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Effect of Bovine Basic Protein Charge Microheterogeneity on Protein-Induced Aggregation of Unilamellar Vesicles Containing a Mixture of Acidic and Neutral Phospholipids†

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ABSTRACT: Two of the charge isomers (components 1 and 2) normally found as microheteromers of myelin basic protein were isolated, and their abilities to aggregate vesicles consisting of mixed phospholipids were studied. Component 1 (the most cationic of the microheteromers) aggregated phosphatidylcholine (PC) vesicles containing 7.8 mol % phosphatidylserine (PS) more rapidly and at lower protein concentrations than component 2, which differs from component 1 by 1 net positive charge. Modification of components 1 and 2 in vitro by phosphorylation with rabbit muscle protein kinase decreased the ability of both components to aggregate vesicles. The greater the extent of phosphorylation, the less effective were the isomers at inducing aggregation. Decreasing the charge of either component 1 or component 2 by removal of the two C-terminal arginyl residues also decreased the ability of the isomers to induce aggregation. Therefore, charge microheterogeneity, whether arising in vivo or generated in vitro, markedly affected the ability of these microheteromers to aggregate PC vesicles containing 7.8 mol % PS. Because a small difference in the charge of the protein had a marked effect on vesicle aggregation, we propose that charge microheterogeneity may play an important and dynamic role in the structure and function of normal myelin.

The myelin sheath is the membranous structure that surrounds the axons of the nervous system. Although derived from the plasma membrane of the oligodendroglia cell, its multilayer structure and its low protein/lipid ratio (about 1/4) make it a unique biological membrane structure. The transmission of impulses along the axon is dependent on the integrity of this multilamellar structure. The role of protein–lipid interactions in generating and maintaining this structure is not fully defined as yet.

One of the major proteins in the myelin membrane is an extrinsic protein called basic protein (BP),[1] which accounts for approximately 30% of the myelin protein (Boggs & Moscarello, 1978). The ability of this positively charged protein to organize phosphatidylglycerol bilayers into multilamellar structures characteristic of myelin (Brady et al., 1981), and to aggregate vesicles containing acidic phospholipids (Lampe & Nelestuen, 1982; Young et al., 1982; Lampe et al., 1983) or those prepared from myelin (Young et al., 1982), suggested that BP may play a structural role in either the development or maintenance of the multilamellar profile of the myelin sheath.

Purified BP with a molecular weight of 18 400 migrates as a single component on high ionic strength sodium dodecyl sulfate (SDS) gels with M_r ~21 000. However, at alkaline pH (10.6), the protein resolves into several components characteristic of microheterogeneity based on net charge (Martenson et al., 1969).

The BP charge microheterogeneity, which results from posttranslational modifications such as phosphorylation (Chou

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[1] Abbreviations: PC, phsophatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; BP, myelin basic protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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et al., 1976; Deibler et al., 1975; Martenson et al., 1983), deamidation (Chou et al., 1976; Martenson et al., 1983), and in some circumstances C-terminal arginine loss (Chou et al., 1977), may alter the capacity of BP to interact with the lipid bilayer. In general, the BP used in previous studies of lipid–protein interactions (Lampe & Nelsestuen, 1982; Young et al., 1982; Lampe et al., 1983) contained all the charge isomers, and so these studies did not address the possibility of modulating effects by this charge heterogeneity on membrane organization. We have investigated this question by comparing the ability of the individual charge isomers of BP to aggregate unilamellar vesicles composed of approximately 8 mol % phosphatidylserine (PS)[1] in phosphatidylcholine (PC) vesicles. In addition, the degree of phosphorylation and of C-terminal arginine loss was selectively altered by incubation of the isomers with an exogenous kinase and with a carboxypeptidase, respectively. These in vitro modified forms of the isomers were then used in the aggregation assay. We found that charge microheterogeneity, whether arising in vivo or generated in vitro, had a marked effect on the ability of BP to initiate vesicle aggregation. Component 1,[2] which is the most cationic isomer, was more effective than any of the other isomers at inducing aggregation of PC vesicles containing approximately 8 mol % PS. The implications of these data, which show that a small change in the net charge of BP markedly alters the interaction of the protein with the lipid, are discussed in relation to myelin structure.

