Investigating the Oligomeric State of Osmoregulatory Transporter ProP of *Escherichia coli* and its Functional Consequences

By

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ABSTRACT

INVESTIGATING THE OLIGOMERIC STATE OF OSMOREGULATORY TRANSPORTER PROP OF ESCHERICHIA COLI AND ITS FUNCTIONAL CONSEQUENCES

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University of Guelph, 2016

Advisor: Dr. Janet Wood

ProP, an osmoregulatory transporter found in *E. coli*, is responsible for the accumulation of osmolytes in response to hyperosmotic stress. ProP is able to dimerize through the formation of a C-terminal coiled coil and the oligomeric state of ProP may play a role in ProP function. The effects of detergents, particularly n-dodecyl-β-D-maltoside, on the oligomeric state of ProP were studied using blue native polyacrylamide gel electrophoresis and laser induced liquid bead ion desorption mass spectrometry. These techniques showed that ProP is primarily monomeric in vitro, implying that ProP studied in proteoliposomes may also exist, at least in part, as a monomer. The functional significance of ProP dimerization was probed by attempting to observe a dominant negative effect in vivo. In vivo work suggested that ProP may not require dimerization to be active and future work will use the same approach to probe the role of dimerization in vitro.
Acknowledgements

I would like to begin by thanking my supervisor Dr. Janet Wood for allowing me to work in her lab as a graduate student and for the opportunities she gave me as an undergraduate student, including giving me my first experience in an academic lab. I am also grateful to her receptiveness to begin a collaboration with an international research group to incorporate into my project, an experience I will always remember. I would also like to acknowledge the support I have received from my fellow Wood lab members, Doreen Culham, Tanya Romantsov and Laura Tempelhagen. In particular to Doreen, thank you for being an excellent teacher in the lab and a pleasure to work with.

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List of Abbreviations

2D-PAGE: two dimensional polyacrylamide gel electrophoresis
ABC: ATP-binding cassette
APS: ammonium persulfate
ATP: adenosine triphosphate
Amp: ampicillin
b: constant inversely related to the slope of a sigmoidal curve
BACTH: bacterial adenylate cyclase two hybrid
BCCT: betaine-carnitine-choline transporter
BCA: bicineholinic acid
BCIP: 5-bromo-4-chloro-3-indolyl-phosphate
BN-PAGE: blue native-polyacrylamide gel electrophoresis
B. pertussis: Bordetella pertussis
BSA: bovine serum albumin
cAMP: cyclic adenosine monophosphate
CAP: catabolite activator protein
CBS: cystathionine β-synthase
C. glutamicum: Corynebacterium glutamicum
CHAPS: 3-((3-cholamidopropyl) dimethylammonio-1-propanesulfonate)
Cm: chloramphenicol
CPM: counts per minute
CRP: cyclic-AMP receptor protein
CV: column volume
CYMAL-6: 6-cyclohexyl-1-hexyl-β-D-maltoside
DDAO: n-dodecyl-N,N-dimethylamine-N-oxide
DDM: $n$-dodecyl-$\beta$-D-maltoside
DM: $n$-decyl-$\beta$-D-maltoside
DMF: N,N-dimethylformamide
DNA: deoxyribonucleic acid
DTME: dithiobismaleimidoethane
ECL: enhanced chemiluminescence
$E.\ coli$: $Escherichia\ coli$
EDTA: ethylenediaminetetraacetic acid
Fos-choline-12: n-dodecylphosphocholine
FRET: fluorescence resonance energy transfer
FSEC: fluorescence size exclusion chromatography
FX Cloning: fragment exchange cloning
GF: gel filtration
GFP: green fluorescence protein
HRP: horse radish peroxidase
HRV: human rhinovirus
IPTG: isopropyl $\beta$-D-1-thiogalactopyranoside
Km: kanamycin
$K_m$: substrate concentration at half maximal transport rate
LB: lysogeny broth
$L.\ lactis$: $Lactococcus\ lactis$
LILBID-MS: laser induced liquid bead ion desorption mass spectrometry
MFS: major facilitator superfamily
MOPS: 3-(N-morpholino)propanesulfonic acid
MTSEA: 2-aminoethylmethanethiosulfonate hydrobromide
NBD: nucleotide binding domain
NBT: nitroblue tetrazolium
NEM: N-ethylmaleimide
Ni-NTA: nickel nitrilotriacetic acid
NMR: nuclear magnetic resonance
OD: optical density at 600 nm
OG: n-octyl-β-D-glucopyranoside
O/N: overnight
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PDB: protein data bank
PL: periplasmic loop
PMSF: phenylmethylsulfonyl fluoride
ProP*: cysteine-less ProP
PVDF: polyvinylidene fluoride
RBS: ribosome binding site
SBD: substrate binding domain
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
Strep: streptomycin
TB: terrific broth
TBS: tris-buffered saline
TEMED: tetramethylethlenediamine
Tet: tetracycline
TLC: thin layer chromatography
TM: transmembrane helix
Tris: tris(hydroxymethyl)aminomethane

TTC: 2,3,5-triphenyltetrazolium chloride

$V_{\text{max}}$: maximum rate of reaction with high substrate concentration

$X_0$: osmolality at which the uptake rate is half maximal

$Y_{\text{max}}$: maximum uptake rate observed when the medium osmolality is high
Chapter 1: Introduction

1.1. Osmotic stress and osmoregulatory transporters

Bacteria reside in diverse and changing environments and therefore require systems that allow them to cope with the variety of stresses that may be encountered. Maintaining cellular hydration levels and the osmotic pressure differential between the external and intracellular environment is essential to survival (Wood, 2007, 1999). Extreme or variable osmotic pressure can result from changes to the external solute concentrations (see Table 1.1 for definition) (Wood, 2007, 1999). Changes in the environment, such as when bacteria transition from the external environment to a mammalian host or as nutrient and hydration levels vary in the soil, can impose osmotic stress on a bacterial cell (Poolman & Glaasker, 1998; Wood, 2007; Wood et al, 2001).

An increase in osmotic pressure, or hyperosmotic stress, causes rapid water efflux from the bacterial cell resulting in dehydration and slow growth (Wood, 1999; Poolman & Glaasker, 1998). If the osmotic upshift is high enough, the cytoplasmic membrane may detach from the cell wall due to cytoplasmic shrinkage, a phenomenon called plasmolysis (Wood, 1999; Poolman & Glaasker, 1998). These effects are avoided by intracellular accumulation of K⁺ and glutamate (Wood, 1999; Poolman & Glaasker, 1998; Wood, 2011a). The Trk proteins are primarily responsible for K⁺ accumulation and have increased activity in response to osmotic stress (see Figure 1.1 for summary of osmoregulatory systems) (Poolman & Glaasker, 1998; Rhoads et al, 1976). Glutamate utilization in the cell is suppressed to balance the influx of cations (Poolman & Glaasker, 1998; Dinnbier et al, 1988; McLaggan et al, 1994). K⁺ and glutamate accumulation prevents the deleterious effects of high osmotic pressure, but high levels of potassium ions can interfere with some cellular processes such as protein-DNA interactions (Wood, 2011a).
Table 1.1: Terminology used to describe osmosensing and osmoregulation. All terms are as defined in (Wood, 2007, 2011b)

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotic Pressure</td>
<td>A pressure equal to the hydrostatic pressure that would be required to prevent water from flowing across a semipermeable membrane into an aqueous solution of a membrane-impermeant solute.</td>
</tr>
<tr>
<td>Osmolality</td>
<td>The osmotic pressure at a particular temperature. Osmolality can be measured not calculated.</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>The sum of the concentrations of osmotically active solutes in solution is an estimate of osmolarity.</td>
</tr>
<tr>
<td>Osmolyte</td>
<td>A small organic molecule that accumulates in cells and protects cellular components against denaturing environmental stresses. Also referred to as a compatible solute.</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>Physiological processes that mitigate changes in cell structure and function caused by changes in extracellular osmotic pressure.</td>
</tr>
<tr>
<td>Osmosensor</td>
<td>A protein that detects changes in water activity (direct osmosensing) or resulting changes in cell structure or composition (indirect osmosensing) and directs osmoregulatory responses.</td>
</tr>
</tbody>
</table>
Figure 1.1: Summary of systems involved in the osmotic stress response in *Escherichia coli*. Aquaporin AqpZ mediates water movement across the cytoplasmic membrane. In response to an osmotic downshift, mechanosensitive channels MscL and MscS release solutes to reduce the cytoplasmic osmolality. One response to increasing osmotic pressure is mediated by K⁺ accumulation by the Trk system and glutamate synthesis. Osmolyte accumulation is facilitated by osmoregulatory systems, ProP, ProU, BetT and BetU. ProU is an orthologue of OpuA from *L. lactis* and BetT and BetU are orthologues of BetP from *C. glutamicum*. Osmolytes are also synthesized in the cell, with enzymes BetAB converting choline to glycine betaine. OtsAB synthesizes the osmolyte trehalose from uridine diphosphate glucose (UDP-glucose) and glucose-6-phosphate. TreA is found in the periplasm and hydrolyzes trehalose to generate glucose. Courtesy of Dr. Janet Wood.
To alleviate hyperosmotic stress and prevent a buildup of potassium ions, many bacteria possess osmoregulatory transport proteins that can import solutes called osmolytes (Table 1.1) (Wood, 1999; Poolman & Glaasker, 1998; Wood, 2011a). Osmolytes are organic, uncharged or zwitterionic compounds that can be accumulated to high levels within the cell without impacting cellular functions (Wood, 1999; Poolman & Glaasker, 1998; Wood, 2011a). The mechanism of osmolyte accumulation is being analyzed through studies of three model systems, OpuA of *Lactococcus lactis*, BetP of *Corynebacterium glutamicum* and ProP of *Escherichia coli* (Wood et al., 2001). These systems are characteristically active at high osmolality and essentially inactive at low osmolality, which is contrary to the trend observed for other transport proteins, such as LacY (Figure 1.2) (Wood, 2006; Culham et al., 2008b).

### 1.2. Potential consequences of the oligomerization osmoregulatory transporters

The structural features that enable these osmoregulatory transporters to be regulated by osmotic stress are major areas of research. One possibility is that homooligomeric interactions may change in response to osmotic stress. Changes to protein-protein interactions have been known to impact protein function and can act as a method of allosteric regulation (Laskowski et al., 2009; Changeux & Christopoulos, 2016). In some cases, protein oligomerization can occur in response to the binding of an allosteric regulator, which can either enhance or inhibit the formation of protein complexes (Laskowski et al., 2009). If complex formation (or disassociation) is crucial for a protein to become active, the stimulus that impacts the quaternary structure of protein complexes contributes to the regulation of protein activity (Laskowski et al., 2009; Changeux & Christopoulos, 2016; Mason & Arndt, 2004). The functional significance of membrane protein oligomerization varies depending on the protein. For example, functional interactions between
Figure 1.2: Effect of osmolality on the activity profiles of transporter ProP and LacY in *E. coli*. The rates of proline uptake by ProP (circles) and lactose uptake by LacY (triangles) are shown as the osmolality increases. The osmoregulatory transporter ProP is activated by increasing osmolality and is inactive at low osmolality, whereas LacY is inactivated by high osmolality and is optimally active under conditions in which ProP is inactive (Culham *et al.*, 2008b). Reprinted with permission from Culham DE, Romantsov T & Wood JM (2008b) Roles of K⁺, H⁺, H₂O, and ΔѰ in solute transport mediated by major facilitator superfamily members ProP and LacY. *Biochemistry* 47: 8176-8185. Copyright 2008 American Chemical Society.

Please see Appendix C for certificate of permission.
subunits of the dimeric lactose/H$^+$ symporter, LacS in *Streptococcus thermophilus*, have been shown to play a role in substrate transport (discussed further in Introduction 1.7) (Geertsma *et al.*, 2005), whereas the functional unit of the dimeric Na$^+/H^+$ antiporter NhaA is a monomer and the quaternary structure is believed to provide structural support, rather than play a functional role (Rimon *et al.*, 2007).

Osmoregulatory transporters become activated by either directly (i.e., sensing water activity) or indirectly sensing osmolality (Wood, 1999). Cellular dehydration caused by hyperosmotic stress leads to many changes in cellular properties simultaneously, making it challenging to identify the specific stimulus sensed by osmoregulatory transporters (see Table 1.2 for list of possible stimuli) (Wood, 1999, 2015). This stimulus may cause a transition from a monomer to an oligomer (or vice versa) resulting in the activation of the transporter. In this case, it is the environmental changes caused by increasing osmolality that could essentially act as an allosteric regulator by affecting oligomerization.

The role of the oligomerization of OpuA and BetP will be discussed briefly in the sections to follow. ProP is the system of interest for this study and its structure and oligomerization will be discussed in detail. The experiments performed in this study have expanded upon the study of the oligomeric state of ProP and begun investigating the functional consequences of ProP oligomerization. There are many approaches to study the significance of protein oligomerization and different approaches can yield different results, even when studying the same protein (discussion of techniques in section 1.6). The methods used to study the role of oligomerization of OpuA and BetP have helped guide the approaches used to study ProP.
Table 1.1: Potential stimuli for activation of osmoregulatory transporters (Wood, 1999)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Stimulus detected/change in\textsuperscript{b}</th>
</tr>
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<tbody>
<tr>
<td>Periplasm</td>
<td>Thickness</td>
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<tr>
<td></td>
<td>Turgor pressure</td>
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<td></td>
<td>Concentration of a specific cosolvent</td>
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<tr>
<td></td>
<td>Macromolecular crowding</td>
</tr>
<tr>
<td></td>
<td>Osmolality</td>
</tr>
<tr>
<td></td>
<td>Ionic strength</td>
</tr>
<tr>
<td>Cytoplasmic Membrane</td>
<td>Osmolality gradient</td>
</tr>
<tr>
<td></td>
<td>Lateral pressure</td>
</tr>
<tr>
<td></td>
<td>Bilayer curvature</td>
</tr>
<tr>
<td></td>
<td>Head group charge density</td>
</tr>
<tr>
<td></td>
<td>Head group hydrogen bonding</td>
</tr>
<tr>
<td></td>
<td>Head group hydration</td>
</tr>
<tr>
<td></td>
<td>Thickness</td>
</tr>
<tr>
<td></td>
<td>Lateral phospholipid distribution</td>
</tr>
<tr>
<td></td>
<td>Intermonolayer phospholipid distribution</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Osmolality</td>
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<tr>
<td></td>
<td>Ionic strength</td>
</tr>
<tr>
<td></td>
<td>Concentration of kosmotropes</td>
</tr>
<tr>
<td></td>
<td>Concentration of a specific cosolvent</td>
</tr>
<tr>
<td></td>
<td>Macromolecular crowding or confinement</td>
</tr>
<tr>
<td>Nucleoid</td>
<td>Turgor pressure</td>
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<td></td>
<td>Counterion composition</td>
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<td></td>
<td>Protein composition</td>
</tr>
<tr>
<td></td>
<td>Macromolecular crowding</td>
</tr>
<tr>
<td></td>
<td>DNA topology</td>
</tr>
</tbody>
</table>

\textsuperscript{a}For certificate of permission see Appendix C

\textsuperscript{b}Bolded entries are properties present in both whole cells and proteoliposomes
1.3: OpuA

OpuA is an osmoregulatory glycine betaine transporter in L. lactis (van der Heide & Poolman, 2000a, 2000b). OpuA is a member of the ATP-binding-cassette (ABC) transporter family with two subunits; the first, called OpuABC, is composed of the membrane permease fused to the substrate binding domain (SBD) and the second subunit, OpuAA, consists of the nucleotide-binding domain (NBD) (Figure 1.3A, Biemans-Oldehinkel & Poolman, 2003). OpuA does not respond to ions in a specific manner and, therefore, senses cytoplasmic ionic strength (Mahmood et al., 2006; Biemans-Oldehinkel et al., 2006).

The sensor domain of OpuA is believed to be located at the C-terminal region of the NBD which possesses tandem cystathionine-β-synthase (CBS) domains followed by a short anionic tail (Biemans-Oldehinkel et al., 2006; Mahmood et al., 2006). The mechanism of activation is not conclusively established, but experiments in proteoliposomes with a defined lipid composition have shown that anionic lipids are needed for OpuA to be active and osmoregulated (Biemans-Oldehinkel et al., 2006; Mahmood et al., 2006). This observation suggested that electrostatic interactions between the CBS domains and anionic lipids may play a role in activation and inactivation of OpuA (Mahmood et al., 2006). Further study directly assessing protein-lipid binding is still required to support this hypothesis.

OpuA is a dimeric protein possessing two substrate binding and two nucleotide binding domains (Figure 1.3B, Biemans-Oldehinkel & Poolman, 2003). To determine whether both SBDs were required for OpuA to function, various OpuA heterodimers were created. Low glycerol concentrations (5%) cause the subunits of OpuA to dissociate; therefore, heterodimers can be generated by mixing two variants of purified OpuA in a low glycerol environment.
Figure 1.3: Osmoregulatory ABC-transporter OpuA from *L. lactis*. A: Organization of subunits in an OpuA monomer. The OpuABC subunit (grey) is made of the SBD and transmembrane domain (TMD). The OpuAA subunit represents the cytoplasmic NBD which possesses tandem CBS domains at the C-terminus (Karasawa *et al.*, 2011). B: Schematic of an OpuA dimer. C: Representation of an OpuA dimer with one SBD chemically blocked. D: OpuA dimer lacking one SBD (Biemans-Oldehinkel & Poolman, 2003).

