Effect of Posttranslational Modifications to Myelin Basic Protein on Its Ability to Aggregate Acidic Lipid Vesicles†

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ABSTRACT: When isolated from central nervous system myelin, myelin basic protein (MBP) exhibits charge microheterogeneity due to posttranslational deamidation, phosphorylation, and deimination of arginine to citrulline. These modifications are known to decrease the ability of MBP to aggregate acidic lipid vesicles and thus could regulate the ability of MBP to mediate adhesion between the intracellular surfaces of myelin. The effects of salt (KCl) concentration and the protein to lipid ratio on the ability of charge isomers of MBP to aggregate large unilamellar vesicles (LUVs) were investigated. Increased salt concentration from 10 to 100 mM caused increasing aggregation of LUVs by low concentrations of all charge isomers but did not eliminate the differences in their abilities to aggregate. All isomers were bound equally up to about 100 mM K^+ but were dissociated at higher K^+ concentrations. The degree of dissociation increased with increasing net negative charge of the isomer. At high concentrations all charge isomers except the form in which six arginine residues are converted to citrulline (C8) aggregated LUVs of phosphatidylcholine/phosphatidylserine (PC/PS) 8:2 (mol/mol) similarly and salt increased the aggregation to the same degree for all. There was less difference in the ability of the charge isomers, including C8, to aggregate LUVs with a lipid composition resembling that of the cytoplasmic leaflet of myelin (Cyt-LUVs) than for PC/PS LUVs. Furthermore, high salt concentrations (400 mM) did not dissociate any of the charge isomers from the Cyt-LUVs. These results suggest that the reason for inhibition of aggregating ability by charge modification is not increased charge repulsion of the protein but rather its reduced multivalency of net positive charge. They indicate further that the lipid composition of the cytoplasmic leaflet is ideally suited to permit MBP-mediated adhesion and that charge modifications of MBP would probably not affect adhesion of the intracellular surfaces of compact myelin where MBP concentration is high. However, charge modifications might affect adhesion in cytoplasm-containing regions of myelin such as the paranodal loops, where MBP concentration is low and where K^+ concentration may vary in the range of 60−75 mM.

About 30% of the protein of central nervous system (CNS) myelin consists of a single protein, myelin basic protein (MBP).[1] This is a water-soluble membrane protein which has been localized to the cytoplasmic side of the myelin membrane (Omlin et al., 1982; Kirschner & Blaurock, 1992). It undoubtedly plays a major role in adhesion of the intracellular surfaces of compact multilayered myelin in the CNS through protein−protein and/or protein−lipid interactions (Smith, 1977; Golds & Braun, 1978a,b; Readhead et al., 1990). It interacts electrostatically with acidic lipids and can aggregate lipid vesicles, especially if they contain acidic lipids (Smith, 1977; Lampe et al., 1983; Walker & Rumsby, 1985; Cheifetz & Moscarello, 1985; Surewicz et al., 1986; ter Beest & Hoekstra, 1993; Jo & Boggs, 1995).

At low protein to lipid ratios where the vesicles have an excess negative charge, MBP-induced aggregation of the vesicles is inhibited by electrostatic double-layer charge repulsion (Cowley et al., 1978) in addition to short-range hydration forces (Rand & Parsegian, 1989). Electrostatic repulsion can be decreased by addition of salt (NaCl or KCl), which shields the charge on the vesicles (Jo & Boggs, 1995). Compact myelin contains such a high MBP to acidic lipid ratio that virtually all of the acidic lipid would be neutralized by binding to protein, and charge repulsion of the intracellular surfaces would probably not be a factor in affecting adhesion. However, the concentration of MBP is much lower in the cytosol-containing paranodal loops of myelin (Brunner et al., 1989; Omlin et al., 1982), which are continuous with compact myelin (Raine, 1977; Kirschner & Blaurock, 1992). Charge repulsion of the intracellular surfaces may be the reason for the lack of compaction and presence of cytosol in these regions. The salt concentration in the cytosol of the paranodal loops may vary with axonal activity since the membrane of the loops contains K^+ channels (Chiu, 1991; Sapirstein et al., 1993) and should be able to take up K^+

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[1] Abbreviations: MBP, myelin basic protein; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; DPPC, dipalmitoylphosphatidylcholine; chol, cholesterol; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; Cyt-LUVs, large unilamellar vesicles consisting of lipids characteristic of the cytosolic side of the myelin membrane; Cit, citrulline.

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released into the extracellular space around the nodes of Ranvier during the nerve action potential, as occurs in oligodendrocytes (Kettenmann et al., 1983). The use of fluorescent dyes whose optical properties are sensitive to changes in membrane potential suggests that this does indeed occur in optic nerve preparations (Lev-Ram & Grinvald, 1986). Increased cytosolic K^+ in the paranodal loops could modulate the ability of MBP to cause adhesion of the intracellular surfaces of the loops and regulate their volume.

Posttranslational modifications to MBP which increase its negative charge, such as deamidation and phosphorylation (Chou et al., 1976; Deibler et al., 1975; Martenson et al., 1983), or which decrease its positive charge by deimination of arginine to citrulline (Wood & Moscarello, 1989), may also affect its ability to cause adhesion of the intracellular surfaces of myelin. MBP isolated from myelin exhibits charge microheterogeneity due to these modifications. These charge isomers are termed C1−C8 in order of decreasing positive charge and increasing degree of modification (Deibler & Martenson, 1973). C2 differs from C1 primarily by deamidation, while C3, C4, and C5 differ primarily by varying combinations of deamidation and phosphorylation. C8 differs from C1 by deimination of at least 6 of the 19 Arg to Cit (Wood & Moscarello, 1989).

