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# Phosphatidylinositol Inhibits Microtubule Assembly by Binding to Microtubule-Associated Protein 2 at a Single, Specific, High-Affinity Site†

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*Received January 22, 1992; Revised Manuscript Received March 31, 1992*

ABSTRACT: The effects of various anionic phospholipids on the in vitro assembly of MAP2/tubulin microtubules has been examined. We show that the potency to inhibit is related to the polarity of the phospholipids and that this is consistent with a mode of action involving the sequencing of microtubule-associated proteins (MAPs) by nonspecific electrostatic interactions. The inhibitory potency of phosphatidylinositol (PI) is, however, considerably larger than predicted by this model. The effects of PI on MAP2/tubulin microtubule assembly have therefore been examined in greater detail by preparing phosphatidylcholine (PC) liposomes doped with increasing amounts of PI. We show that when the PI is sufficiently dispersed by dilution with PC, it inhibits microtubule assembly by binding to MAP2 with an apparent stoichiometry, after correction for the bilamellar nature of the liposomes, of 1:1 mol·mol$^{-1}$ PI:MAP2. Furthermore, we show that the $K_d$ of this interaction is in the submicromolar range.

Numerous studies have indicated that microtubule proteins may interact with membrane components, yet the sequences of neither tubulin nor the major neuronal MAPs[1] contain the long hydrophobic peptides characteristic of integral membrane proteins. Tubulin, for example, has been detected in membrane preparations by colchicine binding (Feit & Barondes, 1970; Bhattacharyya & Wolff, 1975), by polyacrylamide gel electrophoresis (Feit et al., 1971; Blitz & Fine, 1974; Kornguth & Sunderland, 1975), by reactivity with specific antibodies (Walters & Matus, 1975), and by tryptic mapping (Kelly & Cotman, 1978), while a particular tubulin isoform specifically associates with membranes in vivo (Hargreaves & Avila, 1985, 1986). In addition, lipids, and particularly phospholipids, are a contaminant of microtubule protein preparations (Daleo et al., 1974; Lagnado & Kirazov, 1975; Hargreaves & McLean, 1988), and the MAPs bind phospholipids tightly (Murthy et al., 1985), while purified microtubule protein causes stacking/fusion of liposomes (Caron & Berlin, 1979) and the enhanced release of material from liposomes (Klausner et al., 1981).

In vitro microtubule assembly is inhibited by membrane components (Daleo et al., 1977; Reaven & Azhar, 1981), an effect which is not nonspecific since membranes from different cellular structures have differing effects (Reaven & Azhar, 1981). Furthermore, pure phospholipids, particularly phosphatidylinositol, inhibit MAP-dependent microtubule assembly in vitro (Yamauchi & Purich, 1987).

We have investigated the inhibition of microtubule assembly in vitro by both pure phospholipids and phospholipid mixtures. We show that phosphatidylinositol inhibits microtubule assembly due to a specific interaction with MAP2 while the activity of other phospholipids is due to nonspecific anionic sequestering of the MAPs.

## Materials and Methods

All reagents were obtained from Sigma Chemical Co. Unless otherwise stated, phosphatidylinositol (PI) was the ammonium salt of L-α-phosphatidylinositol extracted from bovine liver, which contains primarily stearic and arachidonic acid. Phosphatidylcholine (PC) was L-α-phosphatidylcholine derived from bovine liver. Phosphatidylglycerol (PG) was the ammonium salt of L-α-phosphatidyl-DL-glycerol prepared by the reaction of cabbage phospholipase D with egg yolk lecithin in the presence of glycerol. Phosphatidylserine (PS) was L-α-phosphatidyl-L-serine prepared from bovine brain. Phosphatidic acid (PA) was either the ammonium salt of dioleoyl-L-α-phosphatidic acid monomethyl ester or the sodium

† This work was supported by the Science and Engineering Research Council.

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[1] Abbreviations: MTP, microtubule protein; MAP, microtubule-associated protein; MES, 4-morpholineethanesulfonic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; PIP, phosphatidylinositol 4-monophosphate; $PIP_2$, phosphatidylinositol 4,5-diphosphate; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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salt of dioleoyl-L-α-phosphatidic acid.

