Aggregation of Acidic Lipid Vesicles by Myelin Basic Protein: Dependence on Potassium Concentration†

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ABSTRACT: In the compacted multilayered myelin sheath of the central nervous system, myelin basic protein (MBP) is thought to be responsible for adhesion of the intracellular surfaces by electrostatic interactions with acidic lipids. Noncompacted regions of myelin containing cytosol exist and can take up potassium released into the extracellular fluid after the axonal action potential. Therefore, the effect of K^+ concentration on the ability of MBP to aggregate large unilamellar vesicles (LUVs) containing phosphatidylcholine (PC) and 10−20% acidic lipid was investigated. At MBP to lipid ratios where there was an excess of acidic lipid, physiological increases in K^+ concentration up to about 100 mM greatly increased MBP-mediated aggregation of the LUVs by shielding the negative charge on the vesicle surface. Thus, changes in K^+ concentration during the axonal action potential could regulate MBP-mediated adhesion of the intracellular myelin surfaces of noncompacted regions of myelin such as the paranodal loops. It could thus regulate the volume of these cytosolic regions, allowing MBP to have a dynamic function in myelin. Concentrations of K^+ above 150 mM caused dissociation of MBP from LUVs containing PC and a single acidic lipid. LUVs containing the lipid composition estimated to be characteristic of the cytoplasmic leaflet of myelin (Cyt.-LUVs) were found to interact uniquely with MBP, resulting in greater aggregation, greater sensitivity to K^+ concentration, and resistance to dissociation at high K^+ concentrations. The latter suggested that electrostatic interactions were not the only force involved in binding of MBP to the Cyt.-LUVs. Hydrogen bonding of the protein to the lipid head groups and hydrophobic interactions due to penetration of hydrophobic amino acid side chains into the bilayer could also occur. The greater involvement of hydrophobic interactions of MBP with Cyt.-LUVs compared to PC/acidic lipid LUVs was confirmed from greater labeling of MBP bound to Cyt.-LUVs by the hydrophobic photolabeled TID. Cholesterol and phosphatidylethanolamine together were found to be responsible for the greater MBP-mediated aggregation of Cyt.-LUVs and the greater TID labeling of MBP bound to Cyt.-LUVs compared to PC/acidic lipid LUVs. Thus, the lipid composition of the intracellular surface of myelin is well suited to allow MBP to mediate adhesion of apposing intracellular membranes and to respond in a dynamic way in some regions of myelin, such as the paranodal loops, to changes in K^+ concentration resulting from nerve conduction.

The myelin sheath surrounding the axons of the nervous system has a unique multilamellar structure. In compact internodal myelin, the intracellular surfaces adhere, forming the major dense line, and the extracellular surfaces adhere, forming the intraperiod line [see Raine (1977) and Kirschner and Blaurock (1992) for reviews]. The integrity of this compacted multilamellar structure determines the efficiency of the transmission of nervous signals along the axon. There are also some noncompacted regions containing cytoplasm in the myelin sheath, such as the inner tongue surrounding the axonal internodes, the outer tongue, and the paranodal loops of myelin, and in PNS myelin the Schmidt−Lanterman clefts (Raine, 1977; Kirschner & Blaurock, 1992). These regions of cytoplasm may allow myelin to perform more dynamic functions (Kirschner & Blaurock, 1992).

The weight ratio of lipids to proteins in the myelin sheath is about 3:1. Approximately 30% of the myelin protein of the central nervous system (CNS)[1] is myelin basic protein (MBP) (e.g., see Boggs et al., 1982). MBP is a water-soluble peripheral protein which has been localized to the cytoplasmic side of the myelin membrane by immunoelectron microscopy and X-ray diffraction (Omlin et al., 1982; Kirschner & Blaurock, 1992). It undoubtedly plays an important role in forming the major dense line of CNS myelin by cross-linking the apposed oligodendrocyte cytoplasmic membranes through protein−protein and/or protein−lipid interactions (Golds & Braun, 1978a,b; Smith, 1977; Readhead et al., 1990).

MBP (MW ~18 500) has a high net positive charge and binds electrostatically to negatively charged acidic lipids such

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[1] Abbreviations: CNS, central nervous system; PNS, peripheral nervous system; MBP, myelin basic protein; PG, phosphatidylglycerol; PS, phosphatidylserine; CBS, cerebroside sulfate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DPPC, dipalmitoylphosphatidylcholine; SM, sphingomyelin; chol, cholesterol; SUVs, small unilamellar vesicles; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles; Cyt.-LUVs, LUVs consisting of lipids characteristic of the cytosolic side of the myelin membrane; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine.

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as phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidic acid, cerebroside sulfate (CBS), and myelin lipid extracts (Palmer & Dawson, 1969; London et al., 1973; Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978; Boggs et al., 1981; MacNaughton et al., 1985; Sedzik et al., 1984; Bianco et al., 1992; Sankaram et al., 1991). This electrostatic interaction as well as the ability of MBP to aggregate apposing charged surfaces is expected to be affected by ionic strength due to the involvement of charges. The effect of ionic strength is important especially for understanding the dynamic functions of myelin. During the saltatory conduction causing the action potential, some of the K^+ released by stimulated neurons is taken up by oligodendrocytes and other glial cells and probably by myelin for the homeostasis of extracellular K^+ concentration. Using electron-dense Cs^+, the cytoplasmic space of sciatic nerve myelin has been shown by X-ray diffraction to be accessible to cations (Blaurock, 1971). K^+ uptake is likely to occur at the paranodal cytosolic loops of myelin surrounding the nodes of Ranvier where most axonal Na^+-K^+ pumps are located. K^+ channels exist in oligodendrocytes (Kettenmann, 1987), and they have been found located at the paranodal region in myelin (Chiu, 1991). These may include the myelin protein plasmolipin (Tosteson & Sapirstein, 1981), which has been found in all regions of myelin including the paranodal loops (Sapirstein et al., 1993). Thus, an increase in extracellular K^+ concentration during the action potential causes a corresponding increase in the intracellular K^+ concentration of oligodendroglial cells (Kettenmann et al., 1983). In addition, during compaction of an oligodendrocyte process, the reduction in the cytoplasmic volume may lead to increases in intracellular ion concentrations (Nowak & Berman, 1991). A cytoplasmic bridge between the axon and inner loop of the oligodendrocyte cytoplasm has also been suggested to occur at least transiently in the CNS (Wolman, 1992). This would allow changes in the ionic content of the axoplasm during the action potential to be transmitted directly to myelin cytoplasm. Therefore, it is possible that changes in the concentration of K^+ and other cations in the cytoplasmic regions of the myelin sheath may occur physiologically and affect lipid−MBP interactions and thus modulate the structure and function of myelin.

The ability of MBP to aggregate lipid vesicles can be used as a model system to study factors which might affect its ability to cause adhesion of the intracellular surfaces of myelin. In most previous studies of lipid−MBP interactions, the ability of MBP to aggregate small unilamellar vesicles (SUVs) was investigated at fixed ionic strength (Wood & Moscarello, 1989; Cheifetz & Moscarello, 1985; Lampe et al., 1983; Smith, 1977). However, Walker and Rumsby (1985) showed that an increase in the NaCl concentration increased MBP-mediated aggregation of PS SUVs by shielding the charge on the lipid. At high concentrations, NaCl also increased the MBP-mediated aggregation of PC SUVs at pH 8.9 (Smith, 1977). The intracellular concentration of K^+ in oligodendrocytes is 60−74 mM (Kettenmann, 1987; Kettenmann et al., 1983; Somjen, 1979), and that of Na^+ is 15 mM (Ballanyi & Kettenmann, 1990). In the present study, the concentration of monovalent cation was varied from 20 mM to 410 mM. This range includes the physiological intracellular K^+ concentration of oligodendrocytes. Since K^+ is present at a higher concentration than Na^+, KCl was used for most experiments. However, the effect of NaCl was compared to that of KCl in some experiments.

Large unilamellar vesicles (LUVs), which are geometrically closer to natural membranes with less curvature and more homogeneous in size, were used in this study for studying MBP-induced vesicle aggregation. The lipid to MBP ratio and the content of acidic lipid in LUVs were also varied in a range including their physiological ratios. Furthermore, the effects of these variations on aggregation of LUVs containing the lipid composition estimated by Inouye and Kirschner (1988) to be characteristic of the cytoplasmic leaflet of myelin (Cyt.-LUVs) were studied in order to obtain more physiologically relevant information. This lipid composition was found to interact uniquely with MBP, resulting in greater aggregation, greater sensitivity to K^+ concentration, and higher affinity binding than PC LUVs containing a single acidic lipid. The results indicate that under certain conditions, MBP-induced aggregation of vesicles can be very sensitive to physiological changes in K^+ concentration.