Figure 1.3A: This research was originally published in *The Journal of Biological Chemistry*. Karasawa A, Erken GB, Berntsson RPA, Otten R, Schuurman-Wolters GK, Mulder FAA, Poolman B. Cystathionine β-Synthase (CBS) domains 1 and 2 fulfill different roles in ionic strength sensing of the ATP-binding cassette (ABC) transporter OpuA. *The journal of biological chemistry*. 2011; 286:37280-37291. ©The American Society for Biochemistry and Molecular Biology.

Figure 1.3B-D: See certificate of permission in Appendix C.
(Biemans-Oldehinkel & Poolman, 2003). Subunits will re-associate randomly when the glycerol concentration is raised (Biemans-Oldehinkel & Poolman, 2003). OpuA heterodimers were created that possess only one SBD (Figure 1.3D, Biemans-Oldehinkel & Poolman, 2003). Without the second SBD, the rate of transport was much lower compared to wildtype OpuA dimers with two SBDs, indicating that both SBDs are needed for optimal transport (Biemans-Oldehinkel & Poolman, 2003). The second SBD could play a direct role in transport or may only be important for structural support. OpuA dimers with two SBDs, but with one substrate binding site chemically blocked were used to determine whether the second SBD was primarily required for structural support (Figure 1.3C). Dimers with one functional and one blocked SBD had the same $V_{\text{max}}$ and $K_m$ for glycine betaine as wildtype OpuA (Biemans-Oldehinkel & Poolman, 2003). As transport was not impacted by the inability of one SBD to bind substrate, this indicated that only one SBD is needed for substrate transport, but both SBDs act cooperatively (Biemans-Oldehinkel & Poolman, 2003). The SBD without substrate bound facilitates either docking of the substrate-bound SBD to the permease or the delivery of glycine betaine to the permease (Biemans-Oldehinkel & Poolman, 2003). The effect of these structural changes to OpuA osmoregulation was not tested (Biemans-Oldehinkel & Poolman, 2003).

1.4: BetP

BetP is a trimeric osmoregulatory transporter in the Gram positive soil bacterium Corynebacterium glutamicum (Krämer & Morbach, 2004). BetP is a secondary active transporter belonging to the betaine-carnitine-choline transporter (BCCT) family and transports glycine betaine using the potential energy of the Na$^+$ gradient (Farwick et al, 1995; Krämer & Morbach, 2004; Ziegler et al, 2004). A BetP monomer has 12 transmembrane helices (TM) with extended, cytoplasmic N- and C-termini that both modulate the sensitivity of BetP to osmolality (Ziegler et
BetP is stimulated by intracellular K\(^+\), Cs\(^+\) and Rb\(^+\) levels and its activity depends on the physical state of the membrane (Rübenhagen et al., 2001; Maximov et al., 2014; Schiller et al., 2006). BetP requires the presence of anionic lipids to be active and osmoregulated (Rübenhagen et al., 2001; Maximov et al., 2014; Schiller et al., 2006). A peptide representing the C-terminus has been shown to directly interact with lipids using surface plasmon resonance spectroscopy and changing protein-lipid interactions are believed to play a role in BetP regulation (Güler et al., 2016; Ott et al., 2008).

The trimeric structure of BetP is a potential structural feature involved in BetP activation and regulation. The functional importance of BetP trimerization was probed using two different techniques with conflicting results. For the first technique, a monomeric form of BetP was created and its activity and regulation was examined (Perez et al., 2011). To generate monomeric BetP, six amino acids predicted to be crucial for trimerization were identified using molecular dynamics simulations and in silico alanine scanning (Perez et al., 2011). When two or three of the residues were replaced with alanine, the successful disruption of BetP oligomerization was illustrated using blue native polyacrylamide gel electrophoresis (BN-PAGE) (Perez et al., 2011). The monomeric form of BetP was able to transport substrate but was no longer osmoregulated as the transport rate remained constant regardless of osmolality, suggesting trimerization was needed for its regulation (Perez et al., 2011).

An alternative approach, taken by Becker et al., (2014), involved creating BetP heterotrimers with one active and two inactive subunits (Becker et al., 2014). Multiple inactive BetP variants were examined, with all variants lacking their catalytic abilities and some variants tested also lacked their regulatory capabilities (Becker et al., 2014). The BetP heterotrimers retained both the catalytic and regulatory characteristics of BetP, contradicting Perez et al., (2011) by showing that
BetP monomers can act independently within the trimer and are still osmoregulated (Becker et al, 2014). Becker et al, (2014) proposed that although BetP monomers do not require trimerization to be functional, the environment created by having adjacent BetP subunits may be favourable for transport.

1.5: ProP

ProP is a member of the major facilitator superfamily (MFS) of transporters and facilitates osmolyte uptake using the proton gradient, acting as a H⁺-osmolyte symporter (MacMillan et al, 1999). ProP has a broad substrate specificity that includes common osmolytes such as proline and glycine betaine (Murdock et al, 2014). ProP has twelve transmembrane (TM) helices with an extended C-terminus, with both the N- and C-termini being located in the cytoplasm (Wood et al, 2005).

ProP is regulated at the genetic and protein level. The proP gene is transcribed from two independent promoters, P1 and P2, which regulate proP transcription in response to osmotic stress (Figure 1.4) (Xu & Johnson, 1995). Each promoter is responsible for proP expression at a different growth stage with P1 facilitating a brief increase of proP expression during early exponential phase and P2 causing an increase in proP expression upon entry to stationary phase (Xu & Johnson, 1995). Expression of proP from promoter P2 is dependent on RpoS, a general stress response transcription factor in E. coli, and Fis, a small DNA-binding protein with growth phase dependent expression itself (Xu & Johnson, 1995). Osmotic induction of proP expression from P1 is controlled by cyclic-AMP receptor protein (CRP) (Xu & Johnson, 1995; Landis et al, 1999). The CRP-cAMP binding was found to be sensitive to osmolality and repressed proP expression from the P1 promoter at low osmolality (Landis et al, 1999). This repression was removed at high osmolality (Landis et al, 1999). To control the expression of proP in a more
Figure 1.4: Regulation of proP expression. proP is regulated by two promoters, P1 and P2, both of which are induced by high osmolarity. P2 is RpoS and Fis dependent. The two Fis binding sites and relative position of the promoters are shown (Recreated from Xu & Johnson, 1995). See Appendix C for certificate of permission.
defined manner, experiments reported here utilize a plasmid expression system based on the pBAD24 vector (Guzman et al, 1995) which eliminates the osmotic regulation of proP.

A topology and a three-dimensional homology model (PDB: 1Y8S) of ProP has been published and experimentally verified (Figure 1.5) (Wood et al, 2005; Liu et al, 2007; Keates et al, 2010). The structural model of ProP was generated primarily based on the known crystal structure of the MFS member GlpT, a glycerol-3-phosphate:phosphate antiporter and was further refined and verified based on experimental evidence (Wood et al, 2005; Culham et al, 2008a). LacZ and PhoA ProP fusions and site-directed fluorescence labelling were used to define the membrane-cytoplasm or –periplasm interface and hydrophilic loop regions (Wood et al, 2005; Liu et al, 2007; Keates et al, 2010). Cysteine-scanning and labeling with a membrane-impermeable fluorophore were used to probe the full length of transmembrane helix I (TMI) and TMXII, and supported the placement of these TM helices in the homology model (Keates et al, 2010; Liu et al, 2007).

As expected, the predicted ProP structure is similar to those of GlpT and LacY, with N- and C-terminal helical bundles composed of six TM helices each (Wood et al, 2005). The N-terminal bundle contains a cluster of polar residues deep within the membrane that are highly conserved among ProP homologues (Figure 1.5) (Keates et al, 2010). Replacements of these polar or charged residues reduced or abolished ProP activity, indicating that these residues are likely important for ProP function (Keates et al, 2010). Residues E37, R79 and E144 are essential for ProP activity as even conservative amino acid replacements severely reduced ProP activity (Keates et al, 2010).
Figure 1.5: Experimentally verified homology model of ProP (PDB: 1Y8S). The homology model was constructed based on the crystal structure of MFS member GlpT in a cytoplasmic facing conformation (Wood et al, 2005). The LacZ-PhoA fusions and site-directed fluorescence labelling were used to experimentally verify key elements of structure such as the boundaries of some TM helices. A cluster of polar residues (E37, D40, Y44, R79, K134, Q137 and E144) in the N-terminal bundle proposed to be involved in substrate transport are highlighted in green and red (Keates et al, 2010). Residues E37, R79 and E144 are essential for ProP activity (green). Figure created by Coumoundouros C using PyMOL (The PyMOL Molecular Graphics System, Version 1.5, Schrödinger, LLC).
1.5.1. Alternating Access Transport Model

ProP, like LacY and GlpT, is thought to transport substrate according to an alternating access model of transport in which the transporter alternates between cytoplasmic- and periplasmic-facing conformations (Figure 1.6) (Culham et al., 2008a; Kaback et al., 2011). The change in protein conformation causes the substrate binding site to be exposed alternately to the cytoplasm or periplasm to facilitate substrate binding and release (Culham et al., 2008a). For ProP, the conformational change could occur due to tilting of the N- and C-terminal helix bundles, or more subtle movements of loops and helices, although these scenarios require experimental validation (Liu et al., 2007; Keates et al., 2010).

An additional feature of the transport mechanism of ProP is the activation of ProP in response to increasing osmolality (Racher et al., 2001). The activation of ProP is a reversible process as the level of ProP activity depends only on the magnitude of the most recent osmotic upshift and is not affected if the transporter experiences prior osmotic stress (Racher et al., 2001; Culham et al., 2003). In vitro experiments have shown that the $V_{\text{max}}$ and $K_m$ of ProP for proline both increase as the osmolality increases and ProP is activated (Racher et al., 2001). The stimuli and structural changes that cause the transition between active and inactive ProP will be discussed below.

1.5.2. Osmosensing

ProP is thought to act as an osmosensor rather than a chemosensor, meaning that ProP does not have a binding site where a regulatory ligand could bind to activate transport (Wood, 1999). Studies of ProP activity in proteoliposomes have eliminated many potential stimuli for activation (Table 1.2, non-bold entries) by providing a system without a cell wall or other proteins and the ability to control the internal and external environment (Wood, 1999). Studies utilizing proteoliposomes have determined that ProP is activated by luminal cations (Culham et al., 2012,
Figure 1.6: Alternating access model for ProP activity and activation. The alternating access model is depicted within each box as ProP (P) binds a proton and substrate (S) sequentially and switches conformation from facing the periplasm to cytoplasm. After the substrates are released the conformation again switches. The number of protons required for transport is unknown. The transition between the inactive to active protein occurs in response to increasing osmolality in the case of ProP (Adapted from Culham et al, 2008b). Adapted with permission from Culham DE, Romantsov T & Wood JM (2008b) Roles of K⁺, H⁺, H₂O, and Δѱ in solute transport mediated by major facilitator superfamily members ProP and LacY. Biochemistry 47: 8176-8185. Copyright 2008 American Chemical Society.

Please see Appendix C for certificate of permission.
2016). This activation is not specific for particular cations and is believed to be related to the Coulombic effects of cation accumulation (Culham et al, 2012, 2016). Based on known Coulombic cation effects on DNA helix structure, it is believed the Coulombic effects exerted on ProP by cations cause a clustering of anionic charges which results in ProP activation (Culham et al, 2016). These anionic charges could be anionic residues and/or anionic head groups of phospholipids located in close proximity to ProP (Culham et al, 2016). The possibility that lipids provide the anionic charges is relevant as the cardiolipin content of E. coli cells increases with increased osmolality and anionic lipids have been seen to modulate ProP activity (Romantsov et al, 2008).

In addition to the proposed changes in anion clustering, there is evidence that the hydration state of residues in TMI and periplasmic loop 1 (PL1) also changes in response to osmotic stress (Culham et al, 2008a; Keates et al, 2010). The hydration state of residues can be detected by a change in cysteine reactivity with the reagent N-ethylmaleimide (NEM) (Culham et al, 2008a). Increased hydration of cysteine residues corresponds to the residue becoming more exposed to a polar environment. Along with reduced steric hindrance, this increases cysteine reactivity (Culham et al, 2008a). Residues in PL1 and the periplasmic half of TMI became more NEM reactive when osmotic stress was imposed, proving that ProP does undergo structural changes when transitioning from inactive to active states (Culham et al, 2008a; Keates et al, 2010).

1.5.3. Dimerization of ProP

A second structural change ProP may undergo to cause activation at high external osmolality is dimerization. ProP was predicted to homodimerize at the extended C-terminus via the formation of a coiled coil, a structure formed when the α-helical regions of the C-terminus wrap around each other (Culham et al, 1993). Coiled coils are predicted based on the presence of heptad
repeats with amino acid positions denoted as \(a\ b\ c\ d\ e\ f\ g\), with \(a\) and \(d\) being large hydrophobic residues (usually isoleucine or leucine) and \(b\ c\ e\ f\ g\) being polar or charged residues (Mason & Arndt, 2004). Each ProP possesses six heptad repeats at the C-terminus (Culham et al, 2000; Zoetewey et al, 2003; Hillar et al, 2003). A peptide replica (ProP-p) of the C-terminal region of ProP (residues 456-500) was shown to be \(\alpha\)-helical and possess the ability to form a homodimer (coiled coil), by circular dichroism (CD) spectroscopy and analytical ultracentrifugation (Culham et al, 2000; Zoetewey et al, 2003; Hillar et al, 2003).

The stability of the coiled coil formed by ProP-p was much lower compared to model peptides that are optimized for parallel coiled coil formation and stability, indicating that homodimerization of ProP-p was a weaker interaction (Culham et al, 2000; Hillar et al, 2003). Unlike what is expected in coiled coils, the first, or \(a\), residue of four of the six heptad repeats in ProP are polar (Culham et al, 2000). This should be a major source of instability for the ProP coiled coil and to confirm this, each polar residue was individually replaced by isoleucine, the residue that results in the most stable coiled coil when in the \(a\) position (Culham et al, 2000). These replacements were expected to stabilize the coiled coil, however; the R488I replacement unexpectedly destabilized this structure (Culham et al, 2000). This result provided initial evidence that the coiled coil in ProP may have an anti-parallel rather than parallel orientation as the amino acid replacements did not improve stability (Culham et al, 2000). The anti-parallel orientation of the ProP coiled coil was shown definitively when the NMR structure of a peptide consisting of the last four heptads of the ProP C-terminus (residues 468-497) was obtained (Figure 1.7) (Zoetewey et al, 2003). The solution structure of the ProP C-terminus revealed that residue R488 forms salt bridges with D475 and D478, stabilizing the coiled coil and causing it to favour the anti-parallel orientation (Zoetewey et al, 2003).
Figure 1.7: NMR structure of the anti-parallel coiled coil of ProP using a C-terminal peptide (PDB: 1R48). A peptide replica corresponding to residues 468-497 of ProP was used to obtain the NMR structure of the coiled coil (Zoetewey et al, 2003). The highlighted residues have been key in determining the structure and function of the coiled coil. Residue E480 was used in crosslinking studies as the residues are located in close proximity on the coiled coil and has also been used to generate dimeric ProP (Hillar et al, 2005). Residues R488 and I474 are structurally important residues with the amino acid replacements R488I and I474P causing orientation reversal, or disruption of the coiled coil, respectively (Culham et al, 2000; Tsatskis et al, 2008). Created by Coumoundouros C using PyMOL (The PyMOL Molecular Graphics System, Version 1.5, Schrödinger, LLC).
Two key residues, E480 and K473, of the coiled coil were used as a way to detect the orientation of the coiled coil in vivo. E480 residues are expected to be close together regardless of coiled coil orientation because it is in the middle of the coiled coil, whereas K473 residues are expected to only be close together in a parallel coiled coil (Hillar et al., 2005). This feature was exploited using chemical crosslinking to validate that coiled coil formation, and therefore dimerization of ProP, occurs in vivo. The crosslinking reagent dithiobismaleimidoethane (DTME) was used to monitor dimerization of single cysteine variants of ProP with a cysteine residue at position E480 or K473 (Hillar et al., 2005). Consistent with an anti-parallel coiled coil orientation, crosslinking was observed between E480C residues but not between K473C residues (Hillar et al., 2005). Unlike the E480C amino acid replacement which did not affect ProP activity, the K473C amino acid replacement reduced ProP activity, suggesting that residue K473 is important for ProP function or structure (Hillar et al., 2005). Overall, these results provided evidence that ProP dimerizes in vivo.

