A Uracil Transport Metabolon in *E. coli*:
Interaction between the Membrane Transporter UraA
and the Cytosolic Enzyme UPRT

by

Fan Xia

A thesis submitted in conformity with the requirements
for the degree of Masters of Science

Graduate Department of Biochemistry
University of Toronto

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2016

Abstract

The uracil-H\(^+\) symporter UraA and the uracil phosphoribosyltransferase UPRT are both encoded on the *ura* operon in *E. coli*, creating genetic and functional link between uracil import and its metabolism. Previous studies have determined the crystal structures of UraA and UPRT. However, it is unclear whether a physical interaction exists between UraA and UPRT. Such direct contact would allow channelling of the substrate uracil from the transporter UraA directly to the active site of UPRT, thereby creating a uracil transport metabolon. This study provides evidence for the physical interaction between UraA and UPRT. Manual docking of the two proteins demonstrates that the shape and charges on the potential interacting surfaces complement each other. Various biochemical techniques were used to test for a physical interaction between UraA and UPRT, including a pull-down assay, an electrophoretic mobility shift assay, a liposome floatation assay and bio-layer interferometry. A low-affinity and ionic strength-dependent interaction between UraA and UPRT was detected.
Acknowledgments

I would like to thank my supervisors Dr. Reinhart Reithmeier and Dr. Trevor Moraes for providing me with the opportunity to study and work in their labs. Their constant encouragement and guidance allow me to gain so much research and critical thinking skills that shines light on my research career. Starting from my undergraduate studies, they have been spending so much precious time and energy helping me both academically and personally.

I would also like to thank all the past and present members in the Reithmeier lab and the Moraes lab, especially Jing Li, Dr. Xiaoyun Bai, Chloe Rapp, Christine Lai, Dr. Charles Calmettes, Yogesh Hooda, Anastassia Pogoutse and Dr. Megha Shah. Without their technical training and advice, this work would not have been possible.

I am grateful to my committee members Dr. Walid Houry and Dr. Emil Pai for their helpful suggestions, intellectual insights, as well as challenging me to perfect my work.

I would like to extend my gratitude to Aunt Yang Hou for taking me to various trips, showing me breathtaking sceneries of Canada and making me feel like home.

Lastly, this work is dedicated to my parents. Without their support of my choice to study abroad, none of my scientific adventures would have been possible. Their unconditional love and faith in me provide me with infinite courage to continue my study and explore my future.
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<td>ACP</td>
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</tr>
<tr>
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<td>Anion Exchanger 1</td>
</tr>
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<td>BACterial Two Hybrid</td>
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<td>TBST</td>
<td>Tris Buffered Saline with Tween 20</td>
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<td>TransMembrane</td>
</tr>
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<td>UMP</td>
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<td>Wild Type</td>
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Chapter 1 Introduction
1.1 Metabolons

In 1985, Paul Srere proposed the term “metabolon” referring to a “supramolecular complex of sequential metabolic enzymes and cellular structural elements” (1). A metabolon consists of functionally related enzymes working cooperatively with each other, allowing efficient channelling of metabolites through the metabolic pathway (1, 2). Complexes formed by weak interaction between these enzymes enable efficient transfer of the metabolites from one active site directly to the next, preventing loss of precious intermediates (1, 2). Dynamic interactions allow regulation of the metabolons: the subunits may dissociate when the metabolic function of the metabolon is not in need, and reassemble when the pathway is needed. Early evidence of metabolons includes the organization of the glycolytic enzymes as well as those in the Krebs cycle bound to actin and the mitochondrial inner membrane, respectively (3, 4). Enzymes involved in lipid biosynthesis bound to the endoplasmic reticulum (ER) can also be considered as a metabolon (1). Association of the enzymes with the structural elements of the cell, such as the actin cytoskeleton and membrane surfaces, is thought to be important for the assembly and stability of metabolons (2). Figure 1.1 depicts a general model for a metabolon.
Figure 1.1 A general model for a metabolon. This model depicts a complex of five sequential enzymes comprising a metabolon. The complex (blue shapes) is attached to a structural element such as actin (black strand). Interactions between the subunits allow direct transfer of the metabolite from one enzyme to the next. The interactions might be dynamic: when substrate (S) is limited or the end product (P) of the pathway is not necessary for the cell, the enzyme complex may dissociate. The orange arrow indicates the path of the metabolite through the complex.

1.2 Membrane Transport Metabolons (MTM)

A membrane transport metabolon (MTM) involves a dynamic complex of a membrane transport protein importing a substrate into the cell for the associated enzyme(s) to metabolize (Figure 1.2). Embedded in the biological membrane, the membrane transport protein, or transporter, facilitates the movement of ions, small molecules or macromolecules across the hydrophobic lipid barrier. In a MTM involving an antiporter transporting molecules in opposite directions, the antiporter transports a product of the cytosolic enzyme reaction out of the cell in exchange for its substrate coming into the cell (Figure 1.2B).

Adapted from Figure 9 in Moraes, T. F., and Reithmeier, R. A. F. (2012) BBA. 1818, 2687 – 2706
**Figure 1.2 General models for membrane transport metabolons (MTM).** A membrane transport metabolon consists of a membrane transport protein and cytosolic enzyme(s). A) The transporter may operate as a symporter, importing a proton (bacteria) or a sodium ion (mammals) down its gradient and the energy is used to co-transport the substrate (S) into the cell. The imported substrate is channelled to its cognate enzyme to react with another substrate (X) and generate product (P) and another metabolite (Y). B) In a MTM involving an exchanger or an antiporter, the substrate (S) enters the cell in exchange for another compound, often the product (P) of the cognate enzyme. In this model, the imported substrate (S) is channelled to its cognate enzyme that utilizes another substrate (X) to produce product (P) and another metabolite (Y). In this case, the product (P) does not remain in the cell, but is exported in exchange for the substrate (S). The net result of this scheme is that the substrate (S) is converted to the product (P) outside the cell, while co-substrate (X) is converted to co-product (Y) inside the cell.

### 1.2.1 A bicarbonate transport metabolon

One well-studied MTM in humans is the bicarbonate transport metabolon consisting of carbonic anhydrase II (CAII) and human band 3 (anion exchanger AE1) in the red blood cell (Figure 1.3). Carbon dioxide generated from glycolysis and the Krebs cycle in the tissue cells diffuses into the red blood cell and together with water is converted into bicarbonate and a proton by CAII. The proton produced is buffered by hemoglobin, causing it to release oxygen from the red blood cell to the tissues (Bohr effect). The bicarbonate produced is transported out of the cell by AE1 in 1:1 electroneutral exchange for a chloride ion. As an erythrocyte Cl⁻/HCO₃⁻ exchanger, AE1 controls the entrance and exit of bicarbonate and together with CAII, regulates pH of the red blood cell and the CO₂-carrying capacity of blood (2, 5-7). When blood loaded with CO₂ in the form of HCO₃⁻ travels to the lung, the bicarbonate transport metabolon works in reverse to release CO₂ in exchange for O₂. Previous study has shown that CAII binds to a specific site (-LDDAD-) within the carboxyl-terminal tail of AE1 forming a complex (5, 6). Their physical interaction supports the idea of a bicarbonate transport metabolon. Working together, the complex of AE1 and CAII increases the efficiency of bicarbonate transport and creates a pH microenvironment around the transporter AE1 (2, 7). There is accumulating
evidence for bicarbonate transport metabolons involving the interaction of CAII with other bicarbonate and proton transporters (8).

Adapted from Figure 2 in Moraes, T. F., and Reithmeier, R. A. F. (2012) BBA. 1818, 2687 – 2706

**Figure 1.3 A bicarbonate membrane transport metabolon in the red blood cell.** Carbon dioxide (CO\(_2\)) released from the tissue cells diffuses into the red blood cell and react with water to produce a bicarbonate ion (HCO\(_3^-\)) and a proton (H\(^+\)). This reaction is catalyzed by carbonic anhydrase II (CAII). The produced proton is buffered by hemoglobin. The produced bicarbonate is transported out of the cell by human Band 3, also known as anion exchanger 1 (AE1) or solute carrier SLC4A1, in exchange for a chloride ion. This system increases the blood’s capacity to carry carbon dioxide as plasma bicarbonate.

### 1.3 Bacterial Membrane Transport Metabolons

Membrane transport metabolons (MTMs) also exist in bacteria. An advantage of studying MTMs in bacteria is that genes are organized into operons, suggesting organization at the genetic level. An operon is a transcriptional unit containing one or more genes under control of a single promoter. Transcription produces a single mRNA transcript that is translated into one or more proteins. Genes on the same operon usually encode proteins with related functions. Thus, analysis of bacterial polycistronic operons encoding transporters and cognate enzymes allows prediction of potential MTM components. However, these operons only provide suggestions and
guidance for the study of MTMs. It is also possible that transporters and metabolic enzymes from separate operons form MTMs (2). Previous bioinformatic analysis based on EcoCyc *E. coli* Database predicted that there are 45 polycistronic operons in *E. coli* encoding a transporter and enzyme(s) involved in metabolism of the imported substrate (2) (Table 1.1). Thus, polycistronic operons provide strong evidence for a genetic and functional linkage between transporters and their cognate enzymes. The transporters and metabolic enzymes encoded on each operon have the potential to physically interact with each other at the level of the membrane and form bacterial MTMs.

### Table 1.1 Polycistronic operons in *E. coli* encoding functionally related transporters and enzymes.

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<th>Transport protein</th>
<th>Enzymes</th>
<th>Enzyme activity</th>
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<td>abg</td>
<td>p-Aminobenzyloxy glutamate</td>
<td>AbgT</td>
<td>AbgA/B</td>
<td>p-Aminobenzyloxy glutamate hydrolase</td>
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<td>Acetate/glycolate</td>
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<td>YjcH</td>
<td>Inner membrane protein</td>
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<td>YbbW/Y</td>
<td>AllB</td>
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Transporters in bold letters have known structures. Adapted from Table 1 in Moraes, T. F., and Reithmeier, R. A. F. (2012) BBA. 1818, 2687 – 2706

1.4 Significance and Knowledge Gap

As complexes of sequential enzymes, metabolons ensure that the substrates and intermediates are channelled through specific metabolic pathways and prevent them from diffusing into the cytosol and entering other pathways. This compartmentalisation has obvious kinetic advantages and makes the metabolic systems specific and efficient (3). In smooth muscle, carbohydrate metabolism is compartmentalized, and metabolites from exogenous glucose are channelled through specific pathway, not mixing with the products of glycogen breakdown (9).