Lipid suspensions were prepared from solutions of the desired lipid in either chloroform or chloroform/methanol (3:1) depending on the lipid. Small volumes (<100 μL) of lipid solution were evaporated to dryness in a vacuum desiccator and allowed to slowly form a suspension in MEM buffer (100 mM MES, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT, pH 6.4 with KOH) to a final concentration of 2 $\mu g \cdot \mu L^{-1}$. The reaction mixtures, prior to the addition of MTP, were sonicated (30 min, Decon FS200) to cause the formation of single-layered liposomes. Liposome suspensions were confirmed to contain no multilayered vesicles by low-angle X-ray scattering, while the lack of any diffraction lines on X-ray diffraction corresponding to ordered intralayer spacing showed that the lipids were in the fluid phase.

Microtubule protein (MTP) was purified from bovine brain by two cycles of assembly and disassembly (Burns & Islam, 1981). The protein (≈15 $mg \cdot mL^{-1}$) was stored under liquid nitrogen in MEM/NaCl buffer (MEM supplemented with 67 mM NaCl). Microtubule assembly under these buffer conditions is MAP-dependent: there is no assembly of pure tubulin (Burns, 1991). This therefore allows the extent of microtubule assembly to be used as a functional assay of the effective MAP concentration (Burns, 1990). Appropriate assay mixtures containing MTP, 100 μM GTP, and lipid vesicles in the MEM/NaCl buffer were degassed and transferred to prewarmed cuvettes in a Beckman DU-8 spectrophotometer. The kinetics of microtubule assembly were calculated from the increased turbidity at 350 nm, applying a scattering factor of 44 μM tubulin/absorbancy unit (Burns, 1991). The final extent of assembly was calculated from the instantaneous rate of subunit addition and the concentration of the assembled tubulin, a method which corrects for the effects of dynamic instability (Burns, 1991) and allows for the estimation of an objective error for this measurement. Protein concentrations were determined by the method of Hartree (1972) using bovine serum albumin as a standard.

The binding of the various constituents of MTP was assessed by a lipid sedimentation assay. Aliquots (50 μL) of MTP (30 $mg \cdot mL^{-1}$), clarified by centrifugation (100000*g*, 5 min), were mixed with lipid suspensions (2 $\mu g \cdot \mu L^{-1}$, 0–50 μL) and MEM/NaCl buffer (to 100 μL), incubated at room temperature for 5 min without exogenous GTP, gently layered onto a 30% (w/v) sucrose cushion (100 μL), and centrifuged (100000*g*, 20 min). The pellets and supernatants were analyzed by SDS–polyacrylamide gel electrophoresis on a 10% acrylamide gel, loading equivalent quantities of material to each lane.

## Results

The addition of liposomes of a number of pure phospholipids to MTP prior to assembly decreases the extent of microtubule polymerisation in a dose-dependant fashion (Figure 1a). PC had no detectable effect on microtubule assembly, while PS and PG slightly reduced the final extent of assembly. By contrast, PI and PA were both inhibitors of microtubule assembly. The inhibitory activity of the phospholipids, except for PI, correlates well with their degree of polarity. PC is zwitterionic between the positively charged choline group and the negatively charged phosphate and therefore carries no net charge; PG and PS have an overall negative charge due to the phosphate group, but this is masked by the glycerol and serine residues; PA is negatively charged with a naked phosphate group. The inhibitory activity of these phospholipids is explicable due to nonspecific binding of MAPs to polyanionic substrates as has been observed with DNA (Bryan et al., 1975). This does not account for the observed potency of PI since inositol, like serine and glycerol, is uncharged so that any nonspecific electrostatic effects should be of a similar magnitude to those observed with PS and PG.