The dimerization interface in ProP was further defined using crosslinking to see if dimerization could occur between TMXIIIs of adjacent ProP monomers. Crosslinking between TMXII helices was observed when cysteine replaced residues M419, P420, Y422 and K439 in a cysteine-free ProP background (Liu et al., 2007). These residues are membrane facing according to the homology model, and are located at the periplasmic and cytoplasmic ends of TMXII, respectively (Liu et al., 2007; Wood et al., 2005). This data suggested that TMXII does participate in dimerization (Liu et al., 2007). No crosslinking was observed in the central region of TMXII, possibly due to the non-polar environment which prevents the crosslinking reaction or due to steric hindrance of the crosslinking reagent (Liu et al., 2007).
1.5.4. Functional Role of Coiled-Coil Formation and Dimerization

The function of the coiled coil domain for ProP regulation or transport is not entirely understood, but deletion of the coiled coil domain reduced ProP expression and fully inactivated ProP in vivo (Culham et al., 2000). Coiled coil formation or the α-helical structure of the C-terminus is essential for ProP activity and/or regulation as seen by the low transport activity by the ProP variant ProP-I474P, a variant with a proline substitution in the coiled coil domain that disrupts α-helix formation (Culham et al., 2000). The coiled coil itself does not cause ProP to be osmoregulated as ProP orthologues which lack the coiled coil domain, such as ProP found in Corynebacterium glutamicum, are still osmoregulated (Tsatskis et al., 2005).

The significance of the anti-parallel orientation was investigated using amino acid replacement R488I, which has been shown to cause the orientation of the coiled coil to become parallel (Hillar et al., 2005; Tsatskis et al., 2008). ProP-R488I required higher osmolalities than wildtype ProP to become active and the activation was only transient (Tsatskis et al., 2008; Culham et al., 2000). These results suggest that the coiled coil domain tunes the osmoregulatory response of ProP, enabling ProP to be activated by moderate osmolalities (Culham et al., 2000). The orientation switching of the coiled coil introduced by R488I also showed that the anti-parallel conformation may be important for sustained activity of ProP (Tsatskis et al., 2008).

The E480C amino acid replacement used for crosslinking studies does not impact ProP function itself; however, the ability to crosslink ProP*-E480C (ProP* represents cysteine-less ProP) using DTME facilitated the study of ProP in a dimeric state (Romantsov et al., 2008). Crosslinked ProP*-E80C was still osmoregulated but had increased sensitivity to osmolality and reached maximal activity at lower osmolalities compared to ProP* (Romantsov et al., 2008). It is important to note that not all ProP*-E480C is crosslinked when treated with DTME and
crosslinking locks the protein in a particular conformation which may in itself impact function, but overall this experiment suggests that C-terminal interactions and structure modulates ProP activity and activation (Romantsov et al, 2008).

1.6: Techniques used to study protein oligomerization in vivo and in vitro

The techniques discussed below facilitate either in vivo or in vitro studies of membrane protein oligomeric states, and either have been or will be used to study ProP. Previous lab members used the BACTH system to study ProP oligomerization and their unpublished results are presented below. The use of blue native–polyacrylamide gel electrophoresis (BN-PAGE) and laser induced liquid bead ion desorption mass spectrometry (LILBID-MS) to study ProP will be reported in the results sections of this thesis.

1.6.1: Bacterial adenylate cyclase two hybrid assay

The BACTH system provides a method to detect protein-protein interactions in vivo. The catalytic domain of adenylate cyclase from Bordetella pertussis can be separated into two fragments, T18 and T25, which independently are not able to associate and become active (Figure 1.8) (Karimova et al, 1998). However, if each adenylate cyclase fragment is individually fused with proteins that interact with one another, the T18 and T25 fragments will be brought spatially close together and will form a functional adenylate cyclase (Karimova et al, 1998). The active adenylate cyclase will produce cAMP, which can act as a second messenger to activate transcription of a reporter gene such as lacZ (Karimova et al, 1998). The formation of functional adenylate cyclase when T18 and T25 fusion proteins are co-expressed indicates interaction between the proteins of interest. This technique has been used to study many protein-protein interactions such as the variety of interactions between multiple Fts proteins involved in bacterial cell division (Karimova et al, 2005).
Figure 1.8: Representation of the BACTH method of detecting protein-protein interactions.

The system relies on the *B. pertussis* adenylate cyclase enzyme which converts adenosine triphosphate (ATP) to cAMP (A). Adenylate cyclase can be separated into two fragments, T25 and T18, which when separated cannot reassociate or interact and therefore cannot produce cAMP (B). If the adenylate cyclase fragments are fused to two proteins that do interact (X and Y), the T18 and T25 subunits are brought spatially close together and regain adenylate cyclase activity (C). This activity can be detected when the cAMP that is produced associates with the catabolite gene activator protein (CAP) which interacts with a cAMP/CAP dependent promoter to induce expression of a reporter gene (Karimova *et al*, 1998). Copyright (1998) National Academy of Sciences.
The BACTH was used as a second technique to study in vivo ProP-ProP interactions. Chemical crosslinking can overestimate dimerization as it locks the protein in the crosslinked form, whereas the BACTH system allows proteins to associate and dissociate freely. The T18 and T25 proteins were fused to the N-termini of ProP molecules to test for ProP dimerization in vivo. The reporter gene was lacZ and β-galactosidase activity was measured. When the N-terminal ProP fusion proteins were co-expressed, β-galactosidase activity was observed indicating that two ProP subunits interact, supporting crosslinking experiments (Figure 1.9: Wood Lab, unpublished results).

1.6.2: BN-PAGE

BN-PAGE separates native protein complexes by size, providing information about oligomeric state and complex formation (Wittig et al, 2006). The size separation of BN-PAGE is accomplished by an acrylamide gradient present in the gel (Wittig et al, 2006). The decreasing pore size in the gel will hinder the mobility of proteins once pores become too small to allow passage (Wittig et al, 2006). The negatively charged Coomassie blue G-250 dye added to all samples can bind to membrane proteins, imparting a negative charge to proteins which facilitates their electrophoresis towards the anode (Wittig et al, 2006). The absence of SDS from all components of BN-PAGE experiments causes protein complexes to remain intact and their oligomeric state to be elucidated (Wittig et al, 2006).

BN-PAGE can be used to analyze how oligomerization is impacted by detergents used for membrane solubilisation. The selection of a detergent for solubilisation of membrane proteins can impact the oligomeric state as well as other properties, such as function (Wittig et al, 2006; Seddon et al, 2004). Non-ionic detergents are most often used for studying membrane proteins as they are milder than ionic or zwitterionic detergents, maintaining more native structural
Figure 1.9: Detection of homodimerization of ProP in vivo using the BACTH system. Interactions between proteins were detected using a β-galactosidase activity assay which monitors the activity of LacZ. Coexpression of the T18 and T25 fragments (UT18C + KT25) acted as a negative control showing no interaction between adenylate cyclase fragments. The coiled coil forming sequence of the yeast transcription factor GCN4 (Zip-Zip) was used as a positive control for protein-protein interaction. The C-terminal peptide of ProP (ProPC) was tested for homodimerization, as well as for interaction with the Zip motif, but did not show evidence of an interaction. Full length ProP (ProPF) with the T18 and T25 fragments fused to the C-terminus (ProPFT18 + ProPFT25) showed no interaction, but when the adenylate cyclase fragments were fused to the N-terminus of ProPF (T80ProPF + T25ProPF), protein interaction was observed (Wood lab, unpublished results).
conformations and protein activity (Seddon et al, 2004). The two detergents of interest in regards to BN-PAGE studies with ProP are digitonin and n-dodecyl-β-D-maltoside (DDM). Digitonin is the mildest detergent and has been shown to preserve physiological protein complexes whereas DDM has stronger delipidating properties and can disrupt weaker protein-protein interactions (Wittig et al, 2006; Seddon et al, 2004). This technique was used here to determine the oligomeric state of ProP after solubilisation and to examine whether the detergent type influenced the quaternary structure of ProP.

BN-PAGE offers a quick and efficient way to examine the oligomeric states of membrane proteins. The quantities of protein required are minimal, and many different detergent concentrations and types can be screened simultaneously. BN-PAGE can be compatible with various downstream processes to identify subunits of protein complexes using mass spectrometry and 2D-PAGE systems (Wittig et al, 2006). One disadvantage is that molecular mass analysis of BN-PAGE gels can be challenging as the detergent type and binding of Coomassie dye can impact membrane protein mobility (Wittig et al, 2006). Despite these challenges, it was shown in Heuberger et al, (2002) that it is possible to perform mass analysis with BN-PAGE (Wittig et al, 2006; Heuberger et al, 2002). Heuberger et al, (2002) also showed that BN-PAGE accurately determined the mass and oligomeric state of many membrane proteins, including MFS members LacS and XylP.

1.6.3: LILBID-MS

The oligomeric state of purified membrane protein complexes and the identities of individual components of protein complexes can be determined using LILBID-MS (Morgner et al, 2007). Liquid microdroplets containing purified protein are irradiated by an infrared laser, causing samples to enter the gas phase. The masses of the ions produced by this process are analyzed
using time of flight mass spectrometry. LILBID-MS facilitates the study of protein complexes by controlling the amount of laser power applied to each sample, thereby regulating the degree of complex dissociation. At low laser intensity, large complexes will stay intact allowing for the oligomeric state to be determined by molecular weight analysis. As the laser intensity increases, complexes begin to dissociate and components of the complex will appear in the LILBID-MS spectrum individually.

LILBID-MS is a highly sensitive method to study the oligomeric state of membrane proteins in a detergent environment, only requiring small quantities of protein for analysis (Morgner et al., 2007). This technique can identify individual components of protein complexes and has even revealed preliminary evidence of lipid associations with protein complexes (Morgner et al., 2007; Brandstätter et al., 2011). LILBID-MS was used to analyze the trimeric state of the *E. coli* drug efflux pump AcrB and revealed lipid attachments associated with the protein complex, which were later identified by thin layer chromatography (Brandstätter et al., 2011).

1.7: Dominant negative analysis to study the function of protein oligomerization

To study whether the oligomeric state of proteins plays a functional role, experiments can be designed to detect a dominant negative effect in oligomers with only one active subunit. In the case of a dimeric protein, a heterodimer is a dimer composed of one active and one inactive subunit. Inactive heterodimers would indicate that the protein requires the interaction between two active subunits in order to function, a dominant negative effect. If heterodimers remain active, then the oligomeric state of the protein does not play a functional role and the subunits within a dimer are able to act independently of one another.
There are two main approaches to generate and isolate heterodimers. The first method essentially involves co-expressing the active and inactive forms of the protein and relies on the assumption that the two variants will interact with each other. If purification of heterodimers was desired, different affinity tags would need to be added to the active and inactive protein variants. This would facilitate the isolation of heterodimers by a two step purification with each step targeting one of the affinity tags. This approach was used by Becker et al, (2014) to study the role of trimerization of BetP, by isolating heterotrimers as discussed in section 1.4 (Becker et al, 2014).

An alternative approach is to covalently link the subunits of the oligomer by creating a fusion protein. This approach was used to study heterodimers of LacS, an MFS member that is a dimeric lactose/H⁺ symporter (Geertsma et al, 2005). Heterodimers of LacS were studied by creating a fusion protein with two LacS subunits connected by the addition of a peptide linker and monitoring transport in vivo (Geertsma et al, 2005). With this approach, Geertsma et al, (2005) showed a positive dominant effect in LacS heterodimers, as the active subunit rescued the activity of the inactive subunit (Geertsma et al, 2005). Overall this showed that the subunits within a LacS dimer do interact. This observation was contrary to results obtained by studying LacS heterodimers in vitro (Veenhoff et al, 2001). In this case, LacS subunits functioned independently during proton motive force driven transport (Veenhoff et al, 2001). This difference is believed to be related to the different amino acid replacements used to generate inactive LacS in both papers and the predicted role of those amino acids in transport (Geertsma et al, 2005).

The advantage of using a linker to create heterodimers is that it ensures that the protein of interest is entirely in the heteromeric form desired and facilitates easier analysis of heterodimers in vivo. Co-expression of active and inactive variants of the protein would generate all possible
combinations of subunits, making this technique most effective for in vitro studies when purification can be used to isolate the desired heteromeric form. One disadvantage of purifying heterodimers from co-expression systems is that the protein yield may be low as some of the protein produced will not be heteromeric and therefore not isolated. Although the covalent linkage ensures all protein is heteromeric, the presence of the linker could alter the conformation of the oligomeric protein which could impact activity or regulation.

1.8: Summary of work

The oligomeric state of ProP has primarily been investigated in vivo, but ProP is routinely studied in an in vitro environment which requires purification. BN-PAGE and LILBID-MS were therefore used to examine how the detergent environment impacts the oligomeric state of ProP throughout purification. Since in vivo results show that ProP can dimerize, at least some ProP was also anticipated to exist in a dimeric state in a detergent environment. BN-PAGE also provided an opportunity to investigate and compare the effects of detergent choice on ProP oligomerization by using either digitonin or DDM to solubilise membranes. Dimerization of ProP was expected to be more prevalent when digitonin was used as it is known to be a milder detergent compared to DDM.

As discussed above, the BACTH system was used as a method to detect ProP dimerization and was successful in detecting the homodimeric interaction. To ensure that the ProP variants used in this study were functional, radial streak tests were used to detect glycine betaine uptake by BACTH ProP variants. The addition of the T18 or T25 subunit to ProP was not expected to affect function; therefore, these ProP constructs were expected to be capable of glycine betaine uptake under osmotic stress.
To study whether the oligomeric state of ProP was related to function, a heterodimer approach was used in vivo and in vitro. Both approaches relied on co-expression of active and inactive ProP variants. Co-expression was preferred over adding a covalent linkage between subunits to facilitate native dimer formation and to avoid conformational changes that could be introduced when using a linker. Conformational changes would likely be an issue if using a linker to create ProP dimers as native ProP dimers have essentially a tail to tail orientation and a linker would support a tail to head conformation. As evident through discussion of the relevance of oligomerization to membrane protein function, the role of oligomerization cannot be easily predicted and can have many different effects or none at all, on function. It is therefore difficult to predict whether ProP dimerization is important for function. One possibility is that ProP could alter its oligomeric state to switch between active and inactive states.

1.9: Significance of studying protein oligomerization

The importance of quarternary structure to protein function and/or regulation is unknown for many proteins, particularly membrane proteins (Geertsma et al, 2005). The oligomeric state of a protein varies within protein families and the function of homomeric protein associations varies from increasing protein structural stability, to functional interactions between subunits affecting transport or regulation (Geertsma et al, 2005; Becker et al, 2014; Perez et al, 2011). Studying the functional role of oligomerization has the potential to provide insights into the regulation and transport mechanism of membrane proteins.
Chapter 2: Materials and Methods

2.1: Materials

All products and chemicals were purchased from Fisher Scientific (Ottawa, Ontario, Canada) or Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise indicated. All experiments were replicated at least twice.

2.2: Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2. All strains were stored as glycerol stocks composed of overnight bacterial culture grown in LB (lysogeny broth, 1 mL) combined with glycerol (0.5 mL, 80% (v/v) solution) and were stored at -40°C. Bacterial cultures were grown using either LB or NaCl-free MOPS medium. The LB was composed of NaCl (1% (w/v)), Bacto-tryptone (1% (w/v)) and Bacto-yeast extract (0.5% (w/v)) (Miller, 1972). NaCl-free MOPS was prepared as described in Neidhardt et al, (1974) with the omission of NaCl. The NaCl-free MOPS was supplemented with ammonium chloride (9.5 mM), glycerol (0.4% (v/v)), potassium phosphate dibasic (1.32 mM), L-tryptophan (0.005% (w/v)) and thiamine hydrochloride (0.0001% (w/v)). If catabolite repression of proP expression from the lac operon was desired, the glycerol in the supplemented NaCl-free MOPS medium was replaced with D-glucose (0.2% (w/v)) (Inada et al, 1996). All cultures were grown at 37°C with shaking at 200 rpm unless otherwise specified.

The osmolality of supplemented NaCl-free MOPS medium was adjusted to the desired level for each experiment using NaCl. The osmolality of MOPS media was measured using the VAPRO Vapor Pressure Osmometer 5520 (Wescor (Claremont, Ontario, Canada)). To maintain any plasmids in E. coli strains, ampicillin (Amp, 100 μg/mL) or kanamycin (Km, 50 μg/mL) was
Table 2.1: Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSH4</td>
<td>F trp lacZ rpsL thi</td>
<td>Cold Spring Harbour Laboratories</td>
</tr>
<tr>
<td>DH5α</td>
<td>F φ80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdRI7(rK mK) supE44k thi-1 gyrA recA1</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td>WG210</td>
<td>CSH4 Δ(putPA)101 proU205</td>
<td>Grothe et al, 1986</td>
</tr>
<tr>
<td>WG350</td>
<td>F trp lacZ rpsL thi Δ(putPA)101 ΔproU600 Δ(proP-melAB)212</td>
<td>Culham et al, 1993</td>
</tr>
<tr>
<td>WG708</td>
<td>WG350 pBAD24</td>
<td>Racher et al, 1999</td>
</tr>
<tr>
<td>WG709</td>
<td>WG350 pDC79</td>
<td>Culham et al, 2000</td>
</tr>
<tr>
<td>WG710</td>
<td>WG350 pDC80</td>
<td>Racher et al, 2001</td>
</tr>
<tr>
<td>WG986</td>
<td>WG350 pDC224</td>
<td>Keates et al, 2010</td>
</tr>
<tr>
<td>WG1011</td>
<td>WG350 pDC235</td>
<td>Keates et al, 2010</td>
</tr>
<tr>
<td>WG1368a</td>
<td>WG350 pNS2</td>
<td>Sahtout, 2013</td>
</tr>
<tr>
<td>WG1477</td>
<td>WG210 pDC79</td>
<td>This study</td>
</tr>
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</tr>
<tr>
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<td>WG350 pT18ProP</td>
<td>Wood Lab</td>
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</tr>
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</table>

a E. coli strain WG1368 requires 34 μM of L-arabinose induction to achieve comparable expression levels of ProP-E480C compared to plasmid encoded ProP (Sahtout, 2013).
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD24</td>
<td>Expression vector</td>
<td>Guzman <em>et al</em>, 1995</td>
</tr>
<tr>
<td>pDC79</td>
<td>Encodes ProP</td>
<td>Culham <em>et al</em>, 2000</td>
</tr>
<tr>
<td>pDC80</td>
<td>Encodes ProP-His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Racher <em>et al</em>, 2001</td>
</tr>
<tr>
<td>pDC224</td>
<td>Encodes ProP-E37V</td>
<td>Keates <em>et al</em>, 2010</td>
</tr>
<tr>
<td>pDC235</td>
<td>Encodes ProP-E37Q</td>
<td>Keates <em>et al</em>, 2010</td>
</tr>
<tr>
<td>pDC324</td>
<td>Encodes Flag-ProP and His&lt;sub&gt;6&lt;/sub&gt;-ProP</td>
<td>Culham, D.E</td>
</tr>
<tr>
<td>pNS2</td>
<td>Encodes ProP-E480C</td>
<td>Sahtout, 2013</td>
</tr>
<tr>
<td>pT18ProP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Encodes T18-ProP</td>
<td>Culham, D.E</td>
</tr>
<tr>
<td>pT25ProP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Encodes T25-ProP</td>
<td>Culham, D.E</td>
</tr>
</tbody>
</table>

<sup>a</sup> All plasmids are based on vector pBAD24 unless otherwise specified (Guzman *et al*, 1995).

