Protein-Induced Aggregation of Lipid Vesicles. Mechanism of the Myelin Basic Protein–Myelin Interaction

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Abstract: When the light scattered by suspensions of lipid vesicles during protein-induced aggregation is monitored, changes in the physical size of the vesicle aggregates can be obtained along with kinetic constants for the reaction. The aggregation of phosphatidylserine vesicles, induced by polylysine and by bovine myelin basic protein (MBP), is monitored by this technique. Polylysine rapidly induces high-order aggregation to produce relatively stable vesicle trimers and tetramers. Myelin basic protein is much less efficient at inducing the aggregation reaction, producing dimers that rapidly coalesce to form units of more compact structure. With vesicles prepared from whole myelin suspensions, however, dimers are initially induced by both proteins, and myelin basic protein is much more efficient than polylysine at inducing this dimer formation. The induction of myelin dimers is a rapid equilibrium process displaying simple saturation kinetics with respect to [MBP]. The MBP-induced dimerization is markedly pH dependent, becoming unfavorable in acid, and the dissociation constant follows a simple titration curve with an apparent pK_a of 5.9. It is concluded that the interaction between MBP and the myelin membrane is largely electrostatic. The significance of this pH instability to myelin phagocytosis in demyelinating diseases is discussed.

The cation[1] and protein-[2,3] induced aggregation of lipid vesicles have received much study as models of vesicle fusion events occuring in vivo. When the turbidities of these solutions are monitored during the aggregation reaction, complex kinetic changes are often observed.[1] A problem with the rigorous interpretation of these kinetic data concerns the uncertainty in the nature of the products at each stage of the vesicle polymerization. Light scattering offers a technique by which the turbidity of a vesicle solution, when viewed from several different angles, provides information regarding the shape and size of the scattering particle.[4-6] Thus, aggregation reactions, followed in a light-scattering photometer, offer the potential of providing information regarding both the kinetic time course of the reaction and the accompanying changes in size and shape.

In order to illustrate the kinetic use of light scattering in this type of reaction, we have examined the aggregation of phosphatidylserine vesicles and of vesicles prepared from suspensions of bovine myelin by polylysine and bovine myelin basic protein.

Experimental Section.

Materials. Myelin basic protein was isolated from bovine calf brain by the procedure of Eylar and coworkers,[8] up through the ammonium sulfate step. The ammonium sulfate precipitate was collected by centrifugation, resuspended in 50 mM phosphate buffer (pH 7) and desalted over Sephadex G-25. Protein-containing fractions were combined and lyophilized. The protein was homogeneous by size over a calibrated Sephadex G-100 column and gave a single band on electrophoresis.[9] Protein concentration was determined by biuret assay using bovine serum albumin as standard. Trypsin, phosphatidylserine (98–99%; chromatographically purified from bovine brain), polylysine (M_r 30 000–70 000), and other organic chemicals were from Sigma Chemical Co. (St. Louis, MO) and were used as supplied. The phosphatidylserine was shipped and stored at −20 to −78 °C to avoid decomposition and was homogeneous by thin-layer chromatography

Myelin was isolated by the general procedure of Towes et al.[10] The "purified myelin" was hand homogenized in 50 mM phosphate buffer (pH 7.0) and sonicated at maximum power for 10–60 min by using a Bronwill Biosonik III with a 15-mm probe. The sonications were performed in 30-s bursts, under nitrogen. The samples were cooled to 2°C and were well stirred to avoid denaturation or oxidation. Myelin samples were stored at −23°C until use. Vesicles for light scattering wer sonicated, centrifuged at 20000g for 20 min, filtered through an 8-μm disk, and applied in 10-mL portions to a 2.5 × 25 cm column of Sepharose 4B-200. Fractions of 1 mL were collected with 5 mM Tris buffer, pH 7.5, used as eluant. Each of these fractions were sized by light-scattering methods to determine the radius of gyration; these were typically in the range 700–850 Å. A sample of the vesicle preparation was examined by electron microscopy (glutaraldehyde/OsO_4 fixative, stained en bloc with uranyl acetate/lead citrate). The EM examination showed the vesicles to be closed with single bilayer membranes with a radius consistent with the radius of gyration determined by scattering measurements. The lipid composition of myelin vesicles, isolated in this manner, is well described in the literature.[11] The isolation of myelin lipid followed the general procedure of Folch and Lees[12] with 1% NaCl as the "aqueous phase". Phosphatidylcholine was isolated from egg yolk by standard methods and purified by chromatography on basic alumina.[13] Concentrations of whole myelin are based on lyophilized dry weight.

