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# MYELIN BASIC PROTEIN-ENHANCED FUSION OF MEMBRANES

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**Myelin basic protein caused rapid aggregation of vesicles containing acidic phospholipids. Aggregation could be reversed by trypsin digestion of the myelin basic protein. Aggregated vesicles containing gel phase phospholipids or vesicles containing greater than 15 mol% lysolecithin underwent fusion. The extent of fusion was measured by irreversible changes in the light-scattering intensities or diffusion coefficients of the vesicles. Fusion was also measured by the fluorescence quenching which occurred when vesicles containing a covalently bound fluorophore, *N*-4-nitrobenzo-2-oxa-1,3-diazole, were fused with vesicles containing the covalently bound spin label, 4,4-dimethyl-oxazolidine-*N*-oxyl. The kinetics of fusion were first order in phospholipid and had half-times of 0.5–5 min depending on lysolecithin composition. This protein-enhanced membrane fusion may provide a valuable model system for studying some types of biological membrane fusions.**

## Introduction

Myelin membranes contain approx. 20% protein, of which one-third is myelin basic protein [1]. Myelin basic protein is water soluble, rich in lysine and arginine residues, and associated with anionic lipids [2]. One proposed role for this protein is maintenance of the multilaminar structure of the myelin sheath through membrane cross-linking [3,4].

The association of myelin basic protein with membranes has been studied extensively using a variety of physical techniques [5–9]. Reported properties include the ability to aggregate vesicles containing negatively charged phospholipids [10].

Differentiation between vesicle aggregation and vesicle fusion is difficult. Vesicles containing spin-labeled phospholipids have been shown to quench the fluorescence of membrane-bound fluorescent molecules [11]. Spin-label quenching was used here to detect the fusion-dependent mixing of separate bilayers. Normally stable vesicles containing acidic phospholipid vesicles present in some biological membranes underwent fusion in the presence of myelin basic protein.

## Materials and Methods

PC, lyso-PC, doxyl-PC and NBD-PC were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, U.S.A.). PG, lyso-PG, DPPC, poly(L-lysine) (hydrobromide, mol. wt. 30000) and trypsin (type III, twice recrystallized) were purchased from Sigma Chemical Co. (St. Louis, MO,

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Abbreviations: doxyl, 4,4-dimethyloxazolidine-*N*-oxyl; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine; doxyl-PC, 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine; NBD-PC, 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminodecanoylphosphatidylcholine; PG, phosphatidylglycerol; lyso-PG, lysophosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; PS, phosphatidylserine; HPLC, high-performance liquid chromatography; ANS, 8-anilino-1-naphthalenesulfonic acid; *r*, correlation coefficient; *n*, sample size.

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U.S.A.). The phospholipids were reported to be greater than 98% pure. HPLC chromatography of the phospholipids [12] supported this high purity, and they were used without further purification. Phospholipid concentrations were estimated by organic phosphate measurement [13]. All of the phospholipid components in the vesicles are expressed in mol%.

Myelin basic protein was purified from bovine brains by the method of Eylar et al. [14]. An additional purification step consisted of gel filtration on a Sephadex G-75 superfine column (1.6 × 90 cm) to remove traces of contaminating protein. The final protein contained no contaminants (less than 1% of the major protein band) detected on polyacrylamide electrophoresis. Protein was quantitated using $E^{1\%}_{276.4} = 5.64$ [15].

Phospholipid vesicles were prepared by the ethanol-injection method of Kremer et al. [16]. Phospholipids were dissolved in 0.5 ml of ethanol and injected in 5-$\mu$l aliquots into a stirred 10.0 ml solution of buffer (0.1 M NaCl and 0.05 M Tris-HCl, pH 7.5) at room temperature (40°C for DPPC/PG vesicles). A standardized method of injection was necessary to obtain vesicle populations of consistent size. The same buffer was used throughout these experiments. Dialysis of the vesicles to remove ethanol appeared to have no effect on the vesicle fusion studies, and the vesicles were usually used without dialysis.

Myelin basic protein was added to the cuvette containing vesicles by pipetting the appropriate volume from a 1 mg/ml solution. Where indicated, trypsin was added to the cuvette from a freshly prepared solution (2 mg/ml). The cuvettes were mixed prior to analysis.