In addition to electrostatic and hydrogen bonding interactions of MBP with acidic lipids, a hydrophobic interaction may occur between hydrophobic amino acid side chains of MBP and the lipid bilayer (London et al., 1973; Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978; Boggs et al., 1981, 1988; Sankaram et al., 1989; Mendz et al., 1990). TID hydrophobic photolabeling was used to investigate the degree of hydrophobic interaction of MBP with different lipids. Using Cyt.-vesicles and varying their lipid composition, it was revealed that cholesterol and PE are most responsible for augmenting the binding affinity and the hydrophobic interactions of MBP with vesicles containing acidic lipids.

MATERIALS AND METHODS

Lipids and Protein. Egg L-α-phosphatidylcholine (egg PC) was purchased from Sigma (St. Louis, MO). L-α-Phosphatidylglycerol (PG; prepared from egg PC), bovine brain L-α-phosphatidylserine (PS), bovine brain L-α-phosphatidylethanolamine (PE; mostly plasmalogen form), bovine liver L-α-phosphatidylinositol (PI), and bovine brain sphingomyelin (SM) were obtained from Avanti Polar-Lipids, Inc. (Alabaster, AL). Bovine brain cerebroside sulfate (CBS) was from Matreya, Inc. (Pleasant Gap, PA). Cholesterol (chol) was from Supelco, Inc. (Bellefonte, PA). [^{3}H]Dipalmitoylphosphatidylcholine (DPPC) (58.0 Ci/mmol) was purchased from Dupont-NEN (Boston, MA). Myelin basic protein (MBP) was isolated from bovine brain white matter as described (Cheifetz & Moscarello, 1985).

Preparation of Large Unilamellar Vesicles (LUVs). Lipids were dissolved in chloroform at a concentration of 10−100 mg/mL and stored at −20 °C. Aliquots of solutions of the required lipids were combined in the desired mole ratio. In the case of LUVs containing the lipid composition estimated to be characteristic of the cytoplasmic surface of myelin (Cyt.-LUVs) by Inouye and Kirschner (1988), aliquots of solutions of chol, PE, PS, PC, SM, and PI were combined in the mole ratio 0.44:0.27:0.13:0.11:0.03:0.02. In the case of Cyt.-LUVs containing 5% CBS, the mole ratio of chol, PE, PS, PC, SM, PI, and CBS was 0.42:0.26:0.12:0.11:0.03:0.02:0.05. When either PE or chol was omitted, it was replaced with additional PC. When PI was omitted, it was

replaced with additional PS. A trace amount (1 μCi in 10 μL) of [^{3}H]DPPC was added to the lipid mixture in order to quantitate and normalize the lipid concentration after extrusion. The organic solvents were removed by evaporation under a stream of nitrogen, and the lipid film was evacuated in a lyophilizer for 1 h. The dry lipid film was dissolved in 1−2 mL of benzene, frozen, and lyophilized overnight. Large multilamellar vesicles (MLVs) were prepared by hydrating the dry lipid in 20 mM HEPES buffer, pH 7.4, containing 10 mM KCl and 1 mM EDTA. The concentration of total lipid was 60 mM. The MLV dispersion was vortexed for 15 min at room temperature. The MLVs were freeze-thawed 5 times using a dry ice/acetone bath followed by a warm water bath (40 °C) in order to promote equilibrium transmembrane solute distribution (Mayer et al., 1985). The dispersion (1 mL) was then transferred into an extruder (produced by Lipex Biomembranes, Vancouver, B.C.) which allowed the extrusion of the MLVs through polycarbonate filters with 0.2 μm pore size under nitrogen pressure (Hope et al., 1985; Mayer et al., 1986). After the dispersion was passed through two stacked filters 10 times, the vesicles were extruded again through new filters with 0.1 μm pore size. The resulting large unilamellar vesicles (LUVs) were kept at 4 °C until the aggregation assay.

Aggregation Assay. Two stock solutions (20 mM HEPES containing 1 mM EDTA), one containing 10 mM KCl and one containing 200 or 400 mM KCl, were made. They were adjusted to pH 7.4 with potassium hydroxide (KOH). The final K^+ concentration was determined by flame atomic absorption spectrophotometry (Clinical Biochemistry Laboratory, Hospital for Sick Children) to be 20, 210, and 410 mM, respectively. By combining the required amounts of the above 20 mM K^+ and 210 or 410 mM K^+ solutions, solutions containing varying K^+ concentration were prepared.

Ten to twelve microliters of LUVs containing 0.6 μmol of lipid (7000−8000 cpm) was diluted in 1 mL of 20 mM HEPES buffer containing various concentrations (20−410 mM) of K^+ to give a lipid concentration of 0.6 mM. In order to equilibrate the salt concentration inside and outside the LUVs, the diluted LUVs were sonicated gently in a bath sonicator at 20−25 °C for 10 min and left overnight at 4 °C. MBP solutions were made in 20 mM HEPES buffer at pH 7.4 containing 10 mM KCl (total K^+ concentration is 20 mM) and 1 mM EDTA to give a MBP concentration of 1.5, 0.75, 0.5, or 0.375 mg/mL; 15, 7.5, 5, or 3.75 μg of MBP in 10 μL was added to 0.6 μmol of lipid in a final volume of 1 mL, resulting in a lipid:MBP weight ratio of 30:1, 60:1, 90:1, or 120:1. The cuvettes containing LUVs and MBP were gently inverted twice to mix. In preliminary experiments, aggregation was found to be rapid, reaching a maximum in 2−5 min after mixing; 10−15 min after mixing, the absorbance at 450 nm was measured on a U-2000 spectrophotometer (Hitachi) at room temperature. The absorbance at 450 nm, A_{450nm}, of LUVs in buffer containing 20−410 mM K^+ was measured before adding MBP, and these background values (0.08−0.12) were subtracted from A_{450nm} after MBP-induced aggregation. All aggregation assays were carried out in disposable acryl cuvettes (Sarstedt Inc.). In some experiments, the effect of NaCl was compared to that of KCl.

Reversibility Test of MBP-Induced Aggregation of LUVs. After aggregation of a series of samples of PC LUVs containing 10−20% CBS at different K^+ concentrations, the aggregated samples (1 mL) were divided into two cuvettes (0.5 mL in each), and 0.5 mL of the same KCl solution used for aggregation was added to one set of samples while 0.5 mL of buffer containing 210 mM K^+ was added to the other set of samples. 1:1 (v/v) dilution of samples in buffer containing 20−210 mM K^+ with buffer containing 210 mM K^+ resulted in final K^+ concentrations of 115−210 mM. The cuvettes containing these diluted aggregates were gently inverted twice to mix. After 10 min, the absorbance at 450 nm was measured at room temperature.

In the case of Cyt.-LUVs, 330 μL of the aggregated samples was diluted with 660 μL of the same KCl solution used for aggregation. To another 330 μL of the aggregated samples was added 660 μL of buffer containing 20 mM K^+, resulting in final K^+ concentrations of 20−150 mM.

Quantitation of Lipid to Protein Ratio in Aggregates. Quadruplicate aggregated samples (1 mL in each) in 20, 80, and 210 mM K^+ were transferred into Eppendorf microcentrifuge tubes. They were centrifuged at 10000*g* for 10 min, and the supernatant was decanted. The pellet was washed by adding 1 mL of the respective K^+ solution to the pellet, followed by centrifugation at 10000*g* and resuspension in 1 mL of double-distilled water. Two of the quadruplicate samples were used for quantitation of protein by the Peterson assay (Peterson, 1977) and two for quantitation of lipid. The averaged MBP concentration of the duplicate samples was divided by the original concentration, 15 μg/mL (lipid:MBP = 30:1 or 25:1), to obtain the percent MBP recovered in the aggregates. Since the presence of lipid in the samples caused an increase in A_{750nm}, an additional standard curve was prepared in the presence of 0.6 μmol of 20% PG LUVs or Cyt.-LUVs added to the MBP standard solution to account for the maximum lipid interference. The standard curve in the presence of 20% PG LUVs was 0.02 higher in A_{750nm} than that in the absence of LUVs. The standard curve in the presence of Cyt.-LUVs was 0.22 higher in A_{750nm} than that in the absence of LUVs.

Lipid was quantitated by counting the radioactivity of the resuspended pellets. The averaged value was divided by the original total radioactivity of 0.6 μmol of lipid (7000−8000 cpm) to obtain the percent lipid recovered in the aggregates. The ratio of lipid to MBP (w/w) was then obtained by multiplying the ratio of percent lipid to percent MBP by the original ratio of lipid to MBP.

