Mapping intramolecular distances of the 18.5-kDa Myelin Basic Protein under various conditions by Förster Resonance Energy Transfer

by

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Molecular and Cellular Biology

Guelph, Ontario, Canada
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ABSTRACT

Mapping intramolecular distances of the 18.5-kDa Myelin Basic Protein under various conditions by Förster Resonance Energy Transfer

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The predominant 18.5-kDa isoform of Myelin Basic Protein (MBP) is essential for the development of myelin in the central nervous system (CNS), and is hyper-deiminated in multiple sclerosis. We have previously obtained intramolecular distances between several single Cys-substituted sites in unmodified and pseudo-deiminated MBP variants, and a single internal Trp residue, by Förster Resonance Energy Transfer (FRET). I have obtained additional constraints by FRET of fluorophores attached to double- and single-Cys-substituted residues in the presence of dodecylphosphocholine (DPC) and Zn$^{2+}$ (to mimic the myelin membrane per se). The quenching of Trp fluorescence by the acceptor showed that Zn$^{2+}$ in the presence of DPC at its critical micelle concentration of 1.25 mM affected MBP’s local tertiary fold, but FRET distances indicated a negligible effect on global structure. We showed that DPC, like trifluoroethanol (TFE), caused slight compaction and extension. No significant differences between the two variants were seen.
ACKNOWLEDGEMENTS

This Master’s degree was achieved with the support of a few people that I would like to gratefully acknowledge.

First and foremost, I would like to thank and express my deepest gratitude to my graduate supervisor and mentor, Dr. George Harauz, for not only his support and guidance, but for his patience, time, and most importantly, for giving me the opportunity to work and making my Master’s degree possible. I am truly grateful for having him as my advisor, as he has helped me learn and grow as a scientist in his laboratory.

My sincere appreciation goes to Dr. Vladimir Bamm for all his contribution and guidance to my research. I am thankful for his constructive input throughout my research and helping me improve my knowledge in molecular biology, as well as wrapping my mind around several concepts that I discuss in this thesis. I am also grateful for his patience and his time in training me on a variety of useful laboratory techniques.

I would also like to thank my advisory committee member and co-advisor, Dr. Steffen Graether, for his invaluable contribution of time and expertise to my research.

Last but not least, I could not have done this without the unconditional love and encouragement of my parents and brother. Thank you all for making my graduate education possible.
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<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DABMI</td>
<td>4-dimethylaminophenylazophenyl-4'-maleimide</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DPC</td>
<td>n-dodecylphosphocholine</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IAEDANS</td>
<td>5-(((2-iodoacetyl) amino) ethyl) amino) naphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>IEX</td>
<td>ion exchange</td>
</tr>
<tr>
<td>IDP</td>
<td>intrinsically disordered protein</td>
</tr>
<tr>
<td>IDR</td>
<td>intrinsically disordered region</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein (here, specifically the 18.5-kDa size isoform)</td>
</tr>
<tr>
<td>MoRFs</td>
<td>molecular recognition fragments</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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OLG – oligodendrocyte
PTM – post-translational modification
SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCEP – tris(2-carboxyethyl)phosphine
TFE – trifluoroethanol
UTC1 – unmodified C1 charge component of MBP (untagged, 168 residues)
UTC8 – pseudo-deiminated C8 charge component of MBP (untagged, 168 residues)
Chapter 1.0: Introduction

1.1 Myelin of the Central Nervous System (CNS)

Myelin is a multilayered plasma membrane that is synthesized in the central nervous system (CNS) by oligodendrocytes (OLGs) and wraps around the neuron’s axon (1, 2). It insulates the axons, thereby also clustering sodium channels into the nodes of Ranvier, and causing the action potential (AP) to jump from one node to the other (1). For neuronal function, the myelin sheath is important because it helps to increase the speed at which APs propagate (3).

The CNS myelin sheath is formed by the lipid bilayers and comprises two major regions; the intraperiod line and the major dense line, which are formed by the apposition of the extracellular membrane layers and by the cytoplasmic membrane (3), respectively (Figure 1). The major dense line is held together by a cytosolic, peripheral membrane protein, known as myelin basic protein (MBP), which is the second most abundant CNS myelin protein, after proteolipid protein. Myelin has an unusual molecular composition, comprising nearly 70% lipids by weight as well as myelin-specific proteins in high abundance, and is implicated in various neurological diseases (3).
Figure 1: Schematic diagram of Myelin Basic Protein in the central nervous system. Myelin is a multilayered membrane that is synthesized in the CNS by OLGs, which produce large quantities of plasma membrane that wrap around the neuron’s axon. Myelin has been used for a long time as a model membrane by biochemists and biophysicists because of its unique molecular composition, high abundance, and implication for disease. MBP is an intrinsically disordered protein that undergoes disorder-to-order transitions while interacting with the lipid bilayers. The production of the myelin sheath highly depends on the classic isoforms of MBP. The 18.5-kDa isoform of MBP in the human brain is used as a marker for compact myelin, which joins together the cytoplasmic leaflets of the oligodendrocyte membrane. The two major regions in myelin that are formed by the lipid multilamellae are the intraperiod line and the major dense line, formed by the apposition of the extracellular and cytoplasmic leaflets, respectively. Figure adapted with permission from reference (3).
1.2 Demyelination and Multiple Sclerosis (MS)

Demyelinating diseases include any condition that can damage the myelin sheath (2). Multiple sclerosis (MS) is the most common primary demyelinating disease in humans (1, 4), typically affecting adults 20 to 45 years of age (5). The disease arises by a combination of genetic susceptibility and a non-genetic trigger, such as a virus, altered metabolism, or environmental factors, resulting in immune attacks on the CNS (5). The diagnosis of MS still remains primarily clinical, based on a set of specific criteria and exclusion of other conditions. There have been many different types of criteria used for these tests; however, not every patient fits into these classifications, making the disease difficult to evaluate (4). Impaired saltatory conduction and increasing loss of neurological function occur due to the progressive destruction of the myelin, leading to symptoms such as depression, paralysis, spasticity, and cognitive dysfunction (5).

1.3 Myelin Basic Protein (MBP) and splice isoforms

MBP is important for the development of myelin in the CNS of higher vertebrates (6), particularly the classic isoforms of MBP (7). The classic MBP isoforms come from the third transcription start site of the gene in the oligodendrocyte lineage (golli), range in molecular mass from 14 to 21.5 kDa, and belong to the family of intrinsically disordered proteins (IDPs, discussed below) (8). The predominant 18.5-kDa classic isoform of MBP in the human brain is used as a marker of compact myelin, since it adheres together the opposing cytoplasmic leaflets of the oligodendrocyte membrane (7). The 18.5-kDa MBP is positively charged and interacts with myelin membranes by three amphipathic α-helical segments, representing molecular recognition fragments (MoRFs) (7). This isoform attaches the cytosolic surfaces of oligodendrocyte membranes together, resulting in the formation of a two-dimensional molecular sieve (8, 9). The 18.5-kDa MBP is also involved in “cytoskeletal assembly and membrane extension, binding to
SH3-domains, participation in Fyn-mediated signaling pathways, sequestration of phosphoinositides, and maintenance of calcium homeostasis”, all consistent with roles as a hub and/or and linker (8).

1.3.1 Post-translational modifications (PTMs)

Intrinsically disordered proteins, such as MBP contribute to many essential biological processes, and their binding and structural properties can be modified by post-translational modifications (PTMs). These PTMs are important in regulating the folding of proteins, their targeting to specific cellular compartments, their interactions with other proteins or ligands, and their functional state. PTMs change the local secondary structure in proteins, allowing them (or preventing them) to adopt different conformations that are needed for binding and signalling events (3). This property is seen in IDPs that are similar to MBP, such as tau and α-synuclein (3, 10). Phosphorylation at the MAP-kinase sites is known to affect the formation of α-helical regions in MBP (11), specifically the α2-region (the second of the three amphipathic α-helices) (11). Other PTMs of MBP, besides phosphorylation, are methylation, deimination, and deamidation (8). However, for this isoform of MBP, phosphorylation is the most dynamic (8).

1.3.2 Myelin destabilization by hyper-deimination of MBP

Deimination is the enzymatic conversion of positively charged arginine to neutral citrulline catalyzed by peptidylarginine deiminase (12). For MBP, deimination reduces the net positive charge, hence changing its ability to adhere myelin membranes together tightly, and exposing an immunodominant epitope (13). Previous studies of two-dimensional arrays of MBP variants by cryo-transmission electron microscopy showed a switch from adjacent fibrils to hexagonal close packing of apparent monomers (9). Thus, MBP deimination may alter the arrangement of the molecular sieve, causing the arrangement to become looser (less compact myelin) (9). Deiminated
MBP has reduced ability to polymerize and bundle actin, as well as to tether actin microfilaments to membranes (9). It has been determined that MBP has a role as a PIPmodulin, which is a protein that sequesters phosphoinositides (for instance PI(4,5)P₂ or PI(3,4,5)P₃) (9). Deiminated MBP has a significantly decreased ability to act as a PIPmodulin.

Recent studies have indicated that actin-binding proteins at the outer membrane processes can be released from phosphoinositide sequestration and that deiminated MBP could modulate this function (9). This can be done by altering the number of phosphatidylinositol lipid molecules that are bound to each protein. During demyelination, the overall lipid composition of the myelin membrane becomes significantly modified, which disrupts the ability of all isomers of MBP to maintain the myelin sheath interactively with other components (9).

