Hoppe-Seyler's Z. Physiol. Chem.
Bd. 363, S. 855–864, August 1982

Primary Structure of the *C*-Terminal Cyanogen Bromide Fragments II, III and IV from Bovine Brain Proteolipid-Apoprotein

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(Received 20 April 1982)

Summary: Purified lipophilin from bovine brain white matter was reductively carboxymethylated and then cleaved by canogen bromide into four fragments: CNBr I 18–19 kDa, CNBr II 5 kDa, CNBr III 2.1 kDa and CNBr IV 0.7 kDa. Hydrogenbromide/dimethylsulfoxide and 3-bromo-2-(2-nitrophenylsulfenyl)skatol (BNPS-skatol) cleaved lipophilin into four fragments of molecular masses of approximately 14000 (Trp I), 2100 (Trp II), 5000 (Trp III) and 7000 Da (Trp IV).

Separation and purification of the peptides for liquid phase sequenator degradation was achieved by high performance liquid chromatography. In addition proteolytic cleavage of the Trp IV fragment with trypsin facilitated the alignment of the peptides.

An effective control of the sequenator data came from the partial acid hydrolysis of the Trp IV fragment, which yielded di-, tri- and tetrapeptides. The mixture was *N*-trifluoroacetylated, the amide (peptide) bonds and carboxyl groups were reduced with B_2D_6 (hexadeuterodiborane) and the polyaminoalcohols derivatized with chlorotrimethylsilane. These derivatives were separated and identified by capillary gas-liquid chromatography/mass spectrometry. Extensively overlapping sequences support the data obtained by Edman degradation in a liquid phase sequenator of the CNBr peptides II, III and IV, the 72 amino acid residues containing *C*-terminal sequence of lipophilin of molecular mass (7520 + x) Da, which includes the Trp IV fragment.

Primärstruktur der C-terminalen Cyanbromid-Bruchstücke II, III und IV des Rinderhirn-Proteolipid-Apoproteins

Zusammenfassung: Gereinigtes Lipophilin aus der weißen Substanz von Rinderhirn wurde nach reduktiver Carboxymethylierung mit Bromcyan in vier Fragmente CNBr I 18–19 kDa, CNBr II 5 kDa, CNBr III 2.1 kDa und CNBr IV 0.7 kDa gespalten. Bromwasserstoff-Dimethylsulfoxid und 3-Brom-2-(2-nitrophenylsulfenyl)skatol(BNPS-Skatol)-Spaltung an den drei Tryptophanresten schuf vier Peptide der molekularen Masse Trp I etwa 14000, Trp II 2100, Trp III 5000 und Trp IV 7000 Da.

Enzymes:
Trypsin (EC 3.4.21.4).
Abbreviations:
BNPS-Skatol = 3-bromo-2-(2-nitrophenylsulfenyl)skatol; TosPheCH$_2$Cl = chloro(tosyl-L-phenylalanyl)methane.

0018-4888/82/0363-0855$02.00

Die Peptide wurden durch Hochdruck-Flüssigkeitschromatographie getrennt, gereinigt und anschließend der Sequenatoranalyse unterworfen. Zusätzlich wurde Trp IV mit Trypsin gespalten und die tryptischen Spaltstücke sequenziert.

Eine wirkungsvolle Kontrolle der Sequenator-Daten wurde durch partielle saure Hydrolyse der Trp-IV-Bruchstücke zu Di-, Tri- und Tetrapeptiden mit anschließender *N*-Trifluoracetylierung, Hexadeuterodiboran-Reduktion (B_2D_6) der Amid(Peptid)-Bindungen und Carboxylgruppen zu Aminen und primären Alkoholgruppen erzielt, die als Trimethylsilyl-Derivate der kombinierten Kapillarsäulen-gaschromatographisch/massenspektrometrischen Trennung und Identifizierung unterworfen wurden.

Ausgedehnte überlappende Sequenzen unterstützen voll die durch Edman-Abbau im Flüssigsequenator erhaltenen Sequenzen, die in Folge die CNBr-Peptide II, III und IV als *C*-terminale Sequenz des Lipophilins mit 72 Aminosäureresten der molekularen Masse (7520 + x) Da umfassen, von denen eine 66 Reste umfassende Sequenz das Tryptophanbruchstück IV darstellt.

