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THE PROTECTION OF A_1 MYELIN BASIC PROTEIN AGAINST THE ACTION OF PROTEOLYTIC ENZYMES AFTER INTERACTION OF THE PROTEIN WITH LIPIDS AT THE AIR–WATER INTERFACE

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SUMMARY

1. The specific interaction of bovine myelin A_1 basic protein with lipids at the air–water interface was studied. The interaction was measured by recording the changes in surface pressure and surface radioactivity using ^{131}I-labelled A_1 basic protein. The highest affinity of the A_1 basic protein was found for cerebroside sulphate, a lipid which is characteristic for the myelin lipids.

2. The proteolytic degradation of the A_1 basic protein–lipid complex by proteolytic enzymes such as trypsin, chymotrypsin A_4, subtilopeptidase A and pronase E, showed that specific regions of the protein molecule are protected after the interactions with lipids.

3. The A_1 basic protein–cerebroside sulphate complex was collected from the interface after tryptic hydrolysis. Peptide maps showed that the N-terminal part of the protein molecule (positions 20–113) is preserved in the lipid phase. A schematic model of the A_1 basic protein–lipid interaction is presented.

INTRODUCTION

Monomolecular films of lipid at the air–water interface are the simplest model in which lipid–protein interactions can be readily studied with a high degree of accuracy[1]. Especially a combination of different detection methods can provide detailed information about lipid–protein interactions. The measurement of changes in the surface tension can provide information about a possible penetration of the protein into the lipid region. Measurement of surface radioactivity makes it possible to determine the amount of protein interacting at the interface as well as the adsorption of protein which does not lead to changes in surface pressure. The effect of proteolytic enzymes as described in this paper makes it possible to demonstrate which parts of the protein are involved in the interaction and possibly buried within the lipid core. Also the effects of ions and solutes can be studied by these methods. In the present study we investigated the quantitative effect of proteolytic enzymes on the lipid–protein assembly at the air–water interface, using ^{131}I-labelled basic protein of the central nervous system[2–5], and of lipids derived from the same source. We

intended to answer in more detail the same question as studied recently in bulk studies[6]:

(a) Is there a specific interaction between the A_1 basic protein and the central nervous derived lipids?

(b) Is there a protection of specific regions of the protein from the hydrolytic action of trypsin, after the formation of the lipid–protein assembly at the air–water interface?

This study and other studies on the lipid protein interactions of the A_1 basic protein of the central nervous system myelin and P_1 and P_2 basic proteins of peripheral nerve myelin[6,8] give us a better understanding of the molecular structure of myelin.

MATERIALS AND METHODS

The lipids were extracted and purified from the following sources: cerebroside sulphate, acidic lipid fraction from ox spinal cords; lecithin from egg yolk. The lipid composition of the acidic lipid fraction was as given before[8].

Trypsin (twice crystallized), chymotrypsin free, was purchased from Serva (Heidelberg, Germany). Chymotrypsin A_4 (10000 units/mg) was purchased from Boehringer (Germany). Pronase E (70000 Kunitz proteolytic units/g) was purchased from Merck (Germany). Subtilopeptidase A (crystalline) was purchased from Sigma (U.S.A.). The A_1 basic protein was purified from bovine spinal cords as already described[4]. For the preparation of peptide T (amino acids 117 through 170, mol. wt 5600) and peptide L (amino acids 1 through 116, mol. wt 12400) the method of Burnett and Eylar[9] was used. The two peptides were separated by gel filtration on a superimposed series of Sephadex columns (G-10, G-25, G-50) and found to be pure by using disc electrophoresis[17]. A_1 basic protein was iodinated with ^{131}I and ^{125}I as described before[8]. The interaction of A_1 basic protein and lipid at the air–water interface was performed on a 10^{-2} M Tris–acetate buffer, pH 8.0, or a 10^{-1} M triethylamine buffer, pH 8.0. The latter buffer was used when the complex was collected from the interface and analysed. Basic protein and proteolytic enzymes were injected into the aqueous layer from 1 mg/ml solutions. The changes in surface pressure and surface radioactivity were recorded automatically as described before[8]. Collections of monolayers of lipid–protein complexes were performed after transferring the complex to a protein-free subphase as described before[8]. The peptides were extracted from the lyophilized material, and a two-dimensional peptide map was made[6]. The positions of the iodinated tyrosines in the A_1 basic protein were determined from the known sequence[2] after amino acid analysis of tryptic peptides recovered from the two-dimensional peptide map[6,8] of the ^{125}I-labelled A_1 basic protein.