Quasi-elastic light scattering was used to obtained diffusion coefficients for the vesicle preparations using the methods and apparatus described elsewhere [17]. Light-scattering intensity at 90° (350 nm wavelength) was used to demonstrate qualitatively vesicle aggregation.

Relative 90° light-scattering intensity and fluorescence measurements were performed on a Hitachi Perkin-Elmer Model 44A fluorescence spectrophotometer equipped with a thermostatically controlled, circulating water bath. The measurement of NBD fluorescence used excitation at 350 nm and emission at 534 nm with a 430 nm cut-off filter in front of the emission monochromator. Data points were taken from the fluorescence quenching curves and entered into a computer. Background quenching (see below) was subtracted, and rate constants were obtained from first-order plots of the data.

Phase transition temperatures of vesicle preparations were determined from the inflection which occurs in ANS fluorescence intensity (excitation = 360 nm, emission = 470 nm) as vesicle solutions containing 1% ANS were cooled through the phase transition. The phase transition temperature reported was the midpoint of this inflection [18,19].

## Results

The vesicles formed by the ethanol-injection technique had a weight average diameter of 35–50 nm as determined by diffusion coefficient measurements (Table I). The phase transition temperature determined for PG/DPPC (20 : 80) vesicles was 29.7°C, while all other vesicle preparations had a phase transition below 10°C. In the absence of myelin basic protein, all vesicle preparations were stable for several hours and gave constant light-scattering intensities and diffusion coefficients.

Myelin basic protein caused a large increase in light-scattering intensity to a maximum value in less than 5 s (Fig. 1). Trypsin dissociated the aggregates presumably by digesting the protein cross-links (second arrow, Fig. 1). Vesicles containing low amounts of lyso-PC (less than 15%) returned to the original light-scattering intensities and had the same diffusion coefficients. Vesicles containing higher amounts of lyso-PC or vesicles below their phase transition showed partial irreversibility of these parameters due to vesicle fusion (Fig. 1, Table I). These fused vesicles were no longer small relative to the wavelength of light, and 90° light-scattering intensity was only a qualitative indication of fusion. The diffusion coefficients (Table I) provided a better measure of the increased size of these particles. Vesicle fusion was dependent on the presence of negatively charged phospholipids (cf. 20 and 30% PG, Table I). Above 40 mol% lyso-PC vesicles begin to lose bilayer structure [20]. We observed a sharp

TABLE I

VESICLE LIGHT-SCATTERING MEASUREMENTS

| Vesicles [a] (mol%) | $I_{s1}/I_{s2}$ [b] | $D_{20,w}$ ($\times 10^{-7}$) [c] | |
|---|---|---|---|
| | | 1 | 2 |
| 20% PG, 12.5% lyso-PC, 68% PC | 1.0 | 1.034 | 1.029 |
| 20% PG, 25% lyso-PC, 55% PC | 1.3 | 1.044 | 0.836 |
| 30% PG, 12.5% lyso-PC, 57.5% PC | 1.0 | 0.943 | 0.939 |
| 30% PG, 25% lyso-PC, 45% PC | 5.7 | 1.157 | 0.319 |
| 20% PG, 80% DPPC (19.7°C) | 3.8 | 1.126 | 0.091 |
| 20% PG, 80% DPPC (29.7°C) | 4.9 | 1.126 | 0.395 |
| 20% PG, 80% DPPC (39.7°C) | 1.0 | 1.126 | 1.122 |

[a] 150 μg phospholipid/1.5 ml buffer.
[b] Ratio of light scattering before ($I_{s1}$) and after ($I_{s2}$) addition of myelin basic protein (20 μg) followed by trypsin (40 μg) as in Fig. 1.
[c] $z$-averaged diffusion coefficient before (1) and after (2) addition of myelin basic protein (20 μg) followed by trypsin (40 μg).

decrease in the average vesicle size when lyso-PC exceeded 40 mol% and used a maximum of 35 mol% lyso-PC to study fusion.