Since the stability of metabolons depends on their association with structural elements in cells such as cytoskeleton or membrane, disruption of these structures often results in dissociation of the subunits composing the metabolons. For example, solubilisation of a membrane with detergents may lead to dissociation of the enzymes from the transport protein. The dynamic and specific interactions between metabolon components are therefore hard to capture. In addition, association and disassembly of the structural-functional complexes are dependent on specific environmental conditions. Within the bicarbonate transport metabolon, binding between AE1 and CAII was increased under low ionic strength and acidic environment (5). So far, due to its dynamic nature, there is limited evidence for the physical interactions between metabolon components.
1.5 Uracil Transport Metabolon

Among the 45 *E. coli* operons listed in Table 1.1, the *ura* operon encodes for the uracil phosphoribosyltransferase UPRT (also known as its gene name upp) followed by the uracil-H$^+$ symporter UraA (Figure 1.4). UraA is an inner membrane transport protein with molecular mass of 45.1kDa, and UPRT is a cytosolic enzyme with molecular mass of 22.5kDa (Table 1.2) (Appendix 1). Together, UraA and UPRT are proposed to form a uracil transport metabolon (Figure 1.5), where UPRT converts imported uracil and cytosolic phosphoribosyl pyrophosphate (PRPP) into pyrophosphate (PP$i$) and uridine monophosphate (UMP) (Figure 1.6). UMP is then further synthesized into uridine triphosphate (UTP) or cytidine triphosphate (CTP). The reaction catalyzed by UPRT is a crucial part of the pyrimidine salvage pathway, where nucleobases are recycled from the RNA molecules degraded on the backbone and used for the synthesis of UTP and CTP to build new RNA molecules (Figure 1.7) (10). There are three sources of the substrate uracil for UPRT, one is the exogenous uracil transported into the cell by UraA, another is the uracil regenerated from degradation of RNA molecules in the pyrimidine salvage pathway, as well as the uracil synthesized *de novo* inside the cell (2, 11). Another uracil-H$^+$ symporter RutG exists in *E. coli* as listed in Table 1.1, and together with enzymes RutA-F form another uracil transport metabolon, allowing *E. coli* to use pyrimidines as the sole source of nitrogen (2). Uracil imported by UraA is channelled to pyrimidine biosynthesis, while the uracil imported by RutG is broken down and used as nitrogen supply (2). The specific transporter by which uracil enters the cell directs uracil to go through specific metabolic pathway, supporting the idea of a membrane transport metabolon. That is, the ultimate fate (anabolism versus catabolism) of the imported substrate depends on its entry pathway.
Figure 1.4 *E. coli* ura operon. Genomic context of UraA and UPRT (upp) based on EcoCyc *E. coli* Database shows that these two proteins are encoded on the same polycistronic operon, under control of a single promoter. Downstream of the *ura* operon, there is *hda*, encoding an ATPase regulatory factor involved in DnaA inactivation; upstream of the *ura* operon, there are *purM* and *purN* encode for a phosphoribosylamino-imidazole synthase and phosphoribosylglycinamide formyltransferase 1 respectively, which belong to the *de novo* purine biosynthesis pathway.

Table 1.2 General properties of UraA and UPRT.

<table>
<thead>
<tr>
<th>Name</th>
<th>Mass kDa</th>
<th>Length aa</th>
<th>pI</th>
<th>Structure</th>
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<td>429</td>
<td>8.3</td>
<td>3QE7</td>
<td>Inner Membrane</td>
<td>Dimer?</td>
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<tr>
<td>upp/UPRT</td>
<td>22.5</td>
<td>208</td>
<td>5.3</td>
<td>2EHJ</td>
<td>Cytosol</td>
<td>Tetramer</td>
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</table>

Structures of both UraA and UPRT are available in the PDB (PDB ID 3QE7 and 2EHJ respectively). The structure of UraA was solved as a monomer in detergent solution, but it might exist as a dimer in its native state in a lipid bilayer as explained in Section 1.5.1. UPRT is a tetramer consisting of a dimer of homodimers.
Figure 1.5 A uracil transport metabolon consisting of UraA and UPRT. UraA sits in the inner membrane transporting uracil into the cell driven by the proton gradient. Uracil is channelled to UPRT and reacts with phosphoribosyl pyrophosphate (PRPP) to produce uridine monophosphate (UMP) and pyrophosphate (PPi).

Figure 1.6 Reaction catalyzed by UPRT. The uracil phosphoribosyltransferase UPRT catalyzes the reaction of converting uracil and phosphoribosyl pyrophosphate (PRPP) into pyrophosphate (PPi) and uridine monophosphate (UMP). This is a critical step in the uracil salvage pathway.

**Figure 1.7 The pyrimidine salvage pathway.** The pyrimidine salvage pathway uses the nucleobase uracil, regenerated from degradation of RNA molecule backbones, to synthesize UTP and CTP, which are building blocks of RNA. The substrate and product of UPRT, uracil and UMP, are in red boxes, and UPRT is labeled as number 12.

### 1.5.1 Uracil-proton symporter UraA

In 2011, the structure of the *E. coli* uracil transporter UraA was released (Figure 1.8A) (12), sharing a similar 14 transmembrane (TM) fold to the transporter AE1 in the bicarbonate transport metabolon later solved in 2015 (13). UraA belongs to the nucleobase/ascorbate transporter (NAT) family, which is also known as nucleobase/cation symporter 2 (NCS2). The NAT/NCS2 family consists of secondary active transporters driven by ion gradients and exists in all kingdoms of life (12, 15). Transporters in the NAT/NCS2 family are responsible for the transport of nucleobases coupling a proton in bacteria, plants, fungi and transport of vitamin C
coupling a sodium ion in mammals (12, 14, 15). UraA is the first structurally known member of the family. The structure of UraA was solved by X-ray crystallography at a resolution of 2.8Å (Figure 1.8A) (12). It is observed as a monomer consisting of 14 TM segments divided into a 7+7 TM inverted repeats, with both amino and carboxyl termini in the cytosol. TM13 and TM14 are short helices located on the edge of the protein. Crystal packing of UraA shows close contact of TM13 between monomers, suggesting that UraA likely exists as dimer in the membrane (Figure 1.8B). In addition, the recently solved structure of a fungus homolog UapA, functioning as a purine-H\(^+\) symporter, is observed as a dimer (15).

The structure of UraA is in the uracil-bound form and is in an inward-facing open conformation. Parts of TM3 and TM10 consist of short anti-parallel β-strands at the center of UraA, important for the binding of the substrate uracil. The 14 TM segments are arranged into two domains: a core domain consisting of TM1-TM4 and TM8-TM11, a gate domain consisting of the other six TMs (Figure 1.8A). It was speculated that movement of the gate domain relative to the core domain in an alternating access mechanism allowed transport of uracil (12). The interface between the core and gate domains is hydrophobic, with acidic residues E241, H245 and E290 surrounding the uracil (12). Uptake of uracil by wild type (WT) UraA has a Michaelis constant \(K_M\) of 0.5µM (12). Single mutations of E241, H245 and E290 to alanine abolished UraA binding to uracil and thus, its uptake (Figure 1.8C) (12). These acidic residues may undergo series of protonation and deprotonation and play key roles in proton translocation, driving the transport of uracil (12).

With the crystal structure solved, molecular dynamics (MD) simulations have been performed to study the dynamics of UraA (16). Simulations were done in a native-like bacterial
lipid bilayer, and a preferential binding of anionic lipid cardiolipin (CL) to specific sites of UraA was discovered (16). CL consists of two phosphatidic acids connected by a glycerol backbone. In the CL molecule, only one of the two phosphate groups on the phosphatidic acids is protonated and form a resonance structure together with the hydroxyl group on the glycerol backbone under normal physiological conditions. This special property results in a single net negative charge of a CL molecule, which serves as a proton trap. In the MD simulations, CL in the outer leaflet of the membrane binds to a specific site on UraA close to the transport path, suggesting an important role of CL in capturing a proton and providing it to UraA, driving co-transport of uracil (16). In addition, the MD simulation of UraA was done in the absence of uracil and revealed a novel closed state of the protein (16). MD simulation of the UraA dimer in a lipid bilayer is underway by the same group.
**Figure 1.8 Crystal structure of UraA.** The structure of UraA monomer was solved at a resolution of 2.8Å (PDB ID: 3QE7) (12). Images were generated using PyMOL. A) Side, cytosolic and periplasmic views of UraA. The UraA monomer is colored in rainbow with violet as N-terminus and red as C-terminus. The uracil bound in the center of UraA is colored in magenta. Black circles in the cytosolic and periplasmic views indicate the core domains. B) The UraA dimer image with the periplasmic view is generated based on UraA crystal packing, coordinates available in PDB. Close contact of TM13, pointed by the black arrows, was observed between monomers. C) The UraA monomer placed in an angle to show the central binding site for uracil (colored in magenta). The side chains of the three key residues (E241, H245 and E290) critical for binding were colored in grey.
1.5.2 Uracil phosphoribosyltransferase UPRT (upp)

Although there is currently no published work, the crystal structure of UPRT in \textit{E. coli} is available in PDB at a resolution of 2.8Å (Figure 1.9) (17). The UPRT structure from \textit{E. coli} conforms well to the other known UPRT structures including those from \textit{S. solfataricus} and \textit{T. gondii} (11, 18). All three structures obey the typical phosphoribosyltransferase (PRT) fold which cores consist of \( \alpha \) helices sandwiching a five-stranded parallel \( \beta \) sheet (11, 18). The structure of \textit{E. coli} UPRT is solved as a dimer of homodimers. Each monomer contains an active site, and the binding pocket is on the side of the monomer, away from the interacting surfaces of the oligomers. A highly conserved aspartic residue is found in the binding pocket of uracil, and based on UPRT alignment among the microorganisms, it corresponds to D199 in \textit{E. coli} UPRT (11). D199 is likely the key residue in the active site of \textit{E. coli} UPRT, because the corresponding aspartic residues mutated in \textit{S. solfataricus} UPRT (D209) and \textit{T. gondii} UPRT (D235) resulted in catalytically inactive enzymes (11, 18). In the kinetics study of \textit{E. coli} UPRT, its \( K_M \) for uracil was found to be 0.53\( \mu \)M and for PRPP was 58\( \mu \)M (19). It follows a sequential mechanism with PRPP binding before uracil binding (19). Divalent metal ion \( \text{Mg}^{2+} \) was found to be a cofactor and is required for the stability and efficient activity of \textit{E. coli} UPRT (20, 21). GTP was found to be an allosteric activator for the UPRT of \textit{E. coli} and \textit{T. gondii}, stabilizing the tetrameric state of the enzyme (18, 20, 21). Addition of GTP significantly reduced \( K_M \) for PRPP in \textit{E. coli} UPRT (20). In \textit{T. gondii} UPRT, GTP molecules bind to the positively charged pockets at the interacting surfaces of the oligomers (18).
Figure 1.9 Crystal structure of UPRT. The structure of UPRT tetramer was solved at a resolution of 2.8Å (PDB ID: 2EHJ) (17). Images of the top/bottom and side views were generated using PyMOL. Monomers are depicted in different colors. In the top/bottom view, the active sites are circled in red, with the side chain of the key residue D199 colored in grey. Each monomer contains an active site, but only two of them are visible from one side.
1.6 Objective and Hypothesis

As discussed above, the physical interaction between a transporter and a cognate enzyme makes the metabolon system more efficient by allowing direct entry of the imported substrate into the metabolic pathway. This prevents the loss of precious intermediates often present at low concentration. However, evidence for a direct interaction of transporters and enzymes forming metabolons is lacking. The objective of this study is to provide structural evidence for such direct interaction.

