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LOCALIZATION OF THE BASIC PROTEIN AND LIPOPHILIN IN THE MYELIN MEMBRANE WITH A NON-PENETRATING REAGENT

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Summary

The localization of proteins in myelin was studied by the use of a non-penetrating reagent. Tritiated 4,4′-diisothiocyano-2,2′-ditritiostilbene disulfonic acid was used to label the isolated myelin membrane. The membrane was labelled, the basic protein and the hydrophobic protein, lipophilin, were isolated. After 10 min of exposure to the reagent, the specific activity of lipophilin was found to be 10 times greater than that of the basic protein. Water shock did not alter the specific activities. However, sonication increased the specific activity of lipophilin but not that of basic protein. When the isolated proteins were labelled with ^{3}H-labelled 4,4′-diisothiocyano-2,2′-ditritiostilbene disulfonic acid, the specific activity of the basic protein was 10 times that of lipophilin. We concluded that the low specific activity of basic protein isolated from the labelled membrane was due to the inaccessible position of this protein in the membrane bilayer.

Introduction

The lipid moiety of the myelin membrane is considered to be in the form of a bimolecular leaflet. Interpretation of earlier studies using physical techniques [1,2,3], indicated that the protein moiety formed a monolayer across the polar heads of the lipid molecules at each surface of the bilayer. However, evidence is accumulating that myelin may indeed be organized as either a fluid-mosaic structure [4] or as a protein crystal structure [5] similar to that proposed for plasma membranes.

There are two major myelin protein fractions, the basic and the Folch-Lees proteolipid, together accounting for approximately 70% of the membrane pro-

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-ditritiostilbene disulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid; ANS, 8-anilinonapthalene 1-sulfonate; TNS, 2-*p*-toluidinylnaphthalene 6-sulfonate.

tein. The arrangement of these proteins in the membrane has not been determined. A number of techniques have been employed in order to elucidate the position of the basic protein in the membrane. Wood et al. [6] using trypsin as a metabolic probe, concluded that the basic protein was located in the outside layers of the myelin sheath. As well, the results of Dickinson et al. [7], indicated that basic protein was present at the outer surface (intraperiod line) of myelin. However, the results of Bubis and Wolman [8] based on extraction procedures; Adams et al. [9], using a histochemical technique; and Herndon et al. [10], using an immuno-electron microscopic technique, suggested that the basic protein was located at the cytoplasmic surface (interperiod line) of myelin. These results were in agreement with Lennon et al. [11] who reported that the antigenic determinants of the basic protein were unavailable to circulating antibody in the intact myelin sheath.

More recent studies by Poduslo and Braun [12] and Golds and Braun [13] using a lactoperoxidase-catalysed iodination technique and protein labelling probes (salicylaldehyde and pyridoxal phosphate) respectively showed that the basic protein was exposed only on the cytoplasmic side of myelin from cat spinal cord.

Fewer studies to elucidate the position of the proteolipid protein fraction within the membrane have been undertaken. The results of chemical protein-labelling probes used by Braun and colleagues [12,13] suggested that the proteolipid protein fraction was partially exposed at the extracellular surface of the intact myelin sheath. Feinstein and Felsenfeld [14,15] using fluorescent probes to label both isolated myelin and the two major proteins presented evidence to suggest that the Folch-Lees proteolipid apoprotein was embedded in the lipid bilayer but probably did not span the thickness of a single myelin bilayer.

In this report, the localization of two protein constituents in isolated myelin was studied by the use of a non-penetrating radioactive label 4,4′-diisothiocyano-2,2′-ditritiostilbene disulfonic acid ([^{3}H]DIDS). Evidence is presented to suggest that a homogeneous hydrophobic protein [16] (purified from the proteolipid fraction and termed lipophilin) is at least partially exposed at the surface of the myelin bilayer and partially embedded in the lipid matrix. A portion of the basic protein may be exposed at the surface but is probably less exposed than lipophilin.

Materials and Methods

[^{3}H]DIDS was kindly supplied by Doctors Cabantchik and Rothstein of this Institution. The reagent was described in a recent publication [17]. The specific activity of the material as supplied was 1.8 Ci/mmol and was contained in 1.0 ml of 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.6.

Poly-L-arginine, purchased from Sigma Chemical Co., was used as supplied. Nitromalonyldialdehyde was prepared from mucobromic acid and sodium nitrite [27].

Preparation of myelin

Myelin was prepared from normal human white matter by the method of

Lowden et al. [18]. Lyophilized myelin, stored under vacuum at −20°C, will be referred to as isolated myelin.

