelin formation to disturbed myelin morphology) of PMD of man and the afore-mentioned animal models are similar as a consequence of an impaired function of oligodendrocytes of CNS.

PMD patients, however, exhibit a heterogenous phenotype and divergent modes of inheritance of PMD subtypes have been suggested[8,9,10].

A breakthrough for a molecular definition of the X-chromosomal linked dysmyelinoses in animals and man came from the elucidation of the protein structure of the main integral membrane protein, proteolipid protein (PLP)[11], its gene organization[12] and the complete nucleotide sequence of the 17800-bp PLP locus. The single copy gene was assigned to the long arm of chromosome X q13[12,13].

With this basic knowledge the jimpy mouse defect was allocated to the splice donor site of intron IV of the PLP gene and consists of an A→G tansition which leads to alternative splicing and a frame shift[1]. The md-rat carries a point mutation (A→C transversion) in exon III, which leads to a Thr→Pro exchange[2], the msd-mouse shows a C→T transition in exon IV causing a Ala→Val exchange[5] and the rumpshaker mouse possesses a T→C transition in exon IV introducing an Ile→Thr exchange[6,7].

Like the animal dysmyelinoses some PMD patients show point mutations in the PLP gene[14–19] in different exons. In one case a complete deletion of the PLP locus has been found[20]. No PMD cases have been described so far in which the 5′-regulatory region or the large (8.5 kb) intron I, so far unexploited with regard to the regulation of PLP gene expression, is affected. The PLP mutations in PMD known so far and the mutation described in this paper reward a PMD classification solely restricted to mutations of the 17800 bp of the PLP locus on the X-chromosome.

The recessive trait of the lethal X-linked disease associated with the severe neurological symptoms demands reliable molecular perinatal diagnosis with the definition of the mutation and testing of female carriers within the pedigree showing a vertical inheritance of the PMD defect or even the detection of the spontaneous mutation in the parental line. The fatal prognosis, the prenatal diagnostic chances and the family counselling in PMD demand the conclusive molecular analysis ultimately including the DNA sequence of the mutated PLP gene.

The most reliable screening method of choice for the mutations is the nucleotide sequence determination of the seven exons of PLP with a set of intron primers for PCR amplification and sequencing. We have used this approach in the definition of a PMD mutation described before[17].

We report the molecular analysis of two patients (patients 1 and 2) in two unrelated families and of a PMD pedigree over three generations of two affected brothers (patients 3 and 4). PLP gene rearrangements

were excluded by Southern blot hybridization and overlapping PCR amplification of genomic DNA using intron and exon primers. Reamplified PCR fragments representing the coding exons, the exon-intron boundaries and the promotor region were sequenced.

A point mutation in exon V leading to a $Gly^{216} \rightarrow Ser$ exchange and a *Mae* I RFLP were determined in the pedigree of the PMD patients 3 and 4. The mother is the carrier and two male siblings are affected. Computer modelling indicated that the amino-acid exchange within a highly conserved domain of PLP imposes significant conformational differences between the wild-type and the mutant conformation. The mutation is in the neighborhood of two previously determined PMD mutations[14,18]. The impact of mutations in the different domains of the multispanning integral membran protein PLP for its integration in the myelin membrane is discussed. The molecular analysis of patients 1 and 2 indicated only a known silent mutation in exon IV[18,21], a T→C transition introducing an *Aha* II RFLP. This mutation has no influence on PLP expression and function[18] therefore it is unrelated to the molecular defect responsible for the PMD-like phenotype.

Materials and Methods

DNA of patients and control individuals

Genomic DNA was extracted from whole blood by the procedure of Jeanpierre[22] or from leukocytes by the procedure of Hogan et al.[23]. Leukocytes were obtained from whole blood by a ficoll gradient (15%) centrifugation.

Primers and probes

The oligonucleotides used as primers in the PCR and also used as sequencing primers were synthesized with an Applied Biosystems DNA Synthesizer, Model 381 A. They are listed in Table 1.

Oligonucleotides were labelled with [γ-^{32}P]ATP (Amersham, Braunschweig) and polynucleotide kinase (Gibco BRL, dsDNA Cycle Sequencing System).

Intron I-specific clones SK4 and SK5 and two *Pst* I fragments 743 bp for the 3′- and a 624 bp for the 5′ end of the human PLP cDNA were used for Southern blot hybridization analysis. They were labelled with [α^{32}P]dATP by random priming, using the Random Primed DNA Labelling Kit (Boehringer, Mannheim).