Key words: Lipophilin, CNBr and tryptophan fragments, high performance liquid chromatography, amino acid determination.

Proteolipid-apoprotein of myelin described in 1951 by Folch and Lees[1] as a chloroform/methanol 2:1 (v/v)-soluble but water-insoluble membrane lipoprotein represents a major protein constitutent of central nervous myelin. Its structural and functional role within the myelin bilayer and/or multilayer system is unknown. Only the strong hydrophobicity of this protein and no functional criteria gives guidance to its isolation, purification and characterization (for review: see Boggs and Moscarello[2] and Lees et al.[3]).

A prerequisite for the understanding of interactions of this apoprotein, also named lipophilin[4] because of its avidity to bind to the different brain lipid classes, within the unusual myelin membrane, is the knowledge of the primary structure of the 25 kDa polypeptide chain of proteolipid-apoprotein.

Lipophilin has been reported to be resistant against proteolytic enzymes[1,5]. However, the sequence of a few small peptides, tryptically released from this apoprotein, has been published recently[6].

Preliminary data come from automatic sequence analysis, which comprise the *N*-terminus of proteolipid-apoprotein from rat myelin (30 residues)[6,7], bovine white matter[8,9] (16 and 15 residues, respectively) and human myelin [2,10] (16 and 23 residues; incomplete).

Cyanogen bromide cleavage yielded four fragments, two of which yielded the 19 amino acid *C*-terminal sequence with phenylalanine as *C*-terminus; also 17 residues of CNBr fragment II were obtained[11].

The interesting observation, which comes from a comparison of the *N*-terminal sequences, is the complete homology of the sequences of these three different species, although only a comparison between the first 16 positions is possible due to the lack of further sequence information.

The accompanying paper which describes the isolation, purification and characterization of bovine white matter proteolipid-apoprotein also outlines our strategy to the structural elucidation of the complete amino acid sequence of this hydrophobic protein.

Cyanogen bromide cleavage at methionine residues[12,13] unfortunately yields three small fragments which can easily be separated and sequenced but one very large and still very hydrophobic fragment of molecular mass 18–19 kDa.

Cleavage at tryptophan sites[14–17] of the reductively carboxymethylated lipophilin[18,19] yielded four fragments. Their separation and characterization and the amino acid sequence of tryptophan fragment IV (Trp IV), which resembles the *C*-terminal sequence, will be described in this paper. Automated Edman degradation of the total Trp IV fragment, trypsin fragments and cyanogen bromide fragments of Trp IV elucidated the sequence of the 72 residues containing *C*-terminus. Computer supported analyses of partial acid hydrolysates of Trp IV, which were derivatized

to silylated polyaminoalcohols for separation by capillary gaschromatography/mass spectrometry, confirmed this structure. Trp IV with a computed molecular mass (7520 + x) Da embraces in its sequence the largest part of CNBr II fragment and CNBr III and CNBr IV.

Materials and Methods

Bovine *proteolipid-apoprotein* was isolated as described by Folch et al.[1] and in a forthcoming paper*

The protein was homogeneous by sodium dodecyl sulfate gel electrophoresis (10–15% acrylamide gradient gel) and its molecular mass approximated by comparison with molecular mass standards to 25000 Da.

Trypsin ($TosPheCH_2Cl$-treated) was obtained from Boehringer Mannheim. The protein was converted into the reductively carboxymethylated[18] form or disulfide bonds cleaved by oxidation according to Hirs[19].

Cyanogen bromide cleavage at methionines was performed by the adaptation of the method described in ref.[12–15]: 100 mg freshly carboxymethylated lipophilin was dissolved in 9 ml 98% formic acid with magnetic stirring and 1 ml water added dropwise. 500 mg CNBr was added and the mixture stirred at room temperature in the dark for 24 h. 10–20 volumes of water was added and the sample lyophylized. The residue was dissolved in formic acid for chromatographic separations.