These modifications may have regulatory effects on MBP function. The phosphate groups of MBP turn over rapidly, suggesting they play a dynamic role in myelin structure and function (DesJardins & Morell, 1983). Phosphorylation of MBP in myelinated optic nerve increases in response to the nerve action potential (Murray & Steck, 1984). It also has been reported to increase in cultured oligodendrocytes when they adhere to a substratum (Vartainan et al., 1986) and decrease when the cells are depolarized (Soliven et al., 1994).

Charge modifications have been shown to decrease the ability of MBP to aggregate lipid vesicles and to decrease its ability to form multilamellar structures out of single-layered vesicles under some conditions, as detected by X-ray diffraction (Cheifetz & Moscarello, 1985; Wood & Moscarello, 1989; Brady et al., 1981). However, the dependence on protein to lipid ratio, lipid composition, and salt concentration has not been investigated. In the present study we investigate these factors. The results indicate that these posttranslational modifications could affect the ability of MBP to cause adhesion in cytosolic regions where its concentration is low, such as the paranodal loops, but they are unlikely to have any effect on this function in compact myelin where its concentration is high. In these cytosolic regions the ability of all the charge isomers to mediate adhesion of the intracellular surfaces would likely be regulated similarly by changes in salt concentration. They further indicate that the decreased ability of these more negatively charged isomers to cause aggregation of acidic lipid vesicles persisted in the presence of salt, indicating that it is not directly due to increased charge repulsion by the protein. Rather, it is more likely due to the reduced multivalency of net positive charge of the protein.

METHODS AND MATERIALS

Lipids and Protein. Bovine myelin basic protein (MBP) was isolated from bovine brain white matter according to the method of Cheifetz and Moscarello (1985). It was separated into its charge isomers C1−C5 by ion-exchange chromatography at alkaline pH on CM52 as described (Chou et al., 1976; Cheifetz et al., 1984). Since C8 could not be detected in CM52 fractions of bovine or calf MBP, C8 was isolated from human brain. Human MBP was isolated similarly from human white matter obtained postmortem from Alzheimer's disease patients and supplied by the Canadian Brain Tissue Bank, Toronto, Canada. It was fractionated on a CM52 column and the second peak eluting from the CM52 column before application of the salt gradient was used to purify human C8 by gel-permeation chromatography on Sephadex G75 as described (Koshy & Boggs, 1993). Egg L-α-phosphatidylcholine (egg PC) was purchased from Sigma (St. Louis, MO). Bovine brain L-α-phosphatidylserine (PS), bovine brain L-α-phosphatidylethanolamine (PE), bovine liver L-α-phosphatidylinositol (PI), and bovine brain sphingomyelin (SM) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (chol) was from Supelco, Inc. (Bellefonte, PA). Lipids were chromatographically pure and were used as supplied. [^{3}H]Dipalmitoylphosphatidylcholine (DPPC) (58.0 Ci/mmol) was purchased from Dupont−NEN (Boston, MA).

Preparation of Large Unilamellar Vesicles. Aliquots of chloroform 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), 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. 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 of the LUVs after extrusion. The solvent was removed by evaporation under a stream of nitrogen, and the lipid was redissolved in 1 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 to a final lipid concentration of 30−60 mM. MLVs were formed by vortexing the suspension for 15 min at room temperature followed by freezing and thawing (10 times) in a dry ice/acetone bath and a 40 °C water bath to allow equilibrium transmembrane solute distribution (Mayer et al., 1985). LUVs were prepared with an Avestin LiposoFast extruder (Ottawa, ON) by passing the suspension through 400 nm pore size polycarbonate filters, followed by 200 nm pore filters and then by 100 nm pore filters 19 times under manual pressure (MacDonald et al., 1991). The LUVs were stored at 4 °C until use (within 24 h) and aliquots were taken for phosphorus assay by a modified procedure of Bartlett (1959).

Aggregation Assay. Solutions of unfractionated bovine MBP or purified charge isomers were prepared in distilled water at concentrations of 0.5−1.5 mg/mL. The concentration was determined by Peterson assay (1977) and all solutions were adjusted to the same final concentration. Potassium chloride solutions in 20 mM HEPES containing 1 mM EDTA, adjusted to pH 7.4 with KOH, and containing a final concentration of KCl of 10−410 mM (determined by flame atomic absorption spectrophotometry) were prepared. LUVs were diluted 1/10 with KCl solutions of varying concentration, and small aliquots of the protein solutions containing 5−15 μg of protein were added to 0.3−0.6 μmol of lipid in a final volume of 1 mL in a Sarstedt acryl cuvette (Newton, NC) as described earlier (Jo & Boggs,

1995). The cuvette was gently inverted twice to mix and aggregation was measured 10 min later from the light scattering at 450 nm with a Hitachi U-2000 spectrophotometer at room temperature. Absorbance of protein-free LUVs was also measured at different K^+ concentrations and usually subtracted from that of protein-containing LUVs to give ΔAbs_{450}. Their absorbance was low and KCl had little effect.

Quantitation of Lipid to Protein Ratio in Aggregated and Nonaggregated LUVs. These procedures were carried out essentially as described earlier (Jo & Boggs, 1995). Briefly, aggregated LUVs were sedimented and washed by centrifugation at 10000*g* for 10 min, the pellet was resuspended in 1 mL of double-distilled water and aliquots were taken for lipid and protein assay. Nonaggregated LUVs were assayed by first removing aggregated LUVs as described above by low-speed centrifugation. The supernatant was then centrifuged at 95000*g* in a Beckman L3-50 ultracentrifuge for 2 h to sediment nonaggregated LUVs, the pellet was resuspended in 2.5 mL of double-distilled water, and aliquots were taken for lipid and protein assay. Lipid was quantitated from [^{3}H]-DPPC radioactivity using a Beckman LS 6000IC scintillation counter. Protein was assayed by the method of Peterson (1977).

