Increase in Vesicle Permeability Mediated by Myelin Basic Protein: Effect of Phosphorylation of Basic Protein†

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Received November 27, 1984

ABSTRACT: The two most basic charge isomers of myelin basic protein (BP), components 1 and 2 (C1 and C2), which presumably differ in the degree of deamidation, were purified from bovine BP by cation-exchange chromatography. Two additional specific types of posttranslational modifications were introduced into the purified isomers: (1) C-terminal arginine deficient derivatives of C1 and C2 were prepared by incubating the isomers with a carboxypeptidase, and (2) phosphorylated derivatives of C1 (1.6 and 1.7 mol of phosphate/mol of protein) were prepared by incubating C1 with the protein kinase from rabbit muscle. The ability of these charge isomers to increase the permeability of multilamellar vesicles composed of phosphatidylserine/phosphatidylcholine (1:11.5 w/w) and sphingomyelin/cholesterol/phosphatidic acid (1:1:0.2 w/w/w) was measured by monitoring the release of a water-soluble spin-label (tempocholine chloride) from the vesicles. The increase in vesicle permeability caused by BP was taken as a measure of the degree of perturbation of the bilayer by the protein, most likely by penetration partly into the bilayer. All classes of charge isomers (naturally occurring or generated in vitro) were more effective at increasing vesicle permeability than was poly(L-lysine), a polycation that only interacts electrostatically with the bilayer. Although C1 and C2 and their C-terminal-deficient derivatives did not differ in the amount of marker released, the phosphorylated derivative of C1 caused a smaller increase in vesicle permeability than did the other isomers, suggesting that phosphorylation had altered the ability of the protein to perturb the bilayer. This result, in conjunction with our previous report on the effect of BP charge microheterogeneity on vesicle aggregation [Cheifetz, S., & Moscarello, M. A. (1985) *Biochemistry 24*, 1901–1914], indicates that small changes in the charge of the protein have the potential to significantly influence the interaction of BP with the lipid bilayer.

Myelin basic protein (BP)[1] constitutes approximately 30% of the protein in CNS myelin [reviewed by Boggs & Moscarello (1978a)]. From its ability to induce aggregation of vesicles containing acidic lipids (Lampe et al., 1983; Lampe & Nelsestuen, 1982; Young et al., 1982) and to organize phosphatidylglycerol bilayers (Brady et al., 1981) and myelin lipids (Mateu et al., 1978; Sedzik et al., 1984) into a regular, repeating X-ray pattern reminiscent of the ordering of the myelin lamellae, it has been proposed that BP may be involved in initiating or maintaining the compact structure of myelin. Studies of neurological mutations affecting myelination in mice support a role for BP in the compaction of myelin (Dupouey et al., 1979; Privat et al., 1979).

This proposed role for BP in myelin has led to intensive investigations of the interaction of BP with lipids. Although BP has been shown to interact with lipids predominantly through electrostatic interactions as reflected in its ability to induce phase separation of acidic from neutral lipids in a mixed system, there is strong suggestion that portions of the protein interact hydrophobically with lipids [reviewed by Boggs (1983)]. For example, the ability of BP to decrease the lipid phase transition temperature (Boggs & Moscarello, 1978b; Papahadjopoulos et al., 1975) and to induce leakage of sodium (Papahadjopoulos et al., 1975) and glucose (Gould & London, 1972) trapped inside vesicles has been interpreted as being the consequence of the partial penetration into and/or deformation of the acyl chain packing within the bilayer. The demonstration that BP disrupted the packing of the fatty acid side chains near the lipid polar head groups is consistent with the partial penetration of the protein into the bilayer (Boggs & Moscarello, 1978b).

A characteristic of this protein that has not been extensively investigated with respect to its interaction with lipids is that it exists in situ as a series of charge isomers all of similar molecular weights. These isomers numbered C1, C2, C3, and so on in order of decreasing net positive charge (Deibler & Martenson, 1973) can be resolved at alkaline pH by gel electrophoresis (Martenson et al., 1969) or by cation-exchange chromatography (Chou et al., 1976; Deibler & Martenson, 1973). The charge modifications that give rise to isomers less basic than C1 have been shown to be a combination of partial deamidation (Martenson et al., 1983; Chou et al., 1977, 1976), phosphorylation (Martenson et al., 1983; Chou et al., 1976; Deibler et al., 1975), and, under some circumstances, C-terminal arginine loss (Chou et al., 1977). Phosphorylation, in particular, may play a dynamic role in myelin since the phosphate groups in BP have been shown to be metabolically labile (Desjardin & Morell, 1983). In addition, the degree of phosphorylation was increased by compounds that depolarized nerves (Murray & Steck, 1983) and during conduction of nerve impulses (Murray & Steck, 1984), suggesting some role for the phosphorylation of BP in impulse conduction.

