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SPECIFIC INTERACTION OF CENTRAL NERVOUS SYSTEM MYELIN BASIC PROTEIN WITH LIPIDS

SPECIFIC REGIONS OF THE PROTEIN SEQUENCE PROTECTED FROM THE PROTEOLYTIC ACTION OF TRYPSIN

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SUMMARY

The tryptic hydrolysis of the basic protein of central nervous system myelin (A_1 basic protein) and of A_1 basic–lipid complexes was studied. The tryptic digestion was monitored by "finger printing", column chromatography and amino acid analysis of the resulting pure peptides.

Specific regions of the protein sequence were found to be protected from the hydrolytic action of trypsin only after the protein was recombined with specific lipids. The degree of protection was in the order: cerebroside sulphate > acidic lipid fraction of myelin > phosphatidylserine = total lipid extract of myelin. The protected Lys–X, Arg–X bonds were all situated in the region amino acid 20 to amino acid 113 of the intact protein. This region contains the $(proline)_3$ bend in the protein which is stabilized by interaction with lipids and also the encephalitogenic site for monkey and rabbit.

From the results reported in this publication we would like to suggest a specific interaction between a region of the A_1 basic protein molecule and cerebroside sulphate. Differences in A_1 basic protein–lipid interaction in different animals arising from differences in lipid composition and fatty acid composition of the different lipid species combined with minor changes in the protein sequence could explain the species variability of the encephalitogenic sites of the A_1 basic protein.

INTRODUCTION

The myelin of the central and peripheral nervous system probably consists of a lipid bilayer with little (if any) protein penetrating deep into the hydrocarbon core of the membrane[1]. Very little is known about the interaction of the protein molecules with the lipid constituents of the myelin membrane[2–5]. The A_1 basic protein of the central nervous system myelin is the structural protein factor of the myelin membrane. This protein induces the autoimmune demyelinating disease

Abbreviation: TNBS, 2,4,6-trinitrobenzene sulphonic acid.

denoted as experimental allergic encephalomylitis when injected into animals[6,7] although the effects show species variability[8,11]. The amino acid sequence of the A_1 basic protein has been established[7] and it was suggested that its conformation was an open double chain structure which could be stabilized by interaction with lipids[7,12]. In the present investigation we have studied the tryptic hydrolysis of A_1 basic protein–lipid complexes in order to determine the regions of the protein which will be protected by the lipid from the hydrolytic action of trypsin, and to find out if this protection is specific to a certain lipid and a certain region of the protein. This is related to the occurrence of proteolytic enzymes in the central nervous system and the catabolism of basic proteins in the normal animal[13,15] and in multiple sclerosis[16,17]. A similar study was carried out with A_1 basic protein–lipid complexes at an air–water interface[18] using trypsin as well as other proteolytic enzymes.

MATERIALS AND METHODS

Materials

Trypsin (twice crystallized; chymotrypsin free) was purchased from Serva, Heidelberg, Germany. 2,4,6-Trinitrobenzene sulphonic acid was purchased from B.D.H. England. Sephadex LH-20, Sephadex G-25 fine, Sephadex G-50 fine and Sephadex G-10 were from Pharmacia Fine Chemicals, Sweden. Analytical grade 2-chloroethanol was fractionated by distillation *in vacuo*[19].

Methods

Preparation of A_1 basic protein and central nervous system derived lipids. A_1 basic protein was extracted and purified from bovine spinal cords[4]. The total lipid fraction and the acidic lipid fraction were prepared as previously described[4,5]. The composition of these lipid fractions was determined before[4,5] and is given in Table I. Phosphatidylserine was purified from ox spinal cords[20]. Cerebroside sulphate was purified from the same source. The ether-insoluble lipid fraction was prepared by the method of Folch-Pi[21] and was fractionated by use of DEAE-cellulose column chromatography[22]. The cerebroside sulphate fraction was shown to be more than 99% pure[23].

Preparation of the A_1 basic protein–lipid complexes. The dry lipids were dispersed in 15 ml of water by sonication. Lipid concentration varied from 5 to 15 mg/ml (see Fig. 1). The sonication was performed under nitrogen at a temperature of 0 °C for a constant time (Fig. 1) using a Branson sonifier at an output of 80 to 100 watt. The protein (100 mg) was dissolved in 15 ml of 0.2 M triethylamine–carbonate buffer, pH 8.0. The lipid suspension was added to the protein solution dropwise while stirring. The recombinations were allowed to stand for 15 min at 4 °C, and then centrifuged for 15 min at $35000 \times g$. The sediment was washed three times with 0.1 M triethylamine buffer, pH 8.0, and then suspended in 25 ml of the same buffer. The ratio of lipid to protein in the recombinant was determined by adding different amounts of the sonicated lipid suspension to a constant amount of protein in solution at pH 8.0 followed by measurement of protein recovery in the supernatant after centrifugation, according to the method of Lowry *et al.* For the qualitative and preparative experiments to be described the ratio of lipid to protein was taken to give 100% precipitation of the protein (Fig. 1).