1.4 Intrinsically disordered Proteins (IDPs)

Unlike well-ordered proteins (e.g., most enzymes) that can be studied by X-ray crystallography or scattering, IDPs (of which MBP is a prime example) do not have a stable secondary or tertiary structure (14). IDPs often function as hubs in protein interaction networks involved in transcriptional regulation, translation, and cellular signal transduction (15, 16). Generally, IDPs are characterized by extensive PTMs and alternative splicing, which add complexity to regulatory systems and provide a tissue-specific signaling mechanism (16). Some IDPs may be completely disordered, whereas others can have disorder in some parts of the protein, which are called intrinsically disordered regions (IDRs) (16). Their inability to fold manifests as fluctuation over an ensemble of conformations that go over a range of conformational populations, from extended random coils to collapsed globules (16).
The IDPs play important roles in biological processes that are associated with many diseases and neurodegenerative disorders, such as MS. Their most essential characteristics are the presence of small recognition elements, which fold upon partner binding; a degree of flexibility that enables them to interact with different targets on different occasions; accessible sites for PTMs; the ability to bind partners with high specificity; and kinetic advantages in signaling (16). Since IDPs exist as an ensemble of different structures, X-ray crystallography is not a useful method to study these proteins. Instead, spectroscopic methods such as Fourier-transform infrared (FTIR), electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), and Förster resonance energy transfer (FRET) may be used to analyze IDP dynamics and interactions.

Since IDPs do not have well-defined three-dimensional structures, they can exist as ensembles that can be defined by an average, but within which the individual states are very dynamic; there can even be different ensembles. However, in this study, it is hoped that one ensemble will predominate. Recent studies using EPR and NMR provided more comprehensive information in regards to conformational states of an IDP (17).

1.5 MBP as an IDP and disorder-to-order transitions

All variants of MBP are considered to be IDPs because they have a higher proportion of charged amino acids and lower proportion of hydrophobic amino acids than traditionally-folded proteins (3, 8). In previous studies done using FTIR and circular dichroic (CD) spectroscopies, the 18.5-kDa isoform of MBP was proven to be very flexible and act like a randomly-coiled polymer in aqueous environments, but also gained some ordered secondary structure when associated with detergents and lipids (8).
Techniques such as NMR, EPR, and CD have been used on the unmodified full-length 18.5-kDa protein, to define the three segments (N-terminus, C-terminus, and a central region that is highly conserved) to form amphipathic α-helices (8, 18). NMR spectroscopy has confirmed that these three α-helical segments transition from a disordered-to-ordered state in trifluoroethanol (TFE) solution (18). The central α-helical region contains the α2-helix and a poly-proline sequence (3), and two mitogen-activated protein kinase sites. Phosphorylation at these sites changes MBP’s electrostatic interactions with its binding partners, resulting in conformational changes and making it a central molecular switch (3). Many IDPs experience disorder-to-order transitions, which usually play a role in the way that they function (18). Even though MBP does not show a stable higher-order structure in solution, it still gains secondary structure elements in certain environments such as TFE or phospholipid bilayers (18).

1.5.1 EPR and NMR studies of MBP

Both EPR and NMR techniques have been used to map the secondary structure composition of two α-helical (α2 and α3) anchoring motifs in association with membranes. Previous NMR studies conducted in the Harauz lab have shown that MBP adopted an α-helical structure that was stable in 30% TFE (19). The solvent TFE was used due to its ability to mimic post-synthetic transition from aqueous solution to the membrane surface, and also because it allows MBP to gain ordered secondary structure elements (18, 20). It was also confirmed that MBP did not adopt a significant proportion of a secondary structure in aqueous solution (3, 20).

By using EPR and NMR studies, a loss of structural integrity of an intrinsic α-helix formed by MBP has been shown to occur (3). Deimination results in the α2-helix becoming more exposed to the surface when it is bound to the cytoplasmic leaflet of the myelin membrane, as well as becoming shorter in length (3). Hence, this makes the protein more vulnerable to proteolysis,
compared to the unmodified form (3, 13). Specifically, a previous EPR study of MBP determined that the pseudo-deiminated recombinant C8 charge component of MBP, defined as UTC8 (untagged, 168 residues), created a shorter and more surface-exposed α-helix in comparison to the unmodified recombinant C1 charge component of MBP, defined as UTC1 (untagged, 168 residues) (13). This study also showed that UTC1 and UTC8 variants from a specific region (Val83-Thr92) created a shorter and more surface-exposed helix in UTC8 compared to in UTC1, which was confirmed by solid-state NMR (3, 13). Conformational changes can develop for deimination of UTC1 to yield UTC8, resulting in the α2-helix becoming more susceptible to proteolytic attack and digestion (3, 13). This is due to the cytoplasmic exposure of a Phe86-Phe87 pair, which is a cathepsin D target (3, 13).

### 1.5.2 Previous FRET studies of MBP

Before FRET can be measured, steady-state fluorescence measurements must first be performed (21). FRET is a distance-dependent physical process by which energy is transferred non-radiatively (22). This transfer is from an excited molecular fluorophore, known as the donor, to another fluorophore, known as the acceptor, which indicates that there will be an intermolecular or intramolecular interaction with dipole-dipole coupling (22). It is important to note that the acceptor does not have to be fluorescent and that the donor does not involve emission of light by FRET (23). The FRET efficiency is dependent on the inverse sixth power of the intermolecular separation, which makes it beneficial over distances that are comparable with the dimensions of biological macromolecules (23).

FRET is an important technique for examining various biological processes involving alterations in molecular proximity (23). FRET can provide important information that can be used to study the structure, conformation of proteins, hybridization, and automated sequencing of
nucleic acids (22). This technique can also be used to study the interactions between a receptor-ligand pair and the dimerization of individual receptors on a localized spatial scale (22), as well as the spatial distribution and assembly of protein complexes (23). A basic Jablonski diagram represents the coupled excited state transition that is involved between the donor emission and the acceptor absorbance in FRET. The donor fluorophore can transfer excited state energy to the acceptor without emitting a photon (Figure 2).

![Jablonski Diagram](image)

**Figure 2:** A simple Jablonski diagram of Förster Resonance Energy Transfer. This diagram represents the coupled excited state transition that is involved between the donor emission and the acceptor absorbance in Förster Resonance Energy Transfer (FRET). FRET is a physical process that is distance-dependent by which energy is transferred non-radiatively from an excited molecular fluorophore, known as the donor, to another fluorophore, known as the acceptor, resulting in an intermolecular interaction with dipole-dipole coupling. The donor fluorophore can transfer excited state energy to the acceptor without emitting a photon. Figure created by Author.

There have been previous studies on MBP using FRET, most recently to study the conformational energy landscape of an unmodified charge variant of 18.5-kDa MBP (20). The fluorescent labelling probe 5-(((2-iodoacetyl) amino) ethyl) amino) naphthalene-1-sulfonic acid
(1,5-IAEDANS, henceforth IAEDANS, for simplicity), was used to label all variants of MBP that were being used in this study. A single-Cys residue was incorporated at different locations to allow for labelling with IAEDANS (Figure 3). FRET was used to measure the intramolecular distance between the lone intrinsic tryptophan, used as the donor, and IAEDANS, which served as the acceptor.

![Figure 3: Sequence roadmap of 18.5-kDa MBP (168 residues).](image)

This diagram shows the α-helical regions, residues that were mutated to cysteine, residues that are pseudo-deiminated in MBP-C8, and the intrinsic tryptophan residue are shown. The single-cysteine substitutions that were created are, specifically, S17C, H85C, and S159C. These sites were chosen because one is at the N-terminal beginning, one is in the middle, and one is at the C-terminal end. Here, we intended to use the double-cysteine mutations, which are S17CH85C, S17CS159C, and H85CS159C in MBP-C1 and MBP-C8 variants, as well as S17C, H85C, and S159C for single-Cys MBP variants. Figure adapted with permission from reference (3) which was modified from reference (3, 18).
The data for this previous study were collected in 0%, 15%, and 30% TFE (v/v) concentrations in order to probe global conformational change upon α-helical formation (20). This approach was done for both UTC1 and UTC8 variants of MBP, to determine the effects of net charge reduction due to the deimination on MBP global structure (20). When comparing both variants, a pattern was seen, as FRET efficiency was high (shorter distance) at 10-15% and lower at 0% and 30% TFE concentrations. The contribution of the three conformational species was determined (disordered, intermediate, and α-helical) from measured FRET efficiency (Figure 4) as well as by calculating the Trp-IAEDANS distances that were associated with each of them (20, 24). Overall, compared to more compact conformations, the results suggest that the deimination most likely has a greater structural effect on the expanded conformations of MBP (20).
Figure 4: Representation of disorder-to-α-helical transition of MBP and fractional population of equilibrium conformers. This thermodynamic mechanism shows the disorder-to-α-helical transition of MBP, indicated by the black, red, and blue lines. The fitted thermodynamic values were used to determine the fractional population of each equilibrium species has at different TFE concentrations. It is seen that until approximately 17.5% TFE, the disordered state is present, and the intermediate state is at a maximum fractional population at around 14%. The 18.5-kDa isoform of MBP shows that the α-helical conformation is significantly populated at approximately 15% TFE, and by >30% TFE. Global fitting of the MBP TFE titration curves to a 3-state model was used to obtain the ΔG values and associated populations. Figure adapted with permission from reference (24).
However, the distance constraints obtained in this pioneering study were insufficient to build a three-dimensional model of MBP. It is predicted that 18.5-kDa MBP will adopt either a ‘paperclip’ or ‘hairpin’ conformation (i.e., with either two bends or one, respectively), which will dictate how different α-helical segments (α1, α2, α3) will be arranged on the inner oligodendrocyte membrane leaflets, as shown in Figure 5. By positioning α-helices to interact with membrane leaflets, this conformation could possibly allow for a single molecule of MBP to hold together the major dense line (18).

**Figure 5:** Possible arrangements of the 18.5-kDa MBP isoform between inner oligodendrocyte membrane leaflets. There are a few possible arrangements for the α1, α2, α3 helices. By positioning α-helices to interact with membrane leaflets, this conformation may allow a single molecule of MBP to hold together the major dense line. Figure adapted with permission and modified from reference (18).
1.6 Statement of Thesis

1.6.1 Hypothesis

We hypothesize that a simple tertiary fold (such as a ‘paperclip’ or ‘hairpin’ conformation) is formed as the intrinsically disordered MBP approaches the membrane surface, as mimicked using TFE, DPC, and zinc, in addition to ordered α-helical membrane-anchoring motifs that have previously been defined. This “tertiary fold” is more precisely defined as an average of an ensemble of conformations. Obtaining a set of distance constraints by a variety of spectroscopic approaches, including FRET, will allow us to build a three-dimensional model of this fold. We also hypothesize that there will be significant differences between UTC1 and UTC8 variants of MBP in their ability to adopt that specific fold.