We have been investigating the effect of the charge microheterogeneity of BP on the ability of the protein to interact

† This work was supported by a grant from the Medical Research Council of Canada.

[1] Abbreviations: BP, myelin basic protein; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; SM, sphingomyelin; Chol, cholesterol; MLV's, multilamellar vesicles; RMPK, rabbit muscle protein kinase; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MES, 4-morpholineethanesulfonic acid.

0006-2960/85/0424-5170$01.50/0

with lipids. In an extension of the studies of BP-induced aggregation of vesicles containing acidic lipids, we reported a difference in the ability of the charge isomers to induce vesicle aggregation (Cheifetz & Moscarello, 1985). A decrease in the net positive charge of the protein was correlated with a decreased degree of aggregation of acidic lipid vesicles.

In the present study, we have investigated the effect of charge microheterogeneity arising from deamidation, phosphorylation, and loss of C-terminal amino acids on the abilty of BP to release a water-soluble, spin-label entrapped inside multilamellar vesicles of PS/PC and SM/Chol/PA. In both of these lipid systems, phosphorylation decreased the ability of BP to increase the permeability of the vesicles, suggesting an alteration in the ability of the phosphorylated protein to perturb the bilayer. The possible relationship of these changes to the role of BP in the membrane is discussed.

Materials and Methods

Isolation of Components 1 and 2 from Bovine BP. The procedure used for the isolation of bovine BP from delipidated white matter and for the preparation of C1 and C2 have been described (Cheifetz & Moscarello, 1985). For the studies presented in this paper, C1 and C2 were separately pooled from six columns. The purity of the isomers was checked by SDS gel electrophoresis (Laemmli, 1970) and by the alkaline–urea gel electrophoresis procedure of Deibler et al. (1972), which was modified by the inclusion of a 1-h preelectrophoresis step.

In Vitro Modifications. (A) Removal of C-Terminal Amino Acids. The conditions used to characterize the C-terminal amino acids and to generate derivatives of C1 and C2 lacking the C-terminal tripeptide sequence Ala-Arg-Arg were described previously (Cheifetz & Moscarello, 1985). Briefly, this involved incubating the components with carboxypeptidase S-1 (CPS-1), which had been pretreated with pepstatin to inactivate a contaminating cathepsin activity. The amino acids released by the enzymic digestion were analyzed on a Durrum D-500 amino acid analyzer and the results expressed as a ratio of Arg to Ala. On the basis of the molar ratio of Arg:Ala recovered in the supernatants of the digests, it was possible to estimate the extent of C-terminal arginine loss in the purified isomers. Since the molar ratios of Arg:Ala for both C1 and C2 approached the value of 2:1 expected for an intact C-terminus, it was concluded that each component initially had an essentially intact C-terminus.

As a consequence of the incubation with CPS-1, the treated components now lacked one alanine and two arginines from their C-termini. These proteins, which were collected by TCA precipitation and washed with ether (Cheifetz & Moscarello, 1985), were prepared for use in the permeability studies by dissolving them in water containing 0.1 mM PMSF and boiling for 5 min to ensure complete inactivation of the CPS-1. The control proteins were TCA precipitated, ether washed, and boiled in the presence of pepstatin–enzyme mixtures that had been inactivated with PMSF. Protein concentrations were measured against a BSA standard (Lowry et al., 1951).