BNPS-Skatol cleavage elaborated by Omenn et al.[16] or *HBr-dimethylsulfoxide cleavage at tryptophan* residues described by Wachter and Werhan[17] were adapted to the cleavage of lipophilin: 100 mg carboxymethylated lipophilin, dissolved in 2.5 ml trifluoroacetic acid, was vigorously stirred after the addition of 1.2 ml 12M HCl and 10 µl dimethylsulfoxide. About 100 mg crystalline phenol was added after 30 min, followed by 400 µl conc. HBr and additional 100 µl dimethylsulfoxide. Stirring was continued for 30 min und then 10 ml water added, which precipitated the lipophilin fragments. They were collected by centrifugation in a laboratory centrifuge at 2000 × g, washed several times with water and dried by lyophilization. The residue was dissolved in 0.5 ml trifluoroacetic acid which was evaporated again. 1.8 ml formic acid and 0.2 ml water was added and vigorously stirred (vortex) to keep the mixture completely dissolved. After 24 h at room temperature the solution of the tryptophan fragments was applied to chromatographic separations.

* Stoffel, W., Fischer, H., Hillen, H. & Schroeder, W., in preparation.

Chromatographic separations of fragments

Cyanogen bromide and tryptophan fragments were separated on Biogel P-30, 60, 100 and 150 columns (2 × 120 cm) with 90% formic acid and subsequently subjected to repeated high performance liquid chromatography purification at two coupled Lichrosorb Si 100 (10 µ), Fa. Merck, Si 60 (5 µ) and Si 50 (10 µ) [Nucleosil 50, Macherey & Nagel, Düren] columns (0.9 × 30 cm), equilibrated with 90% formic acid.

The following combination of columns was used;
Molecular mass region 20000 – 5000 Da, Si 100 (0.9 × 30 cm) and Si 60 (0.9 × 30 cm);
Molecular mass region 7000 – 1000 Da, Si 60 (0.9 × 30 cm) and Si 50 (0.9 × 30 cm).

Two small CNBr fragments were separated on Biogel P-30 with 0.5M acetic acid as eluens and Sephadex LH-20[20].

Preparative separations of up to 5 mg per run were achieved isocratically with a flow rate of 1.5 ml/min. The samples were detected at 280 nm or 256 nm with a Kratos-Schoeffel UV-detector SF 770, coupled to a Varian 6000 A-HPLC unit. Peptide fractions were pooled and evaporated at room temperature and freeze-dried if necessary.

Polypeptides elute with reproducible retention times. Molecular masses were related to the following standards: Phe-Tyr-Ser, glucagon, cytochrome *c* and apolipoprotein AI. The almost linear relationship between molecular mass and retention time indicated that this high performance liquid chromatography is an exclusion chromatography (molecular sieve).

Cleavage and derivatization for gas chromatography/mass spectrometry coupling

5 to 10 mg of lipohilin was dissolved in 50 to 100 µl trifluoroacetic acid and 2 ml acetic acid/12M HCl 4:1 (v/v) under nitrogen and vacuum in thick-wall ampoules. The polypeptide solution was heated for 30 min and 110 °C excess HCl evaporated under vacuum and the residue dried in a desiccator over NaOH.

Hexadeuterodiborane (B_2D_6) was prepared according to Maryburg and Larrabel[21].

N-Trifluoroacetylation, hexadeuterodiborane reduction and trimethylsilylation was performed according to Frank et al.[22]. Gas chromatography separation was performed with a Packard gaschromatograph model 427, equipped with the "falling needle" inlet system and capillary columns (0.3 mm i.d., 15 m length) coated with OV-1, purchased from Hewlett-Packard, Frankfurt.

Helium was used as carrier gas, flow rate 2 ml/min. A temperature program between 80° and 300 °C (6°/min) was run.

The tapered capillary column entered the heated (250 °C) coupling part of the double focusing mass-spectrometer MAT 311 A (Finnigan), scanning velocity 10 spectra/min. Ionisation energy: 70 eV. The data system SS 100 was used. All data were accumulated on tapes. The program for the interpretation of the fragmentation pattern was developed in this laboratory and will be described elsewhere.

N-Terminal end group analyses were performed by dansylation according to Gray[23].

Amino acid analyses

In general *hydrolysis* was carried out with 6M HCl for 24 and 72 h at 110 °C in sealed ampoules. The hydrolysis mixture was made 5% in thioglycolic acid for the determination of tryptophan[24,25] or the hydrolysis was performed in 3M mercaptomethanesulfonic acid, according to Penke et al.[26]. Samples which yielded an insoluble residue were hydrolysed in a mixture of propionic acid/12M HCl 1:1 (v/v) for 20 min at 160 °C in sealed ampoules[27]. Solvents were evaporated and the residue dissolved in 0.2M sodium citrate buffer pH 2.2 for amino acid analysis.