Light Scattering. Measurements were obtained using a Brice-Phoenix light-scattering photometer equipped with a thermostated cell holder designed for round, 2-mL scattering cells. A 1-mm slit was used, incident light was 546 nm and unpolarized, and the temperature was maintained at 25° C. Data were collected by using either a galvanometer or a strip-chart recorder at angles ranging between 110° and 35°. Values of R_θ were calculated by using standard methods[14] and calibration constants were obtained by comparison of the micro cell with standard square cuvettes. Calibration was checked further by a molecular weight determination on bovine serum albumin.[15]

Kinetic determinations in the scattering photometer were initiated by addition of small amounts of protein into preequilibrated scattering cells. With a "plumper" mixing device, the total mixing time was approximately 4–5 s. Extreme care was taken to avoid bubble formation during mixing; all scattering solutions were filtered immediately before addition to the cell. The constant $\partial n/\partial c = 0.149$ for phosphatidylcholine[14] was used for the calculation of κ for the myelin vesicles. This value is probably a bit small; however, the calculated radius of gyration is essentially independent of this constant.

First-order rate constants for the aggregation reaction were obtained by standard plots of $A_\infty - A_t$ against time. Such plots were nonlinear, being composed of two distinct linear segments. The magnitude of the "burst" was defined as the difference between the total absorbance change and the total absorbance change attributable to the two first-order processes, extrapolated to zero time.

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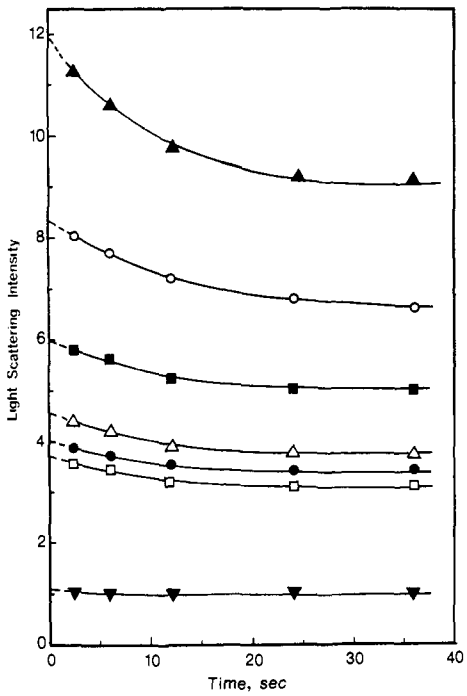

Figure 1. Relative transmittance changes observed as a function of angle for the myelin basic protein induced aggregation of phosphatidylserine vesicles for the following angles: (○) 50°; (■) 60°; (△) 70°; (●) 80°; (□) 90°; (▼) 0°. The data for 0° are ratios of final to initial values. Conditions: [MBP] 15 μg/mL, [PS] 0.5 mg/mL, phosphate buffer, 25 °C.

The addition of MBP alone to buffer blank gave no turbidity changes at concentrations used in our experiments.[16] The observed turbidity changes were heat stable, being unaffected by incubation of the vesicle suspension at 70 °C for 5 min prior to the addition of MBP. In experiments involving tryptic digest of MBP, the protein was incubated with the enzyme (10 mg/mL) for 10 min prior to the addition to the vesicle suspension. The trypsin-treated MBP did not induce the aggregation of the myelin vesicles at the concentrations used in our experiments.