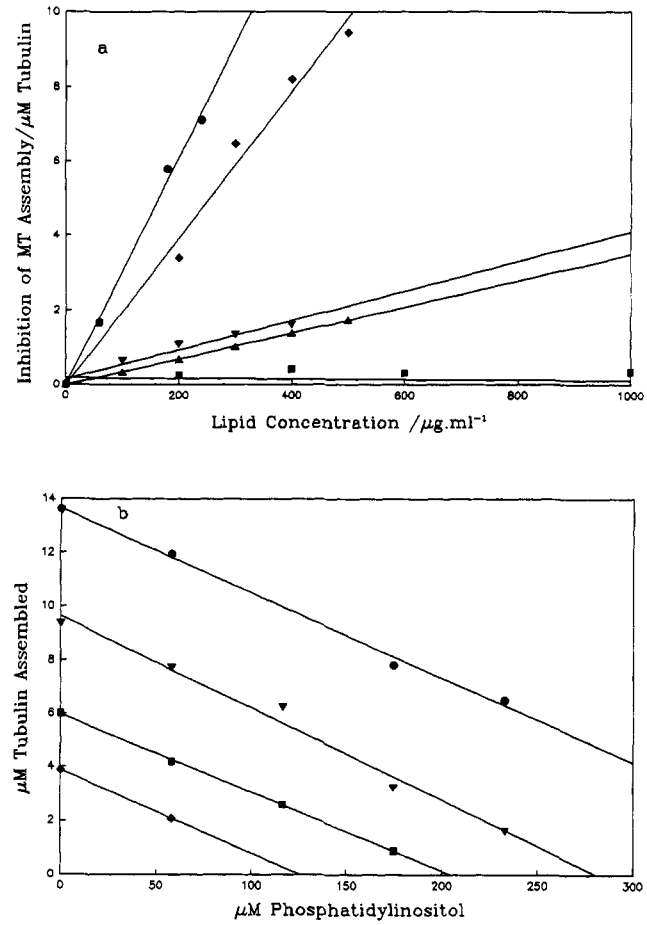


FIGURE 1: Effect of different phospholipids on the final extent of microtubule assembly. MTP (equivalent to 8–12 μM tubulin) was assembled in the presence of (a) differing concentrations of phosphatidylinositol (●), phosphatidic acid (◆), phosphatidylcholine (■), phosphatidylserine (▼), and phosphatidylglycerol (▲) (the inhibition of microtubule assembly by these phospholipids being expressed as the reduction in micromolar concentration of assembled tubule at steady state) or (b) phosphatidylinositol at MTP concentrations equivalent to 14 (●), 10.5 (▼), 7 (■), and 5.25 (◆) μM tubulin. A least-squares regression was plotted through all the data points.

The decrease in the final extent of assembly by PI is directly proportional to the concentration of PI but is independent of the MTP concentration (Figure 1b). The critical concentration governing the extent of assembly is therefore increased while there is no effect on the assembly competency of the protein, as supported by the constant amount of disassembly induced by the addition of a fixed amount of PI at steady state (data not shown). This effect on critical concentration but not on assembly competency suggests that PI binds to the MAPs since the assembly under the selected buffer conditions is MAP-dependent (Burns, 1991). Furthermore, treatment of *taxol*-stabilized microtubules with PI results in the loss of MAP2, while addition of excess MAP2 reversed the PI-induced inhibition of microtubule assembly (Yamauchi & Purich, 1987). The MAP2–PI interaction has been confirmed by incubating PI vesicles (i.e., unsonicated PI suspensions) with MTP under nonassembly conditions, pelleting through a 30% sucrose cushion (100000*g*, 30 min), and fractionation of the resulting pellets and supernatants by SDS–PAGE. The amount of MAP2 present in the vesicle-containing pellet increased with increased lipid concentration, with a concomitant loss of MAP2