Isolation of Nonaggregated LUVs by Ultracentrifugation and Quantitation of the Lipid to Protein Ratio. One hundred microliters of LUVs containing 6 μmol of lipid was diluted in 10 mL of buffer containing either 20 mM K^+ or 210 mM K^+; 150 μg of MBP in 25 μL was added to them, resulting in a lipid:MBP weight ratio of 30:1. COREX tubes containing LUVs and MBP were gently inverted twice to mix. They were centrifuged at 9000*g* (Model J2-21 Centrifuge, Beckman) for 1 h in order to remove the aggregated LUVs in the pellet. Protein-free LUVs did not sediment at this speed. The supernatant, which mostly contained nonaggregated LUVs and MBP, was ultracentrifuged at 95000*g* (Model L3-50 Ultracentrifuge, Beckman) for 2 h. This allowed sedimentation of 90% of protein-free LUVs. Thus, the amount of protein bound to nonaggregated LUVs could be determined. The lipid to protein ratio of the supernatant was also determined. The pellet was resuspended in 800 μL of buffer. Duplicate aliquots (200 μL each) of the suspension and the supernatant were counted for [^{3}H]DPPC

to obtain the amount of lipid while separate duplicate aliquots of the suspension and the supernatant were assayed by the Peterson assay to obtain the amount of protein. The values of percent lipid, percent MBP, and the lipid to protein ratio were calculated as described above.

Photolabeling of MBP-Aggregated Lipid Vesicles with [^{125}I]TID. For some samples, MLVs were used for photolabeling. Six micromoles of the lipid mixture was dried in test tubes, and 150 μg of MBP in 1 mL of 80 mM K^+ solution was added to each tube. When a constant weight of lipid was used, 3.6 mg of the lipid mixture was dried, and 120 μg of MBP in 1 mL of 80 mM K^+ solution was added. The mixtures were vortexed to give MLVs and flushed with nitrogen. For other samples, LUVs were used for photolabeling; 106−122 μL of LUVs containing 6 μmol of lipid was diluted in 10 mL of 80 mM K^+ solution, and 150 μg of MBP in 100 μL was added to them. COREX tubes containing LUVs and MBP were gently inverted twice to mix. They were centrifuged at 9000*g* for 1 h, and the pellet was washed once with 10 mL of 80 mM K^+ solution. Then the pellet was resuspended in 1 mL and flushed with nitrogen. Four microliters of [^{125}I]TID containing approximately 2×10^7 cpm was added to 1 mL of vesicle suspension, and the sample was mixed and equilibrated in the dark at room temperature for 20 min. It was then irradiated for 2 min using a 100 W high-pressure Hg lamp as described (Boggs et al., 1988).

Purification of [^{125}I]TID-Labeled Protein by Lipid Extraction and C-18 Sep-pak Chromatography. After photolysis, the pH of samples containing MBP−lipid vesicles was lowered to pH 1 with 90 μL of 2 N HCl. Four milliliters of acidified chloroform/methanol (1:1) containing 5% 0.1 N HCl was added to the sample (1 mL), and it was vortexed and centrifuged at 2000 rpm for 10 min. The resulting aqueous phase (2.8 mL) was separated from the organic phase and was extracted 3 more times using 2.2 mL of fresh lower phase. The last aqueous phase was lyophilized, taken up in 1 mL of 0.05% TFA, and placed on a C-18 Sep-pak column (Millipore). The protein was eluted with 8 mL of 40% acetonitrile, and the eluate was lyophilized. This procedure was shown to remove TID-labeled acidic lipid from the TID-labeled MBP (Boggs et al., 1988, and unpublished data). The protein was dissolved in 1 mL of H_2O, and aliquots (100 μL × 2) were counted in a γ counter and assayed for protein content by the method of Peterson (1977).

Approximately 10 μg of the above purified proteins was loaded on 16% Tricine gels (NOVEX). Electrophoresis was performed at 100 V (constant voltage) until the tracking dye reached the bottom of the gel. After staining with 0.05% Coomassie brilliant blue R and destaining as described (Ball, 1986), the gel was sliced into strips, and the strips were counted in a γ counter. One milliliter of a color-eluting solution (3% SDS in 50% 2-propanol) was added to the gel strips, and they were incubated at 37 °C for 24 h. The absorbance of the dye at 595 nm was read in a spectrophotometer. Relative specific activities were then obtained (cpm/A_{595nm}).

TID labels lipid as well as protein. The availability of lipid for labeling decreases binding of TID to the protein. Therefore, in order to compare labeling of MBP in solution with that of MBP bound to acidic lipid vesicles, MBP in solution was labeled in the presence of the same amount of PC vesicles, a lipid to which it does not bind (Boggs et al., 1988). The specific activity values for other lipid samples were divided by the values for the MBP−egg PC vesicles in order to allow comparison between different experiments in which different amounts of TID were used.

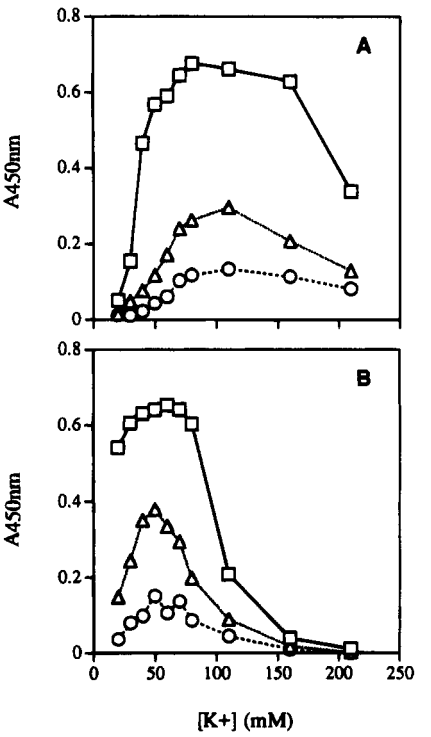

FIGURE 1: Dependence on K^+ concentration of MBP-induced aggregation of PC LUVs containing either 20% PG (A) or 10% PG (B). Aggregation was measured from the absorbance at 450 nm (A_{450nm}). The lipid concentration in the assay system, corrected after extrusion, was 0.6 mM. The final MBP concentrations were 15 μg/mL (squares), 7.5 μg/mL (Triangles), and 5 μg/mL (circles), and the lipid:MBP ratios (w/w) were 30:1, 60:1, and 90:1, respectively.

RESULTS

MBP-Induced Aggregation of PC LUVs Containing 10−20% Acidic Lipid. The effect of salt concentration on the ability of MBP to aggregate large unilamellar vesicles (LUVs) composed of 80 mol % phosphatidylcholine (PC) and 20 mol % phosphatidylglycerol (PG) was determined. The aggregation of LUVs leads to scattering of light, thus increasing the absorbance of the LUV suspension (Freifelder, 1982). Therefore, the degree of aggregation was monitored by measuring the absorbance at 450 nm as in a number of studies (Smith, 1977; Wood & Moscarello, 1989; Cheifetz & Moscarello, 1985; ter Beest & Hoekstra, 1993).

At high protein to lipid ratios, MBP causes rapid aggregation of acidic lipid vesicles even at low ionic strength. MBP is thus able to partially overcome the short-range hydration force and cause relatively close contact of the vesicles, as demonstrated by electron microscopy (Smith, 1977). However, little aggregation was seen at low ionic strength at the low protein to lipid ratios used in this study (Figure 1A). MBP has 31 positively charged and 11 negatively charged residues per molecule at neutral pH. Even at the highest concentration of MBP used (lipid:MBP = 30:1 w/w), the mole ratio of acidic lipid to protein is 150:1, resulting in a large excess of unbound acidic lipid. At low ionic strength, the negatively charged vesicles repel each other due to

Table 1: Maximum Absorbance (max A_{450nm}) and K^+ Concentration Required for the Half-Maximum Absorbance ($[K^+]_{50}$) of MBP-Aggregated PC LUVs Containing Acidic PG, PS, or CBS, or LUVs Containing the Lipid Composition of the Cytoplasmic Leaflet of Myelin (Cyt.)