RESULTS

Fig. 1 gives the recordings of the effect of trypsin on the interfacial complex of acidic lipids from the central nervous system and A_1 basic protein. The increase in pressure after the injection of the ^{131}I-labelled basic protein parallels the increase in surface radioactivity as already shown before[8]. When the maximum pressure increase is reached after A_1 basic protein injection underneath the lipid layer, trypsin is injected underneath. A partial loss of surface pressure and surface radioactivity is

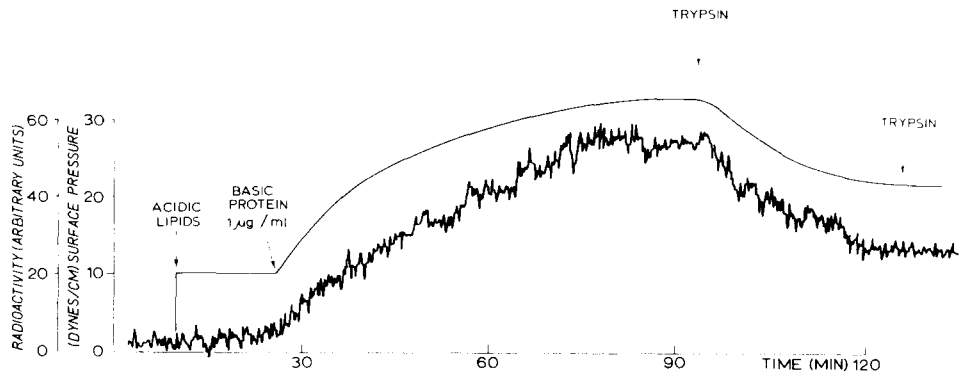

Fig. 1. The effect of trypsin on the surface pressure and surface radioactivity of the interfacial complex of A_1 basic protein–acidic lipid fraction from the central nervous system. A monolayer of acidic lipids was spread on a subphase of 10^{-2} M Tris–acetate, pH 8.0, to an initial pressure of 10 dynes/cm. ^{131}I-labelled A_1 basic protein was injected to a final concentration of 1 μg/ml. Trypsin was injected to final concentrations of 0,05 and 0.10 μg/ml, respectively.

found. About 50% of the original pressure or radioactivity increase after A_1 basic protein injection is preserved. Additional injections of trypsin did not effect a further reduction of the pressure or radioactivity. As is shown in Fig. 2, the pressure increase as well as the radioactivity increase is less extensive when a lecithin monolayer is used. These differences in specificity for different lipid monolayers are in agreement with our previous findings. The rate of reduction of the pressure and radioactivity

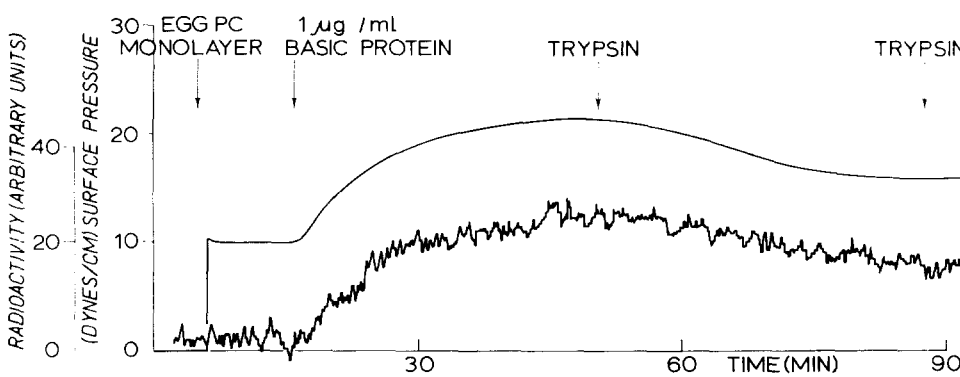

Fig. 2. The effect of trypsin on the surface pressure and surface radioactivity of the interfacial complex of A_1 basic protein–egg lecithin (PC). Details as given in Fig. 1.

