ISOLATION AND TERMINAL SEQUENCE DETERMINATION OF THE MAJOR RAT BRAIN MYELIN PROTEOLIPID P7 APOPROTEIN

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SUMMARY. The major rat brain myelin proteolipid P7 apoprotein has been isolated in pure form by a preparative sodium dodecylsulphate gel electrophoresis system. Automated Edman degradation permitted the establishment of the N-terminal sequence up to the 20th amino acid. The C-terminal sequence was determined by the action of carboxypeptidase A.

Proteolipids are a group of protein-lipid complexes which have been isolated from brain tissue first by Folch and Lees (1) and which have the unusual property of being extracted in chloroform-methanol 2:1 (v/v) together with the lipids. They are especially abundant in nervous tissue; in central white matter, they are mainly localized in the myelin (2,3). It is possible to separate the protein-lipid complexes from adventitious lipids (4-6), and in further steps to prepare a protein moiety free of lipids, i.e. the proteolipid apoprotein (7-10). This almost completely lipid-free apoprotein can be rendered soluble in water, but contains nevertheless 2 to 4 % covalently-bound fatty acids (9,11). The amino acid composition of proteolipid and apoprotein preparations (4,5,7,9) has shown a low proportion of basic and acidic amino acids, an abundance of the non-polar amino acids, and a large amount of methionine and half-cystine, two-third of the latter occurring in disulfide linkages (12). Proteolipids, as well as the apoprotein, have been found resistant to the action of endopeptidases (7,13). The physical and chemical properties of white matter proteolipids have been reviewed (13).

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Up to quite recently, the number of protein species and the molecular weights corresponding to "white matter proteolipids" were unclear (14-21). In a recent study, we have been able to demonstrate by polyacrylamide gel electrophoresis in the presence of SDS[*] that proteolipids of pure myelin (and therefore of white matter), consist at least of three proteins, a major P7, a second P8M and a small P3 (22). Using this system in a preparative scale, we isolated proteolipid P7 apoprotein in pure form in quantities sufficient to permit the beginning of the N-terminal and C-terminal sequence studies.

MATERIAL AND METHODS. *Isolation of myelin.* Myelin was prepared from brains of adult Wistar rats of an inbred strain (200-250 g), by a method adapted of Kurihara *et al.* (23), with some modifications to be published. The pure myelin was lyophilized; an average of 350-400 mg of dry myelin was obtained from 20 brains.

Preparation of myelin proteolipids. Myelin was extracted with chloroform-methanol 2:1 (v/v) (1 ml per mg); the solution was filtered through paper to remove insoluble material (mainly Wolfgram proteins). The basic proteins were precipitated out at 4°C overnight by the addition of 0.05 vol of 0.1 M KCl (24). The precipitate was removed by filtration and the filtrate brought to a known volume with chloroform-methanol 2:1 (v/v) and washed with 0.2 vol of distilled water. The lower chloroform-rich phase was reduced in volume (22) and the proteolipids precipitated by the addition of 4 vol of diethyl ether at -20°C for at least 2 h. The proteolipid pellet after centrifugation at 1,000 *g* for 20 min was resuspended in a mixture of ethanol-diethyl ether 1:1 (v/v) and left again several hours at -20°C. Finally the pellet was dried under a stream of nitrogen and solubilized in a 0.01 M Tris-HCl (pH 7.4) buffer containing 1 % SDS as previously described (22).

Preparative electrophoresis of P7 apoprotein. For the fractionation of the myelin proteolipids, we used an apparatus similar to that described by Waehneldt (25). In the first experiments, the glass tube had an inner diameter of 38 mm, in the latter 48 mm; the total length being 300 mm. The preparation of the resolving and stacking gels was similar to previous described method (25), only the height of the resolving gel was lengthened to 120 mm. The lower and upper buffers were used in substantial volumes (1 liter each) to lower the effects of the electrolysis. 25 to 30 mg of proteolipid proteins for the smaller, up to 40 mg for the bigger gel were loaded on the column. In general, the strong yellow band reached the end of the gel within 48 h under a constant current of 16 mA, or more rapidly if a higher amperage was applied (no more than 24 mA). For fractionation, a collecting device containing a small volume (6-10 ml) of lower buffer was placed on the lower end of the column, and the current then increased to 30 mA. The collection of the fractions was performed automatically under the control of a programmer with the aid of 2 automatic dispensers Fisons, a fraction collector Gilson combined to an absorptiometer Model UV 280 IF allowing the immediate detection of the elution of the proteins. For a more accurate localization of the elution, a selected number of fractions were screened by analytical gel electrophoresis as described (25).

