

Crystallization of Myelin Basic Protein in the Lipidic Cubic Phase

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Abstract

A prominent method by which a protein's function is elucidated is through the study of the protein's three-dimensional structure. Protein structure can be determined by obtaining well-ordered protein crystals. These crystals are thought to represent the most stable and lowest energy state of a protein molecule, and more often than not elucidate the native fold of the protein in question. Crystals that diffract X-rays isotropically to approximately 2-3 angstroms provide structural information that allows for the generation of a three-dimensional structural model of the protein. The lipidic cubic phase offers a continuous three-dimensional lipid bilayer matrix that facilitates the nucleation and growth of protein microcrystals, offering a promising method for the crystallization of Myelin Basic Protein. Solutions of Myelin Basic Protein were overlaid lipidic cubic phase gels of monoolein. Some of these gels were doped with negatively charged acidic lipids because acidic lipids have been previously shown to facilitate crystallization of Myelin Basic Protein on liposomes and lipid monolayers. The electrostatic interactions between the positively charged Myelin Basic Protein and the negatively charged doping lipids may provide the conditions necessary for nucleation of the protein crystal. The results obtained were inconclusive. Although areas of birefringence were noted, none were characteristic of protein microcrystals.

Introduction

Myelin basic protein (MBP) is medically significant because it has been implicated in the etiology of multiple sclerosis (MS). Although the actual mechanism behind MS is not known, it is thought to result from an autoimmune response in which the myelin sheath, the layers of insulation surrounding the nerve axon (Figure 1), of the central nervous system (CNS) is attacked and damaged by lymphocytes that have become sensitized to MBP

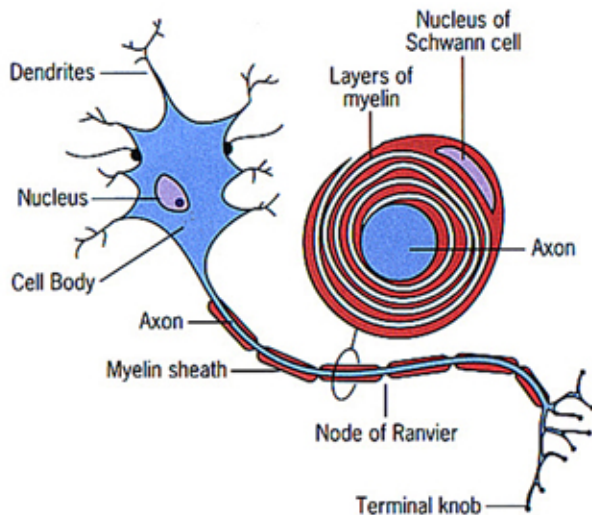


Figure 1. The myelin sheath (Karp 1999)

(International 1999). This etiology is plausible because MBP is well established as a potent antigen in both mice and humans (Menz 1992). In MS, damage to the myelin sheath ultimately slows nerve impulse propagation, resulting in the devastating neurological consequences that affects 2.5 million people worldwide today (International 1999).

In the U.S., MS occurs most frequently in states that are above the 37th parallel. The MS prevalence rate below the 37th parallel is 57 to 78 cases per 100,000 people, while above the 37th parallel, it is 110 to 140 cases per 100,000 people (International 1999). This high prevalence rate suggests that there is some environmental factor playing a role in causation of MS. Identifying MBP's role in the etiology of MS may help identify these environmental factors. In order to understand the function of MBP in the etiology of MS, the three-dimensional (3-D) structure of MBP must be determined. Identifying the protein's physical attributes will elucidate the mechanism by which the immune system's antibodies interact with MBP. A brief overview of protein structure maybe helpful to understand this fully.

A monomeric protein's structure can be defined on three different levels: primary, secondary, and tertiary. The primary structure of a protein is the sequence of amino acids that string together to comprise the protein. This string of amino acids has a main backbone and the interactions between these atoms determine protein's secondary structure.