Materials and Methods

Isolation of BP. Bovine brains, obtained fresh from the abattoir, were placed on ice, and grey matter was separated from the white matter. The latter was stored at −20 °C until processed to isolate BP. Before the BP was extracted, the thawed white matter was delipidated by overnight extraction with chloroform–methanol (2:1 v/v) at 4 °C since this procedure has been shown to minimize the isolation-induced charge heterogeneity in the protein (Chou et al., 1977). The residue from 35 g of delipidated white matter was washed with acetone and then extracted overnight at 4 °C with 100 mL of 0.2 N H_2SO_4 (Lowden et al., 1966). After centrifuging, the 9000 rpm supernatant was mixed with an equal volume of absolute alcohol and left overnight at −20 °C. The precipitate was collected by centrifuging for 90 min at 9000 rpm at −10 °C, washed twice with 90% ethanol, and then resuspended in water containing 10 μM PMSF (Sigma). Any insoluble material was removed by centrifuging. The lyophilized supernatant was stored at −20 °C until it could be processed to isolate the charge isomers.

Isolation of Charge Isomers. The charge isomers were isolated according to the procedure of Chou et al. (1976) on a 0.9 × 30 cm column of carboxymethylcellulose cation-exchange resin (CM52, Whatman, microgranular) at pH 10.5. Components 1 and 2, the last two peaks to elute from the column, were desalted on a Bio-Gel P2 column (1.5 × 60 cm) that had been equilibrated in 0.01 N HCl. Each desalted component was lyophilized, redissolved in water, and dialyzed overnight against 0.1% NH_4HCO_3 and then against water at 4 °C. The dialyzed samples were lyophilized and stored at −20 °C. For the studies presented in this paper, component 1 (C1) from six chromatographic separations was combined and used at the source of C1. Component 2 (C2) was pooled from the same six columns. The purity of the components was tested on a Laemmli SDS gel (Laemmli, 1970) and on alkaline-urea disc gels (Deibler et al., 1972).

In Vitro Modifications. (*a*) *Removal of C-Terminal Amino Acids.* Carboxypeptidase S1 (CPS-1, kindly provided by Dr. T. Hofmann of the University of Toronto) was used to characterize the C-terminal amino acids of the proteins and to generate proteins lacking the three terminal amino acids—Ala, Arg, and Arg. Components 1 and 2, 200 μg of each, were incubated for 3 h at 37 °C with 4 μg of pepstatin-treated CPS-1 (2 min at 37 °C, 1:10 w/w) in 100 μL of a 0.05 M pyridine–formate buffer, pH 4. The digests were stopped by the addition of 500 μL of cold 10% TCA. The resulting pellets and supernatants were extracted 2 and 3 times, respectively, with ether to remove TCA, and the aqueous supernatants were lyophilized. To determine the concentration of the released amino acids, the lyophilized supernatant material was reconstituted in 100 μL of 0.01 N HCl and analyzed on a Durrum D-500 amino acid analyzer. To prepare the proteins for use in the aggregation study, they were redissolved in water containing 0.1 mM PMSF and then boiled for 5 min to ensure complete inactivation of CPS-1. The control proteins were boiled in the presence of the CPS-1 pepstatin mixture that had been pretreated with PMSF. Protein concentrations were measured against a bovine serum albumin (BSA) standard by the procedure of 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 isolated components. Components 1 and 2, 300 μg of each, were incubated at 37 °C for varying times with 20 μg of RMPK in 120 μL of 50 mM MES buffer, pH 6.5, containing 1.2 μM adenosine cyclic 3′,5′-phosphate (cAMP), 25 mM $MgCl_2$, and 2.46 mM ATP (3000 cpm of [γ-^{32}P]ATP/nmol of ATP). The reactions were stopped by the addition of 500 μL of cold 10% TCA and left for at least 2 h and in some experiments overnight at −20 °C. The resulting precipitates were collected by centrifugation at 12000 rpm for 15 min at 4 °C in an Eppendorf centrifuge and then redissolved in 500 μL of water and precipitated again with cold 10% TCA to ensure maximum removal of ATP. The precipitates were collected as before, washed 3 times with ether, and redissolved in water. The amount of phosphate incorporated into the proteins was calculated by comparing the Cerenkov radiation (Haviland & Bieber, 1970) of the sample with that of the stock solution of [γ-^{32}P]ATP of known specific activity, which had been used in the phosphorylation reaction. Nonspecific binding of ATP to the protein, which was monitored by omitting the enzyme from a 24-h incubation, was generally about 0.02 mol/mol of BP. These samples served as the controls in the aggregation assay when phosphorylated proteins were studied. Protein concentrations were determined according to the procedure of Lowry et al. (1951).