content of acidic lipid	lipid:MBP weight ratio	acidic lipid:MBP mole ratio	max A_{450nm}	$[K^+]_{50}$[a] (mM)	ΔA_{450nm}/ 10 mM K^+[b] at $[K^+]_{50}$
20% PG	30:1	~150:1	0.68	36	0.31
	60:1	~300:1	0.30	56	0.05
	90:1	~450:1	0.13	61	0.04
10% PG	30:1	~75:1	0.65	~10[c]	~0.30[c]
	60:1	~150:1	0.38	24	0.10
	90:1	~225:1	0.15	29	0.04
20% PS	60:1	~300:1	0.41	37	0.08
	90:1	~450:1	0.24	40	0.04
	120:1	~600:1	0.16	42	0.03
20% CBS	30:1	~150:1	0.58	29	0.09
	60:1	~300:1	0.24	31	0.04
	90:1	~450:1	0.12	29	0.02
Cyt.±CBS[d] (15−19% acidic lipid)	25:1	~150:1	0.82	26	0.28
	50:1	~300:1	0.83	46	0.13
	75:0	~450:1	0.82	68	0.07
	100:1	~600:1	0.74	86	0.04
Cyt. lacking PE (15% acidic lipid)	50:1	~220:1	0.58	27	0.24
	75:1	~330:1	0.45	33	0.12
	100:1	~440:1	0.31	42	0.09
Cyt. lacking chol (15% acidic lipid)	50:1	~220:1	0.59	37	0.05
	75:1	~330:1	0.26	36	0.07
	100:1	~440:1	0.21	32	0.05

[a] The half-maximum absorbance was obtained by dividing the maximum A_{450nm} by 2. The K^+ concentration required for the half-maximum absorbance, $[K^+]_{50}$, was then obtained by interpolating between the two nearest points on the absorbance curves in Figures 1, 2, and 4. [b] Change in A_{450nm} per 10 mM increase in $[K^+]$ at $[K^+]_{50}$. [c] Aggregation was close to maximal at the lowest K^+ concentration used. [d] In a preliminary study, the MBP-induced aggregation of Cyt.-LUVs without CBS (15% acidic lipid) had similar dependence on K^+ concentration as (Cyt.+CBS)-LUVs (19% acidic lipid). Data shown are actually from (Cyt.+CBS)-LUVs as more detailed measurements were made.

electrostatic double layer repulsion (Cowley et al., 1978) in addition to short-range hydration forces (Rand & Parsegian, 1989). As the salt concentration was increased from 20 mM, the degree of aggregation of the LUVs increased dramatically (shown for KCl in Figure 1A), reaching a maximum at 80−110 mM K^+. NaCl had identical effects as KCl. The cation shields the negative charge on the excess lipid not bound to MBP, facilitating interaction of MBP-bound LUVs with each other and aggregation (Bungenberg de Jong, 1949). As the KCl concentration was further increased from 110 to 210 mM, the degree of aggregation of the LUVs decreased. These K^+ concentrations had an insignificant effect on the absorbance of LUVs in the absence of MBP or on the absorbance of MBP in the absence of LUVs. An increase in ionic strength has been found to cause changes in the Trp fluorescence of MBP suggestive of changes in conformation, a reduction in flexibility, and/or increased self-association (Nowak & Berman, 1991). However, the effects are small at concentrations of KCl or NaCl below 0.2 M.

When the amount of MBP added was gradually lowered from the ratio of lipid:MBP = 30:1 (w/w) to 90:1, the extent of aggregation and the maximum absorbance decreased (Table 1). As the amount of MBP and ratio to PG decreased, the cation concentration required for aggregation increased. The K^+ concentration required for half of the maximum absorbance ($[K^+]_{50}$) is given in Table 1 (20% PG). The dependence of $[K^+]_{50}$ on the amount of MBP can be explained by increased charge repulsion of the vesicles at lower MBP to lipid ratios. Consequently, a higher K^+ concentration was needed to permit aggregation.

The amount of change in A_{450nm} for an increase in $[K^+]$ of 10 mM was determined in the range of K^+ concentrations near the $[K^+]_{50}$ value (Table 1, 20% PG). An increase of 10 mM K^+ caused a significant change in A_{450nm}, especially at low lipid to MBP ratios (30:1). These values allow a comparison of the sensitivity of the MBP-induced aggregation to K^+ concentration under different conditions.

Using LUVs composed of 90% PC and 10% PG, which contain less PG than the above, the degree of maximum aggregation was similar to the above LUVs containing 20% PG (Figure 1B). However, less K^+ was required to permit MBP-induced aggregation than for LUVs of 20% PG since there was less negative charge to be shielded by K^+ (Table 1). Therefore, the aggregation of LUVs depends on (i) the cation (or salt) concentration of the buffer, (ii) the ratio of MBP to acidic lipid, and (iii) the ratio of acidic lipid to neutral lipid.

LUVs containing another type of an acidic phospholipid, phosphatidylserine (PS), were examined next. The values of $[K^+]_{50}$ were lower for PS than PG, and they were less dependent on lipid to MBP ratio, suggesting that K^+ shields the negative charge on PS better than PG (Table 1, 20% PS).

In addition to acidic phospholipids, it was of interest to study the aggregation of LUVs containing an acidic sphingolipid, cerebroside sulfate (CBS), which is present at higher concentration in myelin than in any other mammalian membrane (Karlsson, 1982) (Figure 2). The curves for CBS lacked a lag phase at low K^+ concentration in contrast to the curves for PG (Figure 1A). Thus, the values of $[K^+]_{50}$ were lower for CBS than PG, suggesting that K^+ also shields the negative charge on CBS better than PG (Table 1, 20% CBS). Na^+ had a similar effect as K^+ on this lipid also.

Is the Increase in Absorbance Due to Aggregation of LUVs or Fusion? The increase in A_{450nm} was thought to be the result of the aggregation of LUVs. However, if fusion of LUVs had subsequently occurred, it would have also increased A_{450nm} due to the large size of the fused vesicles. In order to distinguish aggregation from fusion, a high concentration of K^+ (210 mM) was added to the aggregated

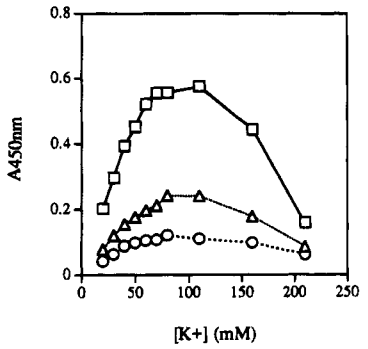

FIGURE 2: Dependence on K^+ concentration of MBP-induced aggregation of PC LUVs containing 20% CBS. The lipid concentration in the assay system, corrected after extrusion, was 0.6 mM. The final MBP concentrations were 15 μg/mL (squares), 7.5 μg/mL (triangles), and 5 μg/mL (circles), and the lipid:MBP (w/w) ratios were 30:1, 60:1, and 90:1, respectively.

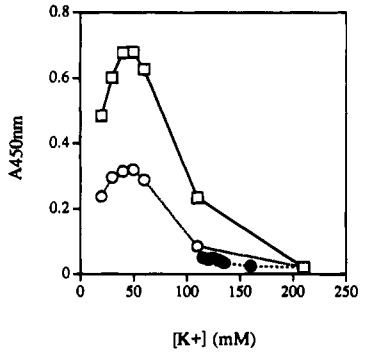

FIGURE 3: Reversibility of MBP-induced aggregation of PC LUVs containing 10% CBS. The lipid concentration in the assay system was 0.6 mM before extrusion. The original MBP concentration was 15 μg/mL, and the lipid:MBP (w/w) ratio was 30:1. The original aggregated LUVs at each K^+ concentration (squares) were diluted 1:1 (v/v) either with the respective buffer containing the same K^+ concentration (open circles) or with buffer containing 210 mM K^+, resulting in final K^+ concentrations of 115−210 mM (filled circles). The decrease in aggregation of samples initially at K^+ concentrations of 20−60 mM, caused by dilution with a high K^+ concentration, indicates that the aggregation was reversible and that fusion had not occurred in the K^+ concentration range 20−60 mM.

LUVs after inducing their aggregation with MBP (shown for PC LUVs containing 10% CBS in Figure 3). On dilution with an equal volume of the same K^+ solution, the A_{450nm} at each K^+ concentration decreased by about 50%. However, on dilution of each aggregated sample with an equal volume of 210 mM K^+, resulting in final K^+ concentrations of 115−210 mM, the A_{450nm} decreased to a value consistent with that expected for the final total K^+ concentration if reversible aggregation and not fusion had occurred. The aggregation was also reversible at higher CBS content and higher concentrations of MBP (data not shown). Therefore, the high A_{450nm} was not due to fusion but due to aggregation of LUVs.

