Exploring unique structural motifs in *Vibrio parahaemolyticus* Thermostable Direct Hemolysin

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A dissertation submitted for the partial fulfillment of
BS-MS dual degree in Science.

Department of Biological Sciences

Indian Institute of Science Education and Research
(IISER) Mohali

April 2019

Certificate of Examination

This is to verify that the dissertation titled **"Exploring unique structural motifs in *Vibrio parahaemolyticus* Thermostable Direct Hemolysin"** submitted by Mr. Abhinav (Reg. No. MS13074) or the partial fulfillment of BS-MS dual degree program of the Institute has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Samarjit Bhattacharyya Dr. Arunika Mukhopadhaya Dr. Kausik Chattopadhyay

(Supervisor)

Dated: April 26, 2019

Declaration

The work presented here in this dissertation has been carried out by me under the guidance of Dr. Kausik Chattopadhyay at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contribution of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussion. Thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Abhinav

Dated: April 26, 2019

In my capacity as the Supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Kausik Chattopadhyay

(Supervisor)

ACKNOWLEDGEMENT

I would like to express my gratitude towards my supervisor Dr. Kausik Chattopadhyay who has been very supportive and understanding. Without his guidance and directions this work would not have been possible. It has been really tireless journey with lots of learning with fun which I enjoyed to the fullest working under him.

I would also take this opportunity to thank Ms Pratima Verma, who has always been there to guide me, support me, make me learn and correct me. It has been a wonderful journey with her.

I would also like to thank Dr. Arunika Mukhopadhaya and her lab members for helping me and allowing me to use lab instruments.

I would also like to thank my other lab members Mr. Anish, Dr. Nidhi, Ms Amrita, Ms. Reema, Mr. Mahendra, Ms Kusum, Ms Shamaita and Ms Tejal, for their kind support and for making the lab environment awesome.

I would also like to thank IISER Mohali for providing me the necessary infrastructure and facilities to complete this project.

I would like to thank Centre for Protein Science, Design and Engineering (CPSDE) and Department of Bio-Technology (DBT) for their funding for my research.

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Abbreviation:

- PFT: Pore-forming Toxin
- PFP: Pore-forming Protein
- TDH: Thermostable Direct Hemolysin
- TRH: TDH-related Hemolysin
- T3SS: Type III Secretion System
- EqtII: Equinatoxin II
- StnII: SticholysinII
- POC: Phosphocholine
- PDB: Protein Database
- PCR: Polymerase Chain Reaction
- WT: Wildtype
- LB: Luria Bertani
- RBC: Red Blood Cells
- O.D: Optical Density
- DNA: Deoxyribonucleic acid
- IPTG: Isopropyl-β-D-thiogalactopyranoside
- PBS: Phosphate Buffer Saline
- Bp: Base pair
- Da: Dalton
- SDS-PAGE: Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis.

CONTENTS

Chapter 1

INTRODUCTION

Prey and predator battle has been going on since the beginning of life. Evolution has played an important role in this battle, and one of the predominant rules of evolution is “the survival of the fittest”. Therefore every organism goes under a constant struggle to be the best and have a stronger weapon in its arsenal to attack and protect itself from surroundings. Every organism has evolved from a simpler organism over a long course of mutations in genome. Therefore, mutations in genome plays a vital role in improving a specie’s survival probability.

Macromolecules in a living organism are one’s mean of survival and all organisms rely on macromolecules for many different purposes (eg, energy consumption, energy utilization, transportation, storage, etc.). Proteins (macromolecule) (comprised of different amino acids which are coded by genes) carry out most of the functions in a cell and are required for structure and regulation of body’s tissues and organs. Mutations in gene leads to mutations in protein.

It is fascinating that bacteria being a prokaryote and primitive organism, has survived for so long in the battle of overcoming more complex systems of advanced organisms such as humans, and mutations in bacterial genome are one to thank. Due to these mutations, bacteria are able to evolve themselves to be equipped with stronger and better

weapons in its arsenal comprising of many different types of proteins. Bacteria use different proteins for many different purposes such as transportation, energy (consumption and production) and making of cell wall to protect themselves from harmful surroundings and many more. Although all these are very important for bacterial survival, bacteria also need an attack mechanism in order to get organic molecules from surrounding cells and make the surroundings suitable for bacteria's survival. Therefore, bacteria have evolved to produce bacterial toxins which help them to kill the host cells [1].

After entering the host organism, bacteria release these toxin which help in bacterial colonization, invasion, and killing of the host cells [2]. There are many different ways in which different bacterial toxins functions as shown in Table 1.

Bacterial Toxin	Functioning Mechanism
Diphtheria toxin	Inhibits protein synthesis
Cholera toxin	Activates secondary messenger pathway
***S. aureus* superantigens**	Activates immune response
***E. coli* hemolysin**	Damage cell membrane
***Clostridium tetani* tetanus toxin**	Activates metalloprotease

Table 1.1. Different bacterial toxins and there mode of action [2].

Largest class among these bacterial toxins is occupied by the "pore-forming toxins" (PFTs) [3]. Cell membrane or plasma membrane is a biological membrane protecting every cell from its surroundings [4]. These membranes (made up of mainly phospholipids, cholesterol, proteins and glycolipids) help cells to maintain potential gradient, help in cells

movement, signaling, and they are also involved in many other important processes which are crucial for cell survival. PFTs are very effective bacterial protein toxins which act by forming pores onto the target cell membrane [5]. These pores provide unwanted pathway to molecules leading them to escape and enter the target cells, which eventually leads to "cell death". Interestingly similar types of proteins are also found in other kingdoms as well, and are known as pore-forming proteins (PFPs)-(eg, Actinoporins produced by the sea anemones) [5, 6].

Most common mechanism of pore-formation by PFTs is: first they are secreted as water-soluble monomers, which then bind to the target cell membrane followed by oligomerization onto target cell membrane after which insertion happens (Figure1.1) [7]. So they change their structure from water-soluble form to transmembrane form [5].

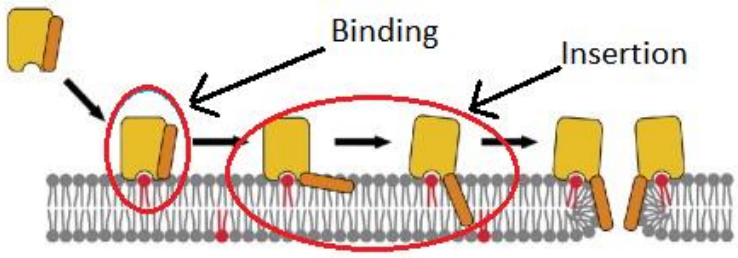

Figure 1.1. Pore-formation mechanism of most of the PFTs [8].