Vesicle Preparation. Egg phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) were obtained from Avanti as dry powders. These lipids were dissolved in chloroform at concentrations of 10–50 mg/mL and stored under nitrogen at −20 °C. Lyso-PC (Sigma) was dissolved in chloroform–methanol (2:1 v/v), and oleic and palmitic acids (Sigma) were dissolved in ethanol. Aliquots of the required lipids or fatty acids were tranferred to acid-washed test tubes, and the organic solvents were removed by a stream of nitrogen to leave the lipid as a film on the sides of the tube. Traces of solvent were removed by vacuum desiccation over pellets of NaOH for 0.5–1 h. Multilamellar vesicles were formed by the addition of 0.5–1 mL of 10 mM HEPES buffer, pH 7.4,

[2] The numbering of the components is according to the system of Deibler & Martenson (1973).

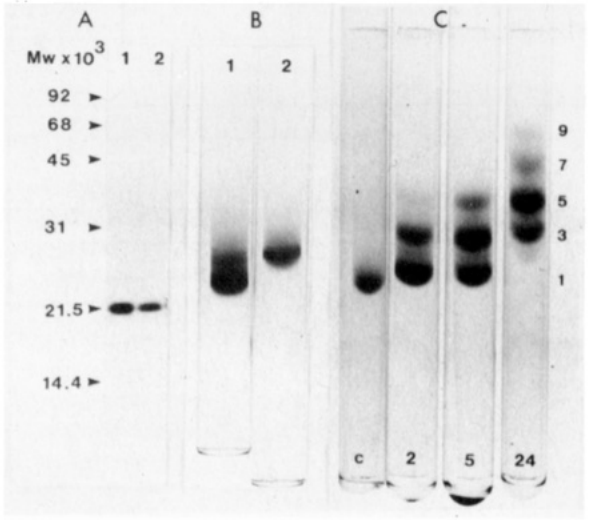

FIGURE 1: (A) SDS gel electrophoresis of 5–10 μg of C1 and C2 on a 15% separating slab gel (pH 8.8) with a 5.7% stacking gel (pH 6.8). Samples were electrophoresed at 10 mA until the dye front was within 0.5 cm of the bottom of the gel. The gel was stained with 0.025% Coomassie Blue R250 in 50% methanol–10% acetic acid and destained in 30% methanol–7% acetic acid. The positions of the molecular weight markers phosphorylase A (M_r 92 000), BSA (M_r 68 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400) are indicated. (B) Alkaline gels at pH 10.4 of C1 and C2. Gels were preelectrophoresed for 1 h at 3.75 mA per gel prior to the electrophoresis of 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 phosphorylation on mobility of C1 in alkaline gels. After 2, 5, and 24 h of incubation with RMPK, the proteins incorporated 0.3, 0.6, and 1.7 mol of phosphorus/mol of C1, respectively. "C" represents the control protein. The bands labeled 3, 5, 7, and 9 contain 1, 2, 3, and 4 mol of phosphate/mol of protein, respectively.

