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Membrane topology of the 22 kDa integral peroxisomal membrane protein

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In order to study the membrane topology and the possible function of the rat liver 22 kDa integral peroxisomal membrane protein (PMP 22) at a molecular level, we have cloned PMP 22 from a λgt11 expression library and sequenced its cDNA. Hydropathy analysis of the deduced primary structure indicates 4 putative transmembrane segments. The accessibility to exogenous aminopeptidase of PMP 22 in intact peroxisomes suggests that the N-terminus faces the cytosol. A model of the topology of PMP 22 in the peroxisomal membrane is discussed. Homology studies revealed a striking similarity with the Mpv 17 gene product. Lack of this membrane protein causes nephrotic syndrome in mice.

Peroxisome; Peroxisomal membrane protein, 22 kDa; cDNA cloning; Membrane topology

1. INTRODUCTION

The membrane of rat liver peroxisomes contains various integral membrane proteins (PMPs) with PMP 69, PMP 26, PMP 36, PMP 22 and PMP 15 as the major components identified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of highly purified peroxisomal membranes [1–5]. None of these PMPs is characterized functionally, and organization as well as biogenesis of the peroxisomal membrane are only poorly understood. Tsukamoto et al. [6] recently identified a 35 kDa peroxisomal membrane protein from rat liver which upon transfection restored peroxisome assembly in a mutant Chinese Hamster Ovary cell line and in human skin fibroblasts of a patient with Zellweger syndrome [7]. Another promising approach to identify membrane components involved in peroxisome assembly is the functional complementation of yeast mutant cell lines. From peroxisome assembly mutants (PAS-mutants) [8] the PAS 3 gene has been cloned and sequenced [9]. The gene codes for a 50.6 kDa membrane protein the lack of which causes the peroxisome-deficient phenotype and mislocalization in the cytosol of peroxisomal matrix proteins.

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Abbreviations: PMS, post-mitochondrial supernatant; PMP, peroxisomal membrane polypeptide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

PMP 69 from rat liver has recently been cloned and its amino acid sequence has been deduced [10]. It is a member of the superfamily of ATP-binding proteins involved in bacterial transport systems that confer multidrug resistance to mammalian tumor cells. It is highly induced in the liver of rats treated with peroxisome proliferators [1–4], and under these conditions becomes the dominant protein in the peroxisomal membrane.

The main component in peroxisomes of untreated rat liver is PMP 22 [2,11]. Its expression is not stimulated by peroxisomal proliferators. The function of PMP 22 has been attributed to the unspecific permeability of the peroxisomal membrane [12–15] since a solubilized and partially purified peroxisomal membrane protein fraction enriched in PMP 22 has been shown to induce pore forming activity in liposomes [13].

In order to get further hints to the possible function of PMP 22 and to study its membrane topology at a molecular level, we cloned PMP 22 from a cDNA expression library and established its nucleotide and deduced amino acid sequence. Based on these and biochemical data a model is proposed of the topology in the peroxisomal membrane of PMP 22.

2. MATERIALS AND METHODS

2.1. *Peptide sequencing*

Limited proteolysis using trypsin of PMP 22 extracted from Coomassie brilliant blue stained SDS-polyacrylamide gels was carried out as described [16]. Peptides were separated by HPLC (Lichrosphere 100 RP 18, Merck, Darmstadt) and peptide sequences determined by gas phase sequencing [17].

2.2. *Immunoscreening of a λgt11 cDNA library*

The λgt11 cDNA library used was prepared by Frain et al. [18].

Immunoscreening was performed by standard techniques [19,20] using a polyclonal anti-PMP 22 antiserum [21] which previously was depleted of anti-*E. coli* antibodies by affinity chromatography [19]. Visualization of positive clones was performed with an affinity-purified peroxidase conjugated goat anti-rabbit IgG antibody and 3,3′-diaminobenzidine–H_2O_2 as the chromogenic substrate [22].

2.3. *Screening of the λgt11 cDNA library with DNA probes*

The 470 base pair insert which contained approximately one-half of the entire PMP 22 cDNA was further used for rescreening the λgt11 cDNA library. For this purpose the insert was radiolabeled with [α-^{32}P]dCTP according to Feinberg et al. [23] and hybridized by standard techniques [19]. Various positive clones were detected from which cDNA inserts were prepared and probed with a synthetic oligonucleotide synthesized according to the peptide sequence SGFWPALQMN. Two of 6 positive clones were used for DNA sequencing.