TABLE I

LIPID COMPOSITION OF CENTRAL NERVOUS TISSUE FRACTIONS

The composition of the lipid fractions used in this study is shown below. The lipids were prepared from bovine spinal cords. Phospholipid, cholesterol and glycolipid values were determined as previously described[4, 18].

Lipid species	*Central nervous system lipids*[9]	*Central nervous system acidic lipids*
Cholesterol	29.3	—
Phospholipid	42.2	
Cerebrosides	28.5	
Cerebroside sulphate		36.5
Serine phosphoglycerides	18.0*	40.0
Ethanolamine phosphoglycerides	37.0	—
Inositol phosphoglycerides	1.7	3.0
Choline phosphoglycerides	21.0	—
Sphingomyelin	19.2	—
Phosphatidic acid	1.2	12.4
Polyphosphoinositides	0.7	5.0
Cardiolipin	—	3.9
Unknown	1.0	2.2

* Percentage of the total phospholipid quantity.

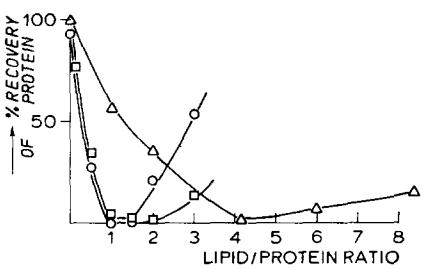


Fig. 1. The recombination of A_1 basic protein with lipid dispersions. Percentage protein recovery in supernatant after recombination at pH 8.0 in 0.1 M triethylamine–carbonate buffer. Details described in the Methods section.

	Sonication time (min)	*mg lipid/mg protein*
△—△, A_1 basic protein–total lipid fraction	5	4
○—○, A_1 basic protein–acidic lipid fraction	2	1.5
□—□, A_1 basic protein–cerebroside sulphate	2	1.5

Trypsin digestion of lipid–protein complexes. In preparative experiments the lipid–protein complexes (5.6 μmoles protein) were digested with 2% trypsin at 37 °C, in 0.1 M triethylamine buffer, pH 8.0, with continuous stirring, for 3 h. Control experiments in which A_1 basic protein free of lipids was hydrolysed by trypsin were carried

out at the same time. The reaction was stopped by addition of 0.5 ml 0.1 M DFP, and the suspension was freeze–dried. In qualitative experiments (0.56 μmole protein) the incubation with trypsin was carried out for various times ranging from 30 min up to 5 h.

Extraction and separation of the peptides. The extraction and separation of the peptides varied with the lipids used in the experiments. In the experiments with the total lipid extract, acidic lipid fraction and phosphatidylserine, the dried material after proteolysis was extracted with dry acetone. The residue recovered after acetone extraction and the dried supernatants were extracted with dry ether. The ether supernatants were combined and dried. The different residues (two ether supernatants and two residues) were extracted for 8 h with 0.1 M HCl at 4 °C. The supernatants were recovered after centrifugation for 20 min at $50000 \times g$, combined, freeze–dried, dissolved in 0.01 M HCl and chromatographed on Sephadex columns as described later. This extraction method was used because it gave high recovery of the basic protein when purified from myelin and in preliminary experiments has been found to be the most efficient extraction technique for these lipids. The combined sediments after the acid extractions were dried and dissolved in 2-chloroethanol–chloroform (2:1, v/v), and the peptides were separated from the lipids on a Sephadex LH-20 column. In the experiment with cerebroside sulphate, part of the peptides were separated from the lipid by extraction with 0.1 M HCl and the residue recovered after centrifugation was fractionated on a Sephadex LH-20 column.