(B) Phosphorylation. Since BP has been shown to be a substrate for the exogenous protein kinase from rabbit muscle (RMPK) (Carnegie et al., 1973), this kinase was used to generate phosphorylated derivatives of C1. The incubation conditions were as follows. Component 1 (300 μg) was incubated for 24 h at 37 °C with 20 μg of RMPK (Sigma) in 50 mM MES buffer, pH 6.5, containing 1.2 μM cAMP, 25 mM $MgCl_2$, and 2.46 mM ATP (~3000 cpm [γ-^{32}P]ATP/nmol of ATP) in a final volume of 120 μL. Two types of control proteins were prepared by incubating C1 for 24 h in a medium that lacked either the kinase or the ATP. All protein samples were then precipitated twice from aqueous media with cold 10% TCA and washed 3 times with ether prior to measuring the amount of [^{32}P]phosphate transferred by monitoring the Cerenkov radiation of the samples (Haviland & Bieber, 1971).

Preparation of Lipid Vesicles. Multilayered vesicles (MLV's) composed of bovine brain phosphatidylserine (PS, Avanti) and egg phosphatidylcholine (PC, Avanti) (1:11.5 w/w) or bovine brain sphingomyelin (SM, Supelco), cholesterol (Chol, Fluka), and phosphatidic acid (PA, Avanti) (1:1:0.2 w/w/w) were used in these studies. Tempocholine was used as the water-soluble spin-label (Kornberg & McConnell, 1971). The vesicles for ESR spectroscopy were prepared by drying the desired lipids (1 mg) under a stream of nitrogen onto the sides of an Eppendorf tube. The tube was left in an evacuated desiccator for 15 min. MLV's containing trapped tempocholine were formed by vortexing the lipid for 2 min in 100 μL of 0.1 or 0.15 M tempocholine in 2 mM HEPES buffer, pH 7.4, containing 10 mM NaCl and 0.1 mM EDTA (Boggs et al., 1983). PS/PC vesicles were formed at room temperature and the SM/Chol/PA vesicles at 45 °C. These vesicles were then diluted to 500 μL with 10 mM HEPES buffer, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, and 0.01% sodium azide (buffer A) in preparation for the gel-filtration steps.

The procedure of Neal & Florini (1973) for desalting small volumes was adapted to faciliate the removal of free spin-label from the MLV suspension. Sephadex G-50 (medium grade) was swollen in buffer A, deaerated, and poured into a 3-mL plastic syringe containing a plug of glass wool and a filter disk until the resin completely filled the syringe. The column was suspended inside a test tube and then centrifuged for 2 min at 500 rpm (International) to remove the external fluid. This resulted in a bed volume of 2.5 mL. After the efficiency of volume recovery was checked by centrifuging 500 μL of buffer A through the column, the diluted vesicles were loaded on the same column and centrifuged as described above. The purification was repeated once more on a second column of Sephadex G-50. These vesicles were maintained at 4 °C and generally used within 24 h of preparation. Since retention of lipid on the gel-filtration resin has been reported (Tyrell et al., 1976), lipid recovery was monitored by comparing the phosphorus content (Bartlett, 1959) of the vesicles before and after the completion of the two chromatographic steps.

Permeability Studies. Aliquots of vesicles were diluted to 45 μL with buffer A and maintained on ice until 5 μL of water (blank) or protein solution prepared in water was added. The basic protein to lipid ratio was ~1:10 (w/w). The effect of basic protein was compared to that of poly(L-lysine) (M_r >20 000) added at the same weight ratio. After being mixed by hand, the samples were taken up in 100-μL micropipets, sealed with Critoseal, and placed at room temperature. The ESR signal was measured after 30 min, on a Varian E-104 spectrometer at a power of 10 mW. The results were expressed as percentages of the maximum signal intensity measured after the vesicles were diluted to 25 μL with buffer A and adding an equal volume of 2-chloroethanol. This disrupted the vesicles and released all of the tempocholine into solution.

Results

Characterization of Isomers. SDS gel electrophoresis (Figure 1A) of the components showed that C1 and C2 contained one major band of M_r 21 000. On alkaline–urea gels (Figure 1B), the mobility of C1 was greater than that of C2. Due to variations in the pooling of the fractions from the