Results and Discussion

Phosphatidylserine Vesicles. When polylysine or myelin basic protein are added to suspensions of phosphatidylserine (PS) vesicles, rapid turbidity changes occur. The magnitudes of the turbidity changes observed at different viewing angles for the aggregation of PS induced by MBP are shown in Figure 1. These data were collected by repeatedly running the aggregation reaction in the scattering photometer with the analyzer at the different angles. Cell cleanliness and accurate placement are critical in these types of experiments. Once compiled, these data present the kinetic course of the reaction as viewed from a number of angles and from zero angle. The ratio of the light scattered at a given angle to the light passing through the sample is all that is necessary in order to define the scattering ratio, and hence the *radius of gyration* (R_G), at any time during the aggregation reaction. A sample of this time-course dependence is shown in Figure 2 for both polylysine and MBP. For polylysine, the starting vesicles, with R_G = 320 Å, rapidly form aggregates of approximately 1180 Å during the mixing time. These appear to be stable on this time scale, since the R_G at 36 s into the reaction is still about 1150 Å. The increase from a radius of gyration, as measured by scattering, of 320 to about 1200 Å would correspond to the formation of aggregates having three to four vesicle monomers. We should point out that light scattering, being a function of the 6th power of the actual radius of a macromolecule (a function of volume squared), measures a "radius of gyration" that corresponds to the gyrating radius of the largest molecules having a significant population in the system. A physical model having

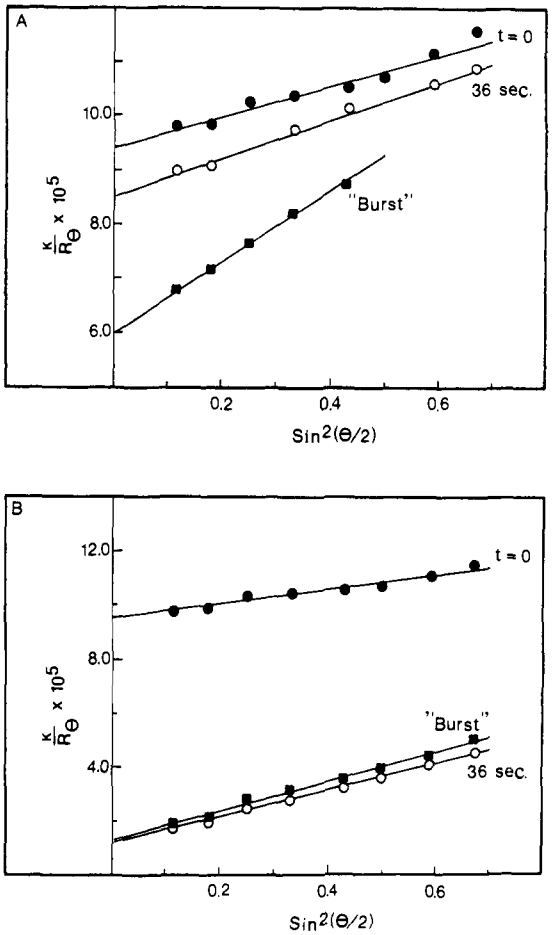

Figure 2. Plot of the scattering function κ/R_θ against the angular function $\sin^2(\theta/2)$ for scattering from phosphatidylserine vesicles: (A) (●) Before addition of MBP, R_G = 320 Å; (■) immediately after mixing, R_G = 590 Å; (○) 36 s after mixing, R_G = 380 Å. Conditions: [MBP] 15 μg/mL, [PS] 0.5 mg/mL, pH 7.0, phosphate buffer, 25 °C. (B) (●) Before addition of polylysine, R_G = 320 Å; (■) immediately after mixing, R_G = 1180 Å; (○) 36 s after mixing, R_G = 1150 Å. conditions: [polylysine] 5 μg/mL, [PS] 0.5 mg/mL, pH 7.0, phosphate buffer, 25 °C.

three or four vesicles bonded nonlinearly would be consistent with the observed data.