Is the Decrease in Absorbance at High [KCl] Due to Dissociation of LUVs or Fusion? However, there is still a possibility that the drop in A_{450nm} value at high KCl concentrations is due to formation of a small number of very large aggregates or fused vesicles. Alternatively, the high ionic strength may dissociate MBP from the lipid and thus prevent aggregation. Hence, the percent of the original lipid and MBP present in the aggregates was quantitated at different K^+ concentrations (Table 2, 20% PG and 20% CBS). LUVs in the absence of MBP cannot be pelleted by centrifugation at 10000*g*. In the presence of MBP, MBP-

Table 2: Quantitation of Lipid and MBP in MBP-Aggregated PC LUVs Containing Acidic PG or CBS, or LUVs Containing the Lipid Composition of the Cytoplasmic Leaflet of Myelin (Cyt.)

content of acidic lipid	K^+ concn (mM)	A_{450nm}	% lipid in aggregates[a]	% MBP in aggregates[b]	lipid:MBP ratio (w/w)[c]
20% PG	20	0.052	11	22	
	80	0.677	101	98	30.9
	210	0.338	41	34	
20% CBS	20	0.203	33	61	
	80	0.557	76	95	24.0
	210	0.159	10	14	
Cyt.+CBS (19% acidic lipid)	20	0.244	34	16	
	80	0.643	101	83	30.4
	210	0.703	98	75	32.7

[a] Percent of total lipid in pellet after 10000*g* centrifugation. [b] Percent of total MBP in pellet after 10000*g* centrifugation. [c] Initial lipid:MBP ratio was 30:1 in cases of 20% PG and 20% CBS, and it was 25:1 in the case of Cyt.+CBS.

aggregated LUVs were pelleted by centrifugation at 10000*g*. At a low K^+ concentration (20 mM), A_{450nm} was low, and the amounts of lipid and MBP which could be easily pelleted were accordingly small. However, at a K^+ concentration of 80 mM where A_{450nm} was maximal, the amounts of pelleted lipid and MBP were close to 100% of the total amounts present in the samples, resulting in comparable weight ratios of lipid to MBP of 30.9 and 24.0, respectively, in the pellets as the original ratio of 30 (Table 2, 20% PG and 20% CBS). As the KCl concentration was increased up to 210 mM, the percentage of lipid and MBP in the aggregates should have remained high if fusion or formation of large aggregates had occurred. However, the percentage of lipid and MBP in the aggregates decreased, consistent with the decrease in A_{450nm} at 210 mM KCl. This suggested that high KCl concentration prevents aggregation of the MBP-bound LUVs, possibly by causing dissociation of MBP from LUVs.

Do Disaggregated LUVs Bind MBP at High [KCl]? In order to investigate whether MBP is bound to the LUVs at 210 mM KCl or dissociated from the LUVs, the ratio of lipid to MBP in the nonaggregated population of 20% CBS LUVs was examined by pelleting them by ultracentrifugation (Table 3). First of all, samples after aggregation at a lipid:MBP ratio of 30:1 were centrifuged at 9000*g* to remove the aggregated population of LUVs (pellet 1). These aggregates had a weight ratio of lipid to MBP of 16:1 and 21:1 at 20 mM KCl and 210 mM KCl, respectively, but those at 210 mM KCl contained less than 15% of the total lipid and protein. The supernatant, which contained nonaggregated LUVs and MBP, was ultracentrifuged at 95000*g* since it was found possible to pellet most of the LUVs by ultracentrifugation in a preliminary study using pure LUVs. The pellet (pellet 2) from ultracentrifugation of the 9000*g* supernatant, which contained most of the nonaggregated LUVs, showed a large decrease in content of MBP on raising the KCl concentration from 20 mM to 210 mM (Table 3). The nonaggregated LUVs at 20 mM KCl were bound to MBP, and the ratio of lipid to MBP was 64:1, which was 3−4 times higher than the smaller population which aggregated and sedimented by centrifugation at 9000*g*. In contrast, the larger population of nonaggregated LUVs at 210 mM KCl did not contain much MBP bound to them, resulting in a much higher lipid:MBP ratio, 2430:1. Accordingly, the supernatant from ultracentrifugation (super. 2) showed a large increase in MBP concentration on raising the KCl concentration from

Table 3: Quantitation of Lipid and MBP in Aggregated (Pellet 1) and Nonaggregated (Pellet 2) PC LUVs Containing 20% CBS at Low and High K^+ Concentrations

step	fraction[a]	K^+ concn (mM)	% lipid[b]	% MBP[b]	lipid:MBP[c] ratio (w/w)
step 1: low-speed centrifugation	pellet 1	20	33	61	16.2
		210	10	14	21.4
step 2: ultracentrifugation of super. 1 at 95000*g*	pellet 2	20	60	28	64.3
		210	81	1	2430.0
	super. 2	20	7	11	19.1
		210	9	85	3.2

[a] Pellet 1 from low-speed centrifugation contains MBP-aggregated LUVs. Pellet 2 from ultracentrifugation of supernatant 1 (super. 1) contains the population of LUVs which did not aggregate. 90% of protein-free LUVs can be sedimented by ultracentrifugation but not by low-speed centrifugation. Supernatant 2 (super. 2) from ultracentrifugation contains mostly unbound MBP. [b] Percent of total lipid or MBP added found in fraction indicated. [c] Lipid:MBP weight ratio in fraction indicated. Initial lipid:MBP ratio was 30:1. MBP and lipid in super. 2 are probably not bound to each other at least in the form of bilayers. If they were, they would have been pelleted during ultracentrifugation.

20 mM to 210 mM (Table 3). Only 11% of the total MBP was recovered from super. 2 at 20 mM KCl whereas 85% of the total MBP was recovered at 210 mM KCl.

Therefore, MBP is bound to the nonaggregated LUVs at low KCl concentrations but not at high KCl concentrations. As the KCl concentration is increased, MBP dissociates from the LUVs, causing their disaggregation or preventing their aggregation. This explains the decrease in A_{450nm} at high KCl concentrations in Figures 1 and 2 and the reversibility of the aggregation in Figure 3. At high KCl concentrations, K^+ shields the negative charge on the acidic lipid to a greater extent, and Cl^- shields the positive charges on MBP, thus preventing MBP from binding to the LUVs (Bungenberg de Jong, 1949).

MBP-Induced Aggregation of LUVs with an Intracellular Myelin Lipid Composition. Next, MBP-induced aggregation of LUVs was studied using a more physiological lipid composition. In the central nervous system (CNS), MBP is thought to be present only on the cytoplasmic side of the oligodendroglial cell and of myelin (Omlin et al., 1982). Therefore, MBP-induced aggregation of model membranes was examined using LUVs with a lipid composition thought to be similar to that of the cytoplasmic (Cyt.) leaflet of myelin: 44 mol % chol, 27% PE, 13% PS, 11% PC, 3% SM, and 2% PI (Inouye & Kirschner, 1988). In parallel, 5% CBS was included in addition to the cytoplasmic lipids in another set of experiments because CBS is a major myelin acidic lipid and it is not known whether CBS is only in the extracellular leaflet of myelin or in both leaflets.

The aggregation of Cyt.-LUVs with and without CBS showed basically the same dependence on KCl and MBP concentrations despite the fact that the former contain 19% total acidic lipid and the latter contain only 15%. Therefore, only the data of Cyt.-LUVs with CBS are shown in Figure 4A and Table 1 (Cyt ± CBS). The values of $[K^+]_{50}$ were dependent on the amount of MBP as expected. At lower MBP to lipid ratios, higher K^+ concentration was needed to cause aggregation, due to greater charge repulsion. In addition, more K^+ was required to shield the charge than for PC LUVs containing PS and CBS, similar to PC/PG LUVs. The sensitivity of aggregation to K^+ concentration was also greater than for PC LUVs containing PS or CBS,