Effect of K^+ on Aggregation of LUVs at Saturating Concentrations of MBP. Varying amounts (2.3−4800 μg) of unfractionated bovine MBP were added to 800 μL of KCL solution containing 10, 50, or 90 mM K^+ in an acryl cuvette. The background absorbance at 450 nm due to the protein was measured at room temperature and subtracted from that after addition of LUVs. PC/PS 9:1 (mol/mol) LUVs (0.28 μmol) in 200 μL of KCl solution was added to the cuvette and gently inverted to mix. The LUVs had been preequilibrated in 10, 50, and 90 mM K^+ solution prior to addition. The absorbance was measured at 450 nm 10 min later at room temperature. For the charge isomers, 200 μg of each was added to 800 μL of either 10 mM K^+ or 80 mM K^+ solution. Background absorbance due to this concentration of protein was only 0.03−0.04. PC/PS 9:1 LUVs (0.3 μmol) were added as above. The sample was inverted twice to mix and the OD at 450 nm was measured at 15 and 25 min following addition of lipid. The cuvettes were inverted again before the absorbance was read to obtain a homogeneous suspension.

RESULTS

The ability of the charge isomers to cause aggregation of PC/PS 8:2 (mol/mol) LUVs at different K^+ concentrations was compared at two protein to lipid ratios, 0.046 and 0.23 mol % protein (Figures 1 and 2, respectively). In both cases the negatively charged lipid is in excess relative to the number of positively charged residues of the protein, 450 PS/MBP (mol/mol) and 86 PS/MBP (mol/mol), respectively. Since C1, the most positively charged isomer, has only 31 positively charged residues at neutral pH (and a net charge of +20), it cannot neutralize all of the charged lipid even at the lower PS to protein ratio. Therefore, as found previously with MBP containing a mixture of isomers, none of the individual isomers could cause much aggregation at low salt concentrations (Jo & Boggs, 1995). Salt shields the charge of the lipid, allowing the vesicles to come closer together so that the bound proteins can overcome the repulsive hydration forces and cause aggregation of the vesicles.

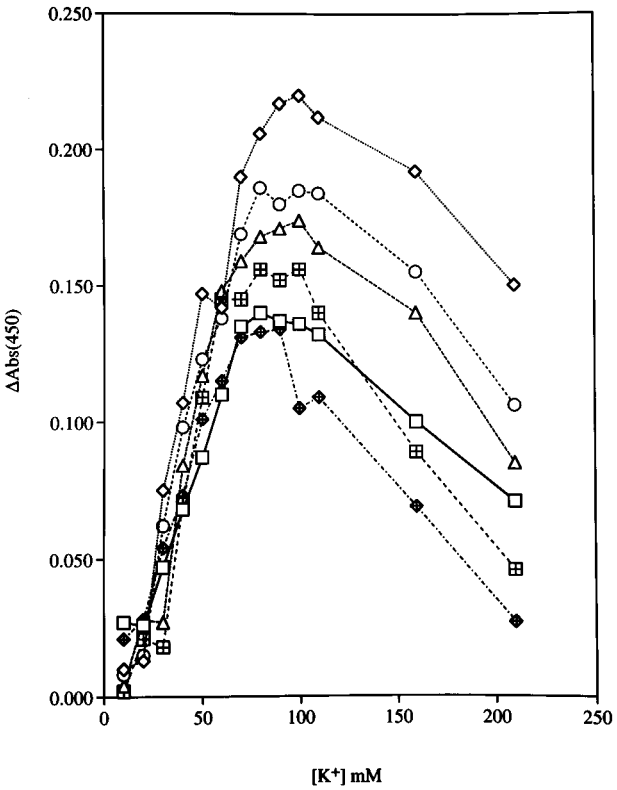

FIGURE 1: Dependence on KCl concentration of absorbance of PC/PS 8:2 LUVs in the presence of 0.046 mol % of unfractionated MBP (□) or different charge isomers, C1 (◇), C2 (○), C3 (△), C4 (plus in box), and C5 (plus in diamond). ΔAbs(450) is the difference in absorbance at 450 nm of protein-bound LUVs from protein-free LUVs. The latter ranged from 0.070 to 0.077.

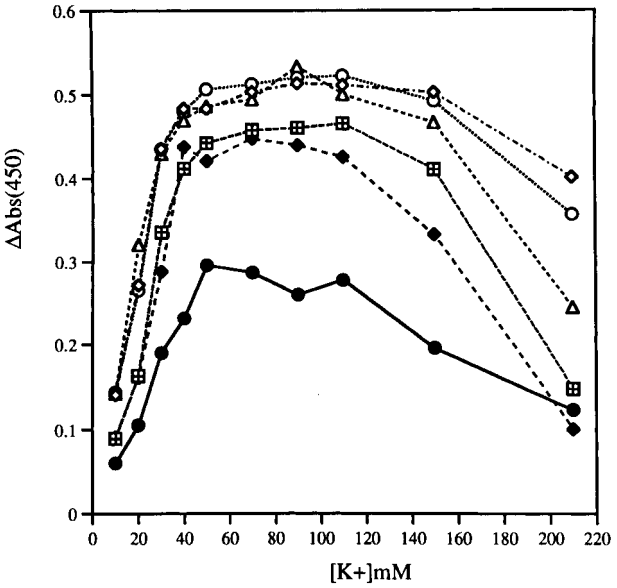

FIGURE 2: Dependence on KCl concentration of absorbance of PC/PS 8:2 LUVs in the presence of 0.23 mol % of different charge isomers, C1 (◇),C2 (○), C3 (△), C4 (plus in box), C5 (◆), and C8 (●). ΔAbs(450) is the difference in absorbance at 450 nm of protein-bound LUVs from protein-free LUVs. The latter ranged from 0.064 to 0.070.