Kinetic analyses of vesicle fusion utilized fluorescence quenching by spin-labeled phospholipid. The experimental conditions were selected to measure the first fusion event. Fig. 2 shows the relative fluorescence intensities of vesicles containing 30% PG, 1% NBD-PC, PC, and varying mole percentages of doxyl-PC. These phospholipids were all mixed in ethanol before vesicle formation. Most of the subsequent kinetic studies used vesicles containing 40 mol% doxyl-PC. Fusion of a vesicle containing 40 mol% doxyl-PC with a fluorescence-labeled vesicle of equal size resulted in a larger vesicle containing 20 mol% doxyl-PC which provided 85% of maximum quenching (Fig. 2). Vesicles containing doxyl-PC were added in a 14-fold excess over those containing NBD-PC so the first fusion of a fluorescence labeled vesicle almost always involved a spin-labeled vesicle. Under these circumstances, the fluorescence quenching observed was primarily a function of the first fusion event. Fused vesicles had fluorescence intensities equal to those of vesicles containing corresponding amounts doxyl-PC and NBD-PC (Fig. 2). These results indicated that fusion caused complete mixing of fluorophore and spin label. Low concentrations of vesicles were used to minimize the effects of turbidity on fluorescence excitation and emission. The maximum absorbance at 350 nm due to turbidity was 0.1.

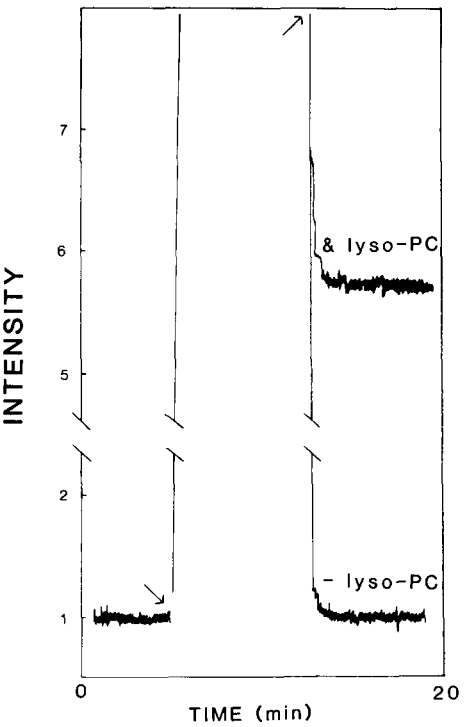


Fig. 1. Effects of myelin basic protein and trypsin on the relative 90° light-scattering intensity. The first arrow indicates the addition of myelin basic protein (20 μg) to phospholipid vesicles (150 μg/1.5 ml buffer). The second arrow shows the addition of trypsin (40 μg) to the mixture. The upper curve shows the result from vesicles of PG/PC/lyso-PC (30:45:25). The lower curve is from vesicles of PG/PC (30:70).

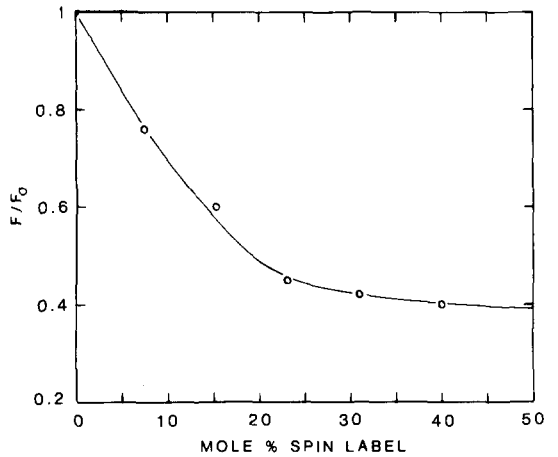


Fig. 2. Relative fluorescence intensity of vesicles as a function of spin label concentration. The fluorescence intensity measured for the given amount of doxyl-PC ($F$) and the fluorescence intensity in the absence of doxyl-PC ($F_0$) were determined for phospholipid vesicles (18.75 μg/1.5 ml buffer) containing PG (30)/NBD-PC (1)/doxyl-PC (indicated amount)/PC (to total 100 mol%).