containing 100 mM NaCl, 1 mM EDTA, and 0.01% sodium azide to 10–20 mg of lipid and subsequent vortexing of the sample under a nitrogen atmosphere. The milky solutions were sonicated in a water-bath sonicator until no further clearing occurred. Vesicles containing approximately 8 mol % or more of an acidic constituent generally required 15–30 min of sonication to clarify. Following dilution to 5 mL with the same buffer, the vesicles were centrifuged for 1 h at 40 000 rpm at 4 °C to remove any large aggregates. Phosphorus was measured according to the procedure of Bartlett (1959), from which the content of phospholipid in the final preparations was determined.

Aggregation Assay. In the basic aggregation assay of PC vesicles containing PS, 25 μg of the test protein was added to 0.32 μmol of phospholipid in a final volume of 500 μL. The absorbance at 450 nm was measured after 10 min at room temperature. When vesicles containing PC plus lyso-PC and a free fatty acid were used, the concentration of protein and lipid was doubled and the absorbance at 450 nm was measured after 24 h at room temperature. All aggregation assays were done in polypropylene tubes to minimize protein loss through adsorption to vessel walls.

RESULTS

Isolation and Characterization of Components. Components 1 and 2 were isolated from bovine BP by cation-exchange chromatography (Chou et al., 1976) as described briefly under Materials and Methods. On SDS gels, C1 and C2 each consisted of a single band of the same mobilities (Figure 1A), demonstrating that the proteins were pure and were of the same size. On alkaline-urea gels, the more cationic C1 had a greater mobility than did the less cationic C2 (Figure 1B),

Table I: Aggregation of PC Vesicles Containing Identical Amounts of Lyso-PC and Free Fatty Acid

addition to PC vesicles (mol %)	OD_{450}[a] palmitic acid	oleic acid
lyso-PC + fatty acid		
5	0.020	
10	0.121	0.039
15	1.38[b]	0.143
20		0.430

[a] Net change in OD_{450} after 24 h. Each assay contained 50 μg of C1 and ~0.64 μmol of phospholipid. [b] OD measured after 5 min.

consistent with a difference of 1 net positive charge (Chou et al., 1976). A small amount of contamination of C1 with C2 was observed as a result of small variations in elution patterns on different columns. Phosphorus analyses indicated that neither protein was phosphorylated. C-Terminal analyses using carboxypeptidase S1 as described under Materials and Methods showed that C1 had an intact C-terminus while approximately 6% of the C2 molecules lacked one Arg.[3] Thus, the difference in the mobility between C1 and C2 on the alkaline-urea gels, which reflects a difference of 1 net positive charge, was most likely due to deamidation (Chou et al., 1976).

Phosphorylation of C1 and C2 by RMPK. Both C1 and C2 were readily phosphorylated by RMPK. The average degree of phosphorylation increased from approximately 0.3 mol of phosphate/mol of protein after a 2-h incubation to approximately 1.6 mol of phosphate/mol of protein after a 24-h incubation. A representative pattern for the time course of phosphorylation of C1 is shown in Figure 1C. On the alkaline-urea gels at pH 10.6, phosphorylation decreased the mobility of the modified proteins relative to the parent compound in the expected manner. The additional slower migrating species relative to C1 observed at the different times of incubation reflected different degrees of phosphorylation and are labeled 3, 5, 7, and 9 in Figure 1C.

Aggregation of Vesicles Containing PC or PC plus Lyso-PC and Free Fatty Acid. Sonicated unilamellar PC vesicles with or without added lyso-PC and a free fatty acid were prepared as described under Materials and Methods. Aggregation was measured by monitoring the absorbance at 450 nm, 24 h after the addition of 50 μg of protein to approximately 0.6 μmol of phospholipid.