PFTs are majorly characterized based on the secondary structure of the membrane-spanning region or the transmembrane region (α-helices or β-barrels) that make the membrane pores [9, 10]. Therefore, PFTs are majorly characterized under αPFTs and βPFTs [5]. Some of the examples of different type of PFTs are illustrated in Table 2.

Type	Family	PFT	Organisms
	Colicin	Colicin A	*Escherichia coli*
		Colicin E1	*Escherichia coli*

αPFTs		Colicin Ia	*Escherichia coli*
	ClyA (CytolysinA)	Cytolysin A (also known as HlyE)	*E. coli, S. enterica, S. flexneri*
		Non-hemolytic tripartite enterotoxin (Nhe)	*B. cereus*
		Hemolysin BL (Hbl)	*B. cereus*
	Actinoporin	EquinatoxinII (EqtII)	*A. equina*
		SticholysinII (StnII)	*S. helianthus*
		FragaceatoxinC (FraC)	*A. fragacea*
βPFTs	Hemolysin	*V. cholerae* cytolysin (VCC)	*V. cholerae*
		V. vulnificus haemolysin (VVH)	*V. vulnificus*
		Necrotic enteritis toxin B (NetB)	*C. perfringens*
	Aerolysin	Aerolysin	*Aeromonas* spp.
		ε-toxin	*C. perfringens*

		Enterotoxin (CPE)	*C. perfringens*
	CDC (cholesterol dependent cytolysin)	Perfringolysin O (PFO)	*C. perfringens*
		Streptolysin O (SLO)	*S. pyogenes*
		Lectinolysin (LLY)	*S. mitis*

Table 1.2. Characterization of different PFTs and their families on the basis of the secondary structure of their membrane-spanning region [5].

Effects of PFTs can be severe for the host organism such as humans.

One of such incidents depicting the severity of harmful effect caused due the outbreak of bacteria secreting PFTs and bringing a new PFT and harmful bacteria into the human's knowledge was in 1950s when Tsunesaburo Fujino discovered *Vibrio parahaemolyticus* which at that time had caused a major outbreak causing illness to 272 individuals and 20 deaths in Japan [11]. Another incident of outbreak of *V.parahaemolyticus* was in Alaska, USA in 2004, when people were getting food poisoning due to consumption of oysters [12]. *V.parahaemolyticus* is a gram-negative bacteria which mostly exist in brackish environment [13]. *V.parahaemolyticus* enters into marine organisms such as fish, oysters etc., which when consumed by humans allows *V.parahaemolyticus* to make their niche in human's intestine causing seafood-borne gastroenteritis [14]. This leads to diarrhea, nausea, abdominal cramps and vomiting and mild fever. It was found that *V.parahaemolyticus* secrets PFTs, Thermostable Direct Hemolysin (TDH) and TDH-related hemolysin (TRH) [15]. TDH and TRH are major virulence factors in *V.parahaemolyticus* arsenal, which help *V.parahaemolyticus* to kill host cells by making host cell membrane porous, leading to cell-death [13]. TDH and TRH show three types of biological activities, hemolytic activity, cardiotoxicity and enterotoxicity based on *V.*

parahaemolyticus secretion system. *V. parahaemolyticus* has two type of type III secretion system (T3SS), T3SS1 and T3SS2. When toxins are secreted via T3SS1, TDH and TRH are involved in cytotoxicity, autophagy and mouse lethality, whereas, T3SS2 is involved in enterotoxicity [16-19]. TDH is a pore-forming toxin, which forms pores onto target cell membrane leading to "cell-death". TDH is composed of 165 amino acids and consist of a β-sandwich domain flanked by two α-helices [20, 21]. TDH is known to show Arrhenius effect which means that on heating TDH upto 60^{o}C, TDH denatures and starts forming amyloid fibrils, and on further heating TDH upto 90-100^{o} C, these fibrils starts clearing, and on rapidly cooling to room temperature it renatures and shows hemolytic activity (hence, Thermostable) [22]. TDH does not show lytic activity (hence, the name-Direct) [23]. Unlike most of the PFTs, TDH exist as tetramer in solution due to the extended C-terminal region. This tetramer binds to the target cell membrane after which pore-formation takes place [20]. TDH makes 23 Å-diameter pore onto target cell membrane [24]. Interestingly, being a prokaryotic PFT, TDH shows no structural similarity with other prokaryotic PFT. But TDH shows high structural similarity with eukaryotic actinoporins like EquinatoxinII, SticholysinII, Fragaceatoxin C (Figure 1.2)[20].

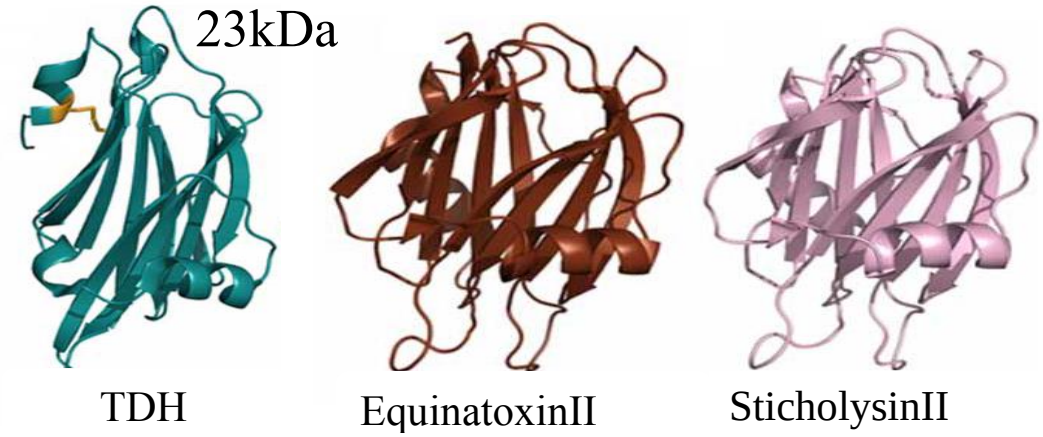

Figure 1.2. Crystal structure of TDH (PDB ID: 3A57), EquinatoxinII (PDB ID: 1IAZ) and SticholysinII (PDB ID: 1GWY) [20].