However, addition of salt does not allow all of the charge isomers to aggregate the vesicles to an equal extent, especially at the high lipid to protein ratio. The maximum aggregation at K^+ concentrations of 50−100 mM was greatest for C1 and decreased in the order C1 > C2 > C3 > C4 > C5 at 0.046 mol % protein (Figure 1). The rate of

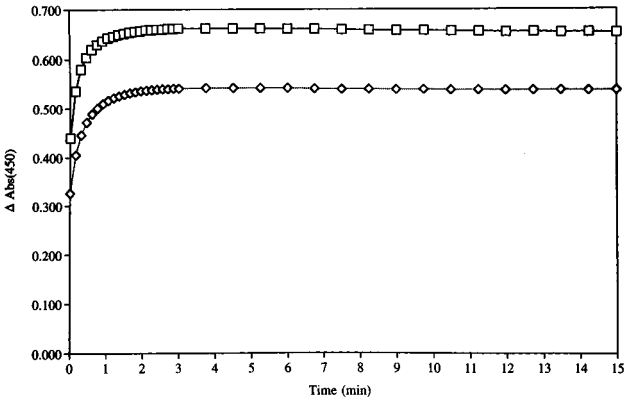

FIGURE 3: Time course of increase in absorbance of PC/PS 8:2 LUVs in the presence of 0.15 mol % C1 (□) and C5 (◇) at 90 mM K^+. The background absorbance of protein-free LUVS of 0.085 has been subtracted to give ΔAbs(450). The protein was added immediately before measurement of absorbance at 0 time.

Table 1: KCl Concentration Required To Cause Aggregation of PC/PS 4:1 (mol/mol) Vesicles by Charge Isomers of MBP and Amount of Protein Bound to Lipid

protein	$[K^+]_{50}$[a] (mM)	$[K^+]_{50}$[b] (mM)	$[K^+]_{50}$[c] (mM)	mol % MBP bound[d]
mixture	43			0.17
C1	40	19	12.5	0.15
C2	43	19	12.5	0.16
C3	44	19	12.5	0.14
C4	45	23	15	0.17
C5	45	23	15	0.16
C8	nd[e]	25	20	nd[e]

[a] Determined at 0.046 mol % of each protein added to PC/PS 8:2 LUVs. [b] Determined at 0.23 mol % of each isomer added to PC/PS 8:2 LUVs. [c] Determined at 0.23 mol % of each isomer added to Cyt− LUVs. [d] Determined at an initial concentration of 0.20 mol % protein and a K^+ concentration of 90 mM. [e] Binding not determined for C8 in this series. However, in another study (Boggs et al., unpublished results) when an initial concentration of protein of 1.7 mol % was added, 0.91 mol % C1 and 0.87 mol % C8 were found bound to PC/PS 4:1 vesicles in 10 mM NaCl. nd, not determined.

aggregation was similar as shown for C1 and C5 at 90 mM K^+ (Figure 3). The effect of unfractionated MBP at the high lipid to protein ratio was in between that of C4 and C5, indicating that its effect was dominated by the more modified isomers (including C8) even though C1 is the predominant charge isomer in the mixture. The amount of K^+ required to permit significant aggregation by the isomers, as indicated by the concentration at which 50% of the maximum aggregation occurred, $[K^+]_{50}$, increased a little in the same order (Table 1, column 2).

At the higher protein concentration (0.23 mol %), all charge isomers caused greater aggregation of PC/PS vesicles and there was less difference in their ability to aggregate (Figure 2). In fact C1, C2, and C3 caused identical aggregation. However, C4 and C5 caused less aggregation than C1, C2, and C3 and required a little higher K^+ concentration to do so (Table 1, column 3). Furthermore, C8, which was also included in the comparison at this protein concentration, caused much less aggregation of PC/PS vesicles than the other isomers. The amount of each isomer bound to the lipid at 90 mM K^+ was similar (Table 1, column 5). The decrease in aggregation at K^+ concentrations above 100−150 mM was shown earlier to be due to dissociation of the protein from the lipid (Jo & Boggs, 1995). However, at these K^+ concentrations, the absorbance decreased more

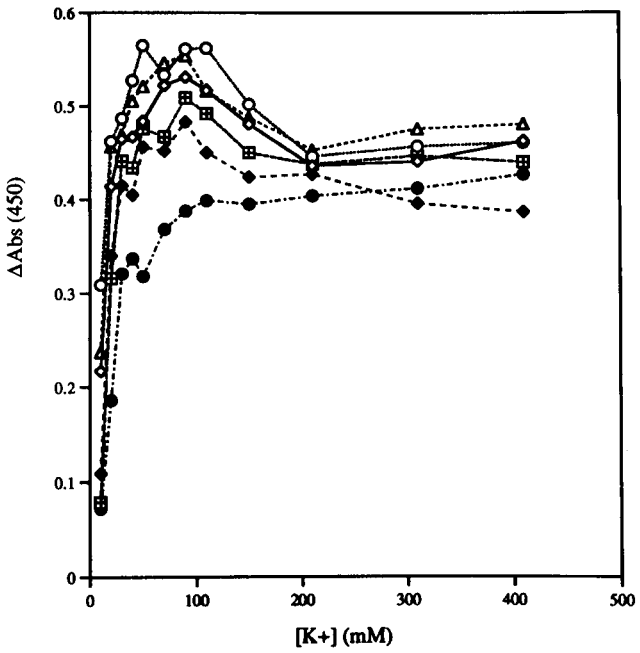

FIGURE 4: Dependence on KCl concentration of absorbance of Cyt-LUVs in the presence of 0.23 mol % of different charge isomers, C1 (◇), C2 (○), C3 (△), C4 (plus in box), C5 (◆), and C8 (●). ΔAbs(450) is the difference in absorbance at 450 nm of protein-bound LUVs from protein-free LUVs. The latter ranged from 0.070−0.085.