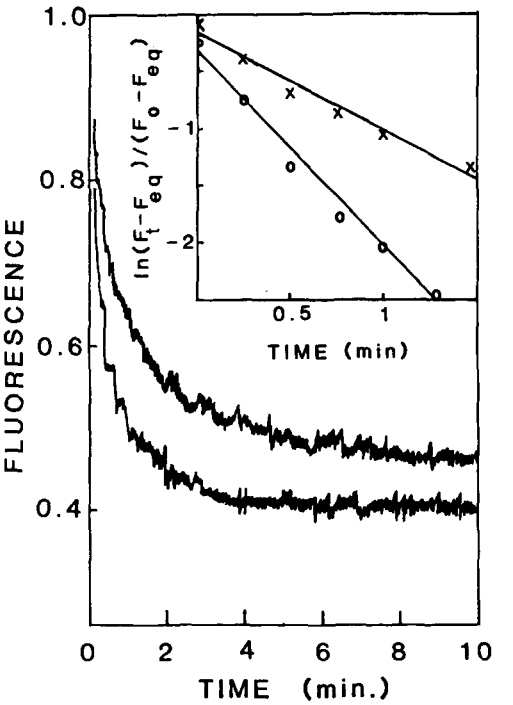


Fig. 3. Myelin basic protein-induced fusion measured by fluorescence decay. Fluorescence is given as the ratio of fluorescence at time $t$ ($F_t$) to fluorescence at time 0 ($F_0$, set at 1.0). The fluorescence decay curves show the results for the addition of 5 μg (upper curve) and 20 μg (lower curve) of protein at time 0 to a mixture of 8.75 μg of vesicles containing PG/lyso-PC/doxyl-PC (30:30:40) plus 0.625 μg of vesicles containing PG/lyso-PC/PC/NBD-PC (30:30:39:1). Inset: first-order rate analysis of fluorescence decays (x, 5 μg; o, 20 μg protein). $F_0$, $F_t$ and $F_{eq}$ (fluorescence at equilibrium) were used to estimate the extent of the reaction.

Addition of myelin basic protein to vesicles containing NBD-PC caused an immediate small increase in fluorescence intensity to a level designated as $F_0$. The cause of this increase is unknown, but it was constant for all experiments. The fluorescence intensity then decreased (Fig. 3). A small decrease was observed in vesicles which, on the basis of diffusion coefficients after trypsin digestion, did not fuse. This decrease was dependent on doxyl-PC and may have resulted from spontaneous transfer of NBD-PC [21] from one vesicle in the aggregated complex to another, a process that occurs at a very slow rate in the absence of myelin basic protein. In any event, the rate of this decay was constant over the range of protein concentrations used and was subtracted as a background from systems undergoing fusion.

Vesicle fusion was detected by a large decrease in fluorescence intensity (Fig. 3). Vesicle aggregation was much faster than fusion, so the fusion kinetics were independent of aggregation. The fluorescence decay was logarithmic (Fig. 3, inset) and the first-order rate constants were the same over a 4-fold range of vesicle concentration. Therefore, fusion was first-order with respect to phospholipid which is anticipated for an event which occurs within vesicle aggregates rather than between free vesicles which must collide. Half-times for fusion were 0.5–5 min depending on vesicle composition. Similar half-times were reported for the fusion of phosphatidic acid/PC vesicles by calcium [22].

The rate of vesicle fusion was saturable with respect to myelin basic protein and required a minimum of 15% lyso-PC (Fig. 4). At saturating protein concentrations, the rate constant for myelin basic protein-induced vesicle fusion was dependent only on the composition of the vesicles. Arrhenius plots showed that the activation energy for fusion was greater for membranes containing low concentrations of lyso-PC. Least-squares analysis estimated the activation energy to be 16 kcal/mol ($r = -0.952$, $n = 7$) for 20 mol% lyso-PC vesicles and 5 kcal/mol ($r = -0.988$, $n = 7$) for 35