Pore-formation mechanism of TDH is not well understood. But earlier studies on the structurally similar EqtII and StnII have shown that in EqtII, there are five different tryptophan residues at three different sites, out of which tryptophan at 116th and 117th position are involved in the binding of EqtII to the hydrophobic region of the target cell membrane, and get deeply buried into the lipid membrane during pore formation [25]. Studies on StnII have shown that StnII forms a pocket in order to bind to Phosphocholine (POC) [26]. Aromatic rings of Tyr111 and Tyr135 interact with positively charged choline moiety via cation-π interaction, and the phosphate groups of the membrane phospholipids interact with phenolic hydroxyl group of both tyrosine residues. Tyr111 in StnII is an important residue for binding to target cell membrane [27].

On superimposing TDH and StnII structures we observed that Tyr111 of StnII superimposes with Tyr87 of TDH (Figure 1.3), and on further examining TDH structure we were able to observe that there exist a hydrophobic pocket at the same site comprising of tyrosine 87 and tryptophan 65 (Figure 1.4). These hydrophobic pockets may possibly facilitate binding of the toxin to the hydrophobic region of the target cell membrane. So, in this work, W65 and Y87 were mutated to alanine and phenylalanine residues in order to examine the significance of this hydrophobic pocket in TDH-binding to target cell membrane.

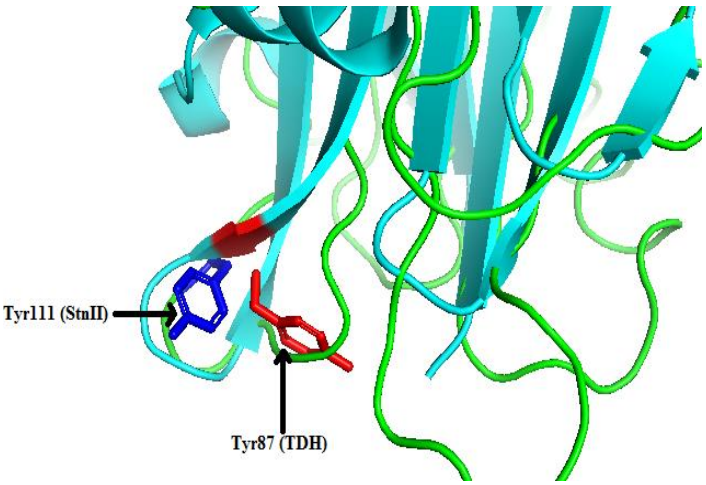

Figure 1.3. Superimposed image of Tyr111 of StnII over Tyr87 of TDH.

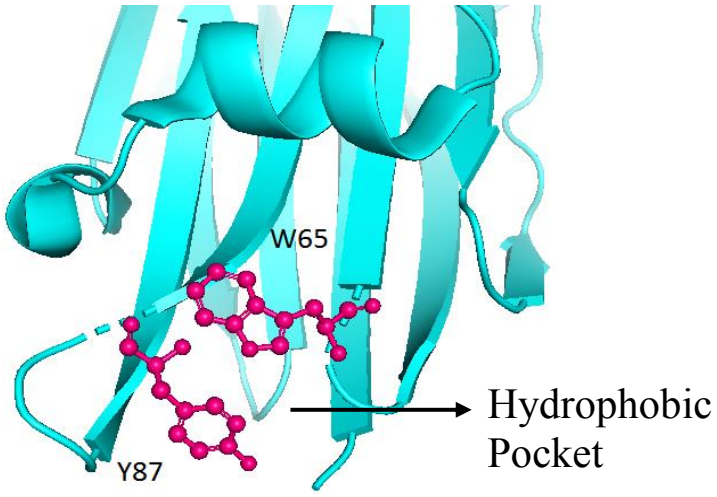

Figure 1.4.
Hydrophobic pocket in TDH comprising of Tyr87 and Trp65.

Chapter 2
Material and Methods

1. **Superimposing TDH and StnII structures:**

   In order to examine any overlapping amino acid of TDH on tyrosine 111 of StnII which might contribute in binding of TDH onto target cell membrane, WinCoot was used to superimpose TDH and StnII structures based on Secondary Structure Matching, SSM. PDB file were taken from Protein Data Bank (http://www.rcsb.org/pdb) of TDH (PDB ID: 3A57) and StnII (PDB ID: 1GWY).

2. **Making cells (Top10 and Origami B) competent via $CaCl_2$ method:**

   In order to make cells competent, cells were incubated in 5 ml LB media overnight at 37^o C at 180 rpm. 10 mL secondary culture was made by adding 200µL (2%) of overnight grown primary culture and incubated at 37^o C at 180 rpm till its $O.D_{600nm}$ reached 0.4 to 0.6. Cells were centrifuged at 4000 rpm for 20 min at 4^{o}C. Supernatant was discarded. Cells were re-suspended in 10 ml of 100 mM $CaCl_2$, which was incubated on ice for 10 min. Then, the cells were centrifuged at 4000 rpm for 20 mins at 4^o C. Supernatant was discarded. Then cells were re-suspended in 5 ml of 50 mM $CaCl_2$ and incubated on ice for 45 min. Then the cells were centrifuged at 4000 rpm for 15 mins at 4^o C. Supernatant was discarded. Pellet was re-suspended in 85% of 100 mM $CaCl_2$ and 15% of absolute glycerol. The re-suspended cells were aliquoted (100 µL each) into 1.5 mL micro-centrifuge tubes. Ca^{2+} role is to neutralize negative charges on both DNA and cell surface (LPS) to avoid electrostatic repulsion between them and incubation on ice will reduce thermal motion.

3. **Making LB-agar Ampicillin (amp) plates:**

LB-agar amp plates were prepared by adding 2.5% LB medium and 2% agar in 200 ml of distilled water. This was autoclaved at 121^{o}C for 15 mins at 15 psi. After autoclave, the media was cooled till its bearable to touch and ampicillin (50µg/mL) was added.