Additionally, each amino acid has a single side chain that interacts with other side chains to comprise the protein's tertiary structure. Altogether, the protein's primary, secondary, and tertiary structure determine the overall structure of the protein, referred to as the protein's native fold (Voet *et al.* 1999). Determining this overall structure can elucidate the characteristics of MBP that cause it to be recognized by the sensitized antibodies of our immune system. Therefore, determination of MBP's 3-D structure may hold the key to understanding MBP's biochemical significance in the etiology of MS. Ultimately, structural determinants could be identified that would delineate the mechanism by which nerve axons are demyelinated in MS.

Study Design MBP is an 18.5 kilodalton, 160-residue protein and is notable for its large number of positively charged basic amino acid residues (10 lysines and 19 arginines). MBP accounts for 20-50% of the protein in CNS myelin and 6-22% of the protein in the peripheral nervous system myelin (Kirschner and Blaurock 1992). Although MBP interacts strongly with the negatively charged head groups of acidic lipids, it is not considered an integral membrane protein because MBP is not incorporated in the lipid bilayer of the cellular membrane. It is instead classified as an extrinsic membrane protein, found in close proximity to the membrane surface (Mendz 1992). Recent research indicates that localization of MBP at the cytoplasmic membrane surface of the myelin sheath suggests that its electrostatic interactions with acidic lipids may be important in forming MBP's secondary and tertiary structure, and therefore, its overall native fold.

MBP is suspected to be in an unfolded random coil conformation in aqueous solution, and appears to adopt a more ordered structure when it interacts with lipids and detergents (Mendz 1992). Circular dichroism spectroscopy, which measures the helical or β -sheet content of the secondary structure of a protein, indicates that the protein changes from a random coil conformation in free solution to one which has 20% α -helix and approximately 12% β -sheet when associated with liposomes composed of negatively charged acidic phospholipids (Keniry and Smith 1981). This suggests that MBP folds into its native conformation upon interaction with negatively charged lipids in a membrane system, which gives insight into a possible crystallization system for MBP.

The crystallization of Myelin Basic Protein has eluded researchers for many years. However, in the Glaeser lab, we have successfully crystallized MBP on negatively charged

acidic cystine-polydiacetylene lipid monolayers, and on tubular lipids comprised of cystine-polydiacetylene and galactose-ceramide lipids. Our ultimate goal has been to obtain two-dimensional crystals that diffract to a high resolution, which would enable high-resolution electron crystallography to be used to obtain a three-dimensional electron density map. This map can then be interpreted in terms of MBP's primary structure, the protein's known amino acid sequence. However, crystallization via this method has produced crystals that only diffract to approximately 30 Å resolution. This resolution is insufficient for accurate placement of MBP's amino acid chain in the generated electron density map because a resolution of 2-3 Å is required. This limitation may be overcome by crystallizing MBP in quasisolid lipidic cubic phase.

The lipidic cubic phase is comprised of complex 3-D lipid arrays (Figure 2). These 3-D lipid arrays provide continuous aqueous channels and hydrophobic support structure for

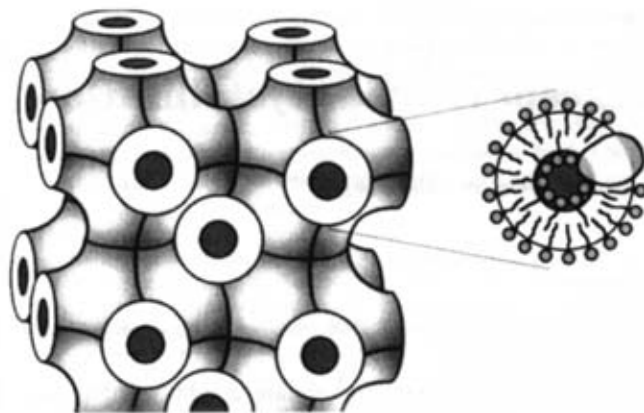


Figure 2. Lipidic cubic phases (Landau *et al* 1996)

nucleation and growth of membrane proteins. These aqueous channels allow for diffusion of the protein, while the lipid support structure mimics the surface of the cellular membrane. Monoolein (MO) or 1-monooleoyl-*rac*-glycerol (C18:1,[*cis*]-9) forms this membrane system of intercommunicating aqueous channels that provide well-ordered diffusion

space for the growth of protein crystals. To encourage nucleation of MBP in the lipidic cubic phase, negatively charged acidic lipids were introduced that showed compatibility with the MO gel matrix. Successful crystallization in the cubic phase would enable the use of X-ray crystallography to determine MBP's 3-D structure to a high resolution, permitting researchers to investigate MBP's structural significance in the demyelination mechanism of MS.