Isolation of myelin proteins

The basic protein was isolated from lyophilized myelin by the method of Lowden et al. [18].

The hydrophobic protein lipophilin, used in these studies, was isolated and purified from the chloroform/methanol soluble proteolipid protein fraction by the method of Gagnon et al. [16].

The conditions of isolation for these two proteins from [^{3}H]DIDS labelled myelin were not altered.

Labelling of myelin

Three 300 mg aliquots of isolated myelin were suspended in 2.0 ml of cold HEPES buffer (5 mM, pH 7.6) and 100 μl of ice-cold [^{3}H]DIDS was added to each vial. The reactions were carried out at 4°C in semidarkness for 2, 10 and 15 min respectively. The labelled samples were then set up to dialyze against cold HEPES buffer containing 10^{-2} M lysine overnight, to remove non-reacted [^{3}H]DIDS. Subsequent dialyses were against distilled water. The retentates were then lyophilized.

Two other myelin samples were disrupted prior to labelling. One 300 mg aliquot was suspended in 1.0 ml of cold HEPES buffer and sonicated, with a Branson Sonifier, for 90 s at 4°C. A second 300 mg aliquot was suspended in 1.0 ml of cold distilled water, the pH of which was adjusted to 7.5 immediately before adding the myelin, for 30 mins at 4°C. The two samples were reacted with [^{3}H]DIDS for 10 min and the labelling procedure continued as above.

Labelling of basic protein and lipophilin

10 mg of each protein purified from unlabelled myelin were labelled for 10 min with [^{3}H]DIDS.

Reaction of basic protein and poly-L-arginine with nitromalonyl dialdehyde

The basic protein and poly-L-arginine were modified with nitromalonyl dialdehyde, a compound which reacts specifically with arginyl residues of proteins [19]. The basic protein was reacted with nitromalonyl dialdehyde (20-fold molar excess with respect to the arginine content of the protein) in 0.25 M NaOH at room temperature. After 45 min, the product was precipitated by the addition of 3 volumes of 0.2 M acetic acid, washed with this solvent and dried in vacuo over sodium hydroxide pellets. Poly-L-arginine was reacted with nitromalonyl dialdehyde (12-fold molar excess with respect to arginine content) in 1 N NaOH for 1 h. The resulting suspension was centrifuged and the precipitate washed with water and dried in vacuo over sodium hydroxide. Both products of reaction with nitromalonyl dialdehyde when dissolved at alkaline pH gave absorption bands at 350 nm indicative of the presence of δ-(5-nitro-2-pyrimidyl)ornithine.

10 mg of each of the modified compounds were labelled with [^{3}H]DIDS for 10 min as described above.

Amino acid analyses

The labelled protein samples were hydrolysed with constant boiling HCl (5.7 N) at 110°C for 20 h under nitrogen in a sealed vial. The HCl was removed by evaporation on a Buchler rotary evaporator. The hydrolysates were taken up in a known volume of distilled water and passed through a 1 × 10 cm Dowex 50W-X8 column (H^+ form). The column was washed with distilled water to remove non-absorbed material. The amino acids which were bound to the column were eluted with 4 M NH_4OH. After removal of the NH_4OH, an aliquot of the amino acid fraction was applied to a Technicon TSM amino acid analyser.

Protein determination

The concentration of protein was measured either by the method of Lowry et al. [20] or by that of Moore and Stein [21].

Results

In the first experiments, the recoveries of the two myelin proteins after [^{3}H]DIDS labelling of myelin were determined and compared with those reported earlier [16]. These are shown in Table I. The weight of the lipophilin and basic protein was calculated as a percentage of the weight of lyophilized myelin. The recoveries of the lipophilin and basic protein were 8.6 and 1.8% respectively. This compares favourably with 6.7 and 2.5% reported originally [16].

The results obtained when the isolated normal human myelin was labelled with [^{3}H]DIDS for various times and when myelin was either sonicated or water shocked prior to labelling are shown in Table II. It can be seen that the specific activities obtained were similar for all five samples. The total dpm incorporated varied between a narrow range of 7.53—9.67 · 10^7 for the different samples. It is noteworthy that sonication did not affect the amount of [^{3}H]DIDS labelling significantly.

In order to determine the extent of labelling of the two proteins, lipophilin and the basic protein were isolated from the membrane [16,18]. The results are shown in Table III.