Unilamellar vesicles containing only PC were generally resistant to BP-induced aggregation though an occasional batch of vesicles prepared from the same lipid stock would aggregate. The inclusion of increasing amounts of lyso-PC plus oleic or palmitic acids in the PC vesicles generated a class of vesicles that were aggregated more readily by the components (Table I). Better aggregation was observed in vesicles containing palmitic acid than in those containing oleic acid.

The effect of charge microheterogeneity generated by phosphorylation of C1 and C2 on the aggregation of PC vesicles containing 10 and 15 mol % lyso-PC plus palmitic acid and oleic acid, respectively, is shown in Table II. Phosphorylation of C1 or C2 had little effect if any on the degree of aggregation of the vesicles.

Aggregation of PC Vesicles Containing 7.8 mol % PS. The ability of C1 and C2 to aggregate unilamellar vesicles composed of PC with 7.8 mol % PS is shown in Figure 2A. Increasing amounts of C1 or C2, depending on the experiment, were added to 0.32 μmol of phospholipid, and the change in

[3] This was determined from the ratio of Arg/Ala, which is 2.0 for an intact C-terminus. The ratio of 1.96 measured for C1 was set to 100%; thus the ratio of 1.84 for C2 arises from 6% arginine loss.

Table II: Effect of Phosphorylation of C1 and C2 on Aggregation of PC Vesicles

addition to PC vesicles	concn of each (mol %)	component	mol of P_i transferred/mol of protein	OD_{450}[a] control	OD_{450}[a] phosphorylated
lyso-PC + palmitic acid	10	C1	1.6	0.595	0.606
		C2	1.5	0.572	0.582
lyso-PC + oleic acid	15	C1	1.6	0.460	0.430
		C2	1.5	0.452	0.346

[a] OD_{450} after 24 h. Each assay contained 50 μg of protein and 0.7 μmol of phospholipid.

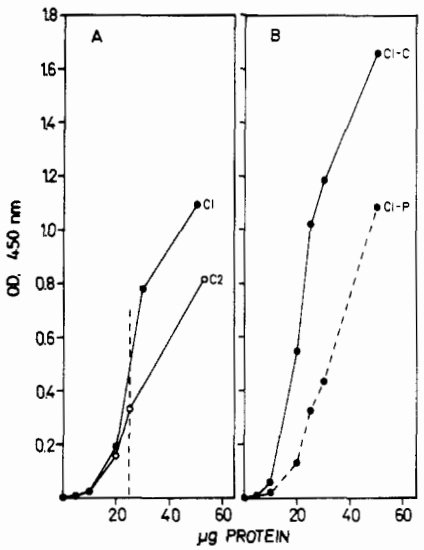

FIGURE 2: Aggregation assay: OD_{450} 10 min after adding varying amounts of BP to 0.32 μmol of 7.8 mol % PS in PC vesicles in a final volume of 500 μL of buffer. Assay variability using the same batch of lipids and proteins was generally between 2 and 10%. (A) C1 (●) and C2 (○). (B) Control C1-C (—) and phosphorylated C1-P containing an average of 1.7 mol of P_i/mol of protein (– –).

the absorbance was measured 10 min after addition of the protein. The shape of the aggregation curves for both components suggested that the interactions were cooperative. The greater effectiveness of C1 in inducing aggregation was reflected in the lower concentration required to attain the cooperative phase. To check the assay variability, the aggregation induced by 25 μg of protein (dotted line in Figure 2A) was measured in triplicate with a second preparation of lipid.[4] The absorbance induced by C1 was 0.177 ± 0.008 while that induced by C2 was 0.120 ± 0.003. The low variability of the assay suggested that the difference in the degree of aggregation induced by 20 μg of C1 and C2 (Figure 2A) was significant, although the differences between the components were more apparent when protein concentrations of 20 μg or more were used. A protein concentration of 25 μg was used in all subsequent comparisons.