PCR amplification, cloning and sequencing of PCR fragments

Amplification of the genomic DNA was carried out in a DNA thermal cycler (Perkin Elmer-Cetus). The sense and antisense primers used for the amplification of the coding exons I to VII were the intron and exon primers described in our previous PMD paper[17].

For the amplification of the GC-rich 5′-untranslated region of the PLP gene 7-deaza-2′-deoxyguanosinetriphosphate (c^7-dGTP) was used[24]. The 100-μl PCR cocktail contained 1mM dATP, dGTP, dCTP, dTTP respectively of 750μM c7-dGTP and 250μM dGTP instead of 1mM dGTP. Sense- and antisense primer concentration were 100mM. The $MgCl_2$ concentration was kept constant at 1.5mM. The following temperature cycle was applied: initial denaturation for 2 min at 94°C, 60°C 2 min annealing, 72°C 2 min synthesis, 35 cycles with 1.5 min denaturation at 94°C. For allele-specific PCR primer annealing and synthesis were carried out in one step of 73°C for 3 min. ds PCR fragments were separated by low melting temperature (lmt) agarose gel electrophoresis (1–1.5%). Lmt-agarose aliquots of the ds PCR fragments were used for a PCR reamplification. The reamplified ds PCR fragments were isolated by agarose gel electrophoresis (1–1.5%) and Quiaex (Diagen, Düsseldorf) extraction for direct ds DNA sequencing or subcloning into *Sma* I, linearized and dephosphorylated pUC19 vector and subsequent double strand sequencing. ss PCR fragments were obtained by asymmetric PCR reamplification and directly sequenced. Then 50 pmol excess primer and 0.5 to 1 pmol second primer, a 3mM $MgCl_2$ concentration and 73°C annealing temperature were used in the PCR. The ss PCR fragments were separated by lmt-agarose electrophoresis. Lmt-agarose aliquots were used for direct sequencing with the Sequenase kit (USB).

ds PCR fragments were phosphorylated in the kinase reaction, blunt-ended by treatment with the Klenow fragment of DNA polymerase and ligated into the *Sma* I-restricted, dephosphorylated pUC19 vector by established methods.

Plasmid sequencing was carried out according to Chen and Seeburg[25] using the T7-sequencing kit (Pharmacia), the Sequenase kit (USB) or the dsDNA Cycle Sequencing System (BRL). The GC-rich 5′ domains were sequenced in the presence of dITP or c^7-dGTP instead of dGTP.

QUIAEX-purified ds PCR fragments were also sequenced directly using the ds DNA Cycle Sequencing System (BRL).

Genomic Southern blot hybridization analysis

10–20 μg genomic DNA was digested in the presence of 100 to 200 U restriction enzyme for 3 to 4 h in a total volume of 200 to 400 μl. DNA was precipitated with ethanol and separated by agarose gel electrophoresis (0.7%), transferred to a gene screen plus membrane by capillary blotting and hybridized with the ^{32}P-labelled denatured DNA probe[26].

Computer modelling

For molecular modelling with energy minimization an ESV3 Unix Workstation (Evans & Sutherland) and the programs Discover 3.8 and Insight 2.5 (Biosym Technologies, Inc.) were used.

Results

The strategy for the analysis of the PLP locus of the PMD patients 1 to 4 consisted of

a) Southern blot analysis and overlapping PCR to search for transpositions, larger deletions or insertions, inversions and partial gene duplications (Fig. 1);
b) PCR amplifications of fragments representing the exons, exon-intron boundaries and the promoter region of the PLP gene;
c) DNA-sequencing of the PCR fragments.