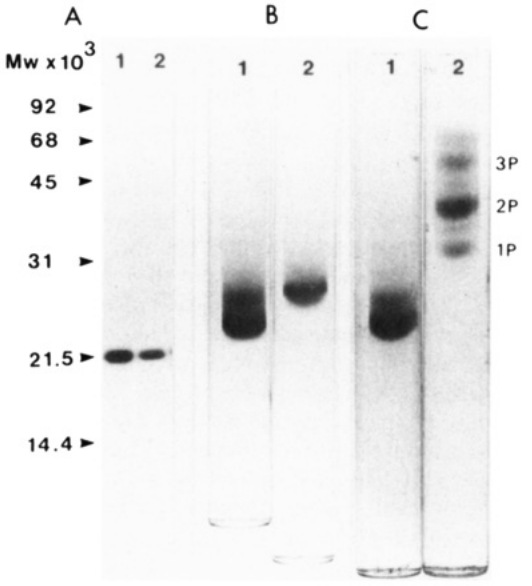

FIGURE 1: (A) SDS gel electrophoresis pattern of C1 (lane 1) and C2 (lane 2) on a 15% separating gel (pH 8.8) with a 5.7% stacking gel (pH 6.8). The migration positions of the molecular weight markers phosphorylase A, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (in order of decreasing molecular weight) are indicated by arrows. The samples were electrophoresed at 10 mA until dye front was within 0.5 cm of the bottom of the gel. The gel was stained with 0.025% Coomassi Blue R250 in 50% methanol/10% acetic acid and destained in 30% methanol/7% acetic acid. (B) Alkaline–urea gels at pH 10.4 of C1 (gel 1) and C2 (gel 2). The gels were preelectrophoresed for 1 h at 3.75 mA/gel prior to the electrophoresis of the samples at the same current for 3.5 h. Gels were stained with 0.5% amido black in 7% acetic acid for 10 min and then destained in 7% acetic acid. (C) Effect of extensive phosphorylation on the mobility of C1 on alkaline–urea gels. The conditions were as described for (B). Gel 1 is the control protein, which was incubated in a medium that contained the ATP but not the kinase. Gel 2 shows the pattern for C1 incubated for 24 h in the complete phosphorylation medium. An average of 1.7 mol of phosphate was transferred per mol of C1.

cation-exchange columns, a small amount of C2 contaminated the C1 preparations.

The nature of the charge modification giving rise to C2 was ascribed to deamidation on the basis of the following considerations. First, the relative mobility and order of migration of C1 and C2 on alkaline–urea gels (Figure 1B) has been shown to result from the loss of one net positive charge in C2 relative to C1 (Deibler et al., 1975). Second, the analysis of the C-terminal amino acids indicated little loss of arginine; therefore, this particular modification could be discounted as a significant contributor to the C2 population isolated for this study. Finally, deamidation has been reported to occur in isomers less basic than C1 (Martenson et al., 1983; Chou et al., 1977, 1976).

Incubation of C1 with RMPK for 24 h resulted in the incorporation of approximately 1.7 mol of phosphate/mol of protein. The alkaline–urea gels in Figure 1C show that this phosphorylation generated several new bands, which, on the basis of their decreasing mobilities relative to the parent compound, contained 1, 2, and 3 mol of phosphate/mol of protein. The control proteins showed no indication of any increase in microheterogeneity resulting from the incubation and subsequent manipulation of the protein (Figure 1C, gel 1).

Permeability Studies. To study the effect of BP charge microheterogeneity on vesicle permeability, MLV's containing trapped tempocholine were prepared. At the high concentrations of tempocholine trapped in these vesicles, exchange

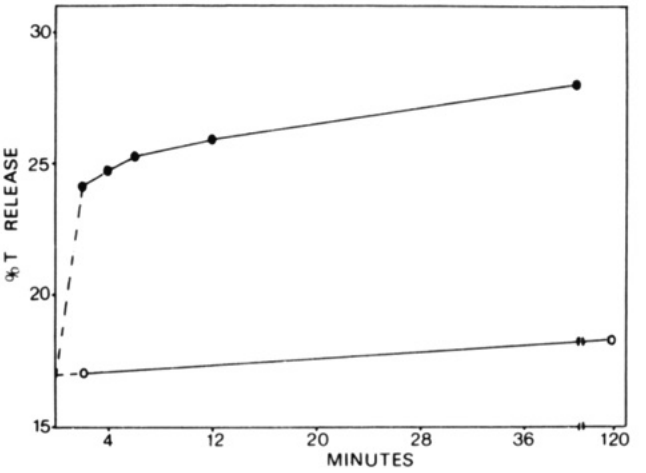

FIGURE 2: Release of tempocholine (T) from PS/PC vesicles. Each assay contained 50 μg of lipid and either 5 μg of C1 (●) or an equivalent volume of water (○). After addition of the protein solution, the ESR signal was measured over a period of 40 min. The sample without protein was measured after 2 min and again after 120 min. The curves have been extrapolated to zero time.