Column chromatography. The LH-20 column[19] (32 cm × 3 cm) was equilibrated and eluted with 2-chloroethanol–chloroform (2:1, v/v). The flow rate was 20 ml/h and 2-ml fractions were collected. The absorbance of the eluate was monitored at 240 nm and at 280 nm. The fractions recovered from the column were also studied by thin-layer chromatography. The fractions containing the peptides were pooled and dried *in vacuo*. Separation of the soluble peptides was carried out by gel filtration on a superimposed series of Sephadex columns consisting of a Sephadex G-10 column (57 cm × 1.6 cm), a Sephadex G-25 fine column (57 cm × 1.6 cm) and a Sephadex

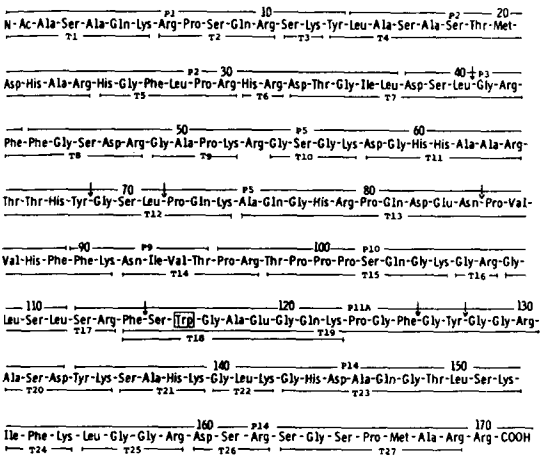
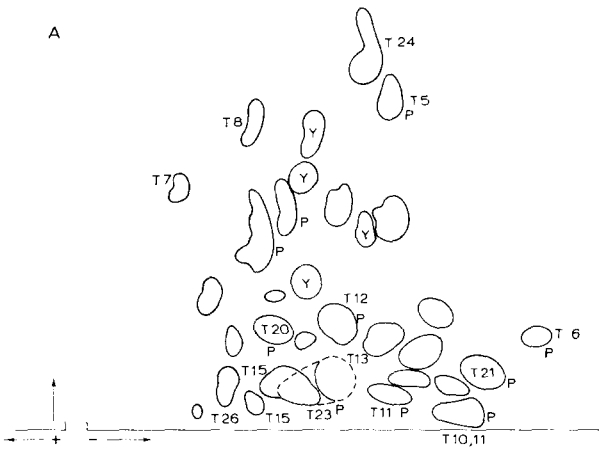


Fig. 2. The amino acid sequence of the A_1 basic protein (reproduced by permission of Dr E.H. Eylar).


A
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T8
Y
T7
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T 12
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T23 P
T 11 P
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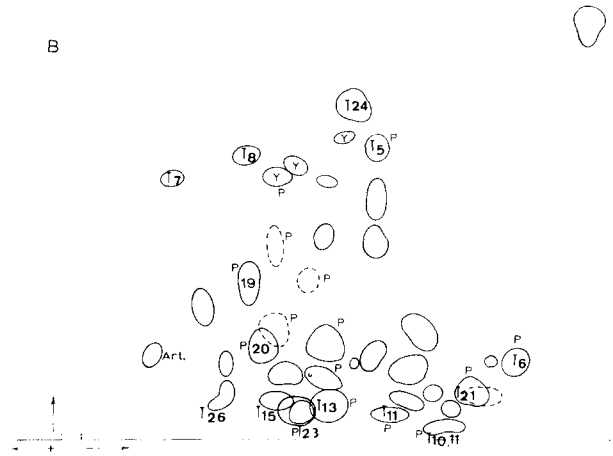
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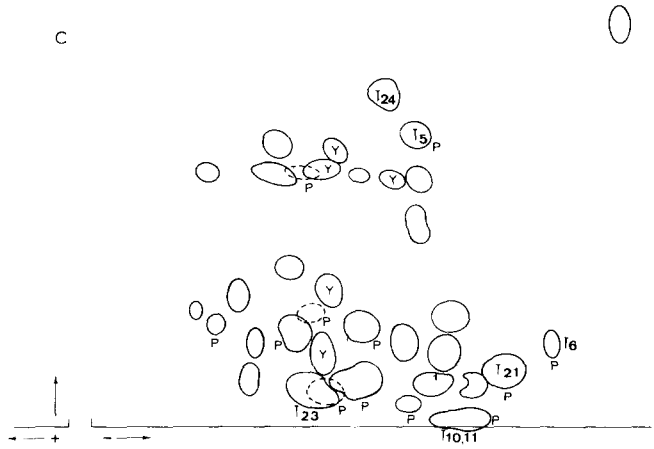
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G-50 column (100 cm × 1.6 cm). The column was eluted with 0.01 M HCl–0.02% sodium azide at room temperature with a flow rate of 20 ml/h and 2-ml fractions were collected. The eluate was monitored at 230 nm and 280 nm. Qualitative peptide mappings were performed on Whatman No. 1 paper while preparative peptide mappings were performed on Whatman 3MM paper[24,25]. The peptides were detected by ninhydrin staining in collidine–acetic acid–*n*-butanol (2:15:33, by vol), Pauli staining technique for histidine and tyrosine[27] and Ehrlich stain for tryptophan[27].