Amino acid compositions were determined with a homemade amino acid analyzer, using components of the Durrum Kit (Durrum Chemical Corporation, Palo Alto/USA) and a Knauer fluorimeter for detection of *o*-phthalaldehyde derivatives of amino acids, combined with the computing Integrator SP 4100 Spectra Physics. The one column method (0.9 × 25 cm column length, Durrum DC-4A resin) allows the separation and detection of all amino acids except proline with a stepwise sodium citrate gradient of pH 3.25, 3.95 and 4.75.

In addition peptides and the protein were analysed on a Beckman Model 119 CL amino acid analyzer. Samples (1–10 nmol) were lyophilized in ampoules and hydrolysed in vacuo at 110 °C for 24 h in 0.5–1.0 ml constant boiling HCl. Cysteine was determined as cysteic acid, following performic acid oxidation[19].

Amino acid sequences were analysed according to the method of Edman and Begg[28] with a Beckman Instrument sequenator (model 890 C).

Fractions were dried under nitrogen and converted to phenylthiohydantoin derivatives with 1M HCl. Separation of these derivatives was effected by mixed bed reversed phase high performance liquid chromatography in an acetate-acetonitrile gradient.* Phenylthiohydantoin derivatives were also identified by thin-layer chromatography on silica gel-coated plates (Merck, Darmstadt), using the solvent systems according to Bridgen[29].

* Stoffel, W. & Deutzmann, R., in preparation.

Results and Discussion

Strategy of sequence determination

The estimate of the molecular mass of proteolipid-apoprotein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10–15% gradient gel) and by gel permeation chromatography in formic acid on CPG-10 (controlled pore glass) was between 25 and 31000 Da.

Although it is possible to attack the aggregated protein in aqueous suspension with trypsin which yielded two fragments, the sequence of which will be reported elsewhere, chemical degradation with CNBr at methionine and HBr/dimethylsulfoxide at tryptophan residue was applied.

Four cyanogen bromide fragments were isolated and four tryptophan fragments were obtained and separated in the following way: Three small CNBr fragments with molecular masses 0.7 kDa, 2.1 kDa and 5 kDa were isolated by column chromatography on Bio Gel P-30 in 0.5M acetic acid or on Sephadex LH-20, using the solvent system of Gerber et al.[20]. The molecular masses were determined by gel permeation high performance liquid chromatography, using silica gels of defined pore size with 90% formic acid as the solvent of choice.

Different methods for the cleavage of a polypeptide chain at tryptophan residues have been described[16,17]. We used a modification of the procedure, described by Wachter and Werhan[17] by substituting acetic acid by trifluoroacetic acid, which kept the protein in solution even after the addition of HCl and HBr.

Four tryptophan fragments were isolated and purified by high performance liquid chromatography and their *N*-terminal residues and molecular masses determined:

Trp I 14 kDa, Trp II 2.1 kDa, Trp III 5.0 kDa and Trp IV 7.0 kDa.

The *N*-terminus of Trp I is leucine, of Trp II proline, of Trp III aspartic acid and Trp IV asparagine.

The amino acid analyses of the four fragments are given in the Table.

Cyanogen bromide released four polypeptides with molecular masses CNBr I fragment 18–19

Table. Stoichiometry of amino acids* of tryptophan fragments I–IV (Trp I–IV).

Fragment		Number of residues														
		Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr**	Phe	Lys	His	Arg	Cys
Trp I	14 kDa	4.5	6.5	6.2	10.1	15.2	13.0	9.2	0.4	5.5	15.2	(9.2)	12.2	5.2	3.1	4.0	(4.8)
Trp II	2 kDa	1.1	2.2	0.3	0.4	2.3	1.2	3.1	0.1	0.9	2.1	(1.1)	0.8	0.8	0.9	0.2	–
Trp III	5 kDa	2.1	3.8	4.9	0.3	4.2	5.1	5.1	0.7	3.4	4.3	(3)	2.2	2.7	0.4	1.4	(3.8)
Trp IV	7 kDa	3.3	6.1	3.2	2.1	5.1	10.0	5.1	2.8	3.1	6.9	(1)	8.1	3.8	1.2	1.0	(2.1)