<sup>b</sup> Plasmid pT18ProP is based on vector pUT18C (Karimova *et al*, 2001).

<sup>c</sup> Plasmid pT25ProP is based on vector pKT25 (Karimova *et al*, 2001).
added to growth media. Expression of \textit{proP} from plasmid-based expression systems was controlled using either L-arabinose (pBAD24-based plasmids) or isopropyl β-D-1-thiogalactopyranoside (IPTG, plasmids pT18ProP and pT25ProP).

The bacterial phenotypes of all strains used in this study were routinely analyzed using plate-based strain tests. Solid media were prepared by adding agar (15 g/L) to the appropriate liquid media. To test for antibiotic resistance, one of the following antibiotics was added to LB medium: Km (50 μg/mL), tetracycline (Tet, 25 μg/mL), streptomycin (Strep, 100 μg/mL), chloramphenicol (Cm, 30 μg/mL) or Amp (50 μg/mL). Plates containing ampicillin or tetracycline were stored at 4°C. Tetracycline is light sensitive; therefore plates containing tetracycline were stored in the dark. MacConkey agar plates containing MacConkey agar (5% (w/v), (Becton, Dickson and Company, Franklin Lake, New Jersey, USA)) were used to test for lactose utilization (MacConkey, 1905). TTC (2,3,5-triphenyltetrazolium chloride) plates tested for utilization of proline (Bochner & Savageau, 1977). The TTC plates contained potassium phosphate dibasic (0.402 M), potassium phosphate monobasic (0.022 M), proteose peptone (0.2% (w/v)), magnesium sulfate (0.00125 M), L-tryptophan (0.005% (w/v)), thiamine hydrochloride (0.0001% (w/v)), TTC (0.0025% (w/v)) and L-proline (0.2% (w/v)).

The NaCl-free MOPS medium based plates were prepared either with (GNTB\textsubscript{1}) or without (GNB\textsubscript{1}) L-tryptophan (0.005% (w/v)) to test for tryptophan auxotrophy. Radial streak tests using GNTB\textsubscript{1} plates with the addition of 0.6 M NaCl were used to test for activity of osmoregulatory transporters in the presence of osmotic stress (Murdock \textit{et al}, 2014). A filter disc was placed in the center of these plates and 100 mM glycine betaine (20 μL) was applied to the disc. Bacterial cultures (1 mL), grown in LB overnight as described above, were washed twice with 0.85% (w/v) NaCl (1 mL) followed by resuspension in 0.85% (w/v) NaCl (0.5 mL). The cell
suspensions were streaked onto the plate as a line from the center to the edge of the plate. When required for plasmid maintenance or induction of protein expression, antibiotics or IPTG were spread on the plates before use.

2.3: Bacterial strain construction

Plasmid DNA was isolated from bacteria using the High-Speed Plasmid Kit (FroggaBio (Toronto, Ontario, Canada)). Restriction endonuclease digestion of plasmid DNA and subsequent analysis using agarose gel electrophoresis was performed as described by Sambrook & Russell, (2001). These techniques were used to confirm the identity of plasmids used in this study. The Invitrogen 1 kb Plus DNA ladder (Burlington, Ontario, Canada) was included in all agarose gels as a reference. Strains constructed for this study were created by transformation of plasmid DNA into the desired strain of *E. coli* following the Hanahan transformation procedure (Hanahan, 1983). Amplification of genes by polymerase chain reaction (PCR) was carried out using the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer (Woodbridge, Ontario, Canada)) as outlined in Brown & Wood, (1992). PCR was used to verify the E480C replacement in ProP by amplifying a portion of ProP and digesting the amplicon with *BclII*. This was necessary as digestion of the entire plasmid cannot differentiate ProP and ProP-E480C due to DNA methylation. The primers used for PCR were AB4451 (5’- GCT ACT TGG TAG TGT TGC CC-3’) and JK2 (5’- CGC AAG CTT TTA TTC ATC AAT TCG CGG-3’).

2.4: Preparation of cell suspensions

The preparation of whole cell suspensions will be described briefly here and is also described in Culham *et al.*, (2003). LB (2 mL) was inoculated using a glycerol stock of *E. coli* and incubated for approximately 7 hours. The resulting culture was used to inoculate 24 mL of supplemented NaCl-free MOPS medium (1% (v/v)) and was incubated for 16-18 hours. The optical density at
600 nm (OD) of overnight cultures was found using the Novaspec II UV/Vis spectrophotometer. Bacteria from overnight cultures were subcultured into fresh supplemented NaCl-free MOPS medium (24 mL) to obtain an OD of 0.17. The volume of overnight culture needed to achieve this starting OD was centrifuged at 5700 rpm for 10 min at room temperature in a Beckman Coulter Allegra X-22 centrifuge. Cell pellets were resuspended in the fresh medium and grown until an OD of 0.4 was reached. If induced expression of plasmid encoded genes was desired, the appropriate inducer was included to achieve the desired concentration in the fresh growth medium. Once cultures reached the desired OD of 0.4, cells were harvested as described above, washed twice with unsupplemented NaCl-free MOPS medium and were finally resuspended in unsupplemented NaCl-free MOPS medium (1 mL). Unsupplemented NaCl-free MOPS medium is composed of NaCl-free MOPS medium lacking all organic supplements, but does contain 35 mM NaCl to adjust the osmolality to the same level as the supplemented medium. If needed, cell suspensions were stored at -40°C for later use. Overnight cultures were also used for plate-based strain testing and, if needed, radial streak tests.

2.5: Preparation of bacterial membranes

Bacterial membranes were prepared on a small scale using a water lysis procedure or on a larger scale by lysing cells with a French press.

2.5.1: Water lysis membrane isolation

To prepare water lysis membranes, bacterial cultures were prepared as described in Section 2.4. Once the bacterial cultures (24 mL) reached an OD of 0.4 and cells were harvested, the pellet was resuspended in 2 mL of lysis buffer containing Tris-HCl (10 mM, pH 7.5), ethylenediaminetetraacetic acid (EDTA, 5 mM, pH 8.0), phenylmethylsulfonyl fluoride (PMSF, 1 mM), egg-white lysozyme (0.3 mg/mL) and bovine pancreatic DNase A (0.04 mg/mL).
Samples were incubated with gentle shaking at room temperature for 15 min, after which they were rapidly diluted with ice cold water (18 mL). Membranes were harvested by centrifugation at 16000 rpm for 20 min at 4°C (Beckman Coulter Avanti J20-XPI Centrifuge). Membranes were washed twice with ice cold water, the final pellet was resuspended in 100 μL of ice cold water, and the suspension was stored at -40°C.

2.5.2: Large scale membrane isolation

The *E. coli* membranes were isolated from large scale cultures as described in Racher *et al.*, (1999). The LB medium (5 mL) was supplemented with Amp (100 μg/mL) and inoculated with the *E. coli* strain WG710 which expresses ProP-His\(_6\). Cultures were incubated for approximately 7.5 hours and were then subcultured (2% (v/v)) into fresh LB medium (150 mL) supplemented with 50 μg/mL Amp. After 16-18 hours of growth, the bacteria was subcultured (2% (v/v)) into fresh LB (6 L) with Amp (50 μg/mL) and were grown to an OD of 0.22. L-arabinose was added to a final concentration of 0.2% (w/v) and the culture was incubated for an additional 2 hours. Cells were harvested by centrifugation in the Beckman Coulter Avanti J20-XPI at 7500 rpm for 20 min at 4°C. Pellets were washed in 100 mM potassium phosphate pH 7.4 (roughly 400 mL), cells were harvested again by centrifugation at 7500 rpm for 20 min at 4°C and the cell pellet was weighed and stored at -40°C. To lyse cells, the cell pellet was resuspended in 5 mL of lysis buffer/g wet weight of pellet. The lysis buffer was composed of postassium phosphate (100 mM pH 7.4) with MgSO\(_4\) (5 mM), DNase I (0.03 mg/mL) and PMSF (0.5 mM). Cells were lysed by passage through a French Press cell at 10000 psi twice. Cellular debris was removed by centrifugation at 10000 rpm for 20 min at 4°C. Membranes were isolated from the supernatant by centrifugation at 42000 rpm for 60 min at 8°C (Beckman Coulter Optima L-100XP Ultracentrifuge). The membrane pellet was homogenized in 1mL of 100 mM potassium
phosphate pH 7.4 for each liter of culture used. Membranes were stored as 1 mL aliquots in liquid nitrogen.

2.6: In vivo crosslinking

Bacterial proteins were crosslinked in vivo using dithiobismaleimidoethane (DTME, Thermo Fisher Scientific) to crosslink cysteine residues and 2-aminoethylmethanethiosulfonate hydrobromide (MTSEA, Toronto Research Chemicals) to block any remaining free cysteine residues after DTME treatment, as described in Hillar et al., (2005). Stock solutions of DTME (64 mM) and MTSEA (30 mM) were prepared in N, N-dimethylformamide (DMF) and were stored at -40°C. Cultures were prepared as described in Section 2.4. At an OD of 0.4, DTME (0.4 mM) was added and the culture was further incubated for 10 min at 37°C with shaking at 200 rpm. This was followed by treatment of the same culture with MTSEA (1 mM) for 10 min at 37°C with shaking. As a negative control for crosslinking experiments, the treatments were performed with DMF. Following the crosslinking treatment, water lysis membranes were prepared as described in Section 2.5.1.

2.7: Protein assay

To quantify the amount of protein in cell extracts or membranes the bicinchoninic acid (BCA) assay was performed as described in Redinbaugh & Turley, (1986) and Smith et al., (1985) using Pierce BCA Protein Assay Reagent A and B. Bovine serum albumin (BSA) was used as a standard and samples were analyzed in 96-well microtiter plates. The absorbance at 562 nm was measured using a Titertek Multiskan PLUS MK II microplate reader.
2.8: Whole cell transport assay

Transport assays were conducted using fresh cell suspensions prepared as described in section 2.4. Initial rates of proline uptake were measured as described in Milner et al, (1988) and Culham et al, (2003). Assays monitored the uptake of L-[\(^{14}\)C(U)] proline (250 mCi/mmol, Perkin Elmer (Waltham, Massachusetts, USA)). Assay media contained the same components as unsupplemented NaCl-free MOPS medium with the addition of D-glucose (10 mM), chloramphenicol (0.09 μg/mL) and NaCl to adjust the osmolality to the desired level. The addition of chloramphenicol prevents the synthesis of new protein during the assay (Rendi & Ochoa, 1962).

Cell suspensions (25 μL) were added to assay buffer (455 μL) and were incubated for 3 min at 25°C with shaking. The addition of 200 μM of L-[\(^{14}\)C] proline (5 mM stock at 5 μCi/μmol) initiated transport. At 20, 40 and 60 seconds after addition of radiolabelled substrate, the reaction mixture (150 μL) was dispensed onto 0.45 μm filters (Millipore). The filter was immediately washed with unsupplemented NaCl-free MOPS medium (5 mL) adjusted with NaCl to have the same osmolality as the assay medium. Filters were placed in 20 mL super polyethylene scintillation vials (Perkin Elmer) and were dried under a heat lamp. Scintanalyzed Xylene containing 0.005% (w/v) 2, 5-diphenyloxazole (5 mL) was added to each vial and counts per minute (cpm) were measured using the Perkin Elmer Tri-Carb 2910 TR Liquid Scintillation Counter (Program 4, Nucleotide: 14C, Quench Indicator: SIS, Pre-count delay: 0 minutes, Count time: 5 minutes, Count mode: High sensitivity). Within each assay, each time point was replicated in triplicate and all assays were repeated at least twice.

The specific activity of the L-[\(^{14}\)C] proline stock used for transport assays was determined by applying 2 μL of the stock solution to a 0.45 μm filter, which was dried and scintanalyzed.
Xylenes containing 2, 5-diphenyloxazole (5 mL) were added. This was performed in triplicate and the average cpm value was divided by the nmol proline (10 nmol) to obtain the specific radioactivity of the stock solution.

A BCA assay was used to determine the protein concentration in cell samples used for each transport assay. The equation shown below was used to determine the nmole proline/mg protein. The total volume of the assay mixture was 500 μL and the 150 μL represents the volume of the assay mixture applied to each filter.

\[
\frac{\text{nmole proline}}{\text{mg protein}} = \frac{\text{cpm of } ^{14}\text{C proline stock}}{\text{nmole}} \times \frac{500 \mu\text{L}}{150 \mu\text{L} \times \text{cpm of sample}} \quad (1)
\]

Linear regression was used to determine the initial rate of proline uptake in nmole proline/mg protein for each transport assay. Within one experiment, each time course was replicated in triplicate resulting in 3 rates to be calculated. The mean of the 3 rates corresponds to the uptake rate in nmole proline/mg protein/minute. The standard error was calculated for each triplicate.

Raw data were analyzed using Microsoft Excel and the uptake rates were further analyzed using SigmaPlot for linear or non-linear regression. Non-linear regression was carried out using the sigmoidal 3 parameter function in SigmaPlot which fits data to equation 2 shown below (Culham et al, 2003). Y represents the proline uptake rate and \( Y_{\text{max}} \) is the maximum rate obtained when medium osmolality is high. The osmolality of the transport assay medium is represented by \( x \) and \( x_0 \) is the osmolality at which the uptake rate is half maximal. The final parameter, b, is a constant which is inversely related to the slope of the sigmoidal curve.

\[
Y = \frac{Y_{\text{max}}}{1+e^{-\left(\frac{x-x_0}{b}\right)}} \quad (2)
\]
2.9: Gel electrophoresis

The mobilities of proteins were analyzed under the denaturing conditions of sodium dodecyl sulfate polyacrylamide gel electrophoresis or under native conditions using blue-native polyacrylamide gel electrophoresis.

2.9.1: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli, (1970) using the BioRad Mini-PROTEAN system (Mississauga, Ontario). All SDS-PAGE gels were prepared using a stock solution of 30% acrylamide with an acrylamide: bis-acrylamide ratio of 37.5:1 (BioRad). The separating gel was composed of Tris-HCl (0.375 M, pH 8.8), SDS (0.1% (w/v)), acrylamide (12% (v/v)), ammonium persulfate (APS, 0.05% (v/v)) and tetramethylethylenediamine (TEMED, 0.05% (v/v)). The stacking gel was composed of Tris-HCl (0.1 M, pH 6.8), SDS (0.08% (w/v)), acrylamide (3.75% (v/v)), APS (0.05% (v/v)) and TEMED (0.1% (v/v)). The running buffer contained Tris-HCl (25 mM), glycine (192 mM) and SDS (0.1% (w/v)).

Samples were prepared for SDS-PAGE analysis by adding sample buffer (0.3 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.125% (w/v) bromophenol blue and 25% (v/v) β-mercaptoethanol) to each sample (1:5 buffer to sample volume ratio) and incubating at 37°C for 30 min. Cells and membranes were solubilised by passing the sample through a tuberculin syringe (1 mL, 26 gauge, 3/8 inches) 15 times, then centrifuged briefly at 13000 rpm to sediment unsolubilised material. A volume of each sample corresponding to a desired protein amount was analyzed using SDS-PAGE gels developed at 150V. Blueye prestained protein ladder (FroggaBio) was used as a reference for all SDS-PAGE gels. When needed as a positive control for Western blot analysis, 15 ng of purified ProP-His$_6$ (purified by DE Culham) was loaded on
SDS-PAGE gels. Gels were either stained using Gelcode Blue (Pierce) or protein was transferred for Western blotting as described in section 2.10.