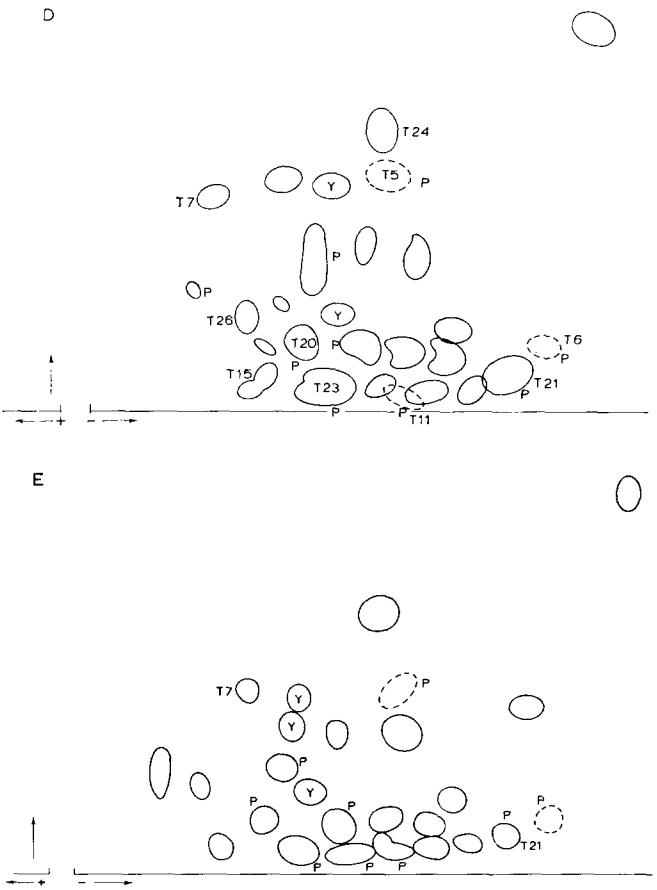
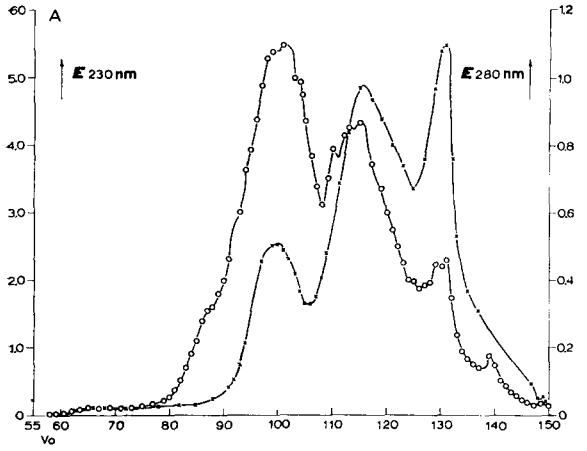


Fig. 3. Peptide maps of A_1 basic protein and A_1 basic protein–lipid complexes. The peptide map was obtained after digestion with trypsin (see Methods). Descending chromatography was carried out for 30 h in butanol–acetic acid–pyridine–water (90:18:60:72, by vol.). Electrophoresis was carried out for 60 min at 3200 V, pH 3.5, pyridine–acetic acid–water (1:10:289, by vol.) The peptide maps were stained with ninhydrin and Pauli reagents. Y, yellow; P, Pauli-positive peptide (tyrosine or histidine). A, A_1 basic protein; B, A_1 basic protein–total lipid fraction. The acid soluble peptides. Art., artefact. C, A_1 basic protein–acidic lipid fraction. The acid soluble peptides. D, A_1 basic protein–cerebroside sulphate. The acid soluble peptides. E, A_1 basic protein–cerebroside sulphate. The acid soluble peptides, and peptides which have been precipitated in acidified chloroform–methanol (2:1, by vol.). The sediment after acid extraction was dissolved in chloroform (2:1, by vol.). The solution was brought to 0.04 M with concentrated HCl. The peptides that precipitated were recovered after centrifugation and pooled with the acid soluble peptides.