TABLE I

RECOVERIES OF PROTEINS AFTER LABELLING NORMAL HUMAN MYELIN WITH [^{3}H]DIDS

Freshly prepared myelin was suspended in 5 mM cold HEPES buffer, pH 7.6, and reacted with [^{3}H]DIDS at 4°C in semi-darkness. The proteins were isolated, dried and weighed.

Material	[^{3}H]DIDS-labelled myelin		Non-labelled myelin *	
	Dry weight (mg)	% of myelin	Dry weight (mg)	% of myelin
Myelin	1500	—	5850	—
Lipophilin	130	8.6	381	6.7
Basic protein	27	1.8	152	2.5

* Obtained from Gagnon, et al. [16].

TABLE II

[^{3}H]DIDS LABELLING OF MYELIN

300-mg aliquots of freshly prepared myelin were suspended in 2.0 ml cold HEPES (5 mM pH 7.6) and 100 μl unlabelled [^{3}H]DIDS (specific activity 1.8 Ci/mmol) was added to each. After various times, the reaction was stopped and the radioactivity determined.

Labelled preparation	Time (min) of reaction with [^{3}H] DIDS	Specific activity (dpm/mg myelin) $\times 10^{-5}$
Myelin	2	3.15
	10	3.22
	15	2.86
Sonicated myelin	10	2.51
Water-shocked myelin	10	2.85

When normal human myelin was labelled for 10 min with [^{3}H]DIDS, the specific activities were $5.7 \cdot 10^5$ dpm/mg protein and $5.1 \cdot 10^6$ dpm/mg protein for the basic protein and lipophilin respectively. The specific activities of the basic proteins were the same, $5.7 \cdot 10^5$, $5.9 \cdot 10^5$ and $6.0 \cdot 10^5$ dpm/mg protein for the three myelin samples. Neither sonication nor water shock affected the exposure of basic protein to the reagent, however, sonication had a marked effect on the specific activity of lipophilin. The specific activity increased from $5.1 \cdot 10^6$ dpm/mg protein for the control to $14.0 \cdot 10^6$ dpm/mg protein for the sonicated sample.

In the next experiments, basic protein and lipophilin were isolated from normal human myelin and then were labelled with [^{3}H]DIDS as described in Methods. These results are also shown in Table III. The specific activity obtained for the basic protein was $2.4 \cdot 10^7$ dpm/mg protein. When compared to the specific activities of $5.7–6.0 \cdot 10^5$ dpm/mg protein obtained for the basic protein isolated from labelled myelin, this represents a 41-fold difference in specific activity. Therefore, the isolated basic protein was readily labelled by the [^{3}H]DIDS, whereas in the membrane, it was labelled with a much lower

TABLE III

[^{3}H]DIDS-LABELLED MYELIN PROTEINS

300-mg aliquots of myelin were suspended in 2.0 ml ice cold HEPES, 5 mM, pH 7.6 and 100 μl of [^{3}H]-DIDS (specific activity 1.8 Ci/mmol) was added to each. After 10 min of labelling, the proteins were isolated and the specific activities (dpm/mg protein) determined. In the case of the isolated basic protein and lipophilin, 10 mg of each were labelled with [^{3}H]DIDS as described in methods.

Labelled preparation	Specific activity	
	Basic protein	Lipophilin
Normal myelin	$5.7 \cdot 10^5$	$5.1 \cdot 10^6$
Sonicated myelin	$5.9 \cdot 10^5$	$14.0 \cdot 10^6$
Water-shocked myelin	$6.0 \cdot 10^5$	$3.6 \cdot 10^6$
Protein extracted from myelin and then labelled with [^{3}H] DIDS	$2.4 \cdot 10^7$	$2.1 \cdot 10^6$

specific activity. On the other hand, when lipophilin from unlabelled myelin was treated with [^{3}H]DIDS and compared with that from labelled myelin, a two-fold difference in labelling occurred. The specific activity was $2.1 \cdot 10^6$ dpm/mg when the isolated protein was labelled as compared to $5.1 \cdot 10^6$ dpm/mg protein when lipophilin was isolated from labelled myelin. It appears that lipophilin is readily accessible to the reagent when it is present in the membrane and therefore must be considered to be exposed.

This difference in specific activity when isolated lipophilin was labelled may be due to conformational factors. A comparison of the labelling of the α-helical form of the hydrophobic protein with the β-conformation (prepared by the method of Moscarello et al. [22]) showed that the α-helical form was more highly labelled. The specific activities were $3.42 \cdot 10^5$ and $5.85 \cdot 10^5$ dpm/μmol amino acid for the α-helical and β-forms respectively. The water soluble preparation of lipophilin used in the above experiments was 70% β-form as determined by circular dichroism. Lipophilin in the membrane would be expected to be highly α-helical.