The uracil MTM consisting of UraA and UPRT was chosen for this study because both proteins can be readily expressed in bacteria and their crystal structures have been determined. In addition, the Reithmeier lab has been working on human Band 3, sharing similar 14 TM 7 + 7 inverted repeat structure as UraA. Preliminary studies were also performed with two other potential MTMs: the lysine-dependent acid resistance (LDAR) system, consisting of the lysine/cadaverine antiporter CadB and the lysine decarboxylase CadA, the arginine-dependent acid resistance (ADAR) system, consisting of the arginine/agmatine antiporter AdiC and the arginine decarboxylase AdiA. Both MTMs are important acid resistance systems in E. coli, as the decarboxylases consume a proton each time they remove the carboxyl group from the imported amino acids (22, 23).

There is evidence for a functional linkage between uracil uptake and metabolism. Previous studies have shown that the uracil uptake level in E. coli is significantly reduced with deletion of upp gene encoding UPRT (Figure 1.10) (24). Blocking uracil metabolism inside the cell may result in decreased uracil uptake due to a smaller uracil gradient. In addition, UPRT and
UraA are encoded on the same operon and under control of a single promoter. The genetic and functional linkage between UraA and UPRT indicates that they likely form a membrane transport metabolon, important for uracil uptake and metabolism in *E. coli*. The hypothesis of this study is that the metabolic enzyme UPRT interacts directly with its genetically and functionally linked transporter UraA, so that the imported substrate uracil is directly channelled from UraA to the active site of UPRT, enhancing substrate uptake and metabolic efficiency.


**Figure 1.10 Effect of UraA and upp gene deletion on *E. coli* uracil uptake.** $^{14}$C-labelled uracil uptake in cells with *UraA* gene deleted (encoding uracil-proton symporter UraA) and/or with *upp* gene deleted (encoding uracil phosphoribosyltransferase UPRT) was compared to that in wild type cells. With UraA and UPRT both present, uracil uptake was in high level (circle). With *upp* gene knocked out, uracil uptake reduced significantly (square). With *UraA* gene knocked out, uracil uptake was lost almost completely (triangle), indicating that UraA is the primary import pathway for uracil in *E. coli.*
1.7 Protein-Protein Interaction (PPI) Methods

Various methods can be used to test PPI as previously reviewed (25, 26). Here, I am listing some popular methods with the potential to detect the physical interaction between UraA and UPRT in three categories: *in vitro, in vivo*, and *in silico*.

1.7.1 *In vitro* – Biochemical – Preliminary Test of PPI

1.7.1.1 Electrophoretic Mobility Shift Assay (EMSA)

Gel electrophoresis is a common technique used to separate macromolecules based on their sizes, charges and shapes to detect purity and integrity of the protein of interest. In an electrophoretic mobility shift assay (EMSA), or a gel shift assay, native gels are used, devoid of the denaturing detergent sodium dodecyl sulfate (SDS), allowing the protein of interest to be maintained in its native state and subunits within protein complexes to remain associated with each other. EMSA is often used to detect protein-nucleic acid interactions (27), but might be useful to detect PPI if the binding is strong enough. To detect an interaction between two proteins, a constant amount of one protein is incubated with increasing molar ratio of the other protein. If the proteins interact with each other, a new band representing the protein complex would appear on the gel with increasing intensity as the molar ratio increases. In addition, the intensity of the band representing the protein that is kept constant is expected to gradually decrease with increasing molar ratio of the other protein. Figure 1.11 compares the expected observation of positive and negative results. This method requires purification of the interacting proteins and a suitable mobility during electrophoresis, but relatively small amounts of proteins are required.
**Figure 1.11 Expected positive and negative results of EMSA.** A constant amount of purified Protein 1 is mixed with increasing molar ratio of purified Protein 2 and the mixtures are run on a native gel. The green arrow indicates the position of Protein 1. The red arrow indicates the position of Protein 2. The blue arrow indicates the position of the protein complex. On the left shows the expected result of two proteins interacting with each other: the band intensity of Protein 1 decreases as more and more Protein 2 is added in the solution, while a band representing the protein complex gradually appears. On the right shows the expected result of two proteins not interacting with each other: the band intensity of Protein 1 stays constant as more and more Protein 2 is added in the solution.

### 1.7.1.2 Affinity Chromatography (Pull-down Assay)

In a “pull-down” assay, cells expressing proteins of interest are harvested and lysed. Affinity chromatography is performed to purify the target protein. The target protein is tagged through genetic modification, and the tag provides high-affinity binding of the protein to specific resins and gets “pulled down”. Other proteins associating with the target protein will also get “pulled down”. To detect the interacting partners of the target protein, immunoblot can be used for proteins of known identity and Mass Spectrometry (MS) can be used to identify the unknown proteins. However, this method involves multiple washing procedures and is not ideal to capture weak or transient interactions. Alternatively, both interacting proteins with different tags can be co-expressed in the same cells. The affinity of the interaction must be high enough to survive dilution during cell lysis and affinity purification, and in the case of membrane proteins, solubilisation of the membrane.
1.7.1.3 Co-immunoprecipitation (Co-IP)

In Co-IP, the idea is similar to the “pull-down” assay, except an antibody against the target protein is used instead of affinity resins to capture the target protein. Antibodies are often highly specific for their target proteins. Proteins interacting with the target protein are immunoprecipitated together and can be detected by immunoblotting. Tags can be added to the proteins to facilitate their detection. Compared to using antibodies against the target protein themselves that may block the interaction sites, using antibodies against tags may be more ideal for Co-IP.

1.7.1.4 Chemical Cross-linking

When studying transient PPI, cross-linking is a great choice because this technique can covalently link interacting proteins in situ, increasing the chance of detecting the protein complex under native conditions. However, no single cross-linker is ideal for every protein, screening of various cross-linkers should be performed considering their membrane permeability, size, functional group and reaction conditions. Smaller cross-linkers such as formaldehyde and glutaraldehyde should be used to ensure the physical interaction is of close contact between the proteins and minimize non-specific cross-linking. In addition, crosslinking of protein components within stable complexes must be distinguished from cross-linking due to transient or random collisional events.

1.7.1.5 Liposome Floatation Assay

In the liposome floatation assay, the membrane protein of interest is reconstituted into liposomes and then is subjected to sucrose floatation ultracentrifugation as previously described (28). The reconstituted proteoliposome is mixed with the soluble protein, either purified or in
crude extracts. The mixture is added to high percentage sucrose solution, with decreasing percentages of sucrose solution layered on top in an ultracentrifugation tube. Since the proteoliposome consists of low-density lipid bilayer, it floats to the top in the low-density sucrose fraction. If the soluble protein interacts with the membrane protein, it would float to the top together with the proteoliposome; otherwise, it would remain at the bottom in the high percentage sucrose solution. Immunoblot can be performed to detect the presence of membrane protein and the soluble protein at each fraction of the sucrose gradient. Figure 1.12 depicts the schematic of liposome floatation assay. Liposomes mimic the native environment of a membrane protein better than detergents, so liposome floatation assay is a great method to study PPI when a membrane protein is involved. The reconstituted protein is likely inserted into the liposome in two orientations with the interacting surface facing inside or outside of the liposome. In addition, a weak interaction may result in dissociation of the interacting proteins during ultracentrifugation with the proteins distributed throughout the gradient.

**Figure 1.12 Schematic of the liposome floatation assay.** In the liposome floatation assay, the membrane protein is inserted into the liposome and incubated with its potential interacting partners. The mixture is put at the bottom of the tube with the highest sucrose percentage in a sucrose gradient. Ultracentrifugation is performed to float the proteoliposome onto the top of the sucrose gradient with low sucrose percentage. Soluble proteins that interact with the membrane protein are pulled to the top; otherwise, they stay at the bottom of the tube. Blue rectangles represent the membrane protein, embedded in the double circle representing the liposome. The red circles represent the soluble proteins as potential interacting partners of the membrane protein.
1.7.1.6 X-ray Crystallography

Co-crystallizing interacting proteins and solve their structures in complex is a very convincing way to show the interaction between two proteins. The structure of a complex between the soluble domain of DauA (YchM), a putative bicarbonate transporter, and acyl carrier protein (ACP) was discovered by X-ray crystallography (29). Their physical interaction links bicarbonate transport and fatty acid metabolism (29). However, the concentration of proteins used for crystallography is very high and may not be representative of protein concentration within a cell. Protein complexes are much more dynamic and heterogeneous than a single homogeneous molecule and therefore, are generally much harder to crystallize. Adding inhibitors, introducing mutations and incubating with stabilizing antibodies might be necessary to lock the complexes in single conformation. Although difficult to achieve, a crystal structure of the protein complex not only provides evidence for the physical interaction, but also gives detailed structural information on how the two molecules interact, which would not be available with other methods. This structural information could be used to disrupt the interaction by mutating key residues on the interacting surfaces and determine the consequences of losing interactions on substrate uptake and metabolism.

1.7.2 In vitro – Biophysical – Obtaining Binding Kinetics

1.7.2.1 Bio-Layer Interferometry (BLI)

BLI is a biophysical method allowing fast and convenient measurement of binding kinetics (Figure 1.13) (30). The Octet Red instrument available from ForteBio measures the change in interference pattern of white light reflected from the reference layer and the biosensor
tip. Binding of molecules to ligands immobilized on the biosensor tip are monitored in real time based on change in interference pattern due to change in the thickness of the biosensor tip. To test PPI, one protein is immobilized onto the biosensor tip. The biosensor tip is coated with Ni, Streptavidin or antibodies to capture the correspondingly modified protein. The loaded biosensor tip is then dipped into solution of the other protein to measure association rates, followed by incubation in buffer to measure dissociation rates. The combination of association and dissociation rates allows accurate calculation of affinities. In addition, the immobilized ligand can be dipped into solutions with various pH values, salt concentrations and other conditions (e.g. presence of substrates or inhibitors) to determine their effect on the interaction. In contrast to other methods such as “pull down” assays that involve washing steps, BLI allows an equilibrium state to be reached on the time course of the experiments. A major factor in dealing with membrane proteins is the need to include detergents to keep the protein soluble and in a native conformation. Thus, if the binding components include a membrane protein, the binding involves the protein and the associated detergent micelle.