2.4. *DNA sequencing*

From a positive clone identified by immunoscreening the DNA was prepared and digested with *Eco*RI using standard techniques [19]. The obtained cDNA insert which was 851 base pairs in size was subsequently cloned into M13mp18 and single-stranded DNA was sequenced by the dideoxy method of Sanger et al. [24] using M13 forward primer (USB, Bad Homburg).

2.5. *In vitro transcription/translation of the cloned gene*

The cloned gene was excised with *Eco*RI from the phage vector M13mp18 and inserted into the *Eco*RI site of the pBluescript vector using standard techniques. In vitro transcription was performed for 1 h as described in [25]. For optimal rate of transcription both GTP and m^7-GpppG (Boehringer, Mannheim) were again added to the incubations 30 min after start of transcription at a concentration of 250 μM. For in vitro translation both the wheat germ and the reticulocyte lysate system (Amersham, Braunschweig) was used. In vivo synthesized PMP 22 was obtained from peroxisomes of isolated hepatocytes [26] labeled with [^{35}S]methionine in a methionine-free RPMI 1640 tissue culture medium containing 25 mM HEPES, pH 7.4, for 45 min at 37°C. Recombinant and in vivo synthesized PMP 22 was isolated by immunoprecipitation using a monospecific polyclonal anti-PMP 22 antiserum and analyzed by SDS-PAGE.

2.6. *Protease treatment of intact isolated peroxisomes*

Peroxisomes were isolated by Nycodenz density gradient centrifugation as described [11]. Centrifugation was performed in a RPV-50T vertical rotor (Hitachi, Tokyo) for 45 min at 50,000 × *g*. Peroxisomes were recovered from the gradients by slowly diluting the peroxisomal fraction with 3 vols. of 0.25 M sucrose, 20 mM glycylglycine, pH 7.4, 1 mM EDTA, pelleting the organelles by centrifugation at 12,000 × *g* for 20 min and resuspending the pellet in the sucrose medium used for dilution. For the protease digestion 400 μl of the peroxisome suspension containing 150 μg peroxisomal protein were treated with 0.5 and 0.7 IU leucine aminopeptidase (Sigma, Munich) at 37°C for 2 h or with 0.1 IU trypsin (Boehringer, Mannheim) at 0°C for 30 min. The trichloroacetic acid precipitated samples were separated by SDS-PAGE and analyzed by immunoblotting as described [27] using anti-PMP 22 antiserum. The PMP 22 cleavage fragments produced by trypsin were visualized by using the ECL system (Amersham, Braunschweig) for developing the immunoblot.

3. RESULTS

3.1. *cDNA cloning and sequencing of PMP 22*

In order to elucidate the primary structure of this major peroxisomal membrane component we cloned its gene from a λgt11 cDNA expression library [18] by screening with a polyclonal anti-PMP 22 antiserum. From one positive clone a 470 base pair insert was obtained coding for the N-terminal portion of the polypeptide. Rescreening of the cDNA library with this insert revealed other positive clones from which the entire nucleotide sequence was obtained. Both the nucleotide sequence and the deduced primary structure of PMP 22 are shown in Fig. 1. The total length of the sequence including the *Eco*RI linkers was 851 base pairs. The initiator codon for methionine was assigned to the nucleotides 1–3. It was followed immediately by the amino-terminal sequence determined from purified PMP 22 by Edman degradation (APAASRLRVESELRSLPK). After tryptic digestion of purified PMP 22, peptides were isolated by high-performance liquid chromatography and the amino acid sequence of three peptides determined (FYPVVTK, NISVF, and SGFWPALQMN). These peptides were identified as parts of the entire sequence (Fig. 1).

The cDNA contains 6 bases of the 5′-noncoding region and 251 bases of the 3′-noncoding region. Nine base triplets upstream from the 3′-end, the consensus polyadenylation signal AATAAA was identified. The open reading frame contains 582 nucleotides encoding 194 amino acid residues. The calculated molecular weight of PMP 22 is 22,447, in close agreement with the value of 22 kDa as estimated by SDS-PAGE. The cloned gene subsequently was inserted into pBluescript phagemid. In vitro transcription resulted in the synthesis of PMP 22 mRNA which could be translated in the cell free in vitro systems derived from wheat germ and reticulocytes. The in vitro translation product was recognized by an antiserum raised against native PMP 22 and thus could be isolated by immunoprecipitation. Comparison by SDS-PAGE of the recombinant PMP22 with that synthesized by isolated hepatocytes revealed identical size of both polypeptides (Fig. 2).