broadening markedly reduced the intensity of the ESR signal (Boggs et al., 1983). As the tempocholine was diluted into the surrounding aqueous environment due to its leakage from the vesicles, the signal was sharpened, was increased in height, and could be quantitated from the height of the peaks (Boggs et al., 1983). For this study, tempocholine was trapped inside MLV's composed of PS/PC or SM/Chol/PA, and these vesicles were used to compare the ability of C1, C2, and their in vitro modified derivatives to induce leakage of the trapped spin-label. The protein to lipid ratios were ~1:10 (w/w).

(A) Permeability of PS/PC Vesicles. The permeability of PC vesicles containing 8% PS, by weight, was monitored after 2 h at room temperature in the absence of protein. The MLV's were apparently sealed, since after 2 h, there was only a 1% increase in the signal intensity over the initial reading taken after ~2 min (Figure 2). In contrast to this low level of leakage in the absence of protein, the addition of 5 μg of C1 to 50 μg of MLV's caused a rapid increase in signal intensity within the first 10 min followed by a period of slow release (Figure 2). To facilitate the processing of a larger number of samples, the signal intensities were measured 30 min after the addition of the protein or water to the vesicles. There was no visible aggregation of the vesicles over this time period.

Table I shows representative results from two measurements of the effect of phosphorylation of C1 on the leakage of spin-label from two preparations of PS/PC vesicles. The non-phosphorylated C1 (C1-C in Table I) caused a release of about 29% of the spin-label from the first batch of vesicles in 30 min. When assayed with the same batch of vesicles (batch 1, Table I), the two preparations of phosphorylated C1 (C1-P), which had incorporated an average of 1.7 and 1.6 mol of phosphate/mol of BP, caused the release of 25% of the spin-label, a 12–13% smaller effect than that induced by the non-phosphorylated C1. Whether the non-phosphorylated C1 was prepared by incubating C1 in the MES buffer lacking one or the other of the kinase or ATP, the percent increase in vesicle permeability induced by the protein was the same (Table I, batch 1). Thus, the difference in vesicle permeability induced by C1-C and C1-P was clearly due to the incorporation of phosphate into C1 and not to the incubation conditions per se.

The standard deviation for the percent release caused by the protein from a particular batch of vesicles was low as indicated in Table I. However, the permeability of different

Table I: Effect of Phosphorylation of C1 on the Permeability of MLV's[a]

vesicle batch	protein batch	% release of spin-label: C1-C +ATP	C1-C +kinase	C1-P	% difference (C1 - C1-P)
1	1	29.2 ± 0.7 (n = 3)		25.4 ± 0.4 (n = 3)[b]	13.0 ± 2.7
	2		28.5 ± 0.8 (n = 3)	25.1 ± 0.8 (n = 3)[b]	11.9 ± 4.0
2	1	8.9 ± 0.6 (n = 3)		7.2 ± 0.4 (n = 3)[c]	19.1 ± 8.1

[a] Assay: 50 μg of lipid (PS/PC, 1:11.5 w/w) with entrapped tempocholine plus 5 μg of protein in water (1 mg/mL) or an equal volume of water (blank). The ESR signal was measured after 30 min at room temperature. The results are corrected for the ESR signal measured in the blank [vesicle 1, 8.7 ± 0.3 (n = 3); vesicle 2, 18.3 (n = 1)]. Phosphorylated C1 (C1-P) prepared as outlined under Materials and Methods, incorporated an average of 1.7 and 1.6 mol of phosphate/mol of protein for preparations 1 and 2, respectively. Non-phosphorylated C1 (C1-C), incubated in complete medium except that the kinase was omitted in one case (+ATP) and ATP in the second case (+kinase), were processed along with the phosphorylated samples. Data were analyzed by Student's t test. [b] Difference between the effect of C1-C and C1-P was significant at $p < 0.01$. [c] Difference between the effect of C1-C and C1-P was significant at $p < 0.05$.