1.6.2 FRET of double-Cys substituted MBP variants

Originally, FRET was intended here to study double-Cys substituted MBP-C1 and MBP-C8 variants for the first time, to obtain additional constraints between different segments by FRET between fluorophores attached to two different Cys-substituted residues. I have designed a double-fluorophore labelling method, containing IAEDANS (donor), and 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI) (acceptor). The IAEDANS and DABMI labels were chosen because they bind specifically only to cysteine residues, are less expensive than other labelling probes, and are presumably small enough not to affect the conformational state of the protein significantly. The technique of CD spectroscopy was used to ensure that site-directed mutagenesis and label incorporation did not change the secondary structure composition of MBP-C1 and MBP-C8 variants significantly. My original goal was that with these new FRET data on double-Cys substituted MBP variants, a better three-dimensional distance map of full-length MBP-C1 and MBP-C8 variants could be determined. Although a successful labelling protocol was
achieved for one of the variants, the yield of labelled protein was so low that only a single FRET experiment could be performed to demonstrate the feasibility (Table A.1). However, the study was evidently impractical within the constraints of an M.Sc. thesis, necessitating switching over to slightly-modified project, which used single-Cys MBP variants instead.

1.6.3 FRET of single-Cys substituted MBP variants

Our laboratory previously obtained intramolecular distances between several single Cys-substituted sites throughout MBP, and a single internal Trp residue, by FRET, under conditions of increasing TFE concentration to mimic the approach of the protein to a membrane surface (18, 22). In this thesis, we repeated these studies under conditions that included the detergent dodecylphosphocholine (DPC), another membrane-mimic that we have previously used (3, 6, 11, 18), as well as to probe the effects of zinc, a divalent cation known to bind to MBP and to stabilize compact myelin (18, 25). The folding of MBP has not yet been studied under either condition by FRET, and my work here was useful to confirm their efficacy and utility.

1.6.4 Significance of study

This study is essential for helping us understand MBP’s topology and various roles in the myelin sheath. Understanding and analyzing the differences between UTC1 and UTC8 will help us better understand the mechanism of demyelination and may help in developing future treatment options for MS, such as inhibitors of peptidylarginine deiminases (9, 12). The increased deimination of MBP is strongly implicated in the structural destabilization of myelin, which leads to a demyelination cascade (12).
Chapter 2.0: Experimental Methods

2.1 Chemicals and Reagents

The reagents 5-((2-[(iodoacetyl)amino]ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS) and 4-dimethylaminophenylazophenyl-4’-maleimide (DABMI) were acquired from Invitrogen Life Technology (Burlington, ON, Canada). Other chemicals and reagents were obtained from Fisher Scientific (Unionville, ON, Canada) or Sigma-Aldrich (Oakville, ON, Canada).

2.2 Transformation, expression, and purification of recombinant full-length 18.5-kDa MBP-UTC1 and MBP-UTC8

Three variants which contained a combination of double-cysteine substitutions (at two of S17C, H85C, and S159C), and two variants which contained single-cysteine substitutions (at one of S17C and S159C), were prepared for each MBP variant: UTC1 and UTC8.

*E. coli* BL21-CP cells were transformed with the pET22b plasmid containing the sequence of the desired MBP variant. The transformed cells were grown in 1.5 L LB media at 37°C, supplemented with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol, until their optical density at 600 nm reached 0.6-0.8 (a.u.). Once the optimal density was achieved (after approximately 2.5 h), each culture mixture was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The proteins were overexpressed at 37°C for 4-5 h post-induction. A 1-mL “pre-induction” sample was collected for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) before the addition of IPTG, and a 1-mL “post-induction” sample was taken for SDS-PAGE after the 4-5 h growth. The samples were centrifuged to pellet cells, supernatant was poured off, and
the cells were resuspended in 100 μL 5 x sample buffer (0.06 M Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, 0.1% bromophenol blue). Each culture was harvested by centrifugation at 6000 rpm (Beckman-Coulter Rotor JA-10) for 5 min at 4°C. The pellets were frozen at -20°C for storage until the next day.

Cells were homogenized and lysed in 45 mL lysis buffer (8 M urea, 100 mM NaH₂PO₄, 500 mM NaCl, 10 mM Tris-base, 1% v/v Tween-20, 12.5 mM β-mercaptoethanol (pH 8.0-10.0). The mixtures were stirred at room temperature for 40 min followed by three sonication cycles (30 sec in the sonication bath and 10 min stirring at room temperature). Cell debris was removed by centrifugation at 25000 x g (Beckman-Coulter rotor JA-25.5) for 20 min at room temperature. The supernatant was collected and dialyzed at room temperature overnight against 2 L ion exchange (IEX) buffer (6 M urea, 80 mM glycine, pH 10.0) using 6-8 kDa molecular weight cut-off (MWCO) dialysis tubing.

Fast protein liquid chromatography (FPLC) was used to achieve purification of each MBP variant by using a BioRad-DuoLogic® apparatus and a 1-mL Pall AcroSep® cation exchange column. The IEX buffer (6 M urea, 80 mM glycine, pH 10), prepared and filtered through a 0.45-μm filter, was the mobile phase. Elution was achieved with a linear gradient of NaCl (0-500 mM) in 50 mL IEX buffer, and 1.5-mL fractions were collected automatically. All fractions were analyzed on the BioRad-DuoLogic® program and the pure fractions were isolated and confirmed via SDS-PAGE using a standard protein ladder. The confirmed protein fractions were combined and dialyzed (with two changes) in the cold room at 4°C overnight against 2 L dialysis buffer (50 mM Tris-HCl, 250 mM NaCl, pH 7.4), followed by four changes of 2 L distilled water. All dialysis
was done using 6-8 kDa MWCO dialysis tubing. The samples were collected and 12.5 mM β-mercaptoethanol was added to each variant to reduce all cysteine residues.

The MBP variants were further purified via reversed-phase HPLC using a Waters apparatus with a Symmetry 300, C18, 5 µm, 4.6 x 250 mm column. The mobile phase was acetonitrile, and the pairing agent was 0.1% trifluoroacetic acid. Detection was at 276 nm and 214 nm, the flow rate was 1 mL/min, and the column temperature was 40°C. The proteins were eluted with a linear gradient of acetonitrile (25-40%, 1%/min rate). An analytical run (50 µL) was conducted for each variant to indicate where the peak of interest could be. A preparative run was then performed to collect the main peak of interest and any other peak of possible interest. These fractions were freeze-fried overnight using a Labconco FreeZone freeze-dryer (Fisher Scientific, Markham, ON, Canada).

### 2.3 Labelling of double-Cys and single-Cys substituted MBP-variants

Dry weight of the freeze-dried protein was measured and then solubilized in distilled water to a final protein concentration of 3 mg/mL. The proteins were aliquoted 1 mg per tube and stored at -20°C.

#### 2.3.1 Labelling of double-Cys substituted MBP with IAEDANS and DABMI

The labelling of double-Cys MBP variants was achieved in a two-step reaction. In the first reaction, ~30-50 µM protein in 20 mM HEPES-NaOH (pH 7.4) was mixed with 1.5-fold molar excess IAEDANS taken from the 20 mM stock in 100% dimethylformamide (DMF). The reaction mixtures were incubated for 3 h at room temperature with gentle agitation. The reaction was terminated using 12.5 mM β-mercaptoethanol, which was removed along with unbound IAEDANS via HPLC (using the same method as for MBP purification). Protein fractions were
collected and analyzed by mass spectrometry to calculate the ratio of unlabelled cysteines and the rest was freeze-dried. Part of that protein served as a donor-alone control and the other part was used for the second reaction. Also, before submitting samples to mass spectrometry, the labelling efficiency (Equation 10) of IAEDANS, and the concentration of protein, were also confirmed using a set of equations derived below (Equations 6-10).

Dry weight of the freeze-dried protein was measured and then it was re-solubilized in distilled water to final protein concentration of 1 mg/mL. The protein concentrations were estimated by absorbance measurements using an extinction coefficient at 276 nm of 13,618 M$^{-1}$ cm$^{-1}$ for MBP-UTC1 and 12,975 M$^{-1}$ cm$^{-1}$ for MBP-UTC8. These values were experimentally determined by resuspending a known mass of protein in a known volume of water and measuring absorbance using the following equation (Beer-Lambert Law):

(Equation 3) \[ A = \varepsilon c l \]

Determining the concentrations of the protein and of IAEDANS in the labelled protein samples was conducted by UV absorption analysis, and the following equations:

(Equation 4) \[ x = [\text{MBP}] = \{A_{\text{total}}^{276} - ([\text{IAEDANS}] \cdot \varepsilon_{276}^{\text{IAEDANS}})\} / (\varepsilon_{276}^{\text{MBP}}) \]

(Equation 5) \[ y = [\text{IAEDANS}] = \{A_{\text{total}}^{336} - ([\text{MBP}] \cdot \varepsilon_{336}^{\text{MBP}})\} / (\varepsilon_{336}^{\text{IAEDANS}}) \]

where $\varepsilon_{\text{MBP-UTC1}}^{276}$ (extinction coefficient of MBP UTC1 at 276 nm) is 13618 M$^{-1}$ cm$^{-1}$, $\varepsilon_{\text{MBP-UTC1}}^{336}$ (extinction coefficient of MBP UTC1 at 336 nm) is 823 M$^{-1}$ cm$^{-1}$, $\varepsilon_{\text{MBP-UTC8}}^{276}$ (extinction coefficient of MBP UTC8 at 276 nm) is 12975 M$^{-1}$ cm$^{-1}$, $\varepsilon_{\text{MBP-UTC8}}^{336}$ (extinction coefficient of MBP UTC8 at 336 nm) is 406 M$^{-1}$ cm$^{-1}$, $\varepsilon_{276}^{\text{IAEDANS}}$ (extinction coefficient of IAEDANS at 276 nm) is 2430 M$^{-1}$ cm$^{-1}$, and
$\varepsilon_{336}^l$ (extinction coefficient of IAEDANS at 336 nm) is 5623 M$^{-1}$ cm$^{-1}$. The values of $\varepsilon_{336}^{MBP-UTC1}$ and $\varepsilon_{336}^{MBP-UTC8}$ were also experimentally determined by resuspending a known mass of protein in a known volume of water and measuring absorbance using Equation 3. The solutions to these equations are:

**Equation 6** \[ [\text{MBP}]_{UTC1} = 75.4 \text{ A}_{276} - 32.58 \text{ A}_{336} \]

**Equation 7** \[ [\text{IAEDANS}]_{UTC1} = 182.6 \text{ A}_{336} - 11.04 \text{ A}_{276} \]

**Equation 8** \[ [\text{MBP}]_{UTC8} = 78.13 \text{ A}_{276} - 33.76 \text{ A}_{336} \]

**Equation 9** \[ [\text{IAEDANS}]_{UTC8} = 180.3 \text{ A}_{336} - 5.64 \text{ A}_{276} \]

The labelling efficiency ($f_A$) was calculated using the following equation:

**Equation 10** \[ f_A = \frac{[\text{IAEDANS}]}{[\text{MBP}]} \]

For the second reaction, dry protein was resuspended in 20 mM HEPES-NaOH, 1 mM TCEP (tris(2-carboxyethyl)phosphine), pH 7.4, to final concentration of 30-50 µM and incubated with 20-fold molar excess of DABMI (taken from 20 mM stock in 100% DMF) for 3 h at room temperature, with gentle agitation. The reaction was terminated again with 12.5 mM β-mercaptoethanol and double-labelled protein was re-purified by HPLC. The labelling efficiency was confirmed by mass spectrometry and the proteins were freeze-dried.
2.3.2 Labelling of single-Cys substituted MBP with IAEDANS

For single-labelling cysteine reactions, the solutions were composed of 5 mM HEPES-NaOH, pH 7.4, 0.5 mM TCEP, pH 7.4, and 30-50 μM unlabelled protein. The IAEDANS in DMF was then added to the protein solution to a final concentration 20-fold in excess of the protein (by moles). This labelling reaction proceeded for 3 h at room temperature, with gentle agitation. Then, HPLC analytical runs were taken each hour, to follow the progress of labelling. The reaction was terminated using 12.5 mM β-mercaptoethanol, which was removed along with unbound IAEDANS via HPLC (same method as for MBP purification). Protein fractions were collected, freeze-dried, and sent to mass spectrometry for labelling efficiency confirmation.

For single-cysteine-substituted MBP, the method of determining the concentrations of labelled and unlabelled protein was the same as above, by using Equations 6-9, as the extinction coefficients for double- and single-Cys MBP variants were equivalent. The labelling efficiency was also calculated using Equation 10.

2.4 Förster Resonance Energy Transfer Measurements

All FRET data were obtained with a Fluoromax Spectrophotometer (Photon Technology International, Edison, NJ, USA) at room temperature. For each double-cysteine-labelled MBP experiment, a DABMI-labelled cysteine was used as the acceptor and an IAEDANS-labelled cysteine was used as the donor. For each single-cysteine-labelled MBP experiment, the lone intrinsic tryptophan (W113) was used as the donor and an IAEDANS-labelled cysteine was also used as the acceptor. Samples consisted of 2.5 μM donor alone (unlabelled protein for the single-Cys experiments and IAEDANS-labelled for double-Cys experiments) or donor-acceptor pair (IAEDANS-labelled for the single-Cys and IAEDANS-DABMI-labelled the double-Cys...
experiments) in 5 mM HEPES-NaOH, 0.5 mM TCEP, at pH 7.4. These samples were measured under several different environmental conditions: with TFE (0-30%), with DPC (0-10 mM) in the presence or absence of zinc, and with zinc (0-100 μM) in the presence or absence of DPC. All measurements were performed using a 5-mm path-length quartz cuvette. In the single-Cys experiment, tryptophan was excited at 295 nm and emission scans were collected from 310 nm to 550 nm; and in the double-Cys experiment excitation was at 336 nm and emission scan was collected in the range of 350-650 nm. Slit widths were set to 4 nm band-pass each. All samples were manually blank-corrected (sample subtracted by corresponding buffer) before data analysis. Each FRET experiment was recorded as an average of three scans, and also was triplicated with samples that were identically prepared. Based on tryptophan fluorescence quenching by IAEDANS, or IAEDANS quenching by DABMI (in the case of double-Cys experiment), the FRET efficiency (E_{FRET}) was determined using the following equation:

\[
\text{(Equation 11)} \quad E_{\text{FRET}} = \left(1 - \frac{D_A}{D}\right)\left(\frac{1}{f_A}\right),
\]

where \(D_A\) is the fluorescence intensity of the donor in the presence of the acceptor, and \(D\) is the fluorescence intensity of donor in the absence of the acceptor. The fractional labelling with acceptor, \(f_A\), normalizes the apparent efficiencies (22). The distance, \(r\), is then calculated, between the donor and the acceptor is calculated by:

\[
\text{(Equation 12)} \quad E_{\text{FRET}} = \frac{R_o^6}{(R_o^6 + r^6)},
\]

where \(R_o\) is referred to as the Förster distance (40 Å for double-Cys and 22.0 Å for single-Cys) (3). This is the distance when the energy transfer efficiency is 50%. All samples from the single-Cys experiments were also analyzed by CD spectroscopy.
2.5 Circular Dichroism Measurements

All CD data were collected on a Jasco J-815 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) by using a quartz cuvette with a 1-mm path length and measured at 22°C using a Jasco PTC-424S/15 Peltier temperature controller (Japan Spectroscopic, Tokyo, Japan). The CD data were acquired from solutions containing 2.5 μM protein, 5 mM HEPES-NaOH, pH 7.4, and several concentrations of either TFE (0-30% vol/vol), DPC (0-10 mM), or Zn²⁺ (0-100 μM). The TCEP reagent was added to each solution to a final concentration 0.5 mM to maintain a reducing environment of cysteine-substituted variants. Each spectrum was collected at a scan rate of 50 nm min⁻¹, which represents an average of three scans. All samples were manually blank-corrected (sample subtracted by corresponding buffer) before data analysis. The CD experiments were also triplicated with samples that were identically prepared.
3.1 MBP Purification and Expression

Double and single-Cys substituted MBP were successfully transformed and expressed in *E. coli* BL21-CP cells. The yield of double-Cys MBP variants produced was very low (Table A.1) due potentially to low expression or aggregation and/or degradation post-synthesis. For this reason, it was necessary to switch over to a slightly modified project, which used single-Cys MBP variants instead. A significant quantity (Table A.2) of single-Cys variants of MBP was produced via induction by IPTG, which was confirmed by SDS-PAGE. Analytical HPLC runs for each MBP variant confirmed if the protein was pure or if there were any minor contaminants from the dialyzed fractions of pure protein from FPLC. If contaminants were observed, another preparative HPLC run was done to separate these fractions, followed by an analytical run to confirm the contaminants were gone. The HPLC protein purification was successful. The UTC1 variants showed a single-peak pattern, whereas UTC8 variants showed a double-peak pattern, indicating that monomers and dimers were present. These dimers are likely the result of disulfide bonds between the introduced cysteine residues. Thus, 12.5 mM β-mercaptoethanol was used to reduce these dimers during the 3 h incubation period, to achieve higher efficiency labelling. Figure 6 shows an SDS-PAGE analysis of an MBP variant after labelling. The reduction of dimers was successful (lanes 4, 5, 6, 8, and 9), which revealed monomeric MBP (one pronounced band). However, lanes 2, 3, and 9 showed dimers, which signifies that the reduction of dimers was not 100% efficient; hence, a post-reduction was done for those samples by adding more β-mercaptoethanol to reduce all dimers (data not shown). Excess β-mercaptoethanol in the samples was removed during HPLC purification. Also, TCEP was added earlier on to the labelling reaction to prevent dimerization/disulphide bond
formation. HPLC was also used to determine the samples’ purity, which was seen to be greater than 95% for all MBP variants.

**Figure 6: SDS-PAGE analysis for MBP variants containing reduction of dimers by β-mercaptoethanol.** Data shown are for labelled and unlabelled MBP variants: UTC1-S17C (Lanes 2-5) and UTC1-S159C (Lanes 6-9), on a 16% SDS-PAGE. Samples were prepared by dissolving variant cell pellets in 5x reducing (R) or non-reducing (NR) loading dye to make 20-μL samples and were boiled at >100°C for 5 min. Lane 1 = Pre-stained Protein Ladder from Precision Plus Protein™ Dual Xtra Standards. Results show variant reduction by 12.5 mM β-mercaptoethanol. Lanes 2, 4, 6, and 8, represent samples mixed 5x reducing buffer for Control-UTC1-S17C (unlabelled), IAEDANS-UTC1-S17C (labelled), Control-UTC1-S159C, and IAEDANS-UTC1-S159C, respectively, indicated at ~18.5 kDa. Lanes 3, 5, 7, and 9 represent samples mixed with 5x non-reducing buffer for Control-UTC1-S17C (unlabelled), IAEDANS-UTC1-S17C (labelled), Control-UTC1-S159C, and IAEDANS-UTC1-S159C, respectively, also indicated at ~18.5 kDa. The gel was stained with Coomassie Brilliant Blue for 20 min and destained with 2% methanol and 1% acetic acid. The reduction of dimers is shown to be successful as indicated by only one pronounced band at ~18.5 kDa in the R and NR lanes (4, 5, 6, 8, 9), signifying that these proteins are monomeric. However, lanes 2, 3, and 7 show two pronounced bands, indication that the reduction of dimers was not 100% efficient; hence, a post-reduction was done for those samples by adding more β-mercaptoethanol to reduce all dimers (data not shown).
3.2 Double-fluorophore MBP labelling

Double-Cys protein labelling was successfully achieved by incubation with 1.5-fold molar excess of IAEDANS taken from the 20 mM stock in DMF. The reaction was terminated after 3 h with 12.5 mM β-mercaptoethanol, which was successfully removed along with unbound IAEDANS via HPLC. The labelling with the second dye DABMI was also effectively done by incubation with 20-fold molar excess of the fluorophore. The reaction was again terminated after 3 h with 12.5 mM β-mercaptoethanol. Mass spectrometry confirmed a labelling efficiency of 85% (Figure A.1). The fractions of interest were collected and freeze-dried.