Southern blot analysis

The genomic DNA of patients 1, 2 and control DNA were digested with *Eco* RI, *Xba* I and *Pst* I. DNA of patients 3 and 4 were digested with *Pst* I. The restriction fragments were separated by agarose gel electro-

PCR primer

Name	Sequence (5'→ 3')	Length (bp)	MP (°C)
1 sense	AACGGATCCT TTTTTCTTGG GGCTGATACA Bam HI	30	73
1 S	CAGATGTCTCTGCAGCAAAG CGAAATTCCA Pst I	30	74
1 anti	*** ACTGAATTCC TGTGTCCTCT TGAATCTTCA ATG Eco RI	33	79
2 S	CTGCTTTCAG AGCCTGTGAC TTCTTGTGTG	30	75
2.45 S	GGCCTGGCAG AGGGGTTTGA GTGGCATGAG	30	79
II S	* * * GTTTGTTAGA GTGCTGTGCT AGATGTCTGC T	31	69
2 A	TGCACAGAGG GAAGACTCGG GAATCCGGTC	30	78
3 S	CTAGAAAATC CCTAGCCTTG TTAAGGTGCT	30	73
wt III A	GGCCCCATAA AGGAAGAAGA AAGAGGCAGT	30	76
2/3 B	CGCCGAATTC TCCCCCACCC CTTGT Eco RI	25	63
3 A	CCAGAACATT GTAAATTTAC CTTGGAGTTA	30	70
4 S	*** AGTGGATCCT CCTCATTCTT CCCCTACCCA TTC Bam HI	33	76
Mut 4N	ATGTCAATCA TTTTAGTTTG TGGGCATCAC	30	71
4 A	** AACTAAGACC CAAGAATTCA TCATCTTATA Eco RI	30	69
5 S	AAGAGGATCC TAATTGTTGG TAGCATCCAA Bam HI	30	72
Mut 5N S	TAGGTGTTCT CCCATGGAAT GCTTTCCCTG	30	73
Mut 5 S	* TAGGTGTTCT CCCATGGAAT GCTTTCCCTA	30	72
5 A	* CGGCGAATTC AAAATCCAGC AAAGGGAAAC Eco RI	30	74
6 S	AATTAGCACA CAGAAAGGAT ATCCAACACA	30	71
VI S	TTCCAAATGA CCTTCCACCT GTTTATTGCT GCATTT	36	87
6 A	TCCCTCCAGC ATTTCAAGGA TGGAAGCAGT	30	75
7 S	AATTCTATAT ACTATCACTA TGGCTCCACT	30	70
7 (301 S)	ATACAGAGTC AAGTAATTTC TCACCTTGTA	30	70
VII E	***** GAATTCAGCT CACCTTCAAG TATTGCTGC	30	58
VII A	GAACTTGGTG CCTCGGCCCA TGAGTTTAAG GACGGC	36	93
7 (901 A)	TAAGAAGAGG GCCAGTTGGT GGCAAAGGCA	30	76
7i S	ATTCAGTCAT CGTAGGTGAT TTGAAGGTCT	30	72
7i A	GAATTATTCT CCAGACATTT CTGATGCAAC	30	71
7 A	TCTTGAGAAG GTACGATTAT TAAGTTTCTA	30	69

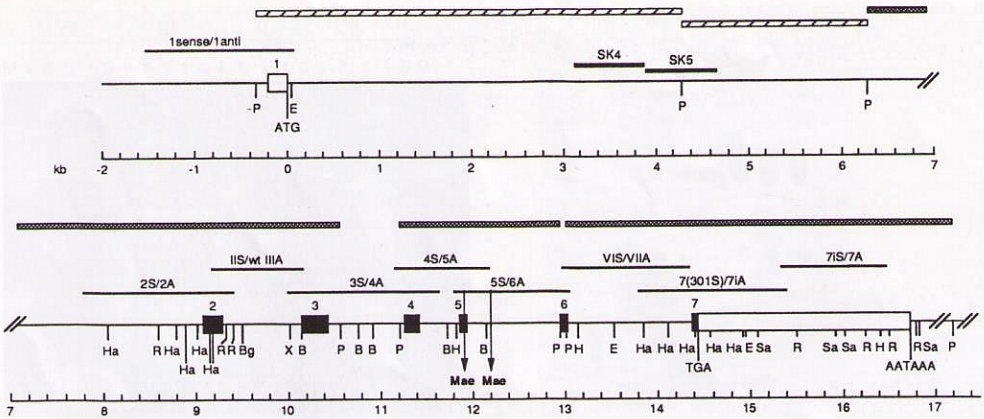

Fig. 1. Restriction map of human PLP gene, overlapping PCR fragments and *Pst* I fragments of PLP hybridizing in Southern blot analysis (see text).