A
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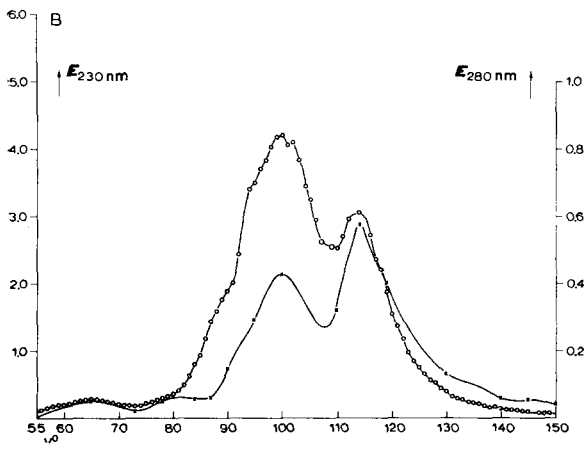
E 280nm
Vo


B
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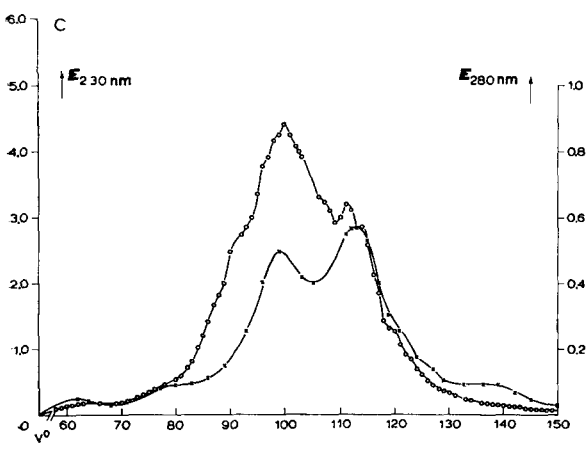

C
E230nm
E280nm
Vo

For preparative work the papers were dipped in 0.2% acetone solution of 2,4,6-trinitrobenzene sulphonic acid (TNBS), dried at room temperature and exposed for 2 h to pyridine–water vapour in a dark chamber[28,29] and the yellow spots were viewed by diffused ultraviolet light[30]. The spots were cut out and eluted with water, and the eluate was lyophilized and hydrolyzed under reduced pressure in constant-boiling 6 M HCl for 24 and 48 h at 110 °C. Amino acid analyses were performed on Beckman-Spinco amino-acid analyser model 120 B.

Nomenclature. Tryptic peptides are designated by T and each amino acid residue is numbered according to Eylar *et al.*[7] (see Fig. 2).