The time course of the aggregation reaction initiated by myelin basic protein is shown in Figure 2B, Again, the starting vesicles are 320 Å, however, the first intermediate (which is formed in the mixing time) has a radius of gyration of 590 Å. This would be consistent with the rapid formation of vesicle *dimers* in the presence of MBP. Further, the dimers appears to be unstable, and 36 into the reaction they have transformed into more compact particles having R_G = 380 Å. The information in Figure 1 allows us to explore this change more closely, since values of R_G can be obtained at any desired time interval. These changes in R_G are plotted logarithimically in Figure 3. The fact that this plot is linear strongly suggests that the observed phenomena is a first-order coalescence. Two physical models are possible, one involving vesicle fusion and mixing of contents and the second involving formation of a tight "myelin-like" junction between the two vesicle surfaces. Further work involving the elegant Tb–dipicolinic acid assay of Papahadjopoulos et al,[17] is in progress in order to resolve the question of the nature of this coalescence. A further slow decrease in the size of the vesicles following the first-order decay is also observed, since the R_G after 36 s is 380 Å, significantly smaller than the 435 Å predicted as the end point in Figure 3.

The concentration dependence of the MBP- and polylysine-initiated aggregation of PS is shown in Figure 4A. The fact that the extent of the initial aggregation (the "burst") follows a simple

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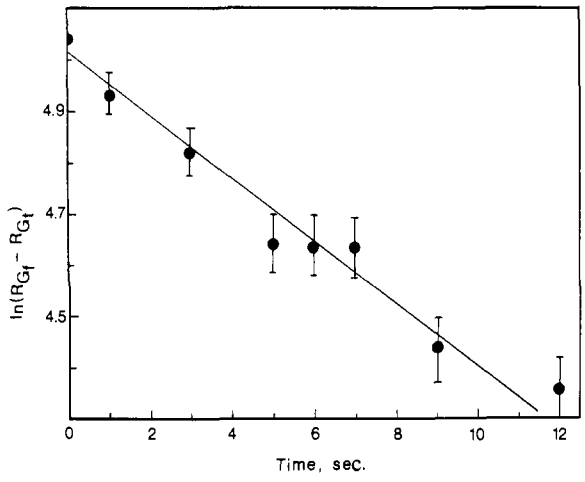

Figure 3. Logarithmic plot of $[R_G(\text{final}) - R_G(\text{time})]$ against time for the first-order process following the dimerization of phosphatidylserine vesicles induced by myelin basic protein. The final radius of gyration was taken to be 435 Å; pH 7.0, phosphate buffer, 25 °C.

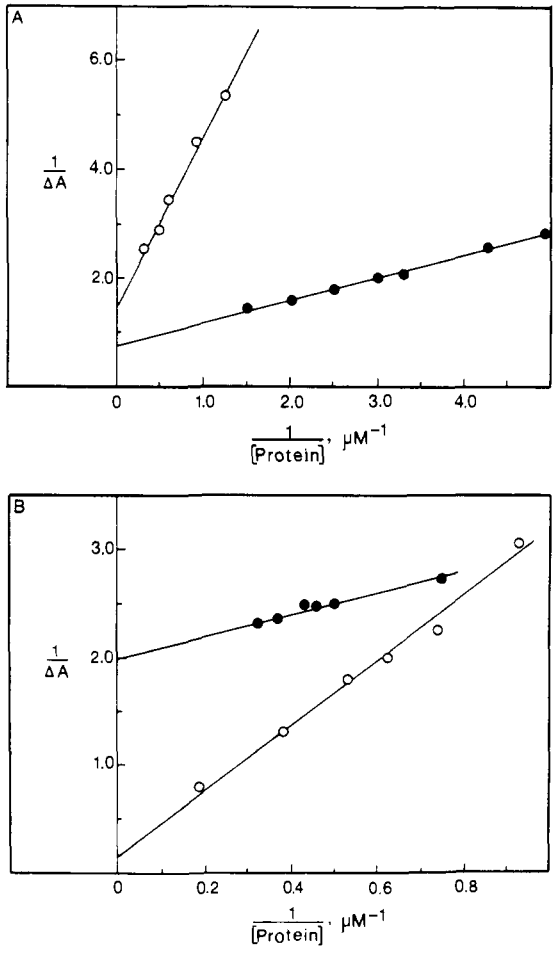

Figure 4. Double-reciprocal plots of initial absorbance change observed on mixing vesicles of (A) phosphatidylserine or (B) whole myelin with myelin basic protein (○) or with polylysine (●). Conditions: [PS] 0.5 mg/mL, [myelin] 0.5 mg/mL, phosphate buffer, pH 7.0, 25 °C.