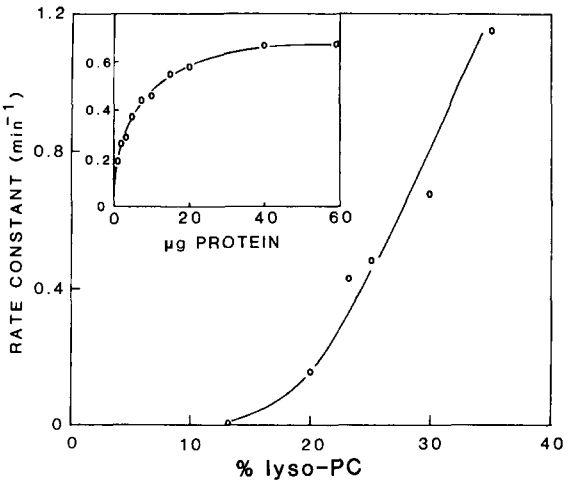


Fig. 4. Effects of lyso-PC and protein on the rate constant for vesicle fusion. Myelin basic protein (40 μg) was added to a mixture of 17.5 μg of vesicles containing PG (30)/doxyl-PC (40)/lyso-PC (amount specified)/PC (to 100 mol%) plus 1.25 μg of vesicles containing PG (30)/NBD-PC (1)/lyso-PC (amount specified)/PC (to 100 mol%). Inset: effects of myelin basic protein concentration on the rate constant for fusion. The amounts of protein shown were added to a mixture of 17.5 μg of vesicles containing PG/lyso-PC/doxyl-PC (30:30:40) plus 1.25 μg of vesicles containing PG/lyso-PC/NBD-PC (30:30:39:1) in 1.5 ml buffer. The rate constants were determined as in Fig. 3.

mol% lyso-PC vesicles (data not shown).

Substitution of the same amount of poly(L-lysine) for myelin basic protein gave similar rates and levels of aggregation of vesicles containing 25% lyso-PC in light-scattering experiments. However, the rate of fusion when poly(L-lysine) (15–200 μg) was mixed with vesicles (PG/lyso-PC/NBD-PC/PC, 30:25:1:44, 1.25 μg; plus PG/lyso-PC/doxyl-PC, 30:30:40, 17.5 μg in 1.5 ml buffer) was too slow to be measured accurately by this fluorescence quenching technique. Poly(L-lysine) was therefore much less effective in the vesicle fusion process. Substitution of PS for PG caused little change in the observations except that PS was about half as effective in the fusion process. Lyso-PG was as effective as lyso-PC in causing vesicle fusion (data not shown).

## Discussion

Previous studies have documented several conditions which promote fusion of phospholipid bilayer membranes. The addition of lyso-PC to vesicles near the phase transition [23] and the addition of calcium to vesicles containing high levels of acidic phospholipids [24] are examples. Membranes in the gel phase are also more susceptible to aggregation and fusion [25,26]. Myelin basic protein-enhanced fusion of PC [27] and phosphatidylethanolamine [28] vesicles prepared by sonication has been reported. We found that PG/PC vesicles prepared by sonication showed evidence of protein-enhanced fusion although the extent varied a great deal with different preparations. No fusion was observed when the vesicles were prepared by the ethanol-injection technique unless lyso-PC was included. We concluded that sonication may sometimes produce sufficient lysolipid to allow protein-enhanced fusion. Stollery and Vail [28] indicate that up to 10% lysolipids could be present after sonication. For this reason, caution should be exercised when interpreting results using sonicated vesicles.

This study demonstrated that myelin basic protein rapidly aggregates vesicles containing low levels of acidic phospholipids and induces the fusion of vesicles of certain compositions. Myelin basic protein binds to phospholipids through ionic and nonionic interactions [29,30] and may destabilize the membranes to aid fusion. This property alone, however, did not promote fusion unless the vesicles contained gel state phospholipids or high (greater than 15%) levels of lyso-PC. Interestingly, a biological membrane known to undergo fusion, chromaffin granules, contains 17% lyso-PC [31].

Two minimum conditions for membrane fusion appear to be bilayer instability and bilayer proximity. Instability can apparently be caused by several factors such as high charge density, the presence of lyso-PC, and membranes in the gel state. Close proximity can be achieved by cross-linking vesicles with multivalent cations or with proteins such as myelin basic protein. The cross-linking of membranes and their subsequent fusion may be an important role of certain proteins. Myelin basic protein may provide a model system for studying this type of membrane fusion. Nerve demyelination in some disease states could be related to myelin basic protein-induced fusion of destabilized myelin membranes.

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