4. **Site-directed Mutagenesis using Splicing by overhang extension polymerase chain reaction (SOE-PCR):**

All the TDH mutants comprising of W65A *tdh*, W65F *tdh*, Y87A *tdh* and Y87F *tdh* were constructed by two round of polymerase chain reactions (PCR) under the method known as splicing by overhang extension PCR. First, PCR program was constructed for TDH as follows:

1. 5 mins initial denaturation at 94^{o} C
2. 30 sec denaturation at 94 ^{o} C
3. 30 sec annealing at 54 ^{o} C
4. 1 min 30 sec amplification at 68 ^{o} C
5. 7 min final extension at 68 ^{o} C

} **30X**

List of primers used in round 1 PCR are depicted in Table 2.1

Primers	Sequence (5'-3')
WT-TDH forward	atattgCTCGAGatgTTTGAGCTTCCATCTGTCCC
WT-TDH reverse complementary	AACATTggatccTTATTGTTGATGTTTACATTCAAA AAACGAT
W65A forward	GTATTCACAACGTCAGGTACTAAAgcgCTGACATC CTACATGACTG
W65A reverse complementary	GTTCACAGTCATGTAGGATGTCAGcgcTTTAGTAC CTGACGTTGTG
W65F forward	GTATTCACAACGTCAGGTACTAAAtttCTGACATC CTACATGACTG
W65F reverse complementary	GTTCACAGTCATGTAGGATGTCAGaaaTTTAGTAC CTGACGTTGTG

Y87A forward	GCGGTGTCTGGCgccAAGCACGGTCATTCTGCTGTGTTCG
Y87A reverse complementary	ATGACCGTGCTTggcGCCAGACACCGCTGCCATTGTATAG
Y87F forward	GCGGTGTCTGGCtttAAGCACGGTCATTCTGCTGTGTTCG
Y87F reverse complementary	ATGACCGTGCTTaaaGCCAGACACCGCTGCCATTGTATAG
T7 forward	TAATACGACTCACTATAGGG

Table 2.1. List of Primers used in TDH mutants preparation.

Round 1 PCR samples were prepared as mentioned in Table 2.2.

	Sample 1	Sample 2	Negative Control	Positive control
Platinum Master Mix	20µL	20µL	20µL	20µL
WT TDH template	1µL	1µL	1µL	1µL
WT TDH Forward primer	0.5 µL	-	0.5 µL	0.5 µL
WT TDH Reverse primer	-	0.5 µL	0.5 µL	0.5 µL
Mutant Forward primer	-	0.5 µL	-	-
Mutant Reverse primer	0.5 µL	-	-	-

Table 2.2. Quantity of Platinum Master Mix, WT TDH template and primers used for different samples in first round of PCR are depicted here.

DNA templates amplified from first round of PCR were run through 0.8% agarose gel (gel electrophoresis). Sample 1 and sample 2 were then extracted from gel using Qiagen gel extraction kit. Second round of PCR was performed with composition depicted in Table 2.3.

Platinum Master Mix	20 µL
WT TDH template	0.5 µL
WT TDH Forward primer	0.5 µL
Sample 1	10ng
Sample 2	10ng

Table 2.3. Composition of round 2 PCR is depicted here.

Product of round 2 PCR (mutated *tdh* template) was run on 0.8% agarose gel and then extracted from gel.

5. **Cloning of mutated *tdh* gene into pET14b vector:**

   Both insert (mutated *tdh* gene) and vector (pET14b) were first digested using restriction enzymes allowing them to form staggered ends with specific sequence. For TDH, 1 µL BamHI and 1 µL XhoI were used as restriction enzymes. Digestion reaction was incubated at 37^{o} C for 3-5 hrs. The product of digestion was run on 0.8% agarose gel and then the specific band (vector at ~500 bp and plasmid at ~4700 bp) was extracted from gel. Further, ligation was carried out for digested vector and insert for 1 hr at 25^{o} C.

6. **Transformation of plasmid into Top10 competent cells and Origami B competent cells:**

   Whole process of transformation was performed on ice. Competent cells were thawed on ice. Plasmid was added to competent cells and incubated for 10 min on ice. Heat shock at 42^{o} C was given on dry bath. Competent cells were immediately put on ice and incubated on ice for 10 min. After incubation, cells were revived in 1 mL 2.5% LB media and incubated at 37^{o} C for 1 hr on shaking. Cells were

centrifuged at 4000 rpm for 3 min. Supernatant was discarded and pellet was re-suspended in 100 µL of LB media and was spread on LB-agar-amp plates containing ampicillin (50 µg/mL) (specifically allowing cells containing ampicillin resistant marker to grow).

7. Colony PCR:

To verify the gene of interest was ligated to the vector (pET-14b), transformed Top10 colonies were picked from the LB-agar-amp plates and dissolved in 10 µL water in PCR tubes. Then, 10 µL of Thermo Mix (2X) was added with 0.5 µL T7 forward primer (to ensure that the insert has been cloned in the right direction) and 0.5 µL WT TDH reverse primer. PCR was carried out and the amplified product was run through 0.8 % agarose gel.

8. Small-scale expression check.

After the transformation of plasmid into origami B cells and plating on LB-agar-amp plates, colonies were picked and transferred to 6 mL of LB media in 15mL tubes and were incubated at 37^{o} C at 180 rpm till their $O.D_{.600nm}$ (optical density) reached 0.4 to 0.6. After this, samples were induced with 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) for 3 hrs at 37^{o} C. Cells were pelleted down after incubation and further resolved on 12.5% SDS-PAGE.

9. Large scale expression of protein.

Seed culture was made by using 200 µL tip and picking plasmid containing origami B cells from its glycerol stock and adding it to 50 mL LB media containing amp (50 µg/mL). This was incubated overnight at 37^{o} C for 16 hrs at 180 rpm. 20 mL of seed culture was added to 1L of LB media containing amp which was further incubated at 37^{o} C at 180 rpm till its $O.D_{600nm}$ reaches between 0.4 to 0.6. 1mM IPTG (0.238 gm in 1 L culture) was added after desired $O.D_{600nm}$ was obtained and was incubated at 30^{o} C at 180 rpm for 3 hrs. Media culture was then transferred to centrifuge bottles and was centrifuged at 4000 rpm for 30 mins at 4^{o} C. Supernatant

was discarded and 3-4 mL P.B.S (phosphate saline buffer- 20mM sodium phosphate buffer) (maintains ph suitable for cells) was used to re-suspend the pellet which was transferred to 50 mL centrifuge tube. Protease inhibitor cocktail (Sigma-Aldrich) (inhibits bacterial protease) (PI) was added to re-suspended pellet (in accordance to 50 µL PI to 1 L culture).