Methods

As a methodological and systematic control, crystallization of Bacteriorhodopsin (BR) was attempted because BR has been successfully crystallized in the lipidic cubic phase using MO gels.

Lipidic Components for BR Crystallization Experiments Bicontinuous cubic phases consisted of monoolein (1-monooleoyl-*rac*-glycerol, C_{18:1c9}, or MO, Sigma) and 25 mM Na/K-P₁ buffer at pH 5.65 with 3 mM NaN₃. NaN₃ is added to prevent bacterial and fungal growth.

Preparation of Cubic Phases and BR Crystallization Experiments Purple membrane (10 mg) was separated on a sucrose density gradient by centrifugation at 40,000 rpm at 5 °C for 30 minutes. The resulting pellet was then resuspended in a mixture of 16.67 ml of 25 mM Na/K-P₁ buffer at pH 6.8 and 20 ml of 12 mg/ml β-octylglycopyranoside. This resuspension was performed by gently pipetting and sonicating the solution in the dark due to the photosensitivity of BR. The solution was then wrapped and left to shake overnight. The following day, 25 mM Na/K-P₁ with 0.1 M HCl was added drop-wise to the solution to obtain a pH of 5.6. The solution was then centrifuged at 40,000 rpm at 5 °C for 30 minutes. The supernatant was then concentrated in an Amicon pressure cell to a final volume of ~1 ml and the concentration was then verified to be ~18 mg/ml on a UV light spectrophotometer at an absorbance wavelength of 550 nm. Cubic phases were prepared by mixing ~13.5 mg of MO with 9 μl of 18 mg/ml BR and 4.5 μl of 25 mM Na/K-P₁ buffer at pH 5.65 with 3 mM NaN₃ to obtain 50% hydration. 50% hydration ensures that the MO gel matrix is bicontinuous at room temperature. The tubes containing these solutions were then centrifuged at 10,000 rpm for 3 hours at room temperature, each tube being rotated 180° every 15 minutes. The tubes were then wrapped with parafilm to ensure freshness and allowed to stand for 1 week at room temperature (Landau *et al* 1996).

Lipidic Components for MBP Crystallization Experiments Bicontinuous cubic phases consisted of monoolein and distilled water with 3 mM NaN₃. In addition to an MO only setup, the following doping lipids were used: octadecanoic acid (stearic acid), trans-9-octadecanoic acid (elaidic acid), cis-9-octadecanoic acid (oleic acid), dioleoyl L-α-phosphatidyl-L-serine (DOPS), dimyristoyl L-α-phosphatidyl-DL-glycerol (C14:0) (DMPG), dimyristoyl L-α-phosphatidic acid (C14:0) (DMPA), and cerebroside sulfate (sulfatides).

The number of setups for each doping lipid tested was limited to 15 due to cost considerations.

Preparation of Cubic Phases and MBP Crystallization Experiments For the MO-only setup and each of the above doping lipids, 100 mg of MO, 10 mg of doping lipid (MO in the case of the MO only-setup), and 110 μ l of distilled water with 3 mM NaN_3 were mixed in a microcentrifuge tube. The mixture was then centrifuged at 10,000 rpm for 3 hours at room temperature, each tube being rotated 180° every 15 minutes. These tubes were then allowed to stand at room temperature for 3 days to allow for the uniform diffusion of the doping lipid in the MO lipidic cubic phase. Then, \sim 1 mg globs of gel were distributed into individual glass tubules and centrifuged for 10 minutes at 10,000 rpm at room temperature. An overlying solution of 100 μ l of 0.1 mg/ml MBP was then added to each tube. The tubes were then allowed to incubate at room temperature for 7 days, after which the setups were inspected for areas of birefringence under a microscope fitted with a polarizing lens.