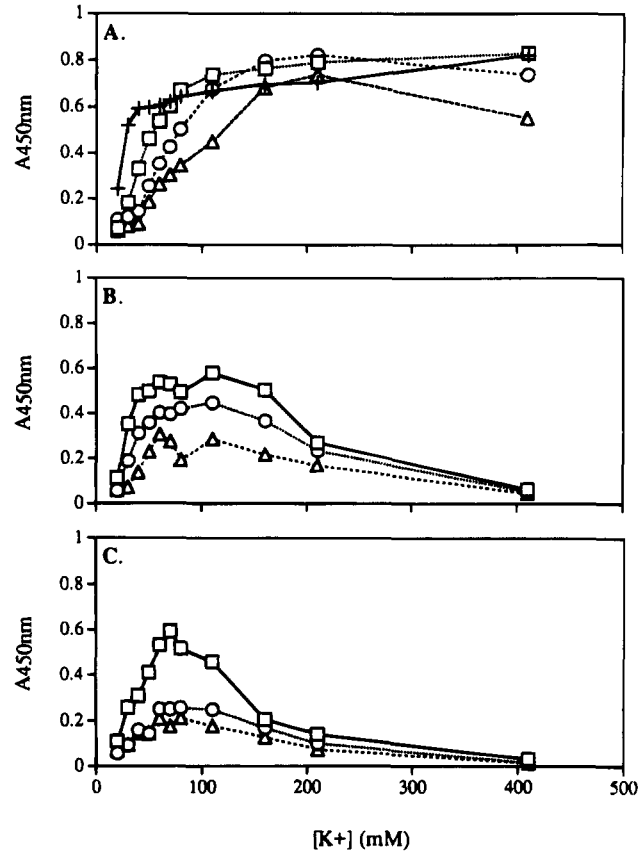

FIGURE 4: Dependence on K^+ concentration of MBP-induced aggregation of LUVs with a lipid composition similar to the cytoplasmic leaflet of myelin. The lipid concentration in the assay system, corrected after extrusion, was 0.6 mM. The final MBP concentrations were 15 μg/mL (crosses), 7.5 μg/mL (squares), 5 μg/mL (circles), and 3.75 μg/mL (triangles), and the lipid:MBP (w/w) ratios were 25:1, 50:1, 75:1, and 100:1, respectively. (A) (Cyt.+CBS)-LUVs composed of a lipid mixture with a composition considered characteristic of the cytoplasmic leaflet of myelin plus 5% CBS. Similar results were obtained when the CBS was omitted. (B) LUVs composed of a lipid mixture with a composition characteristic of the cytoplasmic leaflet of myelin but lacking PE. PC was substituted for PE. (C) LUVs composed of a lipid mixture with a composition characteristic of the cytoplasmic leaflet of myelin but lacking cholesterol. PC was substituted for cholesterol.

as indicated by the values of ΔA_{450nm}/10 mM K^+. Na^+ behaved similarly to K^+ with these vesicles also. K^+ had an insignificant effect on the absorbance of Cyt.-LUVs in the absence of MBP.

Interestingly, with these LUVs, a KCl concentration of 210 mM did not cause a decrease in A_{450nm}. Even the highest KCl concentration used, 410 mM, usually caused only a small decrease in A_{450nm} and only at higher lipid:MBP ratios (Figure 4A). This is distinctly different from the results with PC LUVs containing 10% or 20% of a single acidic lipid. There are two possible explanations for this. The first is a higher affinity of MBP for Cyt.-LUVs than for PC-acidic lipid LUVs. Consequently, the high ionic strength could not disrupt the electrostatic interactions of MBP with lipid by shielding the charge on the acidic lipids and protein to cause dissociation of MBP from Cyt.-LUVs. The second possible explanation for the absence of a decrease in A_{450nm} of Cyt.-LUVs at high K^+ concentration is that fusion of Cyt.-LUVs has occurred. Then, the dissociation of MBP from Cyt.-LUVs would not decrease the size of the fused vesicles or A_{450nm}.

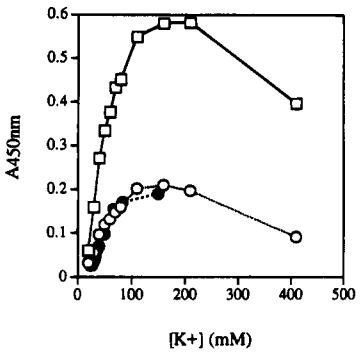

FIGURE 5: Reversibility of MBP-induced aggregation of Cyt.-LUVs. The lipid concentration in the assay system was 0.6 mM before extrusion. The original MBP concentration was 7.5 μg/mL, and the lipid:MBP (w/w) ratio was 50:1. The original aggregated LUVs at each K^+ concentration (squares) were diluted 1:2 (v/v) either with the respective buffer containing the same K^+ concentration (open circles) or with buffer containing 20 mM K^+, resulting in final K^+ concentrations of 20−150 mM (filled circles). The decrease in aggregation of samples initially at K^+ concentrations of 40−410 mM, caused by dilution with a low K^+ concentration, indicates that the aggregation was reversible and that fusion had not occurred in the K^+ concentration range of 40−410 mM.

In order to distinguish between the two possible explanations, a low concentration of KCl (20 mM) was added to each sample of the aggregated LUVs after inducing their aggregation with MBP at 20−410 mM KCl (Figure 5). A_{450nm} of the aggregated LUVs decreased at low final concentrations of KCl (23−150 mM) to the level expected for aggregation at these concentrations, indicating that the aggregated LUVs dissociated when the ionic strength was lowered. Thus, fusion had not occurred. This suggests that Cyt.-LUVs plus MBP also undergo aggregation but not fusion. Disaggregation due to dissociation of MBP occurred only at high KCl concentrations above 210 mM and lower MBP to lipid ratios (Figures 4A and 5). In addition, most of the lipid (98%) and MBP (75%) were still found in the aggregates of (Cyt.+CBS)-LUVs, resulting in the high A_{450nm}, even at the KCl concentration of 210 mM (Table 2, Cyt.+CBS). This confirmed that most of the MBP was not dissociated from (Cyt.+CBS)-LUVs by 210 mM KCl.

Another interesting point to notice with regard to Cyt.-LUVs is that the maximum A_{450nm} is much higher than PC-acidic lipid LUVs (Table 1), especially at high lipid to MBP ratios, suggesting greater maximum aggregation. However, Cyt.-LUVs require more K^+ than the PC-acidic lipid LUVs to achieve this maximal aggregation. Therefore, MBP seems to bind to Cyt.-LUVs with a higher affinity than to PC-acidic lipid LUVs, but K^+ does not shield the charge of the unbound lipid in Cyt.-LUVs as well as in PC-acidic lipid LUVs. This higher affinity must be due to lipids other than PC, PS, and CBS in the lipid mixture. It may result from greater hydrogen bonding of MBP with Cyt.-LUVs than with PC-acidic lipid LUVs since Cyt.-LUVs have additional hydrogen bonding capacity due to hydroxyl groups of cholesterol (44 mol %) and PI and a primary amino group of PE. The higher affinity of MBP for Cyt.-LUVs might also come from greater hydrophobic interaction between MBP and Cyt.-LUVs.

Both PE and Cholesterol in Cyt.-LUVs Are Responsible for High A_{450nm} at High $[K^+]$. In order to determine which lipid component causes the higher affinity binding of MBP to Cyt.-LUVs, either PE or cholesterol was replaced with PC. The aggregation of Cyt.-LUVs lacking PE showed a decrease in A_{450nm} at high KCl concentrations (Figure 4B), contrary to Cyt.-LUVs with and without CBS (Figure 4A). The aggregation of Cyt.-LUVs lacking cholesterol showed an even greater decrease in A_{450nm} (Figure 4C). The KCl dependence of aggregation of these Cyt.-LUVs lacking either PE or cholesterol is more like that of PC LUVs containing any single acidic lipid than like that of Cyt.-LUVs. Therefore, it seems that PE and cholesterol synergistically promote the aggregation of Cyt.-LUVs at high KCl concentrations. To conclude, both PE and cholesterol are required for the high-affinity binding of MBP to Cyt.-LUVs, especially at high K^+ concentrations. This high-affinity binding may be due to greater hydrogen bonding and/or greater hydrophobic interaction of MBP with Cyt.-LUVs containing both PE and cholesterol.

Table 4: Extent of Hydrophobic Photolabeling by [^{125}I]TID of MBP from MBP-Aggregated PC-Acidic Lipid Vesicles and MBP-Aggregated (Cyt.+CBS)-Vesicles

content of acidic lipid		extent of [^{125}I]TID labeling of lipid-bound MBP[a]	
		expt 1 (LUVs)[b]	expt 2 (MLVs)[c]
(I)	20% PG	7.7 ± 0.2	5.2 ± 0.4
(II)	20% CBS	6.9 ± 0.1	3.3 ± 0.1
(III)	Cyt.+CBS (19% acidic lipid)	11.3 ± 0.4	7.1 ± 0.5

[a] The ratio of the specific activity of [^{125}I]TID-labeled MBP bound to lipid vesicles to the control specific activity of [^{125}I]TID-labeled MBP in solution (in the presence of 100% PC vesicles) at 80 mM K^+. The values obtained from duplicate samples run on separate gels were averaged, and the range is indicated. [b] The amounts of lipid in vesicles I, II, and III were 4.2, 3.5, and 3.6 mg, respectively. [c] The amounts of lipid in vesicles I, II, and III were 4.7, 4.7, and 3.7 mg, respectively, and they were 6.0 μmol each.