Figure 1.13 Schematic of BLI. In BLI, the ligand (purple circle) is immobilized onto the biosensor tip surface coated with antibody (dark purple Y shape) or other molecules (e.g. streptavidin) to capture the ligand. The loaded biosensor tip is then incubated in the solution of analyte. Binding of the analyte to the ligand changes the thickness of the biosensor tip, causing change in the reflection pattern of the incident white light from the biosensor tip compared to its reflection from the reference layer. Change in the position of the reflected wavelength is recorded as a function of time and converted to association and dissociation curves to generate binding kinetics.

1.7.2.2 Micro-Scale Thermophoresis (MST)

MST is a quick, easy to prepare and immobilization-free method available from NanoTemper Technologies to detect interactions between biomolecules (Figure 1.14) (31). The NT.115 MST instrument available from NanoTemper Technologies uses an infrared laser to generate a temperature gradient in multiple capillaries containing constant amount of a fluorescently labeled protein with increasing amount of its potential interacting partner. Thermophoresis, the movement of molecules along temperature gradient, would differ significantly between a single protein molecule and a protein complex due to changes in size, charge and entropy. The change in thermophoresis is recorded by the MST machine and used to quantify binding kinetics.
Adapted from NanoTemper Technologies MST guide (2013)

Figure 1.14 Schematic of MST. Initially, fluorescently labeled protein distributes evenly and diffuses freely with its unlabeled potential interacting partner in the solution inside the capillaries. An infrared beam (red ball) is fired onto the protein samples, causing quick diffusion of the proteins away from the heat source. The machine records the diffusion as a quick drop of the fluorescence (red line). After reaching the steady state, infrared laser is turned off, allowing the biomolecules to diffuse back to a homogeneous distribution (sharp increase of fluorescence signal). Interaction of proteins causes change of size, hydration shell, and thus, thermophoresis of the fluorescently labeled protein, which is recorded by the MST machine to measure association rates.

1.7.3 In vivo – PPI under Physiological Conditions

1.7.3.1 Bacterial Two Hybrid (BACTH)

In BACTH, two sub-domains of adenylate cyclase catalytic site from \textit{B. pertussis} are fused to the two proteins in question, respectively. These two fusion proteins are expressed in an adenylate cyclase knockout \textit{E. coli} strain (Figure 1.15) (32). If the two proteins have close
contact with each other, the two fragments of adenylate cyclase catalytic site are brought together to catalyze cyclic AMP (cAMP) synthesis. Generation of cAMP can be detected using a β-galactosidase assay. This method is also good to test transient interactions because as soon as the two fragments are brought together, cAMP is generated to activate the reporter gene. The advantage of this method is the ability to perform sensitive and high-throughput screens within a cellular context. This technique could be used to screen potential interacting partners of membrane transport proteins encoded in polycistronic operons or to determine whether an interaction between a transporter and its cognate enzyme can be detected in situ.


Figure 1.15 Schematic of BACTH. In BACTH, the target proteins and adenylate cyclase catalytic site sub-domains are fused and expressed in adenylate cyclase knockout E. coli. In the diagram, X and Y represent proteins interacting with each other, bringing sub-domains of adenylate cyclase T25 and T18 to close proximity. T25 and T18 together make a complete adenylate cyclase catalytic domain that synthesizes cAMP. Together with catabolite activator protein (CAP), cAMP turns on the expression of reporter genes, such as β-galactosidase. Thus, the interaction of the target proteins can be indicated by β-galactosidase activity.
1.7.3.2 Fluorescence Resonance Energy Transfer (FRET)

FRET utilizes energy transfer from an excited donor fluorophore to an acceptor fluorophore (Figure 1.16) (25). This energy transfer only occurs if the fluorophores are brought to close proximity (2-8nm). To test PPI, the two proteins in question are fused to the donor and acceptor fluorophores respectively and co-expressed in cells. A sensitized emission from the acceptor would be detected if the two proteins interact with each other and brings the fluorophores together. However, the fluorescent tags fused to the proteins should not interfere with the interaction.


Figure 1.16 Schematic of FRET. The diagram illustrates basic principle of FRET. Two proteins (blue cone and orange ball) interact with each other, bringing the fluorophores fused to them respectively to close proximity and allowing energy transfer from the donor fluorophore (blue cylinder) to the acceptor fluorophore (yellow cylinder). Interaction between proteins is detected based on detection of acceptor emission.
1.7.4 *In silico* – PPI Prediction

1.7.4.1 Macromolecular Docking

With structures solved, proteins of interest can be docked manually on computer using Coot and PyMOL programs to predict how they interact and to model protein complexes. Software with pre-written algorithms are also available for macromolecular docking, such as Rosetta Dock (Gray lab, Johns Hopkins University) and DOT (University of California at San Diego Supercomputer Center). However, with rigid-body docking, the molecular details of the interacting surface may not be accurately revealed to the full extent.

1.7.4.2 Molecular Dynamics (MD)

Although it demands high performance computing facility that is not commonly available and requires months of intense simulations, MD is able to predict how the proteins interact in its native environment (33). As the name suggests, MD can present PPI dynamically with animations. Compared to building static protein complex, macromolecular docking by MD allows PPI prediction in biological context and provides insights on the molecular details of the interactions.
Chapter 2 Materials and Methods
2.1 Bacterial Strain

BL21(DE3) competent *E. coli* cells (NEB) were used for the expression of proteins in this study. The BL21 strain originated from *E. coli* B strain. BL21(DE3) cells contain the lambda DE3 prophage, encoding a T7 RNA polymerase highly specific for the T7 promoter. On the other hand, the endogenous RNA polymerase in the host cell would not recognize the sequence of the T7 promoter. This feature allows specific expression of recombinant proteins under control of the T7 promoter to a high level in BL21(DE3) cells.

2.2 pET Expression System

Isopropyl β-D-1-thiogalactopyranoside (IPTG, Bioshop), an analog of lactose metabolite allolactose, triggers expression of T7 RNA polymerase under control of the *lacUV5* promoter, and thus, expression of the recombinant proteins under control of T7 promoters.

2.3 Plasmids

The UraA gene was cloned and inserted into pET21a vector. It was kindly provided by Dr. Nieng Yan from Tsinghua University in Beijing, whose group solved the structure of *E. coli* UraA. UraA gene is under control of T7 promoter and followed by a C-terminal 8x His tag (UraA-His). The UraA plasmid also contains ampicillin resistance gene, allowing selection of cells successfully transformed with the plasmid (pET21a UraA Ct. His AmpR).

The UPRT/upp gene was originally cloned onto a pCA24N vector, kindly provided by Dr. Naoki Kunishima from RIKEN Research Institute in Japan, whose group solved the structure of *E. coli* UPRT. Polymerase chain reaction (PCR) was conducted to amplify the UPRT/upp...
gene with Strep tag II sequence. The sequence of the forward primer was 5’- GGA GAT ATA CAT ATG AAG ATC GTG GAA GTC AAA CAC CC -3’ (NdeI cutting site is in blue), and the sequence for the reverse primer was 5’- GGT GCT CGA GTG CGG CCG CTT ACT TTT CGA ACT GCG GGT GGC TCC AAA GCT TTT TCG TAC CAA AGA TTT TGT C -3’ (NotI cutting site is in blue, Strep tag II sequence is underlined). The PCR product was gel purified, digested with NdeI and NotI (NEB), and ligated to pET41b pre-treated with NdeI and NotI. The ligation product was transformed into DH5α cells for amplification and purified using Mini-prep kit (QIAGEN). To confirm the sequence, the construct was sequenced by ACGT Corp using T7 promoter and terminator primers. Expression of the UPRT/upp gene on pET41b is also under control of the T7 promoter, and the upp gene is followed by the sequence encoding Strep tag II (WSHPQFEK). So UPRT expressed from this plasmid is C-terminally Strep tagged (UPRT-Strep). The UPRT plasmid also contains kanamycin resistance gene, allowing selection of cells successfully transformed with the plasmid (pET41b UPRT Ct. Strep KanR).

For BLI experiments, C-terminally BAP-tagged UPRT was used. BAP stands for Biotin Acceptor Peptide (MSGLNDIFEAQKIEWHE) and is the minimum sequence required for the enzyme BirA in E. coli to recognize it and add a biotin moiety to the lysine residue on the tag (34). Thus, the BAP-tagged UPRT was biotinylated in vivo (34). With the pET41b UPRT-Strep construct as template, back-to-back primers were designed to add the BAP sequence and amplify the whole plasmid in the PCR. The sequence of the forward primer was 5’ – CTC AGA AAA TCG AAT GGC ACG AAT AAT TGA TTA ATA CCT AGG CTG C – 3’ (later half of the BAP DNA sequence is in blue), and the sequence for the reverse primer was 5’ – CCT CGA AGA TGT CGT TCA GAC CAA GCT TTT TCG TAC CAA AG – 3’ (first half of the BAP DNA
The PCR product was then treated with DpnI (NEB) at 37°C for 1 hr to digest the methylated parental plasmid before transformed into the cells.

### 2.4 Cell Growth and Protein Expression

The UraA and UPRT plasmids were transformed separately and together into BL21(DE3) cells with 42°C heat shock for 45 seconds. The transformed culture was plated on agar Luria-Broth (LB) plate with necessary antibiotics (100 µg/mL ampicillin and/or 50 µg/mL kanamycin, Sigma). Antibiotic-resistant colonies grown were picked and inoculated into 50 mL liquid LB media to grow to an OD$_{600}$ of 0.3 at 37°C. 0.5 mM IPTG was added into the culture to induce protein expression, and the cell culture was further grown for overnight at 20°C.

### 2.5 Cell Lysis

Cells were harvested by centrifugation at 4000 rpm for 30 min. Cell pellet collected from the 50 mL liquid culture was re-suspended in 1 mL Tris buffer (150 mM NaCl, 25 mM Tris, pH 8.0). Cells were then lysed by sonication with a 3 mm diameter probe at 80% vibration amplitude and 3 cycles of 45 sec on/1 min off. The samples were kept on ice throughout the sonication procedure.