Hatched bars, *Pst* I fragments hybridized with *Hin*dIII/*Bam*HI fragments isolated from clones SK4 (pos. 3172–3879) and SK5 (pos. 3879–4666) which are indicated by dark bars. Grey bars, *Pst* I fragments hybridized with two *Pst* I fragments: 743 bp of the 3′ end and 624 bp of the 5′ end of the human PLP cDNA. Exons are indicated by boxes, the coding sequences are black. The bars above the gene define the overlapping PCR fragments which are amplified between the primers listed in Table 1. *Mae* I restriction sites within PCR fragment 5S/6A are printed in bold face and indicated by arrows.

phoresis (0.7%), blotted and hybridized with the 743 bp and 624 bp *Pst* I cDNA fragments (data not shown) or with two *Hin*d III-*Bam* HI fragments isolated from the SK4 and SK5 clones corresponding to bp 3172 to 3879 and 3879 –4666 within intron I respectively (Fig. 2). The obtained restriction patterns of the control and PMD patient DNA are identical. In all cases the obtained restriction patterns corresponded to the restriction patterns expected from the mapping.

Overlapping PCR

Nine overlapping PCR fragments were synthesized with the intron and exon primers indicated in Fig. 1, covering 1.3 kb of the 5′-noncoding domain (promoter domain) and all seven exons. The GC-rich sequences between −400 and −700 (A of ATG, the translation start codon, +1) was amplified using c^7-dGTP-dGTP (3:1) to prohibit Hogsteen base pairing. Comparison of control and PMD patient DNA shows no differences in the length of the ds PCR fragments as documented in Fig. 3. The overlapping PCR and the Southern blot hybridization analysis gave no indication for a gene rearrangement. Therefore the coding sequences of the exons were sequenced.

Sequence analysis of PCR amplified coding regions of the PLP gene

The overlapping PCR fragments indicated in Fig. 3 of patients 1 to 4 were reamplified using "inner primers". The corresponding PCR fragments were obtained in high yield and purity. The blunt-ended PCR fragments were cloned blunt end into *Sma* I-linearized, dephosphorylated pUC19 for subsequent plasmid sequencing or directly sequenced. The latter method suppresses possible mutations caused by Taq polymerase and allows direct and simultaneous analysis of different alleles, whereas several clones have to be analysed by the plasmid sequencing to detect different alleles.

These different independent methods revealed in patients 1 and 2 a T→C transition in exon IV, the third base of the Asp^{202} codon. This is a silent mutation leading to an *Aha* II RFLP with no effect on the phenotype[18]. Patients 3 and 4, the two brothers with the clinical diagnosis PMD, showed an identical

◁ Table 1. Synthetic intron and exon derived oligonucleotides used as primers for the PCR amplification of exon I to VII of the PLP gene and for DNA sequencing.

Asterisks above the nucleotides indicate mismatches with the wild type sequence.

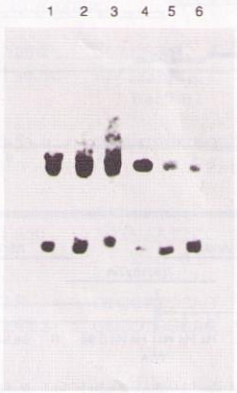

Fig. 2. Genomic Southern blot with random P^{32}-labelled *Hin*d III-*Bam* HI fragments (bp 3172–3879 and 3879–4666) of intron I.

Genomic DNA had been completely digested with *Pst* I and fragments separated by agarose gel electrophoresis (0.7%). Lane 1: control; lanes 2–5: patients 1–4; lane 6: control.

G→A transition in exon V, the first base of the Gly^{216} codon leading to an exchange against serine, Fig. 4. This mutation also introduces a *Mae* I restriction site, indicated in the nucleotide sequence of exon V, Fig. 5, and the agarose gel electrophoresis, Fig. 5B. The exon V sequence of the mother differs clearly from that of her two sons by the A→G double band indicating the G→A transition in one allele only. The other kindreds of the pedigree (Fig. 6) carry only the PLP wild-type allele. Therefore we conclude that this mutation occurred de novo in the mother, the origin of the vertical inheritance. The sequence data of the kindreds of the pedigree were obtained by direct sequencing of an exon V representing PCR fragment.