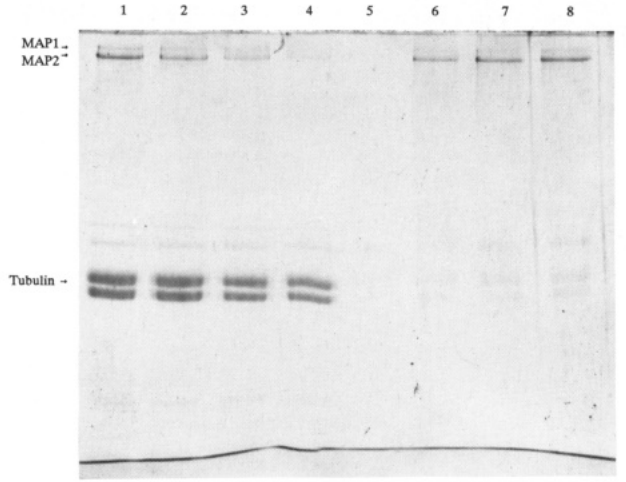


FIGURE 2: Binding of MTP to PI vesicles. Shown is a Coomassie stained 10% polyacrylamide gel of the supernatants (lanes 1–4) and pellets (lanes 5–8) produced by sedimentation of MTP (≈15 μM tubulin) in the presence of 0 (lanes 1 and 5), 0.1 (lanes 2 and 6), 0.2 (lanes 3 and 7), and 1.0 (lanes 4 and 8) mg·mL$^{-1}$ PI vesicles.

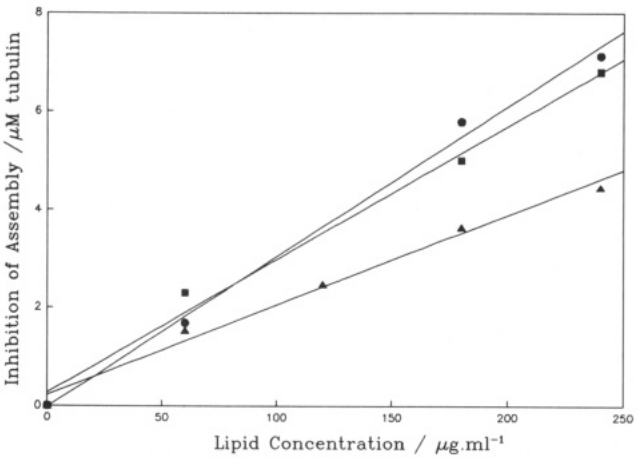


FIGURE 3: Effect of PI from different sources on microtubule assembly. MTP was assembled in the presence of PI from bovine liver (●) and soya bean (■) as well as a cocktail of phosphoinositides (▲). The inhibition of microtubule assembly was assessed as the decrease in final extent of assembly from a control containing no lipid. The extents of the control assemblies were 13.6, 12.2, and 8.3 μM tubulin, respectively. A least-squares regression was fitted through all data points, including the origin.

from the supernatant (Figure 2). By contrast, there was no selective recovery of either MAP1 or tubulin in the pellets, i.e., PI specifically binds to MAP2.

The linearity of the effect of PI on microtubule assembly (Figure 1b) indicates that all potential MAP2 binding sites are occupied, while its slope shows that a decrease of 1 μM in the final extent of assembly results from an increase in PI concentration of 31 μM. Since the MTP consists of a 1:12 stoichiometric mixture of MAP2:tubulin (Burns & Islam, 1984), the implied stoichiometry of PI binding to MAP2 is 370:1. This extremely high apparent stoichiometry might imply that the active component in the PI suspensions is not PI itself but some minor contaminant comprising less than 1% of the preparation. This has been examined by comparing the effectiveness of PI suspensions derived from different sources (bovine liver and soya bean) and a commercial mixture of phosphoinositides (15–20% phosphatidylinositol 4-monophosphate/phosphatidylinositol 4,5-diphosphate (PIP/PIP$_2$); 75–80% PI and PS). The inhibitory activities of the PI from soya bean and bovine liver were identical while that of the phosphoinositide mixture was only slightly reduced from that

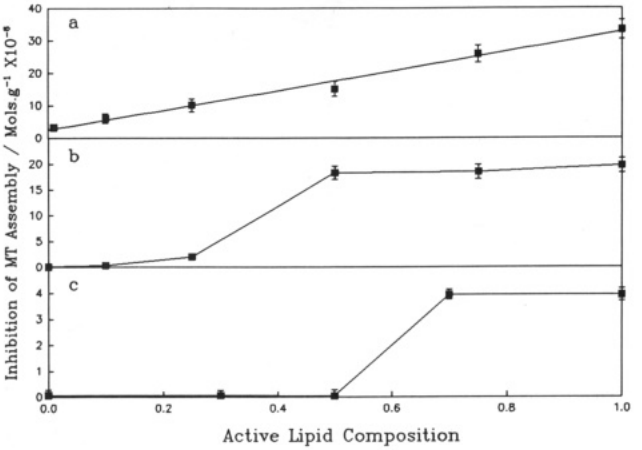


FIGURE 4: Effect of PC liposomes doped with active phospholipids on microtubule assembly. The inhibitory activity of liposomes consisting of varying ratios of (a) phosphatidylinositol, (b) phosphatidic acid, or (c) phosphatidylserine and phosphatidylcholine was assessed as the reduction in assembled tubulin per gram of total lipid added, calculated by least-squares regression (see text).