after trypsin injection is much slower than in the case of a film of lecithin when compared with films of acidic lipids or cerebroside sulphate. The surface pressure of a lecithin–A_1 basic protein complex after the injection of trypsin is also partially reduced. About 50% of the original pressure increase is preserved. Fig. 3 shows the effect of the initial film pressure at which the cerebroside sulphate–A_1 basic protein complex is formed on the trypsin degradation. The highest pressure increase is found at low initial pressures. When trypsin is now injected underneath this complex a large

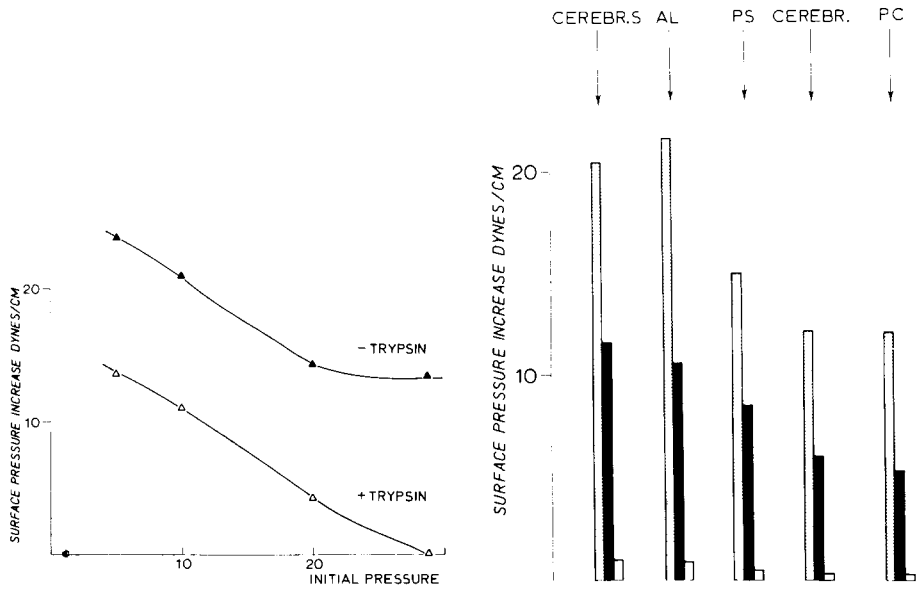

Fig. 3. The effect of the film pressure on the trypsin degradation of the A_1 basic protein–cerebroside sulphate complex. Pressure increase observed after the injection of A_1 basic protein underneath a cerebroside sulphate monolayer at different initial pressures (▲). The remaining surface pressure increase when the complex formed is treated with trypsin (△). Details as given in Fig. 1.

Fig. 4. Comparison of the surface pressure increase of cerebroside sulphate (CEREBR. S), acidic lipid fraction from the central nervous system (AL), phosphatidylserine (PS), cerebroside (CEREBR.), lecithin (PC) monolayers. Dotted columns, pressure increase after injection of A_1 basic protein; black columns, pressure preserved after trypsin degradation of the complex; white columns, pressure increase after injection of peptides formed after preincubation of A_1 basic protein with trypsin. Details are given in Fig. 1.

