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Interactions of dicyclohexylcarbodiimide with myelin proteolipid

(proton transport/liposomes/central nervous system)

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ABSTRACT **Dicyclohexylcarbodiimide (DCCD) is known to bind preferentially to a proteolipid subunit of proton-translocating systems and thereby to inhibit proton transport. In the present study we show that, in an aqueous medium, DCCD binds to the bovine white matter proteolipid apoprotein, the major protein of central nervous system myelin. The binding is dependent on time, temperature, and concentration and is not inhibited by the hydrophilic carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. By contrast, when the incubation is carried out in chloroform/methanol no labeling by DCCD is demonstrable. In isolated rat myelin, DCCD binds specifically to the proteolipid and not to the myelin basic proteins. Liposomes reconstituted with the myelin proteolipid apoprotein transport protons, as assayed by quenching of the fluorescence of 9-aminoacridine. Preincubation of proteolipid-containing liposomes with DCCD results in an inhibition of transport. These studies have important implications for a possible ionophoric function of the myelin proteolipid and for the occurrence of transport processes within myelin.**

Proteolipids are a class of intrinsic membrane proteins that are characterized by their solubility in chloroform/methanol (1, 2). In prokaryotic cells and in the mitochondria of eukaryotic cells, proteolipids associated with the ATPase complex have been shown to be involved in the mechanism of proton translocation (see ref. 3 for review). This proton translocation can be inhibited by dicyclohexylcarbodiimide (DCCD), a lipid-soluble probe that binds to a specific site on proteolipids (4). In the mammalian central nervous system half of the protein of the myelin sheath is a proteolipid protein that is chemically distinct from the DCCD-binding proteolipids identified thus far. Although myelin was for many years considered a metabolically inert membrane that acts only as an insulator around the nerve axon, a reassessment of the evidence suggests an involvement of myelin in active processes. Several enzymes known to participate in ion transport have been identified in myelin (5–7). In addition, voltage-dependent channels are formed upon incorporation of the myelin proteolipid apoprotein into planar lipid bilayers (8), suggesting participation of this protein in ion transport. The amphipathic properties, conformational flexibility (2), and subunit structure (9) of the myelin proteolipid protein are consistent with this concept. In the present communication the specific binding of DCCD to the proteolipid protein has been demonstrated in isolated myelin. Furthermore, we have shown the occurrence of proton transport in reconstituted vesicles containing the apoprotein and the inhibition of this transport by DCCD. These data provide additional support for a functional role for the myelin proteolipid.

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MATERIALS AND METHODS

DCCD was obtained from Aldrich and was added to the incubation medium (see below) in 0.05 vol of absolute ethanol. ^{14}C-Labeled DCCD (50 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from Research Products International, Elk Grove Village, IL. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was obtained from Sigma. Sephadex LH-20 and LH-60 were purchased from Pharmacia. Bovine white matter proteolipid apoprotein was prepared by dialysis of a washed chloroform/methanol tissue extract against neutral and acidified chloroform/methanol (10). The apoprotein contained <0.05% phosphorus and was stored at 4°C in neutral chloroform/methanol at a concentration of 3–5 mg of protein per ml. Lipid-containing proteolipid preparations (approximately 60% lipid) were prepared by emulsification and centrifugation of the solutes from a bovine total lipid extract (11). Sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis of either the apoprotein or lipid-containing preparations showed bands of approximately 25,000 and 20,000 daltons as well as a low molecular mass subunit of 12,000 daltons (11, 12). The chemical similarity of the proteins in the different bands has been documented (12). Rat central nervous system myelin was prepared by the method of Norton and Poduslo (13) and was used immediately after the last osmotic shock step. Protein was measured by the Lowry procedure (14) as modified by Lees and Paxman (15).

Labeling of Proteolipid Apoprotein. The bovine white matter apoprotein was converted to the water-soluble form by the rapid method of Sherman and Folch-Pi (10). [^{14}C]DCCD (20 nmol/mg of protein) was added to the apoprotein (1.25 mg/ml) in 1 mM Tris acetate (pH 7.3). After incubation at 4°C for various periods of time, $NaDodSO_4$ (10%) was added to a final concentration of 0.1%. An aliquot of the solution was immediately applied to a Sephadex LH-60 column (1 × 20 cm) equilibrated with 1 mM Tris acetate buffer (pH 7.3), containing 0.1% $NaDodSO_4$ and eluted at a flow rate of approximately 0.3 ml/min. One-milliliter fractions were collected and monitored for absorbance at 280 nm, and radioactivities were determined in 100-μl aliquots of each fraction. The apoprotein eluted in the void volume, while free DCCD was retained until approximately two bed volumes of solvent had been passed through the column.