sharply for the more modified isomers, C4, C5, and C8, than for C1, C2, and C3, suggesting that salt caused greater dissociation of the less positively charged isomers.

However, for vesicles with a lipid composition thought to be characteristic of the intracellular surface of myelin, as estimated by Inouye and Kirshner (1988), containing 15 mol % acidic lipid (Cyt-LUVs), the degree of aggregation caused by C8 (also at 0.23 mol % protein) was much closer to that of the other charge isomers (Figures 4 and 5), although the amount of K^+ required to induce aggregation by C8 was significantly greater than for C1 (Table 1, column 4). Increased K^+ concentration up to 410 mM did not cause a significant decrease in aggregation for any of the charge isomers, indicating that it did not cause their dissociation from this lipid mixture despite their reduced net positive charge. This difference from the behavior of PC/PS vesicles is especially dramatic for C8. The small decrease in absorbance in Figure 4 between 110 and 210 mM K^+ for C1−C5 was not seen in our earlier study with unfractionated MBP, or in Figure 5 with C1 and C8, and may be due to formation of a smaller number of larger aggregates in the experiment shown in Figure 4. We showed earlier that the aggregation caused by unfractionated MBP was reversible on lowering the K^+ concentration, indicating that significant fusion had not occurred under these conditions (Jo & Boggs, 1995). As the concentration of C8 was lowered from 0.31 and 0.10 mol %, however, it caused much less aggregation of Cyt-LUVs (Figure 5), in contrast to earlier results with unfractionated MBP, which aggregated Cyt-LUVs to a similar extent (at 200 mM K^+) down to protein concentrations of 0.042 mol % (Jo & Boggs, 1995).

If enough MBP is added to the vesicles to saturate all of the acidic lipid, aggregation should occur even at low salt concentrations. Figure 6 (curve 1) shows that this is the case. PC vesicles containing only 10 mol % PS were used in order

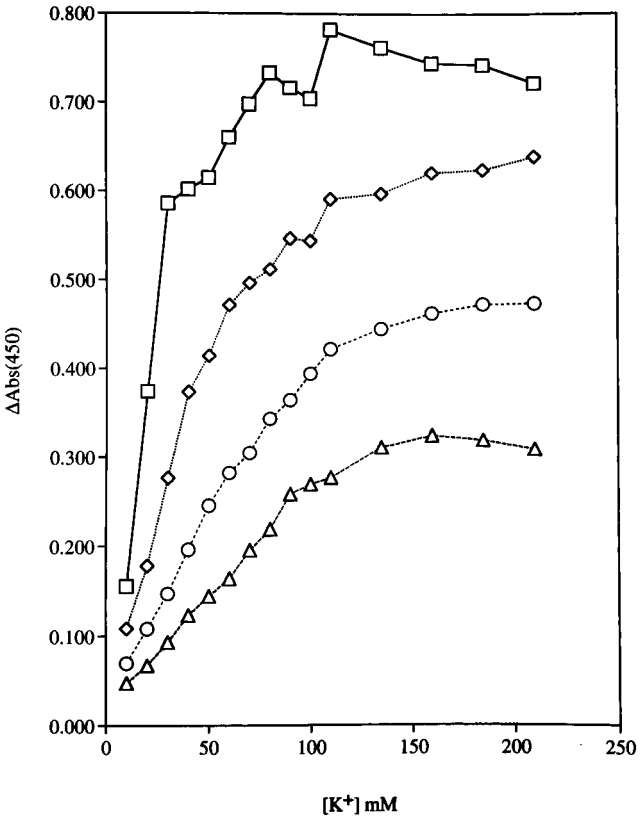

FIGURE 5: Dependence on KCl concentration of absorbance of Cyt-LUVs in the presence of 0.31 mol % C1 (□), 0.31 mol % C8 (◇), 0.15 mol % C8 (○), and 0.10 mol % C8 (△). ΔAbs(450) is the difference in absorbance at 450 nm of protein-bound LUVs from protein-free LUVs. The latter ranged from 0.070 to 0.085.