10. Protein purification via Ni-NTA chromatography and anion exchange chromatography.

Cells from large scale expression were lysed using sonicator and then were transferred to oakridge centrifuge tubes which was centrifuged at 4^{o} C for 30 min at 11900 rpm. Supernatant was collected in 50 mL centrifuge tube and was kept on ice. Meanwhile Ni-NTA column was washed via 500 mM imidazole in PBS (to remove unwanted bonds on Ni as Ni shows high affinity towards imidazole ring) and further by PBS. 1M imidazole was added to protein so that the final concentration of imidazole be 20 mM (helps in avoiding undesired protein to bind to Ni). Protein was loaded into the column. Washing of the column was done with 20 mM imidazole. Protein was eluted in 300 mM imidazole. Column was washed using 500 mM imidazole in PBS and further by PBS. Side by side washing of anion exchange column was done using 500 mM NaCl in 10 mM Tris-Cl and further by 10 mM Tris-Cl. Protein eluted from Ni-NTA column was diluted with 4 X 10 mM Tris-Cl and then loaded into anion exchange column. Washing of anion exchange column was done using 100 mM NaCl in 10 mM Tris-Cl. Protein was eluted in 500 mM NaCl in 10 mM Tris-Cl. Column was washed using 10 mM Tris-Cl.

11. 6 X His-tag cleavage using thrombin enzyme.

In upstream of TDH gene there is a 6 X Histidine gene which helps in purification of the protein, as imidazole ring in histidine shows high affinity towards Ni. After purification, this 6 X His-tag needs to be cleaved in order to make the protein active which is done by thrombin enzyme which is serine protease and cleaves at specific site. This site is present in between 6 X His-tag and our protein gene. So 6 X His-

tag was cleaved by adding thrombin enzyme and incubating at 37^{o} C for 3 hrs at 180 rpm. 0.1 M PMSF (phenylmethylsulfonyl fluoride) (inhibits serine protease) was added so that the final concentration be 1 mM. This was 4 times with 10 mM Tris-Cl. Side by side anion exchange column was washed first with 500 mM NaCl in 10 mM Tris-Cl afterwards with 10mM Tris-Cl. Protein was loaded into the column. Column was washed with 100 mM NaCl in 10 mM Tris-Cl. Protein was eluted in 500 mM NaCl in 10 mM Tris-Cl. Column was washed using 10 mM Tris-Cl.

12. Normalizing protein concentration via SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel)

All the variables of protein, with same calculated protein concentration, were run through SDS-PAGE and the intensity of color of their bands were observed.

Protein	Extinction coefficient (1 mg/mL)
WT TDH	1.28
W65A	0.999
W65F	0.995
Y87A	1.209
Y87F	1.204

Table 2.4. Proteins and their respective extinction coefficient.

13. Hemolytic activity assay (kinetic mode and equilibrium mode).

In order to check the activity of the mutants with respect to WT TDH, two modes of hemolytic activity assay was perform using erythrocytes (RBCs).

i. Kinetic mode: RBCs were washed in PBS by centrifuging at 2500 rpm for 7 mins at 4^{o} C. Blood volume was set so that the O.D at 650 nm (turbidity of the solution) of 1 mL solution in PBS comes in between 0.9 to 1.0. Protein was added in such a way that the final concentration of the protein be 2 μM. Absorbance was observed within 5 mins interval for 1hr. Concentration of protein remains constant and time varies.

ii. Equilibrium mode: RBCs were washed in PBS by centrifuging at 2500 rpm for 7 mins at 4^{o} C. PBS was added to blood in such a way that after lysing cells with Triton X -100 (detergent) in 100 µL flat bottom 96-wells plate, its absorbance at 415 nm (absorbance of hemoglobin) come in between 0.9 to 1.0. Cells were incubated with different protein concentrations (serial dilution) in 100 µL V-bottom 96-wells plate for 1 hr at 37^{o} C. Plate was centrifuged at 2500 rpm for 5 mins at 4^{o} C. Blood was transferred into 100µL flat bottom 96-wells plate and absorbance was observed at 415 nm. Time remain constant and concentration varies.

14. Circular Dichroism in order to check any secondary structural changes.

Protein samples were first dialyzed in 1 mM Tris Cl (reduces NaCl concentration). 2µM protein samples were prepared. Circular Dichroism was performed in 200 µL cuvette, in the range of 200-280 nm.

15. Intrinsic tryptophan fluorescence in order to check any tertiary structural changes.

2 mL fluorimeter cuvette was used. Protein was added to buffer (500 mM NaCl 10mM Tris-Cl) in such a way so that the final concentration of the protein be 2 µM. Excitation wavelength was set at 295 nm (less overlapping of other amino acid excitation wavelength to that of tryptophan at 295 nm) and emission range was set from 310 nm to 450 nm.

16. Flow cytometry based human erythrocytes binding assay (using FACS machine).

Blood was washed in PBS. Side by side, 10 mM Dextran in PBS was prepared by adding 3 gm Dextran (acts as an osmoprotectant) in 30 mL PBS. 10 mM Dextran in PBS was added to blood pellet. This was diluted 10 times with PBS in 1.5 mL mct for cell count via hemocytometer using microscope by pouring 10 µL of this

diluted blood to hemocytometer. Required volume of blood in 10 mM PBS Dextran was calculated from cell count so that the volume of blood added to 100 μL V bottom 96-wells plate contains 10^6 RBC cells (approximately). These RBCs were treated with our protein (total volume made after adding protein in blood was 100 μL) in 100 μL V-bottom 96-wells plate and incubated for an hour at 37^o C. Cells were washed using 10 mM PBS Dextran. Primary antibody (from rabbit) against TDH was added (1 μL antibody in 100 μL 10 mM PBS Dextran) and incubated for 45 mins at 37^o C. Cells were washed using 10 mM PBS Dextran. Secondary antibody (from goat conjugated with FITC (fluorescein isothiocyanate) (fluorophore)) against primary antibody was added (1 μL antibody in 100 μL 10mM PBS Dextran) and incubated for 45 mins at 37^o C. Cells were washed using 10 mM PBS Dextran. Cells were re-suspended in 100 μL 10 mM PBS Dextran which was transferred to 400 μL 10 mM PBS Dextran in FACS tubes. Cells were observed using FACS machine.