Labeling of Myelin. To a freshly prepared myelin suspension (3–4 mg of protein per ml) in 10 mM Tris acetate (pH 7.3) was added between 30 and 60 nmol of [^{14}C]DCCD per mg of protein. After incubation for 21 hr at 4°C, the mixture was diluted

Abbreviations: DCCD, dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

1:5 with cold distilled water and centrifuged as above for 1.5 min. The myelin was washed four times by resuspension and homogenization in water, followed by centrifugation. In experiments in which the myelin was partially delipidated prior to analyses, the washed pellet was resuspended in water and lyophilized, and lipid was extracted five times with cold ethyl ether/ethanol (3:2, vol/vol). The final pellet was suspended in 0.075 M Tris acetate buffer (pH 7.8) at a concentration of 0.5–1 mg of protein per ml.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. The myelin or proteolipid suspensions were dissolved by addition of 0.1 vol of a 10% solution of NaDodSO$_4$ and the mixture was incubated at 37°C for 1 hr. Aliquots were taken for determination of protein and radioactivity. NaDodSO$_4$ disc gel electrophoresis was carried out on 12% gels in 75 mM Tris acetate buffer (pH 7.9). Gels were stained for 30 min with 0.25% Coomassie blue in methanol/water/acetic acid (50:42:8, vol/vol) and destained in water/acetic acid/methanol (88:7:5, vol/vol). Gels were scanned at 540 nm in a Gilford spectrophotometer equipped with a linear scanner. Bovine serum albumin (68,000), chymotrypsinogen (25,000), cytochrome *c* (12,000), and insulin (6000) were used as molecular weight markers.

To determine the distribution of radioactivity, the destained gels were cut into 1-mm slices and digested at 50–55°C overnight with 0.5 ml of 30% (wt/wt) H_2O_2 in loosely capped counting vials containing two slices per vial. Five milliliters of ScintiVerse (Fisher) was added and radioactivity was measured in a Packard Tri-Carb model 2660 scintillation counter.

Preparation of Liposomes. A soybean phospholipid mixture (phosphatidylcholine type IV-S from Sigma) was partially purified by the procedure of Kagawa and Racker (16). Forty milligrams of an ether solution of the phospholipid was evaporated to dryness under N_2. To the dried lipid was added 1 ml of the water-soluble form of the apoprotein (80–400 μg) and an equal volume of 20 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine)/NaOH (pH 8.0) buffer containing 0.5 M sucrose, 0.8 M KCl, and 1.3 mM dithiothreitol. The mixture was sonicated for 15 min in an ice/water bath with a Sonifer Cell Disruptor (Heat Systems-Ultra Sonics, Inc.) at an output of 50 W. The liposomes were dialyzed at 4°C overnight against 200 vol of a 1:1 dilution of the buffer (KCl omitted) with two changes of the dialysis buffer. Control liposomes were prepared similarly but with no added proteolipid.

Assay of Proton Translocation. A 200-μl sample of dialyzed liposomes was diluted with 2.3 ml of 10 mM Tricine/NaOH (pH 8.0) containing 0.3 M sucrose and 6 μM 9-aminoacridine (Aldrich). After addition of 2.5 μl of valinomycin at 10 μg/ml in dimethylformamide, fluorescence was measured at room temperature on an Aminco Bowman spectrophotofluorimeter with excitation and emission wavelengths of 365 nm and 451 nm, respectively.