2.9.2: Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

BN-PAGE was performed as described in Wittig et al, (2006) using precast 4-15% gradient acrylamide gels (Mini-PROTEAN TGX-gels, Bio-Rad) and buffers listed in Table 2.4. Aliquots containing 400 μg of water lysis membranes were centrifuged (20 min, 13000 rpm, 4°C) and the membranes were resuspended in solubilisation buffer A (40 μL, Table 2.3). Detergent was added as outlined below and samples were left to solubilise for 10 min at room temperature. Samples were solubilised using either n-dodecyl-β-D-maltoside (DDM, Anatrace (Maumee, Ohio, USA)) with a detergent: protein ratio of 0.5, 1 or 2 g/g, or digitonin (Millipore) with a ratio of 2, 4 or 8 g/g. After incubation, samples were centrifuged at 13000 rpm for 20 min at 4°C and the supernatant was transferred to a fresh tube. Glycerol (5 μL, 50% (v/v)) was added to each sample followed by Coomassie Blue G-250 to obtain a detergent: dye ratio of 8 g/g. Coomassie blue G-250 was prepared as a 5% (w/v) stock suspended in 500 mM 6-aminohexanoic acid and stored at 4°C.

Cathode buffer B (Table 2.3) was added to the inner chamber of the gel running chamber (BioRad MiniPROTEAN Tetra cell system) and anode buffer was added to the outer chamber. Each sample (50 μg of protein) was loaded to the BN-PAGE gel which was developed at 8 mAmp at 4°C. Once samples had entered approximately one third of the gel, cathode buffer B was removed and replaced with cathode buffer B/10 to reduce the amount of Coomassie Blue dye in the gel as it can compete with protein for binding to polyvinylidene fluoride membranes during Western blotting. Electrophoresis was stopped when the dye front reached the bottom of
Table 2.3: BN-PAGE buffer composition (Wittig et al, 2006).

<table>
<thead>
<tr>
<th></th>
<th>Cathode Buffer B</th>
<th>Cathode Buffer B/10</th>
<th>Anode Buffer</th>
<th>Solubilization Buffer A</th>
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<td>Sodium Chloride (mM)</td>
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<tr>
<td>Tricine (mM)</td>
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<td>Imidazole (mM)</td>
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<tr>
<td>Coomassie blue G250 (%)</td>
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<td>0.002</td>
<td>—</td>
<td>—</td>
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<tr>
<td>EDTA (mM)</td>
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<td>pH</td>
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<td>Storage Temperature (°C)</td>
<td>Room Temperature</td>
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</tr>
</tbody>
</table>
the gel. The BN-PAGE gel was washed twice with water prior to proceeding with Western blotting procedures

2.10 Purification of rabbit anti-ProP antibodies

A crude anti-ProP serum was isolated as outlined in Racher et al., (1999). The crude serum reacts with ProP as well as other *E. coli* proteins; therefore, a second purification step was performed to isolate antibodies that react selectively with ProP (performed by Culham, D.E).

Purified ProP-His<sub>6</sub> was obtained by affinity chromatography as described in section 2.12.2. The purified protein (obtained using 100 mg of *E. coli* membrane protein) was dialyzed into coupling buffer (0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl, 0.04% (w/v) DDM, pH 8.3) using 12-14 kDa cutoff dialysis tubing (Spectrum Labs (Houston, Texas, USA)) with three buffer changes over a roughly 18 hour period at 4°C. Cyanogen bromide-activated sepharose 4B (0.5 g, GE Healthcare (Mississauga, Ontario, Canada)) was washed by filtration with 1 mM HCl (100 mL) followed by a final wash in coupling buffer (2 mL). Washed resin was resuspended in coupling buffer (4 mL) and added to dialyzed ProP-His<sub>6</sub>. This mixture was incubated with gentle mixing for 1 hour at room temperature. The resin was isolated, washed with coupling buffer (10 mL), resuspended in blocking buffer (10 mL, 0.1 M Tris-HCl pH 8.0) and gently mixed for 2 hours at 4°C. Following isolation of the resin, the resin was washed with wash buffer 1 (10 mL, 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0) and wash buffer 2 (10 mL, 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0) and these washes were repeated twice. The resin was further washed with binding buffer (15 mL, 10 mM Tris-HCl pH 7.5) and was finally resuspended in 5 mL of binding buffer.

The washed Sepharose resin was incubated with crude anti-ProP serum (0.5 mL) overnight at 4°C with gentle mixing. The following washes were performed using a chromatography column.
(10 mL volume, 1 cm diameter). The resin was washed with binding buffer (10 mL) and binding buffer with 0.5 M NaCl (10 mL). Purified anti-ProP antibodies were eluted using elution buffer (0.1 M glycine-HCl, pH 2.5) as 10 aliquots (0.5 mL each) into tubes containing neutralization buffer (50 μL, 1 M Tris-Cl, pH 8.0). Eluted anti-ProP antibodies were pooled and dialyzed against phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.4) using 50 kDa cutoff dialysis tubing (Spectrum Labs). The dialyzed antibodies were stored at -40°C until use.

2.11: Western blotting

Transfer of proteins from SDS-PAGE and BN-PAGE gels was carried out as described by Towbin et al, (1979) and Wittig et al, (2006) respectively. Proteins were transferred from SDS-PAGE and BN-PAGE gels to nitrocellulose (0.45 μm, BioRad) or polyvinylidene fluoride (PVDF 0.2 μm, BioRad) membrane, respectively. The PVDF membranes were first activated by soaking in methanol before use. Transfer buffer (16 mM Tris-HCl, 115 mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol) was used in a wet transfer system and proceeded at 80V for 45 min on ice. After the transfer was completed, PVDF membranes were destained in methanol. After this step, both PVDF and nitrocellulose membranes were treated identically.

Anti-ProP

Membranes were rinsed with 10 mL TBS (Tris-buffered saline, 25 mM Tris-HCl, 140 mM NaCl, 0.27 mM KCl) and then were blocked overnight in TBS with 0.1% (v/v) Triton X-100 (TBS-Triton, 25 mL) and skim milk (4% (w/v)). After blocking, the membrane was rinsed once with TBS-Triton and incubated with purified rabbit anti-ProP antibodies prepared as described in section 2.10. Anti-ProP antibodies (60 μL) were diluted in 20 mL TBS-Triton with skim milk
(4% (w/v)) and incubated with the membrane overnight. The membrane was then washed for 15 min in 200 mL of TBS-Triton followed by three, 5 min rinses in 30 mL of TBS-Triton. Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (Sigma Aldrich) were diluted 1:10000 times in TBS-Triton and incubated with the membrane for 1 hour. The washes described above were repeated and the signals were developed using ECL Prime Western Blot Detection Reagent (GE Healthcare). Blots were imaged using the BioRad GelDoc XRS+ or using Amersham Hyperfilm (GE Healthcare) and the Konica Minolta XRS-101A film developer. Densitometry analysis was carried out using ImageLab software (BioRad).

Anti-His

To detect the 6×-His tag, the Penta-His HRP Conjugate Kit (Qiagen, Toronto, Ontario) was used following protocol 7 from the QIAexpress Detection and Assay handbook (Qiagen). Membranes were rinsed with TBS and were blocked overnight in the provided 1x blocking solution with Tween-20 (0.1% (v/v)). After blocking, the membrane was washed twice for 10 min in 30 mL TBS-Tween-Triton (TBS with 0.05% (v/v) Tween-20 and 0.2% (v/v) Triton X-100), and once for 10 min in 25 mL TBS. The Anti-His HRP conjugate was diluted 1:5000 in 1x blocking solution with Tween-20 (0.1% (v/v)) and was incubated with the membrane for 1 hour. Wash steps were repeated and membrane was developed and signals were detected as described for anti-ProP Western blots.

Anti-Flag

For detection of a Flag tag, membranes were rinsed with PBS (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.8 mM KH2PO4, pH 7.4) and blocked in PBS with skim milk (5% (w/v)) for 1 hour. The membrane was washed 3 times for 5 min in PBS with
0.05% (v/v) Tween-20 (PBS-Tween) followed by a 2 hour incubation with 1.5 μg/mL rabbit anti-Flag antibodies (Sigma-Aldrich, product number F7425) diluted in PBS with BSA (1% (w/v)). The membrane was washed 3 times for 5 min in PBS-Tween, and goat anti-rabbit HRP conjugated secondary antibody was incubated with the membrane at a 1:10000 dilution in PBS-Tween for 1 hour. The membrane was washed as before, and was developed and analyzed as described for anti-ProP Western blots.

2.12: Protein purification

ProP was purified using affinity chromatography for both analytical and quantitative analyses.

2.12.1: Analytical scale protein purification

All analytical scale purifications were performed using Micro Biospin chromatography columns (BioRad). These columns were centrifuged at 4600 rpm for 20 second intervals to separate the resin from the buffer.

Ni-NTA resin

Water lysis membranes isolated from 36 mL of culture were resuspended in 950 μL of solubilisation buffer composed of potassium phosphate (100 mM, pH 8.0), NaCl (300 mM) with DDM (1% (w/v)). Samples were rotated at 4°C for 30 min after which they were centrifuged at 13000 rpm at 4°C for 20 min. The supernatant was applied to His-Pur Ni-NTA resin (100 μL packed), which had been washed with buffer 1 (1.5 mL, 100 mM potassium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.04% (w/v) DDM). The supernatant and resin were incubated together for approximately 16 hours at 4°C with rotation. The resin was isolated by centrifugation as described above and was washed with buffer 1 (1.5 mL). The resin was washed with buffer 2 (1.5 mL, 100 mM potassium phosphate pH 8.0, 300 mM NaCl, 30 mM imidazole,
0.04% (w/v) DDM). Protein was then eluted using elution buffer (100 μL, 100 mM potassium phosphate pH 7.4, 300 mM NaCl, 200 mM imidazole, 0.04% (w/v) DDM) which was incubated with the resin for 5 min at 4°C prior to centrifugation.

**Anti-Flag resin**

Water lysis membranes were solubilised and the solubilised membrane protein was isolated as described above. The supernatant was mixed with anti-Flag M2 Affinity Gel (50 μL packed) which had been washed with 1.5 mL of the same buffer used for solubilisation with 0.04% (w/v) DDM. This mixture was incubated for approximately 16 hours at 4°C with rotation. The resin was isolated by centrifugation and was washed with 3 mL of solubilisation buffer with 0.04% (w/v) DDM. Flag-tagged protein was eluted by incubating resin with glycine HCl (100 μL, 0.1 M, pH 3.5) for 5 min at room temperature followed by centrifugation. The protein was eluted directly into potassium phosphate (10 μL, 0.5 M, pH 7.4) with 0.04% (w/v) DDM to immediately neutralize the pH.

**2.12.2: Large scale purification of His-tagged ProP**

Membranes used for a large scale purification of ProP-His$_6$ were obtained as described in section 2.5.2. ProP-His$_6$ was purified as described below and in Racher *et al.* (1999). Roughly 50 mg of membrane protein was solubilised in a 10 mL solution of potassium phosphate (100 mM, pH 8.0) with 1% (w/v) DDM (detergent: protein ratio of 1 g/g) with rotation at 4°C for 30 minutes. Non-solubilised components were separated by centrifugation at 46100 rpm for 41 min at 4°C (Beckman Coulter Optima L-100XP Ultracentrifuge). The Ni-NTA resin (1 mL packed) was washed with at least 10 column volumes (CV) of water, followed by another wash with 10-20 CV of buffer 1 (100 mM potassium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.04%
(w/v) DDM). The isolated supernatant was incubated with the washed resin for 1 hour at 4°C. The resin and supernatant mixture was transferred to a 10 mL disposable column (Biorad) and the flow-through was collected. The resin was washed with 20 CV of buffer 2 (100 mM potassium phosphate pH 8.0, 300 mM NaCl, 30 mM imidazole, 0.04% (w/v) DDM). His-tagged ProP was eluted using 10 mL of elution buffer (100 mM potassium phosphate pH 7.4, 300 mM NaCl, 200 mM imidazole, 0.04% (w/v) DDM).

**Gel Filtration Chromatography**

To prepare affinity chromatography purified ProP-His$_6$ for gel filtration, a buffer exchange was conducted. Purified ProP-His$_6$ was concentrated to a volume of 2 mL using Vivaspin turbo concentrators with a 100 kDa cut-off by centrifugation at 3000 rpm at 4°C (5810R Eppendorf Centrifuge). At least 10 mL of gel filtration buffer (100 mM potassium phosphate pH 7.4, 0.04% (w/v) DDM) was added to the concentrated protein and the sample was again concentrated to 2 mL. This procedure was repeated a second time and the final sample was concentrated to a volume of approximately 1 mL.

Gel filtration was performed using a Superdex 200 10/300 GL gel filtration column (GE Healthcare) with the UPC 900 ÄKTA system (GE Healthcare) and Unicorn 4.12 software. All buffers used for gel filtration were filter sterilized and were stored at 4°C. The column was prepared by washing with of water (2 CV) and gel filtration buffer (2 CV). The protein sample was injected into the column and 0.5 mL fractions were collected. The absorbance at 280 nm was determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific) and the concentration of purified protein was calculated using an extinction coefficient for ProP-His$_6$ of
64080 M/cm (Gasteiger et al, 2005). For long term storage of purified protein, glycerol (20% (v/v)) was added to samples prior to freezing at -40°C.

2.13: Laser induced liquid bead ion desorption mass spectrometry (LILBID-MS)

Samples of protein purified using affinity chromatography followed by gel filtration with a concentration between 9.5-11.5 μM were used for LILBID-MS analysis. All analyses were performed by Nils Hellwig in Dr. Nina Morgner’s laboratory at the Goethe University in Frankfurt am Main, Germany. Buffer exchange was carried out using Zeba spin desalting columns with a 7 kDa molecular weight cut-off and samples were analyzed in either Tris HCl (20 mM, pH 9) with 0.02% (w/v) DDM or ammonium acetate (100 mM) with 0.03% (w/v) DDM. The sample (5 μL) was injected into the LILBID-MS system. The experimental set up of LILBID-MS is described in Morgner et al, (2007). Data was recorded using a user-written labview based program (written by Dr. Nina Morgner) and data was processed and analyzed using Massign (Morgner & Robinson, 2012).
Chapter 3: The Oligomeric State of ProP In Vivo and In Vitro and the Heterodimer Approach to Functional Studies

The oligomeric state of ProP has primarily been studied in vivo using crosslinking and the BACTH system, with both techniques providing evidence of ProP dimerization. The ProP fusion proteins used with the BACTH system were checked for functionality to ensure that the addition of the adenylate cyclase did not prevent membrane insertion of ProP or cause it to become inactive. Knowing that ProP dimerizes in vivo introduces the question of whether the oligomeric state of ProP has a functional role. To study this possibility, systems were created and used to generate ProP heterodimers both in vivo and in vitro. If ProP heterodimers retain the ability to transport proline, this would indicate that dimerization is not needed for ProP to function. The in vivo experiment provided some evidence that dimerization is not essential for ProP activity. However, it was not possible to be certain that heterodimers formed in vivo.

As ProP is often purified for studies in a reconstituted system, it was of interest to investigate heterodimer activity in vitro. To facilitate this approach, heterodimers must be purified. Initial experiments were performed to show that some ProP purifies as a dimer, therefore purification of heterodimers may be possible. To assess the oligomeric state of ProP in vitro in more detail, two in vitro techniques were employed, BN-PAGE and LILBID-MS. The in vitro techniques allowed the oligomeric state of ProP to be studied in a detergent environment which resembles the conditions used for purification of ProP. This experiment will provide initial insights into whether ProP exists as a dimer or monomer after purification.
3.1: The use of the bacterial adenylate cyclase two hybrid system for investigating the in vivo oligomeric state of ProP

The ProP fusion proteins employed to study ProP homodimerization using the BACTH system were analysed to test for expression and functionality. Suspensions of *E. coli* cells expressing either T18-ProP or T25-ProP were prepared as described in the Methods (section 2.4). When *E. coli* cells expressing T25-ProP were grown with glycerol as a carbon source, culture growth stopped during the final growth stage and the culture failed to reach the desired OD of 0.4. It was believed that T25-ProP was overexpressed to such high levels that cell growth was impacted; therefore, all strains used to analyze the expression and function of BACTH ProP fusion proteins were grown in media containing glucose rather than glycerol. This imposed catabolite repression of *proP* gene expression from the *lac* promoter, reducing expression levels of all ProP variants (Inada *et al.*, 1996). Under these conditions, cultures of cells expressing T25-ProP were able to grow for the duration needed for all experiments.