Table II: Permeability of MLV's Induced by C1, C2, and Poly(L-lysine)[a]

vesicle batch	% release of spin-label: C1	C2	poly(L-lysine)
3	6.2 ± 0.3 (n = 3)	6.6 ± 0.4 (n = 3)	0.9 ± 0.3 (n = 3)
4	12.2 ± 0.5 (n = 4)	12.6 ± 0.8 (n = 4)	ND

[a] Assay conditions as described in Table I. The data are corrected for the signal measured in the blanks [vesicle preparation 3, 6.3 ± 0.3 (n = 3); preparation 4, 14.4 (n = 1)]. The difference between C1 and C2 was not statistically significant at $p = 0.05$.

Table III: Effect of Loss of C-Terminal Amino Acids (Ala, Arg, Arg) of C1 and C2 on Permeability of MLV's[a]

component	% release of spin-label: control	CPS-1 treated
C1	14.4 ± 0.5 (n = 3)	15.0 ± 0.5 (n = 3)
		14.6 ± 0.5 (n = 3)
C2	14.8 ± 0.9 (n = 3)	15.0 ± 0.5 (n = 3)
		14.6 ± 0.4 (n = 3)

[a] Components 1 and 2 were treated with a carboxypeptidase S-1 (CPS-1) as outlined under Materials and Methods to remove the amino acids from the carboxyterminus. The results for two different preparations of CPS-1-treated C1 and C2 are shown. The TCA-precipitated controls were processed as outlined under Materials and Methods. Other assay conditions were as described in Table I. The data are corrected for the signal measured in the blank (8.7, n = 1).

preparations of vesicles varied as indicated by the data for a second batch of vesicles shown in Table I. Although the non-phosphorylated C1-C caused release of only 9% of the spin-label from the second batch of vesicles, the phosphorylated protein (C1-P) was 19% less effective, consistent with the results from the first batch of vesicles. When assayed with six different preparations of vesicles, C1-P consistently caused a 12–25% less increase in vesicle permeability than did C1-C. The average difference in the effect between the two isomers on these six independent vesicle preparations was 17.8 ± 4.8%.

The effects of C1, C2, and poly(L-lysine) on the leakage of spin-label from two preparations of PS/PC vesicles are compared in Table II. Since polylysine binds to lipids mainly through electrostatic interactions without significant penetration into the bilayer (Papahadjopoulos et al., 1975), it was used to check the sensitivity of the vesicles to changes in the degree of penetration of the protein. Polylysine released much less spin-label than did C1 and C2, which were equally effective at increasing the vesicle permeability to tempocholine. Since all vesicle preparations gave a positive difference between C1 and phosphorylated C1, the similarity in the ability of C1 and C2 to increase vesicle permeability indicated that deamidation, the most likely cause of the increased negative charge in C2 relative to C1, was not sufficient by itself to alter the degree of penetration of the protein or was not located in a region of the protein that penetrated into the bilayer.

The net charge of C1 and C2 was also reduced by enzymatically removing Ala-Arg-Arg from the C-terminus of the components. These derivatives were used to investigate the specificity of the effect on permeability demonstrated in the study of the phosphorylated protein. Met-166 is not appreciably affected by the interaction of BP with lipids (Hughes et al., 1982; Mendz et al., 1984), suggesting that this region of the protein in the vicinity of the two charged terminal Arg residues does not penetrate into the bilayer. Removal of the Ala-Arg-Arg sequence introduces a negatively charged C-terminal Met into the neutral segment 162–167 and thus should not increase the degree of penetration of this region or affect the ability of BP to increase vesicle permeability. The results from the study of two preparations of carboxypeptidase-treated C1 and C2 are shown in Table III. As expected, the removal of Ala-Arg-Arg from the C-terminus did not significantly alter the ability of the components to release the trapped spin-label.