saturation curve suggests that the binding is a simple rapid equilibrium process. The burst observed at "infinite" MBP is smaller than for polylysine and the observed dissociation constant for MBP is somewhat larger. Two important observations should be made from these data: MBP and polylysine are *different*; and with PS as substrate, polylysine is much more efficient at inducing high-order aggregation than is MBP.

Myelin Vesicles. In vivo, of course, MBP does not interact with homolipid suspensions, but rather interacts with the myelin

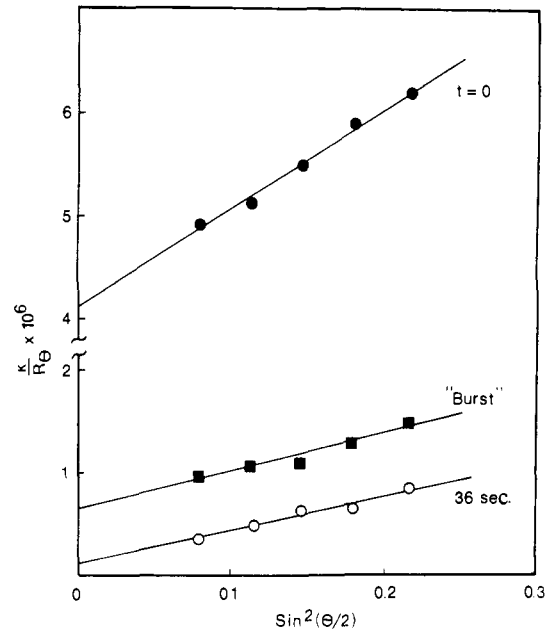

Figure 5. Plot of the scattering function κ/R_θ against the angular function $\sin^2(\theta/2)$ for scattering from vesicles of whole myelin: (●) before addition, R_G = 760 Å; (■) immediately after mixing, R_G = 1500 Å; (○) after 36 s, R_G = 2800 Å.

membrane, a heterogeneous mixture of lipids and proteins.[11] Basic protein is one of the major components of myelin and is exclusively located on the cytoplasmic side, not accessible to the solvent.[18] The role of basic protein in myelin is apparently structural, interacting with the cytoplasmic membranes and stabilizing the characteristic myelin multilamellar membrane assembly. The protein itself is a random coil in solution[19] and is the encephalitogenic agent in allergic encephalomyelitis. Although aggregation of the protein has been described at high pH,[16] it is monomeric under the conditions used in the present work.[16,20] In order to study the mechanism of the interaction of basic protein with the myelin membrane, we have prepared single-walled vesicles from multilamellar myelin suspensions. The myelin vesicles used in these studies contain all of the lipid and protein components that are characteristic of myelin, as judged by $NaDodSO_4$ gel electrophoresis.[9] The surface of the vesicles must be depleted in myelin basic protein, since the addition of MBP or polylysine induces the vesicles to aggregate; in the absence of added proteins they are stable at 4 °C for over 24 h. Using the kinetic light-scattering technique, the data in Figure 5 are obtained. Initially, the R_G of the vesicles is about 760 Å, and, on the addition of MBP, the radius of gyration doubles to about 1500 Å. Coalescence is not observed with the myelin vesicles, and instead, they continue to aggregate, forming "tetramers" after about 30 s. Dimer formation is also observed in the aggregation induced by polylysine. It is not immediately obvious why coalescence is not observed with the myelin membranes, although the high concentration of protein in the vesicles could restrict vesicle fusion or collapse.