DIDS contains two isothocyanate groups which were presumed to react preferentially with the Σ-amino group of lysyl residues to form a covalent bond. Thus the extent of labelling would depend on the number of lysine residues exposed at the surface of the membrane. It was of interest to determine whether other amino acid residues would react with this compound.

The two myelin proteins, which had been labelled before and after isolation with [^{3}H]DIDS, were subjected to acid hydrolysis and then passed through a Dowex 50W-X8 column as described in Methods. The results of the amino acid analyses of the NH_4OH eluates are shown in Table IV.

It can be seen that 7—9 arginyl residues were lost when the basic protein was labelled in situ in the membrane whereas 13—17 arginyl residues were lost when the isolated basic protein was labelled. Ornithine represented 20—30% of the arginine content found on the chromatograms indicating that the guanidyl group was cleaved during hydrolysis. The remainder of the arginine was not accounted for. Although there was no decrease in the number of lysyl residues of basic protein, studies with poly-L-lysine indicated that the reaction had occurred with the Σ-amino group. Acid hydrolysis of the DIDS-lysine adduct would be expected to regenerate the original lysine. Other residues lost as a result of acid hydrolysis of the [^{3}H]DIDS labelled basic protein adduct included variable amounts of tyrosyl and methionyl residues.

On the other hand, there was no loss of arginyl residues or other basic residues from lipophilin. However, there was always a loss of methionyl residues.

To substantiate our findings that the arginyl residues of basic protein bind [^{3}H]DIDS, the basic protein and poly-L-arginine were reacted with nitromalonyl dialdehyde as described in Methods. After acid hydrolysis and column fractionation, it was found that 17 out of 19 arginyl residues were lost from the basic protein molecule and nitromalonyl dialdehyde blocked 90% of the arginyl residues in poly-L-arginine.

Reaction of the modified basic protein with [^{3}H]DIDS resulted in a specific activity of $0.96 \cdot 10^6$ dpm/μmol amino acid. This is compared to a specific activity of $1.88 \cdot 10^6$ dpm/μmol amino acid for the unmodified protein. Basic

TABLE IV

AMINO ACID ANALYSES OF BASIC PROTEIN AND LIPOPHILIN BEFORE AND AFTER REACTION WITH [^{3}H]DIDS

The data are expressed as residues per total residues per mol. Basic protein contains 170 residues (Eylar [26]) and lipophilin 223 (Gagnon et al. [16]). The results are the means of at least 8 independent amino acid analyses in each case.

	Basic protein			Lipophilin	
	Unlabelled	[^{3}H]DIDS-myelin	Labelled with [^{3}H]DIDS	[^{3}H]DIDS-myelin	Unlabelled
Asp	11	11	11	10	10
Thr	8	8	8	19	18
Ser	19	19	19	14	14
Glu	9	9	9	16	16
Pro	12	12	12	6	6
Gly	26	24	24	24	24
Ala	12	12	12	26	26
Val	4	4	4	15	14
1/2 Cys	—	—	—	9	9
Met	2	0—2	0—2	0—1	3
Ileu	4	4	4	10	9
Leu	8	9	9	25	25
Tyr	4	0—4	0—4	11	11
Phe	9	9	9	17	17
Lys	12	12	12	9	9
His	10	10	10	6	5
Arg	19	10—13	2—6	4	4
Trp	1	ND	ND	ND	3

protein contains 19 arginyl, 12 lysyl, 4 tyrosyl and 2 methionyl residues for a total of 37 residues. Blocking 17 arginyl residues with nitromalonyl dialdehyde should reduce the specific activity by about 50%, which was observed.

Discussion

Tritiated DIDS is a non-penetrating reagent which had been successfully used to label erythrocyte membrane proteins [17]. This reagent was assumed to form a covalent bond with the exposed Σ-amino group of the lysyl residues of protein molecules. We have shown that it labels the isolated myelin membrane and that the degree of labelling did not appear to be affected by subjecting the membrane to either sonication or water shock.

After isolation of the two major proteins from the labelled myelin, it was found that the specific activity of lipophilin was ten times greater than that of the basic protein for each of the three reaction times (2, 10 and 15 min). The results would suggest that this protein is more accessible to the label than is the basic protein.