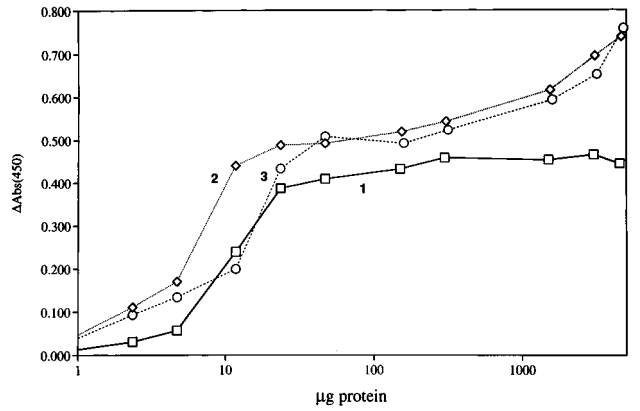

FIGURE 6: Dependence of absorbance on amount of unfractionated MBP added to 0.28 μmol of PC/PS 9:1 LUVs in 1 mL of 10 mM K^+ (□) (curve 1), 50 mM K^+ (◇) (curve 2), and 90 mM K^+ (○) (curve 3). The background absorbance of MBP solutions before addition of LUVs has been subtracted to give ΔAbs(450).

to reduce the amount of MBP required to cause saturation. Maximum aggregation occurred on addition of about 20 μg of MBP to 0.28 μmol of lipid, a mole ratio of PS to MBP of 25, nearly equal to the number of positively charged residues of MBP. Addition of much higher amounts of MBP did not cause dissociation of the vesicles as shown earlier by Lampe et al. (1983). Even if the acidic lipid is completely neutralized by binding to basic residues of MBP at saturation of the lipid, the vesicles will have a net negative surface charge due to the negative residues of MBP (11 per mole for C1 at neutral pH). Addition of 50 mM K^+ (Figure 6, curve 2) was able to increase the maximum aggregation at saturating conditions due to shielding of the charge contributed by the protein, as well as allow lower MBP concentrations to cause maximal aggregation by shielding the charge due to unbound lipid. A further increase to 90 mM K^+ caused no further aggregation, although it increased the MBP concentration required to induce aggregation. This was probably due to increased dissociation of MBP from the lipid, which occurs at a lower K^+ concentration for vesicles containing only 10 mol % PS compared to 20 mol % (Jo & Boggs, 1995).

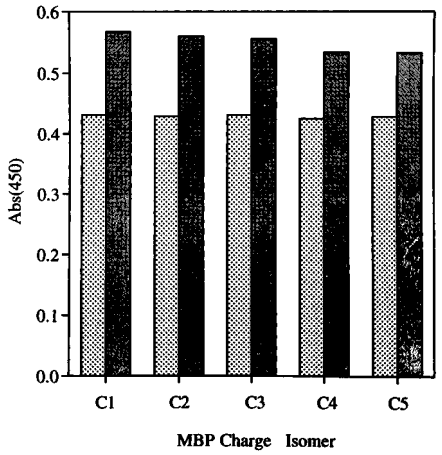

FIGURE 7: Absorbance of PC/PS 9:1 LUVs in the presence of a saturating concentration, 3.7 mol %, of charged isomers C1, C2, C3, C4, and C5 in 10 mM K^+ (dotted bars) and 80 mM K^+ (dark bars) after 15 min. Values were similar after 25 min. Samples were inverted to mix right before measurement of absorbance. Background absorbance of protein solutions before addition of LUVs was 0.032−0.038.

The negative charge contributed by the protein is greater for the more highly modified charge isomers C2−C5. In order to determine if this accounts for the decreased ability of these isomers to cause aggregation, their ability to aggregate PC/PS 9:1 vesicles at a ratio of MBP positively charged residues to PS molecules of 11:1, in excess of the saturating concentration, was compared at 10 and 80 mM K^+. The results show that all five charge isomers caused nearly identical aggregation at this saturating concentration and that 80 mM K^+ increased the degree of aggregation almost to the same extent for all five isomers (Figure 7). Thus increased charge repulsion due to increased negative charge on the protein with increasing modification is probably not a factor in the decreased ability of the charge isomers C1−C5 to cause aggregation at higher lipid to protein ratios.

DISCUSSION

Addition of salt allowed all MBP charge isomers to aggregate acidic lipid vesicles at high lipid to protein ratios where the vesicles have a negative charge, as found earlier for an unfractionated mixture of MBP isomers (Jo & Boggs, 1995). Increasing net negative charge of MBP due to deamidation, phosphorylation, or deimination decreased its ability to aggregate acidic lipid vesicles of PC/PS 8:2 as reported earlier (Cheifetz & Moscarello, 1985; Wood & Moscarello, 1989). However, we show here that the differences in aggregation were greatest at high lipid to protein ratios. The charge of MBP has less effect as the protein to lipid ratio increased, and at saturating concentrations of protein, the charge isomers C1−C5 aggregated similarly. The effect of decreasing positive charge in C8 on aggregation at

saturating conditions was not measured, but at an intermediate protein to lipid ratio, C8 with a net positive charge of 14 aggregated much less well than C5 with a net positive charge of 16. Thus a decrease in the total number of positive residues had a greater effect on the aggregating ability than a decrease in the net positive charge caused by an increase in the number of negatively charged residues.

These results suggest that increasing negative charge due to deamidation or phosphorylation should have little effect on the ability of MBP to maintain adhesion of myelin layers in compact myelin where its concentration is high. Although C8 caused significantly less adhesion of PC/PS 8:2 vesicles than the other charge isomers at a moderate concentration, it aggregated vesicles with a lipid composition thought to be similar to that of the intracellular surfaces of myelin almost as well as the other charge isomers. However, these modifications had a greater effect at lower protein concentrations and thus could have an effect in cytosolic regions of myelin such as the paranodal loops where MBP concentration is low. In those regions, these modifications may regulate the ability of MBP to cause adhesion of the intracellular surfaces and thus regulate the volume of these cytosolic regions. Changes in cytoplasmic salt concentration as a result of nerve activity could exert an additional regulatory role on all charge isomers.