### 2.6 Protein Purification

Lysed cells were centrifuged at 13000 rpm for 30 min to remove cell debris. After centrifugation, the supernatant (~1 mL) was collected, and for samples expressing the membrane protein UraA, 1% n-dodecyl β-D-maltoside (DDM, Anatrace) was added to the solution and incubated at 4 °C with shaking for 15 min. Ni-nitrilotriacetic acid (NTA) agarose beads (QIAGEN) and Strep-Tactin Sepharose beads (IBA), 50 µL for each sample, were equilibrated
by incubating the beads in 1 mL Tris buffer for 5 min with shaking at 4 °C twice. The protein solution was added to the pre-equilibrated beads and incubated with shaking for 1 hr at 4 °C. Samples containing UraA-His were added to Ni-beads, and samples containing UPRT-Strep were added to Strep-beads. Centrifugation at 4000 rpm for 1 min was performed to pellet the beads and the supernatant (flow through) was removed. To wash the beads, 1 mL Tris buffer was added to the bound beads with 10 min of incubation at 4 °C for 3 times. For the UraA-His bound beads, the Tris buffer for washing also contains 0.05% DDM and 30 mM imidazole. After removing the washing Tris buffer from the settled beads, the proteins were eluted by incubating with eluting Tris buffer at 4 °C for 20 min. To elute UraA-His from Ni-beads, the Tris buffer contains 0.05% DDM and 250 mM imidazole. To elute UPRT-Strep from Strep-beads, the Tris buffer contains 5mM d-desthiobiotin. To remove imidazole from UraA-His and desthiobiotin from UPRT-Strep, the eluted proteins went through pre-equilibrated Zeba spin desalting columns (Thermo Fisher).

### 2.7 Gel Electrophoresis and Immunoblots

To test the yield and purity of the proteins obtained as well as to detect the presence of the target protein in various samples, gel electrophoresis was performed using a mini-gel Bio-Rad apparatus. 2x sample buffer (0.13 M Tris, pH 6.8, 20% glycerol, 2% mercaptoethanol, bromophenol blue) with 4% sodium dodecyl sulfate (SDS) was added to each sample in 1:1 ratio. Samples were run on 10% polyacrylamide gels in gel running buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS) at 0.1 amps per two gels for 1 hr. For native gels, SDS was eliminated from all the solutions and buffer system. The gels were then stained with Coomassie Blue R-250 to test protein purity, or underwent immunoblotting to confirm protein identity.
For immunoblotting, proteins on the gels were transferred to a nitrocellulose membrane in transfer buffer (25 mM Tris, pH 8.1, 192 mM glycine, 20% methanol) at 0.3 amps per two membranes for 1 hr. After transfer, membranes were incubated in blocking buffer (3% skim milk dissolved in Tris buffered saline with 0.1% TWEEN 20, TBST) for 1 hr. The membranes were washed with TBST three times for a total of 20 min. The blocked membranes were then incubated in 1:5000 mouse anti-His antibody (Thermo Fisher) to detect UraA-His or 1:5000 mouse anti-Strep antibody (IBA) to detect UPRT-Strep for 1 hr. The membranes were then washed with TBST three times for a total of 30 min, followed by incubation in 1:5000 horseradish peroxidase (HRP) conjugated anti-mouse antibody (Cell Signaling) for 1 hr. All the antibodies were diluted in TBST. After washing in TBST three times for a total of 30 min, enhanced chemiluminescence reagent (GE Healthcare) was added to the membrane. Kodak scientific imaging films were exposed to the membranes to capture the emission of light from the protein bands. The exposed films were processed by automatic film developer (KONICA MINOLTA) to visualize the protein bands.

2.8 Electrophoretic Mobility Shift Assay (EMSA)

Separately expressed and purified UraA-His and UPRT-Strep were mixed in various molar ratios (0:1, 0.5:1, 1:1, 2:1, 1:0). The mixtures were incubated at 4 °C overnight. Samples were run on native gels at pH 8.8 with a running buffer of pH 9.0 to determine whether a protein complex could be detected. Immunoblots of anti-His and anti-Strep were performed to confirm the identity and positions of the bands representing UraA-His and UPRT-Strep respectively. Bovine serum albumin (BSA), with molecular weight of 66.5 kDa and pI of 4.7, was used as a protein ladder on the Coomassie stained native gel.
2.9 Affinity Pull-down Assay

UraA-His and UPRT-Strep were co-expressed in BL21(DE3) cells grown in LB media containing both ampicillin and kanamycin. The proteins were purified using Ni- and Strep-affinity chromatography as described in section 2.6. Anti-His and anti-Strep immunoblots were performed to detect if UraA-His and UPRT-Strep could be pulled down by Ni- and/or Strep-beads.

2.10 Liposome Floatation Assay

In the liposome floatation assay, UraA-His was reconstituted into liposomes, mixed with UPRT-Strep to determine whether the enzyme could associate with the transporter and thus float in a sucrose gradient with the proteoliposome. His-tagged UraA was purified as described above. Purified UraA was incubated with Biobeads SM-2 Adsorbents (Bio-Rad) at 4 °C overnight to remove the detergent DDM from UraA, together with Avanti polar lipids extract from E. coli (67% PE, 23% PG, 10% CL) to insert UraA into liposomes. Purified UPRT-Strep or cell lysate containing UPRT-Strep was then added to the reconstituted UraA-liposome and incubated at 4 °C for 2 hr. Sucrose was then added to the mixture to make 1 mL 60% sucrose solution, followed by 3.5 mL 30% sucrose and 0.5 mL 0% sucrose solutions layered on top of each other in the ultracentrifugation tube. Ultracentrifugation was then performed at 48,000 rpm for 1 hr at 4 °C in a Beckman 50 Ti swinging bucket rotor. Ten fractions, 0.5 mL each, were taken by pipetting from top to bottom of the ultracentrifugation tube. Samples from the ten fractions were taken to perform immunoblots to detect the presence of UraA-His and UPRT-Strep at each fraction. UPRT-Strep was also incubated with protein-free liposome as negative control and went through
the same experimental procedure. After ultracentrifugation, liposomes could be visualized in the top most fractions as a white cloudy layer.

2.11 Bio-Layer Interferometry (BLI)

BLI was performed to quantitatively measure the interaction between UraA and UPRT under equilibrium binding conditions. To test the interaction between UraA and UPRT, the streptavidin biosensor tip was dipped into the cell lysate to pick up BAP-tagged UPRT. The loaded biosensor tip was washed and then incubated with increasing concentrations of purified UraA-His to measure association rates, followed with incubation of the tip in washing buffer to measure dissociation rates. Another loaded sensor was incubated with washing buffer without UraA throughout the experiment and the signals generated acted as baseline and was subtracted from the experimental data. All binding reactions were carried out in Tris buffer with 0.05% DDM, pH 8.0. To test the effect of ionic strength on UraA-UPRT interaction, Tris buffer with 0, 50, 150 and 300 mM NaCl was used. Microsoft Excel and GraphPad Prism were used for data analysis. After correction with baseline signal and normalization to zero, binding data were fitted to a 1:1 Langmuir isotherm model to generate $K_D$ values.

2.12 Macromolecular Docking

The dimeric interface of UraA was predicted using the crystal contacts present in the structure of *E. coli* UraA (PDB ID: 3QE7) (12). Coordinates of two monomers with close TM13 contact were saved as the predicted UraA dimer. *E. coli* UPRT used for docking was in tetrameric state as shown in the crystal structure (PDB ID: 2EHJ) (17). To investigate the possibility of UraA-UPRT interaction, and to predict the geometry of the macromolecular complex, both PDB coordinates of dimeric UraA and tetrameric UPRT were loaded into the
molecular visualization software Coot. The UraA dimer was in a fixed orientation, with its cytosolic side facing the UPRT tetramer. The UPRT tetramer was manually re-oriented to complement the shape the charges of the cytosolic face of UraA dimer. Coordinates of the plausible 3D structure of the macromolecular complex were saved and loaded onto PyMOL for visualization and image generation.
Chapter 3 Results
3.1 Macromolecular docking showed possible interaction between UraA and UPRT.

The crystal structure of UraA is in uracil-bound inward facing conformation, in a state ready to release the substrate to the cytosol, and is likely the conformation interacting with the downstream enzyme UPRT. UapA is a fungus homolog of UraA, and its crystal structure was recently solved as a dimer (15). Close contact between monomers by TM13 indicates that UraA also likely exists as a dimer in its native membrane-bound state (Figure 1.8B). Coordinates of the UraA dimer and the UPRT tetramer were loaded onto Coot software to model the structure of the macromolecular complex. Manual docking to complement the shape and charges of the potential interacting surfaces of UraA and UPRT was performed. Figure 3.1A shows the side view of the predicted UraA-UPRT complex. UraA dimer sits in the inner membrane, with its cytosolic side interacting with UPRT tetramer. Two monomers in the diagonal of the UPRT tetramer have a pair of anti-parallel \( \beta \)-strands (T49-I66) facing out of the concave curvature of the cytosolic side of UraA dimer. These loops may be involved in mediating the interaction between UraA and UPRT. Two pairs of basic residues (R4, R299) and acidic residues (D122, E123) on a UraA monomer and a UPRT monomer respectively are shown as sticks. These residues with opposite charges are in close contact of each other and have the potential to form salt bridges. Other charged residues on the predicted interacting surfaces of UraA and UPRT that might also contribute to their ionic interaction include basic residues R177, K254, K255, K362 on UraA and acidic residues E48, E50, E55, E62, E105, E106, E189 on UPRT. A dimeric UraA structure would allow a higher affinity interaction with UPRT due to an avidity effect.

Figure 3.1B shows the electrostatic potential images of the interacting surfaces of UraA and UPRT. UraA interacting surface mostly consists of positively charged residues (blue) as
listed above, sandwiching a region of negatively charged residues (red) facing out towards the positively charged pocket of UPRT. Surrounding the pocket in the middle of UPRT are mostly negatively charged residues as listed above. The shape and electrostatic properties of the UraA and UPRT interacting surfaces complement well with each other, supporting the idea that these might be the interacting surfaces. Figure 3.1B also illustrates complementation in term of the size. The double-headed arrows with the number in Angstroms illustrate the distance of the key residues involved in uracil binding between UraA monomers (E290, 45Å) and between UPRT monomers (D199, 35Å and 49Å). Manual macromolecular docking shows that UraA and UPRT are of similar size, and they complement each other in shape and charge. These properties support the hypothesis that there is a physical interaction between UraA and UPRT.

A.
Figure 3.1 Size, shape and charge complementation of UraA and UPRT interacting surfaces. Manual docking of macromolecules was performed to generate a model of the UraA-UPRT complex. A) Side view of UraA-UPRT shown in cartoon representation. Dimeric UraA sits in the inner membrane, with its cytosolic face interacting with tetrameric UPRT. Side chains of R4, R299 on UraA monomer and D122, E123 on UPRT monomer are shown as sticks. These oppositely charged residues are in close contact and have the potential to form salt bridges, contributing to ionic interaction between UraA and UPRT. B) Interacting surfaces of UraA and UPRT showing electrostatic potential. The two surfaces are shown as an opened clamshell, with the UraA dimer interacting surface on top and the UPRT tetramer interacting surface at the bottom. Blue represents regions of positively charged residues and red represents regions of negatively charged residues. Interacting surface of UraA is mostly in positive charges, with negatively charged residues sticking out at the center. The interacting surface of UPRT is mostly in negative charges, surrounding a positively charged pocket in the middle. As indicated by the double-headed arrow, the distance between the key residues (E290) in the active sites of UraA...
monomers is 45Å. The distance between the key residues (D199) in the active sites of UPRT monomers in two directions are 35Å and 49Å respectively.