Western blot analysis showed expression of ProP fusion proteins T18-ProP and T25-ProP with no degradation evident (Figure 3.1). Radial streak tests were performed to determine whether T18- and T25-ProP retained the ability to transport osmolytes when exposed to osmotic stress. Glycine betaine stimulated the growth of strains expressing T18- or T25-ProP in the presence of osmotic stress (Table 3.1). For both Western blotting and radial streak tests, positive and negative controls which either do or do not possess the gene encoding wild type ProP were utilized. The presence of ProP in the positive control was observed by Western blot analysis, and these cells were able to grow under osmotic stress in the presence of glycine betaine (Figure 3.1, Table 3.1). Cells lacking the *proP* gene showed no ProP signal on the Western blot and were
Figure 3.1: Western blot analysis of cells expressing BACTH ProP fusion proteins. Strains WG708, WG709, WG1490 and WG1491 were cultivated in supplemented MOPS medium containing 0.2% (w/v) glucose as a carbon source and 50 mM NaCl. These strains expressed no ProP, wildtype ProP, T18-ProP or T25-ProP respectively. Cells were grown either with (+) or without (-) the addition of 0.5 mM IPTG. Cell extracts containing 48 μg of cell protein were loaded onto an SDS-PAGE gel which was then used for Western blotting using anti-ProP antibodies (upper image) (Racher et al, 1999). Purified ProP-His<sub>6</sub> (15 ng) acted as a positive control for the blot. The lower image shows the GelCode Blue stained SDS-PAGE gel which acted as a loading control.
Table 3.1: Radial streak test results of BACTH strains and proP+ and proP- cells. The symbols + and - indicate the presence or absence of IPTG or cell growth.

<table>
<thead>
<tr>
<th>E. coli Strain ProP Content</th>
<th>WG708</th>
<th>WG709</th>
<th>WG1490</th>
<th>WG1491</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM IPTG (+/-)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth (+/-)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
unable to grow in the presence of osmotic stress (Figure 3.1, Table 3.1). This work validates the use of these ProP fusion proteins to study ProP oligomerization.

3.2: In vivo heterodimer analysis

To determine whether dimerization was important to the function of ProP, heterodimers were generated in vivo by co-expressing active and inactive ProP variants in bacterial cells. The active form of ProP was encoded by the chromosome and the inactive ProP variant was encoded by a plasmid with the P_{BAD} promoter. The expression of chromosomally encoded ProP was maximized by adjusting the osmolality of the growth and assay media. The expression of plasmid encoded ProP was titrated using arabinose to control the expression of the inactive ProP variant, thereby also attempting to control the amount of heterodimers formed. The two inactive variants used for these experiments, ProP-E37Q and ProP-E37V, differ in their expression levels, with ProP-E37V having lower expression compared to other plasmid-encoded variants of ProP (Keates et al, 2010).

3.2.1: Optimization of chromosomal proP expression and ProP activity

The proline uptake rate was used to estimate relative expression levels of proP from different expression systems which lack all other proline transport systems. Cells that expressed chromosomally encoded ProP grown without osmotic induction had proline uptake rates of 7.8 and 14.2 nmol/min/mg protein at low and high osmolality (Figure 3.2). When ProP was encoded by a plasmid and the bacteria were cultured without arabinose induction, the proline uptake rates were 75 and 111 nmol/min/mg protein at low and high osmolality (Figure 3.2). The overall lower activity levels of cells containing chromosomally encoded ProP suggested that chromosomal proP was expressed to lower levels than plasmid encoded ProP.
Figure 3.2: Proline uptake by whole cells expressing proP from the chromosome or from a pBAD24 based plasmid. Uptake of $^{14}$C-proline was monitored for whole cells prepared from E. coli strain WG210, expressing proP from the chromosome or from strain WG709, expressing proP from pBAD24 based plasmid, pDC79. Cells were grown in supplemented NaCl-free MOPS medium and initial rates of proline uptake were measured in medium supplemented with 50 mM NaCl (open bars) or 150 mM NaCl (closed bars). Rates represent the average of three replicates and error bars represent the standard error.
Expression of chromosomal \textit{proP} and ProP activity are both enhanced under osmotic stress and this feature was exploited to maximize chromosomal ProP expression (Xu \& Johnson, 1995; Landis \textit{et al}, 1999), thereby minimizing the difference in expression and activity levels of chromosomally and plasmid encoded ProP. Cultures were grown at various salinities to osmotically induce chromosomal \textit{proP} expression. The highest proline uptake rate was obtained when cells were cultivated with 0.4 M NaCl (Figure 3.3), but the high salinity also slowed cell growth and made cells difficult to harvest. For this reason, 0.3 M NaCl was selected as the optimal salt concentration in growth medium for future experiments, because it enhanced ProP activity without significantly impacting growth. The osmolality of the assay medium at which proline uptake rates were measured was also optimized to improve ProP activity. Cells expressing chromosomally encoded ProP, grown with 0.3 M NaCl, obtained the highest proline uptake rate when assayed in medium with an osmolality of 0.65 mol/kg; an osmolality corresponding to the addition of 0.25 M NaCl to the assay medium (Figure 3.4). However, this uptake rate was similar to that achieved with an assay medium osmolality of 0.75 mol/kg (corresponding to the addition of 0.3 M NaCl to assay medium), which keeps the osmolality throughout the experiment constant and would not impose an osmotic downshift on the bacteria. For this reason, it was more desirable to use assay medium supplemented with 0.3 M NaCl for future experiments.

\textit{3.2.2: The effect of ProP dimerization on ProP function in vivo}

All \textit{E. coli} strains used to examine ProP heterodimerization in vivo were characterized by their proline transport activities and ProP expression levels when grown with various concentrations of arabinose. A strain of \textit{E. coli} expressing chromosomal \textit{proP} that contained a pBAD24 vector
Figure 3.3: The impact of growth medium salinity on the proline uptake rate of chromosomally encoded ProP. *E. coli* strain WG210 was cultivated in supplemented NaCl-free MOPS medium with the addition of 0, 0.1, 0.2, 0.3 or 0.4 M NaCl. Cells were harvested and assayed as described in Methods 2.8 in medium with the same osmolality as their respective growth medium. Each point represents an average rate from triplicates and the standard error is shown by error bars.
**Figure 3.4: Proline uptake rate of chromosomally encoded ProP assayed at various osmolalities.** *E. coli* strain WG210 was grown in supplemented NaCl-free MOPS medium with the addition of 0.3 M NaCl, resulting in an osmolality of 0.75 mol/kg. Cells were assayed in assay buffer containing 0, 0.05, 0.1, 0.15, 0.2, 0.25 or 0.3 M NaCl. These salt concentrations corresponded to assay medium osmolalities of 0.15, 0.25, 0.35, 0.45, 0.55, 0.65 and 0.75 mol/kg, respectively. Rates represent the average of a triplicate and the standard error is shown. The line represents a sigmoidal curve fitted to the data using SigmaPlot as outlined in Methods 2.8. The parameters obtained from the fitted curve and their standard error were as follows: the maximum rate of transport ($Y_{\text{max}}$) was 19 ± 0.44 nmol/min/mg protein, the osmolality at which activity was half maximal ($x_0$) was 0.51 ± 0.006 mol/kg and the constant (b) was 0.06 ± 0.005 mol/kg.
acted as a negative control for these experiments. As expected, the negative control strain showed arabinose independent proline uptake rates and ProP expression at all arabinose concentrations (Figure 3.5 and 3.6A respectively). As a positive control, an *E. coli* strain expressing active *proP* from both the chromosome and a pBAD24 based plasmid was utilized to monitor the effect of arabinose on plasmid encoded *proP* expression. As anticipated, the activity of the positive control strain increased as the arabinose concentration increased from 0 to 0.005% (w/v) (Figure 3.5). At an arabinose concentration of 0.02% (w/v), the proline uptake rate showed no further increase and was similar to the uptake rate at 0.005% (w/v) arabinose. The expression of ProP in the positive control increased over the entire range of the arabinose titration (Figure 3.6B).

To create a system which could generate heterodimers, active chromosomally encoded ProP was co-expressed with each inactive plasmid encoded ProP variant, ProP-E37Q or ProP-E37V. In terms of activity, both co-expression systems had a similar trend, with a constant proline uptake rate from 0-0.002% (w/v) arabinose. However, the activity level decreased at higher arabinose concentrations, with the ProP/ProP-E37Q co-expression system showing greater inhibitory effects than the ProP/ProP-E37V system (Figure 3.5). The expression level of ProP in the ProP/ProP-E37Q co-expression system was comparable to that of the positive control strain and increased with increasing arabinose concentrations (Figure 3.6C). Overall, the levels of ProP proteins in the ProP/ProP-E37V co-expression system increased with increasing arabinose concentrations, as anticipated (Figure 3.7A). ProP-E37V is expressed at levels lower than that of other plasmid encoded ProP variants (Keates *et al.*, 2010). It was expected that ProP-E37V would have expression levels comparable to chromosomally encoded ProP. This was verified as the
Figure 3.5: Impact of arabinose induction on the proline uptake rate of ProP co-expression systems. The uptake of $^{14}$C-proline was monitored as the arabinose concentration used for induction of plasmid encoded proP was varied. The arabinose concentration ((w/v) percentage) corresponding with each log [arabinose] value is shown above the plot. WG1477 was used as a positive control as it co-expresses active chromosomal and plasmid encoded ProP (closed circles). WG1479, which expresses chromosomally encoded ProP, acted as a negative control (closed diamonds) and the data for this strain was fitted by linear regression using SigmaPlot. WG1478 and WG1496, co-expressing chromosomally encoded ProP with plasmid encoded ProP-E37Q (WG1478, closed squares) or ProP-E37V (WG1496, open inverted triangles) were used as systems co-expressing active and inactive ProP. All strains were cultivated and assayed in growth medium and assay buffers both supplemented with 0.3 M NaCl. Data was analyzed using SigmaPlot and the standard error is shown.
**Figure 3.6: ProP expression levels in cells used for the in vivo analysis of ProP dimerization** (Figure 3.5). Cells used for Western blot analysis were from cultures grown in minimal medium with 0.3 M NaCl and 0, 0.0002, 0.002, 0.005 or 0.02% (w/v) arabinose. Anti-ProP Western blots (Methods 2.11) show ProP levels in the cell extracts. Extracts of WG708 (15 or 75 μg total protein) which lacks any *proP* genes and purified ProP-His<sub>6</sub> (15 ng) acted as controls for the blot. Gelcode Blue stained SDS-PAGE gels were used as a loading control for each Western blot with the corresponding SDS-PAGE gel shown below each blot respectively. A: Extracts of WG1479 cells (75 μg cell protein, negative control). B: Extracts of WG1477 cells (positive control, 15 μg cell protein). C: Extracts of WG1478 (ProP/ProP-E37Q, test strain, 15 μg cell protein).
Figure 3.7: Comparison of ProP expression in the negative control and ProP/ProP-E37V co-expression systems for the in vivo analysis of ProP dimerization. A: Extracts (75 μg of protein) of WG1479 (negative control) and WG1496 (ProP/ProP-E37V) cells from cultures grown in medium supplemented with 0.3 M NaCl with 0, 0.0002 or 0.002% (w/v) arabinose were analyzed by anti-ProP Western blotting. WG708 (prop, 75 μg protein) and purified ProP-His$_6$ (15 ng) acted as negative and positive controls for the Western blot. A GelCode Blue stained SDS-PAGE gel (shown below) acted as a loading control. B: Multiple lanes of water lysis membranes (75 μg total protein) of WG1479 (negative control) and WG1496 (ProP/ProP-E37V) prepared without arabinose induction were analyzed via anti-ProP Western blotting of an SDS-PAGE gel for densitometric analysis. Purified ProP-His$_6$ (15 ng) was used as a positive control for the blot.
levels of ProP in the ProP/ProP-E37V co-expression system were comparable to the expression levels of chromosomally encoded ProP alone (Figure 3.7) The higher expression of ProP-E37Q compared to ProP-E37V is likely the cause of the more inhibitory effects observed with the ProP/ProP-E37Q co-expression system.

Densitometry was used to compare the relative amount of ProP expressed in the ProP/ProP-E37V co-expression system without arabinose induction and the negative control strain, which only encodes wild type ProP. This showed 2.6 ± 0.6 times more ProP in the co-expression system than in the negative control strain (Figure 3.7B). Co-expression yields two forms of ProP, whereas the negative control strain only expresses one form. Therefore, the higher expression levels of ProP in the co-expression system can be attributed to the addition of the plasmid encoded ProP-E37V. This result supports the conclusion that ProP-E37V is expressed to approximately equal amounts as chromosomal ProP without induction and that arabinose induction creates an increasing excess of the inactive variant.

3.3: Investigating the existence of ProP heterodimers in vitro

Purification of dimers would be required for the analysis of ProP heterodimers in vitro. To investigate this, a bicistronic co-expression plasmid was used, which encodes both Flag-ProP and His₆-ProP as an operon under the control of a single P_BAD promoter (Figure 3.8, constructed by Culham, DE). If a heterodimer consisting of Flag-ProP and His₆-ProP were formed, after a single affinity purification step targeting either of the tags, the ProP variant with the tag not targeted in the purification should co-purify with the alternatively tagged ProP (e.g., if anti-Flag resin is used for purification of Flag-ProP, then His₆-ProP should co-purify and be present in the eluate).
Figure 3.8: Plasmid map of pDC324, a bicistronic plasmid encoding Flag-ProP and His\textsubscript{6}-ProP. A single $P_{\text{BAD}}$ promoter controls expression of both tagged ProP variants and a ribosome binding site (RBS1 and RBS2) precedes both $\text{proP}$ genes. Plasmid map was created using PlasMapper (Dong et al, 2004).
Figure 3.9: SDS-PAGE and Western blot analysis of analytical scale purification of Flag-ProP and His$_6$-ProP using Ni-NTA or anti-Flag resin. *E. coli* strain WG1506 co-expresses Flag-ProP and His$_6$-ProP. WG1506 cells were cultivated in supplemented NaCl-free MOPS medium with 50 mM NaCl and 0.2% (w/v) arabinose. Membranes isolated by water lysis were solubilised in 1% (w/v) DDM and the tagged proteins were purified using either Ni-NTA or anti-Flag resin (Methods 2.12.1). The eluted protein (10 μL unless indicated otherwise) was analyzed by SDS-PAGE and subsequent Western blotting. Purified ProP-His$_6$ (15 ng) was added to some blots as a control. A: Coomassie stained SDS-PAGE gel of protein eluted from Ni-NTA or anti-Flag resin. B, C and D: Anti-ProP, anti-Flag and anti-His Western blots, respectively, showing the presence of various ProP species in the eluted protein samples. E: Anti-His Western blot of Ni-NTA eluted protein and a concentrated version of the anti-Flag eluate. The anti-Flag eluate (60 μL volume before concentration) was concentrated to one third of the original volume. The concentrated eluate (15 μL) was used for anti-His Western blot analysis.
To ensure that heterodimers formed using this co-expression system, single step analytical purifications were performed using either Ni-NTA or anti-Flag resin. The eluates were analyzed by Western blotting to detect the presence of ProP and both affinity tags (Figure 3.9).

SDS-PAGE analysis and anti-ProP Western blot of the eluate of the Ni-NTA and anti-Flag purifications showed that the Ni-NTA purification isolated more ProP compared to the anti-Flag purification (Figure 3.9A and B). An anti-Flag Western blot showed the presence of Flag-ProP in the eluate of both the Ni-NTA and anti-Flag purifications, showing the co-purification of Flag-ProP and His$_6$-ProP (Figure 3.9C). Anti-His antibodies are not as reactive with the N-terminal His tag on ProP so the signal for His$_6$-ProP was weak, even for the Ni-NTA eluate, making it difficult to detect the presence of His$_6$-ProP when anti-Flag resin was used for purification (Figure 3.9D). The presence of His$_6$-ProP in the anti-Flag eluate was only detected when the eluate was concentrated to one third of the original volume (Figure 3.9E).

3.4: Blue native polyacrylamide gel electrophoresis analysis of ProP oligomeric state

The oligomeric state of ProP in detergent solution was assessed using BN-PAGE. Protein standards designed for SDS-PAGE cannot be reliably used for BN-PAGE, as the mobility and mass of membrane proteins are influenced by the type of detergent used, the binding of Coomassie dye and the shapes of protein complexes (Heuberger et al, 2002; Wittig et al, 2006). For this reason, a ProP dimer standard was created to act as a marker on all BN-PAGE gels. Variant ProP-E480C can be crosslinked using DTME, generating a ProP dimer, because the E480 residues located on the C-termini of ProP subunits are located in close proximity when the coiled coil is formed (see Figure 1.7) (Hillar et al, 2005). When membranes containing crosslinked ProP-E480C were solubilised with DDM (1 g/g) the presence of both dimeric and
Figure 3.10: BN-PAGE analysis of the oligomeric state of detergent solubilised ProP. *E. coli* strain WG709 was cultivated in NaCl-free MOPS growth medium supplemented with 50 mM NaCl. Membranes were prepared by water lysis (Methods 2.5.1) and they were solubilised with either DDM or digitonin at the detergent/protein ratio indicated above each lane and in the Methods (2.9.2). Membranes of strain WG1368 that had been treated with the crosslinker DTME or the control solution DMF were solubilised with 1 g/g DDM and served as controls illustrating the mobility of ProP dimers and monomers. The BN-PAGE gel was used for anti-ProP Western blotting for analysis.
monomeric ProP was visualized by anti-ProP Western blotting (the upper and lower band in Figure 3.10 respectively). The ProP-E480C membranes that were not treated with DTME showed the presence of monomeric ProP only (Figure 3.10).

Membranes containing wild type ProP were solubilised using either DDM or digitonin to assess whether detergent type or concentration had an impact on ProP oligomerization. The detergent DDM fully converted ProP to the monomeric form regardless of detergent concentration in the range tested (Figure 3.10). The effects of digitonin on ProP oligomerization differed compared to DDM and also showed a general trend of decreasing electrophoretic mobilities as the digitonin concentration increased (Figure 3.10). Digitonin solubilised membranes contained two ProP species, regardless of detergent concentration used. The species with a lower electrophoretic mobility corresponded to a ProP dimer, whereas the fainter species with higher mobility was that expected of a ProP monomer (Figure 3.10).