3.3 Single-fluorophore MBP labelling

Single-Cys protein labelling was successfully achieved by incubation with 20-fold molar excess of IAEDANS taken from the 20 mM stock in DMF. The reaction was terminated after 3 h with 12.5 mM β-mercaptoethanol, which was successfully removed along with any unbound IAEDANS via HPLC. Mass spectrometry confirmed a labelling efficiency of 95% or higher for all single-Cys MBP variants (Figure A.2). The fractions of interest were collected and freeze-dried.

3.3.1 Extinction coefficient confirmation for MBP_{276}

The extinction coefficient of MBP_{276} was confirmed experimentally by measurement based on dry weight per volume. An alternate method than the one listed in Experimental Methods was used to confirm the concentration of labelled and unlabelled MBP. Firstly, the recalculation of ε^{MBP}_{276} was determined by inputting the following two equations on “QuickMath Automatic Math Solutions” (www.quickmath.com):
\begin{align*}
\text{(Equation 11)} \quad x &= [\text{MBP}] = \left( A_{\text{total}276} - [\text{IAEDANS}] \times e_{276} \right) / e_{\text{MBP}276} \\
\text{(Equation 12)} \quad y &= [\text{IAEDANS}] = [\text{MBP}] = \left( A_{\text{total}336} - [\text{MBP}] \times (e_{\text{MBP}276}/\text{CF}) / (e_{336}) \right)
\end{align*}

where \(e_{276}\) and \(e_{336}\) are the values used in \textbf{Equations 4 and 5}, \(A_{\text{total}276}\) and \(A_{\text{total}336}\) were absorbance values measured from each IAEDANS-labelled variant of MBP, and the correction factor, \(\text{CF}\), represents the ratio of \(A_{276}/A_{336}\) of unlabelled protein; therefore, \((e_{\text{MBP}276}/\text{CF})\) represents the value for \(e_{\text{MBP}336}\). From these two equations, QuickMath provides two new equations for each variant of MBP, one of which calculates the \(e_{\text{MBP}276}\). Since mass spectrometry confirmed 100% labelling efficiency for single-Cys substituted MBP, it can be assumed that the concentration of MBP is equal to the concentration of IAEDANS, and that each variant of MBP will have a different \(e_{\text{MBP}276}\). The second equation provided by QuickMath helps to solve the concentration of IAEDANS-labelled MBP. The newly-solved value of \(e_{\text{MBP}276}\) is used to calculate the concentration of unlabelled MBP using \textbf{Equation 3}, using \(A_{276}\) measured from the unlabelled MBP of each variant. The set of equations for single-Cys MBP used to determine the concentration of labelled MBP is:

\begin{align*}
\text{(Equation 13)} \quad [\text{IAEDANS}]_{\text{UTC1-S17C}} &= 180.3 \ A_{336} - 5.581 \ A_{276} \\
\text{(Equation 14)} \quad [\text{IAEDANS}]_{\text{UTC1-S159C}} &= 178.5 \ A_{336} - 1.451 \ A_{276} \\
\text{(Equation 15)} \quad [\text{IAEDANS}]_{\text{UTC8-S17C}} &= 180.1 \ A_{336} - 5.101 \ A_{276} \\
\text{(Equation 16)} \quad [\text{IAEDANS}]_{\text{UTC8-S159C}} &= 181.2 \ A_{336} - 7.677 \ A_{276}
\end{align*}

From the above equations for labelled-MBP, it can be seen that they are similar to Equations 7 and 8, which were also for labelled-MBP. This similarity confirms that the extinction coefficient determined for MBP_{276} was accurate.
3.4 Determining Labelling Efficiency of MBP

The spectra were collected from 220 to 700 nm for labelled and unlabelled MBP variants. These data showed that the label was successfully linked to the protein. A set of equations was developed to determine the amount of bound label and to subtract the labelled contribution at A276 (or A336 for double-Cys) to determine protein concentration, and also to subtract A276 to calculate the concentration of the amount of bound IAEDANS (or DABMI for double-Cys) label (Section 2.3). Extinction coefficients for MBP UTC1 and UTC8 were determined by resuspending a known mass of protein in a known volume of water and measuring absorbance. The final values calculated are: ε\textsubscript{MBP-UTC1\textsubscript{276}} (extinction coefficient of MBP\textsubscript{UTC1} at 276 nm) is 13618 M\textsuperscript{-1} cm\textsuperscript{-1}, ε\textsubscript{MBP-UTC1\textsubscript{336}} (extinction coefficient of MBP\textsubscript{UTC1} at 336 nm) is 823 M\textsuperscript{-1} cm\textsuperscript{-1}, ε\textsubscript{MBP-UTC8\textsubscript{276}} (extinction coefficient of MBP\textsubscript{UTC8} at 276 nm) is 12975 M\textsuperscript{-1} cm\textsuperscript{-1}, ε\textsubscript{MBP-UTC8\textsubscript{336}} (extinction coefficient of MBP\textsubscript{UTC8} at 336 nm) is 406 M\textsuperscript{-1} cm\textsuperscript{-1}, ε\textsuperscript{I\textsubscript{276}} (extinction coefficient of IAEDANS at 276 nm) is 2430 M\textsuperscript{-1} cm\textsuperscript{-1}, and ε\textsuperscript{I\textsubscript{336}} (extinction coefficient of IAEDANS at 336 nm) is 5623 M\textsuperscript{-1} cm\textsuperscript{-1}. The labelling efficiency was calculated and confirmed by mass spectrometry, and the concentrations of labelled and unlabelled MBP were successfully calculated.
3.5 Characterization of fluorescently-labelled MBP by CD spectroscopy

To ensure that site-directed mutagenesis and label incorporation did not change the secondary structure composition of MBP-C1 and MBP-C8 variants, CD spectroscopy was done. In previous studies, CD spectroscopy has been used to determine that MBP is disordered and monomeric in solution (24). Based on Figure 7, the change in far-UV CD of the disorder-to-α-helical transition of MBP was seen with increasing concentrations of DPC (0-10 mM), which signifies that the α-helical content also increases. At approximately 203 nm, there is an isodichroic point, which suggests a two-state transition. Figure 8 shows the spectral change in CD at 222 nm for MBP in the presence of increasing DPC concentrations from 0-10 mM. The trend is seen to be a saturation curve. Both Figures 7 and 8 show the same concentration of transition with donor and donor-acceptor, as it is transitioning from random coil to α-helical structure. This experiment was repeated under other membrane-mimetic environments (discussed below) to demonstrate that these modifications are not interfering with the propensity of MBP to form α-helical segments. For subsequent CD experiments, only donor-acceptor is shown. The same trend is seen in Figure 9, which represents MBP in the presence of Zn$^{2+}$ with increasing concentrations of DPC; hence, it can be stated that zinc had no further effect than DPC on MBP’s disorder-to-order transitions. As for MBP in the presence of DPC with increasing concentrations of Zn$^{2+}$ (Figure 10), no change of disorder-to-α-helical transition in MBP is seen as all curves are overlapping, with no isodichroic point at 203 nm.
**Figure 7:** Changes in CD spectra of MBP with increasing [DPC] of donor and donor-acceptor. The change in far-UV CD of the disorder-to-α-helical transition of all MBP variants was measured with presence of increasing concentrations of DPC (0-10 mM) from 190-260 nm. This shows that the α-helical content also increases. At approximately 203 nm, there is an isodichroic point, which suggests a two-state transition. These CD spectra of donor and donor-acceptor of MBP indicate that there is evident α-helical content at 10 mM DPC concentration. Measurements were conducted at 22°C. The data shown are for UTC1-S17C and UTC8-S159C, which represents the trend for all other MBP variants (UTC1-S159C and UTC8-S17C). All experiments were performed in triplicate.
Figure 8: Mean residue ellipticity of MBP at 222 nm in the presence of increasing [DPC] of donor and donor-acceptor. This data represents normalized mean residue ellipticity (MRE) at 222 nm against increasing DPC concentrations from 0-10 mM. The trend is a saturation curve for all variants, which indicates a transition of disorder-to-α-helical structure of MBP. The data shown are for UTC1-S17C and UTC1-S159C, which represents the trend for all other MBP variants (UTC8-S159C and UTC8-S159C). All experiments were performed in triplicate.
Figure 9: Changes in CD spectra and Mean residue ellipticity at 222 nm in the presence of Zn$^{2+}$ with increasing [DPC]. The change in far-UV CD of the disorder-to-α-helical transition of all MBP variants was measured in 65 μM Zn$^{2+}$ with increasing concentrations of DPC (0-10 mM) from 190-260 nm. This shows that the α-helical content also increases (Panels A and B). At approximately 203 nm, there is an isodichroic point, which suggests a two-state transition. These CD spectra of MBP portray that there is evident α-helical content at 10 mM DPC concentration. Measurements were conducted at 22°C. Panels C and D represent normalized mean residue ellipticity (MRE) at 222 nm against increasing DPC concentrations. The trend is a saturation curve and also portrays the transition of disorder-to-α-helical structure of MBP. The data shown are for UTC1-S159C and UTC8-S159C, which represents the trend for all other MBP variants (UTC1-S17C and UTC8-S17C). All experiments were performed in triplicate.
Figure 10: Circular dichroism spectra of MBP in the presence of DPC with increasing [Zn$^{2+}$]. No change of disorder-to-α-helical transition in MBP is seen in the presence of DPC with increasing concentrations of zinc (0-100 μM) from 190-260 nm. Measurements were conducted at 22°C. The data shown are for all MBP variants: UTC1-S17C, UTC1-S159C, UTC8-S17C, and UTC8-S159C. All experiments were performed in triplicate.
3.6 Förster Resonance Energy Transfer Measurements of MBP

3.6.1 Double-Cys FRET

FRET experiments were used for the analysis and determination of the intramolecular distance measurements of MBP variants. For double-Cys MBP, the labels IAEDANS and DABMI were chosen because IAEDANS has an excitation wavelength of 336 nm and an emission wavelength of 490 nm, whereas DABMI has an excitation wavelength at 419 nm and does not emit. These properties signify that these two labels are appropriate to use for FRET analysis, since the emission spectrum of IAEDANS will overlap with the excitation wavelength of DABMI.