The concentration dependence of the burst is shown in Figure 4B for both MBP and polylysine. Again, saturation kinetics are observed; however, MBP is now *more efficient* at inducing dimer formation and the dissociation constant for MBP is significantly smaller than for polylysine. The fact that MBP is superior to polylysine in reactions involving myelin vesicles while polylysine is more efficient with regard to PS vesicles strongly suggests a *specificity* in the myelin–MBP interaction that has survived the homogenization process. It is important to note that basic protein is not functioning as a general polycation but appears to specifically

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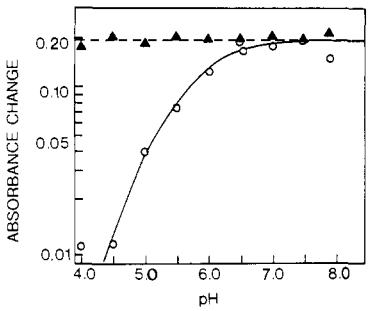

Figure 6. Logarithmic plot of the initial absorbance change that is observed on mixing MBP with whole myelin vesicles (O) and with myelin lipid vesicles (▲) against pH. Vesicle concentrations, 0.5 mg/mL, 25 °C.

Table I. Effect of pH on the MBP-Induced Aggregation of Myelin and Myelin Lipid Vesicles[a]

pH[b]	whole myelin OD_{burst}[c]	myelin lipid OD_{burst}[c]
7.9	0,15	0.22
7.5	0.20	0.20
7.0	0.18	0.21
6.5	0.20	0.20
6.0	0.13	0.21
5.5	0.07	0.21
5.0	0,04	0.19
4.0	0,01	0.18

[a] The concentrations used were MBP 25 μg/mL, myelin vesicles 0.84 mg/mL, and myelin lipid vesicles 0.42 mg/mL. [b] pH maintained with phosphate or citrate buffers at a total concentration of 12 mM. [c] Initial absorbance increase on mixing MBP and vesicles.

interact with some region of the myelin membrane.

The pH dependence of the dimerization reaction is given in Table I and plotted in Figure 6 (open symbols). The striking feature of this figure is the steady decrease in the burst reaction as the pH is lowered below 6. The solid line in the figure is drawn for a simple ionization occurring at pH 5.9. Such a pH profile might be expected if (a) MBP underwent an ionization in this region and the protonated MBP was ineffective at complexing with the myelin vesicles, (b) the lipid components of myelin underwent protonation, neutralizing anionic interaction sties, or(c) if some protein component of whole myelin was ionizing, inhibiting the MBP–myelin interaction.

In order to distinguish between these possibilities, we investigated the interaction of MBP with protein-free myelin lipid. Kinetically, the interaction of MBP with these lipid vesicles is very similar to the interactions observed with whole myelin; the vesicles continue to aggregate, and coalescence is not observed. Since the myelin-lipid vesicles are prepared from evaporated chloroform/methanol extracts, they must be statistically identical in composition and hence kinetically homogeneous. The fact that polyphasic turbidity changes are observed with the myelin lipid vesicles strongly suggests that the similar polyphasic changes that are observed with *whole* myelin vesicles are not the result of microheterogenity of the whole myelin sample.

The pH dependence of the dimerization of myelin lipid vesicles (Table I) is shown as the dark symbols in Figure 6, The important conclusion is that the magnitude of the burst is *pH independent* between pH 4 and 8. Thus, the diminution of the burst that is observed with whole myelin arises due to pH effects on a *protein* component of the myelin membrane. The simplest explanation that is consistent with these data is that the interactin between MBP and myelin is largely electrostatic. At low pH the protein components of myelin would become more cationic, inhibiting the electrostatic association of the polycationic MBP.

If the myelin–MBP interaction is largely electrostatic, then the magnitude of the burst (the dimerization equilibrium) ought to be sensitive to the ionic character of the medium. The effect of ionic strength and phosphate buffer concentration on the magnitude of the burst is given in Table II. As either the ionic strength or [buffer] increases, the interaction decreases monotonically.