(B) Permeability of SM/Chol/PA Vesicles. When used at the same concentration as the PS/PC vesicles, SM/Chol/PA vesicles immediately aggregated upon addition of BP, raising concerns about inhomogeneous mixing of protein with the vesicles. To overcome this problem, the vesicles were diluted to give approximately 10 μg of lipid per assay, and the protein was adjusted to maintain the protein:lipid ratio at 1:10 (w/w). Under these conditions there was no visible aggregation of the vesicles over the time course of the experiments. As for the PS/PC system, the signal intensity was measured after 30 min. Table IV shows the effect of the C1, C2, and modified C1 on the permeability of these vesicles. In the

Table IV: Effect of C1, C2, and Modified C1 on the Permeability of MLV's of SM/Chol/PA (1:1:0.2 w/w/w) to Entrapped Tempocholine[a]

vesicle preparation	experimentally introduced modification	% release of spin-label: C1	modified C1	C2
1	none	45.1 ± 2.0 (n = 3)		44.5 ± 1.7 (n = 3)
1	none	45.7 ± 2.7 (n = 2)		
	minus C-terminal Ala-Arg-Arg		45.2 ± 2.6 (n = 3)	
2	none	36.2 ± 1.2 (n = 3)		
	phosphorylation		33.3 ± 1.1 (n = 3)[b]	

[a] Assay: 10 μg of lipid plus 1 μg of protein (stock solution, 0.2 mg/mL in water) or an equivalent volume of water (blanks). The data are corrected for the signal measured in the blanks [preparation 1, 9.5 (n = 1); preparation 2, 5.6 (n = 1)]. The data were analyzed by Student's t test. [b] The difference between non-phosphorylated C1 (C1) and phosphorylated C1 (modified C1) containing an average of 1.7 mol of phosphate/mol of protein was statistically significant; $t = 3.09$, $p < 0.05$.

absence of protein, only a small percent of the trapped tempocholine was released. The addition of the components of BP caused a release of 33–46% of the entrapped tempocholine in all cases. In contrast, polylysine caused only 12.5% release of spin-label. As was seen in the study of PS/PC vesicles, there was no difference in the amount of spin-label released by C1 and C2 or C1 and its C-terminal deficient derivative. Once more, phosphorylation caused a small but statistically significant decrease in vesicle permeability relative to the control protein.

Discussion

In this paper, we have presented the results from a study of the effect of BP charge microheterogeneity on the ability of this protein to increase vesicle permeability to the entrapped spin-label tempocholine. Two types of MLV's were used—mixed vesicles of PS/PC (1:11.5 w/w) and those containing SM/Chol/PA (1:1:0.2 w/w/w). Components 1 and 2 were purified from bovine BP, and the particular charge modifications studied were those arising from in vitro removal of the carboxyterminal tripeptide from both isomers, in vitro phosphorylation of C1 with an exogenous protein kinase, and in vivo deamidation. The latter modification converts C1 to C2.

Components 1 and 2 and their C-terminal Arg deficient derivatives were found to be equally effective at increasing the permeability of PS/PC and SM/Chol/PA vesicles. In contrast, the incorporation of phosphate apparently decreased the extent to which BP perturbed the bilayer since the phosphorylated protein caused a smaller increase in vesicle permeability than did the non-phosphorylated protein. Although vesicle permeability was reduced in the presence of the phosphorylated protein, it was always greater than that caused by polylysine, a polycation that does not penetrate into the bilayer. Polylysine had little or no perturbing effect on the vesicles. This difference between polylysine and phosphorylated BP indicated that the phosphorylated basic protein still perturbed the bilayer to a degree, likely through interaction of other unaltered regions of the protein with the lipid.

Since Thr-33 and Ser-109, two of the main sites phosphorylated by the exogenous kinase (Carnegie et al., 1974), are located in uncharged sections of the protein that may be capable of penetrating into the bilayer (Boggs & Moscarello, 1978a), there is the potential for phosphorylation to interfere with or reduce the degree of penetration of those regions into the bilayer. In fact, Mendz et al. (1984) have recently shown that His-32 and Thr-35 in porcine BP, which corresponds to His-30 and Thr-33 in bovine BP, are involved in lipid binding. Thus, it is possible that the decreased leakage of tempocholine in the presence of phosphorylated protein is due in part to an altered penetration into the bilayer of the region containing phosphorylated Thr-33. The possible effect of Ser-109 phosphorylation is less certain. Although this residue is located in the uncharged sequence Gly-Leu-Ser-Leu-Ser (residues 107–111), there is conflicting evidence about the interaction of this segment with the bilayer. Mendz et al. (1984) did not detect any interaction between the methylated arginine that preceeds this sequence and the lipid, whereas Littlemore & Ledeen (1979) did detect such an interaction.