extent of the pressure increase is preserved. This means that part of the A_1 basic protein is preserved against the action of trypsin. At initial pressures of 30 dynes/cm a considerable pressure increase is still found after the injection of A_1 basic protein underneath a cerebroside sulphate monolayer. However, the pressure increase is now completely undone so that the extent of protein penetration is not sufficient to protect the protein against trypsin degradation. Fig. 4 gives: (a) the pressure increase after the injection of A_1 basic protein underneath different lipid monolayers, (b) the remaining pressure increase after injection of trypsin, (c) the pressure increase after injection of a preincubated A_1 basic protein with trypsin. The same result is observed when A_1 basic protein and trypsin are incubated first and the monomolecular film is spread after 30 min. For cerebroside sulphate, acidic lipids, phosphatidylserine, cerebroside and lecithin the pressure increase is only partly reduced after trypsin treatment. The ultimate pressure remaining is highest for cerebroside sulphate, but also for the neutral lipids lecithin and cerebroside a high extent of protection is found. It is clear that the trypsin-digested A_1 basic protein is unable to penetrate any of the lipid layers to any extent. The changes in the surface radioactivity (Fig. 5) show approximately the same results as is found with the surface pressure decrease. The

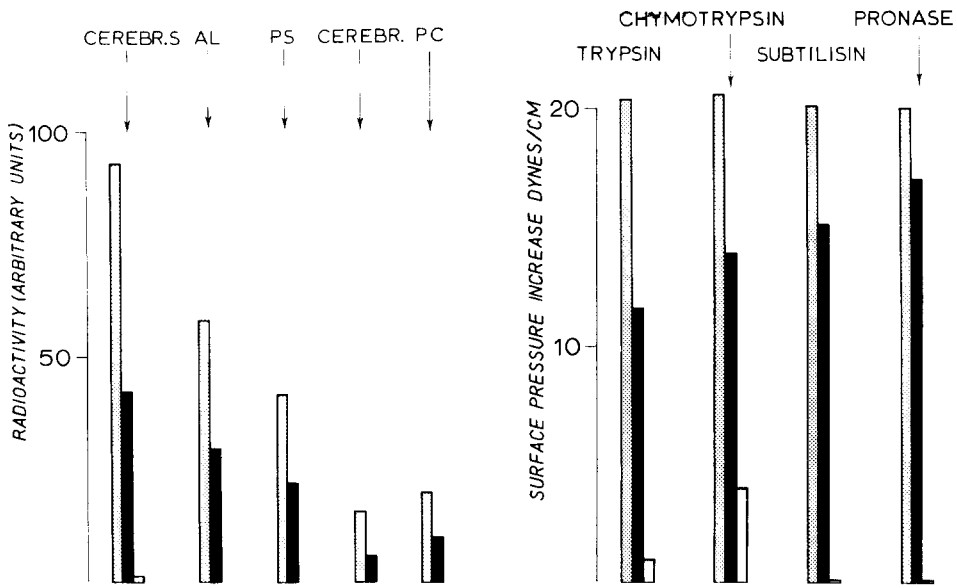

Fig. 5. Comparison of the surface radioactivity increase of the lipids as stated in Fig. 1. See Fig. 4 for meaning of abbreviations and columns.

Fig. 6. The effect of different proteolytic enzymes on the A_1 basic protein–cerebroside sulphate complex. Dotted columns, pressure increase after injection of A_1 basic protein underneath a cerebroside sulphate monolayer; black columns, pressure preserved after degradation of the complex by respectively trypsin, chymotrypsin A_4, subtilopeptidase A and pronase E; white columns, pressure increase after injection of peptides formed after incubation of A_1 basic protein with the respective proteolytic enzymes. Details as given in Fig. 1.

effects of other proteolytic enzymes on the cerebroside sulphate–A_1 basic protein complex are given in Fig. 6.