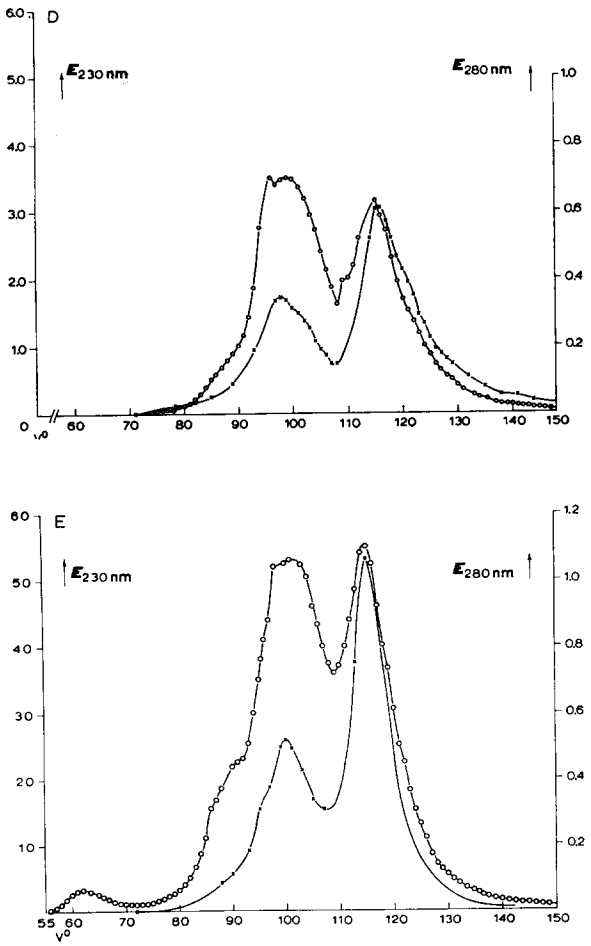


Fig. 4. The gel filtration elution pattern is shown for the tryptic peptides of A_1 basic protein and A_1 basic protein–lipid complexes (see Methods) on a superimposed series of Sephadex columns eluted with 0.1 M HCl. ○—○, absorbance at 230 nm; ×—×, absorbance at 280 nm. A, A_1 basic protein; B, A_1 basic protein–total lipid fraction, the acid soluble peptides; C, A_1 basic protein–acidic lipid fraction, the acid soluble peptides; D, A_1 basic protein–cerebroside sulphate, the acid soluble peptides; E, A_1 basic protein–cerebroside sulphate, the acid soluble peptides, and peptides which have been precipitated in acidified chloroform–methanol (2:1, by vol.).

RESULTS

The time course of proteolysis of the A_1 basic protein was studied at time intervals ranging from 30 min to 5 h, and showed that there were no differences between the tryptic peptide map of the protein after 90 min of proteolysis and a longer time of hydrolysis. As our intention was to compare the "finger prints" of the A_1 protein when complexed with lipids and the soluble, lipid-free protein, we performed the same experiment but with A_1 basic protein–lipid complexes. We chose 180 min of hydrolysis because by this we could then exclude changes in the "finger prints" due to an inhibition or delay in the enzyme action caused by the aggregated from of the complexes.

Extraction of the peptides

The extraction method used for the recovery of the peptides was found to give complete recovery of the peptides free from lipids. In a control experiment the peptides after 180 min trypsinolysis were recombined with the lipids and recovered after acid extraction. The peptide maps before and after the extraction of the peptides were compared and no differences were found between them. The presence of artefacts, due to ninhydrin positive contaminants in the lipid fractions was checked in two dimensional "finger prints" of the lipids alone and lipid treated with trypsin. Only in the total lipid fraction was contaminant found (see Fig. 3B).

A_1 basic protein–total lipid complex

There were differences between the 180 min tryptic maps of the experimental sample (Fig. 3B) and the control (Fig. 3A). The differences found were mainly a reduction in the quantities of some of the peptides, mainly peptide T_{18} and an increase in the quantity of peptide T_{19}. There was inhibition of cleavage of the Arg-122–Pro-123 bond although the two peptides were found[7]. All the peptides

TABLE II

THE SEQUENCES OF THE PEPTIDES RECOVERED FROM THE PEPTIDE MAPS OF A_1 BASIC PROTEIN–CEREBROSIDE SULPHATE (SEE FIG. 7) AND ACIDIC LIPID FRACTION–A_1 BASIC PROTEIN PROTEOLYSATES

Peptide	*Sequence*
$T_{4,5}$	Tyr^{23}–Leu–Ala–Ser–Ala–Ser–Thr–Met–Asp–His–Ala–Arg–His–Gly–Phe–Leu–Pro–Arg^{30}–
$T_{6,7}$	His^{31}–Arg–Asp–Thr–Gly–Ile–Leu–Asp–Ser–Leu–Gly–Arg^{42}
Arg $T_{10,11}$	Arg–Gly^{54} ---Arg^{64}
$T_{10,11}$	Gly^{54}–Ser–Gly–Lys–Asp–Gly–His–His–Ala–Arg^{64}
T_{12}	–Thr^{65}–Thr–His–Tyr–Gly–Ser–Leu–Pro–Gln–Lys^{74}–
T_{13}	–Ala^{75}–Gln–Gly–His–Arg–Pro–Gln–Asp–Glu–Asn–Pro–Val–Val–His–Phe–Phe–Lys^{91}–
$T_{14,15}$	Asn^{92}–Ile–Val–Thr–Pro–Arg–Thr–Pro–Pro–Pro–Ser–Gln–Gly–Lys^{105}
T_{16A}	Gly^{106}–Arg*–Gly–Leu–Ser–Leu–Ser–Arg^{113} | Methyl

were extracted with 0.1 M HCl and no peptides were recovered after chromatography on Sephadex LH-20 eluted with 2-chloroethanol–chloroform (2:1, by vol). The elution pattern on the combined Sephadex columns (Fig. 4B) was different from the control experiment (Fig. 4A) mainly in the disappearance of the third peak which contains mainly peptide T_{18} (a tryptophan peptide) and small peptides. This peak appears in the elution pattern of the total lipids A_1 protein proteolysate only as a shoulder (Fig. 4B).