[*]Abbreviations : SDS, sodium dodecylsulphate; PTH, phenylthiohydantoin.

To obtain the native apoprotein of P7, the fractions containing pure P7 were pooled and the solution acidified by 0.1 vol of glacial acetic acid to lower the pH to 2-3 and extracted 5 times with 0.5 vol of diethyl ether. The SDS was partitioned in the upper phase, whereas the apoprotein precipitated at the interface. After the last extraction, the diethyl ether remaining was evaporated under a stream of nitrogen and some ethanol added to permit an easier deposition of the apoprotein by centrifugation. The protein precipitate was then washed successively by chloroform-methanol 1:1 (v/v), ethanol and water. Finally, the protein was completely dissolved in 98 % formic acid, further diluted with water and lyophilized.

Analytical methods. The amino acid composition was determined with a Technicon Autoanalyzer after total hydrolysis (6 M HCl at 110°C for 18, 48 and 72 h under vacuum). The tryptophan content was established according to Spies and Chambers (26).

For reduction and alkylation of the protein, the pooled fractions were lyophilized. The large precipitate was then dissolved in a small amount of water and dialyzed for 48 h against 0.2 M Tris-HCl (pH 8.0) buffer containing 0.1 % SDS. Reduction and S-carboxymethylation was carried out according to Lux *et al.* (27) with iodoacetic acid. The carboxymethylated protein was separated from the reaction products and excess reactants by a one-day dialysis against 0.01 M ammonium bicarbonate buffer (pH 8.0) followed by chromatography on a column of Sephadex G-25 fine (1 x 90 cm) with the same buffer. The method described by Nicot *et al.* (28) was also used for the carboxymethylation without the chromatography of the reduced protein on Biogel step. The protein was purified by precipitating with a mixture of acetone-N HCl 39:1 (v/v) and dialysis against 0.01 M ammonium bicarbonate.

The oxidation of the protein was performed following the method of Hirs using performic acid during 2 h at 4°C (29).

Automated Edman degradation (30) was carried out in a Socosi Sequencer, Model PS-100, by the Quadrol method. The thiazolinones were converted into PTH-amino acids and these latter were characterized by thin-layer chromatography (chloroform-methanol 90:10 or 80:20 v/v), by gas-chromatography (Beckman GC 45 Chromatograph) or with an amino acid Autoanalyzer after regeneration of the free amino acids (150°C, 6 M HCl containing 1/2,000 mercaptoethanol for 24 h).

For the determination of the C-terminal sequence, the carboxymethylated protein was submitted to the action of a suspension of carboxypeptidase A (EC 3.4.2.1) in 0.1 M ammonium bicarbonate during 24 h at 37°C. The liberated amino acids were characterized with an Autoanalyzer.

The N-amino terminal residue of P7 apoprotein was first identified by dansylation following the technique of Gros and Labouesse modified by Zanetta *et al.* (31). The dansyl-amino acids were separated on pre-coated TLC Silica gel 60 F_{254} plates (Merck) using a bidimensional solvent system (benzene-pyridine-acetic acid 80:20:5 v/v/v; toluene-chloro-2 ethanol-25 % ammonia 100:80:6.7 v/v/v).

2'3'-cyclic AMP 3'-phosphohydrolase activity was assayed by the technique of Kurihara and Tsukada after activation with deoxycholate (32). Protein was determined by the method of Lowry *et al.* (33) with serum albumin as standard.

RESULTS. The purity of myelin samples was ascertained essentially by electron microscopic examination. The lamellar structure was well preserved with fully intraperiod lines. 2'3'-cyclic AMP 3'-phosphohydrolase, regarded as a

myelin marker, showed a 4-5 times enrichment of its activity in comparison to total homogenate. The reduction of the level of the bands in the high molecular weight region in polyacrylamide gels in the presence of SDS was also claimed to be a criterion for the evaluation of myelin purity (34). In this respect, our preparations seemed to be highly purified (Fig. 1).