A single FRET experiment for a single-Cys MBP variant (UTC1-H85CS159C) was successfully completed, as there was not enough protein to work with for more experiments. TFE changed the interaction of the donor and acceptor for double-Cys labelled UTC1-S17CH85C. Figure 11 shows this trend, as the distance (Table 1) between donor and donor-acceptor are closer together at 15% TFE than at 0% or 30% TFE, reflected by a larger difference between the donor and donor-acceptor emission spectra at 15%.

The quenching of IAEDANS with increasing concentrations of TFE is shown in Figure 12 and was seen that the quenching of IAEDANS increases from 0-15% TFE and decreases from 15-30% TFE. The labelling efficiency was 85%, which was confirmed by mass spectrometry (Figure A.1) and also by using Equation 10. This result means that 85% of the molecules had the donor and donor-acceptor together, while 15% of the molecules did not have the acceptor, so the labelling efficiency was corrected (Donor is 15% higher) at 500 nm, which can be seen in Figure 13A.

The intramolecular distances of this MBP variant in the presence of increasing TFE concentrations (0-30% v/v) can be seen in Figure 13B, which was produced using Equations 11
and 12. The higher the FRET efficiency, the shorter the distance (Table 1), which signifies the shape of the inverted bell-curve. The $R_o$ was 40 Å for the IAEDANS-DABMI pair (26, 27). It is seen that at 15% TFE, it is compact, as discovered in previous studies (3). It is also evident that as DABMI gets closer, emission is reduced.
Figure 11: Emission spectra of donor vs. donor-acceptor in the presence of increasing [TFE]. These figures show the interaction between the donor (blue) and donor-acceptor (red) with increasing concentrations of TFE (0-30% v/v) for UTC1-H85CS159C. The 0%, 15%, and 30% TFE points are shown. The donor and donor-acceptor are closer together at 15% TFE than at 0% or 30% TFE. This is shown by the larger difference between donor and donor-acceptor emission spectra at 15%. Measurements were conducted at the excitation wavelength of 336 nm and at 22°C.
Figure 12: Quenching of IAEDANS by the presence of acceptor with increasing [TFE]. The difference spectra for UTC1-H85CS159C show the quenching of IAEDANS with increasing concentrations of TFE (0-30% v/v) by the presence of the acceptor. Here, 85% of the molecules had the donor and donor-acceptor together, while 15% of the molecules did not have the acceptor, so the labelling efficiency was corrected (donor is 15% higher). Measurements were conducted at 22°C.
Figure 13: Normalized Quenching of IAEDANS by DABMI under TFE conditions at 500 nm and Distance between cysteines vs. [TFE]. Both graphs represent the MBP variant UTC1-H85CS159C. Panel (A) shows the normalized fluorescence intensity difference (donor-acceptor subtracted from donor) at 500 nm, in the presence of increasing concentrations of TFE (0-30% v/v). Panel (B) shows the distance (Å) versus increasing TFE concentrations (0-30% v/v). The labelling efficiency was 85% according to mass spectrometry. Refer to Table 1.
Table 1: FRET measurements of double-Cys labelled MBP in the presence of increasing [TFE]. These values are for the double-Cys-substituted MBP variant UTC1-H85CS159C, which was successfully double-labelled with IAEDANS and DABMI. The labelling efficiency was 85%. Samples contained 2.5 μM protein, 5 mM HEPES-NaOH, pH 7.4, and 0.5 mM TCEP. Measurements were conducted at 22°C. The excitation wavelength for IAEDANS was at 336 nm and the emission scan was collected in the range of 350-650 nm. The FRET efficiencies and distances were calculated from donor (IAEDANS) quenching with DABMI at different positions in 18.5-kDa MBP as indicated. Data are shown as a single experiment since there was not enough protein for another experiment.

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3.6.2 Single-Cys FRET

The FRET experiments for single-Cys MBP variants (UTC1-S17C, UTC1-S159C, UTC8-S17C, and UTC8-S159C) under various membrane-mimetic conditions were successfully completed. The emission spectra of donor and donor-acceptor with increasing concentrations of DPC can be seen in Figure 14, in the presence and absence of Zn$^{2+}$. This fluorescence spectrum of Donor shows that the emission maximum is at 340 nm and is also consistent with the presence of Tryptophan residues exposed to solvent. It is seen that the emission at 340 nm decreases, and another peak around 500 nm is evident, which represents the resonance energy transfer between the donor, Tryptophan, and the acceptor, IAEDANS. No difference is seen in regard to the absence (Figure 14A-C) or presence (Figure 14D-F) of zinc. The emission of donor is highest at 10 mM DPC for both conditions. This is because the donor is in a hydrophobic environment; hence, it will fluoresce more. Tryptophan sits in a more hydrophobic environment at 10 mM DPC; hence, the protein becomes more folded, whereas the protein structure is still random coil at 0 mM DPC, indicating that MBP is less exposed as the concentration of DPC increases from 0 to 10 mM.

The emission spectra of donor and donor-acceptor with increasing concentrations of zinc, in the presence and absence of DPC, can be seen in Figure 15. Panels A to C, which represent increasing concentrations of zinc without DPC, show no hydrophobic effect from 0 to 100 μM Zn$^{2+}$. No changes are also seen from 0 – 100 μM Zn$^{2+}$ in the presence of 1.25 mM DPC or 8 mM DPC; however, emission at ~340 nm is further apart. This fluorescence spectrum of Donor also shows that emission maximum is at 340 nm and is also consistent with the presence of Tryptophan residues exposed to solvent. The emission at 340 nm decreases, and another peak around 500 nm is seen, which is also depicted in Figure 14.
Figure 14: Emission spectra of donor vs. donor-acceptor in the presence and absence of zinc with increasing [DPC]. These figures show the interaction between the donor (black) and donor-acceptor (red) with increasing concentrations of DPC (0-10 mM). Panels A-C represent donor and donor-acceptor in the absence of Zn$^{2+}$ and panels D-F represent in the presence of 65 μM Zn$^{2+}$. The 0, 1.25, and 10 mM DPC points are shown for each condition. Measurements were conducted at the excitation wavelength of 336 nm and at 22°C. The shown data are for UTC1-S17C, which represents the trend for all other MBP variants (UTC1-S159C, UTC8-S17C and UTC8-S159C). All experiments were performed in triplicate.
**Figure 15: Emission spectra of donor vs. donor-acceptor in the presence and absence of DPC with increasing [Zn$^{2+}$].** These figures show the interaction between the donor (black) and donor-acceptor (red) with increasing concentrations of Zn$^{2+}$ (0-100 μM). Panels A-C represent donor and donor-acceptor in the absence of DPC, panels D-F represent the same with the presence of 1.25 mM DPC, and panels G-I are with the presence of 8 mM DPC. The 0, 10, and 100 μM Zn$^{2+}$ points are shown for each condition. Measurements were conducted at the excitation wavelength of 336 nm and at 22°C. The shown data are for UTC1-S17C, which represents the trend for all other MBP variants (UTC1-S159C, UTC8-S17C, and UTC8-S159C). All experiments were performed in triplicate.
The quenching of Tryptophan with increasing concentrations of DPC, as well as increasing concentration of DPC with the presence of zinc, is shown in Figures 16A and 16B, respectively. A similar trend that was seen with double-Cys TFE (Figure 12) is also seen here. For increasing concentrations of DPC in either the absence and presence of zinc, Trp quenching is increasing from 0-1.25 mM DPC, and decreasing from 1.5-10 mM DPC (Figure 16A-B). Zinc is thus likely to be stabilizing MBP in a DPC environment.

The quenching of Trp with increasing concentrations of zinc (Figure 16C), and in 8 mM DPC with increasing concentrations of zinc (Figure 16E), show no trend or effect on MBP. However, a trend with 1.25 mM DPC in the presence of increasing concentrations of DPC is evident (Figure 16D). The quenching of Trp starts to increase from 0-20 μM, and then decreases from 50-100 μM. Therefore, zinc only has an effect when in the presence of 1.25 mM DPC with increasing concentrations of zinc.
Figure 16: Quenching of tryptophan by the presence of acceptor under various conditions. These difference spectra show the quenching of Trp by the presence of acceptor in different environments, which are as follows, Panel A: increasing [DPC], Panel B: 65 μM Zn^{2+} with increasing [DPC], Panel C: with increasing [Zn^{2+}], Panel D: 1.25 mM DPC with increasing [Zn^{2+}], and Panel E: 8 mM DPC with increasing [Zn^{2+}]. The DPC concentrations range from 0 to 10 mM and zinc concentrations range from 0 to 100 μM. Measurements were conducted at 22°C. The shown data are for UTC8-S17C, which represents the trend for all other MBP variants (UTC1-S17C, UTC1-S159C and UTC8-S17C). All experiments were performed in triplicate.
The fluorescence intensity difference (donor-acceptor subtracted from donor) at 340 nm for all MBP variants can be seen in Figures 17A-D. The same pattern is seen in Figures 17A-C, resembling a bell shape, with a maximum between ~1.25-1.75 mM DPC, which makes sense because the critical micelle concentration of DPC is at 1 mM. However, Figure 17D (UTC8-S159C) shows a different pattern, leading to saturation at the end of the curve, resembling a sigmoidal shape, with a maximum at ~5 mM. Equation 11 followed by Equation 12, was used to produce the distance curves, shown in Figures 17E-H. These also resemble a bell shape, except for Figure 17H, which was the corresponding distance curve to Figure 17D. The Ro was 22 Å for the Tryptophan-IAEDANS pair (28).