3.2 UraA and UPRT were purified.

UraA and UPRT were over-expressed in E. coli for PPI studies and purified using affinity chromatography as described in section 2.4, 2.5 and 2.6. The Coomassie Blue stained SDS-PAGE gels (CB) show the efficiency of UraA and UPRT purification (Figure 3.2 A and C). Both UraA-His and UPRT-Strep can be purified, as many bands in the sonicated samples (T) cannot be seen in the eluted samples (E). In lane E, the major band (indicated by the arrow) runs close to the molecular mass of the target protein: 40 kDa for UraA (Figure 3.2A) and 25 kDa for UPRT (Figure 3.2C). Compared to the expected molecular mass of 45 kDa, UraA migrated faster than predicted in the SDS-PAGE gel, likely due to binding to excess SDS at its hydrophobic regions or incomplete unfolding of the protein. One major contaminant band exists in the eluted sample of UraA at ~27 kDa. The band was excised, digested with trypsin and sent for mass spectrometry (MS) analysis (SickKids SPARC BioCentre, Toronto). The contaminant band was identified to be the sensitive to lysis D (SlyD) protein from E. coli. SlyD belongs to the FK506-binding protein (FKBP) family and possess a chaperone-like peptidyl-prolyl cis-trans isomerase activity. Previous study has shown that SlyD interacts with nickel ions, and its activity is modulated by Ni (35). Thus, instead of binding to UraA and helping its folding as an isomerase, SlyD might just be binding non-specifically to the Ni-beads as a major purification contaminant.

As for UPRT purification, only one major band close to the predicted molecular mass of UPRT monomer (23 kDa) was present in the eluted sample, indicating more efficient expression.
and purification of UPRT than UraA. Besides better purity, the cytosolic enzyme UPRT also had greater yield than the membrane protein UraA with 3.5 mg/L culture versus 0.9 mg/L culture.

To confirm the identity of the purified proteins, Western Blots (WB) with anti-His and anti-Strep antibodies were performed to detect UraA-His and UPRT-Strep respectively (Figure 3.2 B and D). In each immunoblot, only one major band representing the protein monomer was detected and it was at the same position as that in the respective Coomassie stained gel. Thus, identity of the bands representing UraA-His and UPRT-Strep was confirmed. An upper band was detected in both anti-His blot (~80 kDa) and anti-Strep blot (~55 kDa). Considering the molecular mass of monomeric UraA and UPRT, the upper bands were likely the dimeric forms of UraA and UPRT, respectively.
**Figure 3.2 UraA and UPRT can be purified.** Samples of sonicated cells were taken as "T" for total. Centrifugation was performed to remove cell debris and samples were taken as "S" for supernatant and as "P" for pellet. Supernatants were then incubated with Ni-beads for UraA or Strep-beads for UPRT, and the unbound samples were taken as "F.T." for flow through. Samples from the first wash and the third wash were taken as "W1" and "W3". The eluted samples were taken as "E". Molecular mass (kDa) of the protein ladder is labeled on the left of each image. Red arrows indicate the positions of UraA monomer, and green arrows indicate the positions of UPRT monomer. A) Coomassie Blue stained SDS-PAGE gel (CB) showing UraA purification. B) Anti-His Western Blot (WB) identifying UraA-His. C) CB showing UPRT purification. D) Anti-Strep WB identifying UPRT-Strep.

### 3.3 Minimal interaction between purified UraA and UPRT was detected using EMSA.

Separately expressed and purified UraA-His and UPRT-Strep were mixed in various molar ratios (0:1, 0.5:1, 1:1, 2:1, 1:0) as shown in Figure 3.3. The mixtures were run on a native gel to determine whether a protein complex would form as described in section 1.7.1. Figure 3.3A shows the Coomassie stained native-PAGE gel, and Figure 3.3B and C show the anti-His blot detecting UraA-His and anti-Strep blot detecting UPRT-Strep respectively. Since the buffer system used was pH 9.0, the acidic UPRT (indicated by the green arrow) migrates well into the gel towards the anode, while the basic membrane protein UraA (indicated by the red arrow) barely enters the separating gel (Table 1.2). Some UraA also likely aggregated due to its instability to an overnight incubation and remained at the bottom of the loading wells (Figure 3.3A last 4 lanes). As increasing molar ratio of UraA was added to constant amount of UPRT (1 µg), there was slight decrease in the intensity of the bands representing UPRT. Neither an upper shift of UPRT nor a protein complex was observed, providing no evidence for the interaction between UraA and UPRT. Immunoblots of anti-His and anti-Strep were performed to confirm the identity of the bands representing UraA and UPRT respectively. BSA, with molecular mass of 66.5 kDa and pI of 4.7, was acting as a ladder on the Coomassie stained native gel. With
48 monomeric molecular mass of 23 kDa and similar pI as BSA, UPRT ran at ~100 kDa, suggesting its native tetrameric state during gel electrophoresis, consistent with its crystal structure (17).

Figure 3.3 Minimal gel mobility shift of UPRT was observed when incubated with increasing molar ratio of UraA. Increasing amount of UraA was added to 1µg of UPRT with molar ratios 0:1, 0.5:1, 1:1, 2:1 as labeled on top of the gel images. Lanes labeled “UraA” had a UraA:UPRT molar ratio of 1:0. A) Coomassie stained native gel with BSA acting as protein ladder. B) Anti-His immunoblot detecting UraA-His. C) Anti-Strep immunoblot detecting UPRT-Strep. Red arrows indicate the positions of UraA, and green arrows indicate the positions of UPRT tetramer.

3.4 Interaction between co-expressed UraA and UPRT was detected using pull-down assay.

If the affinity of the interaction between UraA and UPRT is low, their interaction may not be able to be detected using purified proteins because of the high concentrations required to establish binding. Therefore, the two proteins were co-expressed in E. coli to facilitate their interaction in a native environment. In this case, UraA is in a native membrane and UPRT is present in the cytosol at a high concentration, promoting their interaction. In addition, co-
expression allows the interaction to take place in situ, an important feature if other factors are required for the interaction. These factors may include scaffold proteins, metabolites or the native conformational state of the target proteins. Co-expressed UraA-His and UPRT-Strep were purified using both Ni- and Strep-affinity chromatography to test if the partner protein could be co-purified (Figure 3.4 E to G). Anti-His and anti-Strep immunoblots were performed to detect if the differently tagged proteins can be pulled down by the same beads due to PPI. UraA-His (Figure 3.4 A and B) and UPRT-Strep (Figure 3.4 C and D) were also separately expressed and purified with both beads to act as controls. Figure 3.4 A, C and E are Coomassie stained SDS-PAGE gels showing total proteins present in each sample. Figure 3.4 B and F are anti-His immunoblots detecting the His-tagged UraA, and Figure 3.4 D and G are anti-Strep immunoblots detecting the Strep-tagged UPRT. As expected, UraA-His expressed alone was pulled down by the Ni-beads (Figure 3.4 B lane 2), but not by the Strep-beads (Figure 3.4 B lane 3). However, separately expressed UPRT-Strep was pulled down by both Ni- and Strep-beads (Figure 3.4 D), indicating non-specific binding of Strep-tagged protein to the Ni-beads. When UraA-His and UPRT-Strep were co-expressed, Ni-beads were able to pull down UPRT-Strep (Figure 3.4 G lane 2) together with UraA-His (Figure 3.4 F lane 2), but due to non-specific binding of Strep-tagged UPRT to Ni-beads, the result was not reliable. However, Strep-beads had no non-specific binding to the His-tagged UraA, but was able to pull down UraA-His (Figure 3.4 F lane 3) together with UPRT-Strep (Figure 3.4 G lane 3). In conclusion, UraA-His, when co-expressed with UPRT-Strep, could be captured by Strep-affinity chromatography, indicating interaction between UraA and UPRT.
Figure 3.4 UraA-His was pulled down by the Strep-beads when UPRT-Strep was present. Separately expressed and co-expressed UraA-His and UPRT-Strep were purified with both Ni- and Strep-beads. “Input” refers to cell lysate samples before incubating with the beads, and “output” refers to the eluted samples from the Ni- or Strep-beads. Molecular mass (kDa) of the protein ladder is labeled on the left of each image. Red arrows indicate the positions of UraA monomer, green arrows indicate the positions of UPRT monomer. A) Coomassie stained gel showing samples from UraA-His expressed alone. B) Anti-His immunoblot detecting UraA-His expressed by itself. C) Coomassie stained gel showing samples from UPRT-Strep expressed alone. D) Anti-Strep immunoblot detecting UPRT-Strep expressed by itself. E) Coomassie stained gel showing samples from co-expressed UraA-His and UPRT-Strep. F) Anti-His immunoblot detecting UraA-His co-expressed with UPRT-Strep. F) Anti-Strep immunoblot detecting UPRT-Strep co-expressed with UraA-His.
3.5 Interaction between UraA and UPRT was detected using liposome floatation assay in cell lysate.

The detection of an interaction between co-expressed UraA and UPRT via a Strep pull-down posed the question whether the interaction required lysate containing external factors or UraA to be membrane-bound. Thus, UraA was incorporated into liposome composed of *E. coli* polar lipids extract to test its interaction with UPRT, either purified or in a cell lysate. After floatation of the liposomes in the sucrose gradient with ultracentrifugation, anti-His and anti-Strep immunoblots were performed to detect the presence of UraA-His and UPRT-Strep in fractions 1 to 10 from the top to the bottom of the tube. As shown in Figure 3.5, UraA-His (indicated by the red arrows) can only be detected in the top fractions together with the liposome, suggesting successful incorporation of UraA into the liposome. UPRT-Strep, purified (Figure 3.5A) or in a whole cell lysate (Figure 3.5B) were tested to capture the interaction between UraA and UPRT. In either case, the majority of the UPRT-Strep remained at the bottom of the tube (fractions 8 to 10). As shown in Figure 3.5A, purified UPRT was absent at the top fractions, indicating that it did not interact with either UraA-liposome (green arrow) or empty liposome (black arrow) to float in the sucrose solutions. However, when UPRT in cell lysates was incubated with UraA-liposome, a portion of UPRT interacted with UraA-liposome and floated to the top fractions (Figure 3.5B, green arrow), while those incubated with empty liposomes all stayed at the bottom of the tube (Figure 3.5B, black arrow). A parallel experiment performed with UPRT in a crude lysate however showed a similar interaction with an unrelated membrane protein, the lysine/cadaverine antiporter CadB (Appendix 2), indicating a lack of specificity of the interaction between UPRT and UraA. To summarize the results from liposome floatation assay, an interaction between UraA-liposome and UPRT was captured with cell lysate, but not purified protein, indicating possible involvement of additional factors present in the cell lysate in
promoting the interaction. In addition, besides the majority of UPRT-Strep remaining at the bottom (fractions 8 to10) and a small amount present in fraction 1 with UraA-liposome, some portions of UPRT-Strep were also present along the middle fractions 2 to 7 (Figure 3.5B, green arrow). This observation indicates weak and dynamic interaction between UraA and UPRT as some UPRT dissociated from UraA as the proteoliposome migrated through the sucrose solutions, towards the top of the tube.