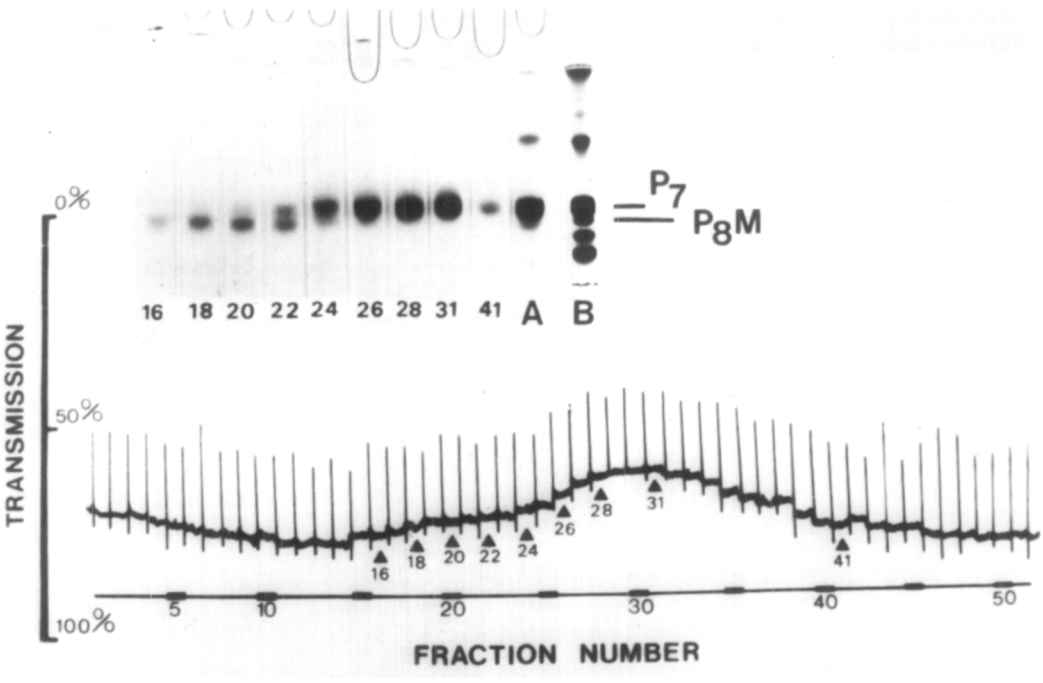

Fig. 1. Elution profile of P8M and P7 apoproteins by preparative gel electrophoresis. The purity of the fractions was checked by analytical gel electrophoresis.
A : total rat brain myelin proteolipids.
B : total rat brain myelin proteins

The feasible isolation of some brain myelin proteins by polyacrylamide gel electrophoresis in the presence of a ionic detergent in a milligram-scale has been demonstrated (25). We adopted this method for the preparative separation of the 2 important proteolipids P7 and P8M of rat brain myelin (22). The elution profile and the analytical gels showed that the 2 proteolipid proteins overlapped in some fractions (Fig. 1), due to the fact that their molecular

weights are too similar, 23,500 for P7 and 19,500 for P8M. These molecular weights were roughly determined by comparing the rate of migration on SDS gels with that of known proteins : cytochrome C, myoglobin, chymotrypsinogen A, pepsin and ovalbumin.

Amino acids analyses have been performed on purified P7 preparations using three times of hydrolysis (Table 1). The calculated molecular weight from the values reported in Table 1 is 23,623.

TABLE 1. - Amino acid analysis of the major rat brain myelin proteolipid P7 apoprotein. Residues/mole of molecular weight 23,500.

Amino acid	Time of hydrolysis			Nearest integer
	18 h*	48 h	72 h	
Asp	10.3	9.5	9.5	11
Thr	16.5	16.2	13.0	17
Ser	11.2	10.9	8.8	12
Glu	12.6	12.2	12.2	13
Pro	6.0	5.4	5.1	6
Gly	21.9	18.9	19.1	22
Ala	24.0	24.0	24.0	24
Val	16.6	16.3	17.0	17
(Cys-)	8.0	7.4	4.7	8
Met	2.5	2.2	2.2	3
Ile	12.0	10.8	10.9	12
Leu	24.0	22.7	20.0	24
Tyr	9.7	8.1	8.1	10
Phe	17.1	16.0	16.7	17
Trp				2***
Lys	9.7	9.0	8.6	10
His	5.2	5.1	4.6	5
Arg	5.9	5.3	5.3	6
Total				219*****

*Residues per mole calculated on the basis of 24 residues of Ala.
***Characterized following the procedure of Spies and Chambers (26).
*****Calculated molecular weight : 23,623.