Mae *I RFLP*

The PCR fragment embracing exon V is 1210 bp long with one *Mae* I restriction site, yielding an 812 and 398 bp fragment. The G→A transition in exon V of the afflicted males introduces an additional *Mae* I site. The digestion of this PCR fragment with *Mae* I yielded three fragments, 812, 233 and 165 bp long. Fig. 5 demonstrates this RFLP in the heterozygous mother and the two PMD sons. The results have also been confirmed with allele-specific primers marked in the sequence of exon V of Fig. 5A.

Computer modelling

The effect of the Gly^{216}→Ser exchange on the protein conformation was examined by molecular modelling

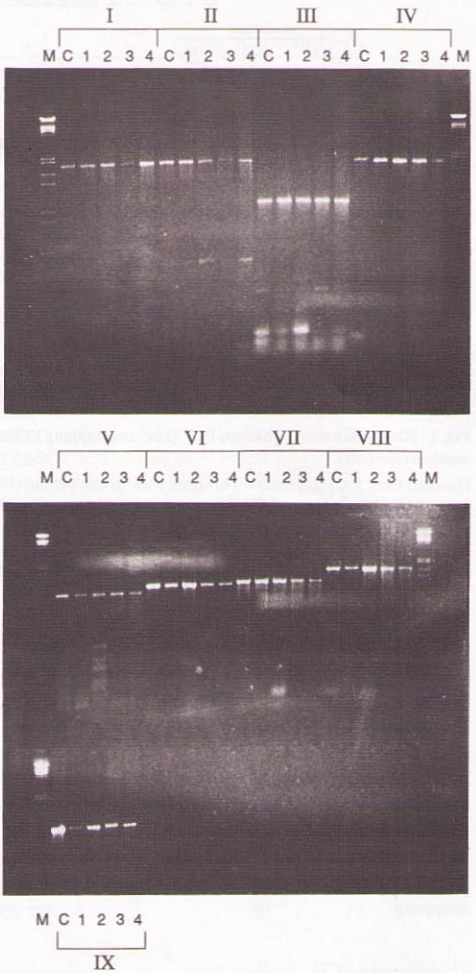

Fig. 3. Overlapping PCR fragments as shown in Fig. 1.

M: marker (λ-*Eco*RI/*Hin*dIII); C: control; lanes 1–4: patients 1–4. PCR fragments: I: 1sense/1anti (1.79 kb); II: 2S/2A (1.76 kb); III: IIS/wt IIIA (1.0 kb); IV: 3S/4A (1.7 kb); V: 4S/5A (1.07 kb); VI: 5S/6A (1.3 kb); VII: VIS/VIIA (1.3 kb); VIII: 7(301S)/7iA (1.65 kb); IX: 7iS/7A (1.08 kb).

with energy minimization of the extracytosolic polypeptide chain from Leu^{200} to Gly^{220}. The comparison of the predicted conformation of the wild type and mutant PLP sequence indicates a considerably tighter packing of the mutated polypeptide chain (model not shown). This conformational change could influence regular PLP expression and function.