Chymotrypsin, subtilisin and pronase showed even less effective degradation of the lipoprotein complex than was found with trypsin. However, when the A_1 basic protein was incubated in the subphase with these proteolytic enzymes before an interaction could take place with cerebroside sulphate, the peptides formed were not able to penetrate the monolayers. After treatment of A_1 basic protein with BNPS-skatole [2-(2-nitrophenylsulphenyl)-3-methylindole], the molecule is split into peptides T and L. Both these parts of the A_1 basic protein are tested on their ability to penetrate the lipid monolayer and to increase the surface pressure (Fig. 7). Peptide L, consisting of the amino acids 1–116, gives an appreciably higher pressure increase than peptide T which consists of the amino acids 117–170. This could mean that peptide L penetrates to the lipid core to a greater extent than peptide T and a greater protection of this peptides should be obvious. To check which parts of the protein are hydrolysed by trypsin, the interfacial cerebroside sulphate–A_1 basic protein complex is collected and analysed after the hydrolytic action of trypsin. The interaction of cerebroside sulphate with A_1 basic protein was first carried out until maximum pressure increase was reached. The complex was then transferred to a protein-free interface after which trypsin was injected. The ultimate reduction in pressure

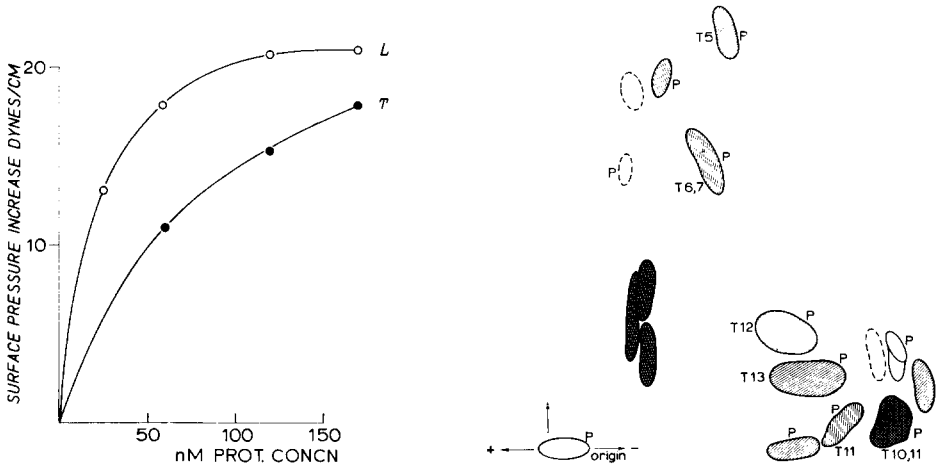

Fig. 7. Surface pressure increase after the injection of different amounts of peptide L and T underneath a monolayer of cerebroside sulphate. Details as given in Fig. 1.

Fig. 8. Peptide mapping of the A_1 basic protein–cerebroside sulphate complex after tryptic hydrolysis at the air–water interface. Details as given in Fig. 1 and ref. 6.

after the action of the enzyme was reached after 30 min. The monomolecular film was transferred to a clean subphase, then compressed and at the same time sucked from the interface. The peptide mapping of the peptide material after separation from the lipid is given in Fig. 8. The peptide mapping of A_1 basic protein after incubation with trypsin in bulk is given in Fig. 9. It is clear that both mappings show a completely different picture. The peptides found in the lipoprotein collected from the monolayer, are T_5, $T_{6,7}$, $T_{10,11}$, T_{11}, T_{12}, T_{13} and very probably T_{14}– T_{16A}. Parts of the protein beyond 113 are not found (see Fig. 10). Also the experiments with the ^{131}I-labelled

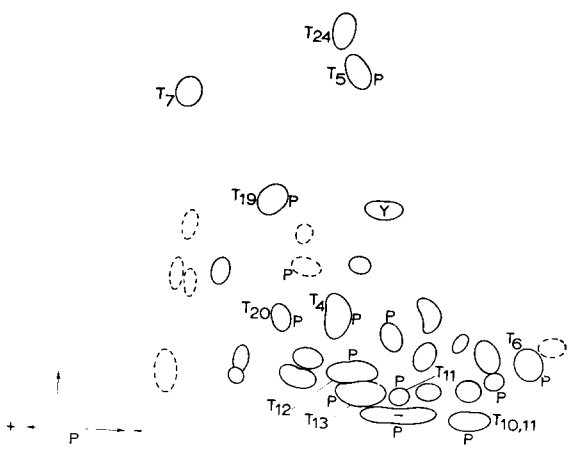

Fig. 9. Peptide mapping of the A_1 basic protein after tryptic hydrolysis in the absence of lipids. Details as given in Fig. 1 and ref. 6.