Wüthrich & Steck (1981) investigated the effect on the permeability of myelin vesicles of incubation with MgATP in order to phosphorylate BP by the endogenous kinase. Although they found a decrease in vesicle permeability after phosphorylation, the difference could be duplicated in heat-inactivated (non-phosphorylating) vesicles. Thus, they concluded that the phosphorylation of BP per se was not the cause of the altered vesicle permeability.

Table V: Summary of Results for Aggregation and Leakage Experiments[a]

modification	aggregation of PS/PC	leakage from PS/PC and SM/Chol/PA
deamidation	C1 > C2	C1 ~ C2
C-terminal Arg deficient	C1 > C1*	C1 ~ C1*
	C2 > C2*	C2 ~ C2*
phosphorylation	C1 > C1-P	C1 > C1-P
	C2 > C2-P	

[a] Difference in net positive charge of C1 and C2 was tentatively ascribed to deamidation. See text. C1* and C2* were treated with CPS-1 as outlined under Materials and Methods to remove C-terminal tripeptide Ala-Arg-Arg. C1-P and C2-P were incubated with RMPK as outlined under Materials and Methods to phosphorylate the proteins at selected Ser and Thr residues. Aggregation data were taken from Cheifetz & Moscarello (1984).

In our model membrane system, phosphorylation of BP was definitely correlated with a decrease in membrane permeability and not due to residual MgATP. One interesting question that remains to be investigated is whether the differences in the sites phosphorylated by the exogenous and endogenous kinases (Carnegie et al., 1974) could also affect the ability of the protein to alter vesicle permeability.

We have recently reported that charge modifications also affected the ability of BP to aggregate acidic unilamellar vesicles (Cheifetz & Moscarello, 1985). In that study, all three types of modifications—deamidation, C-terminal Arg loss, and phosphorylation—decreased the extent to which BP aggregated unilamellar vesicles composed of PS/PC (1:11.5 w/w). The demonstration that phosphorylation altered two in vitro properties of BP (or its ability to cross-link vesicles and to increase membrane permeability) may be of particular significance in vivo since myelin contains protein kinases and phosphatases capable of changing the level of phosphorylation of BP [reviewed by Martenson (1980)]. Thus, a phosphorylation/dephosphorylation mechanism involving BP provides a possible mechanism for dynamic change in myelin. The recent demonstrations that the phosphorylation of BP was increased by the electrical activity of the myelinated nerve (Murray & Steck, 1984, 1983) supports such a hypothesis. Since ion flow has been detected in the myelin sheath during impulse conduction (Funch & Faber, 1984), it is possible that phosphorylation of BP is directly involved in altering the membrane permeability as suggested by our in vitro studies. Alternatively, phosphorylation may affect the function of myelin through more complex mechanisms mediated by an altered interaction of BP with the lipid bilayer.

In addition to its possible role in the normal function of myelin, alterations in site or level of phosphorylation may lead to myelin dysfunction. For example, the results reported in this study suggest the phosphorylation could alter the penetration of certain segments of BP into the bilayer. Thus, phosphorylation at sites other than those normally modified in vivo, as in the phosphorylation of BP by vaccinia virus (Tschannen et al., 1980), might affect the antigenicity of the protein by causing exposure to the aqueous environment of normally buried regions of the protein. In addition, phosphorylation could destabilize the membrane through altered cross-linking of the bilayer as pointed out in our earlier study (Cheifetz & Moscarello, 1985).

In summary, this study of BP-mediated permeability of vesicles and the earlier report on vesicle aggregation induced by this protein (Cheifetz & Moscarello, 1985) summarized in Table V have shown that the modification of the charge of BP had a definite effect on these two properties when measured in model membrane systems. Assuming that the

in vitro ability to increase membrane permeability and induce vesicle aggregation are a reflection of the in vivo roles of the protein, then alteration of the charge of BP or perhaps the relative proportions of the isomers may affect the ability of the protein to carry out those functions. Whether the ultimate expression of the changes are of a regulatory or pathological nature would likely depend on the nature of the modifications, their location within the protein, and the relative distribution of the various classes of charge isomers. These results are in agreement with recent X-ray evidence that a small change in net charge can have dramatic effects on the organization of multilayers (Brady et al., 1985).

Registry No. Chol, 57-88-5.

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