Untreated + Stained (primary + secondary antibody)	Untreated + Secondary antibody	Untreated Unstained
WT TDH Stained	WT TDH + Secondary antibody	WT TDH Unstained
Alanine mutant Stained	-	-
Phenylalanine mutant Stained	-	-

Table 2.5 Treatment composition for binding assay.

Chapter 3

Results and Discussion

1. **SDS-PAGE/Coomassies staining of the TDH variants:**

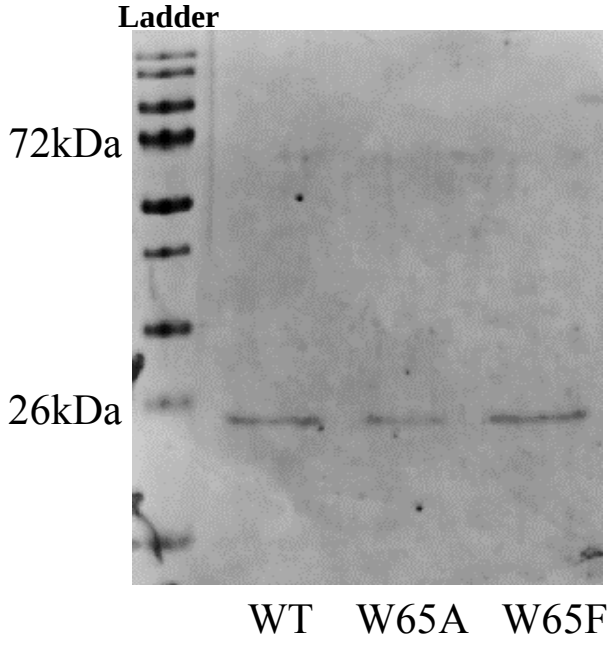

Figure 3.1. SDS-PAGE/Coomassie staining showing normalized protein concentration.

TDH band comes at around 25 kDa. All the sample's extinction co-efficient was first measured using Expasy protein-parameter tool, which was used to calculate their concentrations after checking their absorbance at 280 nm with a spectrophotometer.

2. **Hemolytic activity assay of the TDH variants with mutation of Trp65:**

a. Kinetic mode **b. Hemolytic mode**

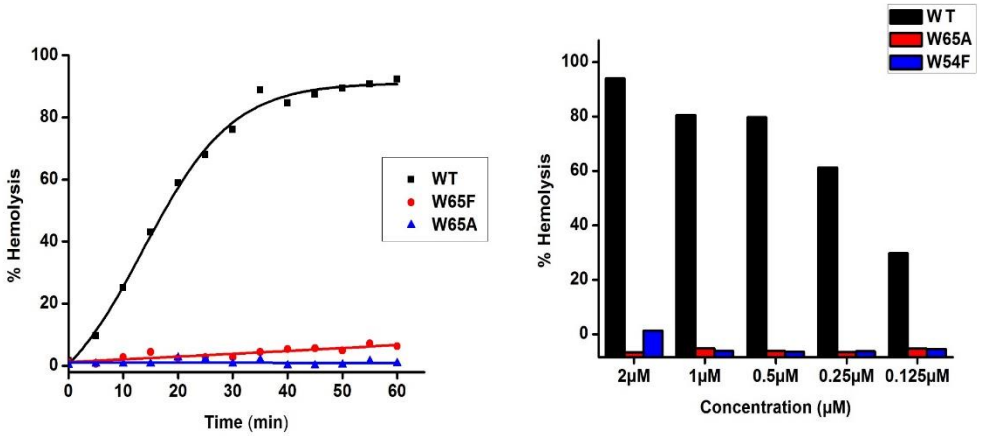

Figure 3.2. Hemolytic activity assay of Trp65A and Trp65F TDH variants as compared to that of wild type TDH (WT)

a. Extent of hemolytic activity over 1 hour time period with same concentration of protein (2μM).

b. Extent of hemolytic activity over a period of 1 hour, with varying concentration of proteins.

W65A and W65F, both mutants show reduced hemolytic activity suggesting that pore-formation mechanism was interrupted. As tryptophan is an aromatic/hydrophobic residue, and on mutating tryptophan to alanine both these characteristics are gone. Therefore, as alanine mutant shows highly reduced hemolytic activity, this shows that aromaticity and hydrophobicity are playing an important role at 65^{th} position of TDH. On mutating W65 to phenylalanine, hydrophobicity has been restored as phenylalanine is more hydrophobic than tryptophan but it is less aromatic then tryptophan. Therefore, this suggests that aromaticity at 65^{th} position is majorly involved in pore-formation mechanism of THD.

3. Circular Dichroism of TDH variants with mutation of Trp65:

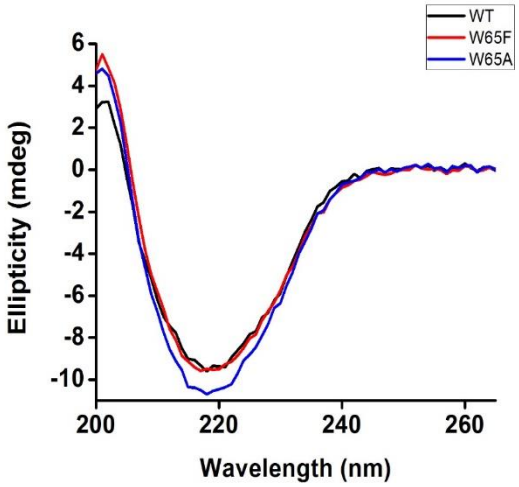

Figure 3.3. Circular Dichroism/Far UV Spectra of both the W65 mutants with respect to WT TDH.

TDH is having a β-sandwich structure, which means that there are β-sheets between two α-helices. TDH is mostly comprised of β-sheets. In far-UV CD spectra, β-sheets show negative ellipticity at around 218-219 nm. Therefore, on mutating tryptophan to its variant if this would lead to any secondary structural changes then that change will be visible in CD spectra. But both the mutant showed no variation from that of WT TDH. Therefore no secondary structural change was observed suggesting that mutating W65 does not affect the secondary structure of TDH.