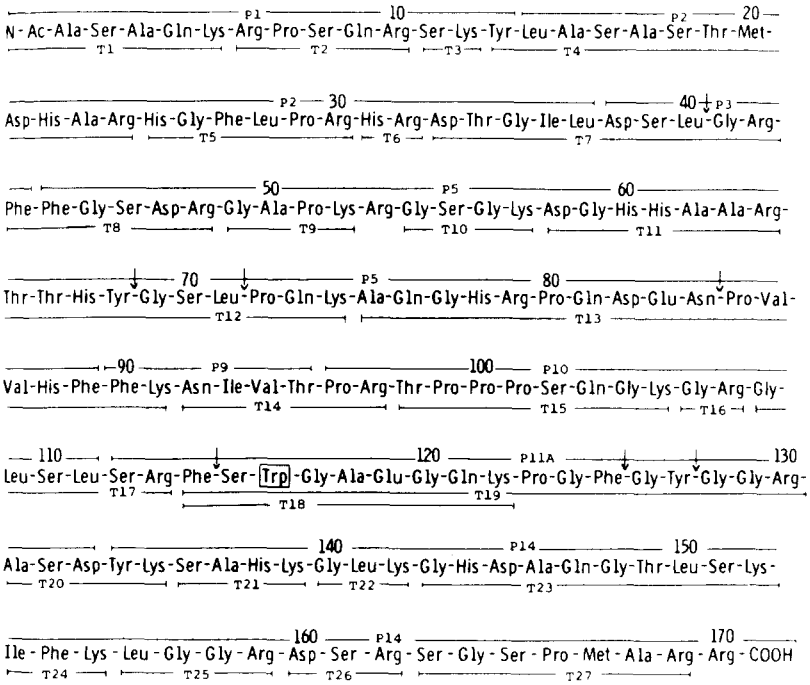

Fig. 10. The amino acid sequence of the bovine A_1 basic protein. Reproduced with the kind permission of Dr. E. H. Eylar.

A_1 protein with the labels on Tyr 68 (peptide T_{12}) and Tyr 134 (peptide T_{20}) show that about 50% of the label is released after the trypsin degradation. This means that the peptide containing the ^{131}I label at 134 is released from the lipid monolayer.

DISCUSSION

The preceding paper[8] has given clear evidence for a specific interaction of the A_1 basic protein with, in the myelin present, cerebroside sulphate. The interaction was measured by the change in surface pressure. At the same time the actual amount of protein in the lipid layer was determined by surface radioactivity using ^{131}I-labelled A_1 basic protein. Less charged lipids present in the myelin membrane such as phosphatidylserine showed a much smaller interaction with the A_1 basic protein. Neutral lipids such as cholesterol, lecithin, sphingomyelin and cerebrosides showed only a moderate affinity for this protein. A positively charged lipid, lysylphosphatidylglycerol from *Staphylococcus aureus*, showed no interaction at all. It can be concluded from the previous study that ionic forces are involved in the interaction of A_1 basic protein and lipids. The affinity of the protein is directed towards negatively charged lipids. The pressure increase is reduced in the presence of 1 M NaCl. Ca^{2+} is expelled from the lipid interphase when the A_1 basic protein is injected. On the other hand, hydrophobic forces are also very much apparent. The interaction is affected by the fatty acid chain length and by the presence of KCNS. The penetration of the protein