Studies using proteoliposomes rely on using ProP-His₆ purified using DDM. To ensure that the addition of a hexahistidine tag to the C-terminus of ProP did not impact the trends in ProP oligomerization observed in Figure 3.10, the analysis was repeated using membranes containing ProP-His₆. The addition of the hexahistidine tag did not alter the oligomeric state of ProP. DDM solubilisation resulted in solely monomeric ProP and digitonin solubilisation preserved two ProP species (Figure 3.11). This observation raises the question about the oligomeric state of ProP-His₆ in a reconstituted system after it has undergone DDM solubilisation.

To determine whether the time that solubilised membrane protein spent in a solution with 1 g/g DDM impacted the oligomeric state of ProP, samples were prepared followed by storage at 4°C for up to 4 days. Throughout this storage period, ProP remained monomeric (Figure 3.12).
**Figure 3.11: BN-PAGE analysis of the oligomeric state of detergent solubilised ProP-His$_6$.**

*E. coli* strain WG710 was cultivated in minimal medium with the addition of 50 mM NaCl. Water lysis membranes (Methods 2.5.1) of WG710 were solubilised with either 1 or 2 g/g of DDM or 2 or 4 g/g digitonin. Membranes of WG1368 which contain ProP-E480C were solubilised with 1 g/g DDM and acted as a control for the mobility of a dimeric or monomeric ProP depending on whether membranes had been treated with DTME. The BN-PAGE gel was used for anti-ProP Western blotting for analysis.
Figure 3.12: Analysis of the effect of storage time on the oligomeric state of ProP by BN-PAGE. Water lysis membranes derived from *E. coli* strain WG709 were solubilised with 1 g/g DDM and were stored at 4°C for up to 4 days. Glycerol (5 μL, 50% (v/v)) and Coomassie G-250 dye (1 μL, 5% (w/v)) were added to samples prior to loading onto the BN-PAGE gel. The gel was used for anti-ProP Western blotting. Membranes containing ProP-E480C were included in the gel as a standard for the mobility of ProP dimers and monomers depending on whether cells had been treated with DTME. All membranes were derived from cells cultivated in minimal media supplemented with 50 mM NaCl.
Figure 3.13: Gel filtration elution profile of ProP-His$_6$. Affinity purified ProP-His$_6$ was injected into the Superdex 200 gel filtration column and eluted with potassium phosphate (100 mM, pH 7.4) containing DDM (0.04% (w/v)). The absorbance at 280 nm was monitored as purification proceeded and 0.5 mL fractions were collected.
3.5: Examination of the oligomeric state of ProP in vitro using LILBID-MS

ProP-His\textsubscript{6} was purified using affinity and gel filtration chromatography with the detergent DDM and was analyzed using LILBID-MS as described in Methods 2.13. The gel filtration profile showed two major peaks (Figure 3.13). The first peak with an elution volume of approximately 10.6 mL corresponded to the elution of ProP-His\textsubscript{6} and the fractions contained in this peak (fractions from 9.5 to 12 mL, pooled volume of 2.5 mL) were pooled for LILBID-MS experiments. The SDS-PAGE analysis confirmed that the pooled fractions contained ProP-His\textsubscript{6} and that the purified ProP-His\textsubscript{6} was sufficiently pure for LILBID-MS analysis (Figure 3.14).

The LILBID-MS spectrum of purified ProP-His\textsubscript{6} was obtained at both low and high laser powers. At low laser power, two main peaks were seen representing ProP-His\textsubscript{6} species with molecular masses of approximately 65 and 125 kDa (Figure 3.15, red). At high laser power, one main peak was observed corresponding to a molecular weight of 54.8 kDa (Figure 3.15, black). The spectrum showed the addition of 3.5 kDa followed by the addition of 7 kDa to the 54.8 kDa species. Two other peaks were seen in the spectrum; one is a broad, small peak with molecular weights ranging from 110-130 kDa and the second is a single peak with a molecular mass of 3.5 kDa. This peak pattern was generally reproducible (see Appendix Figure A.1).

3.6: Discussion

The oligomeric state of ProP was investigated using in vivo and in vitro techniques. Previous work using the BACTH system showed that ProP homodimerizes in vivo (Figure 1.9) (Wood lab, unpublished). The ProP constructs used for BACTH experiments were shown to be expressed and intact using Western blot analysis (Figure 3.1). The effects of IPTG induction of both BACTH ProP variants were not strongly seen, likely due to the catabolite repression that was imposed during cell growth and induction. T18- and T25-ProP were shown to be capable of
Figure 3.14: SDS-PAGE analysis of purified ProP-His$_6$. ProP-His$_6$ was initially purified by Ni-NTA affinity chromatography targeting the hexahistidine tag, which was followed by gel filtration chromatography. The purified protein from the pooled fraction in Figure 3.13 (5.2 μg) was loaded on an SDS-PAGE gel. The Blueye prestained protein ladder acted as a reference.
Figure 3.15: LILBID-MS profile of purified ProP-His$_6$. As described in the Methods (2.13), gel filtration purified ProP-His$_6$ (Figure 3.14) with a concentration of 11.6 μM was analyzed in ammonium acetate (100 mM) with 0.03% (w/v) DDM for LILBID-MS. Samples were analyzed at both low (red) and high (black) laser power and the mass/charge ratio was obtained.
glycine betaine transport as evident by growth on minimal medium plates with 0.6 M NaCl with glycine betaine supplied on a filter disc (Table 3.1). These experiments have shown that the addition of the T18 or T25 fragment of adenylate cyclase to the N-terminus of ProP does not prevent incorporation of ProP into the membrane in a transport competent form. This validates the use of the BACTH system for studying ProP-ProP interactions.

To test whether the dimerization of ProP had a functional role, heterodimers were generated in vivo and in vitro. To study ProP heterodimers in vivo, the level of active, chromosomally encoded ProP was fixed while the expression of the inactive ProP variant was titrated utilizing an arabinose controlled expression system. As expected, the chromosomally encoded ProP expressed in the strain used as a negative control for the analysis of ProP heterodimers showed constant ProP expression and activity that was independent of the arabinose concentration. When chromosomally encoded ProP was co-expressed with active, plasmid encoded ProP, the activity and expression of ProP increased with increasing arabinose concentrations. The high expression of plasmid encoded ProP without arabinose induction compared to chromosomally encoded ProP was noticeable by the high proline uptake rate of the positive control without induction, compared to the negative control in Figure 3.5. The proline uptake rate of the positive control strain did level off when the two highest arabinose concentrations were used (0.005 and 0.02% (w/v)), although Western blot analysis showed that ProP expression continued to increase over this range of arabinose concentrations. It is possible that the high level of ProP overexpression at high arabinose concentrations may have had a non-specific effect on the ability of ProP to perform transport, making data obtained at these high arabinose concentrations unreliable.

To generate heterodimers, inactive ProP variants were selected for expression from an arabinose inducible plasmid. The two variants selected have different amino acid replacements at the same
residue in ProP, E37, located in TMI which is not in the immediate vicinity of the site of dimerization, making this residue unlikely to impact the ability of ProP to dimerize. The inactive variants were chosen for their different expression levels, as ProP-E37Q has expression levels similar to most plasmid encoded forms of ProP, whereas ProP-E37V has lower expression levels compared to other variants of plasmid encoded ProP, a trend that was also observed in this study (Keates et al, 2010). The E. coli strains predicted to generate heterodimers had similar proline uptake rates, regardless of which inactive variant was co-expressed with active chromosomally encoded ProP. Overall, these strains had proline uptake rates similar to the negative control, but their activity levels decreased at high arabinose concentrations with the ProP-E37Q system showing greater inhibitory effects. As discussed above, the high overexpression of ProP at the highest arabinose concentrations used in this study impacted ProP activity, therefore this decrease is not believed to be related to the effects of heterodimer formation. In addition, it is reasonable to assume that in the ProP/ProP-E37Q co-expression system, even without arabinose induction, there is an excess of the plasmid encoded ProP-E37Q compared to the chromosomally encoded ProP. This means that if heterodimers formed, any dominant-negative effects caused by heterodimer formation should be immediately apparent, even when no arabinose was provided. This in vivo study supports the conclusion that ProP dimerization is not required for ProP activity, as the co-expression of active and inactive ProP did not impact ProP activity. However, this conclusion depends upon the assumption that heterodimers have successfully formed in the in vivo co-expression system, which would require further study.

In vitro studies showed the co-purification of Flag-ProP and His6-ProP after a one step purification targeting either the Flag or His tag. This showed that the bicistronic expression system led to the formation of heterodimers of ProP with each subunit possessing a different
affinity tag. These results also showed that some ProP purifies as a dimer, although it is not clear whether the dimeric protein in the sample is of significant quantities compared to monomeric ProP. In addition to issues regarding quantity, the ProP purified using Ni-NTA resin yielded a very impure sample, making it possible that the Flag-ProP that co-purified with His\textsubscript{6}-ProP may have been present as an impurity rather than reflecting a true interaction between both ProP variants. The BN-PAGE analysis (discussed below) further validated this concern as it was determined that DDM solubilisation yielded monomeric ProP (see Figure 3.11). Regardless, further experiments could be conducted to test the yield of heterodimers after a larger scale, two-step purification. This would determine whether large enough quantities of ProP heterodimers form to facilitate in vitro analysis of heterodimers in a reconstituted system. This would also confirm whether heterodimer interactions are truly formed during purification, or if the co-elution of Flag-ProP and His\textsubscript{6}-ProP was only an artifact due to impurities in the eluted protein.

The oligomeric state of ProP in a detergent environment was studied using BN-PAGE and LILBID-MS. The BN-PAGE analysis of solubilised membranes containing ProP showed that DDM solubilisation yielded monomeric ProP whereas solubilisation with digitonin showed the presence of two ProP species (Figure 3.10). When membranes were solubilized with digitonin, the band with higher mobility is likely monomeric ProP and the band with lower mobility is consistent with dimeric ProP. Digitonin is a milder detergent than DDM and is often used to preserve protein-protein interactions, which explains how ProP homodimers might remain after solubilisation using digitonin (Wittig et al, 2006). The overall slightly lower mobility of samples solubilised with digitonin compared to DDM may also be caused by the mild nature of digitonin. It is possible that additional lipids may not be removed from the protein when
digitonin was used, or alternatively, digitonin molecules may become attached to the ProP complex, impacting the mobility of ProP species.

The detergents had the same impact on the oligomerization of ProP-His$_6$, showing that the addition of the hexahistidine tag did not impact ProP’s behaviour in DDM and digitonin. The time solubilised membrane protein spent in 1 g/g DDM did not alter ProP oligomerization and no aggregation or dimerization was evident, even when samples were left at 4°C for 4 days. This observation suggested that the high concentration of detergent kept ProP in a stable monomeric state and prevented ProP-ProP interactions.

The state of samples used for BN-PAGE most closely reflects the state of ProP after the membrane solubilisation step of a purification, when the detergent concentration is high. To assess the oligomeric state of ProP after purification, when the detergent concentration is lowered, LILBID-MS was employed. LILBID-MS showed that purified ProP-His$_6$ was primarily monomeric. At low laser power, the peak believed to correspond to monomeric ProP-His$_6$ had a molecular mass of approximately 65 kDa, which is higher than the expected molecular mass of 55.6 kDa. This discrepancy was explained by the presence of attachments to ProP, which were more clearly seen when the same sample was analyzed at high laser power. The high laser power spectrum showed the presence of a 54.8 kDa species, which is likely monomeric ProP-His$_6$ stripped of all attached molecules. The nearby peaks suggested the addition of attachments to ProP, first a 3.5 kDa attachment followed by a 7 kDa attachment. Additional experiments are needed to determine the identity of these attachments, but they could be residual lipid or detergent molecules that remain bound to ProP.
The final significant peak seen at both low and high laser power showed a ProP species in the range of 110-130 kDa, which roughly matches the expected molecular weight of a ProP-His$_6$ dimer (111.2 kDa). This suggested that, in addition to monomeric ProP, some ProP in the sample may have dimerized or aggregated. This higher oligomeric state species was not observed on BN-PAGE gels when samples were treated with DDM. LILBID-MS uses a ProP sample in a lower concentration of DDM compared to BN-PAGE (0.04% (w/v) compared to 1-2% (w/v)), which may explain why higher molecular weight mass were visible with LILBID-MS. In addition to this, samples for LILBID-MS were put into a buffer more compatible with LILBID-MS immediately prior to analysis which may have impacted the stability of the sample.
Chapter 4: Conclusions and Future Directions

The goal of this study was to investigate the oligomeric state of ProP in a detergent environment and to use a heterodimer approach to probe the function of ProP dimerization. Understanding how the detergent environment impacts ProP oligomerization is important because ProP is often studied in a reconstituted system that utilizes purified ProP-His<sub>6</sub>. Both BN-PAGE and LILBID-MS analysis of ProP in DDM showed that ProP is primarily monomeric in the detergent environment (Figure 3.10 and 3.15). Culham <i>et al</i>, (2016) were unable to determine whether reconstituted ProP was a monomer or dimer based on mathematical modelling of transport assay data, as models based on both oligomeric states fit ProP activity data equally well. The results reported in this study imply that when ProP is reconstituted into proteoliposomes, the majority of ProP is monomeric. It is possible that the lipid environment may affect the equilibrium between monomeric and dimeric ProP, either enhancing or reducing the amount of oligomerization possible. To explore the oligomeric state of ProP in proteoliposomes, cryo-electron microscopy could be employed (Wang & Sigworth, 2009).

BN-PAGE has also provided insights into the oligomeric of ProP in vivo through the use of the mild detergent digitonin. Due to the mild nature of digitonin, it is better able to maintain native protein complexes compared to DDM and therefore, may more accurately reflect the protein complexes that occur in vivo. The presence of both monomeric and dimeric ProP in digitonin solubilised samples (Figure 3.10) leads to the hypothesis that ProP can exist as both a monomer and a dimer in vivo. In vivo dimerization is experimentally supported by BACTH studies (Figure 1.9).

The LILBID-MS analysis of purified ProP identified the presence of attachments, possibly lipids that are associated with ProP (Figure 3.15). The identity of these attachments could be evaluated
using thin layer chromatography (TLC), which would separate the lipids/detergents present in purified ProP samples and lipids could be identified by comparison with lipid standards. This is the same approach taken by Brandstätter et al, (2011) to identify the attachments observed by LILBID-MS analysis of the *E. coli* drug efflux pump AcrB (Brandstätter et al, 2011).

The functional role of ProP was probed by attempting to generate ProP heterodimers in vivo. In vivo results suggested that dimerization was not essential for ProP to be active, but this approach did not facilitate the direct detection of heterodimer formation. To show that heterodimers formed in vivo, FRET (fluorescence resonance energy transfer) could be used to detect the protein-protein interactions between the two different subunits (Pollok & Heim, 1999).

A similar approach to studying the purpose of ProP dimerization was used in vitro with the aim of purifying ProP heterodimers. Analytical scale purifications showed that ProP heterodimers formed during the purification of ProP. To determine whether the quantity of heterodimers present is large enough to facilitate reconstitution of heterodimers into proteoliposomes, a large scale, two step purification must be attempted to test the heterodimer yield. If the yield is high enough, heterodimers can be reconstituted into proteoliposomes and assayed for activity. Purification using different detergents could be tested in an effort to improve heterodimer yield. If heterodimers are active, this would support the in vivo results that dimerization is not essential for ProP activity.

Overall, this study has shown that ProP exists as both a monomer and dimer in vitro, with the monomeric form dominating, which leads one to propose that ProP may exist in both forms in vivo as well. Although further study is required to conclusively show whether dimerization plays...
a functional or regulatory role, these experiments have suggested that dimerization may primarily have a structural rather than functional role.
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Appendix A

The replicate LILBID-MS spectrum of Figure 3.15 is shown below (Figure A.1). Overall it has a similar peak pattern as in Figure 3.7. Two major ProP species are seen at low laser power with masses of approximately 61 kDa and 120 kDa. These masses are higher than anticipated for the molecular weight of a ProP monomer and dimer, suggesting detergents or other molecules are attached to ProP. At high laser power, the presence of multiple small peaks within the broad peak around 60 kDa further indicates the presence of attachments to the ProP protein.

Figure A.1: LILBID-MS spectrum of purified ProP-His$_6$. As outlined in the Methods (2.13), ProP-His$_6$ was purified by affinity and gel filtration chromatography. Pure protein (concentration of 9.5 μM) was analyzed by LILBID-MS in Tris-HCl buffer (20 mM, pH 9) with DDM (0.02% (w/v)) at both low (red) and high (black) laser power. The mass/charge ratio was obtained.
Appendix B

The aim of the work described below was to create ProP expression systems that could be used to optimize the purification of ProP using pre-crystallization screening techniques. FX Cloning (Geertsma & Dutzler, 2011) was used to create multiple expression systems for either ProP-His$_{10}$ or ProP-GFP-His$_{10}$ (GFP stands for green fluorescent protein). The fluorescence of GFP was used to monitor ProP-GFP-His$_{10}$ expression and to optimize growth and detergent solubilisation conditions (Drew et al, 2006; Kawate & Gouaux, 2006). The effectiveness of this screening process was assessed by performing a large scale purification according to the optimized cultivation and solubilisation conditions. The monodispersity of the protein obtained in this way was evaluated using gel filtration chromatography.