RESULTS

Binding of DCCD to Apoprotein. Incubation of the proteolipid apoprotein with radioactive DCCD resulted in a binding of the DCCD to the protein that was dependent on time, temperature, and concentration. After a rapid initial labeling, binding of DCCD continued to increase. Between 15 min and 1 hr at room temperature, the amount of labeling doubled, with a continuing slow increase for many hours. At 1 hr, binding was twice as great at room temperature as at 4°C. At a fixed time and temperature, the extent of labeling increased with DCCD concentration over a wide concentration range. At the highest reagent concentration tested (20 mM), approximately one mole of DCCD was bound per mole of apoprotein (25,000 daltons). Electrophoretic analysis showed that the ^{14}C label coincided with the stained protein bands (Fig. 1*A*). The densitometric trace of the stained gel showed the typical pattern for the apoprotein with the major band of 25,000 daltons, a shoulder of 20,000 daltons, and low and high molecular weight bands representing subunits and aggregates, respectively. Thus, interaction with DCCD did not alter the mobility of the protein. The additional radioactive peak, which moved close to the dye front, corresponded to free DCCD, as seen in the control gel, Fig. 1*B*. Competition experiments showed that the amount of radioactivity incorporated was reduced by the presence of unlabeled DCCD in a concentration-dependent manner (Fig. 2). By contrast, the water-soluble carbodiimide EDAC did not block DCCD binding even at a concentration 80 times that of the [^{14}C]DCCD.

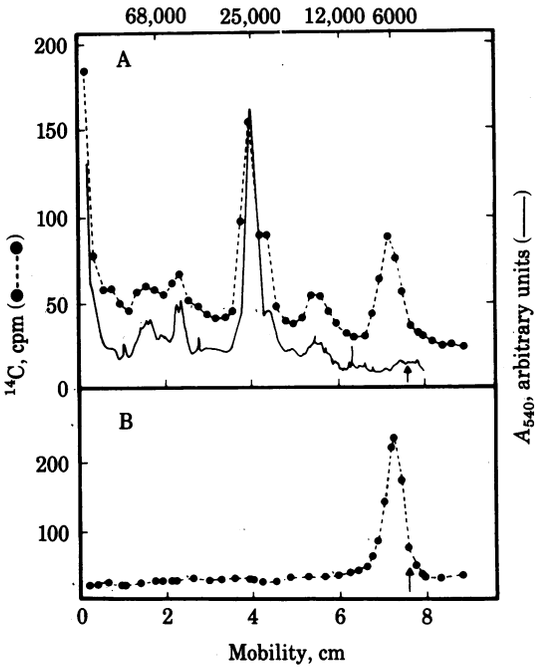

FIG. 1. Incorporation of [^{14}C]DCCD into proteolipid. (*A*) A proteolipid preparation containing lipid was incubated with 10 nmol of [^{14}C]DCCD per mg of protein for 21 hr at 4°C in 10 mM Tris/acetate buffer (pH 7.3). After incubation of the suspension, the protein was pelleted by centrifugation for 5 min in a bench-top Eppendorf centrifuge. The pellet was washed four times and resuspended in the same buffer at a concentration of 1 mg/ml. (*B*) Control, in the absence of proteolipid. After NaDodSO$_4$ gel electrophoresis the stained gel was scanned at 540 nm and sliced, and radioactivity was determined. Arrow indicates the position of the tracking dye. Numbers on top correspond to the position of molecular weight markers.

The solubility properties of the apoprotein made it possible to investigate the interaction of the protein with DCCD in an organic solvent as well as in the aqueous medium. The chloroform/methanol-soluble form of the apoprotein was incubated with [^{14}C]DCCD in chloroform/methanol (2:1, vol/vol). Upon chromatographic separation of free DCCD from protein, the protein peak was clearly separable from the radioactive peak (Fig. 3) and no labeling of the protein by DCCD was observed even after 66-hr incubation, the longest time period studied. Thus, binding of DCCD to the apoprotein occurred only in an aqueous medium and not in an organic solvent mixture.

Binding of DCCD to Myelin Proteins. To assess whether these observations have physiological relevance, the binding of DCCD to isolated myelin membranes was studied. After incubation of freshly prepared rat myelin with [^{14}C]DCCD, the protein components were separated by gel electrophoresis and