3.2. *Sequence homology to Mpv 17*

Comparison of the entire PMP 22 sequence with sequences stored in the Gen EMBL nucleotide data library using the TFASTA algorithm of Lipman and Pearson [28] revealed in a 155 amino acid overlap 26.5% identical amino acid residues and a striking sequence similarity of 74.8% with Mpv 17 (Fig. 3), a polypeptide of 176 amino acids. Mpv 17 was characterized in a mutant mouse line [29]. Mice homozygous for a retroviral insert at the Mpv 17 locus lack the expression of Mpv 17. After 2–3 months of age the homozygotes start to develop a glomerular disease that clinically resembles the nephrotic syndrome in man.

3.3. *Hydropathy analysis of PMP 22*

The biochemical properties of PMP 22 already suggested the existence of several transmembrane regions to be contained in the molecule. We further investigated the topology of PMP 22 in the peroxisomal membrane by hydropathy analysis using the algorithm of Kyte and Doolittle [30]. Choosing a window size of 10 amino acid

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-20  aattcggcacgagcgcgatggcacctgccgcgtcaaggctgagggtggaatccgagctcc   39
                     M  A  P  A  A  S  R  L  R  V  E  S  E  L  R

 40  ggtcgcttcccaagcgagcgctcgcccaatacttgctgtttctaaagttctatccggtgg   99
      S  L  P  K  R  A  L  A  Q  Y  L  L  F  L  K  F  Y  P  V  V

100  tcaccaaagctgtcagcagtggcattttgtcagcccttggaaaccttctggcccagatga   159
      T  K  A  V  S  S  G  I  L  S  A  L  G  N  L  L  A  Q  M  I

160  ttgagaagaagcagaaaaaagactctcgaagtctagaagtcagtgggcttctcagatatt   219
      E  K  K  Q  K  K  D  S  R  S  L  E  V  S  G  L  L  R  Y  L

220  tagtttatgggttgtttgtcacaggtcccctgagtcactacctctacctcttcatggagt   279
      V  Y  G  L  F  V  T  G  P  L  S  H  Y  L  Y  L  F  M  E  Y

280  actgggtccctcctgaggttccctgggccagagtcaagaggctcctgctggatcgcctgt   339
      W  V  P  P  E  V  P  W  A  R  V  K  R  L  L  L  D  R  L  F

340  tctttgccccaacctttctgctgctgttcttcttcgtcatgaacttgctggagggaaaaa   399
      F  A  P  T  F  L  L  L  F  F  F  V  M  N  L  L  E  G  K  N

400  acatcagtgtctttgttgccaagatgaggagtggcttctggcctgctctgcaaatgaact   459
      I  S  V  F  V  A  K  M  R  S  G  F  W  P  A  L  Q  M  N  W

460  ggaggatgtggacacccttacagttcatcaacatcaactatgtgcccttgcagttccggg   519
      R  M  W  T  P  L  Q  F  I  N  I  N  Y  V  P  L  Q  F  R  V

520  tgctctttgccaacatggcagctctgttctggtatgcctacctggcctctctggggaagt   579
      L  F  A  N  M  A  A  L  F  W  Y  A  Y  L  A  S  L  G  K

580  gatgtctgatacaccgtggatgaggtgtggggagatccaaccaccagcaggagagagcag   639

640  aatccatcattaaggacacctcatgactaaattatgtgattcaagatgccttaaaattac   699

700  agagggaggaacgtgtcagtgtcggaatcatgtgcttcaaagagacagcactagtctcgg   759

760  tggagttaatcttagttgtcactgcaaacaggtaaaggtcaataaacagtgatgcagcct   819

820  cgtgccgaattc  831
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Fig. 1. Nucleotide and translated amino acid sequence of PMP 22 cDNA. The underlined sequences have been confirmed by Edman analysis. The polyadenylation signal is underlined two-fold. The nucleotide sequence is numbered on the left starting from the first residue of the initiating ATG codon. Nucleotides in the 5'-untranslated region are numbered negatively.

residues 4 potential transmembrane segments were recognized (Fig. 4). They are located between the amino acid residues 30–45, 73–93, 115–133 and 171–192, i.e. they are spread over the entire molecule. The algorithm of Engelman et al. [31] produced a quite similar hydropathy profile. Comparison of the hydropathy plot of PMP 22 with that of Mpv 17 (Fig. 4) again revealed a striking similarity of the relative location of the transmembrane segments suggesting Mpv 17 to be embedded in its target membrane in a way similar to PMP 22.