* Fluorometric detection with *o*-phthalaldehyde; proline not detected.
** Tyrosine is derivatized to *o*-bromotyrosine by the cleavage reagent (HBr/dimethylsulfoxide), the quantitation of which is not accurate.
Cysteine, carboxyamidylated cysteine and methionine residues are partly decomposed under the condition of the cleavage reaction; these numbers of cysteine residues are therefore only approximative.

kDa, CNBr II 5 kDa, CNBr III 1.5 kDa and CNBr IV 0.7 kDa.

Here we report the primary structure of the CNBr fragments II, III and IV, which include Trp IV, the *C*-terminal of the four Trp fragments. The primary structure was determined by combination of HBr/dimethylsulfoxide cleavage at tryptophan residues and of cyanogen bromide fragmentation at methionines, trypsin proteolysis and gas-liquid chromatography/mass spectrometry of partial acid hydrolysates which were derivatized to volatile products. The polypeptides, separated and purified by high performance liquid chromatography, were subjected to Edman-degradation in a liquid phase (spinning cup) sequenator.

The CNBr fragments and Trp fragment were also partially hydrolysed to di-, tri- and tetrapeptides which were derivatized for gas chromatography/mass spectrometry coupling[22,30–32].

One example is documented in Fig. 1, in which the recording of the ionisation current of the hydrolysate of CNBr II, separated as silylated polyaminoalcohols by capillary gas chromatography, is given. A typical fragmentation pattern of the tripeptide Ala-Val-Leu as an example is given in Fig. 2. On the basis of all available data about the CNBr fragments II, III and IV and the Trp IV fragment the following sequence of the 72 residues containing *C*-terminal sequence is proposed, Fig. 3.

The Trp IV fragment must comprise the *C*-terminal sequence for the following reason: CNBr II (molecular mass 5000 Da), 19 residues of which were sequenced by automated Edman degradation, overlaps with 12 residues of Trp IV, which was sequenced over 23 cycles. Trypsin cleavage at lysyl 23 yielded a fragment, which again overlapped CNBr III. CNBr III and IV were separated by Bio-Gel P-30 chromatography (0.5M acetic acid) and their sequences established by automated Edman degradation. The results confirm the structure of CNBr III and IV, published by Jollès et al.[11].

Cysteine at position 23 of this sequence must be involved in a cystine bridge to the large CNBr fragment of about 19 kDa (CNBr I), because only upon reductive carboxymethylation or also performic acid oxidation releases CNBr II from

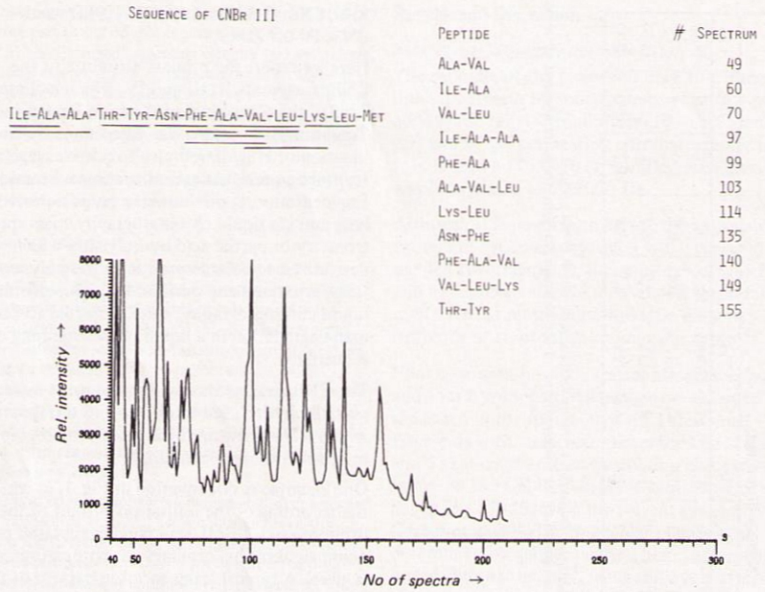

Fig. 1. Separation of dideuteroethyl polyaminoalcohols as *O*-trimethylsilyl derivatives of peptides liberated from CNBr III by acid hydrolysis.