In the above experiments vesicle aggregation was shown to be affected by the charge on the protein. However, vesicle aggregation was also sensitive to the surface charge on the vesicle. Thus, lowering the amount of PS in the vesicles decreased the aggregation. PC vesicles containing 3.9 mol % PS gave an OD_{450} of 0.127 compared to an OD_{450} of 1.137 when the PC vesicles contained 7.8 mol % PS.

Effect of Phosphorylation of C1 and C2 on Aggregation of PC–PS Vesicles. Phosphorylating C1 and C2 with RMPK and ATP as described under Materials and Methods decreased

[4] Since the absolute levels of aggregation varied between different preparations of lipid vesicles, all comparisons of treated and control proteins for a particular experiment were done with the same batch of vesicles. Aggregation assays involving different types of modifications were not necessarily done with the same batch of vesicles.

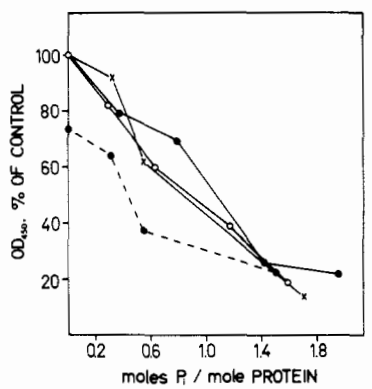

FIGURE 3: Aggregation assay conditions as in Figure 2 except that each assay point contained 25 μg of protein. C2 (●---●); C1, preparation 1 (○—○); C1, preparation 2 (●—●); C1, preparation 3 (×—×). OD_{450} is expressed as a percentage of the OD_{450} of the control C1.

the ability of both charge isomers to aggregate PC vesicles containing 7.8 mol % PS (Figures 2B and 3). The effect of increasing concentrations of C1 (control) and phosphorylated C1 on the aggregation is shown in Figure 2B. The control protein was prepared by omitting the kinase from the 24-h incubation as outlined under Materials and Methods. At concentrations of 10 μg and higher the control protein aggregated the vesicles in a cooperative manner. The phosphorylation of C1 decreased its effectiveness in aggregating the vesicles; thus higher concentrations of phosphorylated protein were required to aggregate the vesicles.

Aggregation was also very sensitive to the degree of protein phosphorylation (Figure 3). In this study, C1 and C2 were phosphorylated to different extents, and the effect of the degree of phosphorylation on the aggregation of PS–PC vesicles induced by 25 μg of protein was studied. Since there was variation in the absolute degrees of aggregation induced by various preparations of phosphorylated proteins, the OD_{450} values are expressed as percentages of the control, i.e., nonphosphorylated C1. For both C1 and C2, increasing the extent of phosphorylation decreased the ability of the proteins to aggregate the vesicles.

Effect of Removal of C-Terminal Arginines on Vesicle Aggregation. Components 1 and 2 were treated with a carboxypeptidase as outlined under Materials and Methods, to generate a class of charge isomers that were deficient in C-terminal arginine. The ability of 25 μg of these derivatives to aggregate PS–PC vesicles was compared with that of an equal amount of the control proteins. The controls were prepared by boiling the protein in the presence of the enzyme and PMSF as outlined under Materials and Methods. The removal of the three C-terminal residues (two arginines and one alanine) of C1 decreased the aggregation induced by this component from an OD_{450} of 0.78 to that of 0.36. Similar treatment of C2 decreased the OD_{450} from 0.59 to 0.37. Thus, aggregation was sensitive to modifications of the C-terminal portion of the protein. The removal of two arginine residues affected the aggregation of the vesicles in a manner similar

to the addition of 1 mol of phosphate (Figure 3). Both of these modifications decreased the net positive charge on the protein by 2.