of the same mass concentration of pure PI (Figure 3). These results strongly suggest that the inhibitory activity is not a minor contaminant of the PI preparations. Furthermore, the liposomes themselves are the active component since the activity is abolished by inclusion of nonionic detergent (results not shown).

The similar activity of the mixture of phosphinositides and pure PI (Figure 3) suggests that the inhibition of microtubule assembly is determined by the concentration of total lipid rather than PI, even though liposomes of pure PC had no effect on microtubule assembly at concentrations several times in excess of those at which PI significantly reduced microtubule assembly (Figure 1a). The effect of the active lipid content was therefore examined in greater detail by preparing PC liposomes doped with increasing amounts of PI, PS, and PA. Such mixed liposomes inhibited microtubule assembly in the same fashion as liposomes of pure lipid, i.e., the liposome-dependent reduction in final extent of assembly was directly proportional to the concentration of lipid added and was independent of the concentration of MTP (results not shown). The inhibitory activity of these liposomes could thus be expressed as reduction in assembled tubulin per lipid added (mol·g$^{-1}$, calculated using a least-squares linear regression). The inhibitory activity of these liposomes was dependent upon the active lipid composition defined as the weight fraction of active to total lipid (Figure 4). Surprisingly, this dependency was qualitatively different in the case of PI (Figure 4a) as compared to PA and PS (Figure 4b,c). Liposomes of low PA/PS compositions had no effect on microtubule assembly, while above a certain composition (≈40% for PA and ≈60% for PS) the liposomes inhibited microtubule assembly with a constant activity. This is comparable to similar experiments performed with PC liposomes doped with cardiolipin (diphosphatidylglycerol; Reaven & Azhar, 1981), which only inhibited microtubule assembly above ≈50% cardiolipin. PA and PS therefore appear to inhibit microtubule assembly by sequestering MAPs through nonspecific electrostatic interactions, and this requires a minimum charge density. This effect is not observed with PI: the graph of inhibitory activity against PI composition was a straight line of slope 30 ± 1 μmol·g$^{-1}$ over the range 1–100% PI with ordinate intercept of 2.5 ± 0.8 μmol·g$^{-1}$. This positive intercept implies that an infinite dilution of PI has a significant effect on microtubule assembly even though the concerted action of a number of PI

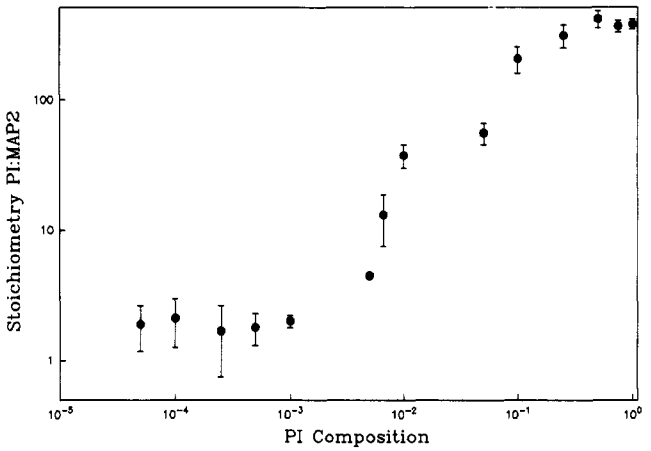


FIGURE 5: Effect of dilution of PI with PC on apparent PI:MAP2 stoichiometry. The apparent stoichiometry of binding of MAP2 to PI was determined from the inhibition of microtubule assembly of liposomes containing various dilutions of PI with PC.

molecules would be impossible. Intriguingly, the microtubule assembly inhibiting activity of PS liposomes doped with PI is independent of PI composition over the range 1–100% PI and equal to that of pure PI liposomes (results not shown). It is therefore clear that the linear dependence of inhibitory activity with PI composition for PC liposomes over the same range (Figure 4a) stems from the interactions of PI with the surrounding lipid rather than the specific interaction of PI with MAP2.