The total ion current has been recorded. Mass spectra were recorded every 6 s, stored in the Varian MAT SS 100 data system. The data were processed according to the program developed in this laboratory by the Cyber 72 Computer (Rechenzentrum der Universität zu Köln). 11 Di- and tripeptides given in the insert were extracted from the data accumulated for the control of the sequenator data.

the CNBr I which appears in the void volume on high performance liquid chromatography separation.

Methionine residue 30 requires some comment. This residue is followed by threonine. The nucleophilic attack by the secondary alcohol group of the neighbouring threonine leads to cyclisation and prohibits cleavage with homoserine formation, an observation described before by Schroeder et al.[33]. Therefore despite four methionine residues are being found, only four polypeptide fragments are being formed.

The sequence of the 72 amino acid *C*-terminus. described in this paper, suggests a number of considerations regarding its secondary structure and interactions with the lipid bilayer of the myelin membrane.

A positively charged *C*-terminus of nine amino acid residues (2 Lys, 1 Arg) and a hydrophilic *N*-terminus of 33 amino acids border a 30 residue uninterrupted stretch of uncharged, hydrophobic amino acids.

The hydrophobicities of the over-all amino acid composition of the peptide, which have been calculated according to a hydrophobicity scale[34,35] most obviously indicate that the central sequence with 30 amino acids, with the average hydrophobic free energy[36] of -5.1 kJ/mol residue

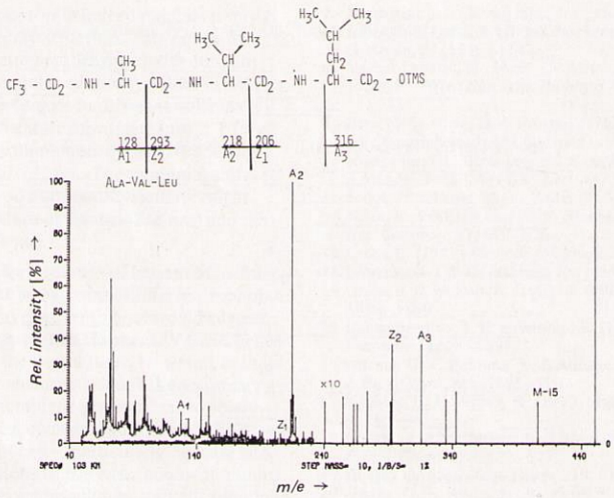

Fig. 2. Mass spectrum of dideuteroethyl polyaminoalcohol *O*-trimethylsilyl (OTMS) derivative of tripeptide Ala-Val-Leu, a component of the acid hydrolysate of CNBr III.

The fragment (5–10 mg) was hydrolysed, derivatized and separated by capillary gaschromatography with open coupling to the mass spectrometer as described under Materials and Methods.

versus 7.6 kJ/mol residue of the *C*-terminal sequence and +0.9 kJ/mol residue of the hydrophilic *N*-terminal sequence, represents the membrane integrated (penetrating) segment which interacts with the hydrocarbon phase of the bilayer structure of myelin complex lipids.

Empirical rules have been derived and successfully applied to the prediction of the secondary structure of globular proteins[37–43]. These rules also proved to be applicable to apolipoproteins of serum high-density lipoprotein[44–46]. The α-helical content calculated according to these rules agreed well with those derived experimentally from circular dichroism measurements.

The rules have also been used for the prediction of the secondary structure of the hydrophobic amino acid sequence of the transmembrane protein glycophorin spanning the core region of the erythrocyte membrane. An α-helix structure has been assigned to this sequence[35,47]. However, our own data reported previously[48] make a β-sheet structure likely.

These contradictory interpretations make clear, that the application of the predictive rules to membrane proteins might not be unambiguous.

When the probability values for α-helical and β-sheet structures were derived for the 72 amino acid *C*-terminus reported here by computer-assisted calculations* according to programs of Chou and Fasman[37–39], Robson[40] and Nagano[41–43], the two structures had almost the same weight. Therefore an α-helical or β-structure could be assigned to the hydrophobic segment of tryptophan fragment IV of lipophilin.