Table II. Effect of Ionic Strength and Phosphate Buffer Concentration on the MBP-Induced Aggregation of Myelin Vesicles[a]

ionic strength[b]	k_1/s^{-1} [c]	OD_{burst}[d]
0.005	0,13	0.23
0.045	0.16	0.14
0.057	0.19	0.08
0.069	0.21	0.08
[buffer]/mM		
5.0	0.14	0.21
12.0	0.16	0.18
24.0	0.19	0,15
36.0	0.18	0,08
48.0	0.15	0,05

[a] At pH 7.0, 25 °C; the concentrations of whole myelin and MBP were 0.84 mg/mL and 25 μg/mL, respectively, [b] pH maintained with 12 mM phosphate buffer; ionic strength adjusted with KCl. [c] Observed rate constants for absorbance increase following the burst. [d] Initial increase in absorbance observed on mixing MBP and myelin vesicles.

Further, the decrease in the burst is a direct linear function of the square root of the ionic strength of the medium. This is the behavior predicted for the Debye–Hückel shielding parameter, κ, consistent with the electrostatic model.[4] The present data are insufficient to attempt a more rigorous interpretation of the free-energy changes associated with the electrostatic interaction, and further work is in progress.

The data in Figure 6 also allow an upper limit to be placed on the contribution of pH-independent hydrophobic interactions to the myelin–MBP association equilibrium. This upper limit is given by the ratio of the observed burst at high pH to the magnitude of the burst seen at pH 4. The observed ratio is about 0.05; thus, pH-independent hydrophobic interactions are contributing less than 5% to the observed binding constant. These data do not eliminate the possibility of hydrophobic interactions existing; however, if they do contribute, they must be electrostatically induced.

Our conclusion that the MBP–myelin interaction is largely electrostatic or electrostatically induced is at odds with the report by Smith[3] that MBP causes the aggregation of phosphatidylcholine vesicles. Since these are neutral, it would seem likely that hydrophobic interactions are responsible for this aggregation. We have found, however, that phosphatidylcholine vesicles sonicated under a nitrogen atmosphere *do not* aggregate in the presence of MBP, while vesicles sonicated in air do aggregate. Based on our experience, we would conclude that the reactions observed by Smith are possibly the result of anionic impurities in the lipid preparations.

Using calorimetric techniques, Boggs et al.[21] have concluded that both the N- and C-terminal peptides of basic protein interact electrostatically and hydrophobically with phosphatidylglycerol vesicles. Likewise, Deber and Young,[22] using NMR, have concluded that the encephalitogenic nonapeptide from basic protein interacts with anionic vesicles by electrostatic means. Since we have found that trypsin-treated MBP does not aggregate myelin vesicles, it would appear that none of these peptides, singly, or in combination, is sufficient to induce cross-linking of the myelin membranes. The interesting suggestion by Boggs[21] that C- and N-terminal interactions within the same protein may be responsible for linking two adjacent myelin membranes would be consistent with our observation of rapid equilibrium dimer formation from MBP and both myelin and PS vesicles.

A striking feature of the pH profile in Figure 6 is how *fragile* the interaction of whole myelin with MBP appears to be, a relatively small pH decrease giving rise to a large change in association constant. The "buffering capacity" of the membrane to absorb electrostatic alteration appears to be very small. If the

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surface of the myelin vesicles in our experiments bears a significant resemblance to the myelin membrane in vivo and the interaction of MBP and myelin is largely electrostatic, then anything that perturbs the charge or charge density of the membrane will lead to decreased binding of MBP. In the present case, the charge is perturbed by the protonation of protein components, In vivo, perturbation could result from a number of mechanisms including enzyme-catalyzed cleavage of anionic residues from membrane phospholipids or sulfatides. This suggests that the primary events in inflammatory demyelination may be associated with increases in these enzyme activities. A simpler mechanism of destabilization of the myelin lamellae would involve a simple pH effect, such as seen in Figure 6. Lysosomal granules, at about pH 3–4, lysed in the immediate vicinity of the sheath would provide a high local concentration of lactic and other small organic acids. Diffusion of these across the lamellae would drastically lower the pH within the confines of the major density region, allowing the lamellae to separate. This type of internal "splitting" along major densities is often observed in electron micrographs during the phagocytosis of myelin.[23]

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Registry No. Polylysine, 25104-18-1; polylysine, repeating unit, 38000-06-5.