When the PI doping of PC liposomes is reduced to less than 0.1%, the inhibitory activity is less than predicted for that at infinite dilution (Figure 4a), yet it remains directly proportional to the concentration of lipid added and is independent of MTP concentration (data not shown). Calculation of the PI:MAP2 stoichiometry under these conditions yields a constant value of ≈2:1 PI:MAP2, while the value increases to ≈400:1 PI:MAP2 when the PI content exceeds 10% of the total lipid (Figure 5). These calculations of the PI:MAP2 stoichiometries assume that PI binds to MAP2 (Figure 2; Yamauchi & Purich, 1987) and that the MAP2:tubulin stoichiometry is 1:12 mol·mol$^{-1}$ (Burns & Islam, 1984).

## Discussion

Various negatively charged phospholipids inhibit microtubule assembly (Yamauchi & Purich, 1987; Reaven & Azhar, 1981; Figure 1a). This results from nonspecific electrostatic interactions as the potency of this inhibition is related to the degree of polarity of the phospholipids. Indeed, PC liposomes doped with small amounts of negatively charged phospholipids other than PI have no effect on microtubule assembly, while liposomes with greater than a critical composition of negative phospholipid inhibit microtubule assembly by the same amount as liposomes of pure negative phospholipid (Reaven & Azhar, 1981; Figure 4b,c).

The qualitative difference in behavior of liposomes doped with PI (Figures 4 and 5) from those doped with other negatively charged phospholipids clearly demonstrates that the inhibition of microtubule assembly by PI is different from that caused by other phospholipids. This PI-induced inhibition is an intrinsic property of PI rather than of a minor contaminant (Figure 3), is reversed by detergent treatment, and is a direct consequence of binding to MAP2 (Yamauchi & Purich, 1987; Figure 2). The potency of pure PI vesicle is 32 ± 2 μmol·g$^{-1}$ [compared with an equivalent value of around 90 μmol·g$^{-1}$ calculated from the data of Yamauchi and Purich (1987)], yielding an apparent PI:MAP2 stoichiometry of about 400:1 mol·mol$^{-1}$. This stoichiometry could be lowered to about 2:1 mol·mol$^{-1}$ by reducing the effective PI concentration by doping PC vesicles (Figure 5).

The PI concentration required to inhibit microtubule assembly greatly exceeds the probable critical micelle concentration, which equals 0.47 nM for dipalmitoylphosphatidylcholine (Smith & Tanford, 1972), so that the inhibition is due to the single-bilayered lipid vesicles. As MAP2 molecules are unlikely to be able to cross lipid bilayers, they can only have access to outer layer of lipid molecules, i.e., the apparent stoichiometries for inhibitory activity will be twice the actual binding values. The stoichiometries therefore range between 200:1 PI:MAP2 for pure PI liposomes to 1:1 for PC vesicles doped with small amounts of PI (Figure 5). The higher values are interpreted in terms of each MAP2 molecule binding tightly to a single PI molecule but covering about 200 of the surrounding molecules. Dispersal of the individual PI molecules by increasing the PC:PI ratio allows MAP2 to bind to each individual PI molecule, with the consequential decrease in PI:MAP2 stoichiometry to 1:1. PC liposomes with PI compositions greater than 10% appear to deviate from the predictions of this "footprint" model. These liposomes yield constant stoichiometries of around 200:1 PI:MAP2 despite the varying PC content. This effect may be due to a gross change in the nature of the liposomes, such as the addition of PC reducing the average cross-sectional area of the lipid molecules, the asymmetric distribution of PI and PC between the inner and outer faces of the liposomes, or lipid clustering such that the liposomes are effectively a mosaic of pure PI and pure PC regions.

This deviation from the predictions of the footprint model is not observed with PI doped PS liposomes. These have an activity independent of PI composition above 1% PI and therefore a constant footprint of around 200 lipid molecules. Since PI and PS have similar electrostatic properties by virtue of their uncharged headgroups, the anomalous effect in PC liposomes may be due to the difference in the electrostatic properties of PI and PC. This suggests that in addition to the specific interaction of PI with MAP2 additional PI molecules are clustered under the MAP2 by the same nonspecific interactions which cause the inhibitory activity of PS and PG.