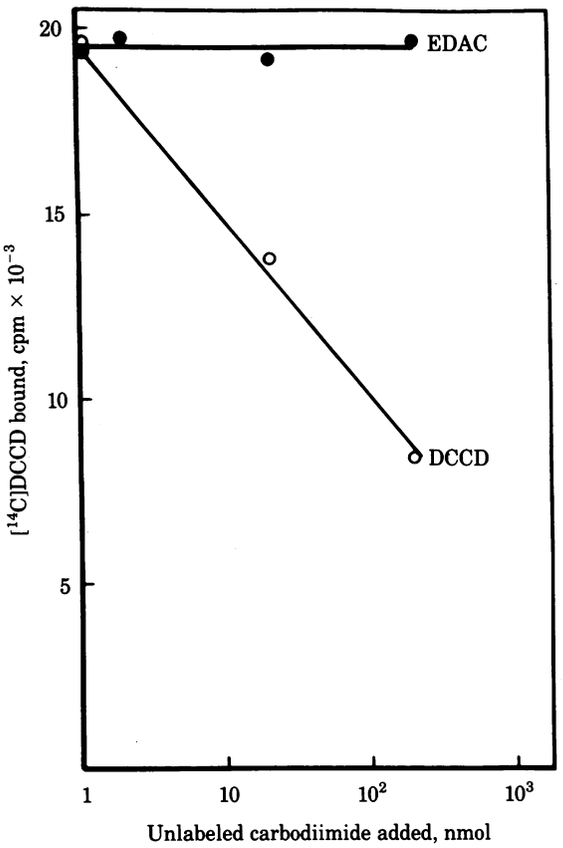

FIG. 2. Effect of unlabeled carbodiimides on [^{14}C]DCCD binding to apoprotein. Proteolipid apoprotein (125 μg in 100 μl of 1 mM Tris/acetate buffer, pH 7.3) was incubated with 2.5 nmol of [^{14}C]DCCD and various amounts of unlabeled carbodiimide. After incubation for 66 hr at 4°C, free DCCD was removed by chromatography and bound radioactivity was determined in the eluate.

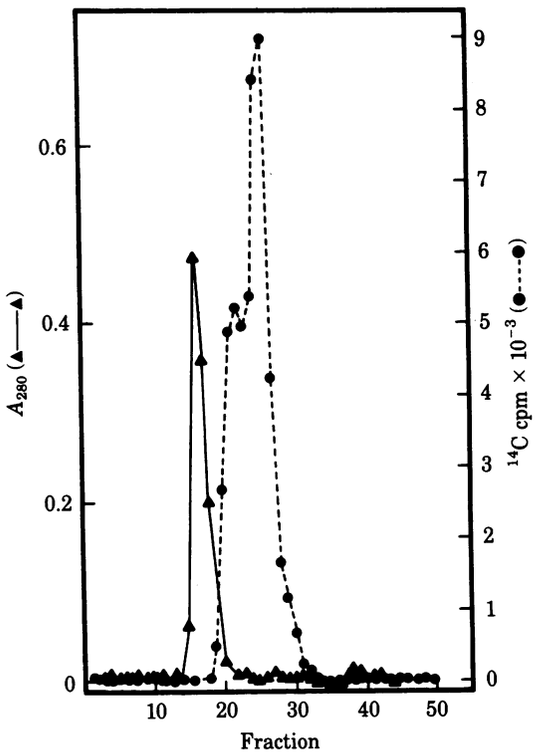

FIG. 3. Chromatography of proteolipid and [^{14}C]DCCD on Sephadex LH-20. Proteolipid apoprotein (500 μg in 500 μl of chloroform/methanol, 2:1 by vol) was incubated with 1 nmol of [^{14}C]DCCD at 4°C for 16 hr. The mixture was chromatographed on a Sephadex LH-20 column (1 × 20 cm) equilibrated and eluted with chloroform/methanol. Fractions (500 μl) were collected and absorbance at 280 nm was determined. Individual fractions were dried under N_2 and their radioactivities were measured after addition of 5 ml of ScintiVerse.

the profile of the stained gels was compared with the distribution of radioactivity (Fig. 4). The bulk of the radioactivity comigrated with the major proteolipid band of 25,000 daltons; no labeling of the two myelin basic proteins (18,000 and 14,000 daltons) was observed. The minor radioactive peaks in the high molecular weight region apparently correspond to oligomers of proteolipid because they were also observed in gels of the isolated proteolipid (Fig. 1*A*). The radioactive peak close to the tracking dye comigrated with free DCCD (Fig. 1*B*). The same radioactivity profile as in Fig. 4 was observed after delipidation of the DCCD-treated myelin, indicating that under these conditions essentially no binding of DCCD to lipids occurred.

To gain insight into the specificity of the carbodiimide binding, myelin was incubated with different concentrations of the radioactive probe. Labeling of the proteolipid protein band continued to increase up to 60 nmol of DCCD per mg of protein, the highest concentration tested. Even at this concentration no detectable radioactivity was associated with the myelin basic proteins. Similarly, when incubation was carried out for longer periods of time, there was increased labeling of the proteolipid but no labeling of myelin basic proteins.