**Figure 3.5 UPRT floated to the top of the sucrose gradient together with UraA-liposome when cell lysate was present.** UPRT, purified or in cell lysate, was incubated with UraA reconstituted into the liposome. Numbers 1 to 10 on top of the images refer to samples of the ten fractions from the top to the bottom of the ultracentrifugation tubes. Molecular mass (kDa) of the protein ladder is labeled on the left of each image. Red arrows indicate the positions of monomeric UraA-His detected by the anti-His immunoblot. Green arrows indicate the positions of monomeric UPRT-Strep incubated with UraA-liposome and detected by the anti-Strep immunoblot (experiment). Black arrows indicate the positions of monomeric UPRT-Strep incubated with empty liposome and detected by the anti-Strep immunoblot (control). A) Purified UPRT-Strep was incubated with UraA-liposome or empty liposome. B) UPRT-Strep in cell lysate was incubated with UraA-liposome or empty liposome.
3.6 Low affinity and ionic strength-dependent interaction between UraA and UPRT was detected using BLI.

To quantify the physical interaction and to study the binding kinetics of UraA and UPRT, BLI was performed under equilibrium binding conditions. Biotinylated \textit{in vivo}, UPRT in whole cell lysates was immobilized onto the Streptavidin biosensor tip, and the loaded tip was dipped into various concentrations of purified UraA. Figure 3.6A shows a representative image aligning baseline-corrected association and dissociation curves (separated by the red vertical line) with five different concentrations of UraA as labeled at the bottom of the image. Shifts in the association curves were observed upon incubation with UraA in a concentration-dependent manner. Two independent biological replicates were performed, generating $K_D$ of values 1.1 µM and 0.76 µM, suggesting weak binding between UraA and UPRT.

To test if the interaction was dependent on ionic strength as predicted in the docking experiments, the loaded biosensor was incubated with different concentrations of UraA present in various salt NaCl concentrations (Figure 3.6 B to D). At each specific concentration of UraA (indicated at the top of each image), higher association signals were generated under lower salt conditions (peach and light green), indicating the interaction between UraA and UPRT was ionic strength dependent. As shown in the electrostatic potential images (Figure 3.1B), interacting surfaces of UraA and UPRT complement each other with regions of oppositely charged residues. Excessive ions in high salt buffers would mask the charges on the interacting surfaces, interrupting the physical interaction between UraA and UPRT.
Figure 3.6 Weak and ionic interaction between UraA and UPRT was detected with BLI. Biotinylated UPRT was loaded onto Streptavidin biosensor tip and dipped into buffers containing various concentrations of UraA and salt NaCl. In each image, incubation time period is recorded on the x-axis, and y-axis shows change of wavelength positions in nanometers. The red vertical straight lines separate the association curves from the dissociation curves. A) Association and dissociation curves of UPRT-loaded tip incubated with various concentrations of UraA (pink-0.5 µM, orange-1 µM, green-2 µM, blue-3 µM, black-4 µM). B, C, D) Association and dissociation curves of UPRT-loaded tip dipped in various concentrations of NaCl (peach-0 mM, light green-50 mM, dark green-150 mM, black-300 mM) with presence of 0.5 µM, 1 µM, 2 µM UraA respectively.
Chapter 4 Discussion
Proteins usually do not exist in isolation in cells. In order to remain stable and be fully functional, they commonly attach to a structural element in the cell or interact with other proteins to form a complex. Research on sequential metabolic enzymes forming supramolecular assemblies started early with Krebs cycle enzymes. Based on previous work, Paul Srere first proposed the concept of a “metabolon”, referring to a protein complex efficiently channelling metabolic intermediates and regulated by association and dissociation of the enzymatic components (1). Since it was first introduced to the scientific public, 30 years has passed, but the concept of metabolon still remains an interesting theory. Very limited work has been done to provide evidence for enzymatic assemblies in boosting metabolic efficiency. Membrane transport metabolons (MTMs) involving transporters and their cognate enzymes are even less studied due to the hydrophobic nature of the membrane proteins. The bicarbonate transport metabolon consisting of human Band 3 and CAII in the red blood cells is a well-studied example of a MTM. Loss of interaction between Band 3 and CAII has been linked to decreased transport activity of Band 3 (6), providing evidence for the functional assembly of the metabolon components. In *E. coli*, soluble domain of a putative bicarbonate transporter DauA, also known as YchM, was found to be interacting with the acyl carrier protein ACP, an essential part of the fatty acid biosynthesis (FAB) pathway (29). Their assembly indicates functional linkage between bicarbonate transport and fatty acid metabolism (29).

This study aims to provide further evidence to support the metabolon theory by investigating MTM in a bacterial system. Having functionally related transporters and enzymes organized in the same operons allows prediction of MTM components in bacteria. Previous study has systematically reported 45 such polycistronic operons encoding potential MTMs in *E. coli*, including the *ura* operon (2). The *ura* operon is bicistronic and encodes for the uracil
phosphoribosyltransferase UPRT followed by the uracil-H\(^+\) symporter UraA. UraA and UPRT are important for uracil transport and metabolism in *E. coli*. Both genetically and functionally linked, UPRT and UraA potentially form a uracil transport metabolon. As components of a uracil transport metabolon, UPRT and UraA are hypothesized to physically interact with each other, allowing efficient channelling of the substrate uracil into its metabolic pathway. Interaction between UPRT and UraA would allow UPRT to associate with the cytosolic surface of the inner membrane. Such an interaction could be dynamic, depending on the affinity of the interaction and its regulation.

With both crystal structures known (12, 17), it is possible to predict how UraA interacts with UPRT using macromolecular docking. To start, a model of the UraA dimer was built based on close contact of TM13 between UraA monomers in crystal packing. The dimeric state of UraA would allow two points of contact with UPRT, resulting in an avidity effect. Since UraA is a membrane protein and UPRT is a cytosolic enzyme, the cytosolic side of UraA must be its interacting surface. For UPRT, because of its symmetrical nature, the top and the bottom of the UPRT tetramer are identical. To build the model of UraA-UPRT complex, the UPRT tetramer was rotated in three dimensions to find a surface that best complement the shape of the cytosolic side of the UraA dimer. To verify the predicted interacting surfaces of UraA and UPRT, their electrostatic potential images were generated and general complementation of charges was found between the interacting surfaces. As a result, the model of UraA-UPRT complex was built based on shape and charge complementation through manual docking.

However, limitations exist in manual docking of macromolecules. Unlike macromolecular docking using computational algorithms where various parameters were
systematically evaluated to generate a model with the lowest intermolecular energies (36), manual docking is solely based on the judgement of the researcher. In cells, proteins undergo conformational changes to fit perfectly in shape with their interacting partners. Manual docking uses rigid bodies of the crystal structures and may not reflect subtle changes and molecular details of the interacting surfaces. Thus, the macromolecular model built in this study should only be used as a guide for general picture of the interaction, and details in the predicted model should be considered and used carefully.

Before testing the interaction between UraA and UPRT, both proteins were purified with good quality. The enzyme UPRT had higher yield and better purity than the membrane protein UraA. A major contaminant of purified UraA was found to be SlyD, a chaperone that binds to Ni and eluted from the Ni-beads together with the His-tagged UraA (35). In addition, an immunoblot-detectable band was observed with a molecular mass equivalent to the protein dimer for both purified UraA and UPRT. These dimers are only small portions of the purified proteins that might resist full dissociation in SDS. Alternatively, the dimers could arise from the non-specific association of the denatured monomers. Both tags of UraA and UPRT were constructed on their C-termini, so that the tags were translated last and should not affect the proper folding of the proteins. In the 3D structure, the Strep tags are flanked to the side of the UPRT tetramer, away from the predicted interacting surface and should not affect its interaction with UraA. The His tags are in the middle of the interacting surface of the UraA dimer, but considering that the tags are small and would not affect the general charges on the surface, they should have little effect on the PPI. However, it would be worthwhile to remove the His tag from purified UraA to eliminate confounding effects of the tag in future experiments.
With purified UraA and UPRT, their interaction can be tested using gel electrophoresis in a native system. Native gel systems lack the denaturant SDS and allow the proteins to remain in their native folded states and the protein complexes to remain intact. In this study, UraA and UPRT have the opposite pIs, and with a buffer system of basic pH, only the acidic UPRT migrated well towards the anode. However, inefficient migration of UraA into the gel should not affect detection of the interaction. If the interaction exists, band shift of UPRT should be observed, as the portions of UPRT interacting with UraA would not migrate into the gel as efficiently as UPRT by itself. In addition, consistent with the structure solved, UPRT ran at ~100 kDa as a tetramer on the native gel compared to BSA with similar pI, suggesting that the UPRT purified was likely in its native state. It would be useful to perform enzyme assays of purified UPRT to ensure its functional state.

Minimal interaction was detected in native gels with purified proteins. This might be due to lack of important factors present in the cell lysate. These factors could be another protein, a small molecule metabolite (e.g. the substrate uracil or the product UMP), or the proper folding of conformational state of the target proteins. Thus, UraA and UPRT were co-expressed in the same cells and their interaction was detected using a pull-down assay. Due to non-specific binding of UPRT-Strep to the Ni-beads, detection of UPRT-Strep pulled down together with UraA-His by the Ni-beads was not solid evidence for their interaction. On the other hand, without non-specific binding, UraA-His was pulled down together with UPRT-Strep by the Strep-beads and served as evidence for the interaction between UraA and UPRT. However, the interaction captured had poor reproducibility. When repeating with the same experimental conditions and procedure, UraA-His was either not pulled down by Strep-beads with UPRT-Strep, or it was detected as a band at a much lower position likely due to protein degradation.
Instead of using membrane proteins in detergent micelles, those detergents can be removed to incorporate the membrane proteins into the liposomes (28, 37). Liposomes are spherical lipid bilayers that better mimic the native environment of the membrane proteins than the detergent micelles do. UraA reconstituted into liposomes and UraA dissolved in detergent DDM were both treated with the cross-linker glutaraldehyde and detected using immunoblot (Appendix 3). The result showed that the amount of cross-linked UraA dimers (~75 kDa) incorporated into the liposome was significantly higher than those dissolved in detergent DDM. Liposomes enhanced stability of membrane protein oligomerization, and UraA likely existed as a dimer in its native membrane-bound state as predicted (Figure 1.8B). Thus, UraA was reconstituted into liposomes in this study, and liposome floatation assay was performed to detect the interaction between the membrane protein UraA and the soluble enzyme UPRT. In the liposome floatation assay, interaction between UPRT and UraA-liposome was captured when cell lysate was present, but not with purified samples, indicating requirement of other factors such as scaffold proteins or small molecules for UraA-UPRT interaction. UPRT had no interaction with the empty liposome, but it was found to be interacting non-specifically with an unrelated transporter CadB incorporated into the liposome (Appendix 2). Although structurally unknown, CadB may follow the “positive-inside rule” as other membrane proteins (38), with plenty of positive charges populated on its cytosolic side complementing the negative charges on the interacting surface of UPRT.