For MBP in the presence of zinc with increasing concentrations of DPC (0-10 mM), the normalized quenching of Tryptophan for all MBP variants was also analyzed at 340 nm, which can be seen in Figures 18A-D. These also have the same pattern as Figures 17A-D (absence of zinc with increasing [DPC]) also resembling a bell shape, with a maximum between ~1.25-1.75 mM DPC. The corresponding distance curves are shown in Figures 18E-H, which indicate that the shape of the transition is similar to DPC in the absence of zinc, seen in Figure 17. However, zinc with increasing concentrations of DPC is shown to induce the overall compact state of UTC8 variants when compared to DPC in the absence of zinc, as can be seen by the distances in Tables 2 and 3. Therefore, it appears that DPC in the presence of zinc affects the distance for UTC8 MBP variants, as the trend of transition for the protein becomes shorter, hence the protein is becoming more compact.
Figure 17: Normalized quenching of tryptophan in the presence of increasing [DPC] at 340 nm and the Distance between Trp and Cys vs. [DPC]. Panels A-D represent the fluorescence intensity difference (donor-acceptor subtracted from Donor) at 340 nm for all MBP variants: UTC1-S17C, UTC1-S159C, UTC8-S17C, and UTC8-S159C. Panels E-H represent the corresponding distance (Å) versus increasing DPC concentrations (0-10 mM). The labelling efficiency was greater than 95% according to mass spectrometry. Note that the y-axis scales for each variant are different, and Panel F has an x-axis scale from 1-2.5 mM due to negative FRET efficiencies. All experiments were performed in triplicate. Refer to Table 2.
Table 2: FRET measurements of single-Cys labelled MBP in the presence of increasing [DPC]. These values are for single-Cys-substituted UTC1 and UTC8 variants, which were successfully labelled with IAEDANS. The labelling efficiency was approximately 95-100%. Samples contained 2.5 μM protein, 5 mM HEPES-NaOH, pH 7.4, and 0.5 mM TCEP. Measurements were conducted at 22°C. The excitation wavelength for Trp was at 295 nm and was collected from 310 nm to 550 nm. The FRET efficiencies and distances were calculated from donor (W113) quenching with IAEDANS at different positions within 18.5-kDa MBP as indicated. Data are shown as averages of 2-3 independent measurements, and the error bars represent the standard error.

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<th>[DPC] (mM)</th>
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<td>* 0</td>
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* 0: This is zero because Trp was in a much higher hydrophobic environment (fluctuations in the concentration); therefore, based on donor quenching, FRET cannot be calculated.
* ND: Distance is not determined (ND) due to FRET efficiency.
Figure 18: Normalized quenching of tryptophan in the presence of 65 μM Zn²⁺ with increasing [DPC] at 340 nm and the Distance between Trp and Cys vs. [DPC]. Panels A-D represent the fluorescence intensity difference (donor-acceptor subtracted from Donor) at 340 nm for all MBP variants: UTC1-S17C, UTC1-S159C, UTC8-S17C, and UTC8-S159C. Panels E-H represent the corresponding distance (Å) versus increasing DPC concentrations (0-10 mM) with the presence of 65 μM Zn²⁺. The labelling efficiency was greater than 95% according to mass spectrometry. Note that the y-axis scales for each variant are different. All experiments were performed in triplicate. Refer to Table 3.
Table 3: FRET measurements of single-Cys labelled MBP in the presence of 65 μM Zn$^{2+}$ with increasing [DPC]. These values are for single-Cys-substituted UTC1 and UTC8 variants, which were successfully labelled with IAEDANS. The labelling efficiency was approximately 95-100%. Samples contained 2.5 μM protein, 5 mM HEPES-NaOH, pH 7.4, and 0.5 mM TCEP. Measurements were conducted at 22°C. The excitation wavelength for Trp was at 295 nm and was collected from 310 nm to 550 nm. The FRET efficiencies and distances were calculated from donor (W113) quenching with IAEDANS at different positions within 18.5-kDa MBP as indicated. Data are shown as averages of 2-3 independent measurements, and the error bars represent the standard error.

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<th>[DPC] (mM)</th>
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For increasing concentrations of Zn$^{2+}$ (0-100 μM), the normalized quenching of Tryptophan for all MBP variants was also analyzed at 340 nm, which can be seen in Figures 19A-D. The corresponding distance curves are shown in Figure 19E-H. No changes or trends are seen; therefore, it can be stated that zinc does not have an effect under these conditions.

The normalized quenching of Trp in the presence of DPC with increasing zinc concentrations at 340 nm, as well as the distances between Trp and Cys (Figure 20), shows that there is also no change between the donor and the acceptor. Zinc did not appear to show any effect at the point where DPC provides its maximal effect, which is at 1.25 mM.

Another type of analysis for increasing DPC concentrations with the presence and absence of zinc was conducted, as this method provided less noise than the fluorescence intensity difference spectra. The ratio of donor-acceptor at 340 nm to donor-acceptor at 500 nm was plotted against the concentration of DPC (Figure 21). This verifies that no change with zinc is seen and all curves show a saturation trend, indicating that zinc does not affect the tertiary structure of MBP under these conditions. This method provides a more definitive answer to the effect of zinc.
Figure 19: Normalized quenching of tryptophan in the presence of increasing [Zn$^{2+}$] at 340 nm and the Distance between Trp and Cys vs. [Zn$^{2+}$]. Panels A-D represent the fluorescence intensity difference (donor-acceptor subtracted from donor) at 340 nm for all MBP variants. Panels E-H represent the corresponding distance (Å) versus increasing zinc concentrations (0-100 μM). The labelling efficiency was greater than 95% according to mass spectrometry. Note that the y-axis scales for each variant are different. The data shown are for all MBP variants: UTC1-S17C, UTC1-S159C, UTC8-S17C, and UTC8-S159C. All experiments were performed in triplicate. Refer to Table 4.
Table 4: FRET measurements of single-Cys labelled MBP in the presence of increasing \([\text{Zn}^{2+}]\). These values are for single-Cys-substituted UTC1 and UTC8 variants, which were successfully labelled with IAEDANS. The labelling efficiency was approximately 95-100%. Samples contained 2.5 \(\mu\)M protein, 5 mM HEPES-NaOH, pH 7.4, and 0.5 mM TCEP. Measurements were conducted at 22°C. The excitation wavelength for Trp was at 295 nm and was collected from 310 nm to 550 nm. The FRET efficiencies and distances were calculated from donor (W113) quenching with IAEDANS at different positions within 18.5-kDa MBP as indicated. Data are shown as averages of 2-3 independent measurements, and the error bars represent the standard error.

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* ND: Distance is not determined (ND).
Figure 20. Normalized quenching of tryptophan in the presence of DPC with increasing [Zn$^{2+}$] at 340 nm and the Distance between Trp and Cys vs. [Zn$^{2+}$]. Panels A-D represent the fluorescence intensity difference (donor-acceptor subtracted from donor) at 340 nm for all MBP variants. Panels E-H represent the corresponding distance (Å) versus increasing DPC concentrations (0-10 mM) with the presence of zinc. The labelling efficiency was greater than 95% according to mass spectrometry. Zinc concentrations range from 0 to 100 μM, and 1.25 mM and 8 mM concentrations of DPC were used. The data shown are for UTC1-S17C and UTC8-S159C, which represents the trend for all other MBP variants (UTC1-S159C and UTC8-S17C). Note that the y-axis scales for each variant are different. All experiments were performed in triplicate, except for UTC8-S159C, as there was not enough material to perform another experiment. Refer to Tables 5 and 6.
Table 5: FRET measurements of single-Cys labelled MBP in the presence of 1.25 mM DPC with increasing [Zn\(^{2+}\)]. These values represent single-Cys-substituted MBP of UTC1 and UTC8 variants, which were successfully labelled with IAEDANS. The labelling efficiency was approximately 95-100%. Samples contained 2.5 μM protein, 5 mM HEPES-NaOH, pH 7.4, and 0.5 mM TCEP. Measurements were conducted at 22°C. The excitation wavelength for Trp was at 295 nm and was collected from 310 nm to 550 nm. The FRET efficiencies and distances were calculated from donor (W113) quenching with IAEDANS at different positions within 18.5-kDa MBP as indicated. Data are shown as averages of 1-3 independent measurements, and the error bars represent the standard error, if any.

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<th>[Zn(^{2+})] (μM)</th>
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<th>UTC8-S17C</th>
<th>UTC8-S159C</th>
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<td>Distance (Å)</td>
<td>FRET efficiency (%)</td>
<td>Distance (Å)</td>
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<td>31.5±6.5</td>
<td>59.6</td>
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<td>34.9</td>
<td>24.4</td>
</tr>
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<td>24.8</td>
<td>37.3</td>
<td>24.0</td>
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<td>29.7</td>
<td>33.3</td>
<td>24.7</td>
</tr>
<tr>
<td>8</td>
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<td>34.2±3.4</td>
<td>37.4</td>
<td>24.0</td>
</tr>
<tr>
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<td>33.8</td>
<td>20.2</td>
<td>27.7</td>
</tr>
<tr>
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<tr>
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<td>23.9</td>
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<td>35.1±8.5</td>
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<td>24.4±11.8</td>
<td>28.3±9.2</td>
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</table>

* 0: This is zero because Trp was in a much higher hydrophobic environment (fluctuations in the concentration); therefore, based on donor quenching, FRET cannot be calculated.
* ND: Distance is not determined (ND) due to FRET efficiency.
Table 6: FRET measurements of single-Cys labelled MBP in the presence of 8 mM [DPC] with increasing [Zn²⁺]. These values are for single-Cys-substituted UTC1 and UTC8 variants, which were successfully labelled with IAEDANS. The labelling efficiency was approximately 95-100%. Samples contained 2.5 μM protein, 5 mM HEPES-NaOH, pH 7.4, and 0.5 mM TCEP. Measurements were conducted at 22°C. The excitation wavelength for Trp was at 295 nm and was collected from 310 nm to 550 nm. The FRET efficiencies and distances were calculated from donor (W113) quenching with IAEDANS at different positions within 18.5-kDa MBP as indicated. Data are shown as averages of 1-3 independent measurements, and the error bars represent the standard error, if any.