3.4. *Membrane topology of PMP 22*

For a more detailed view on how PMP 22 is inserted in the peroxisomal membrane we determined the sidedness of the N-terminus of the protein. In intact isolated peroxisomes PMP 22 was found to be susceptible to cleavage by exogenously added trypsin and aminopeptidase (Fig. 5). Incubations with increasing amounts of aminopeptidase produced the appearance of a fragment of PMP 22 with apparent molecular weight of 21,500. As a control the cleavage by aminopeptidase of 3-ke-

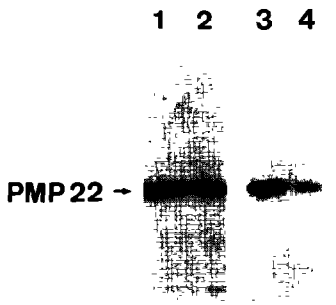

Fig. 2. In vitro transcription/translation of PMP 22. The PMP 22 gene, recloned into pBluescript, was used to synthesize an active mRNA which was translated in the wheat germ in vitro translational system. Both total translation products (lane 1) and PMP 22 immunoprecipitated after in vitro translation (lane 2) were analyzed by SDS-PAGE and fluorography. In order to compare the size of the recombinant PMP 22 with that of the native one, the in vitro translation product (reticulocyte lysate, lane 3) and the immunoprecipitate obtained from peroxisomes of [^{35}S]methionine labeled hepatocytes (lane 4) were analyzed by SDS-PAGE.

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                20        30        40        50        60        70
pmp22           RLRVESELRSLPKRALAQYLLFLKFYPVVTKAVSSGILSALGNLLA-QMIEKKQKKDSRS
                                              :::::| | ::|:::: |::|::  :: ::
mpv1-mouse                 MALWRAYQRALAAHPWKVQVLTAGSLMGVGDMISQQLVERRGLQQHQA
                                     10        20        30        40

                    80        90       100       110       120       130
pmp22           LEVSGLLRYLVYGLFVTGPLSHYLYLFMEYWVPPEVPWARVKRLLLDRLFFAPTFLLLFF
                  : :::: | :| || ||:    | ::: :| ::    :|::|||:  ||| ||  |:
mpv1-mouse      GRTLTMVS-LGCG-FV-GPVVGGWYKVLDHLIPGTTKVHALKKMLLDQGGFAPCFLGCFL
                50          60          70        80        90        100

                    140       150       160       170       180       190
pmp22           FVMNLLEGKNISVFVAKMRSGFWPALQMNWRMWTPLQFININYVPLQFRVLFANMAALFW
                 ::::|:|::     ||::::: ||  |: :|:::|: |:  |||::|:  :: :|: |
mpv1-mouse      PLVGILNGMSAQDNWAKLKRDYPDALITNYYLWPAVQLANFYLVPLHYRLAVVQCVAIVW
                  110       120       130       140       150       160

                    200
pmp22           YAYLASLGK
                 :||:
mpv1-mouse      NSYLSWKAHQF
                  170
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Fig. 3. Comparison of the cDNA deduced primary structure of PMP 22 and Mpv 17. In an overlap of 155 amino acid residues 26.8% of amino acids are identical (vertical strokes) and 74.8% are similar (double points).

toacyl-CoA thiolase, a peroxisomal matrix enzyme, was investigated. It turned out that the enzyme was not at all susceptible to the protease unless detergent was added to the peroxisomes (not shown). Trypsin treatment of isolated peroxisomes produced PMP 22 fragments of the expected molecular weight of approximately 10–11 kDa that weakly reacted with the anti-PMP 22 antiserum whereas treatment with carboxypeptidase Y did not shorten PMP 22 to any perceptible extent (not shown).