Investigation MBP's diffusion in MO and MO plus doping lipid gels After the 7 day wait prescribed above, the protein concentration in each tube was determined by UV light spectrophotometry at an absorbance wavelength of 280 nm. Comparison to the initial concentration will reveal whether or not MBP is actually diffusing into the MO lipidic cubic phases.

Results

BR crystallization BR containing bicontinuous cubic phases, formed with MO, produced preparations that were initially completely purple in color, suggesting that the protein was uniformly distributed within the MO matrix. Within a week, a shower of birefringent, hexagonally shaped, purple microcrystals formed in the MO gel matrix. As these crystals formed, the preparations turned uniformly clear, suggesting that BR is diffusing within the gel matrix, and then aggregating, allowing for crystal growth. Crystal growth seemed symmetric, and did not seem to adversely affect the gel matrix. The matrix appeared to maintain its transparency and elasticity.

MBP crystallization *Stearic acid setups* Stearic acid was found to be immiscible in the MO phase. Even after centrifugation, the stearic acid remained randomly mixed as a solid

white wax throughout the MO gel. I did not pursue the use of this lipid further because the transparency of the gel is essential for the identification of MBP microcrystals.

Elaidic acid setups Elaidic acid was miscible to some extent in MO, however, the resultant gel was opaque. As explained above this condition prevents the use of elaidic acid as a doping lipid.

Oleic acid setups Oleic acid, a liquid at room temperature, would not mix with MO to form the cubic phase. The result was a slurry with the MO gel suspended in the liquid lipid. These conditions do not allow oleic acid to meet the requirements necessary for use as a doping lipid.

DOPS setups DOPS was completely miscible with MO, and once centrifuged, formed a clear gel that was slightly yellowish in color. The lipid's miscibility was encouraging, because DOPS is purified from bovine brain, as is our MBP. However, few areas of birefringence were noted in any of the 15 tubes. Tubes containing birefringent areas were monitored for an additional three weeks but no additional growth of these areas was apparent. These areas of altered light refraction were most likely a result of contamination, or self-crystallization of the MO matrix. Overall, the gels retained their clarity, yellowish color and firmness, which suggest that the lipid matrix is stable in the presence of the overlying protein solution. Sampling of the overlying solution for protein content revealed an unexpected increase in protein concentration. On average, UV light spectrophotometry at an absorbance wavelength of 280 nm indicated an increase in protein content to 3.27 mg/ml from the original concentration of 0.17 mg/ml.

DMPG setups DMPG was also completely miscible with MO, and once centrifuged, formed a clear gel that had no color. Tubes containing birefringent areas were also monitored for an additional three weeks. Many of the birefringent areas showed significant growth. However, these areas proved to be characteristic of areas of MO self-crystallization. These gels also retained their clarity and firmness, confirming the stability of this lipid matrix in the presence of the overlying protein solution. Sampling of the overlying solution for protein content again revealed an unexpected increase in protein concentration. UV spectrophotometry readings indicated an increase in protein content to 0.23 mg/ml from the original concentration of 0.17 mg/ml.

DMPA setups DMPA was also completely miscible with MO, and once centrifuged, also formed a clear gel that had no color. Birefringent areas were monitored for an additional three weeks and some areas of birefringence showed growth. These areas of altered light refraction were identified to be a result of contamination or self-crystallization of the MO matrix. These gels retained their clarity and firmness, suggesting again that this lipid matrix is stable in the presence of the overlying protein solution. An increase in protein concentration was noted upon sampling of the overlay solution. UV spectrophotometry readings indicated an increase in protein content to 0.23 mg/ml from the original concentration of 0.17 mg/ml.

Sulfatides setups Sulfatides were also completely miscible with MO, and once centrifuged, also formed a clear gel that had no color. Miscibility of this lipid is especially encouraging because sulfatides consist primarily of galactocerebroside sulfate, which comprises approximately 6% of the total lipid in the bovine brain; and is localized to the myelin sheath (Sigma 2000). Tubes containing birefringent areas were monitored for an additional three weeks. Few areas of birefringence showed growth. These areas of altered light refraction were again most likely a result of contamination, or self-crystallization of the MO matrix. The gels retained their clarity and firmness, which suggest again that this lipid matrix is stable in the presence of the overlying protein solution. Sampling of the overlying solution for protein content revealed a slight decrease in protein concentration. UV light spectrophotometry at an absorbance wavelength of 280 nm indicated a decrease in protein content to 0.15 mg/ml from the original concentration of 0.17 mg/ml.