The intracellular myelin lipids also differed from PC/PS 8:2 in that none of the charge isomers including C8 were dissociated from the lipid at high KCl concentrations despite the fact that this mixture has only 15 mol % acidic lipid in contrast to 20 mol % for the PC/PS mixture. Since C8 has a net positive charge of only 14 in contrast to 20 for C1, this supports our earlier suggestion that MBP may interact with this lipid mixture by hydrogen-bonding and hydrophobic interactions in addition to electrostatic interactions (Jo & Boggs, 1995). In that study we showed that both PE and cholesterol in the mixture were necessary to cause the unique interaction of MBP with this liquid mixture. Thus the intracellular myelin membrane appears to have an ideal lipid composition to permit adhesion to these surfaces by all charge isomers of MBP.

The decreased ability of MBP to mediate adhesion of PC/PS bilayers with increasing charge modification of the protein, at lower protein concentrations, could be due to several mechanisms: (i) reduced binding due to reduced multivalency of net positive charge, (ii) increased charge repulsion of the vesicles contributed by negatively charged sites on the protein, (iii) a conformational change, or (iv) steric hindrance by the modifying group, especially phosphate. The possible involvement of some of these depends on whether MBP causes adhesion as a monomer or a dimer. The latter is supported but not proven by the fact that aggregation is not inhibited at high MBP concentrations well above those necessary to saturate all binding sites on the vesicles. However, this fact does not rule out aggregation by monomers since a monomer of MBP bound to one vesicle may be displaced by exchange with a second binding site on a monomer bound to another vesicle, resulting in cross-linking of the two vesicles by a monomer.

Inhibition of aggregation increased with increasing net negative charge of the protein regardless of the type of modification, deamidation, phosphorylation, or deimination, or the sites modified. Although no isomer containing 1 mol of phosphate/mol of MBP was used in this study (C3, C4, and C5 all contain a mixture of phosphorylated and deamidated species), Cheifetz and Moscarello (1984) have shown that in vitro phosphorylation of bovine C1 and C2 with 1.5−1.6 mol of phosphate/mol of MBP reduced their ability to aggregate. Furthermore, in their study, removal of the two C-terminal arginines had a similar effect as addition of 1 mol of phosphate. These facts favor mechanisms (i) and (ii). If mechanisms (iii) and (iv) were involved, the effect of the modification would be expected to depend more on the type of modification as well as on the location of the sites modified.

(i) Decreased Binding. Increased charge modification did not decrease binding of MBP to PC/PS vesicles up to 90 mM K^+. However, MBP could cause aggregation as a monomer if a second site on the protein binds to another vesicle. Increased charge modification might decrease binding of this second site by decreasing the multivalency of net positive charge of the protein. Phosphorylation or replacement of neutral residues with acidic ones in peptides containing a number of basic residues has been found to significantly reduce the binding affinity of the peptides to acidic lipid vesicles (Kim et al., 1994). The MBP charge isomers were dissociated more easily by higher salt concentration as their net positive charge decreased. Although MBP can be phosphorylated on a number of sites using different kinases in vitro, when isolated from bovine myelin it is obtained phosphorylated mainly on Thr 97 and Ser 164 and deamidated mostly at Gln 102 and 146 (Chou et al., 1976). C8 is deiminated at two Arg in the N-terminal half and four Arg in the C-terminal half. Thus most of the modifications in the charge isomers isolated from bovine myelin (human for C8) occur in the C-terminal half. Both halves in C1 have an equal distribution of positively charged residues. If both the N- and C-terminal halves bind independently to lipid vesicles, these modifications could affect interaction of the C-terminal half with a second vesicle without affecting MBP binding via the N-terminal half. Thus a reduction in the net positive charge accounts for the decreased ability of MBP charge isomers to cause aggregation. Reduced positive charge multivalency can occur due either to a reduction in the net positive charge caused by modification of the protein with negatively charged groups or to a reduction in the number of positively charged groups by conversion to neutral groups. The latter would be expected to have more effect; this is supported by the fact that C8 caused less aggregation than C5. Reduced interaction of the C-terminal half with other vesicles could be overcome by an increase in protein concentration which would contribute more cross-links to stabilize the aggregate.

(ii) Charge Repulsion. The increased negative charge of the modified isomers could cause increased charge repulsion of the vesicles, thus inhibiting aggregation. However, the increased charge of the vesicles due to the protein could be shielded by salt and the charge modification should have little effect unless it is at a site directly involved in cross-linking of bilayers. In that case, only certain modifications should have an effect. At high lipid to protein ratios, aggregation still decreased with increasing net negative charge of the isomers even at 50−100 mM K^+, where the negative charge contributed by the lipid is shielded. Under saturating conditions, where all the negative charge due to the acidic lipid is neutralized by basic residues of the protein, increased K^+ concentration did allow increased aggregation

by MBP, indicating that it shielded the negative charge contributed to the vesicles by the acidic residues of the protein. However, all charge isomers of MBP caused similar aggregation even without salt under these conditions. Thus increased charge repulsion due to the modifications is not likely to be a factor in their inhibitory effect on MBP-induced aggregation.

(iii) Conformational Change. A conformational change resulting from the modification could be a factor in reduced aggregating ability regardless of whether a monomer or dimer is involved. Conformation would probably not be affected by salt concentration over the range studied according to a study based on the tryptophan fluorescence of MBP in solution (Nowak & Berman, 1991). However, there is no correlation between the effect of phosphorylation and deamidation on the secondary structure (Ramwani et al., 1989; Deibler et al., 1990) and its aggregating ability. Furthermore, it is unlikely that C-terminal arginine removal would have any effect on the secondary structure, yet it decreases the cross-linking ability.

(iv) Steric Hindrance. If MBP causes aggregation by dimerization, inhibition of aggregation by phosphorylation could be caused by steric hindrance of dimerization due to binding of a phosphate group to the dimerization site. This would not be affected by salt concentration, but other modifications at other sites would not be expected to have the same effect.