A_1 basic protein–acidic lipid fraction complex

Here we have found quantitative and qualitative differences between the experiment and control (see Fig. 3C). Peptide $T_{10,11}$ is increased in quantity as was seen by the intensity and recovery from the preparative peptide maps and there is the appearance of a new peptide $T_{14,15}$ (see Fig. 7, Table II). The quantity of peptid $T_{6,7}$ is also increased. This peptide appears also in the control experiment but with a yield of only 4%[7] and cannot be seen in the "finger prints" of the control experiment.

Some of the peptides were not recovered after the acid extraction or by any other extraction technique tried. They could be recovered from the LH-20 column eluate (see Fig. 5). These were peptides $T_{10,11}$, $T_{14,15}$ and $T_{6,7}$ (see Table III). The elution pattern of the acid soluble peptides. (Fig. 4C) is different from the control experiment (Fig. 4A).

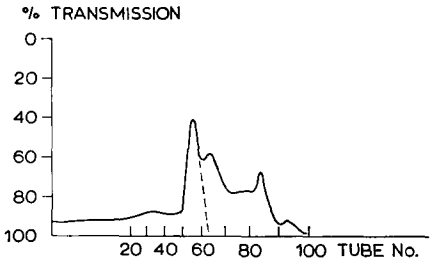


Fig. 5. The gel filtration elution pattern on Sephadex LH-20 in 2-chloroethanol–chloroform (2:1, by vol.), is shown for A_1 basic protein–acidic lipids fraction (see Methods). The broken line is the peptide containing peak.

TABLE III

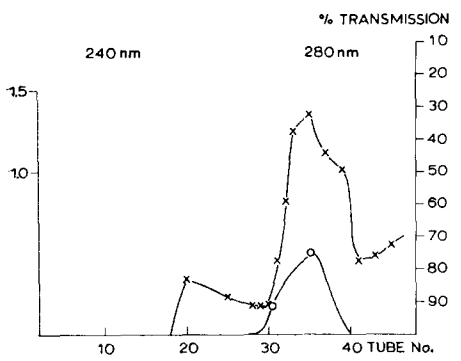
THE DIFFERENT PEPTIDES FORMED AND RECOVERED AFTER TRYPSINOLYSIS OF A_1 BASIC PROTEIN–LIPID COMPLEXES

Lipid complexed with A_1 protein	*New peptides formed*	*Peptides recovered from LH-20 column*
Total lipids	—	—
Acidic lipids	$T_{10,11}$, $T_{14,15}$, $T_{6,7}$	$T_{10,11}$, $T_{14,15}$, T_{15}, $T_{6,7}$
Cerebroside sulphate	$T_{10,11}$, $T_{14,15}$, $T_{6,7}$, $T_{4,5}$, Lys^{73}–Gln^{75}	$T_{10,11}$, Arg–$T_{10,11}$, $T_{6,7}$, $T_{4,5}$, T_{16A}, T_5, T_{13}, T_{15}, T_{11}, T_{12}

A_1 basic protein–cerebroside sulphate complex

Here we found the most pronounced differences between the control and the experiments (Figs 3D, 4D, 4E, Table II). The peptides $T_{6,7}$, $T_{10,11}$, $T_{14,15}$ were recovered in high yields. Also recovered were the peptides Arg-53–$T_{10,11}$ and a chymotryptic peptide Lys-73–Ala-74–Gln-75 which was not found in the control experiment (Fig. 7). Peptide $T_{4,5}$ (Fig. 7, Table II) was recovered with a low yield.

Certain peptides could not be completely recovered from the lipid by the extraction techniques tried including acidified chloroform–methanol (2:1, by vol.) (Figs 3E and 4E) but were recovered by Sephadex LH-20 column chromatography (see Fig. 6). These were peptides T_{12}, T_{13}, T_{15}, T_{11}, $T_{6,7}$, $T_{10,11}$, $T_{14,15}$, T_{16A} and peptide T_5 (see Table II, Fig. 7).



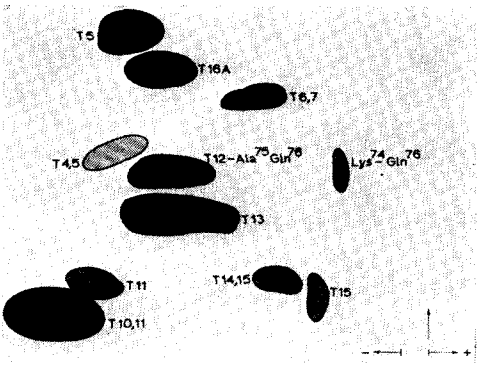


Fig. 6. The gel filtration elution pattern on Sephadex LH-20 in 2-chloroethanol–chloroform (2:1, by vol.) is shown for A_1 basic protein–cerebroside sulphate (see Methods). ○—○, % transmission at 280 nm; ×—×, absorbance at 240 nm.