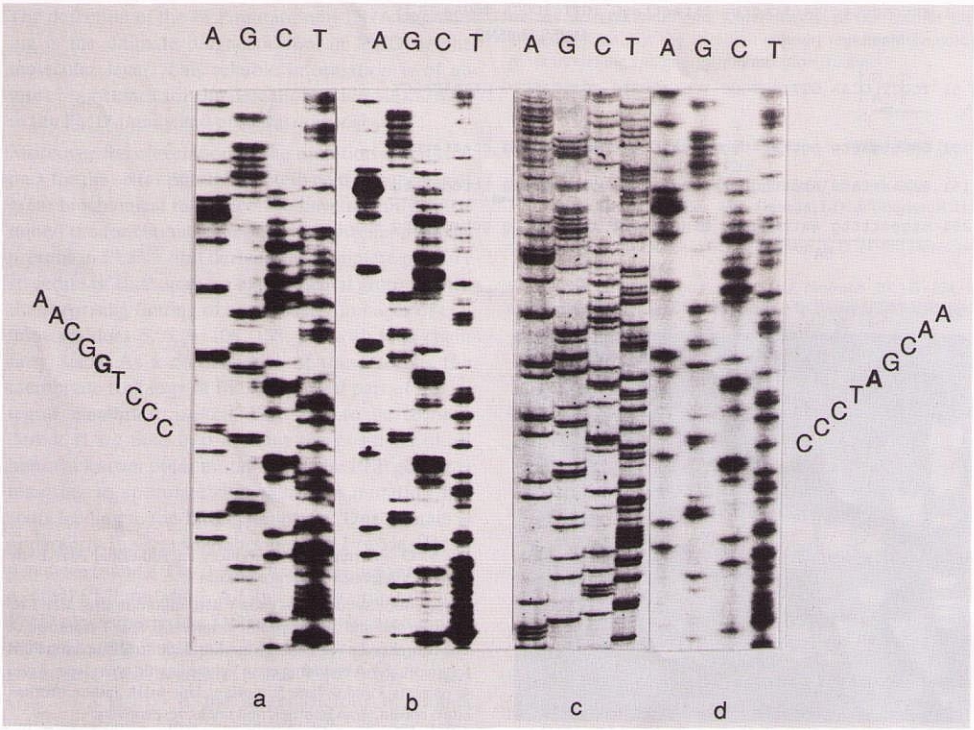

Fig. 4. Nucleotide sequence of exon V of PLP.
a) Wild type (control); b) sequence of the mother; c) and d) of the affected male patients 3 and 4.

Discussion

The PLP gene located on the X chromosome (Xq 13–22) encodes the main integral membrane protein of CNS myelin, proteolipid protein (PLP), an extremly hydrophobic 276 amino-acid residues long polypeptide (molecular mass 30 kDa) and its DM-20 isoform (241 amino acids) which results from alternative splicing by the activation of a cryptic splice donor site within exon II[27].

PLP has an extremely conserved structure with few amino-acid exchanges even between widely divergent species[28]. This indicates that minor mutational events inevitably lead to conformational alteration of this membrane protein not compatible with its function.

The molecular basis of dysmyelinosis such as inherited slowly progressive leukodystrophies have been elucidated in mouse, rat, dog and man. The neurodegenerative disorders of CNS primarily affect the spatially and timely regulated myelination. Most of these leukodystrophies can be referred to mutations within the X-chromosomal PLP locus. These mutations appear as pleiotropic mutations due to the defect of the oligodendrocytes. Whereas the PLP mutations in the afore-mentioned animal models are well defined single point mutations due to the homogeneity of these mutant animal strains, the PMD presents itself as a clinically heterogenous neurodegenerative disorder affecting the white matter of CNS. Several attempts have been made to extract a classification from genetic, clinical, neuropathological and recently CNS MR imaging findings. However on the basis of the recent molecular genetic analysis of the leukodystrophy, type Pelizaeus Merzbacher, a definition of the disease as mutated PLP locus would be logical. The mutations might occur in the coding or noncoding regions of the PLP gene. Several mutations within the PLP gene have been found in clinically tentatively diagnosed PMD patients. These mutations are scattered in different positions of the PLP gene, many of them in sequences coding putative transmembrane

```
 1 accctgcttg ctttttgtgt cttacttagG TGTTCTCCCA TGGAATGCTT
     Mae I                          Mut5S/Mut5NS
51 TCCCTGCAA GGTTTGTGGC TCCAACCTTC TGTCCATCTG CAAAACAGCT
101 GAGgtgagtg ggttatttgg gttattttac aagggagtag ctaatacçat
151 acaaattaca cccatggcct tcaattttaa ggactgaaag tttccctttg
201 ctggattttg aattagccga ttgccttcta caacatgttg gctaagtgtg
    5A
```

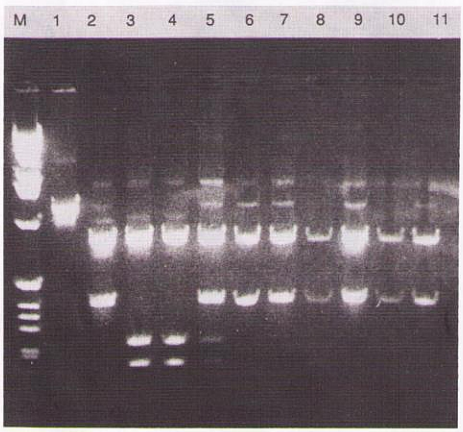

Fig. 5. G→A transition introduces an additional *Mae* I site into the nucleotide sequence of exon V.