Proton Transport in Proteolipid-Containing Vesicles. The specific binding of DCCD to the myelin proteolipid suggests a possible role for this protein in proton transport. To test this possibility, direct measurement of proton conductance was carried out in phospholipid vesicles preloaded with K^+ and reconstituted with proteolipid. Valinomycin was added to induce K^+ diffusion from the liposomes, and the proton countertransport was measured by using the fluorescent probe 9-aminoacridine as an indicator of ΔpH. In the presence of a pathway for proton movement, protons enter the vesicles to compensate for the

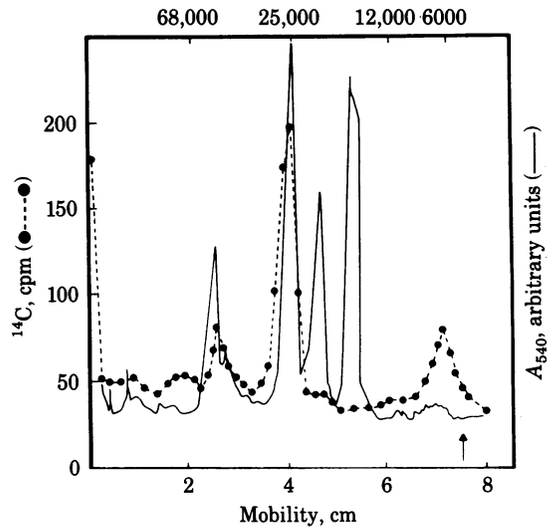

FIG. 4. Comparison of electrophoretic and radioactive patterns of [^{14}C]DCCD-labeled rat myelin. Freshly prepared rat myelin was incubated with 30 nmol of [^{14}C]DCCD per mg of protein for 21 hr at 4°C. An aliquot containing 50 μg of protein was subjected to $NaDodSO_4$ gel electrophoresis. The stained gels were scanned at 540 nm and sliced and radioactivity was determined. Arrow indicates the position of the tracking dye. Numbers on top correspond to positions of molecular weight markers.

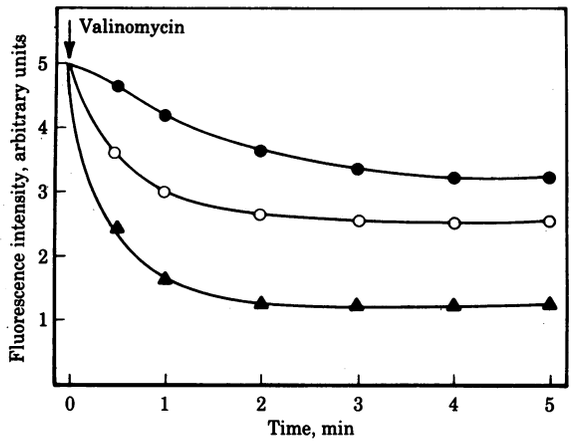

FIG. 5. Effect of proteolipid concentration on liposomes containing a constant amount of phospholipid. Preparation of liposomes and assay for proton translocation were as described in the text. Liposomes were prepared in the absence of proteolipid (●) and with the addition of 80 μg (○) or 400 μg (▲) of proteolipid.

valinomycin-induced release of K^+ with concomitant quenching of the 9-aminoacridine fluorescence. It was observed that, in the absence of proteolipid, only a minimal, slow change was obtained in the fluorescence of the liposomes. However, upon incorporation of proteolipid apoprotein into the liposomes, the rate of fluorescence quenching increased in direct proportion to the amount of proteolipid added during reconstitution (Fig. 5). When liposomes into which proteolipid was incorporated were treated with DCCD, the initial rate of fluorescence quenching was dramatically reduced (Fig. 6). DCCD thus inhibits the proton translocation induced by incorporation of the myelin proteolipid apoprotein into liposomes.