is also demonstrated by the big area increase after the interaction of the protein with the lipid layer. From the experiments described in this paper it can be concluded as to which parts of the protein molecules are particularly involved in the interaction. Comparing Figs 1 and 2, and from previous experiments, it is clear that negatively charged lipids and especially cerebroside sulphate bind at least twice as much A_1 basic protein as lecithin or cerebroside. After the injection of trypsin the loss of pressure or surface radioactivity is much faster for the negatively charged lipids than for the neutral lipids. Probably the ionically adsorbed proteins or protein attached to the interface by protein–protein interactions, are released first and thereafter the regions of the protein molecule which are not protected by the lipid core. Experiments with other proteolytic enzymes, chymotrypsin A_4, pronase E and subtilopeptidase A, also show a high degree of protection of the protein by the lipid layer. The respective peptides which can be formed in the absence of lipid, are not able to interact with lipid monolayers.

From the amino acid sequence of the A_1 basic protein it was suggested by Eylar *et al.*[2] that the protein consists of an open double chain with a sharp bend at the Pro–Arg–Thr–Pro–Pro–Pro position 96–101 (Figs 10 and 11).

The N-terminal part of the molecule (positions 1–116) contains far more nonpolar amino acids at pH 8.0 than the C-terminal part of the molecule (positions 117–170) mainly because of the asymmetric distribution of the histidines which are neutral at pH values above 7.0 and then form hydrophobic structures.

Since ionic as well as hydrophobic interactions are involved it is most likely that the peptide L part of the molecule (positions 1–116) is primarily interacting with the lipid layer. This view is in agreement with the results in Fig. 7 where a higher pressure increase is shown for peptide L than for peptide T. The peptide mappings of the lipid–protein complex collected after tryptic degradation give the most convincing evidence that primarily the peptides T_5, $T_{6,7}$, $T_{10,11}$, T_{11}, T_{12}, T_{13} and probably T_{14}, T_{16A} remain in the lipid phase. A schematic model of the A_1 basic protein–lipid interaction is represented in Fig. 11. When ^{131}I-labelled A_1 basic protein was used, the tyrosine at position 68 and position 134 was labelled. The measurements of the surface radioactivity showed a reduction of about 50% after the action of trypsin. The peptide mapping showed that the tyrosine at position 134 is released into the interface and that the tyrosine at position 68 remains in the lipid layer. It was also found that the amount of Arg–(dimethyl) (position 107) collected with the cerebroside sulphate monolayer was enriched by 25 to 35% when compared with the amount of this amino acid in the lipid-free protein. Chymotrypsin which hydrolyses peptides, amides and esters especially at bonds involving the carboxyl groups of aromatic L-amino acids, tyrosine, phenylalanine, tryptophan and at a lower rate leucyl, methionyl, glutaminyl, asparginyl and histidinyl bonds, showed a less effective action on the A_1 basic protein–lipid complex at the air–water interface. The enzyme will cleave the A_1 molecule in the absence of lipids at bonds which were shown to be in protected regions after the interaction with lipids, *e.g.* Met^{20}–Asp^{21}, Phe^{43}–Phe^{44}, Tyr^{68}–Gly^{69}, Phe^{89}–Phe^{90} and therefore the enzyme will be less effective than trypsin, which acts on the more polar parts of the protein molecule. Subtilopeptidase A shows a very wide specificity but attacks the amide bonds at a lower rate. From the sequence (Fig. 10) we can see that glutamine and asparagine are concentrated mainly in the N-terminal part of the molecule, the hydrophobic binding site. Pronase cleaves 87% in bulk of all