The purity of P7 apoprotein was ascertained by dansylation of its N-terminal amino acid. Only glycine was detected. In further experiments, the N-terminal sequence was established in details by the use of a sequencer (Table 2). When the native apoprotein was used as starting material, only the N-terminal

tetrapeptide Gly-Leu-Leu-Glu- could be characterized, whereas with the reduced protein the N-terminal decapeptide was identified. In a third series of experiments employing the oxidized protein, a longer sequence was determined : Gly-Leu-Leu-Glu-$CySO_3H$-$CySO_3H$-Ala-Arg-$CySO_3H$-Leu-Val-Gly-Ala-Pro-Phe-Ala-X-Leu-Val-Ala-... (X : unidentified amino acid). The quantitative figures indicated in Table 2 for the first 4 steps of the reduced and oxidized protein were lower than those obtained with the native protein ; indeed the solubility of this latter was higher in the usual reaction mixture.

When the reduced apoprotein was digested during 24 h by carboxypeptidase A, only Phe (23 %) was liberated. When higher amounts of mercaptoethanol were used for the reduction of the protein, this latter was more easily attacked by the enzyme; under similar experimental conditions, Phe (22 %), Gly (13 %) and Ser (10 %) were characterized; this experiment suggests ...-Ser-Gly-Phe as C-terminal sequence of the apoprotein.

TABLE 2. - N-terminal sequence of P7 apoprotein determined with a sequencer.

Residue N°		1									10
		Gly	Leu	Leu	Glu	Cys	Cys	Ala	Arg	Cys	Leu
Native	(a)	+	+	+	+						
	(b)	22	22	22	22						
Reduced	(a)	+	+	+	+	⁂	⁂	+	+	⁂	+
	(b)	11	11	11	7				2.8		2.5
Oxidized	(a)	+	+	+	+			+			+
	(b)	13	16.5	17	10	2⁂⁂	2⁂⁂	8	1	2⁂⁂	12
	(c)		7	8				8			11

Residue N°		11									20
		Val	Gly	Ala	Pro	Phe	Ala	X	Leu	Val	Ala
Oxidized	(a)	+	+	+	+	+	+		+	+	+
	(b)	8	8	6.5	2	5	5		4	3.7	5
	(c)	5			2	5	5		5	7	

X = unidentified amino acid.
(a) PTH-amino acid determined by thin-layer chromatography.
(b) amino acid determined with an Autoanalyzer after regeneration, % recovery.
(c) PTH-amino acid determined by gas-liquid chromatography, yield %.
⁂traces of S-carboxymethylcysteine.
⁂⁂determined as cysteic acid.

DISCUSSION. For a better understanding of the unusual physical and chemical properties of the proteolipids, the primary structure of the apoprotein must be determined. We have therefore purified the most important myelin proteolipid P7 by preparative disc-gel electrophoresis. The molecular weight values obtained by us are in agreement with those published by others using a similar system (21,28). Higher values have been determined with phenol-formic acid-water (14:3:3 w/v/v) (18) or 98 % formic acid (35) as solvents. Much higher values have been obtained when the apoprotein was placed in water (35). Only the primary structure of the protein can lead to the knowledge of the exact molecular weight.

The amino acid composition showed no great difference from that determined by several authors for a total proteolipid protein preparation of whole white matter. This is easily explained since P7 apoprotein represents near 60 % of the sum of the 3 proteolipid proteins bands in myelin (22).

As far as we know, this is the first time that the N-terminal sequence of a proteolipid protein has been established. The purity of the protein made it possible to sequence the first 20 amino acids. Two remarks concerning this N-terminal sequence must be made : 1) 17 out of 19 amino acid residues are hydrophobic assuming that the three half-cystine residues are implicated in disulfide bonds; 2) three half-cystine residues were characterized; their presence might explain the difficulties encountered when the native apoprotein was submitted to chemical or enzymic treatments. For example, the carboxymethylation of the half-cystine residues was never complete.

The present study has been directed towards the protein moiety. Some preliminary results agree with the presence of fatty acids associated to the apoprotein P7 after electrophoresis, as earlier demonstrated for total proteolipid apoprotein (11). The high content of apolar amino acids, combined with the possible association of fatty acids makes it a good candidate as "bimodal protein". Such a protein is defined as one with regions of apolar separated by short regions of polar amino acids (35,36). The apolar region can fold up

into helical conformation, thereby fitting into the hydrophobic environment of the membrane. A part of this 20-amino acid N-terminal sequence fulfills such conditions.

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