A nucleotide sequence of exon V with mutation site, *Mae* I restriction site and allele-specific primers. B *Mae* I digestion of PCR amplified exon V. M: kb marker; lane 1: 1210 bp long PCR fragment 5S/6A including exon V; lane 2: wild type; lanes 3 and 4: patients 3 and 4; lane 5: mother; lane 6–11: father, brother, aunt, cousin, grandmother, and uncle, respectively.

helices[15-17,19], others within hydrophilic domains[14,18,19]. The Gly^{216}→Ser exchange described here occurs also in a putative extracytosolic domain within a strongly conserved sequence. Computer modelling of the mutated domain demonstrated clearly, that this amino-acid exchange introduces a considerable conformational change which could influence protein-protein- and protein-lipid interactions affecting expression or function of the proteolipid protein.

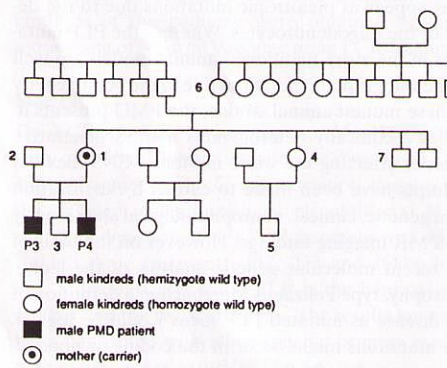

Fig. 6. Pedigree of PMD family of this study. The vertical transmission starts with the mother (1), two sons of F1 (P3 and P4) are affected. DNA from 1 mother, 2 father, 3 brother, 4 aunt, 5 cousine, 6 grandmother, 7 uncle, and patients 3 and 4 (P3, P4) was analysed.

The G→A transition leading to the Gly^{216}→Ser exchange is present in the heterozygote mother as carrier, in which the primary mutation event has occurred and in two affected sons (patient 3 and 4), whereas the alleles of the other kindreds have the wild type sequence. No previous history of PMD in this family is known. Therefore the G→A transition in exon V and the Gly^{216}→Ser exchange is causing PMD in this family.

The T→C transition in exon IV which has been found in patients 1 and 2 is a silent mutation with no influence on PLP expression and function[18]. Therefore this mutation can be excluded as cause for the diagnosed leukodystrophy but not a mutation in the noncoding regions of the PLP gene not sequenced here and which might affect regular splicing. Alternatively a mutation in another locus involved in myelination could have caused the PMD-like phenotype.

The detection of the PLP mutation by DNA sequencing is the ultimate diagnostic tool of PMD on the molecular level. This reliable information is of utmost importance for the detection of female carriers in the PMD-family and prenatal diagnosis.

Moreover the correlation of the mutation and its impact for the so far putative protein conformation is of great biochemical relevance. We have recently determined the functional state of the 14 cysteine residues present in PLP[29] and derived the complete primary structure of PLP on these experimental grounds with the surprising finding of six thioester linkages of cysteine residues 5, 6, 9, 108, 138, 140 with long-chain fatty acids. As a consequence of these results the membrane topology of the N-terminal part of the integral membrane protein PLP had to be reconsidered. If we take into account the positions of all hitherto known point mutations of the PLP gene it is tempting to speculate that there are mutation hot spots leading to the PMD phenotype. One domain is apparently the transmembrane loop 2 (exon III) in which we found a $Thr^{75} \rightarrow Pro$ exchange in the md-rat, a lethal PLP mutation. A $Gly^{73} \rightarrow Arg$ mutation in a PMD case reported by Doll et al.[19] is located in juxtaposition. Similary the highly conserved domain between the two disulfide bonds $Cys^{183}-Cys^{227}$ and $Cys^{200} - Cys^{219}$[29] is the target of four mutations $Val^{218} \rightarrow Phe$[18], $Pro^{215} \rightarrow Ser$[14], $Gly^{216} \rightarrow Ser$ (this paper) and $Asp^{202} \rightarrow His$[19]. Unfortunately neither information about the history, life expectancy and clinical symptoms of these PMD families are available nor about the degree of myelination, myelin structure and number and functional state of oligodendrocytes on the biochemical level.

It is, however, tempting to assume a crucial function of this extracytosolic domain of the proteolipid protein within the oligodendrocyte plasma membrane or myelin processes of the plasma membrane, either for inter- or intramolecular protein-protein or protein-lipid interactions.

It cannot be decided whether these PLP mutations exert their deleterious destructive function on the transcriptional, translational or posttranslational level. Animal models which mimick these mutations and which can be generated by homologous recombination will be of great importance for our understanding of the molecular processes in the affected oligodendrocytes.