To quantify the binding kinetics of UraA and UPRT, BLI was used. BLI is very sensitive and requires very small amount of proteins. It also monitors molecular interactions in real time, which can progress to equilibrium conditions. Previous study has shown that the Streptavidin biosensor tip of BLI is efficient in selecting the biotinylated protein from the cell lysate, allowing
successful determination of binding constant with protein captured from crude extracts (34). In this study, C-terminally biotinylated UPRT in cell lysate was immobilized onto the Streptavidin biosensor tip and incubated in purified UraA with various concentrations followed by incubation in buffer to determine the association and dissociation rates respectively. The $K_D$ ($k_{\text{off}}/k_{\text{on}}$) was determined to be around 1µM with two biological replicates. Alternatively, $K_D$ can be determined with steady state analysis. Typically, 180 sec was enough to allow the association curves to reach steady state (34). However, in this study, the association curves did not plateau after 400 sec of incubation of the loaded biosensor tip with purified UraA, likely due to non-specific binding of the aggregated UraA onto the biosensor tip. To stabilize UraA, it can be reconstituted into the liposomes. However, few experiments have been done on BLI with proteoliposomes (39). The major obstacle is that the size of a proteoliposome is too big to allow detection of PPI with high resolution. In addition, increase in salt concentration was associated with decrease in association signal, suggesting that the interaction between UraA and UPRT was ionic, consistent with the prediction from manual docking. In the future, it would be interesting to study the effect of uracil and other metabolites on the interaction between UraA and UPRT.

Comparing the negative result in EMSA and the positive result in pull-down assay with co-expressed proteins, as well as the result from liposome floatation assay, additional factors present in the cell lysate might be required for the interaction between UraA and UPRT. The additional factors might be scaffold proteins, small molecules such as co-factors and intermediates, or the native environment that keeps the proteins properly folded in a particular conformational state. To identify the additional factor as a protein, mass spectrometry (MS) can be performed to identify the unknown bands present with the co-expressed UraA and UPRT on the electrophoretic gel. Cell lysate with UraA and UPRT co-expressed can be run on gel
filtration, and if the additional factor involved is a scaffold protein, the UraA-UPRT complex would be eluted faster from the column together with the scaffold protein. To test if the cell lysate is required for the proper folding and activity of UraA and UPRT, functional assay can be performed with radiolabelled uracil, comparing its uptake into the reconstituted UraA-liposome with the presence of purified UPRT or the presence of UPRT in cell lysate.
Chapter 5 Conclusion and Future Directions
5.1 Result Summaries and Conclusion

This study aimed to examine if there is physical interaction between UraA and UPRT, creating a uracil transport metabolon in *E. coli*. The following evidence has been collected to support the hypothesis: 1) UraA and UPRT are encoded on the same operon and are genetically linked. 2) UraA and UPRT have related functions. Both of them are involved in uracil metabolism, with UraA importing the substrate uracil into the cell for the enzyme UPRT to convert to UMP. 3) Structurally, UraA and UPRT have interacting surfaces that complement the shapes and charges of each other. 4) Physical interactions between UraA and UPRT were detected under certain conditions using a pull-down assay, a liposome floatation assay and BLI. The interaction was found to be weak and ionic strength dependent. Other factors present in crude cell lysate might be required for the interaction.

5.2 Future Directions – Confirmation of the Interaction

To confirm the interaction between UraA and UPRT detected in this study, other PPI methods mentioned in section 1.7 can be used. Since a metabolon is regulated through association and dissociation of its components, interaction between UraA and UPRT consisting the uracil transport metabolon might be transient and hard to capture. Many *in vivo* methods are ideal for detecting dynamic interactions, such as BACTH and FRET introduced in sections 1.7.3, because as soon as the proteins interact with each other, signals are immediately generated in terms of turned on reporter gene or fluorescence. In addition, *in vivo* methods test PPI in the native environment inside live cells that ensure the presence of other factors necessary for the interaction. As mentioned in section 1.7.3.1, BACTH can be performed to test the interaction between UraA and UPRT fused to T18 and T25 domains respectively on either terminus.
BACTH can also be used as a high-throughput screen for PPI. It would be worthwhile to carry out high throughput BACTH screens on all transporters listed in Table 1.1. Any positive “hits” would be validated using direct measurements of PPI such as “pull-down” assay, with purified components or co-expressed transporter and its cognate enzyme in *E. coli*.

### 5.3 Future Directions – Functional Assay

Metabolons are functional assembly of enzymes. In the long term, the effect of the physical interaction between UraA and UPRT on their activities should be tested. In this study, charged residues that might be key to the interaction between UraA and UPRT have been predicted using manual docking and are located on the interacting surfaces. These residues can be mutated to alanines to produce interaction-perturbing mutants. Significance of the physical interaction between UraA and UPRT can be studied by comparing uracil uptake efficiency and metabolic rate between wild type (WT) cells and cells expressing interaction-losing mutants with radiolabeled uracil. An interesting experiment would be switching the charged residues on UraA and UPRT so that the interacting surface of UraA is negatively charged and the interacting surface of UPRT is positively charged. Such mutations are not expected to affect the interaction between UraA and UPRT, and thus, are not expected to affect their activities. However, it should be validated first that switching the charges do not affect proper folding and functionality of the proteins.

As a first step, the effect of knocking out *upp* gene encoding UPRT on the cellular uptake of uracil should be determined. As demonstrated by previous data with UraA and UPRT, deletion of the *upp* gene resulted in a significant reduction in uracil uptake (24). Completing this
study would also involve deletion of the uraA gene and the removal of both uraA and upp genes. In addition, mutated UPRT losing the interaction with UraA while still being active could be tested for the effect on substrate uptake. It would then be interesting to compare uracil uptake between cells expressing WT UPRT, interaction-losing but active UPRT and interaction-preserved but catalytically inactive UPRT (mutated active site).

A competition experiment having the catalytically inactive UPRT competes with WT UPRT for the binding site on UraA would be important. With the competition from the interaction-preserved but catalytically inactive UPRT, uracil uptake is expected to decrease in cells expressing mutated UPRT compared to WT cells. Over-expressing active UPRT enhances uracil uptake, while over-expressed inactive UPRT may bind to UraA and block its interaction with the endogenous UPRT, resulting in decreased uracil uptake. It would also be interesting to compare the fate of the imported uracil into pools of UMP and UTP.
References


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Appendices

Appendix 1. Amino acid sequences of *E. coli* UraA and UPRT

**UraA**

MTRRAIGVSRPPLLQTIPLSLQHLFAMFGATVLPVLFHINPATVLLFNGGTLHLYLFI 60
CKGKIPAYLGSSFAISPVLILLPLGYEVALGGFIMCGLFLCVSFIVKAGTGWLDVF 120
PPAAMGAIVAVIGLELAGVAAGMAGLLPAEGQTPDSKTIISITTLAVTLGSLFRGFL 180
AIIPIILIVLGYALSFGMIVDTIIINAHWFALPTLYTPRFREWFAILTILPAALVIA 240
EHVGLLVTVANIVKDLRDPGLHRSMFANGLSTVSGFFGSPNTTYGGINGVMAITRSVS 300
YSTWVIGGAIFAILLSCVGLAAAIQMIPLPVMGGVSLLGYGVGASGIRVLIIESKVDY 360
NKAQNLILTSVILIIGVSGAVNIGAAELKGMALATIVGIGLZIFKLISVLRPEEVL 420
AEDADITDK 429

**UPRT**

MKIVEVKHPLVHKGLMREQDISTKRFRELASEVGSSLTYEATADLETKEVTIEGWNGP 60
VEIDQIKGKKTIVVPIRLAGMGMDVLENSARISVGMYRNEETLVPVFQKLVSNIEN 120
IDERMALIVDPMATGSSVIAITIDLLKKAQCSSIKVLVVAPEGIANKHDPVEYT 180
ASIDQGLNEHGYYIIPGLGDAGDIFGTK 208

**Appendix 1. Amino acid sequences of *E. coli* UraA and UPRT.** In the amino acid sequence of UraA, the 14 TM helices are highlighted in yellow, with key active site residues E241, H245, E290 in red letters. In the amino acid sequence of UPRT, the key active site residue D199 is in red letter.
Appendix 2. Liposome floatation assay with CadB as non-specific binding control

A.                                             B.

Appendix 2. UPRT in cell lysate floated to the top of the sucrose gradient together with UraA-liposome or CadB-liposome. UPRT in cell lysate was incubated with UraA or CadB reconstituted into the liposome. Numbers 1 to 10 on top of the images refer to samples of the ten fractions from the top to the bottom of the ultracentrifugation tube. Molecular mass (kDa) of the protein ladder is labeled on the left of each image. A) Samples from UPRT incubated with UraA-liposome. B) Samples from UPRT incubated with CadB-liposome. Red arrows indicate the positions of monomeric His-tagged transporters detected by the anti-His immunoblot. Green arrows indicate the positions of monomeric UPRT-Strep incubated with the proteoliposome and detected by the anti-Strep immunoblot.

Appendix 3. Dimerization of UraA in liposome versus in detergent

Appendix 3. The amount of cross-linked UraA dimers significantly decreased in detergent compared to those incorporated into the liposome. Purified UraA-His was reconstituted into the liposome. Half of the proteoliposome sample was dissolved in DDM (lane “+”), while the other half was not treated with the detergent (lane “-”). Both samples were treated with the cross-linker glutaraldehyde and detected using anti-His immunoblot. One major band was detected on both lanes at 75 kDa, close to the expected molecular mass of UraA dimer. However, the band intensity was higher for UraA incorporated into the liposome than those dissolved in the detergent, suggesting enhanced stability of UraA dimerization in the liposome. Monomeric UraA was not detected on the anti-His immunoblot.