Thus the inability of increased salt concentration to overcome the decreased ability of different charge isomers to aggregate vesicles and the fact that a variety of modifications inhibit aggregation supports mechanism (i) for the mechanism of effect of the charge modifications. It also favors the idea that the protein mediates aggregation as a monomer rather than as a dimer.

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REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.
Brady, G. W., Fein, D. B., Wood, D. D., & Moscarello, M. A. (1985) *Biochem. Biophys. Res. Commun. 126*, 1161−1165.
Brunner, C., Lassmann, H., Waehneldt, T. V., Matthieu, J.-M., & Linington, C. (1989) *J. Neurochem. 52*, 296−304.
Cheifetz, S., & Moscarello, M. A. (1985) *Biochemistry 24*, 1909−1914.
Cheifetz, S., Moscarello, M. A., & Deber, C. M. (1984) *Arch. Biochem Biophys. 233*, 151−160.
Chiu, S. Y. (1991) *Glia 4*, 541−558.
Chou, F. C.-H., Chou, C.-H. J., Shapira, R., & Kibler, R. F. (1976) *J. Biol. Chem. 251*, 2671−2679.
Cowley, A. C., Fuller, N. L., Rand, R. P., & Parsegian, V. A. (1978) *Biochemistry 17*, 3163−3168.
Deibler, G. E., & Martenson, R. E. (1973) *J. Biol. Chem. 248*, 2392−2396.
Deibler, G. E., Martenson, R. E., Kramer, A. J., Kies, M. W., & Miyamoto, E. (1975) *J. Biol. Chem. 250*, 7931−7938.
Deibler, G. E., Stone, A. L., & Kies, M. W. (1990) *Proteins: Struct., Funct., Genet. 7*, 32−40.
DesJardins, K. C., & Morell, P. (1983) *J. Cell Biol. 97*, 438−446.
Golds, E. E., & Braun, P. E. (1978a) *J. Biol. Chem. 253*, 8162−8170.
Golds, E. E., & Braun, P. E. (1978b) *J. Biol. Chem. 253*, 8171−8177.
Inouye, H., & Kirshner, D. A. (1988) *Biophys. J. 53*, 247−260.
Jo, E., & Boggs, J. M. (1995) *Biochemistry 34*, 13705−13716.
Kettenmann, H., Sonnhof, U., & Schachner, M. (1983) *J. Neurosci. 3*, 500−505.
Kim, J., Blackshear, P. J., Johnson, J. D., & McLaughlin, S. (1994) *Biophys. J. 67*, 227−237.
Kirschner, D. A., & Blaurock, A. E. (1992) in *Myelin: Biology and Chemistry* (Martenson, R. E., Ed.) pp 1−78, CRC Press, Boca Raton, FL.
Koshy, K. M., & Boggs, J. M. (1993) *Anal. Biochem. 208*, 375−381.
Lampe, P. D., Wei, G. J., & Nelsestuen, G. L. (1983) *Biochemistry 22*, 1594−1599.
Lev-Ram, V., & Grinvald, A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6651−6655.
MacDonald, R. C., MacDonald, R. I., Menco, B. P. M., Takeshita, K., Subbarao, N. K., & Hu, L. (1991) *Biochim. Biophys. Acta 1061*, 297−303.
Martenson, R. E., Law, M. J., & Deibler, G. E. (1983) *J. Biol. Chem. 258*, 930−937.
Mayer, L. D., Hope, M. J., Cullis, P. R., & Janoff, A. S. (1985) *Biochim. Biophys. Acta 817*, 193−196.
Murray, N. I., & Steck, A. J. (1984) *J. Neurochem. 43*, 243−248.
Nowak, M. W., & Berman, H. A. (1991) *Biochemistry 30*, 7642−7651.
Omlin, F. X., Webster, H. deF., Palkovits, C. G., & Cohen, S. R. (1982) *J. Cell. Biol. 95*, 242−248.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346−356.
Raine, C. S. (1977) in *Myelin* (Morell, P., Ed.) pp 1−49, Plenum Press, New York.
Ramwani, J. J., Epand, R. M., & Moscarello, M. A. (1989) *Biochemistry 28*, 6538−6543.
Rand, R. P., & Parsegian, V. A. (1989) *Biochim. Biophys. Acta 988*, 351−376.
Readhead, C., Takasashi, N., Shine, H. D., Saavedra, R., Sidman, R., & Hood, L. (1990) *Ann. N.Y. Acad. Sci. 605*, 280−285.
Sapirstein, V. S., Durrie, R., Nolan, C. E., & Marks, N. (1993) *J. Neurosci. Res. 35*, 83−91.
Smith, R. (1977) *Biochim. Biophys. Acta 470*, 170−184.
Soliven, B., Takeda, M., & Szuchet, S. (1994) *J. Neurosci. Res. 38*, 91−100.
Surewicz, W. K., Epand, R. M., Epand, R. F., Hallett, F. R., & Moscarello, M. A. (1986) *Biochim. Biophys. Acta 863*, 45−52.
ter Beest, M. B. A., & Hoekstra, D. (1993) *Eur. J. Biochem. 211*, 689−696.
Vartanian, T., Szuchet, S., Dawson, G., & Campagnoni, A. T. (1986) *Science 234*, 1395−1398.
Walker, A. G., & Rumsby, M. G. (1985) *Neurochem. Int. 7*, 441−447.
Wood, D. D., & Moscarello, M. A. (1989) *J. Biol. Chem. 264*, 5121−5127.

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