Discussion

Though the charge microheterogeneity of basic protein is well established, the biological role of this heterogeneity is unknown. It has been shown that BP interacts with acidic lipids through both ionic and hydrophobic forces and that, in mixed-lipid systems, the protein preferentially interacts with acidic lipids inducing phase separation of these lipids from the neutral ones [reviewed in Boggs (1983)]. Thus, alterations in the net charge on the protein have the potential to affect the lipid–protein interactions both at the ionic level, due to the introduction of a new charged residue, and at the hydrophobic level, due to the introduction of a charged residue in or adjacent to a hydrophobic segment of the protein.

In a biphasic lipid system, acetylation of 8 of 13 lysyl residues in bovine BP decreased the number of lipid molecules that interacted with BP (Steck et al., 1976). Jones & Epand (1980) reported a tendency of the higher numbered components (less cationic isomers) to interact with less PS in a biphasic system though the statistical significance of the differences was questioned. Thus, the charge microheterogeneity of the protein has the potential to affect the membrane by modulating the amount of acidic lipid available for interaction with the other lipids or proteins in the membrane.

In the present study, we have compared the ability of the charge isomers, either naturally occurring or experimentally produced, to induce vesicle aggregation. In our initial studies, we attempted to extend Smith's study (Smith, 1977) of BP-induced aggregation of PC vesicles. In the absence of any added constituents such as lyso-PC and fatty acid, there was generally very little aggregation of these vesicles, which agrees with the work of Young et al. (1982).

Young et al. (1982) attributed the BP-induced aggregation of PC vesicles reported by Smith (1977) to an anionic impurity generated during lipid sonication. Though they did not characterize the nature of the impurity, the work of Stollery & Vail (1977) indicated that lipid hydrolysis could occur during sonication, thus generating anionic impurities in the lipid. To study the possible effects of lipid hydrolysis on the aggregation, we prepared PC vesicles containing lyso-PC plus oleic or palmitic acids, thus stimulating lipid hydrolysis. Although these vesicles were aggregated by the charge isomers, there was not a significant difference in the degree of aggregation induced by the phosphorylated proteins relative to the controls (Table II).

In contrast, when the PC vesicles contained approximately 8 mol % PS, we found that small changes in protein charge, whether due to phosphorylation, C-terminal arginine loss, or deamidation, had a dramatic effect on the ability of the protein to induce aggregation. Each type of modification decreased the degree of aggregation induced in PS–PC vesicles. The concentration curves reflected the apparent cooperative nature of the aggregation reaction. Similar sigmoidal aggregation curves were reported by Ohki et al. (1982) in their study of divalent cation induced aggregation of PS–PC vesicles.

The exogenous kinase RMPK, which was used to phosphorylate the isomers, recognized one site (Ser-54)[5] shown to be phosphorylated in vivo (Carnegie et al., 1974; Martenson et al., 1983). The two other sites, Ser-109 and Thr-33, do not appear to be phosphorylated in vivo although Ser-109 is near the in vivo sites at Ser-114[6] (Martenson et al., 1983) and Thr-97 (Chou et al., 1976; Martenson et al., 1983). Therefore, protein phosphorylation by RMPK can be used to probe the possible consequences of in vivo phosphorylation. A preliminary study of PC vesicles containing approximately 8 mol % PA indicated that the nature of the lipid can affect the aggregation. The substitution of PA for PS in the PC vesicles rendered the vesicles less sensitive to the effect of the degree of protein phosphorylation. A decrease in the aggregation was generally apparent only after the incorporation of more than 1 mol of phosphorus/mol of protein whereas the aggregation of PS–PC vesicles was sensitive to lower levels of phosphorylation. This suggested that there was a difference in the interactions of the modified proteins with these two types of mixed vesicles and may be a reflection of the greater degree of penetration of BP into PA vesicles than into PS vesicles (Stollery et al., 1980).