<table>
<thead>
<tr>
<th>[Zn²⁺] (μM)</th>
<th>UTC1-S17C</th>
<th>UTC1-S159C</th>
<th>UTC8-S17C</th>
<th>UTC8-S159C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FRET efficiency (%)</td>
<td>Distance (Å)</td>
<td>FRET efficiency (%)</td>
<td>Distance (Å)</td>
</tr>
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<td>36.1</td>
<td>8.4</td>
<td>32.7</td>
</tr>
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<td>35.0</td>
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<td>29.9±15.9</td>
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<td>1.1±0.9</td>
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<tr>
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<td>4.4±1.6</td>
<td>36.8±1.6</td>
</tr>
<tr>
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<td>36.0</td>
<td>2.0</td>
<td>42.3</td>
</tr>
<tr>
<td>20</td>
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<td>32.4±12.2</td>
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<tr>
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<td>43.0±1.9</td>
<td>25.9</td>
<td>26.2</td>
</tr>
<tr>
<td>80</td>
<td>4.0±1.7</td>
<td>37.4±1.7</td>
<td>2.1</td>
<td>41.7</td>
</tr>
<tr>
<td>100</td>
<td>2.9±3.2</td>
<td>40.4±3.2</td>
<td>3.9±1.2</td>
<td>37.5±1.2</td>
</tr>
</tbody>
</table>

* 0: This is zero because Trp was in a much higher hydrophobic environment (fluctuations in the concentration); therefore, based on donor quenching, FRET cannot be calculated.
* ND: Distance is not determined (ND) due to FRET efficiency.
Figure 21: Donor-acceptor at 340 nm / donor-acceptor at 500 nm ratio comparison in the presence or absence of zinc with increasing [DPC]. The DPC concentrations range from 0 to 10 mM, with the absence (A-D) or presence (E-H) of zinc. The shown data are for all MBP variants: UTC1-S17C, UTC1-S159C, UTC8-S17C, and UTC8-S159C. All experiments were performed in triplicate.
Chapter 4.0: Discussion

4.1 Disorder-to-order transitions of MBP in lipid and membrane-mimetic solvents

In this study, circular dichroism was used to confirm that DPC is an effective membrane-mimetic condition for the 18.5-kDa isoform of MBP. An α-helical transition is induced and stabilized in the presence of DPC (0-10 mM) for both C1 and C8 single-Cys variants of MBP (Figure 7) in regions that already have α-helix-forming propensity (18, 24). This was also seen with TFE in previous studies with single-Cys MBP variants (3). The same transition is seen for both donor and donor-acceptor, as shown by the mean residue ellipticity data at 222 nm (Figure 8), which is the wavelength commonly used for quantifying the α-helical content of protein. A two-state transition is suggested, as an isodichroic point is evident at approximately 203 nm, which is the approximate wavelength where peptides and proteins have an unchanged ellipticity while experiencing disorder-to-order transition. The same random-coil to α-helical structure is seen for MBP in the presence for Zn$^{2+}$ with increasing concentrations of DPC (Figure 9). This observation suggests that Zn$^{2+}$ has no effect (with DPC) on MBP’s secondary structure, as data trends are similar. Although zinc is an abundant metal in the brain and has been previously shown to induce hydrodynamic compaction in MBP, CD spectroscopies has indicated that MBP in the presence of only zinc concentrations shows no disorder-to-order transition, as there is also no isodichroic point at 203 nm (Figure 10).

The FRET distances of MBP have successfully been calculated for UTC1 and UTC8 variants under various conditions. For the double-Cys variant of MBP, UTC1-S17CH85C, it was seen that the distance of donor and donor-acceptor are closer together at 15% TFE than at 0% or
30% TFE, which is portrayed by the larger distance between donor and donor-acceptor for the emission spectra at 15% (Figure 11). This was also the same trend for previously studied single-Cys MBP variants with TFE (3). The quenching of IAEDANS with increasing concentrations of TFE (Figure 12) demonstrates that IAEDANS quenching is increasing from 0-15% TFE and decreasing from 15-30%.

It has long been known that MBP is a zinc-binding protein, and zinc is believed to stabilize the protein’s interaction with lipids, which can help form compact myelin in the CNS (25). In previous studies, it has been seen that zinc showed no significant changes to MBP in aqueous solution. In this study, FRET was used to determine the quenching of Trp in the presence of the acceptor, and it was seen that there was an effect of zinc at the point where DPC provided the maximal effect, which was close to the critical DPC micelle concentration of 1.25 mM (Figure 16D). The quenching of Trp was seen to increase from 0-20 μM and decrease from 50 to 100 μM. No effect of zinc on MBP’s tertiary structure was seen at 8 mM DPC (Figure 16E), as this is where DPC was shown to reduce in the presence of increasing zinc concentrations. Although this was the case for Trp quenching in the presence of IAEDANS, FRET distances showed that Zn$^{2+}$ did not show any effect on MBP in the presence or absence of increasing concentrations of DPC (Figures 18-20 E-H).
4.2 Conclusions and Future Directions

Intrinsically disordered proteins, such as MBP, have no secondary structure, as they are highly flexible and dynamic, which allows them to undergo a variety of conformations. There are several directions of future research that can be studied to extend this project. First, a quantitative analysis can be done using thermodynamic analysis, as the ellipticity curves at 222 nm for each variant can be fit to a two-state model (disorder-to-α-helical) (3, 24). This can be used to determine the Gibbs free energy, as well as the stoichiometry and dissociation constant (Kₐ). Another interesting avenue of future research would be to study the interaction of MBP variants under other conditions. We have previously used TFE, which is a lipid-mimetic solvent, and DPC, which is a membrane-mimetic solvent; however, it would be interesting to perform FRET to see the interaction of MBP with something closer to the myelin structure, such as large unilamellar vesicles (LUV), which are bilayered. Other interactions to study could be with signaling proteins, such as calmodulin (regulates calcium flux), and cytoskeletal proteins, such as actin and tubulin. We may know how these interact with MBP; however, the way MBP’s structure changes with these interactions is unknown. The effects of other PTMs such as phosphorylation can also be studied on the disorder-to-α-helical transitions of MBP via FRET measurements. In addition, a successful protocol has been designed for labelling double-Cys variants and these proteins should be studied under other conditions (DPC with and without Zn²⁺) as well, provided that yields can be improved.
REFERENCES


APPENDICIES

A.1 Protein yields for double- and single-Cys MBP variants

Table A.1: Protein yields for double-Cys MBP variants. UTC1-H85CS159C was the only double-Cys MBP variant that was efficiently purified and expressed, whereas others yielded less. This variant provided enough material for one single FRET experiment. All protein yields shown are from 1 L of culture.

<table>
<thead>
<tr>
<th>MBP Variant</th>
<th>Protein Yields from 1 L Culture (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTC1-S17CH85C</td>
<td>0.14</td>
</tr>
<tr>
<td>UTC1-S17CS159C</td>
<td>0.08</td>
</tr>
<tr>
<td>UTC1-H85CS159C</td>
<td>0.80</td>
</tr>
<tr>
<td>UTC8-S17CH85C</td>
<td>0.16</td>
</tr>
<tr>
<td>UTC8-S17CS159C</td>
<td>0.12</td>
</tr>
<tr>
<td>UTC8-H85CS159C</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table A.2: Protein yields for single-Cys MBP variants. All single-Cys MBP variants were efficiently purified and expressed, and yielded high amounts of protein. All protein yields shown are from 1 L culture.

<table>
<thead>
<tr>
<th>MBP Variant</th>
<th>Protein Yields from 1 L Culture (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTC1-S17C</td>
<td>7.3</td>
</tr>
<tr>
<td>UTC1-S159C</td>
<td>9.6</td>
</tr>
<tr>
<td>UTC8-S17C</td>
<td>3.3</td>
</tr>
<tr>
<td>UTC8-S159C</td>
<td>4.8</td>
</tr>
</tbody>
</table>
A.2 Mass spectrometry results

**Figure A.1:** Mass spectrometry result for double-Cys MBP (UTC1-H85CS159C) after labelling with IAEDANS and DABMI. The molecular weight of IAEDANS-DABMI pair with MBP is ~18,964 Da and 2-IAEDANS with MBP is ~18,950 Da, as highlighted above. The total abundance value was calculated for ‘2-I’ and ‘I-D,’ and then the abundance for each was divided by the total to determine the labelling efficiency. The 2-IAEDANS was determined to be labelled 15% and IAEDANS-DABMI to be 85%. This was the only double-Cys MBP variant that was labelled, as the others did not yield sufficient amounts of protein.

**Figure A.2:** Mass spectrometry result for single-Cys MBP (UTC1-S159C) after labelling with IAEDANS. The molecular mass of IAEDANS with MBP is ~18,679 Da for UTC1 and ~18,538 Da for UTC8 variants. The shown data are for UTC1-S159C, which indicates 100% labelling efficiency, judged based on the detectable level of unlabelled protein. Labelling efficiency for other single-Cys MBP variants (UTC1-S17C, UTC8-S17C, UTC8-S159C) was ~95-100%.