The current assay has used the final extent of assembly of tubulin to measure the MAP2 concentration not bound to PI. This assay is limited to MTP concentrations exceeding the critical concentration for assembly (i.e., >0.5 μM tubulin; 0.04 μM MAP2; Burns, 1991), while significant decreases in MT assembly are only measurable at MTP concentrations equivalent to ≈10 μM tubulin (0.8 μM MAP2). Thus this assay is inappropriate for studying the interaction of PI with submicromolar MAP2 concentrations. The direct relationship between the reduction of extent of microtubule assembly and the concentration of lipid, even for liposomes with extremely low PI compositions, indicates the binding of all of the added PI. MAP2 must therefore have a high affinity for PI, and the $K_d$ must be significantly below the current MAP2 concentrations, i.e., less than $1 \times 10^{-6}$ M.

An interaction of this affinity strongly suggests that it may have some physiological significance. Indeed it has been observed that the distributions of MAP2 and tubulin in some neurons do not completely overlap (Matus et al., 1986), and this may be due to the association with membranes of a fraction of the total MAP2. One fascinating possibility is that the in vivo modulation of the MAP–PI interaction may control the extent of the neuronal microtubule cytoskeleton in a manner analogous to the control of the actin cytoskeleton via

the interaction of profilin with $PIP_2$ (Goldschmidt-Clermont et al., 1991). Such modulation of the PI-MAP2 interactions may constitute a link by which extracellular factors influence the microtubule cytoskeleton.


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# Bacteriorhodopsin Can Be Refolded from Two Independently Stable Transmembrane Helices and the Complementary Five-Helix Fragment†


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*Received January 10, 1992; Revised Manuscript Received April 7, 1992*



ABSTRACT: This paper describes experimental tests of the hypothesis that bacteriorhodopsin (BR) can fold by the association of independently stable transmembrane helices. Peptides containing the first and second helical segments of BR were chemically synthesized. These two peptides and the complementary five-helix fragment of BR were reconstituted in three separate populations of native-lipid vesicles which were then mixed and fused to allow the fragments to interact. After addition of retinal, absorption spectroscopy of the reconstituted BR and X-ray diffraction of two-dimensional crystals of this material showed that the native structure of BR was regenerated. The first two helices of BR can therefore be considered as independent folding domains, and covalent connections in the loops connecting the helices to each other and to the rest of the molecule are not essential for the appropriate association of the helices.


Predicting the folded structure of a protein on the basis of its amino acid sequence is a major goal of modern biology. Achieving this goal with membrane proteins may prove to be easier than with water-soluble proteins because of the constraints on secondary structure imposed by the hydrophobic environment of the membrane. Although $\beta$ structure is present in some instances (Weiss et al., 1990; 1991), in the majority of integral membrane proteins the membrane-spanning regions appear to be $\alpha$-helical (Popot & de Vitry, 1990). These segments are localized in the membrane because of the hydrophobicity of their amino acid side chains, and they are helical because of the strong energetic favorability of backbone hydrogen bonding in the nonpolar environment (Engelman et al., 1986).

Bacteriorhodopsin (BR)[1] is an integral membrane protein that is composed of seven transmembrane helices connected by short extramembranous loops (Henderson et al., 1990). It has been hypothesized that the secondary structure of the


† This research was supported by grants from the NIH (GM22778 and GM39546), the NSF (DMB8805587), and the National Foundation for Cancer Research.



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[1] Abbreviations: BR, bacteriorhodopsin; BSA, bovine serum albumin; KB, 30 mM potassium phosphate, 150 mM KCl, 0.025% $NaN_3$, pH 6; KB/5, 6 mM potassium phosphate, 30 mM KCl, 0.005% $NaN_3$, pH 6; SDS, sodium dodecyl sulfate; SDS buffer, 50 mM sodium phosphate, 5% SDS, 0.025% $NaN_3$, pH 6, 7, or 8; UV-CD, ultraviolet circular dichroism.


0006-2960/92/0431-6144$03.00/0