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Molecular Structure of 18-Deoxyaldosterone and Its Relationship to Receptor Binding and Activity[1]

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Abstract: 18-Deoxyaldosterone possesses one-third the binding affinity of aldosterone for the cytoplasmic mineralocorticoid receptor and exhibits an approximate 2:1 antagonist to agonist ratio. Crystals of 18-deoxyaldosterone contain two molecules that differ significantly from one another in 17β-side-chain orientation, 4-en-3-one conjugation, and hydrogen bonding. The C(20)–O(20) bond is synperiplanar to C(17)–C(16) in molecule I and to C(17)–C(13) in molecule II. The latter conformation, previously observed only in the presence of a 16β-methyl or -halo substituent, has been stabilized by the fact that epoxide formation draws the C(18) methyl away from the D ring. The two conformers must be in equilibrium in solution. Both molecules of 18-deoxyaldosterone resemble aldosterone in the overall shape of the A, B, C, and E rings. Molecule II and aldosterone have similar hydrogen bonding to O(3) and nearly planar 4-en-3-one conformations. Although the D-ring composition of aldosterone and 18-deoxyaldosterone is different, the side-chain orientation of molecule I of 18-deoxyaldosterone comes closest to approximating that of aldosterone in shape and potential hydrogen-bonding geometry. Analysis of the conformations and activity of aldosterone, 18-deoxyaldosterone, and spironolactone is in agreement with the model which proposes that the A-ring end of the steroid is primarily responsible for initiating and maintaining receptor binding and D-ring variation governs agonist–antagonist response. Molecule II appears to have an A ring ideally suited to receptor binding and a side-chain orientation that would elicit little or no subsequent activity, while molecule I has the side-chain orientation that most likely contributes to the partial agonism exhibited by the molecule. The crystal structure of the hemihydrate of 18-deoxyaldosterone (a = 19.878 (3) Å, b = 30.341 (4) Å, c = 5.9951 (5) Å, $P2_12_12_1$) was determined by direct methods and refined to a final R index of 0.072.

18-Deoxyaldosterone (21-hydroxy-11β,18-oxido-4-pregnene-3,20-dione) is an analogue of the natural mineralocorticoid hormone aldosterone in which the aldehyde hemiacetal structure is replaced by a stable 11β,18-oxide ring (Figure 1). Removal of the 18-oxo group from aldosterone transforms it from an agonist into an antagonist. The 18-deoxy derivative possesses one-third of the binding affinity of aldosterone for the cytoplasmic mineralocorticoid receptor and exhibits an approximate 2:1 antagonist to agonist ratio in toad bladder and adrenalectomized rat bioassay systems.[4]

An equilibrium between the structural isomers of Figure 2 has been proposed on the basis of chemical studies of aldosterone. Because isomers b and c introduce two new chiral centers into the structure, a total of seven isomers are possible. X-ray analysis of the only stable crystal form of aldosterone revealed it to be a monohydrate of the 18(R)-acetal-20(S)-hemiketal isomer (c).[5]

Removal of the 18-hydroxy group from the hemiacetal form of aldosterone would be expected to relieve some of the strain observed in the 18(R)-acetal-20(S)-hemiketal isomer.[5] Nevertheless it is difficult to predict how this change will affect the corticoid side-chain orientation and the overall shape of the molecule. The X-ray crystal structure of 18-deoxyaldosterone was undertaken in order to compare the conformation with that of aldosterone and to attempt to define the structural basis for the difference in binding affinity and activity between the two steroids.

Results

Crystals of 18-deoxyaldosterone contain two crystallographically independent molecules that differ significantly from one another

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