Greater Hydrophobic Interaction of MBP with Cyt.-Vesicles than with PC-Acidic Lipid Vesicles. The hydrophobic photolabel [^{125}I]TID was used to label MBP in aqueous solution and bound to lipid vesicles of different composition. In a previous study, MBP was labeled to some extent by [^{125}I]TID when in aqueous solution (in the presence of PC vesicles added to maintain a similar lipid concentration as when acidic lipids were used), indicating that it has a hydrophobic site which can bind the reagent (Boggs et al., 1988). However, MBP was labeled 2−4 times more when bound to acidic lipids than when in solution in the presence of PC vesicles. It was also labeled more than polylysine bound to acidic lipids. Therefore, although not a membrane-spanning intrinsic protein, when bound to acidic lipids, MBP was labeled from the lipid bilayer rather than from the aqueous phase (Boggs et al., 1988). Accordingly, the extent of hydrophobic photolabeling by [^{125}I]TID might indicate the degree of penetration of hydrophobic amino acid side chains of MBP into the bilayer. In the present study, the hydrophobic photolabeling of MBP-aggregated vesicles of different lipid composition was examined in order to compare the extent of hydrophobic interaction between MBP and lipid vesicles of different lipid composition.

First of all, PC LUVs containing either 20% PG or 20% CBS and (Cyt.+CBS)-LUVs were aggregated by MBP at 80 mM K^+, and they were photolabeled by [^{125}I]TID. Typically 0.08−0.1% of the TID added was transferred to MBP bound to Cyt.-LUVs at the lipid to MBP ratios used. The ratio of the specific activity of [^{125}I]TID labeling of MBP bound to lipid vesicles to that of the control is presented in Table 4. MBP was labeled much more (3−11 times) when

Table 5: Extent of Hydrophobic Photolabeling by [^{125}I]TID of MBP from MBP-Aggregated PC-Acidic Lipid MLVs and MBP-Aggregated Cyt.-MLVs with Varying Lipid Composition[a]

	lipid composition	extent of [^{125}I]TID labeling of lipid-bound MBP[b]
(I)	20% PS	1.4 ± 0.3
(II)	20% CBS	1.7 ± 0.2
(III)	Cyt.+CBS (19% acidic lipid)	2.3 ± 0.2
(IV)	Cyt. (15% acidic lipid)	2.3 ± 0.2
(V)	Cyt. lacking chol[c] (15% acidic lipid)	1.0 ± 0.1
(VI)	Cyt. lacking PE[c] (15% acidic lipid)	1.6 ± 0.1
(VII)	Cyt. lacking PI[d] (15% acidic lipid)	2.5 ± 0.1

[a] The amounts of lipid in vesicles I−VII were 3.6 mg each. K^+ concentration was 80 mM. [b] See footnote *a* in Table 4. [c] The cholesterol and PE omitted from the Cyt.-lipid mixture were replaced with PC. [d] The PI omitted was replaced with PS.

bound to lipid vesicles than when in solution (in the presence of PC). MBP bound to (Cyt.+CBS)-LUVs or MLVs was labeled by [^{125}I]TID significantly more than when bound to PC-acidic lipid LUVs or MLVs (Table 4). However, the weight of lipid present in the photolabeling reaction mixture differed slightly among the three samples (I−III) although they all contained 6 μmol of lipids in expt 2. This difference in weight might account for the greater labeling of MBP in the presence of (Cyt.+CBS)-vesicles since these vesicles have the least weight amount of lipid (in expt 2); thus, more [^{125}I]TID is available for labeling MBP.

Therefore, the same weight of lipid was used for each type of vesicle in the third experiment (Table 5). The ratio of labeling of lipid-bound MBP to that in solution was less than in the previous two experiments. However, MBP bound to (Cyt.+CBS)-MLVs was still labeled by [^{125}I]TID significantly more than when bound to PC-acidic lipid MLVs (Table 5, I−III). Similar results were obtained for Cyt.-MLVs lacking CBS (Table 5, IV) even though Cyt.-MLVs contained less total acidic lipid (15%) than (Cyt.+CBS)-MLVs (19%). This is consistent with the results of the aggregation assay in which Cyt.-LUVs with and without CBS were aggregated similarly by MBP. The results presented in Table 5 (I−IV) suggest that hydrophobic side chains of MBP may penetrate into the bilayer of Cyt.-MLVs more than into PC-acidic lipid MLVs. This greater hydrophobic interaction must be due to lipids other than PC, PS, and CBS in the lipid mixture.

In order to determine which lipid component in Cyt.-MLVs allows greater hydrophobic interaction with MBP and greater [^{125}I]TID labeling, one of the lipid components in Cyt.-MLVs was eliminated at a time, and the effect on [^{125}I]-TID labeling was determined. When 44 mol % cholesterol was replaced with another neutral lipid, PC, in the lipid mixture of Cyt.-MLVs, the extent of hydrophobic labeling of MBP dropped dramatically almost to the level of MBP in solution (Table 5, IV and V). When 27 mol % PE was replaced with PC, the extent of hydrophobic labeling of MBP also decreased (Table 5, IV and VI). On the other hand, the replacement of 2% PI with another acidic lipid, PS, did not cause a significant difference (Table 5, IV and VII).

Therefore, the greater extent of hydrophobic interaction of MBP with Cyt.-MLVs than with PC-acidic lipid MLVs seems to result mainly from cholesterol and PE in the lipid mixture. Greater hydrophobic and hydrogen bonding interactions of MBP with Cyt.-vesicles caused by cholesterol and PE could contribute to its greater ability to aggregate these vesicles and to its higher binding affinity, making it resistant to the dissociating effects of high KCl concentrations.

DISCUSSION

The interaction of MBP with membranes has been intensively studied since MBP is believed to be responsible for maintaining the intercytosolic membrane interactions of the myelin sheath in the CNS through membrane cross-linking. This is probably mediated by dimerization of MBP monomers which are bound by electrostatic and other interactions to apposing cytoplasmic leaflets. Although MBP monomers are able to cross-link detergent micelles (Smith & McDonald, 1979), other studies of MBP association with phospholipid vesicles demonstrate that aggregation of vesicles is not inhibited at high MBP concentrations, suggesting that MBP probably cross-links apposed bilayers as a dimer (Lampe et al., 1983). We have confirmed this with the lipid systems used in this study (not shown). Self-association of MBP occurs and is enhanced by detergent binding (Smith, 1982). Dimerization of MBP in myelin has been detected using cross-linking reagents (Golds & Braun, 1978a,b).

Although many studies have focused on the electrostatic interaction of MBP with membranes containing acidic lipids, the effect of a physiological change in ionic strength on the ability of MBP to cross-link apposed bilayers has not been extensively considered especially at a low MBP to lipid ratio. In order to determine what factors promote or perturb MBP−membrane and MBP−MBP interactions, we employed a model system for cross-linking of membranes, in which MBP-induced aggregation of LUVs was assessed by measuring light scattering. By varying (i) the ionic strength, (ii) the MBP concentration, (iii) the type and content of acidic lipid, and (iv) the lipid composition, the effect on the degree of MBP-induced aggregation was examined. Since the K^+ concentration in the oligodendrocyte is much higher than that of Na^+, the effect of K^+ was emphasized, assuming that similar concentrations of K^+ and Na^+ occur in the paranodal loops and other cytoplasmic enclosures in myelin. However, the effect of NaCl was also measured and was found to be similar to that of KCl. Thus, the K^+ and Na^+ in the cytoplasmic regions will have an additive effect.

First of all, MBP-induced aggregation of LUVs was dependent on the K^+ concentration in all experiments involving acidic lipids. Generally, as K^+ concentration was increased, the degree of MBP-induced aggregation increased up to a maximum and then decreased. A significant increase in A_{450nm} was observed for an increase of K^+ concentration of 10 mM at physiological intracellular K^+ concentrations (60−74 mM), particularly for the Cyt.-LUVs.

The intracellular K^+ concentration in oligodendrocytes rises when the extracellular K^+ concentration is increased *in vitro* (Kettenmann et al., 1983). In the CNS, K^+ uptake by oligodendrocytes or myelin paranodal loops might help regulate the extracellular K^+ concentration which increases after the axonal action potential. The change in intracellular K^+ concentration may be no more than 2−5 mM. On the other hand, Chiu (1991) has calculated that the K^+ concentration in the extracellular nodal space could increase by as much as 54 mM if all the leakage current causing repolarization of the axon is due to K^+. This would cause a greater increase in the K^+ concentration in the myelin cytosolic paranodal loops around nodes of Ranvier *in vivo*. Therefore,

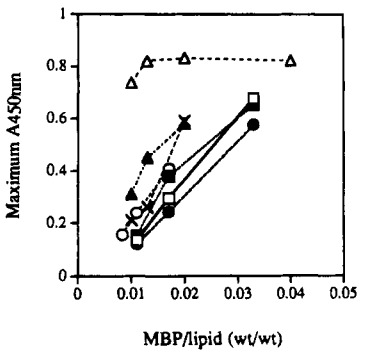

FIGURE 6: MBP concentration dependence of MBP-induced maximum aggregation. The maximum A_{450nm} values in Table 1 are plotted against the MBP to lipid ratio for PC LUVs containing 20% PG (open squares), 10% PG (filled squares), 20% PS (open circles), and 20% CBS (filled circles), and for (Cyt.+CBS)-LUVs (open triangles), Cyt.-LUVs lacking PE (filled triangles), and Cyt.-LUVs lacking cholesterol (X).

it could affect the degree of MBP-induced adhesion between the cytosolic surfaces in the loops by altering the degree of shielding of the negatively charged membranes. This could regulate the cytosolic volume of the loops.