4. Intrinsic Tryptophan Fluorescence of W65 mutants:

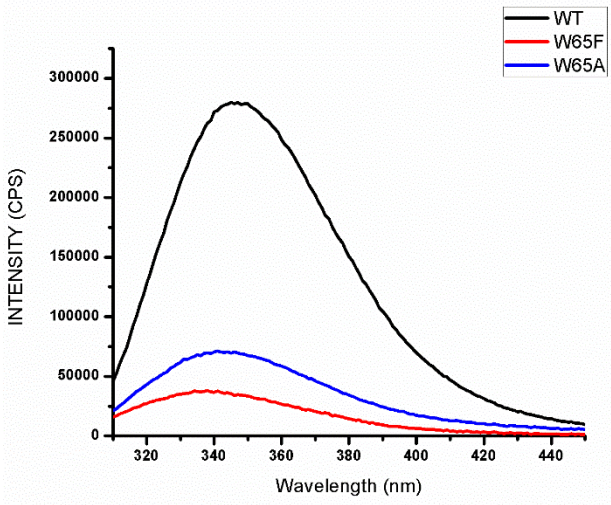

Figure 3.4. Intrinsic tryptophan fluorescence of W65 mutant as compared to WT TDH. Excitation at 295 nm.

Intrinsic tryptophan fluorescence is used to analyze the tertiary structural features of protein. As we mutated tryptophan itself we were expecting decrease in intensity. As TDH has only two tryptophan, the intensity has drastically reduced on mutating one of them.

5. Flow cytometry-based human erythrocytes binding assay of W65 mutants:

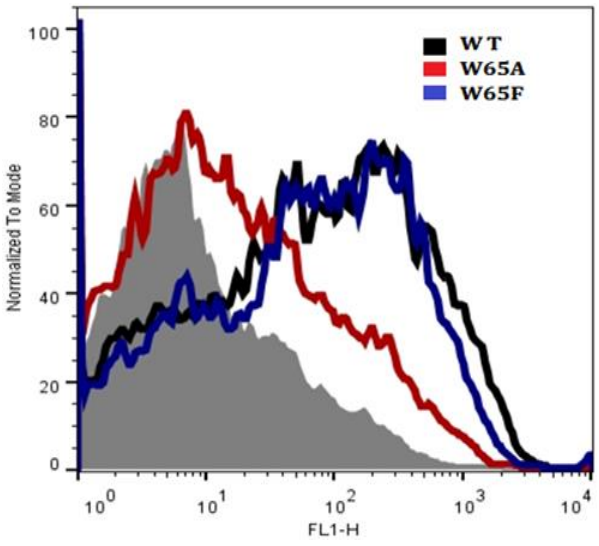

Figure 3.5. Binding of W65 mutants with human erythrocytes with respect to WT TDH determined by the flow cytometry-based assay.

Flow cytometry-based RBC binding assay of tryptophan mutants shows that on mutating tryptophan to alanine, the binding of TDH has reduced drastically. But on mutating tryptophan to phenylalanine, the binding of TDH has been restored. This suggest that with hydrophobicity a 65th position been restored, binding of TDH to target cell membrane also has been restored. Therefore, hydrophobicity at 65th position is important to TDH binding to target cell membrane.

6. SDS-PAGE/Coomassies staining of the TDH variants:

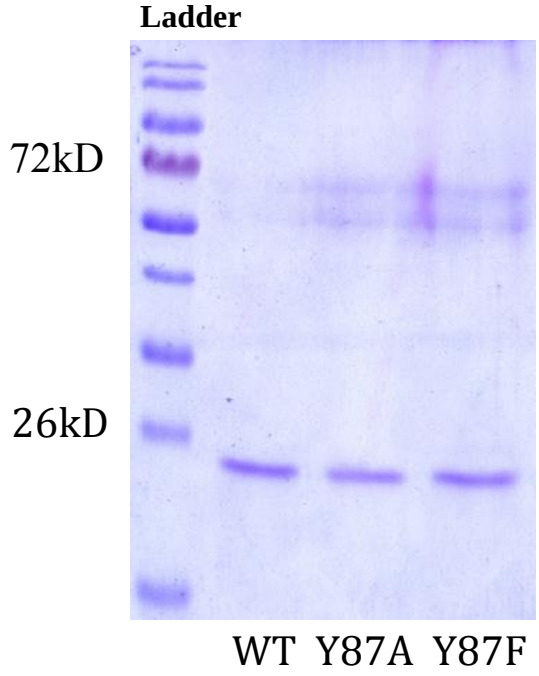

Figure 3.6. SDS-PAGE/Coomassie staining showing normalized protein concentration.

Both the tyrosine mutants seems to be of same concentration to that of WT TDH.

7. **Hemolytic activity assay of the TDH variants with mutation of Tyr87:**

Kinetic mode: **Hemolytic mode:**

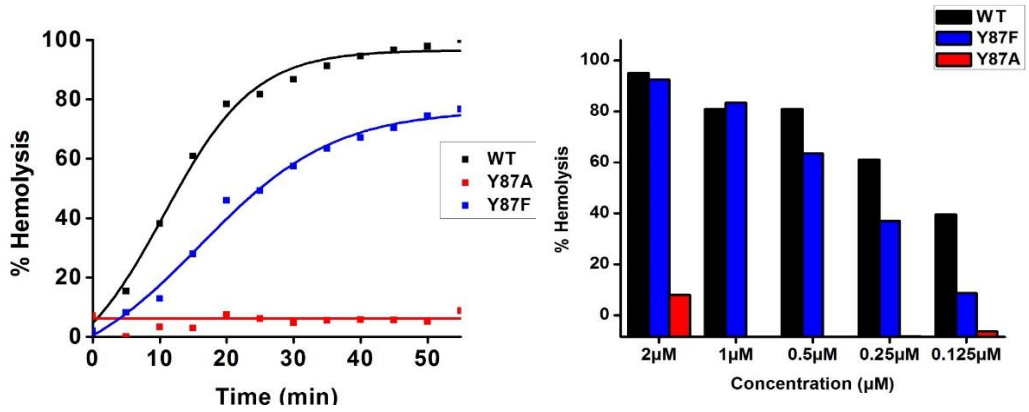

Figure 3.7. Hemolytic activity assay of Tyr87A and Tyr87F TDH variants as compared to that of wild type TDH (WT)

a. Extent of hemolytic activity over 1 hour time period with same concentration of protein (2μM).
b. Extent of hemolytic activity over a period of 1 hour, with varying concentration of proteins.