The removal of C-terminal arginines, which may not occur in vivo (Chou et al., 1976), allowed the formation of a set of charge isomers with well-defined modifications. In addition, since there is an in vivo phosphorylation site near the C-terminal at Ser-164 (Chou et al., 1976; Martenson et al., 1983), the removal of the C-terminal arginines in vitro (residues 168 and 169) allowed an investigation of the consequences of a change in charge localized to the C-terminal portion of the molecule. This modification was found to decrease the ability of C1 and C2 to induce vesicle aggregation.

The final type of modification investigated was deamidation. Chou et al. (1976) have shown that partial deamidation of Gln-102 and -145 accounts for some of C2. Partial deamidation of Asn-91, Gln-72, and Gln-120 has also been detected (Eylar et al., 1971; Martenson et al., 1983). Since the C1 and C2 in our study had essentially intact C-terminals, and according to the alkaline gel patterns, they differed by 1 net positive charge, it was likely that the charge microheterogeneity in C2 was due to deamidation. In the aggregation assay of PS–PC vesicles, C2 was less potent than was C1 in inducing aggregation. Thus, deamidation can also affect the ability of BP to induce vesicle aggregation.

Lampe et al. (1983) proposed that vesicle aggregation was a two-step process consisting of protein–vesicle interaction followed by protein-mediated vesicle aggregation. Thus, a decrease in aggregation could be due to a decrease in the initial binding of the protein to the vesicles or to an alteration in the subsequent vesicle cross-linking. Due to difficulties in separating the lipid-bound protein from that which was free in solution, we have been unable to determine which step in this model was most sensitive to the alteration in charge.

The sensitivity of vesicle aggregation to the degree of protein phosphorylation may have particular significance since myelin contains both kinase and phosphatases that recognize BP as a substrate (Martenson, 1980; Petrali & Sulahke, 1982), and so the level of protein phosphorylation can be modulated in vivo. For example, McNamara & Appel (1977) have reported that membrane-bound phosphatase activity was elevated in rapidly myelinating rat brain relative to that of the adult brain, suggesting that phosphorylation may have a role in development. On the other hand, Desjardin & Morell (1983) found no difference in the in vivo incorporation of radiolabeled phosphorus in BP isolated from adult or developing rat brain.

[5] The numbering of the residues is based on the sequence of bovine BP reported by Eylar et al. (1971) as revised by Brostoff et al. (1974).

[6] This site has been reported in rabbit BP only where the endogenous sites of phosphorylation have been extensively studied (Martenson et al., 1983). The corresponding sequence number in the rabbit protein is Ser-113.

They reported that the phosphate in BP was rapidly metabolized and proposed that "phosphorylation may be involved in keeping cytoplasmic incisures open in the otherwise compact structure of myelin...". If the differences in aggregation detected in the model membrane systems used in our study occur in vivo, then it is conceivable that alterations in the degree of protein phosphorylation could affect the apposition of the membrane bilayers.

These studies on vesicle aggregation correlate with recent liquid diffraction X-ray studies in which C1 was found to be more effective than C2 in inducing sharp X-ray reflections, consistent with "crystalline" packing of the multilayers (Brady et al., private communication). From the aggregation and X-ray studies, one of the roles of BP appears to be to induce and maintain the compact multilamellar structure of myelin. It is surprising that a small change in overall charge of the molecule has such a dramatic effect on the compaction of the model membranes. Since BP consists of a number of charge isomers, the relative proportion of these isomers may reflect the need for a delicate balance of overall charge for the stabilization of normal myelin. Alternatively, the ability to vary the overall net charge on BP may reflect a requirement of myelin membrane for different degrees of compaction, e.g., a predominance in the proportions of C1 may produce a more compact myelin that may be required for specific functions, possibly in specific areas of the brain.

Registry No. Oleic acid, 112-80-1; palmitic acid, 57-10-3.

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