Fig. 7. Peptide map of A_1 basic protein–cerebroside sulphate tryptic peptides recovered from the Sephadex LH-20 column (see Methods).

Peptide T_{16A} was recovered with high yield free of the lipid only after Sephadex LH-20 chromatography in 2-chloroethanol–chloroform. The ratio of arginine to arginine-(methyl)$_2$ in this peptide was found to be 3.7 as compared to 4.7 as reported by Eylar[31].

When the A_1 basic protein was recombined with phosphatidylserine, there were no differences in the peptide maps between the control and the experiment, except for the yield of peptide T_{18} which was minimal.

DISCUSSION

The capacity of the myelin basic protein and other highly basic proteins to form complexes with acidic, negatively charged lipids has already been studied and documented[2,32,33]. Cerebroside sulphate, which is a lipid specific to the myelin[34,35] was found to protect specifically certain regions of the A_1 basic protein molecule from hydrolysis by trypsin. Other lipids such as brain phosphatidylserine and the

total lipid extract of myelin gave little or no protection. The acidic lipid fraction composed mainly of phosphatidylserine and cerebroside sulphate showed protection of almost the same regions of the protein molecule as did the sulphatides though to a lesser extent. The regions in the protein which were "protected" or showed specific interaction with cerebroside sulphate were all in the region of amino acid 20 through amino acid 113 of the protein sequence (Fig. 2).

This part of the A_1 protein molecule is characterized by the presence of eight of the total 10 proline residues, five of which are rather evenly spaced through the chain so that only a relatively short part of the chain would be able to form an α-helical conformation. The highly charged amino acids lysine, arignine, aspartic acid and glutamic acid are also evenly spaced and tend to be paired, Arg-32–Asp-33, Asp-47–Arg-48, Lys-57–Asp-58 and Asp-82–Glu-83. There is a high content of histidine (8 residues from the total of 10) which at pH values above 7 will be neutral and so will be non-polar. These histidines tend to be paired either with another histidine residue, His-60–His-61 or with the charged residues arginine, lysine and aspartic acid, Asp-21–His-22, Arg-24–His-25, Arg-30–His-31 and His-78–Arg-79.

From the results reported here and from the study of the interaction of A_1 basic protein with lipids at the air–water interface[18] and low angle X-ray diffraction studies of these lipid–protein complexes[5] we can suggest that this region of the peptide chain interacts specifically with cerebroside sulphate.

From the results reported here and from the sequence we would like to suggest that after the interaction with the sulphatides this region of the protein could take the form of a β-conformation with penetration of amino acid residues into the lipid matrix and *vice versa*. We have also a strong indication for the double chain conformation of the A_1 protein stabilized by the interaction between the methylated arginine 107 (peptide T_{16A}) and the phenylalanines 89–90 (peptide T_{13}). Only after interaction with lipid was it possible to preserve the sequence of peptide $T_{14,15}$ from cleavage by trypsin and peptides T_{13} and T_{16A} were recovered with high yield with the lipids. Peptide T_{16A} which was recovered from the cerebroside sulphate–A_1 basic protein hydrolysate had a lower ratio of arginine to dimethyl arginine compared to that reported by Eylar[31]. This segment of the peptide chain is probably a binding site for lipids and its structure is stabilized by this interaction. The primary structure of the A_1 basic protein assumes special significance in its immunological interactions[31], as this protein is encephalitogenic when injected into animals[31]. The 46-residue fragment (residues 45 to 90) was shown to be encephalitogenic in rabbit and in monkey, but not in guinea pig[9], while the tryptophan region (residues 114–122) is highly active in both guinea pig and rabbit but not in monkey[31]. This species variability[37,38] could rise from differences in A_1 basic protein–lipid interactions in the different animals arising from differences in lipid composition and fatty acid composition of the different lipid species, combined with minor changes in the protein sequence. Thus different regions on the protein molecule will form strong specific interactions with the lipids and as a result, different regions of the sequence will be exposed and accessible to attack by sensitized lymphocytes or by antibody produced locally by them. The method described in this publication can be used for the studies of lipid–protein interactions of the peripheral nerve basic proteins and other membrane proteins with lipids using trypsin and other proteolytic enzymes such as brain neutral and acid proteinases[13].

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