* We are very grateful to *Dr. B. Wittmann-Liebold*, Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, for performing these calculations.

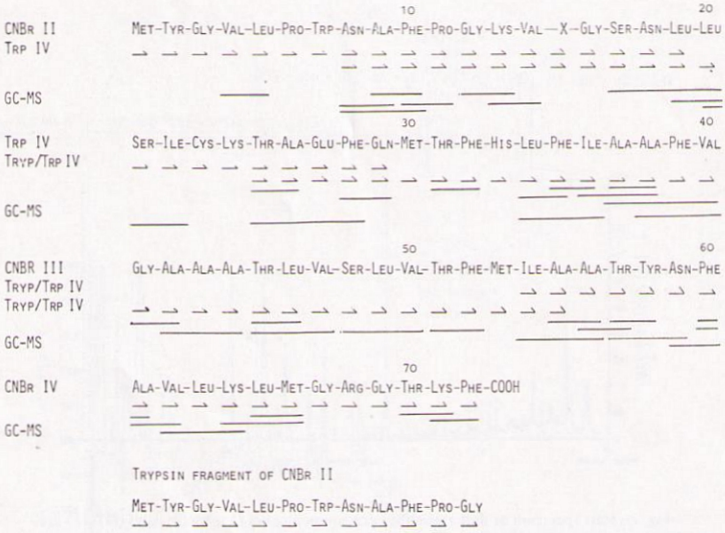

Fig. 3. Amino acid sequence of 72 residues containing CNBr fragment II, III and IV including Trp fragment IV. Arrows indicate overlapping sequences determined by automated Edman degradation by the spinning cup sequenator. Full lines indicate peptide sequences established by gas chromatography/mass spectrometry (GC/MS) analysis.

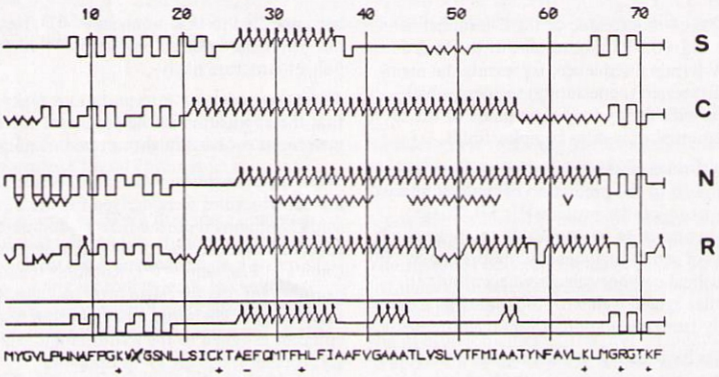

Fig. 4. The secondary structure of the 72 amino acid residues polypeptide according to the predictive rules of Scheraga (S), Chou and Fasman (C)[37–39], Nagano (N)[41–43] and Robson (R)[40].
The probability of α-helix and β-sheet structures for positions 21 to 56 are very similar, 40.87 and 40.72 respectively.

Proteolipid-apoprotein indeed exhibits a very flexible secondary structure. This can be demonstrated by circular dichroism, the dramatic changes of the molar ellipticity values in which this protein undergoes in different solvents*: 80% α-helix in chloroform/methanol 2:1 (v/v), 60% in 0.1% sodium dodecyl sulfate solution with minima at 222 nm. The minimum shifts to 211 nm in 1% sodium dodecyl sulfate might indicate a mixture of β-form (218 nm) and random coil (200 nm).

The 80% α-helix structure of lipophilin in chloroform, a solvent which mimicks the hydrophobic phase of the myelin membrane, would suggest that within tryptophan fragment IV the α-helices may override the β-structures. However, as indicated above, conclusions about the secondary structure of membrane proteins, taken solely from theoretical considerations, should be drawn with great reluctance, particularly because additional interactions of the basic nonmembranous sequences with acidic sphingo- and phospholipids might occur at the water interphase.

The hydrophobic part of the Trp IV fragment described here represents one out of four hydrophobic regions distributed over the complete lipophilin sequence, which will be reported in the subsequent paper.

We gratefully acknowledge the support of this project by the *Deutsche Forschungsgemeinschaft* and the *Thyssen-Foundation*.

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