DISCUSSION

Myelin is an elaboration of the plasma membrane of the oligodendroglial cell and, as such, can be expected to share some general features of plasma membranes. Plasma membranes function in part to modulate the transport of metabolites in or out of the cell and can therefore be regarded as permeability barriers. The high lipid content (70%) and multilamellar structure of myelin have led to particular emphasis on its role as an insulator and as a barrier to the passage of ions and molecules. However, these properties do not exclude the possibility that dynamic processes are occurring within parts of the lamellar structure. Evidence has accumulated for myelin-associated enzyme activity and for interactions between myelin and the axon in the course of myelin development and maintenance. For example, movement of specific lipids between the axon and myelin has been demonstrated, and this phenomenon may be important for the turnover of myelin components (17–19). Several enzymes in myelin are concerned with the transport of small molecules (5–7), suggesting that these enzymes may participate in the removal of metabolites from the axon. Specifically, the myelin-associated carbonic anhydrase (5, 6) might be involved in dissipating the CO_2 produced by axonal metabolism. The removal of protons and bicarbonate ions formed in this process would require (*i*) the capability of forming ion channels within the bilayer and (*ii*) the presence of an aqueous compartment into which the ions could be moved. Electron microscopy (20) and especially freeze-fracture studies (21) have demonstrated that compartmentation features of myelin and the presence of regions of cytoplasm intercalated within the compact structure. Thus, evidence for an aqueous compartment already exists. The present data demonstrate proton movement by the myelin proteolipid apoprotein and supplement previous evidence of a voltage-dependent conductance change induced by the apoprotein in planar lipid bilayers (8). Both approaches suggest an involvement of this proteolipid in some type of channel formation. The present demonstration of an ionophoric property of the apoprotein in reconstituted liposomes is indirect evidence for an additional role of this protein in myelin over and above its accepted role as a structural protein.

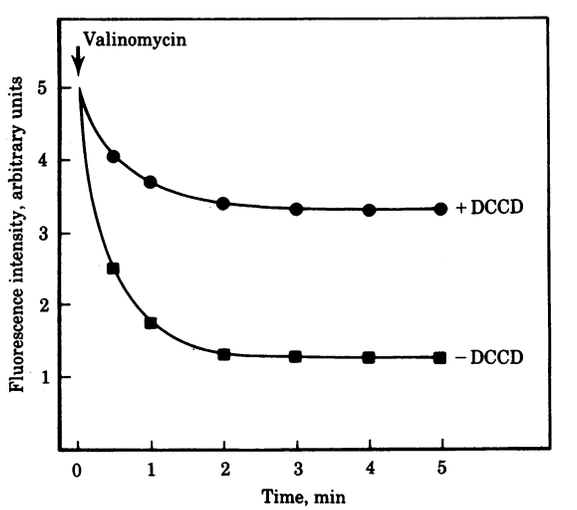

FIG. 6. Effect of DCCD on liposomes reconstituted with proteolipid. A 0.2-ml aliquot of proteolipid-containing liposomes (protein-to-lipid ratio 1:100 by weight) was incubated with 1 μl of DCCD at 10 mg/ml in ethanol at room temperature for 1 hr prior to assay for proton translocation. The control sample was incubated with 1 μl of ethanol without DCCD.

The inhibition of proton movement by DCCD in reconstituted liposomes is in agreement with extensive observations on proton transport mediated by proteolipids in other systems (3). DCCD is a well-established inhibitor of proton translocation in ATP-linked systems and has been shown to act through covalent binding to a specific carboxylate group on the proteolipid subunit of the ATPase complex (4). The observation that DCCD binds selectively to the proteolipid of myelin and not to other myelin proteins suggests that the myelin proteolipid has structural features analogous to those of other proteolipids. The binding appears to be covalent, because the label is not removed by washing with organic solvents or ionic detergents. In addition, the protein and the radioactive probe could not be separated from one another by either chromatography or $NaDodSO_4$ gel electrophoresis. However, the stoichiometry of the binding remains to be established.

Although the present data do not show whether DCCD binding occurs at only a single site on the protein, they are consistent with binding in a hydrophobic region(s) of the molecule and not in polar regions. First, EDAC, a highly reactive, water-soluble carbodiimide that interacts covalently with many proteins (22, 23) does not interfere with DCCD binding. Thus, the binding site is apparently inaccessible to the external aqueous phase. Second, myelin basic proteins, the major water-soluble extrinsic proteins of myelin, are not labeled by the reagent even with the highest DCCD concentration used in the study. Third, no labeling by DCCD of the isolated proteolipid is apparent if incubation is carried out in chloroform/methanol. Presumably, under these conditions, there is no driving force for the probe to partition into hydrophobic regions of the protein because the

hydrophobicity of the medium masks the hydrophobicity of the protein.

The present data add significantly to the growing body of evidence for an active participation of myelin in the overall events concerned with the maintenance of the normal nervous system. DCCD should be a useful probe for elucidating functional aspects of the proteolipid in the myelin membrane of the central nervous system.

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