B.1: Methods

B.1.1: Bacterial strains and growth conditions

All strains and plasmids used in the experiments outlined in Appendix B are shown in Tables B.1.1 and B.1.2. Cells were cultivated in LB (Methods 2.2) or terrific broth (TB, 1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 17 mM KH$_2$PO$_4$ and 72 mM K$_2$HPO$_4$) (Tartoff & Hobbs, 1987) at 37°C with shaking at 200 rpm unless otherwise indicated. OD measurements used to monitor cell growth were obtained using the Ultospec 10 cell density meter (GE Healthcare).

B.1.2: FX Cloning

The vectors and plasmids used for FX Cloning are listed in Table B.1.2 and were isolated as described in Methods 2.3. To prepare the insert for use in FX Cloning, the proP gene from plasmid pDC79 (Table 2.2) was PCR amplified using the primers listed in Table B.1.3. The PCR
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<td>DB 3.1 pINITcat</td>
<td>Lab of Dr. K.M. Pos</td>
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### Table B.1.2: Plasmids

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### Table B.1.3: Primers

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cycle began with a 2 minute treatment at 98°C, followed by a cycle of denaturation at 98°C for 20 seconds, annealing for 30 seconds at 65.2°C and extension for 1 minute at 72°C which was repeated 35 times. Finally, the sample was left at 72°C for 10 min followed by a temperature decrease to 4°C.

All cloning and transformations were carried out as described in Geertsma & Dutzler, (2011). In brief, the insert was ligated into the pINITcat vector and the resulting plasmid, pINITcat-proP, was used for sub-cloning of proP into multiple expression vectors. Plasmids with names beginning with p7X are Km resistant and respond to IPTG induction, and plasmids named pBX are Amp resistant and respond to L-arabinose induction. Plasmids were sequenced with primers shown in Table B.1.3 and sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany).

**B.1.3: Expression screening of GFP tagged proteins**

Small scale expression screening was conducted with strains expressing ProP-GFP-His<sub>10</sub> as described in (Drew et al., 2006).

**Screen 1**

Bacteria expressing ProP-GFP-His<sub>10</sub> (see Table B.1.1) were cultivated in LB broth (1 mL) supplemented with Amp (100 µg/mL) or Km (50 µg/mL) as needed for approximately 16 hours (Methods 2.2). The first expression screen tested for optimal cell type and inducer concentration. A 96 Deep Well Microplate (VWR) was used for small scale screens and the plate was covered with a Breathe-Easy membrane. Each well contained overnight culture (7 µL) and fresh LB with antibiotics (700 µL). The plate was incubated at 37°C with shaking at 900 rpm for 2 hours (OD of roughly 0.6), after which, cells were induced as desired. The induction levels tested were 0.2,
0.5, 0.7 and 1 mM IPTG or 0.0002%, 0.002%, 0.02% and 0.2% (w/v) L-arabinose. Wells lacking inducer acted as controls. Incubation was continued for 3 hours at 25°C with shaking at 900 rpm. Cells were harvested by centrifuging the 96 well plate (4000 rpm, 20 min, 4°C) and pellets were resuspended in PBS (200 μL, Methods 2.11).

Final OD measurements were obtained using 100 μL of the cell suspensions and the Infinite M200 reader (Tecan, Mainz, Germany). Fluorescence measurements were obtained using cell suspensions (100 μL) in a black 96 well plate (BrandTech, Connecticut, USA, product number 781668) and GFP fluorescence was measured with the Infinite M200 reader (Tecan) with an excitation wavelength of 485 nm and emission wavelength of 535 nm. Relative fluorescence values were obtained by dividing the fluorescence by the OD for each sample.

**Screen 2**

Overnight cultures were prepared as described for Screen 1. The resulting culture (100 μL) was added to LB or TB (10 mL). Cultures were incubated at 37°C until an OD of 0.6 was obtained, at which point cultures were induced and grown at either 37 or 25°C. Aliquots of cells corresponding to a normalized OD of 1 (normalized by letting an OD of 1 equal 1 mL) were taken at various time points (0, 2, 4 or 16 hours post-induction). Cells were harvested by centrifugation (13000 rpm, 5 minutes) and were resuspended in PBS (200 μL). Final OD and fluorescence measurements were obtained as described for Screen 1.

If SDS-PAGE analysis of cell samples was desired, cells were harvested from the 200 μL cell suspension used for OD and fluorescence measurements and pellets were stored at -20°C until use. To analyze samples by SDS-PAGE, the cell pellets were resuspended in 1X sample buffer (100 μL, 0.06M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.025% (w/v)...
bromophenol blue and 5% (v/v) β-mercaptoethanol) and an aliquot (10 μL) was loaded onto the SDS-PAGE gel.

B.1.4: SDS-PAGE analysis

SDS-PAGE gels were prepared and developed as outlined in the Methods (2.9.1). The PageRuler prestained protein ladder was used as a reference. If SDS-PAGE samples contained fluorescent protein, they were incubated at 37°C for 5 minutes (compared to 30 minutes) to preserve the fluorescence signal. In-gel fluorescence was detected using the ImageQuant LAS 4000 (GE Healthcare) set to detect GFP fluorescence.

B.1.5: Anti-His Western blotting

Protein from SDS-PAGE gels was transferred to nitrocellulose membrane (0.2 μm, Carl Roth (Germany)) using a semi-dry transfer system (Trans-Blot Turbo Transfer System, Biorad) and transfer proceeded for 30 min at 25V. The membrane was blocked for 1 hour at room temperature in blocking solution (25 mL TBS with bovine serum albumin (BSA, 3% (w/v)). The membrane was washed in TBS-Triton (30 mL, 3 washes, 5 minutes each). The membrane was incubated with monoclonal anti-polyhistidine-alkaline phosphatase produced in mouse (Sigma Aldrich) diluted 1:4000 in TBS-Triton with BSA (1% (w/v)) for 1 hour at 4°C. The wash step was repeated and the membrane was then washed 3 times for 5 minutes in TNM buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂). The membrane was developed in TNM buffer (20 mL) with nitroblue tetrazolium (NBT, 90 μL of a 75 mg/mL stock prepared in 70% (v/v) DMF) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 90 μL of a 50 mg/mL stock prepared in DMF). The reaction proceeded until bands appeared, at which time the developing solution was discarded and the membrane was washed with water. For details of buffer composition not described above, see Methods (2.11).
B.1.6: Detergent screen

Detergent screening was carried out as outlined in Kawate & Gouaux, (2006) and will be described below. LB supplemented with Km was inoculated with *E. coli* strain C41 p7XC3GH-proP and incubated at 37°C for 16-18 hours. The resulting culture (200 μL) was added to TB medium (20 mL) supplemented with Km and the culture was grown to an OD of 0.6. The culture was then induced with IPTG (0.7 mM) and grown for an additional 16-18 hours. Cells were harvested by centrifugation (4300 rpm, 10 min, 4°C) and resuspended in cold lysis buffer (1.2 mL, 50 mM potassium phosphate pH 7.2, 1 mM MgSO₄, 10% (v/v) glycerol, 1 mM PMSF and trace amount of DNase). Glass beads (600 mg, 0.1 mm beads) were added to the cell suspension and cells were lysed using a FastPrep-24 instrument (2, 20 second intervals, force 6), an instrument which disrupts cells through vigorous shaking. Samples were cooled on ice for 5 minutes between treatments. Once the glass beads settled, aliquots of the supernatant (90 μL) were combined with a detergent (10 μL of 10% (w/v) stock) and incubated at 4°C with rotation for 1 hour. The detergents tested include: DDM, digitonin, n-dodecyl-N,N-dimethylamine-N-oxide (DDAO), n-octyl-β-D-glucopyranoside (OG), 6-cyclohexyl-1-hexyl-β-D-maltoside (CYMAL-6), n-decyl-β-D-maltoside (DM), 3-((3-cholamidopropyl) dimethylammonio-1-propanesulfonate) (CHAPS), n-dodecylphophocholine (Fos-choline-12). Solubilised samples (100 μL) were centrifuged (90000 rpm, 10 min, 4°C) and the supernatant was transferred to a well of a 96 well plate (0.5 mL, polypropylene, Agilent Technologies, CA, USA) which was covered with a Nunc 96 well cap mat.

Fluorescence size exclusion chromatography (FSEC) was performed using the Agilent 1200 Series HPLC system (Agilent Technologies) and the Superdex 200 5/15 GL increase column (GE Healthcare). The column was washed with 2 CV of water followed by a wash with 2 CV of
FSEC buffer (50 mM potassium phosphate pH 7, 200 mM NaCl and 0.04% (w/v) DDM). Each sample (50 μL) was injected into the column and the eluate was monitored for GFP fluorescence (excitation wavelength (480 nm) and emission wavelength (530 nm)).

B.1.7: Large scale purification

Cultures were prepared according to the optimal growth conditions identified using the growth screens. LB supplemented with Km (50 μg/mL) was inoculated with *E. coli* strain C41 p7XC3GH-proP or C41 p7XC3H-proP and incubated at 37°C for 16-18 hours. The cultures were subcultured (1% (v/v)) into fresh TB medium (4 L) and after reaching an OD of 0.8, IPTG (0.7 mM) was added to the culture and growth continued for another 16-18 hours. After cultivation of the bacteria, samples were treated as described in Methods 2.5.2 to isolate cell membranes and solubilise protein.

ProP-His₁₀ and ProP-GFP-His₁₀ were purified by affinity chromatography followed by gel filtration chromatography as described in Methods 2.12.2. One additional step was added to the purification of ProP-GFP-His₁₀ to cleave the GFP-His₁₀ tag using human rhinovirus (HRV) 3C protease (Cordingley *et al*, 1989). After ProP-GFP-His₁₀ was purified using Ni-NTA resin and a buffer exchange was conducted to transfer the sample into GF buffer, HRV 3C protease (prepared by Cuesta Bernal J) was added to the sample at a 1:3 protein to protease molar ratio. The protein/protease mixture was incubated for 16 hours at 4°C with rotation.

The Ni-NTA resin (1 mL packed) was washed with water and buffer 1 as described in Methods 2.12.2 and was incubated with the protein/protease solution for 2 hours at 4°C. ProP was isolated by collecting the flow through when the resin and protein mixture was transferred to a 10 mL disposable column. The isolated ProP was concentrated to a volume of 500 μL and was then
purified by gel filtration chromatography as described in Methods 2.12.2 using a Superdex 200 10/300 GL increase gel filtration column (GE Healthcare).
B.2: Results

All experiments discussed below were not replicated unless otherwise indicated.

B.2.1: Optimization of Cultivation Conditions

Relative fluorescence values from growth screens 1 and 2 showed that ProP-GFP-His$_{10}$ is optimally expressed from the p7XC3GH vector in *E. coli* strain C41 ΔacrAB (Table B.2.1). Expression was highest when cells were grown in TB broth with 0.7 mM IPTG at 37°C overnight (O/N), which corresponds to 16-18 hours of growth post-induction (Table B.2.2). The identification of the optimal cultivation conditions was supported by in gel fluorescence analysis of cell suspensions, which showed that ProP-GFP-His$_{10}$ from cells grown as described above was expressed to levels that were higher or comparable to that in other samples cultivated at various growth conditions that had high relative fluorescence values (Figure B.2.1, lower).

GFP fluorescence can be used as an indicator of properly folded protein and it has been observed by Geertsma *et al.*, (2008) that comparison of in-gel fluorescence and anti-His Western blots can reveal the presence of unfolded protein (Geertsma *et al.*, 2008). The fluorescent protein observed by in-gel fluorescence will also appear on an anti-His Western blot and non-fluorescent, misfolded protein will also appear on the anti-His Western blot as a band above the band corresponding to the folded protein (Geertsma *et al.*, 2008). In Figure B.2.1, the band with a molecular weight of 72 kDa on the anti-His Western blot corresponds to the fluorescent band observed by in-gel fluorescence and therefore represents properly folded ProP. The second significant band observed on the Western blot with a molecular weight of roughly 85 kDa represents unfolded protein as the band is not observed by in-gel fluorescence. The proportion of unfolded ProP-GFP-His$_{10}$ was highest when ProP-GFP-His$_{10}$ was expressed from strain MC 1061 pBXC3GH-proP. The proportion of folded compared to unfolded ProP-GFP-His$_{10}$...
### Table B.2.1: Relative fluorescence values for growth screen 1

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<th>IPTG (mM)</th>
<th>C41 p7XC3GH-proP</th>
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### Table B.2.2: Relative fluorescence values for growth screen 2<sup>a</sup>

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<th>MC1061 pBXC3GH-proP</th>
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<sup>a</sup> MC1061 pBXC3GH-proP was induced with 0.02% (w/v) L-arabinose and C41 p7XC3GH-proP was induced with 0.7 mM IPTG.
Figure B.2.1: SDS-PAGE and Western blot analysis of ProP-GFP-His\textsubscript{10} isolated under optimal expression conditions. Samples from the best conditions identified by relative fluorescence screening were loaded onto an SDS-PAGE gel (Methods B.1.3). In-gel fluorescence (lower image) was used to image the SDS-PAGE gel prior to Western blotting using anti-His antibodies (upper image). This experiment was performed twice.
expressed from strain C41 p7XC3GH-proP was not greatly affected by the growth conditions; therefore, the optimal cultivation conditions identified above were employed for all future experiments.

**B.2.2: Optimization of detergent solubilisation conditions**

Membranes of *E. coli* strain C41 p7XC3GH-proP were solubilised using various detergents and FSEC was used to monitor the gel filtration profile (Figure B.2.2). Overall, the GF profiles were dominated by a single peak for all detergents used except CHAPS. The peaks observed when either OG or DDAO was used are shifted to the left compared to other profiles, with a retention time close to 6 minutes. This shorter retention time may indicate misfolding of the protein, so these detergents would likely be poor candidates for use in a purification. All other detergents tested appeared to be appropriate candidates for use in future purifications. The detergent DDM was selected for use in large scale purifications as it has previously been used to purify ProP-His$_6$ (Racher *et al*, 1999).

**B.2.3: Large scale purification**

The success of pre-purification screening was evaluated by performing large scale purifications using the optimized conditions identified through growth and detergent screens. Purifications were performed using strains C41 p7XC3GH-proP and C41 p7XC3H-proP, with the only difference between strains being that the former expresses ProP-GFP-His$_{10}$ whereas the latter expresses ProP-His$_{10}$. The gel filtration profile of ProP-GFP-His$_{10}$ showed a largely monodisperse peak with some shoulder regions (Figure B.2.3, upper). The GF profile of ProP-His$_{10}$ showed a broad peak that was not monodisperse (Figure B.2.3, lower). Overall, both purifications could
Figure B.2.2: Detergent screen to determine optimal solubilisation conditions for the purification of ProP-GFP-His\textsubscript{10}. *E. coli* strain C41 p7XC3GH-proP was cultivated and membranes were isolated as described in Methods B.1.6. The detergents (1% (w/v)) used for solubilisation are as follows: DDM (orange), digitonin (red), CYMAL 6 (green), DM (purple), OG (teal), CHAPS (light blue), Fos-choline-12 (dark blue), DDAO (pink). The solubilised protein was analyzed by FSEC and GFP fluorescence was detected.
Figure B.2.3: Gel filtration profile of purification of ProP (GFP-His\textsubscript{10} tag cleaved) and ProP-His\textsubscript{10}. Affinity purified ProP-GFP-His\textsubscript{10} treated with HRV 3C protease (upper image) or ProP-His\textsubscript{10} (lower image) was injected into a Superdex 200 increase gel filtration column and eluted with potassium phosphate (100 mM, pH 7.4) containing DDM (0.04% (w/v)). The absorbance at 280 nm was monitored as purification proceeded and 0.5 mL fractions were collected.
be further optimized to improve the monodispersity of the protein preparation; for example, different detergents could be tested, such as Fos-choline-12 or buffer compositions could be altered.

**B.3: Conclusions**

The optimal cultivation and solubilisation conditions identified through pre-crystallization screening was to use the *E. coli* strain C41 p7XC3GH-proP, grown in TB with 0.7 mM IPTG overnight at 37°C to generate membranes which were solubilised using DDM (1% (w/v). This led to the successful purification of ProP-GFP-His\textsubscript{10}, with the gel filtration profile showing that the protein was monodisperse (Figure B.2.3, upper). The growth and detergent screens also provided information as to what other conditions could be tested if further optimization of the purification of ProP-GFP-His\textsubscript{10} was desired, for example the growth temperature could be reduced or a different detergent, such as Fos-choline-12 or DM, could be used for solubilisation. The different gel filtration profiles obtained for ProP-GFP-His\textsubscript{10} and ProP-His\textsubscript{10} have shown that conditions optimized for the purification of ProP-GFP-His\textsubscript{10} were not optimal for the purification ProP-His\textsubscript{10}, even though the only difference between the proteins was the addition of GFP to the C-terminus of ProP.
References


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Figure 1.2 and Figure 1.6

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