We are grateful to *Brigitte Kisters-Woike* of the Institut für Genetik, Köln, for the excellent support regarding the computer modelling and energy minimization studies.

References

1 Nave, K., Lai, C., Bloom, F. & Milner, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9264–9268.
2 Boison, D. & Stoffel, W. (1989) *EMBO J.* **8**, 3295–3302.
3 Yanagisawa, K., Moller, J.R., Duncan, I.D. & Charles, R.H. (1987) *J. Neurochem.* **49**, 1912–1917.
4 Nadon, N.L., Duncan, I.D. & Hudson, L.D. (1990) *Development* **110**, 529–537.
5 Gencic, S. & Hudson, L.D. (1990) *J. Neurosci.* **10**, 117–124.
6 Griffiths, I., Scott, I., McCulloch, M., Barrie, J., McPhilemy, K. & Cattanach, B. (1990) *J. Neurocytol.* **19**, 273–283.
7 Schneider, A., Montague, P., Griffiths, I., Fanarraga, M., Kennedy, P. & Nave, K.-A. (1992) *Nature (London)* **358**, 758–762.
8 Baraitser, M. (1990) *The Genetics of Neurological Disorders*, Oxford Univ. Press, Oxford.
9 Seitelberger, F. (1979) in *Handbook of Clinical Neurology*, vol. 10 (Vinken, P.J. & Bryott, C.W., eds.) pp. 150–202, North Holland Publ. Comp. Amsterdam.
10 Bulloche, J. & Aicardi, J. (1986) *J. Child Neurol.* **1**, 233–239.
11 Stoffel, W., Hillen, H. & Giersiefen, H. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5012–5016.
12 Diehl, H.-J., Schaich, M., Budzinski, R.-M. & Stoffel, W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9807–9811.
13 Willard, H. & Riordan, J. (1985) *Science* **230**, 940–942.
14 Gencic, S., Abuelo, D., Ambler, M. & Hudson, L. (1989) *Am. J. Hum. Genet.* **45**, 435–442.
15 Hudson, L., Puckett, C., Berndt, J., Chan, J. & Gencic, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8121–8131.
16 Trofatter, J.A., Dlouhy, S.R., DeMyer, W., Conneally, P.M. & Hodes, M.E. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9427–9430.
17 Weimbs, T., Dick, T., Stoffel, W. & Boltshauser, E. (1990) *Biol. Chem. Hoppe-Seyler* **371**, 1175–1183.
18 Pham-Dinh, D., Popot, J.-L., Boespflug-Tanguy, O., Landrieu, P., Deleuze, J.-P., Boue, J. & Jollès, P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7562–7566.
19 Doll, R., Natowicz, M.R., Schiffmann, R. & Smith, F.I. (1992) *Am. J. Hum. Genet.* **51**, 161–169.
20 Raskind, W.H., Williams, C.A., Hudson, L.D. & Bird, T.D. (1991) *Am. J. Hum. Genet.* **49**, 1355–1360.
21 Trofatter, J.A., Pratt, V.M., Dlouhy, S.R. & Hodes, M.E. (1991) *Nucleic Acids Res.* **19**, 6057.
22 Jeanpierre, M. (1987) *Nucleic Acids Res.* **15**, 9611.
23 Hogan, E.L., Constantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
24 McConlogue, L., Brow, M.A.D. & Innis, M.A. (1988) *Nucleic Acids Res.* **110**, 6869.
25 Chen, E.Y. & Seeburg, P.H. (1985) *DNA* **4**, 165–170.
26 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York.
27 Stoffel, W. (1990) *Angew. Chem., Int. Ed. Engl.* **29**, 958–976.
28 Schließ, F. & Stoffel, W. (1991) *Biol. Chem. Hoppe-Seyler* **368**, 419–425.
29 Weimbs, T. & Stoffel, W. (1992) *Biochemistry*, in press.

B. Otterbach and W. Stoffel*, Institut für Biochemie, Medizinische Fakultät, Universität zu Köln, Joseph-Stelzmann-Str. 52, W-5000 Köln 41;

V. Ramaekers, Kinderhospital, Medizinische Fakultät, RWTH Aachen, Pauwelsstr., W-5100 Aachen.

* To whom correspondence should be addressed.