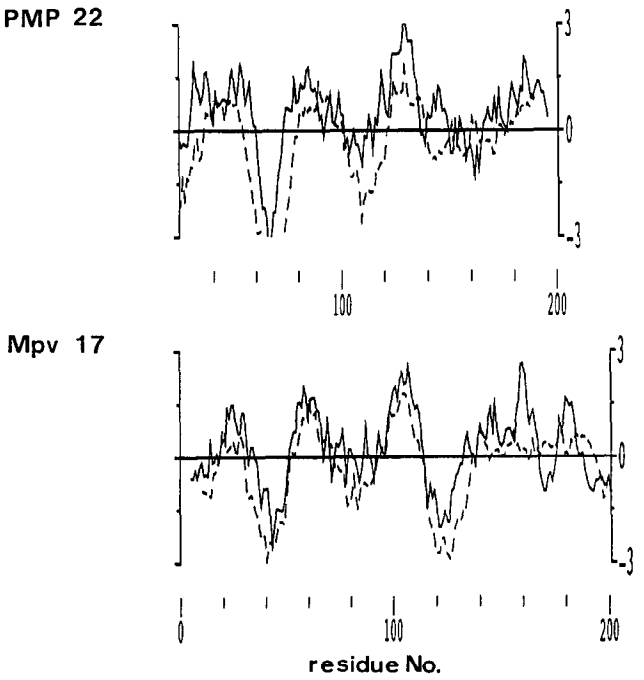

Fig. 4. Hydropathy profile comparison of the predicted amino acid sequences of PMP 22 and Mpv 17. Hydropathy analysis was performed according to Kyte and Doolittle ([30], full lines) and Engelman et al. ([31], dashed lines). The hydrophobicity index as defined by Kyte and Doolittle is indicated on the right ordinate. In both molecules 4 transmembrane spanning regions are recognized, the relative location of which is strikingly similar.

4. DISCUSSION

4.1. *Molecular properties and membrane topology of PMP 22*

To gain more insight into the molecular properties of PMP 22 its gene was cloned and sequenced and from the nucleotide sequence the primary structure of the protein deduced (Fig. 1). In vitro transcription/translation of the cloned gene resulted in the synthesis of a translation product which was recognized by the antiserum raised

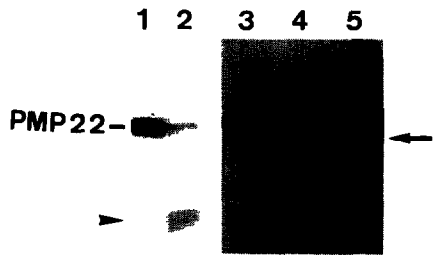

Fig. 5. Accessibility of PMP 22 to proteolysis by exogenously added proteases. Intact isolated peroxisomes (150 μg, lanes 1 and 3) were incubated with trypsin (0.1 IU, lane 2) and leucine aminopeptidase (lanes 4 and 5) and the proteolytic processing of PMP 22 was analyzed by SDS-PAGE and immunoblotting using a horseradish peroxidase coupled second antibody and the ECL system (lanes 1 and 2) or a chromogenic substrate [22] for the visualization reaction. Trypsin treatment caused a considerable reduction of the native PMP 22 and produced fragments of the expected molecular weight of approximately 10–11 kDa (arrowhead) whereas leucine aminopeptidase produced a PMP 22 fragment of 21.5 kDa (arrow).

against the native protein and which was electrophoretically indistinguishable from the native molecule. Thus in vitro transcription/translation along with a suitable in vitro import system offers the possibility to study in more detail the mechanism of the in vitro insertion of PMP 22 into the peroxisomal membrane, e.g. the identification of putative targeting signals. The SKL-signal present in some peroxisomal matrix enzymes [32] is not found in PMPs [33] and no cleavable presequences containing a targeting signal as for peroxisomal 3-ketoacyl-CoA thiolase [34] are present. Thus the underlying mechanism of PMP-targeting is completely unknown.

Hydropathy analysis of the protein suggested the presence of four transmembrane stretches distributed along the entire molecule with the N-terminal 36 amino acids protruding from the membrane (Fig. 4). In order to determine the sidedness of the molecule the accessibility of the N-terminus to exogenously added aminopeptidase and of the C-terminus to carboxypeptidase Y was investigated. Cleavage of PMP 22 was observed only with aminopeptidase (Fig. 5) suggesting that the N-terminus is facing the cytosol. The existence of 4 transmembrane stretches, as indicated by the hydropathy plot, implies that the C-terminal end of the polypeptide must also be directed towards the cytosol. From the C-terminal part of PMP 22 possibly only the C-terminal Lys^{193} protrudes out of the membrane which explains the unaccessibility in intact peroxisomes of the C-terminus to carboxypeptidase Y.