The specific activity obtained for lipophilin from labelled myelin was $5.1 \cdot 10^6$ dpm/mg protein. In contrast, a specific activity of $2.1 \cdot 10^6$ dpm/mg protein was obtained when the isolated protein was labelled. The extent of labelling did not differ by more than 2-fold, suggesting that the DIDS reactive groups of lipophilin must be accessible at the surface of the bilayer. It was also

shown that a 2-fold increase in labelling was obtained for the α-helical form of this protein when compared with the β-structure. Although the conformation of lipophilin in myelin is not known, the conformation in phosphatidylcholine vesicles is highly α-helical (unpublished data). Thus it is likely that the conformation of lipophilin in myelin is α-helical. In comparison, the conformation of the isolated protein in HEPES buffer was 70% β-structure.

Earlier studies using freeze fracture [23] and differential scanning calorimetry [24] of lipid vesicles in which lipophilin had been incorporated showed that it was embedded in the lipid matrix. These conclusions are in agreement with those of Feinstein and Felsenfeld [14,15]. The results of their studies on isolated myelin and the isolated protein constituents using the fluorescent probes ANS and TNS suggested that the hydrophobic protein was partially embedded in the lipid matrix and probably did not span the thickness of a single bilayer. Braun and his colleagues [12,13] employed chemical probes to label the proteins of an intact and disrupted myelin nerve bundle, as well as isolated myelin. Their results also suggested that the hydrophobic protein was partially embedded in the lipid matrix and probably did not span the thickness of a single bilayer. Braun and his colleagues [12,13] employed chemical probes to label the proteins of an intact and disrupted myelin nerve bundle, as well as isolated myelin. Their results also suggested that the hydrophobic protein was partially exposed at the membrane surface. The results of our labelling studies presented here show that although lipophilin is embedded in the lipid matrix, it is nevertheless exposed to the hydrophilic environment.

The position of the basic protein within the myelin membrane has attracted a great deal of interest because of its implication in demyelinating disorders such as experimental allergic encephalomyelitis and multiple sclerosis. It was thought that breakdown of the basic protein may be involved early in the pathogenesis of these demyelinating diseases [25].

Histochemical [9], immunochemical [11], and immuno-electron microscopic [10] studies suggested that the basic protein was located at the cytoplasmic surface of the membrane. It was not established whether the protein lies "external" to the lipid bilayer at this surface.

The studies in our laboratory have shown that the basic protein was not readily accessible to the non-penetrating reagent DIDS. Since [^{3}H]DIDS binds to the Σ-amino group of lysyl residues, failure to react must mean that these groups were interacting with other constituents of the membrane. In support of this view were the results obtained when isolated basic protein was labelled with [^{3}H]DIDS. In this case, the specific activity obtained was forty-one times greater than that for the basic protein isolated from labelled myelin. Thus the majority of the reactive groups of the basic protein in myelin were not available for reaction with DIDS. On the other hand, a small segment of the molecule may be exposed to the hydrophilic environment since some labelling occurred.

Poduslo and Braun [12] and Golds and Braun [13] employed several chemical probes to label intact cat spinal cord. They found no labelling of the basic protein. However, the basic protein was labelled when both isolated myelin and delipidated myelin was used. Thus, they concluded that basic protein must be located at the cytoplasmic surface of cat spinal cord myelin.

It is difficult for us to compare our results with theirs [12,13] since we used

isolated myelin from human brain, as well as a different labelling reagent. In addition, both basic protein and lipophilin were isolated and characterized. Our conclusions regarding the relatively non-exposed location of basic protein in the membrane pertains only to our system. As a result of our isolation procedure, the myelin was disrupted. However, the lipid bilayer with its protein components was intact. Our labelling studies therefore have meaning, only if the relative positions of the two proteins in the lipid bilayer are considered. The relative ease with which the isolated basic protein was labelled compared to the difficulty of labelling it as a component of the membrane, lead us to conclude that it is not completely exposed to the hydrophilic environment.

The reagent [^{3}H]DIDS was shown to react avidly with lysyl and arginyl residues of proteins and polypeptides. Since both these residues represent a larger percentage of the total residues in basic protein than in lipophilin, the fact that lipophilin is more readily labelled is interpreted to mean that it is more exposed to the hydrophilic environment than basic protein.

In summary, the two conclusions from our labelling experiments are (i) human brain myelin basic protein is more difficult to label with a non-penetrating reagent [^{3}H]DIDS in situ than the isolated protein, (ii) although lipophilin has fewer [^{3}H]DIDS reactive group than basic protein, it is labelled with a higher specific activity than basic protein, implying that it is more exposed than basic protein.

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