Similar effects might occur in PNS myelin which also contains some MBP and has more cytoplasm than CNS myelin. Interestingly, the Schmidt–Lanterman clefts containing cytoplasm in compact PNS myelin have been observed to open and close by time-lapse photography (Gitlin & Singer, 1974), indicating a dynamic process in myelin. Both MBP and another protein, PO, are probably responsible for adhesion of cytosolic surfaces in compact PNS myelin since mutants lacking either MBP (Shiverer) or PO still have some compact PNS myelin with a major dense line (Inouye et al., 1985; Martini et al., 1995). However, it is not known which proteins are present in the paranodal loops and Schmidt–Lanterman clefts in the PNS to possibly regulate opening and closing under some conditions. These clefts and other cytoplasmic enclosures are more frequent in the Shiverer PNS myelin than in normal mouse myelin (Gould et al., 1995; Rosenbluth, 1980), suggesting the absence of MBP might be directly responsible. Ca^{2+}-induced swelling of the cytoplasmic space of fish CNS myelin involving a metabolic effect has been observed and also suggested to play a role in the formation of cytoplasmic openings in compact myelin (Blaurock et al., 1986).

An inverse dependence of MBP-induced aggregation on ionic strength was observed at high KCl concentrations. After a maximum aggregation was reached, further increase in K^+ concentration caused MBP to dissociate from PC-acidic lipid LUVs. However, lipid vesicles containing the lipid composition characteristic of the intracellular surface of myelin were particularly resistant to the dissociating effects of high salt. Consistent with this result, high salt does not cause swelling of myelin at either the intracellular or the extracellular appositions (Melchior et al., 1979; Hollingshead et al., 1981; Blaurock, 1971). Rather, further compaction at both surfaces was observed due to exclusion of proteins from the compacted areas.

The second factor that determines the extent of MBP-induced aggregation of LUVs is the MBP concentration. To compare the maximum aggregation of various LUVs upon increasing the amount of MBP, the maximum A_{450nm} was plotted against the MBP to lipid ratio (Figure 6). For PC LUVs containing 10–20% PG, PS, or CBS, the maximum extent of aggregation was in proportion to the MBP:lipid ratio, and the dependence on MBP:lipid ratio was relatively similar for all three acidic lipids. However, the dependence on MBP:lipid ratio was much different for the (Cyt.+CBS)-LUVs. Much less MBP was required to cause maximal aggregation of (Cyt.+CBS)-LUVs than of PC LUVs containing an acidic lipid. Cyt.-LUVs lacking cholesterol or PE behaved more like PC-acidic lipid LUVs. This might be due to a higher affinity of MBP for (Cyt.+CBS)-LUVs, or it might be due to an altered conformation which is better able to cause vesicle aggregation through protein dimerization.

Although in myelin there are other proteins which might affect the extent of MBP–membrane interaction, the actual weight ratio of lipid to MBP in isolated compact myelin is about 10:1, which is much lower than in our experimental system. At this ratio, LUV aggregation would be complete even in the absence of salt and would be insensitive to salt concentration in the range below 100 mM. Thus, changes in salt concentration would not affect MBP-induced adhesion of compact myelin, consistent with X-ray diffraction studies which show no effect on the cytoplasmic spacing of PNS and CNS myelin (Kirschner & Blaurock, 1992). However, electron microscopic immunocytochemical studies showed that MBP concentration was much lower in the paranodal loop regions than in compact myelin (Brunner et al., 1989; Omlin et al., 1982). Therefore, the ratio of lipid to MBP in the paranodal loops, where a change in K^+ concentration is most likely to occur, may be much higher than 10:1. Then, the lipid:MBP ratio of 25:1 to 120:1 in our experiments might be in a reasonably physiological range. Hence, both the MBP concentration dependence and the K^+ concentration dependence of MBP-induced aggregation indicate possible factors that may affect the dynamic functions of myelin.

According to our results, we would expect low salt to cause an increase of the cytoplasmic volume of the paranodal loops and other regions where the MBP to lipid ratio is low, similar to the swelling which occurs at the extracellular surfaces under hypotonic conditions (Kirschner & Blaurock, 1992). However, X-ray diffraction studies show that a small decrease in the cytoplasmic spacing of compact PNS myelin actually occurs under hypotonic conditions (Blaurock, 1971; Inouye et al., 1989). This has been attributed to movement of salts and their water of hydration out of the cytoplasmic space or to increased dipole–dipole attraction at low ionic strength. Lack of swelling must be due to adhesion of the cytosolic surfaces by protein. This phenomenon observed in compact myelin is thus different from the type of effect which is expected in the paranodal loops where MBP content is low, provided that the cytoplasmic surfaces have a net charge.

The third factor that affects the extent of MBP-induced aggregation of LUVs is the type and content of acidic lipid in LUVs. As was seen in Table 1, $[K^+]_{50}$ values of different LUVs varied in the order of Cyt. > 20% PG > 20% PS > 20% CBS > 10% PG. The differences in $[K^+]_{50}$ among 20% PG, PS, and CBS might be due to the greater efficiency of K^+ in shielding the negative charge on CBS or PS than PG.

Furthermore, the composition of neutral lipid in LUVs is also a determinant of the extent of MBP-induced aggregation of LUVs. When LUVs with a lipid composition thought to be similar to that of the cytoplasmic leaflet of myelin (Cyt.-

LUVs) were examined, the maximum A_{450nm} was higher than for PC LUVs containing any single acidic lipid. In addition to having a higher affinity for Cyt.-LUVs or a greater ability to aggregate and/or dimerize when bound to these LUVs, MBP was not dissociated as readily at high K^+ concentrations, indicating that electrostatic interactions were not the only force involved in binding of MBP to Cyt.-lipids. Additional interactions of MBP with a myelin lipid extract, besides electrostatic interactions, have also been suggested by Sedzik et al. (1984). Since different lipids would contribute different properties to the binding sites on the lipid bilayer for MBP, MBP could bind to Cyt.-LUVs by a greater variety of interactions than to PC-acidic lipid LUVs. Among the lipid components in Cyt.-LUVs, PE and cholesterol were found to be most responsible for the greater MBP-induced aggregation and for the higher affinity binding of MBP to Cyt.-LUVs at high K^+ concentrations. Others have found that cholesterol increases the ability of MBP to aggregate PC, PS, and PC/PE liposomes (ter Beest & Hoekstra, 1993; Walker & Rumsby, 1985; Surewicz et al., 1986). Although PE is a zwitterionic lipid and binds less MBP than acidic lipids (Boggs & Moscarello, 1978), MBP aggregates and fuses SUVs of pure PE (Stollery & Vail, 1977). Therefore, the effect of cholesterol and PE on LUVs containing acidic lipids in our study is consistent with earlier studies. Cholesterol and PE could interact with MBP by hydrogen bonding interactions. The greater TID labeling of MBP bound to Cyt.-LUVs indicates that greater hydrophobic interaction also occurs in the presence of cholesterol and PE. Thus, not only electrostatic interaction but also hydrogen bonding and hydrophobic interactions between MBP amino acid side chains and lipids may contribute to the compaction of the myelin sheath and resistance to dissociation of MBP by salt.

Therefore, the lipid composition of the intracellular surface of myelin is well suited to allow MBP-mediated adhesion of apposing intracellular membranes. MBP-mediated aggregation of Cyt.-LUVs was also more sensitive to changes in K^+ concentration in the physiological intracellular K^+ concentration range than other PC-acidic lipid LUVs. Thus, this lipid composition is also well suited to allow MBP interactions to respond in a dynamic way to changes in the salt environment resulting from nerve conduction.

To conclude, there are at least four factors that affect MBP-induced adhesion of bilayers and possibly stabilization of the multilayered structure of myelin. They are (i) the KCl concentration, (ii) the MBP concentration, (iii) the type and content of acidic lipid, and (iv) the lipid composition. Most of all, the sensitivity to salt concentration of MBP-induced aggregation may be physiologically important since extracellular K^+ is taken up by oligodendrocytes and paranodal myelin loops into the cytoplasm during the action potential. This provides a potential role for MBP in subtle regulation of myelin dynamics.

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