Based on these data a model of the membrane topology of PMP 22 is proposed (Fig. 6). The most important features of this model are the existence of 4 transmembrane spanning regions with one hydrophilic protein loop exposed to the cytosol and two others to the lumen of the peroxisomes. The N-terminal part of the molecule protruding from the membrane between Arg^{19} and Lys^{29} contains a hydrophobic stretch of 9 amino acids which may be attached to or partially be inserted into the bilayer and thus may explain the protection of this N-terminal part from proteolytic attack by proteinase K and subtilisin as observed previously [2]. The consideration of one hydrophilic protein loop between Met^{92} and Leu^{113} oriented to the cytosol is compatible with the appearance of tryptic fragments of approximately 10–11 kDa.

4.2. *PMP 22 is a Type IIIb transmembrane protein*

According to Singer [35] PMP 22 belongs to integral membrane proteins of Type IIIb with more than one transmembrane stretch and the N-terminus exposed to the exterior surface of the membrane. Such a classification is crucial when the mechanism of the protein's insertion into the membrane is considered. This may involve several insertion steps which most probably are arranged sequentially [36] and are mediated by one or more signal sequences and stop transfer sequences [37–40]. It has been noted previously that the hydrophobic transmembrane stretches are flanked frequently by one or more charged residues and that these charges are important for the transmembrane orientation [41,42]. The charged segments might facilitate electrostatical binding of the protein to the membrane thus enabling insertion as a loop [41]. Charged residues surrounding the putative transmembrane segments are also found in PMP 22 and are depicted in Fig. 6. Viewed from the N-terminus to the C-terminus the transmembrane segments most interestingly are preceded immediately by a positively charged amino acid residue (lysine or arginine) and terminated by a glutamic acid residue. According to the data collected on the membrane topology of bacterial membrane proteins, a hydrophobic segment with positively charged residues toward its C-terminus is oriented with its N-terminus periplasmically [41]. A corresponding situation is observed with the N-terminal transmembrane segment of PMP 22 and the exterior orientation of its N-terminus. We believe that PMP 22 serves as a suitable model protein to study mechanistic aspects of the insertion of eukaryotic Type IIIb proteins.

4.3. *Sequence similarity between PMP 22 and Mpv 17*

Comparison of the primary structure of PMP 22 with the known structure of other proteins demonstrated a striking sequence similarity with Mpv 17 (Fig. 3), a 176 amino acid polypeptide also containing four transmembrane regions distributed along the entire molecule (Fig. 3). The physiological function of Mpv 17 is still not known. Studies on transgenic mice lacking the expression of Mpv 17 revealed the development of adult onset nephrotic syndrome and chronic renal failure [29]. The Mpv 17 gene product seems to be important for the

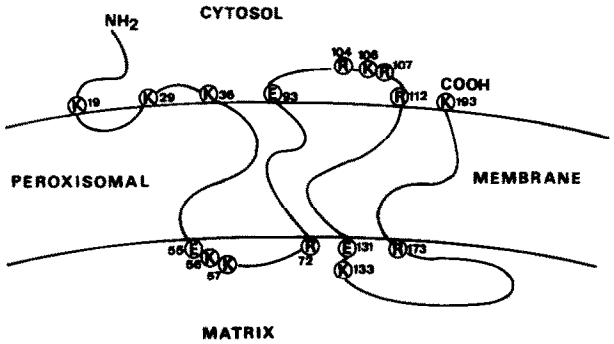

Fig. 6. Hypothetical topology of PMP 22 in the peroxisomal membrane. The model is distinguished by the presence of 4 hydrophobic transmembrane segments spanning the peroxisomal membrane and the orientation of both the amino- and the carboxy-terminus to the cytosol. Between Lys^{19} and Lys^{29} there is a hydrophobic stretch of 9 amino acids which might be loosely associated with the bilayer. Polar amino acid residues are limiting the transmembrane segments. Most interestingly the positively charged amino acids lysine (K) or arginine (R) and the negatively charged amino acid glutamic acid (E) are alternately flanking the membrane spanning segments. According to the model one loop of the polypeptide chain between Glu^{93} and Arg^{112} faces the cytosol and contains additional positively charged residues (Arg^{104}, Lys^{106}, Arg^{107}) permitting tryptic attack.

correct functioning of the renal visceral epithelial cells that, due to lack of turnover, become progressively damaged in the mutant animal.

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