Monoolein-only setups No doping lipids were added to this setup. Tubes containing birefringent areas were monitored for an additional three weeks. No areas of birefringence showed growth, which suggests that these areas of altered light refraction, were again most likely a result of contamination, or self-crystallization of the MO matrix. Overall, the gels retained their clarity and firmness, which suggest that this lipid matrix is stable in the presence of the overlying protein solution. Sampling of the overlying solution for protein content revealed a slight decrease in protein concentration to 0.14 mg/ml from the original concentration of 0.17 mg/ml.

Discussion

BR crystallization Successful crystallization of BR in the lipidic cubic phase is encouraging, however, BR is a membrane protein and the mechanism of its crystallization in the lipidic cubic phase differs from that of a soluble protein like MBP. BR integrates itself into the lipid matrix of the gel while MBP would associate with the surface of the lipid support structure. As BR crystals grow, they displace the lipid support structure. I envisage that MBP crystals would grow within the aqueous channels of the lipidic cubic system. Regardless, as a methodological and systematic control, successful crystallization of BR in a MO gel is promising, and suggests a satisfactory technique.

MBP crystallization Octadecanoic acid (stearic acid), trans-9-octadecanoic acid (elaidic acid), and cis-9-octadecanoic acid (oleic acid) were shown to be incompatible with the lipidic cubic phase and were not used as doping lipids.

Dioleoyl L- α -phosphatidyl-L-serine (DOPS), dimyristoyl L- α -phosphatidyl-DL-glycerol (C14:0) (DMPG), dimyristoyl L- α -phosphatidic acid (C14:0) (DMPA), and cerebroside sulfate (sulfatides) all formed well-ordered gels for experimental use. Although many gels produced areas of birefringence, none of these areas were consistent with those expected of protein microcrystals. Protein microcrystals, as previously explained, would be expected to grow as uniform structures, possibly resulting in a shower of birefringent areas. This was not the case however, and many of the areas shown to have birefringence were erratic in shape and size. Areas that did not grow were probably a result of some sort of contamination, possibly from dust fibers in the air, or glass shards leftover from the glass-tube manufacturing process. Areas that did show growth were characteristic of the self-crystallization process of the MO gel itself. These MO crystals are disordered, and are identified by their long, slender, spike-like arms originating from a central crystalline body.

In addition to the lack of protein microcrystals, it was also determined that protein diffusion into the lipid matrix could not reliably be determined by UV light spectrophotometry. Although results show that there was a decrease in protein content to 0.14 mg/ml from the original concentration of 0.17 mg/ml in the MO-only setup, many of the other setups actually showed protein concentration increasing in the overlay solution. It seems possible that the doping lipids may be diffusing into the overlay solution resulting in a

change in absorbance at 280 nm. I suspect now that accurate readings might be obtained by using the doping lipid in the spectrophotometer blank.

This preliminary investigation into the crystallization of MBP in the lipidic cubic phase has shown that MBP will not be easily crystallized in MO based gels. Many factors, most of which are beyond the scope of this experiment, need to be taken into account to thoroughly investigate the propensity of crystallizing MBP in the lipidic cubic phase. Varying pH, doping lipid concentrations, hydration percentages and other conditions would allow for a more thorough investigation of the crystallization of MBP in the lipidic cubic phase.

Ultimately, if well-ordered crystals can be obtained, X-ray crystallography could be performed to acquire the data necessary to generate a three-dimensional electron density map of the MBP crystal. X-ray crystallography requires that an X-ray beam incident upon an MBP crystal. The resulting diffraction pattern is then assessed by a computer via Fourier transform, and an electron density map of the molecule is generated. MBP's amino acid sequence is then fitted to this density map and a three-dimensional model of MBP is produced. The validity of this model can then be assessed, determining whether or not this structure adequately explains MBP's function in the myelin sheath.

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