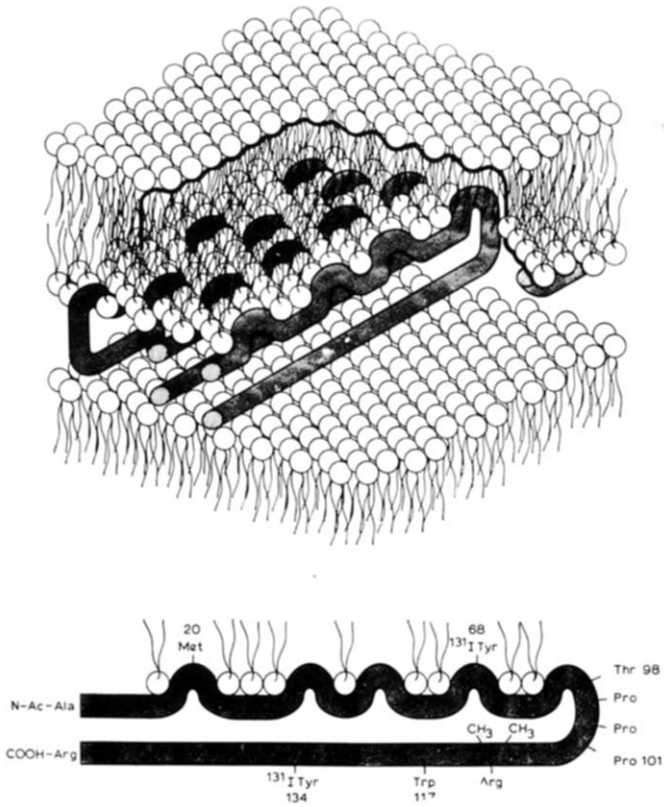

Fig. 11. Schematic representation of the specific binding sites and conformation of the A_1 basic protein after complex formation with lipids.

peptide bonds but like subtilopeptidase A it has a bigger molecular size compared to trypsin and chymotrypsin and therefore probably also suffers from steric hindrance because of the penetration of part of the protein into the lipid core. Past studies on the myelin membrane using low angle X-ray diffraction[10,11], neutron diffraction[12], swelling experiments[10,13,14] and studies on myelogenesis[14,16] indicate that the myelin membrane is asymmetric. The asymmetry was assumed to be protein symmetry[11] between the cytoplasmic or major dense line and the extracellular side, or intraperiod line, of the membrane. Another assumption was protein and cholesterol asymmetry[12]. Our past[4,6,8,18] and present studies on the interaction of myelin basic protein[7,19], mainly the A_1 basic protein of the central nervous system[19], with lipid, show rather conclusively that the basic protein interacts specifically, electrostatically and hydrophobically with negatively charged lipids. From these lipids, the interaction is most specific with cerebroside sulphate. The Folch–Lees apoprotein[20], which accounts for 55% of the total protein of myelin[21] shows a relatively different specificity for lipid when studied at the air–water interface[22]. This protein shows a higher affinity for cholesterol[22]. Dickinson *et al.*[23] suggest that the only protein present in the intraperiod line of the lamellar myelin is the basic protein. Swelling experiments[10,13,14] showed

that the intraperiod line swells to a much greater extent as compared with the major dense line. The formation of the characteristic structure of the multilamellar compact myelin appears to occur only when the basic protein and Folch–Lees protein are detectable[24,25] and the appearance of these proteins correlates well with the synthesis of cholesterol, cerebroside and cerebroside sulphate[24,26,27]. The basic proteins not detectable in the "myelin-like" fraction[28,29] or in the oligodendroglial cell membranes. We have showed in the present study, and in the study on the interaction of Folch–Lees protein with lipids, differences in lipid specificities of the two proteins which determine 85% of the total protein of myelin. These results support the thesis of lipid and protein asymmetry in the myelin membrane. We would like to propose as a working hypothesis that the basic protein is the organizer molecule and the initiator of the spiral winding of the myelin around this axon. The basic protein extruded on the myelin membrane will cause asymmetric charge distribution to give a more polar side of the membrane which will tend to swell. The Folch–Lees apoprotein by its different lipid specificity will cause asymmetric distribution of cholesterol and the more amphoteric lipids, phosphatidylethanolamine and sphingomyelin[22] and as a result of which a more hydrophobic compact cytoplasmic side of the myelin membrane. This asymmetry will cause the myelin mechanically to wrap around its axon. At the end of myelogenesis the basic protein probably acts mainly as a structural stabilizer of the membrane. An attack on this protein as occurs in multiple sclerosis[30] will cause demyelinization.

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