Tyrosine is an aromatic as well as hydrophobic amino acid. So on muatating tyrosin with alanine, both these characteristics are gone. On mutating tyrosine with phenylalanine long hydrophobic side chain has been restored but phenylalanine is still less aromatic as compared to tyrosine. Alanine mutant of Y87 shows highly reduced hemolytic activity and on mutating Y87 to phenylalanine hemolitic activity has been restored to around 70-80%. This suggest that hydrophobicity at 87th position is playing an important role in TDH pore-forming mechanism.

8. Circular Dichroism of Y87 mutants:

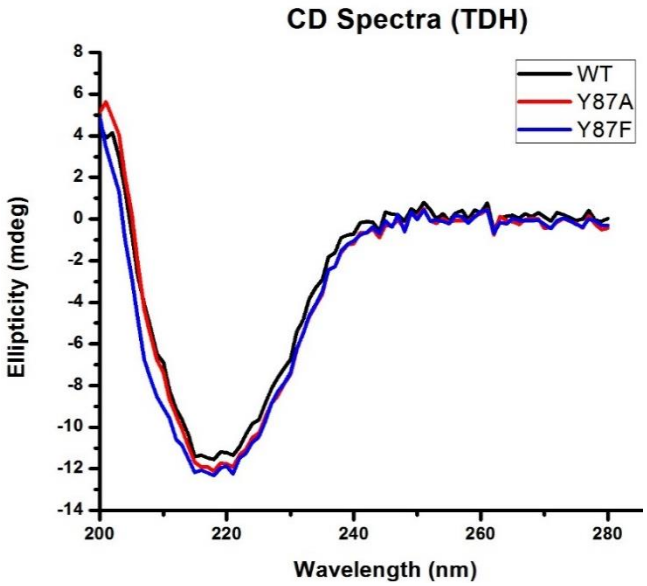

Figure 3.8. Circular Dichroism/Far UV Spectra of both the Y87 mutants with respect to WT TDH.

Both tyrosine mutants (alanine and phenylalanine) shows no secondary structural variation from WT TDH. This suggest that mutating Y87 does not affect secondary structure of TDH.

9. Intrinsic Tryptophan Fluorescence of Y87 mutants:

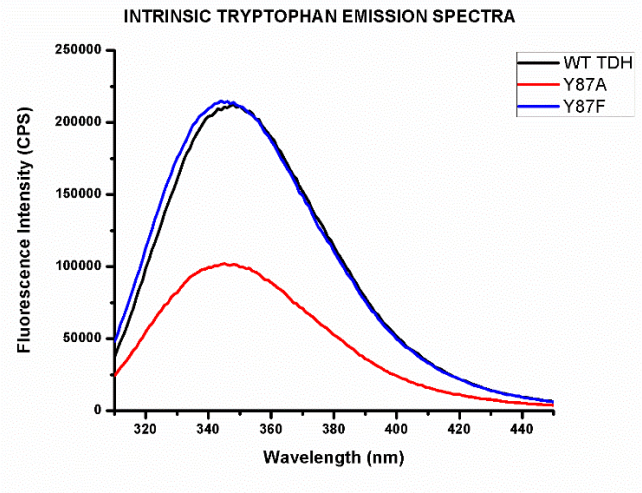

Figure 3.9. Intrinsic tryptophan fluorescence of Y87 mutant w.r.t WT TDH. Excitation at 295 nm.

No blue or red shift is observed on mutating tyrosine to alanine and phenylalanine.

10. Flow cytometry based human erythrocytes binding assay of Y87 mutants:

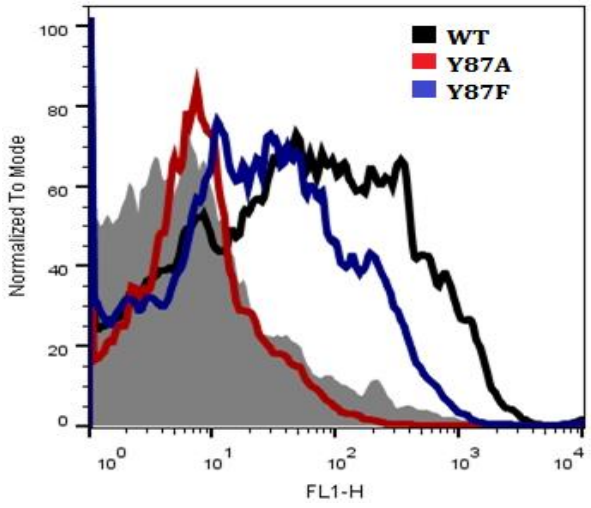

Figure 3.10. Binding of Y87 mutants with human erythrocytes with respect to WT TDH determined by the flow cytometry-based assay.

On mutating Y87 to alanine, binding of TDH to target cell membrane has drastically reduced and on mutating tyrosine to phenylalanine, binding has been restored. This suggest that hydrophobicity at 87th position is important for TDH binding to target cell membrane.

Conclusion

- W65 is an important residue in pore-formation mechanism of TDH onto target cell membrane.
- Mutating W65 to alanine or phenylalanine reduces TDH hemolytic activity.
- Mutating W65 to alanine reduces TDH binding to RBCs, but on mutating W65 to phenyl alanine the binding has been restored.

  All these points suggest that hydrophobicity at 65^{th} position is important for TDH binding to target cell membrane, BUT TDH is still not able to make pore when tryptophan's aromatic character has been reduced. Therefore, aromaticity at 65^{th} position is of high importance for TDH for its pore-formation mechanism.

- Y87 is an important residue in pore-formation mechanism of TDH onto target cell membrane.
- Mutating Y87 to alanine reduces its hemolytic activity drastically but it is restored on mutating Y87 to phenylalanine.
- Mutating Y87 to alanine reduces TDH binding to RBCs, but on mutating Y87 to phenyl alanine the binding has been restored.

  Since, on mutating Y87 to alanine TDH shows drastic reduction in hemolytic activity and also reduction in binding, suggesting that Y87 is an important residue in pore-formation mechanism of TDH. But on mutating Y87 to phenylalanine hemolytic activity and binding both has been restored suggesting that hydrophobicity as well as aromaticity are playing an important role at 87^{th} position of TDH.

Overall, hydrophobic pocket comprising of W65 and Y87, is highly involved in binding of TDH to target cell membrane but data also suggest that this pocket is also involved in further steps of pore-forming mechanism of TDH after binding.

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